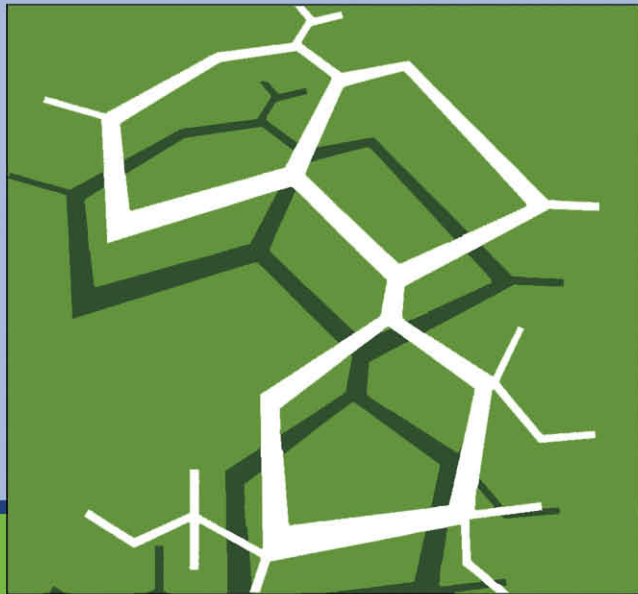


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Adenosine in the Nervous System

Edited by
Trevor Stone



Adenosine in the Nervous System

NEUROSCIENCE PERSPECTIVES

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Adenosine in the Nervous System

edited by

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Series Preface

The driving force for the production of this series lies in my own inability to keep up with the advances occurring in those areas of neuroscience in which I am especially interested. So many times I have been frustrated by being unable to find a current review of an important research area. Even when I resort to bothering colleagues who are experts in a particular field, I am told, more often than not, that such an overview does not exist. In my own area of expertise I frequently send away empty handed students who have asked me to direct them to a definitive article on a well researched topic.

Although regrettable, perhaps this situation is not surprising since the neurosciences are one of the most diverse and rapidly advancing areas in the biological sphere. By definition, research in the neurosciences encompasses anatomy, pathology, biochemistry, physiology, pharmacology, molecular biology, genetics and therapeutics. Indeed, there are few individuals capable of maintaining a grasp of the literature in all these aspects of their own research interests let alone in other fields.

My answer was to establish *Neuroscience Perspectives* and to develop gradually a series of individual edited monographs dealing in depth with issues of current interest to those working in the neuroscience area. Each volume is being designed to bring a multidisciplinary approach to the subject matter by pursuing the topic from the laboratory to the clinic. As a consequence I have asked the editors of the individual volumes to produce a balanced critique of their topic which will be read, understood and enjoyed by as wide an audience as possible within the realm of neuroscience.

The choice of the topics for the series is a difficult matter. In the first instance these were largely dictated by my own interests or by my awareness of important and fundamental work being undertaken by colleagues. More recently, I have been recruiting subject matter and editors through attending a variety of diverse symposia in the neuroscience area. However, the choice of topics should reflect the needs of the audience reached by the series. So I invite you to let me know of areas which you feel are of importance and to give me suggestions for individuals who would be keen to edit a book for *Neuroscience Perspectives*.

Finally, it only remains to thank those individuals at Academic Press who have already worked for several years to develop *Neuroscience Perspectives*. In particular, Dr Carey Chapman who has the unenviable task of recruiting the editors that I suggest and then harassing them for the completed work. My hope is that the series will fill the gap that I perceive and provide for my

Series Preface

colleagues in the neurosciences a collection of interesting books which will become reference volumes in their field. I hope you will enjoy *Neuroscience Perspectives*.

Peter Jenner

Preface

As with many growth areas in biological research, the recruitment of new workers, the expansion of ideas and the development of potential applications have spawned a number of international meetings on various aspects of the biochemistry, pharmacology and physiology of adenosine. These are, of course, invaluable to those already working in the area and who can use the opportunities presented to build upon their own background knowledge to develop new ideas as well as renew social contacts. But for those scientists new to the field it becomes increasingly difficult to 'break in' to the jargon and background and, especially, to perceive new results in perspective. It is also a sad reflection of the current pressures on time and money that work is rapidly regarded as somehow outdated, and full recognition is often not given to those workers who made original and crucial, if isolated, observations.

This book is partly an attempt to rectify some of these difficulties by presenting within a single volume overview chapters by some of the most consistent and original major contributors to purine research. It is fitting that, within the context of the *Neuroscience Perspectives* series, it should be concerned largely with adenosine in the nervous system, since it was here that Al Sattin and Ted Rall produced in 1970 some of the first evidence for receptor-mediated actions of extracellular adenosine, and made the critical discovery that methylxanthines would block those receptors. The present volume explores most aspects of adenosine function developed largely since that time, including work on uptake and metabolism, release and electropharmacology, biochemistry and therapeutic potential. It is hoped that the book will find a useful home as an easily readable reference source of information for established workers and their students, as a summary of knowledge as it stands at the beginning of the 'Decade of the Brain'.

Trevor Stone

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CHAPTER 1

ADENOSINE TRANSPORT IN NERVOUS SYSTEM TISSUES

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Introduction

The tissue levels of adenosine, which are almost certainly controlled by uptake and release processes, help to regulate a multitude of physiological functions (Phillis and Wu, 1981). Accordingly, an understanding of where, and the mechanisms by which, adenosine levels are regulated may be pivotal to the formulation of concepts concerning its utilization as a neuroregulatory

substance or 'retaliatory metabolite' (Newby, 1984). Moreover, the benefit of such an understanding might be the development of new strategies for drug design whereby adenosine re-uptake inhibitors are used in a site- and event-specific manner to increase the endogenous tissue levels of adenosine in order to, for example, enhance the quality of sleep, decrease seizures and prevent CNS damage secondary to hypoxia/ischaemia. However, even though adenosine transport (uptake) inhibitors have been shown under *in vivo* and *in vitro* experimental conditions to affect adenosine's ability to depress neuronal excitability, alter cAMP levels, produce antinociception and decrease locomotor activity, it is not completely clear whether their actions are mediated, completely or in part, through adenosine transport inhibition. Although adenosine transporters are selectively expressed in discrete populations of cells and neural systems in the CNS (Geiger and Nagy, 1990), and may represent potential sites for pharmacological manipulation (Deckert *et al.*, 1988a), adenosine transport processes have not been well-characterized and this has almost certainly hindered pharmaceutical developments in this area. Because much of what we infer about CNS adenosine transporters comes from research conducted on other tissues, the extent to which these data can be extrapolated to human CNS is uncertain. It is our intention here to review the literature on CNS transporters in order to stipulate what is known, to delineate what needs to be described, and to unmask dogma that presently lacks experimental support.

Adenosine transport

Classification of adenosine transporters

The transport of adenosine across plasma membranes in the CNS was first described over two decades ago (Santos *et al.*, 1968). Until recently, purine and pyrimidine nucleosides were thought to be transported at normal physiological levels by equilibrative facilitated diffusion via specific transporter proteins or, at higher than normal levels, by non-specific passive diffusion through the lipid bilayer. Research conducted mainly on peripheral tissues and cultured cells has shown that adenosine flux across membranes cannot be so simply classified. Indeed, it now appears that a family of nucleoside transporters exists which can be subcategorized according to their (a) relative affinities (high and low) for adenosine as a substrate, (b) relative selectivity for adenosine over other endogenous purine and pyrimidine nucleoside substrates, (c) sensitivity or resistance to the blocking effects of nitro-benzylthioinosine (NBI), dipyradamole (DPR) and other nucleoside transport

inhibitors, and (d) ability to translocate nucleosides across cell membranes by passive and facilitated equilibrative processes, or by sodium-dependent and concentrative transport systems.

The classical and best-characterized means by which adenosine crosses cell membranes is by facilitated diffusion through a nucleoside transport system. The facilitated diffusion process has a high affinity for adenosine, a very rapid turnover rate especially in human erythrocytes, and the ability to transfer nucleosides bidirectionally down concentration gradients and hence the term equilibrative (Plagemann and Wohlhueter, 1980; Paterson *et al.*, 1981; Young and Jarvis, 1983). These transporters have molecular weights ranging from 55 to 64 kDa and have been identified with SDS-PAGE as a band-4.5 polypeptide (Jarvis and Ng, 1985; Agbanyo *et al.*, 1990). Even though adenosine transporters in brain have molecular properties similar to those characterized in peripheral tissues, pharmacological differences between central and peripheral sites suggest that differences exist as well.

Criteria for measurement of adenosine transport

It is difficult to measure initial rates of adenosine transport (henceforth referred to as adenosine transport) because of the apparently rapid permeation of adenosine across cell membranes and metabolism by enzymes such as adenosine kinase (AK), adenosine deaminase (ADA) and *S*-adenosylhomocysteine hydrolase. Therefore, stringent criteria must be met whereby measurements of adenosine transport must be made using very short incubation times, non-metabolized substrates for the transporter, or tissues deficient in the enzymes responsible for metabolizing the transported substrate (Paterson *et al.*, 1985). However, it is difficult to compare transport rates in nervous system tissues with those in peripheral tissues, in part, because data were not or could not be expressed on an activity per cell or nerve ending basis. Because few similarly extensive studies have been conducted with nervous system tissues we are left to draw analogies with results from tissues where these phenomena have been better characterized.

Where it has been measured, adenosine influx through the transporter appears to be more rapid than its subsequent intracellular metabolism. Therefore, measurement of adenosine transport across the plasma membrane in cells capable of metabolizing this substrate can only be accurately measured following very brief reaction intervals (initial zero-trans transport). Importantly, if this criterion is not met, phosphorylation of adenosine by, for example, AK with its high affinity for adenosine ($0.2\text{--}2\text{ }\mu\text{M}$) will lead to the intracellular formation of phosphorylated derivatives of adenosine and trapping of radiolabelled adenosine. Therefore, under prolonged incubation conditions the measured K_T values for adenosine accumulation may reflect

more the activity of AK. Alternatively, deamination of adenosine by ADA may affect transport measurements. Although ADA in comparison with AK has a lower affinity for adenosine (Daval and Barberis, 1981; Stefanovich, 1983), adenosine levels in CNS tissues increase in response to, for example, hypoxia/ischaemia and seizures, to levels known to produce substrate inhibition of AK and promote deamination of accumulated adenosine (Nagy *et al.*, 1990). Under these conditions, although not yet shown for CNS tissues, intracellular accumulated adenosine and/or inosine may act as competitive substrates for the transport process, thereby affecting both influx and efflux of adenosine down its concentration gradients. One way to stop the metabolism of transported adenosine is to add, to incubation media, inhibitors of AK and ADA such as 5'-iodotubercidin and 2'-deoxycytidine, respectively. However, problems of data interpretability may arise because these inhibitors can serve as substrates for nucleoside transporters. Thus, if one observes less uptake in the presence of these inhibitors it is necessary to differentiate between a metabolic effect or direct inhibition of the transporter. Furthermore, under conditions of prolonged incubations where phosphorylation is favoured, inhibition of ADA could lead to increases in levels of intracellular adenosine below that which can inhibit AK and consequently could enhance the conversion of adenosine to 5'-AMP. This would further confound data interpretations.

Adenosine accumulation

If CNS adenosine transporters are similar to their counterparts in other tissues, then data from the bulk of studies performed to date on adenosine accumulation into CNS tissues, where prolonged incubations were used, most likely represent uptake (transport plus metabolism) rather than initial rates of transport. In Table 1 we have summarized the kinetic data from adenosine uptake and transport experiments on neural tissues. The K_m values of [^3H] adenosine uptake were, for example, 0.9 and 21 μM for rat and guinea-pig cortical synaptosomes, respectively (Barberis *et al.*, 1981; Bender *et al.*, 1981), and ranged from 9.6 to 140 μM for mouse and guinea-pig brain slices (Shimizu *et al.*, 1972; Banay-Schwartz *et al.*, 1980; Davies and Hambley, 1986), and from 3.4 to 6.5 μM for primary cultures of mouse astrocytes and neurones (Hertz, 1978; Bender and Hertz, 1986). In studies of more rapid accumulation of [^3H] adenosine where incubations conducted for 15 s at 37°C produced substrate metabolism of less than 10%, both high- and low-affinity accumulation processes were found with K_T values of 0.8 and 259 μM , respectively (Geiger *et al.*, 1988). However, even under these conditions measurement of initial rates of transport was still uncertain. It has been estimated that incubations at 37°C for longer than 5 s may be inappropriate to accurately determine

initial rates of transport (Wu and Phillis, 1984). Lee and Jarvis (1988b) used incubation conditions such that adenosine was not significantly metabolized and reported K_T values of $17\ \mu\text{M}$ for guinea-pig cerebral cortex synaptosomes. In primary cultures of chick neurones depleted of ATP to inhibit AK activity, adenosine transport *per se* in neuronal cells had a reported K_T value of $13\ \mu\text{M}$ (Thampy and Barnes, 1983a). In cultured LRM55 astroglial cells, as incubation times increased from 30 s to 30 min, K_m values decreased from 1200 to $46\ \mu\text{M}$, respectively (Shain and Madelian, 1987). Thus, as with peripheral tissues and cultured cells, when accumulated adenosine is highly metabolized in nervous system tissues, K_T values will likely be lower (high affinity) and under some conditions may be approximately equal to K_m values for AK.

Alternate substrates for adenosine transporters

Due to the complex interactions between transport and metabolism, investigators have sought alternate, more metabolically stable substrates with which to study nucleoside transporters. Two such substrates have now been used: uridine, a pyrimidine nucleoside, and L-adenosine, the stereo-enantiomer of physiological D-adenosine. Importantly in terms of satisfying the criteria for transport measurements (see above), these nucleosides were not extensively metabolized under the incubation conditions used. However, because of the relatively low affinity of the transporter for uridine ($K_m\ 300\ \mu\text{M}$) (Stefanovich, 1983; Lee and Jarvis, 1988a), this substrate may be useful for the study of some, but not all adenosine transporter systems (Geiger *et al.*, 1988). Alternatively, [^3H] L-adenosine may be used as a substrate for passive and, to a far lesser extent, facilitated diffusion systems in mouse erythrocytes and L-1210 cells (Gati *et al.*, 1989). However, in contrast to the results of Gati *et al.* (1989), rat brain synaptoneurosomes accumulated [^3H] L-adenosine equally as well as [^3H] D-adenosine. Thus synaptoneurosomes do not appear to display stereoselectivity for the ribose moiety of adenosine. High micromolar ($100\ \mu\text{M}$) concentrations of NBI and D-adenosine both inhibited [^3H] L-adenosine accumulation by only about 50% (Gu *et al.*, 1991). If, as previously suggested (Gati *et al.*, 1989), the inhibitor-resistant component of [^3H] L-adenosine accumulation represents passive diffusion and because L- and D-adenosine both appear to be substrates for the same transporter in rat brain synaptoneurosomes, then even low micromolar concentrations of D-adenosine may be transported by a passive-diffusion-‘like’ process. It is therefore possible that CNS inhibitor-resistant processes, which have higher K_T values (lower affinity) for adenosine than do inhibitor-sensitive systems (Lee and Jarvis, 1988b), are mediated by passive rather than facilitated diffusion.

Table 1 Kinetic parameters for adenosine uptake in preparations of nervous system tissues

Tissue preparation	Adenosine uptake		Assay condition		Reference
	K_m (μM)	V_{\max}^a	Time (s)	Temperature ($^{\circ}\text{C}$)	
Rat					
Synaptosomes	0.9	10.5	60	37	Bender <i>et al.</i> , 1981
Synaptosomes	9	6	600	37	Premont <i>et al.</i> , 1979
Synaptosomes ($K_{T(H)}$)	1.0	1.7	900	37	Bender <i>et al.</i> , 1980
($K_{T(L)}$)	5.3	6.8	900	37	Bender <i>et al.</i> , 1980
Blood-brain barrier	18	NA	NA	NA	Cornford and Oldendorf, 1975
Capillaries	4.7	2.1	600	NA	Wu and Phillis, 1982
Dissociated cells ($K_{T(H)}$)	0.8	24.8	15	37	Geiger <i>et al.</i> , 1988
($K_{T(L)}$)	259	8868	15	37	Geiger <i>et al.</i> , 1988
Mouse					
Brain slices	140	75 ^b	3600	37	Banay-Schwartz <i>et al.</i> , 1980
Astrocytes	3.4	360	3600	37	Hertz, 1978
Astrocytes	6.5	160	1800	37	Bender and Hertz, 1986
Neurones	6.1	110	1200	37	Bender and Hertz, 1986
Endothelium	5	1150	300	37	Beck <i>et al.</i> , 1983a
Smooth muscle	10	950	300	37	Beck <i>et al.</i> , 1983b
Dissociated cells ($K_{T(H)}$)	0.34	14	15	37	Johnston and Geiger, 1990
($K_{T(L)}$)	407	2452	15	37	Johnston and Geiger, 1990
Guinea-pig					
Synaptosomes	21	2573	1–30	30	Barberis <i>et al.</i> , 1981
Synaptosomes	17 ^c	168	5	22	Lee and Jarvis, 1988b
Synaptosomes	68 ^d	366	5	22	Lee and Jarvis, 1988b
Brain slices	19	NA	600	37	Shimizu <i>et al.</i> , 1972
Dissociated cells ($K_{T(H)}$)	1.5	18.4	15	37	Johnston and Geiger, 1990
($K_{T(L)}$)	488	7168	15	37	Johnston and Geiger, 1990

Other					
<i>Torpedo</i> electric organ	2	300	1800	22	Zimmermann <i>et al.</i> , 1979
<i>Torpedo</i> electric organ	2.4	17.3	1800	22	Meunier and Morel, 1978
Astrocytoma	1.0	140	600	NA	Lewin and Bleck, 1979
LRM55 Astroglia	1200	NA	30	NA	Shain and Madelian, 1987
LRM55 Astroglia	254	NA	600	NA	Shain and Madelian, 1987
LRM55 Astroglia	46	NA	1800	NA	Shain and Madelian, 1987
Chromaffin cells	1.0	~13 ^e	60	22	Torres <i>et al.</i> , 1987
Chromaffin cells	4.6	66.7 ^e	60	37	Delicado <i>et al.</i> , 1990
Bovine microvessels	1.9	0.2	600	37	Stefanovich, 1983
Chick glia					
(+ inhibitors)	370	10 300	<25	37	Thampy and Barnes, 1983b
(- inhibitors)	12	340	600	37	Thampy and Barnes, 1983b
Chick neurones					
(+ inhibitors)	13	150	<25	37	Thampy and Barnes, 1983a
(- inhibitors)	6.4	160	<25	37	Thampy and Barnes, 1983a
Spiny lobster olfactory organ	7.1	0.6	900	22	Trapido-Rosenthal <i>et al.</i> , 1987

NA = data not available.

^a All V_{\max} values are expressed in units of pmol/mg protein/min unless otherwise indicated.

^b $\mu\text{mol/ml}$ tissue $\text{H}_2\text{O/min}$.

^c Inhibitor-sensitive nucleoside transport system.

^d Inhibitor-resistant nucleoside transport system.

^e pmol/ 10^6 cells/min.

Radioligand binding to adenosine transporters

In large part, due to the difficulties in measuring adenosine transport in CNS tissues, [^3H]NBI (Geiger and Nagy, 1990) and [^3H]DPR (Bisserbe *et al.*, 1985, 1986; Deckert *et al.*, 1987, 1988b; Marangos and Deckert, 1987) are being increasingly used as putative ligands for the study of nucleoside transport binding sites. [^3H]NBI and [^3H]DPR bind to, in most cases, a single class of high-affinity binding sites. For [^3H]NBI binding, the dissociation constants (K_d) and apparent maximal number of binding sites (B_{\max}) values have been determined in brain tissues obtained from rat, mouse, guinea-pig, dog, rabbit and human; the K_d values were relatively consistent between laboratories and across species (see Geiger and Nagy, 1990). The affinities of binding sites in brain for [^3H]DPR were at least ten-times lower and the number of sites two-times higher compared with those labelled by [^3H]NBI (Marangos *et al.*, 1985; Marangos and Deckert, 1987). Some evidence for a low-affinity [^3H]DPR-binding site has been observed (Marangos and Deckert, 1987). Moreover, [^3H]DPR may label more nucleoside transport sites than does [^3H]NBI. This may be due to the highly lipophilic nature of [^3H]DPR which allows it to label cell surface as well as intracellular transporters, because more than one molecule of [^3H]DPR binds to each nucleoside carrier, or because [^3H]DPR binds to proteins other than nucleoside transporters like, for example, glucose transporters (Torres *et al.*, 1990). [^3H]NBI either labels a smaller subset of these sites, or alternatively, [^3H]NBI sites are located only on the cell surface. A third radiolabelled nucleoside transport inhibitor, [^3H]dilazep, has now been introduced which labels both high (K_d 0.2 nM) and low (K_d 10 nM) affinity sites in S49 mouse lymphoma cells (Gati and Paterson, 1989). The high-affinity [^3H]dilazep-binding sites appear to be similar to those labelled by [^3H]NBI. [^3H]Dilazep-labelled sites in nervous system tissues have not been reported on as yet. Thus, although certain problems exist with [^3H]NBI as a label for adenosine transporters, it still represents the best ligand currently available, mainly due to findings that [^3H]DPR-binding sites have higher levels of non-specific binding (up to 50% for [^3H]DPR in comparison with about 10% for [^3H]NBI), have millimolar potency for adenosine as opposed to low micromolar values for [^3H]NBI, and have not been detectable in mouse and rat (Deckert *et al.*, 1988b).

Distribution of adenosine transporters

The CNS distributions of adenosine transporter sites labelled by [^3H]NBI and [^3H]DPR have now been successfully demonstrated both by autoradio-

graphic and membrane binding methods (Geiger and Nagy, 1984, 1985; Bisslerbe *et al.*, 1985; Geiger, 1986). High levels of [^3H]NBI sites were found in midbrain, superficial layers of the superior colliculus, area postrema, choroid plexus, lateral, third and fourth ventricles, hypothalamus, striatum, accumbens, nucleus of the solitary tract, pyriform cortex and dorsal spinal cord. Furthermore, within brain areas [^3H]NBI site densities were not evenly distributed; in thalamus higher levels of binding were found in the mediodorsal nucleus, and in hypothalamus binding in the tuberomammillary nucleus was about twice that found in more anterior portions. Although [^3H]DPR-labelled sites were greater in number and more evenly distributed throughout brain than were [^3H]NBI sites, regional differences did occur. High levels of [^3H]DPR binding were observed in the supraoptic nucleus, nucleus of the solitary tract, hypothalamus and in superficial layers of the superior colliculus. The very high levels of [^3H]DPR-binding sites in the non-neuronal ependyma cell lining of the lateral ventricles and choroid plexus suggests that these sites might be involved in the exchange of nucleosides across the blood-brain and CSF-brain barriers (Bisslerbe *et al.*, 1986). In contrast with [^3H]NBI, high levels of [^3H]DPR-labelled sites were found in the superficial layers of cerebral cortex, the interpeduncular nucleus, the central grey matter, on brainstem arteries and on cerebellar Purkinje cells in guinea-pig (Geiger and Nagy, 1984; Bisslerbe *et al.*, 1985; Deckert *et al.*, 1987).

The most worthwhile comparison, of course, would be the distribution of adenosine transporter-binding sites with that of regional adenosine transport capabilities. However, as mentioned earlier, very little is known about adenosine transport in CNS tissues and regional levels of adenosine transport have not as yet been reported. Where adenosine uptake has been examined in guinea-pig and rat brain, only small differences among brain areas were noted (Davies and Hambley, 1986; R. Shank, pers. commun.; unpublished observations). This uniformity is, however, probably not too surprising given that uptake, not transport, was being measured and that glial cells have high uptake capacities. Thus, such comparisons will only be informative once the various subtypes of adenosine transporters have been identified and the regional levels of these sites measured.

The significance of adenosine transporter distribution in the CNS in terms of understanding the actions of adenosine must await consensus as to whether all receptor and transporter subtypes are being labelled and whether markers for adenosine receptors are valid indicators of sites of adenosine action (Deckert *et al.*, 1988b; Geiger and Nagy, 1990). It is presently unclear as to whether there is a necessary anatomical and neurochemical relationship between nucleoside transport capability and all adenosine receptor-mediated events. This issue is likely to remain unresolved until some of the complexities

surrounding adenosine transport sites are better understood and comparisons are made in human brain.

Subclasses of nucleoside transporters

Nucleoside transport inhibitor-sensitive and -resistant sites

Nucleoside transport inhibitors, such as NBI and DPR, inhibit nucleoside transport with competitive, non-competitive and mixed inhibition patterns (Young and Jarvis, 1983). In addition, it is clear that tissues do, to varying degrees, express nucleoside transporters that are very resistant to the blocking actions of these inhibitors regardless of the mechanism. The concept that such sites exist, and are species-specified, originated in part from studies demonstrating the presence of NBI-resistant adenosine transporters with K_i values (micromolar) for NBI inhibition of adenosine uptake that are orders of magnitude higher than the subnanomolar K_d values for [^3H]NBI binding (Deckert *et al.*, 1988b). However, the usefulness of [^3H]NBI as a label for adenosine transporters has been bolstered by recent findings that high-affinity [^3H]NBI-binding sites are responsible for inhibitor-sensitive adenosine transport in rat and guinea-pig brain preparations (Morgan and Marangos, 1987; Geiger *et al.*, 1988; Lee and Jarvis, 1988b). NBI-resistant transporters are nevertheless blocked by DPR and other transport inhibitors (Davies and Hambley, 1986). Furthermore, subnanomolar concentrations of NBI completely inhibited the binding of [^3H]NBI but only partially inhibited the binding of [^3H]DPR (Deckert *et al.*, 1987; Marangos and Deckert, 1987) and the potencies with which NBI competed for [^3H]DPR binding varied significantly among brain regions; NBI (500 nM) inhibited [^3H]DPR binding by approximately 10% in ependyma and cerebellar Purkinje cells, but by greater than 65% in anterior hypothalamus, nucleus tractus solitarius, area postrema and brainstem arteries (Deckert *et al.*, 1987). Of the adenosine transporters in guinea-pig brain labelled by [^3H]DPR only about 40% are of the NBI-sensitive type.

The precise relationship of [^3H]NBI sites with the transporter is complicated by the presence of different types of adenosine transporters in nervous system tissues and observations that in some other tissues adenosine transporters are: (a) sensitive to inhibition by NBI and contain high levels of [^3H]NBI-binding sites; (b) less sensitive to NBI and contain few [^3H]NBI-binding sites; and (c) less sensitive to NBI but retain high numbers of [^3H]NBI-binding sites (Paterson *et al.*, 1987). These findings have contributed to the emerging concept of heterogeneous populations of adenosine transporters and, as a

result, the association of [^3H]NBI and [^3H]DPR with nucleoside transporters must be determined for each tissue where these sites are studied.

Adenosine specificity

The specificity with which endogenous purine and pyrimidine nucleosides other than adenosine are used as substrates by nucleoside transporters in the CNS is important in the development of concepts related to transport as a mechanism for maintenance of adenosine levels. If the transporters are truly selective for adenosine then these sites may be helpful in identifying those cells and neural systems involved in distinct neuroregulatory actions of this purine. Indeed, adenosine selectivity for uptake systems and [^3H]NBI-labelled transporter sites has been reported in some peripheral tissues and cultured cells (Plagemann and Wohlhueter, 1980), rat cerebral cortex synaptosomes (Bender *et al.*, 1980; Wu and Phillis, 1984), primary cultures of chick neurones (Thampy and Barnes, 1983a), bovine cerebral cortex microvessels (Stefanovich, 1983), and in rat brain dissociated cells (Geiger *et al.*, 1988). These transporters specific for adenosine have been characterized as high-affinity systems, whereas low-affinity adenosine transporters show little selectivity for adenosine over other nucleosides such as inosine and uridine (Thampy and Barnes, 1983b; Geiger *et al.*, 1988). Broader substrate specificities have been found in primary cultures of chick glia (Thampy and Barnes, 1983b) and in some peripheral tissues (Plagemann and Wohlhueter, 1980). As mentioned earlier, only at very high concentrations (K_i 1.8 mM) and with little selectivity did adenosine compete for [^3H]DPR-binding sites in guinea-pig brain (Marangos and Deckert, 1987) and thus [^3H]DPR may label a subset of non-selective nucleoside transporters. Whether neural systems that express adenosine-selective transporters may be considered wholly or in part 'purinergic' has yet to be determined.

Sodium dependence

It is perhaps not surprising, given the diversity of adenosine's actions, that different types of transporters in addition to simple facilitated diffusion systems are expressed in different cell types to maintain adenosine concentrations under physiological and pathological situations. Such precedence has already been described for other compounds, such as amino acids, glucose and succinate, which are transported across cell membranes by active sodium gradient-dependent concentrative systems in addition to equilibrative transport systems. Active, sodium-dependent concentrative nucleoside transport systems are present in renal brush border vesicles, hepatocytes, isolated intestinal epithelial cells, cultured IEC-6 intestinal epithelial cells (see Johnston and

Geiger, 1989), and in mouse spleen lymphocytes, leukaemia cells, fibroblasts and macrophages (Plagemann and Aran, 1990). The active transport systems appear to be electrogenic in that changes in membrane potential in the absence of sodium gradients provide enough energy to produce concentrative nucleoside transport (Williams *et al.*, 1989). Sodium-dependent transport has a stoichiometry of 1:1 (sodium:nucleoside) and a K_m for sodium of about 2 mM (Franco *et al.*, 1990). Subclasses of sodium-dependent nucleoside transporters that have different substrate specificities exist in the same cells (mouse intestinal epithelial cells) (Vijayalakshmi and Belt, 1988). In rat renal proximal tubule cells, in contrast to facilitated diffusion systems, sodium-dependent transporters were found to be adenosine-specific (Williams *et al.*, 1989). Where both facilitated and sodium-dependent transporters have been identified in the same tissues, the affinity of the sodium-dependent system for nucleoside substrate was over 10-times greater (Jarvis *et al.*, 1989). Depending on the tissue, sodium-dependent transport may be either a minor or a major contributor to total nucleoside transport capacities (Plagemann and Aran, 1990). Some evidence for potassium-driven nucleoside transport has appeared with a stoichiometry of 3:2 (potassium:nucleoside) (Williams *et al.*, 1989).

As early as 1978, evidence suggestive of the existence of sodium-dependent adenosine transport systems in nervous system tissues appeared. This evidence consisted of reports that adenosine accumulation in the absence of sodium was 25% less in rat cerebral cortex synaptosomes (Bender *et al.*, 1980), 15–55% less (varied with substrate concentration) in mouse brain slices (Banay-Schwartz *et al.*, 1980), and 20% less in synaptosomes of the electric organ of *Torpedo* (Meunier and Morel, 1978). We later confirmed and extended the earlier findings by showing that sodium increased adenosine accumulation by about 20% and that sodium significantly decreased the K_T in rat, but not guinea-pig or mouse, and left unchanged the V_{max} values for both the high- and low-affinity adenosine transport systems (Johnston and Geiger, 1989, 1990). These findings and those of Jarvis *et al.* (1989) for [3H]uridine transport in renal brush border vesicles are consistent with a model of transport, similar to those for glucose and succinate transport, where sodium binds first to the transporter and thereby produces allosteric modification and increased affinities of the substrate recognition sites (Johnston and Geiger, 1989). Indirect support for the existence of sodium gradient-dependent transporters in peripheral and CNS tissues is derived from results that in assay systems containing sodium, both ouabain, an inhibitor of Na^+, K^+ -ATPase, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, inhibit adenosine accumulation to levels found when assays were conducted in the absence of sodium (Bender *et al.*, 1980; Schwenk *et al.*, 1984; Johnston and Geiger, 1989). Findings that NBI and DPR were effective in inhibiting sodium-dependent adenosine accumulation in some (Johnston and Geiger, 1989; Franco *et al.*,

1990) but not all tissues (Jarvis *et al.*, 1989) has contributed to suggestions that as with the equilibrative systems, so too a family of concentrative nucleoside transporters may be expressed. The physiological significance of the sodium-dependent transporters has yet to be clearly established.

Pharmacology and therapeutic implications

In vitro and *in vivo* actions of adenosine transport inhibitors

Nucleoside transport inhibitors can be classified into four categories according to their chemical structures: purine ribosides (NBI), pyrimidopyrimidine derivatives (DPR), substituted piperazines (mioflazine, soluflazine), and tertiary amine diazepine compounds (dilazep). These compounds have been widely used in *in vivo* (Table 2) and *in vitro* (Table 3) assay systems, and have either undergone clinical trials or are presently being used clinically. DPR (2,6-bis(diethanolamino)-4,8 dipiperidinopyrimido-(5,4*d*)-pyrine) has been used clinically as a dilator of coronary and cerebral arteries, for platelet anti-aggregation, and for the treatment of certain neoplasms (Solvary *et al.*, 1984; Dawicki *et al.*, 1985; Rhodes *et al.*, 1985). Dilazep (1,4-bis-[3-(3,4,5-trimethoxybenzoyl-oxy) propyl]perhydro-1,4-diazepine), also a vasodilator, decreases coronary and total vascular resistance, and increases coronary blood flow in patients diagnosed with angina (Marzilli *et al.*, 1984). Dilazep is rapidly metabolized by ester hydrolases and thus its clinical potency is substantially reduced in the absence of hydrolase inhibitors (Ijzerman and Voorschuur, 1990). Although not used clinically, NBI (6-[(4-nitrobenzyl)-mercapto]purine ribonucleoside) has proven to be a valuable probe for nucleoside transport systems at the whole animal, *in vitro* and molecular levels. It should be mentioned that NBI is not metabolically stable; the *p*-nitrobenzyl moiety can be split from its purine base through the actions of purine nucleoside phosphorylase, the activity of which is high in brain and has been localized to glia and endothelial cells (Van Reempts *et al.*, 1988). Following such cleavage, 6-mercaptapurine with its immunosuppressive, toxic and mutagenic properties may accumulate in the animal and may interfere with pharmacological studies where NBI is chronically administered. Finally, mioflazine and soluflazine are prototypes of drugs that inhibit adenosine transport, produce vasodilation, decrease tissue damage secondary to myocardial ischaemia, cause sedation, increase the quality of sleep, and appear to be the only adenosine transport inhibitors capable of entering the brain following parenteral administration (Wauquier *et al.*, 1987).

The observed influence of adenosine transport inhibition on physiological and biochemical processes typically mediated by adenosine, some of which are listed in Tables 2 and 3, may be considered as at least indirect support for the notion that the pharmacological effects of these inhibitors are mediated through adenosine. The strength of this argument depends, of course, on the degree of certainty that the inhibitors are specific for adenosine transporters and that adenosine is principally involved in mediating the physiological response. Although adenosine transport inhibition has been shown to increase adenosine levels under certain conditions, it remains uncertain whether the physiological potentiations result directly from increased levels of adenosine. If adenosine transport inhibitors are specific for adenosine transporters then it becomes apparent from Tables 2 and 3 that the use of these inhibitors evoke a variety of physiological effects, as wide as adenosine itself, in various experimental species. For instance, under *in vivo* situations (Table 2), DPR, papaverine, lidoflazine and flunarizine all enhanced the hypoxia/ischaemia-evoked rise in blood flow in a variety of neural tissues. In conscious animal preparations, NBI, nitrobenzylthioguanosine and papaverine all diminished the animals' locomotion while NBI also enhanced morphine-induced antinociception. Under *in vitro* conditions (Table 3), DPR, NBI and R-E 244 all diminished the release of neurotransmitters such as acetylcholine, GABA, noradrenaline and serotonin; DPR, NBI, dilazep, papaverine and hexobendine enhanced adenosine-induced cAMP accumulations; and DPR, NBI and hexobendine either decreased field potentials or increased adenosine-evoked inhibitions of endplate potentials. In many of these studies, transport inhibitors by themselves, even in high doses did not produce measurable changes. Thus, despite the limitations associated with the use of adenosine transport inhibitors, these agents may be useful in potentiating the actions of adenosine, especially at locations where purine nucleoside levels are rising either as a result of increased neuronal activity or because of decreased cellular energy charge, i.e. in a site- and event-specific manner.

Effects of adenosine receptor agonists on adenosine transporters

Adenosine receptor agonists interact with adenosine transporters. The adenosine receptor agonists 2-chloroadenosine (CADO), cyclohexyladenosine (CHA), R-phenylisopropyladenosine (R-PIA) and to a lesser extent *N*-ethylcarboxyamidoadenosine (NECA) compete for [^3H]NBI and [^3H]DPR-labelled transport sites with K_i values in the low micromolar range (Geiger *et al.*, 1985; Marangos and Deckert, 1987). R-PIA, CHA and cyclopentyladenosine, relatively selective adenosine A_1 receptor agonists, inhibited the accumulations of [^3H]adenosine in rat retinal cells and in LRM55 astroglial cells (Schaeffer and Anderson, 1981; Shain and Madelian, 1987).

Table 2 Pharmacological effects of adenosine transport inhibitors *in vivo*

Nucleoside transport inhibitor	Dose	Response	Species	Reference
DPR	100 mg QID	↑ headaches	Human	Hawkes, 1978
	0.25 mg/kg	(-) seizure	Mouse	Morgan <i>et al.</i> , 1989
	5 mg/kg	↑ immobility	Mouse	Kulkarni and Mehta, 1985
	1–2 mg/kg	↓ neuronal firing	Cat	Salter and Henry, 1987
	0.25 mg/kg	↑ blood flow	Rabbit	Puiroud <i>et al.</i> , 1988
	20 μ M/min	↑ blood flow	Rabbit	Heistad <i>et al.</i> , 1981
	500 μ g/kg	↑ blood flow	Rat	Phillis <i>et al.</i> , 1989
	10 mg/kg	↓ locomotion	Mouse	Crawley <i>et al.</i> , 1983
	0.25 mg/kg	↓ seizures	Mouse	Morgan <i>et al.</i> , 1989
	25 nmol i.t.	↑ antinociception	Mouse	DeLander and Hopkins, 1987
NBG	10 mg/kg	↓ locomotion	Mouse	Crawley <i>et al.</i> , 1983
Papaverine	32 μ g i.c.v.	↓ locomotion	Mouse	Coffin <i>et al.</i> , 1984
	25–50 mg/kg	↓ seizure	Rat	Dragunow <i>et al.</i> , 1985
	25 mg/kg	↓ kindling	Rat	Dragunow and Goddard, 1984
	1 mg/kg	↑ blood flow	Rat	Phillis <i>et al.</i> , 1984
	200 mg	↑ blood flow	Human	Jayne <i>et al.</i> , 1952
Dilazep	50 mg/kg	↓ locomotion	Rat	Clark and Dar, 1988
Lidoflazine	0.01 and 0.05 mg/kg	↑ blood flow	Rat	Phillis <i>et al.</i> , 1985
Flunarizine	0.05 and 0.1 mg/kg	↑ blood flow	Rat	Phillis <i>et al.</i> , 1985
R75231	100 μ g/kg	↓ ventricular fibrillation	Pig	Wainwright <i>et al.</i> , 1990

Table 3 Pharmacological effects of adenosine transport inhibitors *in vitro*

Nucleoside transport inhibitor	Dose (μM)	Response	Species	Tissue	Reference
DPR	4	↓ ACh release	Rat	Cortex	Pedata <i>et al.</i> , 1983
	10	↓ ACh release	Rat	Cortex	Pedata <i>et al.</i> , 1984
	10	↓ GABA release	Rat	Cortex	Hollins and Stone, 1980b
	50	↓ field potentials	Rat	Cortex	Motley and Collins, 1983
	50	↑ cAMP	Rat	Cortex	Motley and Collins, 1983
	50	(–) EAA release	Rat	Cortex	Motley and Collins, 1983
	25	↓ EAA release	Rat	Hippocampus	Burke and Nadler, 1988
	25	(–) GABA release	Rat	Hippocampus	Burke and Nadler, 1988
	3	↑ cAMP	Rat	Hippocampus	Fredholm <i>et al.</i> , 1982
	100	(–) ganglionic transmission	Rat	SCG	Alkadhi <i>et al.</i> , 1984
	3	↑ cAMP	GP	Brain slices	York and Davies, 1982

NBI	5	↓ endplate potentials	Rat	Neuromuscular junction	Sebastiao and Ribeiro, 1988
	1	↓ EPSP	Rat	Hippocampus	Haas and Greene, 1988
	10–50	↓ seizures	Human	Cortex	Kostopoulos <i>et al.</i> , 1989
NBG	1	↓ NA release	Rat	Caudal artery	Shinozuka <i>et al.</i> , 1988
Dilazep	3	↑ cAMP	Rat	Hippocampus	Fredholm <i>et al.</i> , 1982
	3	↑ cAMP	GP	Brain slices	York and Davies, 1982
Papaverine	3	↑ cAMP	Rat	Hippocampus	Fredholm <i>et al.</i> , 1982
	200	↑ cAMP	GP	Brain slices	York and Davies, 1982
Hexobendine	3	↑ cAMP	Rat	Hippocampus	Fredholm <i>et al.</i> , 1982
	2	↑ cAMP	GP	Brain slices	York and Davies, 1982
	25	↓ field potentials	Rat	Hippocampus	Dunwiddie and Hoffer, 1980
Inosine	100	↑ vasodilation	Rat	Pial vessels	Ngai <i>et al.</i> , 1989
R-E 244	3	↓ 5-HT release	Rabbit	Hippocampus	Feuerstein <i>et al.</i> , 1985

EAA = excitatory amino acids.

GP = guinea-pig.

CV-1808 (2-phenylaminoadenosine), a relatively selective adenosine A₂ receptor agonist, inhibited [³H]uridine uptake into guinea-pig erythrocytes (Balwierczak *et al.*, 1989). CADO, a relatively non-selective adenosine receptor agonist, inhibited adenosine uptake into cerebral smooth muscle cells (Beck *et al.*, 1983b), cultured cerebral endothelial cells (Beck *et al.*, 1983a), and in rat renal brush border vesicles (Belloni *et al.*, 1987; Franco *et al.*, 1990). In contrast, NECA at low concentrations has been found to increase adenosine transport in dissociated brain cells (Geiger *et al.*, 1988) and cultured chromaffin cells (Delicado *et al.*, 1990). Although it is presently unclear as to whether all the analogues in addition to inhibiting transport also are substrates for the transporter, it has been shown that, for example, R-PIA can be accumulated by rabbit retinal cells by an NBI-sensitive nucleoside transporter (Blazynski, 1991), and that CADO can be carried across plasma membranes by NBI-sensitive nucleoside transport systems in human erythrocytes (Jarvis *et al.*, 1985) and guinea-pig myocytes (Heaton and Clanachan, 1987). However, in contrast, although R-PIA was observed to equilibrate rapidly across plasma membranes of Novikoff rat hepatoma cells, this process was not carrier-mediated and was not blocked by papaverine, DPR or uridine (Plagemann and Wohlhueter, 1984). As a consequence of this increasing body of evidence, it is imperative that investigators consider the possibility that at least part of the biological actions of adenosine analogues are due to interactions with adenosine transport processes.

Do nucleoside transport inhibitors block adenosine release?

Recent evidence suggests that a close relationship may exist between transport and release processes for a variety of neuro-active substances (Fillenz, 1984; Woodward *et al.*, 1988). In the case of adenosine, increased extracellular levels as a consequence of depolarizing stimuli and hypoxia may originate as adenosine *per se* or alternatively as released adenine nucleotides following their metabolism by ectonucleotidases. For adenosine transporters in neural tissue that are bidirectional and equilibrative, adenosine efflux out of cells can only occur if intracellular adenosine levels rise to sufficiently high levels so as to cause it to flow down its concentration gradient. Evidence for the efflux of adenosine through its transporter in neural tissues comes only indirectly from findings that nucleoside transport inhibitors including dilazep, DPR and NBI inhibit the basal release of adenosine from rat cerebellar synaptosomes (Clark and Dar, 1989), murine neuroblastoma cells deficient in AK (Green, 1980), primary cultures of rat glial cells (Caciagli *et al.*, 1988) and in primary cultures of chick neurones and glia (Meghji *et al.*, 1989). Moreover, these same inhibitors decreased the evoked release of adenosine from purified cholinergic nerve terminals of rat caudate nucleus (Richardson and Brown, 1987),

cultured sympathetic neurones (Tolkovsky and Suidan, 1987), rat hippocampal and hypothalamic slices (Fredholm and Jonzon, 1981; Jonzon and Fredholm, 1985), and rabbit retina (Perez *et al.*, 1986). In contrast, indirect evidence suggests equally strongly that purines are not released through transporters; DPR, papaverine and NBI increased (Heller and McIlwain, 1973; Green, 1980; Daval and Barberis, 1981; McIlwain and Pull, 1986; Haas and Greene, 1988; Phillis *et al.*, 1989) and dilazep, DPR and solufazine failed to affect (Hollins and Stone, 1980a; Daval and Barberis, 1981; Fredholm and Jonzon, 1981; Jonzon and Fredholm, 1985; Caciagli *et al.*, 1988; Clark and Dar, 1989; Phillis *et al.*, 1989) the release of purines from various CNS preparations. Thus, the literature is roughly equally divided as to the effectiveness of nucleoside transport inhibitors in affecting the release of adenosine. In attempting to interpret these findings it is important to note that the conditions under which the release measurements were made probably determined to a large extent whether adenosine or adenine nucleotides were released. Situations where the transport inhibitors were not effective in inhibiting release or caused increases in extracellular adenosine has, under certain circumstances, been interpreted as evidence for release and uptake occurring from separate cellular compartments (Phillis *et al.*, 1989). As the biochemical characteristics and locations of adenosine transport and release sites become clearer, so too will be possible functional relationships between them.

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CHAPTER 2

ADENOSINE PRODUCTION AND METABOLISM

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Adenosine levels in the brain increase when the supply:demand ratio for oxygen decreases in many instances such as ischaemia, hypoxia and hypoperfusion (Rubio *et al.*, 1975; Winn *et al.*, 1980; Van Wylen *et al.*, 1986). Adenosine produces vasodilation in most vascular beds, suggesting that it plays a role in the local regulation of blood flow (Berne *et al.*, 1981). In addition, adenosine has a variety of other actions in the nervous system, including suppression of spontaneous neuronal firing, inhibition of synaptic transmission and transmitter release (Phillis and Kostopoulos, 1975; Harms *et al.*, 1979; Fredholm and Hedqvist, 1980; Hollins and Stone, 1980; Fredholm and Dunwiddie, 1988). These effects of adenosine may modulate synaptic transmission both presynaptically and postsynaptically. Adenosine levels in the brain are elevated during seizures and it has been postulated that adenosine

may have a protective role in seizures and post-ischaemic neural damage in brain by inhibiting release of excitatory amino acids (Dragunow and Faull, 1988). The rate of adenosine formation rises during periods of increased neuronal activity (Winn *et al.*, 1980; Jonzon and Fredholm, 1985). Adenosine is a more potent inhibitor of excitatory than inhibitory neurotransmitter release (Duner-Engstrom and Fredholm, 1988). Adenosine has been implicated in sedation, antinociception and analgesia. All these effects of adenosine fit into an overall protective role in re-establishing energy balance and thus adenosine has been described as a 'retaliatory metabolite' (Newby, 1984; Newby *et al.*, 1990). Adenosine also has a more restricted role as a neuromodulator.

Although ATP can be catabolized rapidly to adenosine by ectonucleotidases (Pearson *et al.*, 1980; Maire *et al.*, 1984; Grondal and Zimmermann, 1986), many of its effects which are due to P₂ purinoceptors, are distinct from those of adenosine. Adenosine produces vasodilation in most vascular beds, including the brain, acting via A₂ receptors. In contrast ATP-induced vasodilation is mediated via prostacyclin and endothelium-derived relaxing factor released by ATP action on P_{2Y} receptors on endothelial cells (Olsson and Pearson, 1990). Topically applied ATP produced vasodilation in the pial arteries of the cat at concentrations as low as 0.1 nM (Forrester *et al.*, 1979). ATP also selectively activates dorsal horn and dorsal root ganglion neurones, an effect which is not mediated by adenosine receptors (Jahr and Jessell, 1983).

Adenosine production

Adenosine is formed by degradation of AMP. Consistent with this, electric shock produced a dramatic increase in AMP content of rat brain which was followed by a rise in adenosine content (Schultz and Lowenstein, 1978). 5'-Nucleotidase enzymes exist in two different cytosolic forms and as a membrane-bound ecto-enzyme. A comparison of the kinetic properties of ecto-5'-nucleotidase and cytosolic 5'-nucleotidases is shown in Table 1. It is clear that there are potentially two distinct sources of extracellular adenosine depending on the metabolic route followed (Fig. 1). Adenosine formation may be linked to the balance between energy supply and demand and thus formation and degradation of cytoplasmic ATP, and hence only be indirectly related to neurotransmission. Adenosine formed by this route must then exit the cell to reach receptor sites which are generally extracellular. It can then act on the adenosine-forming cells, neighbouring neurones or other cells, including the cerebral vasculature, to restore energy balance. Alternatively, adenosine formation may be directly linked to neurotransmission, probably

Table 1 Comparison of the kinetic parameters of three 5'-nucleotidases

	Plasma membrane enzyme	AMP-specific cytosolic enzyme	IMP-specific cytosolic enzyme
Structure and size	Homodimer 148 kDa	150 kDa	Allosteric protein with four subunits 200 kDa
AMP	K_m 5–40	K_m 1200–5200	K_m 3000–14 000
IMP	K_m 8	K_m 800–20 000	K_m 100–1200
ATP	K_i 1.5–4.4	Activator K_{app} 150	ATP > ADP activates in the millimolar range
ADP	K_i 0.08	Activator K_{app} 50–90	ADP < ATP activates in the millimolar range
Mg ²⁺	Not required	Required	Required
AOPCP	K_i 0.006	No effect	No effect
Antibody to ecto-5'-nucleotidase	Complete inhibition	No inhibition	No inhibition
P _i	Not inhibited	10% inhibition by 10 mM P _i	> 90% inhibition by 5 mM P _i
pH optimum	7.5	7.0	6.5

All K values are μM ; P_i, orthophosphate; AOPCP, α,β -methylene ADP. Data from references in text.

by release of adenine nucleotides together with other transmitters by exocytosis followed by ectonucleotidase activity. The adenosine thus formed then acts directly at extracellular receptors to modulate the activity of the neurones from which it arises, as well as neighbouring neurones.

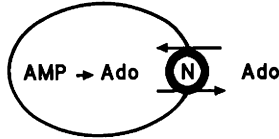
Ecto-5'-nucleotidase

Ecto-5'-nucleotidase is a homodimer linked to the plasma membrane through a glycosyl-phosphatidylinositol lipid anchor, with its active site facing outside the cell (Stanley *et al.*, 1980; Baron *et al.*, 1986; Bailyes *et al.*, 1990). It is internalized by endocytosis and is present on the extra-cytoplasmic face of

INTRACELLULAR

All Cells

↓ energy supply or ↑ energy demand = ATP catabolism



EXTRACELLULAR

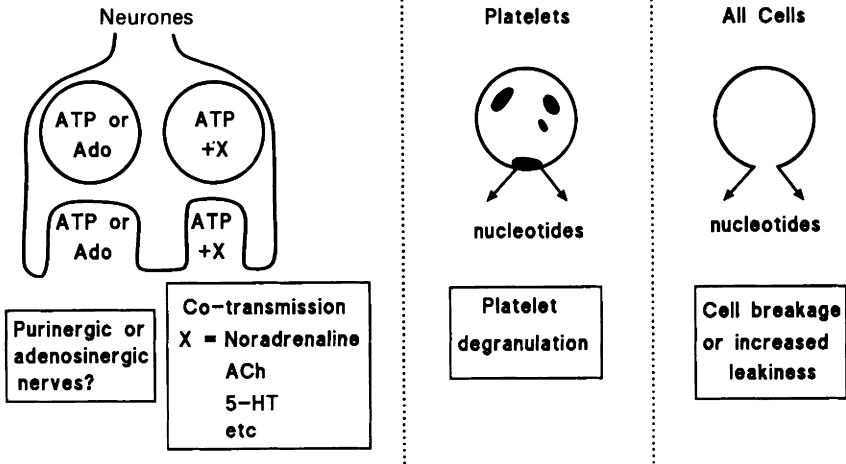


Figure 1 Sources of extracellular adenosine. Ado, adenosine; N, nucleoside transporter.

the vesicles (Stanley *et al.*, 1980). Recently, Misumi *et al.* (1990) have cloned and sequenced the cDNA of rat liver ecto-5'-nucleotidase. These authors have also confirmed the extracellular location of the catalytic site of this enzyme. Thus, this enzyme is inaccessible to cytoplasmic nucleotides. Ecto-5'-nucleotidase in sheep brain, chick glial and neural preparations, like ecto-5'-nucleotidase in other tissues, is inhibited by ADP, ATP and by α,β -methylene ADP (AOPCP) (Burger and Lowenstein, 1975; Meghji *et al.*, 1989). This enzyme has a preference for AMP over IMP (Naito and Lowenstein, 1981) and has a K_m for AMP in the micromolar range. Even if ecto-5'-nucleotidase had access to intracellular nucleotides it would be most strongly inhibited when adenosine is being produced, since levels of ADP (which is a more potent inhibitor than ATP) rise during hypoxia and ischaemia. A 'cytosolic'

5'-nucleotidase which is inhibitable by nucleotides and AOPCP has been detected in bovine brain (Mallol and Bozal, 1983). It is likely, however, that this activity is due to contamination of the cytosolic fraction with cell membranes (Meghji *et al.*, 1988a) or is a result of release of ecto-5'-nucleotidase by phospholipase C (Baillyes *et al.*, 1990).

Inhibition of ecto-5'-nucleotidase by AOPCP did not reduce adenosine production or release by amphibian ganglia (Rubio *et al.*, 1988), brain slices (Pons *et al.*, 1980), synaptosomes (Daval and Barberis, 1981), rabbit non-myelinated vagus nerve preparation (Maire *et al.*, 1984) and embryonic chick neural and glial cultures (Meghji *et al.*, 1989), ruling out the involvement of the ecto-enzyme in adenosine production. Since the active site of this enzyme faces the interstitial space these studies also provide evidence for an intracellular site of adenosine formation. The demonstration of adenosine release from rabbit vagus nerve preparation (Maire *et al.*, 1984) and from glial cells (Meghji *et al.*, 1989) shows that release of adenosine is not restricted to nerve endings.

This does not rule out the possibility that adenine nucleotides may also be released from neurones. ATP may be released by itself, from postulated purinergic neurones (Burnstock, 1972) or as a co-transmitter together with other transmitters (Burnstock, 1986a). Quinacrine, a fluorescent compound which binds to ATP, has been used to localize postulated purinergic nerves in a variety of tissues including gut, bladder and brain (Burnstock *et al.*, 1978, 1979; Crowe and Burnstock, 1984). The use of quinacrine has been criticized as its binding is non-specific (Phillis and Wu, 1981). However, evidence other than quinacrine staining has accumulated for purinergic transmission in the peripheral nervous system (Burnstock, 1986b).

Non-neural cells may also release purines when electrically stimulated. Using tetrodotoxin it was demonstrated that only 30–50% of the electrically evoked release of radiolabelled compounds from the superfused taenia coli (Rutherford and Burnstock, 1978), cerebral cortical slices (Di Iorio *et al.*, 1988) and guinea-pig cerebral tissues is neuronal in origin (Pull and McIlwain, 1973). The possibility that the remainder of the release was due to increased glycolysis was excluded in one study only (Pull and McIlwain, 1973). If nucleotides rather than adenosine are released from non-neural cells then the mechanism of release is not known.

ATP is certainly stored with other transmitters such as noradrenaline, acetylcholine and 5-HT. Release of nucleotides has been demonstrated from nerve terminals (Holton, 1959; Silinsky, 1975; Potter and White, 1980; Lew and White, 1987). In a recent study, the release into interstitial fluid of one pool of adenosine and AMP from guinea-pig perfused hearts was inhibited by both AOPCP and chemical sympathectomy, suggesting that the AMP was produced from ATP released from sympathetic nerves (Imai *et al.*, 1989). In another study, inhibitory antibodies of 5'-nucleotidase decreased the formation

of adenosine from ATP released by depolarization of purified rat striatal cholinergic synaptosomes and also prevented the inhibitory effect of ATP on ACh release (Richardson *et al.*, 1987). However, cortical terminals, which are autoinhibited by acetylcholine but not by adenosine (Marchi *et al.*, 1983), lack both presynaptic adenosine receptors and ectonucleotidases (Richardson *et al.*, 1987), implying a role for ecto-5'-nucleotidase in adenosine formation and responsiveness. ATP is stored with 5-HT in platelet granules and is released during platelet activation. Vascular endothelial cells have been shown to release ATP *in vitro* when treated with thrombin or trypsin by as yet unexplained mechanisms (Pearson and Gordon, 1979). Cytosolic ATP is present at concentrations of approximately 5 mM, this may also be released during tissue damage. However released, ATP is rapidly hydrolysed by an ecto-enzyme cascade including 5'-nucleotidase (Stefanovic *et al.*, 1976; Pearson *et al.*, 1980; Maire *et al.*, 1984), and in this way ectonucleotidases may play a role in adenosine production and neuromodulation.

Ecto-5'-nucleotidase is widely used as a plasma membrane marker; nonetheless, the distribution of 5'-nucleotidase in the brain is heterogeneous. In cytochemical studies at the electron microscope level, ecto-5'-nucleotidase activity was found to be associated with plasma membrane of glial cells and astrocytic processes with ecto-5'-nucleotidase frequently found to engulf synaptic complexes which could therefore metabolize nucleotides released from synaptic terminals (Schubert *et al.*, 1983). 5'-Nucleotidase has been found to be predominantly located on glial cells (Schoen *et al.*, 1987) with some activity on neuronal sites (Kreutzberg *et al.*, 1986; Nagata *et al.*, 1984). 5'-Nucleotidase in *Torpedo* electric organ is associated with Schwann cell membranes along the entire length of the axon (Grondal *et al.*, 1988). In contrast, ATPase is not associated with these membranes. Electron microscopic studies show that the axon, the terminal axon region or the postsynaptic membrane contain ATPase but not 5'-nucleotidase (Grondal *et al.*, 1988). The absence of ecto-5'-nucleotidase from nerve terminals was further confirmed in a complement-fixation assay using antiserum to 5'-nucleotidase in cholinergic neurone terminals (Richardson, 1983). The distribution of ecto-5'-nucleotidase appears to be non-neuronal.

Schubert *et al.* (1979) have shown a correlation between ectonucleotidase localization and transneuronal transport of radioactive adenine derivatives in the rat hippocampus. A correlation has been demonstrated between highest densities of ecto-5'-nucleotidase and adenosine A₁ receptors in the rat brain (Schubert *et al.*, 1983; Snyder, 1985). However, exceptions were found where high binding was found in areas of low enzyme activity (Schubert *et al.*, 1983), and this correlation is still controversial. A study on the brains of the rat, mouse, guinea-pig and cat reported a poor correlation between the distribution of A₁ adenosine receptors and ecto-5'-nucleotidase and a marked difference

in species distribution of the enzyme (Fastbom *et al.*, 1987). It may be that although adenosine has been demonstrated to be a presynaptic and postsynaptic neuromodulator, in some instances ATP acts directly on the postsynaptic cell and that ectonucleotidases may function only to terminate the physiological action of ATP. In any case the absence of a tight correlation between A_1 receptors and ecto-5'-nucleotidase is not surprising since adenosine formation is not restricted to this enzyme.

Cytosolic 5'-nucleotidases

Degradation of ATP during increased metabolic activity results in a rise in intracellular AMP concentration. Since adenylate kinase catalyses a near-equilibrium reaction and the initial concentration of ATP is approximately 50 times higher than that of AMP, a small percentage fall in ATP concentration can lead to a large rise in AMP concentration. AMP may then be further degraded to adenosine via cytosolic 5'-nucleotidases, of which two have been characterized. The K_m values of these enzymes, termed AMP- and IMP-specific 5'-nucleotidases, for AMP are similar and are much higher than the likely concentrations of AMP (0.1–0.5 mM) in the cytoplasm. Both enzymes can therefore respond to an increase in AMP concentration with a proportional rise in the rate of adenosine production, thus providing a direct link between ATP breakdown and adenosine formation (Worku and Newby, 1983; Newby, 1984) and correcting any mismatch between energy demand and supply.

The nucleoside transporter is essential to facilitate diffusion of cytoplasmically formed adenosine to its receptor sites on the cell in which it is formed and neighbouring cells (Fig. 2). Inhibition of the symmetric nucleoside transporter in a variety of preparations including hypothalamic synaptosomes, hippocampal slices and embryonic chick neural and glial cultures (Fredholm *et al.*, 1980, 1983; Jonzon and Fredholm, 1985; Meghji *et al.*, 1989), has resulted in a decrease in release of adenosine and an increase in intracellular accumulation of adenosine consistent with an obligatory role of the nucleoside transporter in adenosine efflux. These studies also establish without doubt the existence of an intracellular location of adenosine formation.

IMP-specific cytosolic 5'-nucleotidase

An IMP-specific cytosolic 5'-nucleotidase has been characterized from a variety of tissues. It is an allosteric protein with four subunits (Itoh, 1982) which hydrolyses AMP (K_m 3–14 mM) but IMP is the preferred substrate (K_m 0.1–1.2 mM) (Van den Berghe, 1977; Itoh, 1981a; Itoh *et al.*, 1986). It is activated by Mg^{2+} , high ionic strength, ATP more potently than ADP and is inhibited by P_i in the millimolar range (Itoh, 1981a,b; Lowenstein *et al.*,

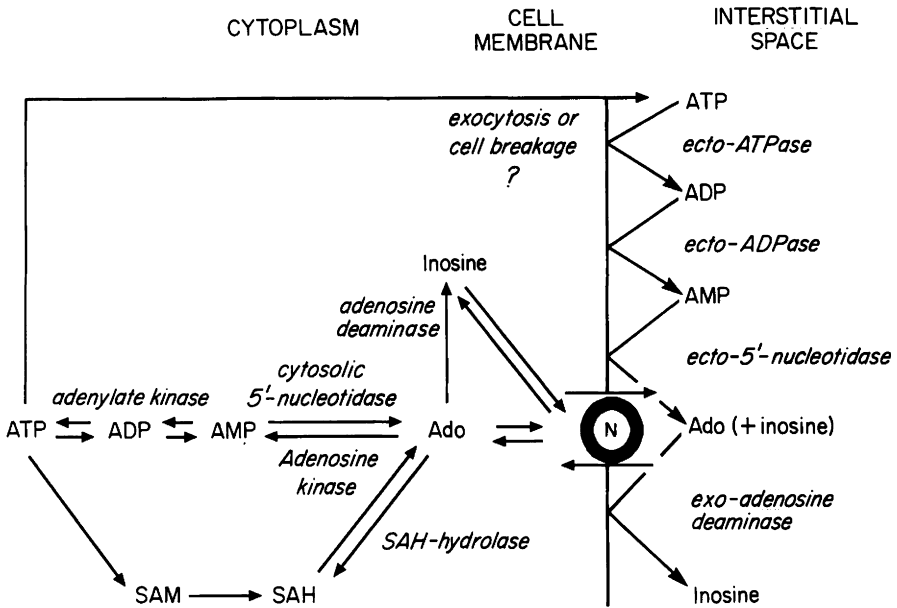


Figure 2 Pathways of adenosine formation and metabolism. Ado, adenosine; SAH, S-adenosylhomocysteine; SAH-hydrolase, S-adenosylhomocysteine-hydrolase; SAM, S-adenosylmethionine; N, nucleoside transporter.

1983). The IMP-specific enzyme is therefore maximally active under resting conditions in the cytoplasm and is inhibited during ischaemia and hypoxia when P_i levels rise (Itoh, 1981b; Itoh *et al.*, 1986). In addition, IMP would competitively inhibit hydrolysis of AMP. Calculations indicate that in the heart the activity of this enzyme towards AMP is probably insufficient to account for the ischaemia-induced adenosine formation (Meghji *et al.*, 1988a). However, an imbalance in energy supply and demand results in a reduction in energy charge $[ATP + 1/2ADP]/[ATP + ADP + AMP]$, and this reduction in energy charge has been shown to activate the IMP-specific cytosolic enzyme, suggesting that it may play a role in adenosine production (Itoh, 1981b; Worku and Newby, 1983; Itoh *et al.*, 1986). Indeed, in polymorphonuclear leucocytes the activity of the IMP-specific enzyme is sufficient to account for the rate of adenosine production observed (Worku and Newby, 1983). Furthermore, the rate of adenosine production is decreased by inhibition of the enzyme in intact cells with 5'-deoxy-5'-isobutylthioadenosine or 5'-deoxy-5'-isobutylthioinosine (Skladanowski *et al.*, 1989).

AMP-specific cytosolic 5'-nucleotidase

A soluble 5'-nucleotidase which is 15–20-fold more active with AMP than with IMP has recently been identified in pigeon (Newby, 1988), rat (Truong *et al.*, 1988), and rabbit hearts (Collinson *et al.*, 1987). This enzyme is activated by ADP but not by ATP at physiological concentrations of AMP (Yamazaki *et al.*, 1989; Skladanowski and Newby, 1990) and is not inhibited by AOPCP (Newby *et al.*, 1987; Newby, 1988; Skladanowski and Newby, 1990). It is less potently inhibited by P_i than is the IMP-specific enzyme (Itoh *et al.*, 1986; Newby, 1988; Truong *et al.*, 1988). These properties, in comparison with the IMP-specific enzyme, suggest that the AMP-specific enzyme is activated to a greater extent during ATP catabolism. Interestingly, the pigeon heart, which lacks ecto-5'-nucleotidase but has an active AMP-specific cytosolic 5'-nucleotidase, is able to produce adenosine (Meghji *et al.*, 1988a; Newby, 1988).

Both AMP- and IMP-specific enzymes are present in rat heart (Meghji *et al.*, 1988a; Newby, 1988; Truong *et al.*, 1988). However, rat polymorphonuclear leucocytes only contain the IMP-specific enzyme (Skladanowski *et al.*, 1989) and the pigeon heart only the AMP-specific enzyme (Skladanowski and Newby, 1990). Most of the studies on mechanisms of adenosine formation have concentrated on cardiac tissues and very little information is available on the cytosolic 5'-nucleotidases in neural tissues. The presence of the IMP-specific enzyme, however, has been demonstrated in bovine and rat brain (Montero and Fes, 1982; Newby *et al.*, 1987; Bontemps *et al.*, 1989). It remains to be seen if the AMP-specific enzyme is also present in the brain and whether the activities of these cytosolic enzymes are sufficient to account for the observed rates of adenosine formation.

S-Adenosylhomocysteine hydrolase

Production of adenosine may also occur by hydrolysis of S-adenosylhomocysteine (SAH) (Fig. 2). SAH is formed from S-adenosylmethionine (SAM) in the course of the transmethylation pathway by the action of SAH-hydrolase. This enzyme has been immunocytochemically localized in the neocortex, hippocampal formation, cerebellum and olfactory tubercle of rat brain (Patel and Tudball, 1986). The involvement of SAH-hydrolase in adenosine formation has not been tested in the brain. In well-oxygenated hearts, a substantial proportion of adenosine is derived from hydrolysis of SAH (Lloyd *et al.*, 1988). However, during hypoxia and ischaemia this pathway, which is oxygen-insensitive, is not accelerated and the major metabolic route is breakdown of ATP to AMP (Lloyd *et al.*, 1988).

Adenosinergic neurones

It has recently been suggested that adenosine may be stored in neuronal cells. Using specific antisera against adenosine, neuronal cells have been localized in rat brain and retina of various species (Braas *et al.*, 1986, 1987) and it has been suggested that adenosine may be a neurotransmitter. It is not clear yet whether this adenosine is stored in vesicles or which stimuli are able to release it. Hypoxia increased the intensity of staining although it did not alter the pattern of staining (Braas *et al.*, 1986). The possibility that the cells being stained are ones that are especially sensitive to hypoxia cannot be completely ruled out.

Multiple pathways

More than one mechanism of adenosine formation may operate under different conditions. This has been demonstrated for synaptosomes where inhibition of ecto-5'-nucleotidase has shown that most of the basal accumulation of extrasynaptosomal adenosine is derived from released nucleotides, probably ATP (MacDonald and White, 1985). However, about half of the veratridine-evoked accumulation of adenosine and most of the K^+ -evoked extrasynaptosomal adenosine is adenosine release *per se* rather than production from released nucleotides (MacDonald and White, 1985).

Adenosine inactivation

Adenosine must be taken up by neurones or neighbouring cells before it is inactivated either by phosphorylation by adenosine kinase or deamination by adenosine deaminase (ADA), since the location of both these enzymes is usually cytosolic (Fig. 2).

Nucleoside transport

The importance of adenosine uptake in its inactivation is emphasized by observations that potent nucleoside transport inhibitors produce vasodilation, potentiate the ability of adenosine to decrease locomotor activity (Crawley *et al.*, 1983; Sanderson and Scholfield, 1986), depress neuronal activity (Motley and Collins, 1983), increase nociceptive thresholds in animals (Yarbrough and McGuffin-Clineschmidt, 1981), exert anticonvulsant effects

(Dragunow and Goddard, 1984) and induce sleep in dogs (Wauquier *et al.*, 1987).

Adenosine is thought to be taken up into cells by a facilitated diffusion process which is largely regulated by the concentration gradient for adenosine. It is non-concentrative and exhibits directional symmetry (Plagemann and Wohlhueter, 1983). Using isolated cells in culture, nucleoside transport systems have been shown both in glia and neurones (Bender and Hertz, 1986; Meghji *et al.*, 1989).

In an effort to identify adenosine-forming and -inactivating regions of the brain, binding studies have been carried out using tritiated nitrobenzylthioinosine (NBI), a nucleoside transport inhibitor. Studies have, however, been complicated by the finding that both NBI-sensitive and NBI-insensitive sites exist in a number of cell lines (Belt, 1983; Plagemann and Wohlhueter, 1984), guinea-pig brain (Marangos and Deckert, 1987), dog brain (Deckert *et al.*, 1988) and rat cerebral-cortical synaptosomes (Lee and Jarvis, 1988). Both transport components are sensitive to inhibition by dipyrindamole, another nucleoside transport inhibitor (Plagemann and Wohlhueter, 1984; Lee and Jarvis, 1988). Plagemann and Wohlhueter (1984) have suggested that a single transporter molecule can exist in a NBI-sensitive or -insensitive form depending on its orientation in the plasma membrane. The sensitive form may be made up of the substrate-binding site plus a hydrophobic region which interacts with the lipophilic nitrobenzyl group of NBI, the latter site being inaccessible in the NBI-insensitive transporters. These results suggest that NBI binding may not be a good marker and that a lack of high-affinity NBI-binding sites on cells does not prove the absence of functional nucleoside transporters. Certain cell lines that lack high-affinity [^3H]NBI binding transport nucleosides as efficiently as cells that possess high-affinity binding sites (Belt, 1983; Plagemann and Wohlhueter, 1984). Furthermore, only 40% of the transport sites in guinea-pig brain (Marangos and Deckert, 1987) and rat cerebral-cortical synaptosomes (Lee and Jarvis, 1988) are NBI-sensitive, and so the use of NBI binding alone as a marker for nucleoside transport in brain is likely to be misleading.

Sites labelled by [^3H]NBI show a heterogeneous distribution in the brain (Geiger and Nagy, 1984; Bisserbe *et al.*, 1985; Deckert *et al.*, 1988). Attempts have been made to correlate adenosine inactivation sites with adenosine receptor sites. [^3H]NBI-binding sites in rat (Bisserbe *et al.*, 1985) or guinea-pig brain (Deckert *et al.*, 1988) did not correlate well with the distribution of A_1 receptors. Use of [^3H]dipyridamole as an additional ligand probe for adenosine uptake sites revealed more transport sites but only partly improved the correlation between A_1 adenosine receptor sites and nucleoside transport sites (Deckert *et al.*, 1988).

Adenosine kinase

Once taken up, adenosine may be phosphorylated by adenosine kinase or deaminated by ADA. The K_m values of adenosine kinase for adenosine range from <0.4 to $10\ \mu\text{M}$ whereas the values of ADA for adenosine range from 7 to $54\ \mu\text{M}$ in a variety of preparations including brain and brain neuronal cultures (Arch and Newsholme, 1978; Phillips and Newsholme, 1979; Brosh *et al.*, 1990). In most preparations, the values of K_m of adenosine kinase for adenosine are between one and two orders of magnitude lower than those for the deaminase (Arch and Newsholme, 1978). The rates of phosphorylation of adenosine are therefore maximal in the micromolar range and they decrease with further increase in adenosine concentration (Fisher and Newsholme, 1984). It is likely in general, therefore, that adenosine is phosphorylated at low adenosine concentrations and that deamination is the more important pathway at high adenosine concentrations. Consistent with this, adenosine (0.5 – $17\ \mu\text{M}$) is converted mainly to nucleotides in brain slices, synaptosomes or cultured neurones (Kuroda and McIlwain, 1974; Barberis *et al.*, 1981; Reddington and Pusch, 1983; Wolinsky and Patterson, 1985). The distribution and properties of brain adenosine kinase in various species have been extensively studied (Arch and Newsholme, 1978; Phillips and Newsholme, 1979). This enzyme has a cytosolic location. There were no marked differences in the activities of crude homogenates from cerebral cortex, cerebellum and hind brain of the rat brain nor was there any marked difference in activities measured in nine different areas of human brain.

Matz and Hertz (1989) have reported major differences in adenosine ($10\ \mu\text{M}$) metabolism between astrocytes and neurones isolated from newborn Swiss mice. In astrocytes, the main metabolic route was formation of nucleotides with little deamination. On the other hand neurones incorporated less label into ATP and there was considerable deamination.

The situation is, however, complex. The rates of adenosine formation and influx will determine the free cytosolic concentration of adenosine and this, together with the total activities of the adenosine kinase and adenosine deaminase will then determine the proportion of adenosine phosphorylated or deaminated.

Adenosine deaminase

Deoxycytosine (an ADA inhibitor) produces adenosine-like sedative and hypnotic effects in rats (Radulovacki *et al.*, 1983) and man (Major *et al.*, 1981), demonstrating the importance of ADA in the inactivation of adenosine.

The distribution of ADA has been studied in rat and human brain (Phillips and Newsholme, 1979). There were no marked differences in the various

regions of the rat brain; however, in the human brain the hind brain had a lower activity of ADA compared to the rest of the brain. An extensive plexus of ADA-containing neurones has been immunocytochemically localized in the rat basal hypothalamus that innervates the striatum, amygdala and cortex (Nagy *et al.*, 1984). A correlation was found between the regional distribution of ADA-immunoreactive neurones and NBI-binding sites in the rat brain (Nagy *et al.*, 1985) consistent with the need for adenosine to be taken up before it is deaminated. Attempts to correlate the distribution of ADA with adenosine receptors have not been as successful. The hippocampus and cerebellum, which are very rich in adenosine receptors, contain little ADA activity (Geiger and Nagy, 1986). ADA-immunoreactive neurones have also been found in mouse and hamster brain (Yamamoto *et al.*, 1987). There appear to be wide species variations. Although the rabbit and the cat brain contain large amounts of ADA activity, no specific immunolabelled neuronal systems were found (Patel and Tudball, 1986; Schrader *et al.*, 1987). Schrader *et al.* (1987) have shown that ADA is restricted to oligodendroglia and endothelial cells in rabbit brain.

Although ADA has largely been considered to be a cytosolic enzyme, there have been several reports of an extracellularly located ADA or an exo-ADA in a variety of cell types (Andy and Kornfeld, 1982; Hellewell and Pearson, 1983; Meghji *et al.*, 1988b). Evidence for extracellular deamination has also been obtained in the desheathed rabbit vagus nerve (Maire *et al.*, 1984). This enzyme may be bound to a membrane-associated ADA-binding protein (Andy and Kornfeld, 1982; Schrader *et al.*, 1983, 1984). However, this is controversial since ADA was detected in oligodendroglia and in endothelial cells lining blood vessels of rabbit brain, whereas complexing protein was found in neurones (Schrader *et al.*, 1987). Franco *et al.* (1986) have demonstrated a membrane-associated ADA in rat brain subfractions. An exo-ADA would allow direct inactivation of adenosine.

S-Adenosylhomocysteine hydrolase

Another possible pathway for adenosine inactivation is reaction with L-homocysteine to form SAH (Fig. 2) since SAH-hydrolase equilibrium favours the synthesis reaction (Fox and Kelly, 1978; Schrader, 1983). In the rat hippocampal slice preparation the main route of adenosine metabolism was incorporation into nucleotides with SAH synthesis only being a minor pathway (Reddington and Pusch, 1983). L-Homocysteine availability limits this pathway, which can be accelerated therefore by added L-homocysteine (Schrader, 1983; McIlwain and Poll, 1986). This strategy has been used to determine free cytosolic adenosine concentration and to localize adenosine

formation in mammalian neocortical tissues (McIlwain and Poll, 1986) and perfused hearts (Deussen *et al.*, 1988).

Conclusions

Extracellular adenosine formation is likely to take place by two routes. First, ATP can be released as a co-transmitter together with other transmitters. In this case it is catabolized to form adenosine via the ectonucleotidase cascade. The presence of ATPase has been demonstrated on neurones. 5'-Nucleotidase on the other hand has a strictly non-neuronal distribution. The ability to secrete nucleotides under physiological conditions appears to be restricted to a few cell types such as neurones and platelets. The intracellular concentration of ATP is about 5 mM and it is likely that cell damage may result in an increase in extracellular ATP concentration.

Alternatively, adenosine may be produced intracellularly during hypoxia and ischaemia as a result of an imbalance between energy demand and energy supply. There is a rise in intracellular concentration of AMP followed by adenosine production. Studies on the mechanisms of adenosine formation in neural tissue have lagged behind those of other tissues. Although an intracellular site of formation has been established, the enzymes involved have not been identified. However, by analogy with heart muscle and polymorphonuclear leucocytes this route of adenosine formation may involve the IMP-specific and the AMP-specific cytosolic 5'-nucleotidases.

For either case of adenosine production, adenosine can be inactivated by uptake into neurones and neighbouring cells through the nucleoside transporter. It is then either phosphorylated by the action of adenosine kinase or deaminated by the action of ADA. The kinetic parameters of these enzymes are such that adenosine is likely to be phosphorylated at low adenosine concentrations, the deamination pathway becoming important at higher adenosine concentrations only. Adenosine deaminase also exists in an extracellular membrane-associated form. In this case adenosine may be deaminated directly without prior need to be taken up.

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CHAPTER 3

ATP RECEPTORS AND THEIR PHYSIOLOGICAL ROLES

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Introduction

Research into the pharmacology of purine compounds is largely hampered by the lack of specific competitive antagonists and of specific agonists, which are needed for the solid characterization of receptors. Consequently, knowledge of the physiological significance of P₂ purinoceptors is limited. Physiological studies are even more hampered than pharmacological studies because

Table 1 Organs within which there is evidence that ATP is a neurotransmitter

Gastrointestinal tract	
Gizzard	Burnstock <i>et al.</i> , 1970
Stomach	Burnstock <i>et al.</i> , 1970; Beck <i>et al.</i> , 1988
Small intestine	Ahmad <i>et al.</i> , 1978; White, 1982; White and Leslie, 1982; White and Al-Humayyd, 1983; Al-Humayyd and White, 1985; Manzini <i>et al.</i> , 1985, 1986a; Hammond <i>et al.</i> , 1988
Large intestine	Su <i>et al.</i> , 1971; Kuchii <i>et al.</i> , 1973; Burnstock <i>et al.</i> , 1978b; Satchell, 1981, 1982; Hedlund <i>et al.</i> , 1983; Manzini <i>et al.</i> , 1986b
Gall bladder	Takahashi <i>et al.</i> , 1987
Cardiovascular system	
Heart	Bulloch and McGrath, 1988; Flavahan <i>et al.</i> , 1985; Schlicker <i>et al.</i> , 1989
Arteries	Hoyle and Burnstock, 1986; Bramich and Campbell, 1989; Bramich <i>et al.</i> , 1989
	Katsuragi and Su, 1980, 1982; Ishikawa, 1985; Kennedy and Burnstock, 1985b; Kügelgen and Starke, 1985; Sneddon and Burnstock, 1985; Cheung and Fujioka, 1986; Kennedy <i>et al.</i> , 1986; Neild and Kotecha, 1986; Burnstock and Warland, 1987a; Muramatsu and Kigoshi, 1987; Warland and Burnstock, 1987; Westfall <i>et al.</i> , 1987; Åstrand <i>et al.</i> , 1988; Machaly <i>et al.</i> , 1988; Nagao and Suzuki, 1988; Muramatsu <i>et al.</i> , 1989
Veins	Burnstock <i>et al.</i> , 1979; Flavahan and Vanhoutte, 1986
Capillaries	Holton, 1959
Genito-urinary tract	
Bladder	Burnstock <i>et al.</i> , 1972, 1978a, b; Dean and Downie, 1978; Choo and Mitchelson, 1980; Choo, 1981; Theobald, 1982, 1986; Kasakov and Burnstock, 1983; Westfall <i>et al.</i> , 1983; Hourani, 1984; Holt <i>et al.</i> , 1985; Moss and Burnstock, 1985; Theobald and Hoffman, 1986; Fujii, 1988; Hoyle <i>et al.</i> , 1989, 1990

ATP receptors and their physiological roles

Vas deferens	Fedan <i>et al.</i> , 1981; Sneddon <i>et al.</i> , 1982; Sneddon and Burnstock, 1984; Sneddon and Westfall, 1984; Stjärne and Åstrand, 1985a; Allcorn <i>et al.</i> , 1985; Kirkpatrick and Burnstock, 1987; Åstrand <i>et al.</i> , 1988; Cunnane and Manchada, 1988; Bulloch and McGrath, 1988
Skin	
Chromatophores	Kumazawa and Fujii, 1984, 1986; Kumazawa <i>et al.</i> , 1984; Fujii, 1986; Fujii and Oshima, 1986
Peripheral ganglia	
Superior cervical	Potter <i>et al.</i> , 1983; Wolinsky and Patterson, 1985; Furshpan <i>et al.</i> , 1986; McCaman and McAfee, 1986; Tolkovsky and Suidan, 1987
Central nervous system	
Brain	Schubert and Kreutzberg, 1974; Barberis and McIlwain, 1976; Schubert <i>et al.</i> , 1976; White, 1978; Potter and White, 1980; White <i>et al.</i> , 1980; Richardson and Brown, 1986
Spinal cord	Fyffe and Perl, 1984; Salter and Henry, 1985; White <i>et al.</i> , 1985; Sweeney <i>et al.</i> , 1989

physiological evaluation of P₂ purinoceptor subclasses requires that whatever putative agonists or antagonists are used, they must be non-toxic at useful doses. So far, much of the information about the involvement of P₂ purinoceptors has come from extrapolation of results from pharmacological experiments. As yet there is little information about the involvement of P₂ purinoceptors in homeostatic mechanisms, although there is increasing evidence for the utilization of ATP by peripheral and central neurones, as a neurotransmitter or co-transmitter. Although the presence of receptors on central and autonomic effector cells suggests involvement of ATP and P₂ purinoceptors in physiological processes, well-defined examples of physiological significance are limited.

In the autonomic nervous system ATP is used a transmitter or co-transmitter by sympathetic and parasympathetic nerves, and also by those neurones that belong to intrinsic ganglia of many viscera and that are neither strictly sympathetic nor parasympathetic, exemplified by the myenteric plexus of the

Table 2 Distribution of subtypes of receptors for ATP*P_{2X}* Purinoceptors

Smooth muscle	
Gastrointestinal tract	Hedlund <i>et al.</i> , 1983; Meldrum and Burnstock, 1985
Cardiovascular system	
Arteries	Ishikawa, 1985; Kennedy and Burnstock, 1985b; Kennedy <i>et al.</i> , 1985, 1986; Kügelgen and Starke, 1985; Mathieson and Burnstock, 1985; Sneddon and Burnstock, 1985; Cheung and Fujioka, 1986; Neild and Kotecha, 1986; Burnstock and Warland, 1987a, b; Houston <i>et al.</i> , 1987; Muramatsu <i>et al.</i> , 1981, 1989; Muramatsu and Kigoshi, 1987; Ramme <i>et al.</i> , 1987; Machaly <i>et al.</i> , 1988; Nagao and Suzuki, 1988
Veins	Kennedy and Burnstock, 1985a; Reilly and Burnstock, 1987
Vascular beds	Flavahan <i>et al.</i> , 1985; Bertrand <i>et al.</i> , 1987; Fleetwood and Gordon, 1987; Hopwood and Burnstock, 1987
Genito-urinary tract	
Urinary bladder	Theobald, 1982, 1986; Kasakov and Burnstock, 1983; Westfall <i>et al.</i> , 1983; Holt <i>et al.</i> , 1985; Hourani <i>et al.</i> , 1985; Hoyle and Burnstock, 1985; Moss and Burnstock, 1985; Theobald and Hoffman, 1986; Moss <i>et al.</i> , 1987; Fujii, 1988; Hoyle <i>et al.</i> , 1989
Vas deferens	Fedan <i>et al.</i> , 1981, 1982; Meldrum and Burnstock, 1983; Sneddon and Westfall, 1984; Sneddon and Burnstock, 1984; Stjärne and Åstrand, 1984, 1985a; Allcorn <i>et al.</i> , 1985; Åstrand <i>et al.</i> , 1988; Bulloch and McGrath, 1988
Nictitating membrane	Duval <i>et al.</i> , 1985
Cardiac muscle	Hoyle and Burnstock, 1986; Bramich and Campbell, 1989
Fibroblasts	Okada <i>et al.</i> , 1984

P_{2Y} Purinoceptors

Smooth muscle

Gastrointestinal tract

Gough *et al.*, 1973; Satchell and Maguire, 1975; Kerr and Krantis, 1979; Crema *et al.*, 1983; Beck *et al.*, 1988

Cardiovascular system

Arteries

Mathieson and Burnstock, 1985; Burnstock and Warland, 1987b; Muramatsu and Kigoshi, 1987

Veins

Kennedy and Burnstock, 1985a; Reilly *et al.*, 1987

Vascular beds

Fleetwood and Gordon, 1987; Hopwood and Burnstock, 1987

Cardiac muscle

Björnsson *et al.*, 1989

Endothelial cells

Kennedy *et al.*, 1985; Houston *et al.*, 1987

Erythrocytes

Boyer *et al.*, 1989; Cooper *et al.*, 1989

Hepatocytes

Keppens and Wulf, 1986

Pancreatic β cells

Chapal and Loubatières-Mariani, 1983; Bertrand *et al.*, 1987; Loubatières-Mariani and Chapal, 1988

Pulmonary alveolar type II cells

Rice and Singleton, 1987, 1989

Sensory neurones

Krishtal *et al.*, 1983; Krishtal and Marchenko, 1986

P_{2T} Purinoceptors

Platelets and megakaryocytes

Haslam and Rosson, 1975; Cusack *et al.*, 1979, 1985

P_{2Z} Purinoceptors

Blood cells

Erythrocytes

Parker and Snow, 1972

Lymphocytes

Schmidt *et al.*, 1984

Macrophages

Cameron, 1984; Steinberg and Silverstein, 1987

Mast cells

Cockroft and Gomperts, 1979a, b, 1980; Bennett *et al.*, 1981; Tatham *et al.*, 1988

Neutrophils

Kuhns *et al.*, 1988

Ascites tumour cells

Hempling *et al.*, 1969

Protozoan contractile vacuole

Pothier *et al.*, 1987

Table 2 *continued****P_{2S} Purinoceptors***

Guinea-pig ileum

Wiklund and Gustafsson, 1988a, b

P₃ Purinoceptors

Sympathetic nerve terminals

Tail artery

Shinozuka *et al.*, 1988

The receptor classifications shown were not necessarily made by the original authors. The P_{2X} purinoceptor classification was made according to antagonism by ANAPP₃, α,β -methylene ATP or agonist potency order. The P_{2Y} purinoceptor classification was made according to antagonism by reactive blue 2, or agonist potency order. The P_{2Z} purinoceptor classification was made according to activation by ATP⁴⁻, antagonism by divalent cations, or lack of activity of analogues of ATP with altered phosphate chains.

gut and intramural ganglia of the urinary bladder. Table 1 contains a summary of organs in which purinergic transmission has been claimed. They include many types of blood vessel, the heart, viscera of the genito-urinary tract and gastrointestinal tract, autonomic ganglia and the brain. There is also good evidence that ATP can be released from the peripheral and central ends of some sensory nerves.

The receptors at which ATP acts are called P_2 purinoceptors, as distinct from P_1 purinoceptors which are selective for adenosine (Burnstock, 1978). Since this original definition, the P_2 purinoceptor has been subdivided into several classes, each with their own distinctive pharmacology. The major divisions are the P_{2X} and P_{2Y} subclasses, originally identified by Burnstock and Kennedy (1985a), and also the P_{2Z} and P_{2T} subclasses, identified and defined by Gordon (1986). A fifth subclass, and P_{2S} purinoceptor, has been named on the basis that the pharmacological profile at this receptor is different from the other subtypes of P_2 purinoceptor (Wiklund and Gustafsson, 1988a, b). Also, a P_3 purinoceptor has been proposed (Shinozuka *et al.*, 1988) which is claimed to be activated by ATP but blocked by P_1 purinoceptor antagonists; however, this needs confirmation. The distribution of these subtypes of P_2 purinoceptor is given in Table 2.

P₂ Purinoceptors in the cardiovascular system

Control of vascular tone by purines has been well-studied, particularly *in vitro*. In addition to neurally released ATP affecting the mechanical tone of a vessel,

ATP from diverse sources, for example endothelial cells, platelets or other blood-borne cells, can also affect vascular tone. ATP may act directly on vascular smooth muscle, or may act via P_2 purinoceptors to stimulate endothelial cells, platelets and mast cells, causing them to release vasoactive substances, and thereby ATP can also have an indirect action on the vascular tone.

Vasoconstriction and vasodilatation

In many peripheral arteries, stimulation of sympathetic nerves causes vasoconstriction. This pressor response is often only partially blocked by adrenoceptor antagonists, notably phentolamine (a general α -adrenoceptor antagonist) or prazosin (an α_1 -adrenoceptor antagonist). The non-adrenergic component can be blocked in many cases by either desensitization of P_{2X} purinoceptors with α,β -methylene ATP or by antagonism with the photoaffinity analogue of ATP, arylazidoaminopropionyl ATP (ANAPP₃); this shows that the non-adrenergic component is purinergic. In the mesenteric artery from several species (Ishikawa, 1985; Kügelgen and Starke, 1985; Muramatsu and Kigoshi, 1987; Machaly *et al.*, 1988; Nagao and Suzuki, 1988; Muramatsu *et al.*, 1989), rabbit and guinea-pig saphenous artery (Cheung and Fujioka, 1986; Burnstock and Warland, 1987a; Warland and Burnstock, 1987), and in rabbit ear artery (Kennedy and Burnstock, 1985b; Kennedy *et al.*, 1986), the purinergic component is frequency-dependent. That is to say: the purinergic pressor response increases with increasing frequency of sympathetic nerve stimulation. However, the proportion of the purinergic component relative to the adrenergic component decreases as the stimulus-frequency increases. Further, purinergic vasoconstrictor responses are not well-maintained: in the presence of adrenoceptor blockade, during prolonged periods of stimulation the vessel will relax from its peak of contraction. This may be a manifestation of desensitization of the P_{2X} purinoceptors by ATP released from nerves – at higher frequencies more ATP is liberated, thus accelerating desensitization (Kennedy *et al.*, 1986). In contrast, adrenergic responses, although slower in onset are well-maintained. In many vascular beds there is a sympathetic tone due to adrenergic activity: because such tonic activity cannot be maintained by the purinergic sympathetic component, it has been suggested that physiologically the purinergic component is more important when an acute response is needed, such as in stress.

In the pithed rat, an experimental model which is more physiological than isolated blood vessels, stimulation of the thoracic or thoracolumbar sympathetic outflow evokes a pressor response which is only partially suppressed by adrenoceptor antagonists (Bulloch and McGrath, 1988; Schlicker *et al.*, 1989). The residual response, which is also the initial part of the pressor response,

is blocked after tachyphylaxis or desensitization with α,β -methylene ATP has been induced, which blocks pressor responses due to ATP (Bullock and McGrath, 1988; Schlicker *et al.*, 1989). The initial pressor response is also blocked by suramin (Schlicker *et al.*, 1989), which is an antagonist of P_{2X} and P_{2Y} purinoceptors (Hoyle *et al.*, 1989). These results indicate that ATP, released from sympathetic nerves, contributes to raised peripheral resistance and therefore an increase in blood pressure, and suggests that such purinergic transmission can be invoked as a physiological control mechanism.

Stimulation of a specific hypothalamic region in the rabbit brain evokes alert-responses and a large increase in blood flow to skeletal muscle due to a vasodilatation of the muscular vascular bed (Shimada and Stitt, 1984). This vascular response is mimicked by intra-arterial infusion of adenosine or ATP, but responses to adenosine are blocked by aminophylline. Responses to ATP are insensitive to aminophylline, but are blocked in parallel with responses to hypothalamic stimulation by antazoline, thus indicating that a purinergic mechanism is involved in the reflexly commanded vasodilatation (Shimada and Stitt, 1984).

One of the acute responses to cold-exposure is a reduction in cutaneous blood flow which serves to reduce loss of body heat. This is a sympathetic reflex. In the dog saphenous vein there is little purinergic contribution to venoconstriction at 37°C, but at colder temperatures the purinergic component becomes more effective, and the sympathetic response becomes more powerful (Flavahan and Vanhoutte, 1986). This suggests that purinergic constriction may be invoked as part of a physiological response to cold-stress.

In the hepatic artery, there is a large purinergic component which is dominant to the adrenergic component throughout a large range of stimulation frequencies, but as in other blood vessels, the responses fade rapidly, possibly due to autodesensitization of the P_2 purinoceptors by released ATP (Brizzolara and Burnstock, 1990). *In vivo*, stimulation of the sympathetic hepatic nerve causes only a transient vasoconstriction (Greenway *et al.*, 1967; Greenway and Stark, 1971), and it has been suggested that this is due to the self-limiting purinergic pressor responses and that this is one of the physiological mechanisms that serve to prevent large decreases in total hepatic blood flow (Brizzolara and Burnstock, 1990).

There is good evidence that sensory nerves release ATP and evoke vasodilatation in the rabbit ear capillary bed. In this vascular system, antidromic stimulation of the sensory nerves supplying the ear, which run in the great auricular nerve, causes dilatation of the capillary bed supplied by the central ear artery (Holton and Perry, 1951); with correct stimulus parameters the larger arterial elements are unaffected (Hilton and Holton, 1954). The capillary dilatation is mimicked by ATP and, like the action of ATP, responses to sensory nerve stimulation are antagonized by physostigmine

(Holton and Holton, 1953). When dilatation is evoked by nerve stimulation, purine compounds including ATP are found in the venous effluent. Mechanical stimulation of the cutaneous afferents in the ear itself causes capillary dilatation and concomitant release of ATP (Holton and Holton, 1953; Holton, 1959). Extracts of the dorsal root ganglia, which contain the nerve cell bodies that supply these sensory nerves, contain high concentrations of ATP (Holton and Holton, 1954) while a microassay puts the concentration of ATP in single cells isolated from dorsal root ganglia at around 1.7 mM (Fukuda *et al.*, 1983). Although the subclass of the P_2 purinoceptor in the rabbit ear capillary bed is undefined, it is more likely to be P_{2Y} than P_{2X} , because the responses are relaxant rather than contractile.

ATP causes several types of blood vessel to relax. This action is sometimes mediated via P_{2Y} purinoceptors of the vascular smooth muscle: for example, the rabbit mesenteric artery and portal vein both display a P_{2Y} purinoceptor-mediated relaxant response (Kennedy and Burnstock, 1985a; Mathieson and Burnstock, 1985; Burnstock and Warland, 1987b; Reilly *et al.*, 1987), as do the dog basilar and middle cerebral arteries (Fleetwood and Gordon, 1987; Hopwood and Burnstock, 1987). The dog basilar artery relaxes in response to sympathetic purinergic nerve stimulation (Muramatsu and Kigoshi, 1987), while in the rabbit portal vein the purinergic inhibition is effected via parasympathetic nerves (Burnstock *et al.*, 1979; Kennedy and Burnstock, 1985a; Reilly *et al.*, 1987). In most blood vessels, however, the site of P_2 purinoceptor-mediated vasodilator activity is the endothelial cell rather than the smooth muscle. This action was first shown for ATP in the dog femoral artery (De Mey and Vanhoutte, 1981), and has since been shown in the dog coronary artery, pig aorta and rat femoral artery, in which cases a P_{2Y} purinoceptor on the endothelial cell is involved (Gordon and Martin, 1983; Kennedy *et al.*, 1985; Martin *et al.*, 1985; Houston *et al.*, 1987). Activation of this receptor stimulates the endothelial cell to release endothelium-derived relaxing factor (EDRF). EDRF is thought to be free radicals of nitric oxide, generated from L-arginine (Palmer *et al.*, 1987, 1988). Relaxant actions of ATP may be important components in the complex physiological regulation of vascular tone. For example, during exercise, when there is an increased demand by skeletal muscle for oxygen, there is a vasodilatation in the muscular vascular bed, and an increase in levels of ATP in the venous outflow (Forrester and Lind, 1969).

In vessels that contract in response to neurally released ATP acting on P_{2X} purinoceptors, but relax in response to exogenous ATP acting on P_{2Y} purinoceptors, it is suggested that there is an anatomical separation of these populations of purinoceptors, which allows for their independent activation due to different physiological stimuli. In most blood vessels the sympathetic innervation is predominantly adventitial and these nerve fibres do not

penetrate deeply into the muscle, and it is these more peripheral muscle cells that possess P_{2X} purinoceptors, activated physiologically by sympathetic activity. The more intimal muscle cells possess P_{2Y} purinoceptors, which are activated by ATP delivered from a luminal source. Similarly, P_{2Y} purinoceptors on endothelial cells, which when activated induce release of EDRF, are also activated by a luminal source of ATP. As ATP has a very short half-life in whole blood, it is thought that these P_{2Y} purinoceptors are activated by ATP released locally from endothelial cells or blood-borne cells such as platelets, due to physiological or pathophysiological stimuli, such as hypoxia or damage to the vascular wall.

Hyperaemic responses

In many vascular beds, particularly the coronary circulation, episodes of hypoxia cause vasodilatation that permits an increase in blood flow (i.e. hyperaemia). ATP is stored in high concentrations in cardiac endothelial cells, much higher than that in the muscle cells, or that of the purines ADP, AMP and adenosine (Nees and Gerlach, 1983). Although ATP appears in the coronary outflow following hypoxia, ATP is broken down or removed from the circulation so rapidly that the local concentrations of ATP are much higher than would be suggested by the measurements of ATP in perfusates (Paddle and Burnstock, 1974). Adenosine has been proposed as the mediator of hyperaemia (Berne, 1963) because it, and its metabolites, are found in high concentrations in coronary effluent following hyperaemia. However, taking into account the greater potency of ATP compared with adenosine (Toda *et al.*, 1982; see Burnstock and Kennedy, 1985b), the fact that ATP is released from endothelial cells during hypoxia and that ATP can act on endothelial cells to cause release of EDRF and the rapid metabolism of ATP, it is highly probable that ATP is an important mediator of hypoxic (or ischaemic) hyperaemia (see Burnstock, 1989). This is also supported by the observations that antagonists of P_1 purinoceptors affect neither vasodilatation due to ATP nor ischaemia (Eikens and Wilcken, 1973; Giles and Wilcken, 1977; Olsson *et al.*, 1978) while blocking adenosine-induced vasodilatation. Cerebral arteries are also more sensitive to ATP than to adenosine (Hardebo and Edvinsson, 1979; Forrester *et al.*, 1979; Toda *et al.*, 1982) and it has been suggested that the reactive hyperaemia associated with headaches in migraine could involve an initial vasodilatation due to ATP (Burnstock, 1981).

Haemostasis

ATP is involved in many of the haemostatic mechanisms that are continuously in play and that become prominent at sites of vascular injury. When collagen

becomes exposed due to endothelial damage, platelets begin to adhere to it, aggregate and release ADP, which promotes further aggregation via activation of P_{2T} purinoceptors. ADP can also act on P_{2Y} purinoceptors, either on endothelial cells or on exposed vascular smooth muscle cells, and thereby evoke dilatation. Thrombin, which is formed during clot-formation, is a powerful stimulant of irreversible platelet aggregation, during which platelets degranulate and undergo morphological changes, releasing ATP, ADP, other vasoactive purines such as adenine dinucleotides (Flodgaard and Klenow, 1982; Lüthje and Ogilvie, 1983), and other vasoactive substances such as 5-hydroxytryptamine (Mills *et al.*, 1968). ATP is used as an energy source for mechanisms of clot-retraction, but it can also act on the local P_{2Y} purinoceptors. Platelet aggregation is inhibited by ATP and adenine dinucleotides, acting on the platelet P_{2T} purinoceptor (Harrison *et al.*, 1975; see Hoyle, 1990), so high local concentrations of these compounds arising as a result of degranulation, could serve to control the extent of aggregation. Thrombin also induces release of ATP from endothelial cells, and causes endothelium-dependent vasodilatation (Pearson and Gordon, 1979; De Mey *et al.*, 1982), and 5-hydroxytryptamine can cause endothelium-dependent vasodilatation or a direct vasoconstriction. Thus, when haemostatic mechanisms are invoked during vessel injury, the state of the endothelium will be important for determining the effect on vascular tone. It would appear that if an injured vessel maintains its endothelial cells, local vasodilatation will occur, but if endothelial cells are damaged badly, as might occur in a severe injury, then vasoconstriction would be favoured.

P₂ Purinoceptors in the vas deferens

The motor innervation of the vas deferens is sympathetic (Burnstock, 1970), and the physiological significance of P_2 purinoceptors in this tissue is that they are postjunctional to the nerve terminals which release ATP, employing ATP and noradrenaline as co-transmitters. In the rat, mouse and guinea-pig vas deferens, ATP and noradrenaline are released concomitantly in response to electrical field stimulation; both these transmitters are known to be secreted from neurones because their release can be prevented by tetrodotoxin, guanethidine, 6-hydroxydopamine (6-OHDA) or by removal of extracellular calcium (Westfall *et al.*, 1978; Fedan *et al.*, 1981; Levitt *et al.*, 1984; Stjärne and Åstrand, 1985a; Kirkpatrick and Burnstock, 1987; Kasakov *et al.*, 1988). Tetrodotoxin blocks neuronal action potential discharge, guanethidine and 6-OHDA cause degeneration of sympathetic nerves, and removal of calcium prevents exocytotic release mechanisms from working. In the guinea-pig vas

deferens there is no evidence that ATP is released from non-neuronal cells, such as smooth muscle (Kirkpatrick and Burnstock, 1987; Kasakov *et al.*, 1988), but in the rat vas deferens it has been suggested that ATP can also be released as a consequence of release of noradrenaline, from nerves or muscle (Fredholm and Hedqvist, 1980; Fredholm *et al.*, 1982; Vizi and Burnstock, 1988). In other tissues ATP may be released in this secondary fashion also, for example in the rat tail artery, heart and kidney (Fredholm and Hedqvist, 1978; Westfall *et al.*, 1987).

In the vas deferens, both ATP and noradrenaline cause contractions, but while the response to noradrenaline is a sustained contracture, the response to ATP is a phasic twitch-like transient contraction. In response to sympathetic stimulation there is an initial rapid twitch, followed by a sustained contracture. This initial phase is blocked by blocking P_{2X} purinoceptors with either ANAPP₃ or desensitization with α,β -methylene ATP, and the secondary phase is blocked by α -adrenoceptor antagonists (McGrath, 1978; Fedan *et al.*, 1981; Sneddon *et al.*, 1982; Brown *et al.*, 1983; Meldrum and Burnstock, 1983; Sneddon and Burnstock, 1984; Sneddon and Westfall, 1984; Stjärne and Åstrand, 1984, 1985a; Allcorn *et al.*, 1985; Cunnane and Manchanda, 1988). Guanethidine and 6-OHDA abolish neurogenic contractions, but reserpine, which selectively depletes noradrenaline from sympathetic nerves, causes abolition of only the secondary contraction (Kirkpatrick and Burnstock, 1987). Also, in the pithed rat, twitch responses of the vas deferens evoked by stimulation of spinal sympathetic outflow are blocked by pretreatment with α,β -methylene ATP (Bulloch and McGrath, 1988).

The physiological significance of the release of ATP from neuronal or non-neuronal tissues, secondarily to release of noradrenaline from sympathetic nerves supplying the rat vas deferens, is obscure. It seems as if there is a positive feedback mechanism, whereby an excitatory signal is enhanced (i.e. excitatory sympathetic activity induces release of ATP and noradrenaline which both cause contraction of the smooth muscle, and noradrenaline additionally evokes a supplementary release of ATP which further contributes to the contractile response). This action of noradrenaline is mediated via α -adrenoceptors, and when they are blocked there is a reduction in the purinergic component of the neurotransmission process (Vizi and Burnstock, 1988). It is also likely that ATP contributes to a negative feedback mechanism, because ATP is rapidly broken down by ecto-5'-nucleotidase (Vizi and Burnstock, 1988), and the resultant adenosine can act on prejunctional P_1 purinoceptors, mediating inhibition of further release. When the time courses of these actions are considered it is apparent that transmission to the vas deferens would be geared for short bursts of activity, as occur during ejaculation, resulting in fast twitches, with a component of positive feedback, followed by a self-inhibiting offset phase with a component of negative

feedback. Although it has been shown that adenosine inhibits neurogenic contractions in the vas deferens *in vitro* (Hedqvist and Fredholm, 1976; Paton *et al.*, 1978; Müller and Paton, 1979; Sneddon *et al.*, 1984), such activity has not been shown *in vivo*.

P₂ Purinoceptors in the urinary bladder

The parasympathetic nerves that supply the urinary bladder run in the pelvic nerve to parasympathetic ganglia situated peripherally on the bladder, and to ganglia within the wall of the bladder. These ganglia project to the bundles of smooth muscle and to each other, forming an intramural plexus. The sympathetic supply comes from the inferior mesenteric and hypogastric ganglia, and runs in the hypogastric nerve, passing into the wall of the bladder to innervate the parasympathetic ganglia, and to contribute to the vesicular plexus (Petras and Cummings, 1978). Stimulation of the pelvic nerve results in a contraction of the bladder body (Langley and Anderson, 1895; Henderson and Roepke, 1934). Stimulation of the hypogastric nerve usually leads to contraction of the bladder sphincter accompanied by a relaxation of the bladder body. Stimulation of the hypogastric nerve also inhibits the action of stimulation of the pelvic nerve (Theobald, 1982). In excised strips of smooth muscle, from the detrusor or bladder body, electrical field stimulation of the intramural, postganglionic parasympathetic neurones causes contractile responses (Ambache and Zar, 1970). The physiological significance of P₂ purinoceptors in the urinary bladder is that they are postjunctional to intramural purinergic motoneurones.

The P₂ purinoceptors in the urinary bladder, activated by ATP and its analogues, belong to the P_{2X} subclass (Burnstock and Kennedy, 1985a). Typical features of this subclass are the susceptibility to antagonism by ANAPP₃ or desensitization by α,β -methylene ATP, along with the greater potency of α,β -methylene ATP and β,γ -methylene ATP than ATP itself, at evoking contractile responses. L- β,γ -Methylene ATP is also a potent spasmogen in the bladder, and can cause desensitization accompanied by antagonism of responses to exogenous ATP and non-cholinergic nerve stimulation (Hourani, 1984; Hourani *et al.*, 1985). This compound may be a specific ligand for the P_{2X} purinoceptor because it is inert in the guinea-pig taenia coli, which is populated with P_{2Y} purinoceptors. The binding characteristics of the P₂ purinoceptor in the rabbit detrusor have been evaluated, using [³H]ATP (Levin *et al.*, 1983). The binding is independent of divalent cations, is displaced by β,γ -methylene ATP, has a pH optimum between pH 5.0 and 7.0, and a K_D of 180 nM. These purinoceptors have a concentration of 9.5–15 pmol/mg

protein, which is very high when compared with that of 22 fmol/mg protein and 91 fmol/mg protein for muscarinic cholinceptors and β -adrenoceptors, respectively (Levin and Wein, 1979; Levin *et al.*, 1980, 1983).

In the rat urinary bladder [^3H] α,β -methylene ATP has been used to localize P_2 purinoceptors autoradiographically (Bo and Burnstock, 1989) and to determine the presence of high and low-affinity binding sites in rat urinary bladder detrusor smooth muscle (Bo and Burnstock, 1990). As in the rabbit, the binding is displaced by β,γ -methylene ATP, but in contrast, it is inversely dependent on the concentration of Mg^{2+} . The high-affinity sites have a K_D of 8.5 nM and the low-affinity sites have a K_D of 120 nM (in the presence of Mg^{2+} at 2 mM), and the concentrations of the B_{max} are 7.0 and 34.9 pmol/mg protein, respectively (Bo and Burnstock, 1990).

The neurogenic excitation of the bladder is in part due to acetylcholine, and in part due to a non-cholinergic neurotransmitter, which is probably ATP. The relative contributions of the cholinergic and purinergic transmission vary according to the frequency of applied stimulation and the actual species. Generally, single pulses of electrical field stimulation, less than 1 ms duration, evoke contractions that are insensitive to cholinergic antagonists. As the stimulus frequency is raised, the contractile response increases and becomes partially sensitive to atropine. Thus, at low frequencies of stimulation (below 8 Hz) the neurogenic response is largely non-cholinergic, at higher frequencies it is largely cholinergic (Ambache and Zar, 1970; Krell *et al.*, 1981; MacKenzie and Burnstock, 1984; Moss and Burnstock, 1985).

In the majority of species studied, the atropine-sensitive component of excitatory nerve stimulation increases with increasing frequency of the applied stimulus, and maximally accounts for 40–60% of the contractile response. This is so for the rat, rabbit, guinea-pig, marmoset (*Callithrix jacchus*), monkey (*Macaca fascicularis* and *Cebus capella*), ferret, ringtail possum and pig (Burnstock and Campbell, 1963; Johns and Paton, 1977; Dean and Downie, 1978; MacKenzie and Burnstock, 1984; Maggi *et al.*, 1984; Sibley, 1984; Moss and Burnstock, 1985; Craggs and Stephenson, 1986). However, in cats, the cholinergic component may account for only 20% of the neurogenic response (Langley and Anderson, 1895; Craggs and Stephenson, 1982), while in the human the neurogenic response may be more than 80% cholinergic (Hoyle *et al.*, 1989), although some have claimed that it is wholly cholinergic (Sibley, 1984). In baboons and rhesus monkeys the cholinergic component may also be very dominant, accounting for over 90% of the contractile response evoked by stimulation of excitatory nerves (Brindley and Craggs, 1975; Craggs and Stephenson, 1986). It has been suggested that the non-cholinergic element of neurotransmission, present in New World primates (e.g. marmoset and cebus monkey) and in non-primates, has evolved for territory-marking behaviour (Craggs and Stephenson, 1986). This is compatible with the suggestion that

the non-cholinergic element of transmission is more important for the initiation of contraction of the bladder, rather than for maintenance of bladder-tone during voiding (Krell *et al.*, 1981). Thus animals that mark territory by urinating need to have a controlled phasic voiding, as evoked by purinergic nerve stimulation, whereas animals that do not have need for frequent short-duration voiding have a less pronounced purinergic component, and tonic cholinergic-type bladder control is more appropriate.

Of several transmitter substances that have been proposed as an alternative to ATP as the non-cholinergic transmitter in the bladder, such as noradrenaline, histamine, prostaglandins, vasoactive intestinal polypeptide, substance P, neurotensin and enkephalins, none is suitable. This is because either it does not mimic the neural contraction, or when its action is modified by pharmacological manipulation there is no parallel effect on the response to nerve stimulation (Taira, 1972; Downie and Larsson, 1981; MacKenzie and Burnstock, 1984; Hourani, 1984; Meldrum and Burnstock, 1985; Callahan and Creed, 1986).

Release of ATP from intramural neurones in the bladder has been measured, using the luciferin–luciferase assay. This release is sensitive to tetrodotoxin, and to removal of calcium from the extracellular medium (Burnstock *et al.*, 1978a). ATP is released from nerve terminals following invasion by action potentials, and is released into the junctional cleft where it acts on postjunctional P_{2X} purinoceptors on the smooth muscle membrane. The stable analogue of ATP, α,β -methylene ATP, causes rapid desensitization of purinoceptors in the bladder, blocking responses to exogenous ATP and non-cholinergic nerve stimulation simultaneously (Kasakov and Burnstock, 1983). In combination with atropine, desensitization of P_{2X} purinoceptors by α,β -methylene ATP abolishes responses to postganglionic parasympathetic nerve stimulation in the guinea-pig, rabbit, pig, marmoset, ferret and human urinary bladder (Kasakov and Burnstock, 1983; Hoyle and Burnstock, 1985; Moss and Burnstock, 1985; Katsuragi *et al.*, 1986; Fujii, 1988; Hoyle *et al.*, 1989). At an electrophysiological level, atropine in combination with desensitization of P_2 purinoceptors causes abolition of the non-cholinergic excitatory junction potential in rabbit detrusor muscle and selective antagonism of depolarization induced by exogenous ATP (Hoyle and Burnstock, 1985). Similar results have been obtained in other species too (Fujii, 1988).

Perhaps the best evidence for a physiological activation of the P_2 purinoceptor in the urinary bladder has been demonstrated by Theobald (1983) and Theobald and Hoffman (1986), who have shown that in the cat *in vivo*, contractile responses in the bladder, evoked by preganglionic parasympathetic pelvic nerve stimulation, are antagonized by ANAPP₃. This also blocked responses to close intra-arterial application of ATP and β,γ -methylene ATP, but not noradrenaline. *In vitro*, ANAPP₃ blocks the non-cholinergic response

to electrical field stimulation of the intramural neurones in the guinea-pig urinary bladder detrusor (Westfall *et al.*, 1983).

In the urinary bladder, ATP can induce synthesis of prostaglandins, which may contribute to physiological responses of ATP. In the rabbit, inhibition of prostaglandin synthesis selectively antagonizes responses to exogenous ATP and non-cholinergic nerve stimulation (Dean and Downie, 1978; Husted *et al.*, 1979). In the monkey bladder, synthesis of prostaglandins, possibly induced by ATP, plays a significant part in the neurogenic response (Johns and Paton, 1977). Prostaglandins can obscure the pharmacological activity of ATP, which also makes physiological elucidation difficult. For example, in the presence of indomethacin, ATP causes a concentration-dependent inhibition of contractions evoked by activation of cholinceptors, but in the absence of indomethacin ATP may potentiate such activation (Brown *et al.*, 1979). The relationship between ATP and prostaglandins may be important in pathophysiological conditions. It has been shown in cases of chronic neurogenic vesicle dysfunction that a supersensitivity towards prostaglandins develops (Vaidyanathan *et al.*, 1982).

P₂ Purinoceptors in the gastrointestinal tract

An arylazidoaminopropionyl ATP (ANAPP₃) is a photoaffinity analogue of ATP, and when activated by ultraviolet light it covalently binds within the domain of its attachment. ANAPP₃ has been shown to block responses to ATP or its analogues, mediated via P_{2X} purinoceptors (Hogaboom *et al.*, 1980; Fedan *et al.*, 1982; Frew and Lundy, 1982a; Westfall *et al.*, 1983), but has not been shown to antagonize ATP to a useful extent in P_{2Y} systems (Westfall *et al.*, 1982; Frew and Lundy, 1982a,b). Further, ANAPP₃ may interact with P₁ purinoceptors (Frew and Lundy, 1986).

The anthraquinone sulphonic acid derivative, reactive blue 2, has been used as an antagonist of responses mediated via P₂ purinoceptors (Kerr and Krantis, 1979; Choo, 1981; Crema *et al.*, 1983; Manzini *et al.*, 1985, 1986b). However, it is only useful within a narrow range of concentrations, above which it has non-specific effects. Although reactive blue 2 can selectively inhibit P_{2Y}-mediated responses in the gut and some blood vessels (Manzini *et al.*, 1985, 1986b; Burnstock and Warland, 1987b; Reilly *et al.*, 1987; Houston *et al.*, 1987; Hopwood and Burnstock, 1987; Hopwood *et al.*, 1989; Taylor *et al.*, 1989), it also antagonizes P_{2X} purinoceptors in the bladder (Choo, 1981). In combination with agonist potency studies, reactive blue 2 has been used to

identify P_{2Y} purinoceptors in other systems, such as rat alveolar type II cells and rabbit stomach (Rice and Singleton, 1987, 1989; Beck *et al.*, 1988).

In most regions of the gastrointestinal tract ATP causes relaxation, typically mediated via P_{2Y} purinoceptors. Unfortunately, good competitive antagonists are lacking, but reactive blue 2 and suramin have been used to provide supporting evidence for parasympathetic purinergic neuromuscular transmission. Reactive blue 2 selectively antagonizes responses to ATP, or α,β -methylene ATP, and non-adrenergic, non-cholinergic neurogenic responses in the rabbit stomach, rat duodenum and caecum, and in guinea-pig internal anal sphincter and colon (Kerr and Krantis, 1979; Crema *et al.*, 1983; Manzini *et al.*, 1985, 1986b; Beck *et al.*, 1988). Suramin antagonizes relaxant responses to ATP and nerve stimulation in the guinea-pig taenia coli.

Incubation of tissues with the enzyme nucleotide pyrophosphatase selectively antagonizes responses to exogenous ATP and electrical stimulation of intramural nerves in the guinea-pig taenia coli and rat duodenum (Satchell, 1981, 1982; Manzini *et al.*, 1985). Again this suggests that ATP is a neuromuscular transmitter in these preparations.

The trypanocidal drug, suramin, has recently been discovered as a P_2 purinoceptor antagonist, but it is effective at both P_{2X} and P_{2Y} purinoceptors in the vas deferens, urinary bladder and taenia coli (Dunn and Blakeley, 1988; Den Hertog *et al.*, 1989a,b; Hourani and Chown, 1989), and it does not have any selectivity for either subtype (Hoyle *et al.*, 1990). Potentiation of P_{2X} -mediated events but not P_{2Y} -mediated events has also been reported (Hourani and Chown, 1989; Hoyle *et al.*, 1990).

In the guinea-pig taenia coli, following pre-incubation with [3H]adenosine, electrical stimulation of the perivascular sympathetic nerves causes relaxation and overflow of [3H]ATP. The relaxation and release are blocked by the neurotoxin, tetrodotoxin and the sympatholytic, guanethidine (Su *et al.*, 1971; Kuchii *et al.*, 1973). In varicosities isolated from the guinea-pig myenteric plexus, although ATP and noradrenaline are not always released in parallel, it is evident that a proportion of ATP is co-stored and co-released with noradrenaline (White and Al-Humayyd, 1983; Al-Humayyd and White, 1985; Hammond *et al.*, 1988). Hence in sympathetic nerves supplying the intestine, ATP and noradrenaline co-exist and may co-transmit.

In the rat distal colon *in vivo*, in the presence of atropine, stimulation of the pelvic nerves causes contractions which are mimicked by high doses of ATP and α,β -methylene ATP. Following tachyphylaxis to α,β -methylene ATP, these contractile responses are lost, and inhibitions are unmasked. This implies that either at the neuromuscular junction or perhaps within a parasympathetic ganglion, ATP acts as a transmitter onto P_{2X} purinoceptors (Hedlund *et al.*, 1983).

P₂ Purinoceptors in chromatophores

Rapid changes in colour in many animals are due to the activity of dermal chromatophores. In several species of teleost fish, stimulation of sympathetic nerves causes aggregation of chromatophores, which disperse rapidly when stimulation ceases. Noradrenaline, or acetylcholine (depending on species), is released from these nerves, along with ATP, and mediates this aggregation. ATP causes dispersion of chromatophores, and this action is sensitive to blockade by methylxanthines, which indicates that ATP is acting via metabolism to adenosine. Because noradrenaline, or acetylcholine, is rapidly inactivated by catabolism and uptake, while ATP is broken down into active products, when sympathetic nerve traffic ceases the pigment-aggregating stimulus is quickly lost and the pigment-dispersal stimulus pervades (Kumazawa and Fujii, 1984, 1986; Kumazawa *et al.*, 1984; Fujii, 1986; Fujii and Oshima, 1986). Thus there is a rapid control of colour changes. This mechanism is not confined to chromatophores (light-absorbing cells) but may also work in motile leucophores (light-reflecting cells) (Fujii and Oshima, 1986; Oshima *et al.*, 1986).

P₂ Purinoceptors in peripheral ganglia

Responses to exogenous ATP or its analogues have been observed at an electrophysiological level in several types of peripheral ganglia, including the sensory ganglia and intrinsic ganglia of the heart, urinary bladder and gut. In these autonomic ganglia there is histological evidence of the presence of purinergic nerves, and it is therefore possible that within these ganglia the P₂ purinoceptors lie postjunctionally with respect to preganglionic neurones.

Intrinsic cardiac, vesicular and myenteric ganglia

Neurones from intracardiac ganglia and intramural bladder ganglia can be grown in culture (Hassall and Burnstock, 1986; Pittam *et al.*, 1987). In both of these types of cultures, the actions of ATP have been investigated electrophysiologically, and in both systems ATP has excitatory actions (Burnstock *et al.*, 1987; Allen and Burnstock, 1990). In the cardiac neurones the responses to ATP have been examined in detail, and the receptor involved appears to be of the P_{2Y} subtype (Allen and Burnstock, 1990).

In the myenteric plexus *in situ*, ATP has both excitatory and inhibitory activity, in S- and AH-type neurones respectively (Katayama and Morita, 1989). This excitatory action is unaffected by P₁ purinoceptor antagonists,

indicating a true P_2 purinoceptor-mediated event, but the inhibitory action on AH-type neurones is blocked by P_1 purinoceptor antagonists, suggesting the involvement of adenosine following the degradation of ATP (Katayama and Morita, 1989).

Sympathetic ganglia in the bullfrog, *Rana catesbiana*, depolarize in response to applied ATP (Siggins *et al.*, 1977; Akasu *et al.*, 1983a,b; Akasu and Koketsu, 1985), and as in mammalian sensory ganglia (above), adenosine is without effect (Akasu and Koketsu, 1985). This indicates the presence of P_2 purinoceptors and the absence of P_1 purinoceptors on postsynaptic ganglionic cell bodies.

Some sympathetic neurones may release adenosine rather than ATP, as determined from preparations of sympathetic ganglia co-cultured with rat atrial myocytes, the activity of which can be used as an *in situ* bioassay of substances released from the sympathetic nerves (Potter *et al.*, 1983; Furshpan *et al.*, 1986). However, it should be pointed out that in the rat atrium, the actions of ATP can be blocked by P_1 purinoceptor antagonists (Burnstock and Meghji, 1983), so in the co-culture system the bioassay could have been detecting ATP in addition to, or even instead of, adenosine.

Results obtained with histochemical techniques indicate that there are populations of neurones in the intrinsic cardiac ganglia, intramural ganglia and myenteric plexus which contain high concentrations of ATP, and which may therefore be purinergic (Burnstock *et al.*, 1978a; Wilson *et al.*, 1979; Crowe and Burnstock, 1981a,b, 1982). Thus it is plausible that ATP released from nerve terminals within ganglia, i.e. from preganglionic neurones or interneurones, can act on the P_2 purinoceptors on ganglionic nerve cell bodies.

Sensory ganglia

In the vestibular, nodose and trigeminal nuclei of the rat, all of which are sensory, and in the nodose ganglion of the cat, ATP and its analogues, α,β -methylene ATP and β,γ -methylene ATP, cause depolarization of populations of nerve cell bodies with characteristics of a P_2 purinoceptor (Krishtal and Marchenko, 1984, 1986; Krishtal *et al.*, 1983, 1988; Marchenko *et al.*, 1988). There does not appear to be an involvement of P_1 purinoceptors because adenosine and AMP are without agonistic effects (Krishtal *et al.*, 1983; Krishtal and Marchenko, 1986).

P_2 Purinoceptors and neuromodulation in the periphery

Neuromodulation by any substance can occur at several different sites. Perhaps the most common or best-known type of modulation is prejunctional inhibition,

whereby an agent acts on nerve terminals and prevents further release of neurotransmitter. Neuromodulation may occur postjunctionally, and a substance may act on its own receptor and potentiate the response of another agent also acting on its own receptor. With ATP the situation is often complicated because of its rapid hydrolysis by ecto-enzymes which can make it difficult to determine whether ATP is acting *per se*, or whether the pharmacological activity is due to its degradation to adenosine or AMP.

Prejunctional modulation by purines

In the guinea-pig ileum and cat urinary bladder, exogenous ATP inhibits release of acetylcholine and prevents cholinergic excitatory neuromuscular transmission (Takagi and Takayanagi, 1972; Vizi and Knoll, 1976; Dowdle and Maske, 1980; Moody and Burnstock, 1982; Theobald and De Groat, 1988). In both these organs, the actions of ATP and its more stable analogue, β,γ -methylene ATP, are prevented by P_1 purinoceptor antagonists such as theophylline or 8-parasulphophenyltheophylline (8PSPT) (Sawynok and Jhamandas, 1976; Vizi and Knoll, 1976; Dowdle and Maske, 1980; Moody and Burnstock, 1982; Frew and Lundy, 1986; Theobald and De Groat, 1988). Further, it has been shown that the inhibitor of nucleoside uptake, dipyridamole, potentiates the action of these nucleotides (Moody and Burnstock, 1982; Katsuragi *et al.*, 1985), while incubation with adenosine deaminase or AMP deaminase inhibits their activity. These results, therefore, indicate that ATP is acting following its degradation. However, in the guinea-pig ileum it has been suggested that ATP acts on a prejunctional P_2 purinoceptor, because inhibition of 5'-nucleotidase potentiates responses to exogenous ATP (Moody and Burnstock, 1982; Wiklund and Gustafsson, 1986).

There may be a prejunctional P_{2X} purinoceptor in the guinea-pig ileum, activation of which evokes release of acetylcholine (Moody and Burnstock, 1982). This observation is supported by results of Reese and Cooper (1982), who showed that release of acetylcholine from ileal synaptosomes is inhibited by adenosine and low concentrations of ATP, in a theophylline-sensitive manner, but that higher concentrations of ATP stimulate release of acetylcholine.

In the rat tail artery, release of noradrenaline from sympathetic nerves is inhibited by adenosine, ATP, ANAPP₃, β,γ -methylene ATP and the stable analogue of adenosine, 2-chloroadenosine (Shinozuka *et al.*, 1988). These actions are blocked by 8PSPT, but only adenosine is potentiated by a nucleoside uptake inhibitor. This implies that the nucleosides are acting directly on the 8PSPT-sensitive receptor, and that their actions do not involve nascent adenosine. This leads to the proposal of a P_3 purinoceptor, since it is

neither characteristically a P_1 purinoceptor nor a P_2 purinoceptor. However, it should be pointed out that α,β -methylene ATP, which is more resistant to degradation than β,γ -methylene ATP, does not produce such inhibition.

Sympathetic release of noradrenaline in the mouse vas deferens is also inhibited by ATP (Kügelgen *et al.*, 1989). Unlike the situation in the rat tail artery, this action of ATP is not blocked by 8PSPT. The analogue of ATP, ATP γ S, which is resistant to ecto-enzymes (Cusack *et al.*, 1983) mimics the effects of ATP, but β,γ -methylene ATP does not. Although α,β -methylene ATP is without effect, the receptor involved does not appear to be a P_{2Y} purinoceptor because it is not blocked by reactive blue 2 or suramin (Kügelgen *et al.*, 1989).

In the rat, mouse and guinea-pig vas deferens, using stimulus parameters that preferentially stimulate the purinergic component of sympathetic transmission, it has been shown that ATP, and some related compounds, cause prejunctional inhibition (Stone, 1981b, 1985). However, it is not clear what the subclass of the receptor is, even though it is blocked by P_1 purinoceptor antagonists, and it has been suggested that this inhibition is secondary to evoked release of adenosine from an extraneuronal source (Stone, 1981b).

Prejunctional inhibition of release of noradrenaline in the rabbit pulmonary artery is sensitive to theophylline (Katsuragi and Su, 1982), and as in the guinea-pig ileum, theophylline alone enhances release of transmitter (Katsuragi and Su, 1982). It has been suggested that ATP released from nerve terminals could be responsible for this type of inhibitory tone (Katsuragi and Su, 1982).

Postjunctional modulation by ATP

The earliest demonstration of postjunctional modulation by ATP was probably that of Buchtal and Kahlson (1944), who showed that in addition to adenine nucleotides being able to cause tetanic contractions in the feline tibialis anterior muscle, ATP could also potentiate the responses to acetylcholine. ATP potentiates cholinergic activity in the rat diaphragm (Ewald, 1976) and a possible physiological source for this ATP is from the terminals of the phrenic nerve (Silinsky and Hubbard, 1973; Silinsky, 1975). Likewise, in the frog sartorius muscle, ATP potentiates acetylcholine (Saji *et al.*, 1975; Akasu *et al.*, 1981).

In the autonomic nervous system, in frog sympathetic ganglia, ATP depresses release of acetylcholine (Akasu *et al.*, 1983a; Silinsky and Ginsborg, 1983) but enhances the affinity of the postjunctional nicotinic receptor for acetylcholine (Akasu *et al.*, 1983b; Akasu and Koketsu, 1985). In the frog spinal ganglia ATP potentiates responses to γ -aminobutyric acid (GABA)

(Morita *et al.*, 1984). None of these responses involves conversion of ATP to adenosine or activation of P_1 purinoceptors.

In the vas deferens, subthreshold concentrations of ATP potentiate responses to noradrenaline and sympathetic nerve stimulation (Kázić and Milosavljevic, 1980; Huidoboro-Toro and Parada, 1988). Similar results were not obtained by Holck and Marks (1978), who found that adenosine and AMP, but not ATP, could potentiate the actions of noradrenaline. In the mesenteric arteries of rabbits and rats, ATP and noradrenaline show post-junctional synergistic interactions (Krishnamurty and Kadowitz, 1982; Lukackso and Blumberg, 1982), and in the rat this seems to involve the P_{2X} purinoceptor and α_1 -adrenoceptor (Ralevic and Burnstock, 1990). In the guinea-pig and rat portal vein, ATP potentiates responses to noradrenaline (Kennedy and Burnstock, 1986).

P_2 Purinoceptors in the central nervous system

Surprisingly, there is comparatively little information available on the presence and roles of P_2 purinoceptors in the central nervous system, compared with the periphery. This may be because there is a lack of P_2 purinoceptor-mediated activity within the brain and spinal cord. Other than the P_2 purinoceptors involved in sensory pathways in the dorsal horn of the spinal cord, in most regions of the central nervous system ATP has activity mediated via P_1 purinoceptors, following its degradation to AMP or adenosine.

Brain

ATP is present in high concentrations within the brain, varying from approximately 2 mM in the cortex, to 4 mM in the putamen and hippocampus (Kogure and Olonso, 1978). Release of ATP from synaptosomal preparations of discrete areas of the rat and guinea-pig brain, including cortex, hypothalamus and medulla (Barberis and McIlwain, 1976; White, 1978; Potter and White, 1980; White *et al.*, 1980) has been measured. In cortical synaptosomes a proportion of the ATP appears to be co-released with acetylcholine, and a smaller proportion with noradrenaline, but most is not released from either cholinergic or adrenergic varicosities (Potter and White, 1980; White *et al.*, 1980). These results imply that ATP can be released from neurones within the central nervous system, but it has been suggested that neuronally released ATP is rapidly broken down by specifically distributed 5'-nucleotidases which result in the local formation of adenosine (Schubert *et al.*, 1979). Synaptosomes prepared from the rat cortex can rapidly

incorporate [^3H]ADP, with a radiolabelled purine nucleus, which implies that central neurones can uptake or salvage purine nucleotides or nucleosides (Csillag *et al.*, 1982), although it is likely that ADP has to be dephosphorylated to adenosine before it can be taken up (Pull and McIlwain, 1972a,b; Richardson and Brown, 1986).

In preparations of affinity-purified cholinergic nerve terminals from the rat cuneate nucleus, ATP and acetylcholine are co-released (with a ratio of ATP to acetylcholine of approximately 1:10), and 90% of extrasynaptosomal ATP is hydrolysed within 2 min (Richardson and Brown, 1986). Further, 25% of released ATP is taken up again as adenosine. This uptake of adenosine derived from released ATP is inhibited when 5'-nucleotidase is inhibited by the binding of a specific antibody, under which conditions degradation of ATP is also halted (Richardson and Brown, 1986).

The passage of [^3H]adenosine, which is taken up by neurones and converted into nucleotides, has been traced in the brain. In the manner of a neurotransmitter, the radiolabelled substances are transported rapidly along the axons, are released from terminals, and are taken up by postsynaptic neurones (Schubert and Kreutzberg, 1974; Schubert *et al.*, 1976). Nerve terminals, *in vivo* or *in vitro*, take up adenosine, which is then mostly converted into nucleotides, predominantly ATP and cyclic AMP (Pull and McIlwain, 1972a,b; Kuroda and McIlwain, 1974).

As long ago as 1967, ATP was shown to cause excitation of neurones in the cuneate nucleus, when applied extremely focally by iontophoresis from a microelectrode (Galindo *et al.*, 1967). ATP also causes excitation of rat cerebellar Purkinje cells (Hoffer *et al.*, 1971) and of cells in the sensory vestibular and trigeminal nuclei (Krishtal *et al.*, 1983; Salt and Hill, 1983). The response to ATP in the trigeminal nucleus is not always only excitatory, but is often biphasic with an initial transient excitation being followed by a period of depression (Salt and Hill, 1983). Excitatory or biphasic responses are also evoked by ATP in the cortex (Phillis *et al.*, 1979). The excitatory responses evoked by ATP are likely to be mediated via P_2 purinoceptors rather than P_1 purinoceptors, because in these systems adenosine and AMP are either inert or inhibitory (Krishtal *et al.*, 1983; Salt and Hill, 1983). The subtype of P_2 purinoceptor has not been classified, but it is P_{2Z} -like in that it is activated by ATP^{4-} , which is the ion species of ATP ejected during iontophoresis (Phillis *et al.*, 1979), and it is antagonized by elevating the extracellular concentration of divalent cations (Salt and Hill, 1983). Further, ATP is two orders of magnitude more potent than its isostere, β,γ -methylene ATP (Krishtal *et al.*, 1983). In corticospinal cells, ATP can cause excitation but its predominant action is that of inhibition (Phillis *et al.*, 1974), and in CA1 hippocampal pyramidal neurones, ATP appears to have only an inhibitory action, as does AMP (Di Cori and Henry, 1984). Similarly, in

recordings of dorsal cortical neurone activity in the rat brain, both ATP and adenosine cause a depression (Stone and Perkins, 1981).

In several regions of the brain, ATP can cause an inhibition of synaptic transmission, but this is likely to be due to activation of P_1 purinoceptors following its rapid degradation to adenosine by ectonucleotidases. This is so, for example in the olfactory cortex, cerebral cortex and CA1 hippocampal neurones (Okada and Kuroda, 1975, 1980; Kuroda and Kobayashi, 1975; Phillis *et al.*, 1979; Lee *et al.*, 1981; Artemenko and Gerasimov, 1983). Adenosine is thought to mediate these responses because responses to ATP are blocked by adenosine deaminase (Lee *et al.*, 1981) and P_1 purinoceptor antagonists (Okada and Kuroda, 1980; Stone and Cusack, 1989). Further, neither stable analogues of ATP such as α,β -methylene ATP, nor the P_{2X} - and P_{2Y} -specific analogues L- β,γ -methylene ATP and 2-methylthio- (β,γ -difluoro)-ATP, respectively, have any activity (Phillis *et al.*, 1979; Stone and Cusack, 1989). Thus there appear to be presynaptic P_1 purinoceptors, but not presynaptic P_{2X} nor P_{2Y} purinoceptors within the brain.

It would appear that there are postsynaptic P_2 purinoceptors in the brain, and the presumption must be that they are involved in physiological systems of central transmission. As yet, central purinergic pathways involved in a particular behavioural or homeostatic mechanisms have not been identified. It is possible that ATP can modulate central transmission involving glutamatergic or noradrenergic nerves. ATP causes inhibition of high-affinity glutamate uptake by cortical synaptosomal preparations (Warner *et al.*, 1980; Arbuthnot and Cantrill, 1985). This action is specific for glutamate, as opposed to aspartate, and involves a P_{2Z} -like purinoceptor because adenosine and β,γ -methylene ATP are both inactive (Arbuthnot and Cantrill, 1985). In a noradrenergic model, both ATP and adenosine can stimulate tyrosine hydroxylase in cortical synaptosomes (Kuroda and Kobayashi, 1978a), and the suggestion here is that ATP released from neurones is degraded to adenosine and feeds back onto the presynaptic nerve terminals, where its action mediated via P_1 purinoceptors is to increase activity of tyrosine hydroxylase. The physiological significance of both the modulatory mechanisms is obscure, but both are further evidence of the complexity of the role of ATP in the central nervous system.

Spinal cord

Advanced pharmacological and electrophysiological techniques have added to the evidence that ATP and adenosine may be neurotransmitters from sensory nerves in the spinal cord. Using microelectrodes inserted into nerve cell bodies in the cat spinal cord at laminae I and II of the substantia gelatinosa, which receive inputs from sensory afferent nerve fibres, it has been

determined that those cell bodies which are postsynaptically activated in response to mechanical stimulation of the skin (due to C-fibre afferent input) are also excited by ATP (Fyffe and Perl, 1984). In the same study, dorsal horn cells which selectively receive a cutaneous nociceptive input displayed little or no response to ATP. Similarly, ATP has been found to be mostly excitatory on non-nociceptive-receiving nerve cell bodies, rather than nociceptive or wide-dynamic range units in the cat spinal cord (Salter and Henry, 1985).

In synaptosomal preparations of nerve terminals from the dorsal horn of the spinal cord, from guinea-pigs and rats, ATP can be released in response to depolarizing stimuli or the sensory neurotoxin capsaicin (White *et al.*, 1985; Sweeney *et al.*, 1989), but it was also observed that the amount of ATP released is low relative to the population of sensory nerves. This implies that ATP is only contained in a proportion of sensory nerve terminals in the spinal cord. In dissociated cell cultures of dorsal horn neurones, ATP excites only a subpopulation of 25–30% of cells, which would be consistent with only a subpopulation of neurones secreting ATP within the spinal cord (Dodd *et al.*, 1984; Jahr and Jessell, 1984).

The purine-handling enzyme, fluoride-resistant acid phosphatase (FRAP) has been found in a population of small type B cells in dorsal root ganglia (Knyihar, 1971; Nagy and Hunt, 1982; Nagy *et al.*, 1984; Nagy and Daddona, 1985). FRAP is transported from the cell body to the dorsal horn (Knyihar, 1971) and is found in nerve terminals in the deeper layers of the substantia gelatinosa (Nagy and Hunt, 1982). Although the presence of FRAP is not evidence that these neurones utilize ATP as a transmitter, there is a correlation between the projection of these enzyme-containing neurones and the layers of the substantia gelatinosa in which postsynaptic responses to ATP have been observed.

Summary

Several roles for ATP receptors have been described, including: maintenance of vascular tone, where ATP is released as a co-transmitter with noradrenaline from sympathetic nerves; reactive hyperaemia, where ATP released from endothelial cells upon hypoxia leads to vasodilatation via EDRF produced following occupation of endothelial P_{2Y} purinoceptor; haemostatic mechanisms where ATP released from endothelial cells and blood cells acts on vascular smooth muscle, endothelial cells and blood cells to produce net vasodilatation or vasoconstriction. In the genito-urinary and gastrointestinal tracts roles of ATP have been described where it has been shown that ATP acts as a

neurotransmitter, released from autonomic nerves. The involvement of ATP in colour-changing in fish chromatophores has been detailed: here ATP is released as a co-transmitter with noradrenaline or acetylcholine and is responsible for pigment dispersal. This is also an example of a process that is possibly unique to ATP as a neurotransmitter, in that following its release from nerves it is broken down into active substances (adenosine and AMP).

Receptors for ATP have been described which are distributed within peripheral ganglia and the central nervous system. At the level of the spinal cord P_2 purinoceptors appear to be involved in pathways of sensory transmission, but for the most part physiological processes involving central P_2 purinoceptors are yet to be investigated.

The widespread distribution of P_2 purinoceptors and potent effects of ATP on a variety of cells, including hepatocytes, pancreatocytes, immune cells and blood cells, suggests that further examples of the physiological significance of ATP are likely to emerge during the next few years.

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CHAPTER 4

ADENOSINE RECEPTOR SUBTYPES: CLASSIFICATION AND DISTRIBUTION

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Historical aspects – interactions of adenosine with adenylate cyclase

The powerful effect of adenosine on the cardiovascular system has been known since 1927 (Drury and Szent-Györgyi, 1927). Its importance in intercellular communication in the nervous system, however, has only been appreciated since the early 1970s from investigations on the effects of various neurohumoural substances on cyclic AMP metabolism in the brain. The formulation of the second messenger hypothesis by Sutherland in the mid-1960s (Sutherland *et al.*, 1965) provided an enormous stimulus to studies on the mechanism of

action of hormones and neurotransmitters in various cells and tissues, including the central nervous system. It was soon discovered that various biogenic amines such as noradrenaline and histamine, as well as electrical pulses, were potent stimulators of cyclic AMP accumulation in brain slices *in vitro* (Rall and Kakiuchi, 1966). Inclusion of an inhibitor of cyclic AMP breakdown, such as theophylline, with the biogenic amines led to the expected enhancement of cyclic AMP accumulation (Kakiuchi and Rall, 1968a, b). In contrast, the enhanced cyclic AMP content of brain slices observed after electrical stimulation was paradoxically inhibited by theophylline (Kakiuchi *et al.*, 1969). A search for endogenous substances in brain extracts that might mediate the action of electrical pulses led to the discovery that adenosine was a potent stimulator of cyclic AMP accumulation, and that this effect was inhibited competitively by theophylline (Sattin and Rall, 1970). These observations on the stimulation of cyclic AMP accumulation by adenosine and the adenosine antagonist function of theophylline proved to be seminal for elucidating the actions of adenosine in various tissues and in the development of potent adenosine antagonists (see Chapter 7). Thus progress in the field of adenosine in the nervous system has from the start been intimately associated with studies on cyclic AMP metabolism.

Historically, the first neurohumoural effect of adenosine to be reported in the nervous system was its stimulation of cyclic AMP accumulation. Investigations into the action of adenosine as an antilipolytic agent in fat cells, on the other hand, also demonstrated an adenosine-induced inhibition of cyclic AMP generation (Ebert and Schwabe, 1973; Fain, 1973). On the basis of the pharmacological profiles of these responses to various nucleoside derivatives, Londos *et al.* (1980) as well as Van Calcar *et al.* (1979) working with astrocyte cultures, independently proposed the existence of two adenosine receptor classes for adenosine, one mediating an inhibition, the other a stimulation of adenylate cyclase. The receptor classification scheme proposed by these authors has continued to provide a useful framework for approaching the physiological actions of adenosine at the receptor level.

Classification of adenosine receptors

The classification of adenosine receptors emerged from studies on different cells and tissues by different groups of researchers, a fact that has led to the existence of different classification schemes. The broadest classification is that proposed by Burnstock (1978) which differentiates primarily between the actions of adenine nucleotides and adenosine. In this scheme, purinergic receptors are divided into those preferring adenosine, referred to as P₁, and

those preferring ATP, known as P_2 . Nucleotide receptors have been dealt with in Chapter 3. The physiological receptors for adenosine discussed below fall into the P_1 category of Burnstock.

The basis of the further subclassification of the adenosine receptors is provided by the twofold action of adenosine and its derivatives on adenylate cyclase. The first evidence that adenosine can influence adenylate cyclase in different ways was obtained by Londos and Wolff (1977), who demonstrated both stimulatory and inhibitory effects of adenosine on adenylate cyclase. Screening of various adenosine derivatives for their relative potencies as stimulators or inhibitors showed that a stimulation of adenylate cyclase required an intact ribose ring but was tolerant of modifications of the purine moiety. In contrast, the inhibition of adenylate cyclase required an unmodified purine ring. This led to the designation of two sites known as the R site (the stimulatory site) and the P site (which mediates the inhibitory effect). It is important to note that the designation of this inhibitory site with the letter P refers to the dependence on the purine ring and should not be confused with the use of the terms P_1 and P_2 for purinergic receptors proposed by Burnstock. In contrast to the R site, which represents a true, G protein-related receptor class, the P site appears to be located on the catalytic subunit of adenylate cyclase at an intracellular site; its physiological significance is unclear.

It is now known that adenylate cyclase can be regulated by two different adenosine receptor types, both of which fall into the category of R sites, i.e. they are rather intolerant of modifications of the ribose ring, especially in the 2' and 3' positions. These receptors are responsible for the dual physiological regulation of adenylate cyclase by adenosine, which can either stimulate or inhibit cyclic AMP production via G protein-mediated mechanisms. These two receptor types were described independently by Londos *et al.* (1980) in fat cells and by Van Calker *et al.* (1979) in cultured astrocytes from mouse brain. On the basis of their R site nature and their mediation of either an inhibitory or stimulatory action on adenylate cyclase, Londos *et al.* (1980) referred to these as R_i and R_s . Van Calker *et al.* (1979), on the other hand, proposed the designation A_1 and A_2 for the inhibitory and stimulatory receptors, respectively. Although both schemes were originally based upon studies of cyclic AMP synthesis, the A_1/A_2 classification has become more common since this nomenclature is free of any direct implication of an involvement of cyclic AMP as second messenger. This has proved to be an advantage since adenosine can act via cyclic AMP-independent mechanisms (see Chapter 6) and the receptors mediating various physiological actions of the nucleoside can be approached without implying a specific mechanism of action. The general properties of these receptors relevant for their classification will be considered below and are summarized in Table 1.

Table 1 Schemes for the classification of purinergic receptors

Classification scheme	General properties	Action on adenylate cyclase
P_1/P_2	ATP \gg Adenosine	None
P/R	P: Requires unmodified purine moiety	Inhibitory
	R: Requires unmodified 2' and 3' ribose positions	Inhibitory or stimulatory
A_1/A_2 (R_i/R_a)	A_1 : CHA/R-PIA > 2-CADO > NECA > S-PIA	Inhibitory
	A_2 : CGS 21680 = NECA > = CADO > R-PIA	Stimulatory

A_1 and A_2 receptors

The initial reason for proposing two receptor classes was the observation that the relative dose–response curves for various adenosine derivatives differed for the inhibitory and stimulatory actions on cyclic AMP synthesis. The most commonly used agonists for studying adenosine receptors are the N^6 -substituted adenosine derivatives such as N^6 -phenylisopropyladenosine (PIA), N^6 -cyclohexyladenosine (CHA) and N^6 -cyclopentyladenosine (CPA) and the N -alkylcarboxamido derivatives such as N -ethylcarboxamidoadenosine (NECA) and N -cyclopropylcarboxamidoadenosine. Also of importance is the halogenated derivative, 2-chloroadenosine. Although other derivatives have been developed which show a high affinity for A_1 receptors, those mentioned above have been most commonly employed in physiological systems when attempting to define the receptors involved. A detailed description of the agonists and antagonists available for studying adenosine receptors is beyond the scope of this chapter and is to be found in Chapter 7. Only the substances most commonly used as a basis for classifying adenosine receptors will be considered here.

The most frequently employed criteria for distinguishing between A_1 and A_2 receptors are the absolute and relative activities of the agonists R-PIA and NECA. In general, the A_1 receptors are sensitive to low concentrations of adenosine derivatives in the nanomolar range, whereas micromolar concentrations are required for A_2 receptor activation. The relative activities of R-PIA and NECA provide a major criterion for distinguishing between

these receptor types. Thus the affinity of the A₁ receptor for R-PIA is greater than that for NECA, whereas the reverse is true at the A₂ receptor. It is important to note, however, that NECA, despite its relatively greater affinity for A₂ receptors compared to R-PIA, still has a high affinity for A₁ receptors. It is therefore essential to perform comparisons of the actions of various derivatives and not to rely only on the action of NECA or related compounds as a means of identifying A₂ receptors.

In earlier studies, the stereoselectivity for R-PIA over its enantiomer S-PIA was suggested as a criterion for identifying A₁ receptors, but the wide range of relative affinities observed for these substances has made them less useful than a comparison between R-PIA and NECA. More recently, a relatively specific agonist for A₂ receptors has been introduced, CGS 21680 (Hutchison *et al.*, 1989). This provides an important addition to the range of substances used to study adenosine receptor subtypes.

No receptor classification can rely entirely on a comparison of the relative actions of only two agonists, and in many studies the effects of several agonists have been compared. In general there is good agreement between the relative dose-response curves of a broad range of adenosine analogues on A₁-like nucleoside actions in various cells and tissues (Ukena *et al.*, 1987; Williams, 1987). One exception is, however, 2-chloroadenosine, whose activity relative to NECA can differ (Bruns *et al.*, 1986). For instance, a discrepancy is found between the relative actions of these substances on evoked potentials in a hippocampal slice preparation and their ability to displace A₁ receptor ligands from brain membrane preparations (Reddington *et al.*, 1982). Discrepancies in dose-response relationships involving 2-chloroadenosine have also been observed in peripheral tissues (Collis, 1985). This, together with the observation that the pharmacology of A₁-like responses at presynaptic sites differs from other systems has led to the proposal of a third adenosine receptor type, designated A₃ (Ribeiro and Sebastião, 1986).

The A₂ adenosine receptor class is the one that was first identified as mediating the stimulatory action of adenosine in intact cell preparations. Although this action has been reported in many cells and tissues, attempts to find an adenosine-stimulated adenylate cyclase in cell-free systems from brain tissue have been less successful. Significant stimulatory effects of adenosine analogues on adenylate cyclase in crude membrane fractions from brain tissue have only been found in a very few regions (Premont *et al.*, 1979). Membranes from other regions, such as cerebral cortex or hippocampus show no adenosine-stimulated adenylate cyclase, despite the strong stimulation of cyclic AMP accumulation by adenosine observed in slice preparations from these areas. The reason for this is unknown. Pharmacologically, there are some differences between the responses of cyclic AMP to adenosine derivatives. The affinities of various adenosine analogues are generally higher in the cell-free

systems than in intact cells and some differences are observed in the relative actions of agonists and antagonists. This has led to the suggestion that the terms A_{2a} and A_{2b} should be used for the A_2 receptor subclasses that mediate the stimulatory actions of adenosine in striatal membranes and in cerebral cortical slices, respectively (Daly *et al.*, 1983). Indeed, as will be described later, radioligand-binding techniques only allow detection of binding sites in those areas characterized by A_{2a} receptors, indicating that there are important differences in the molecular properties of these receptor subtypes.

In the above discussion only the actions of agonists have been considered. The most important class of adenosine receptor antagonists are the methylxanthines. Indeed, one of the earliest pieces of evidence for the existence of adenosine receptors was the observation that theophylline is an antagonist of the stimulatory action of adenosine on cyclic AMP accumulation in brain slices (Sattin and Rall, 1970). The same is true of other alkylxanthines such as caffeine and isobutylmethylxanthine. These have provided the basis for developing more potent antagonists, the most commonly used of which is 8-cyclopentyl-1,3-dipropylxanthine (DPCPX*). This shows an approximately 700-fold selectivity for A_1 receptors (Lohse *et al.*, 1987) and has provided a valuable tool for characterizing the receptors mediating the electrophysiological actions of adenosine, which have in most cases been found to be of the A_1 type. At present no antagonists of comparable specificity are available for the A_2 adenosine receptor class.

Approaches to receptor characterization

Characterization of adenosine receptors *in situ*

The A_1/A_2 receptor classification has provided a useful framework for approaching the pharmacology of adenosine action. However, the practical application of this classification scheme does present some difficulties. The most obvious problems are encountered when applying adenosine receptor ligands *in vivo* and arise because of the chemical nature of the available agonists and antagonists. In studies of the central nervous system, especially, the poor

* This substance was originally referred to as 1,3-dipropyl-8-cyclopentylxanthine by Lee and Reddington (1986a) rather than the chemically correct 8-cyclopentyl-1,3-dipropylxanthine. This has led to the use of the abbreviation DPCPX, and the radioactive form of this compound is marketed under this name. Although this substance has also been described as CPDPX (Olsson and Pearson, 1990) or CPX (Bruns *et al.*, 1987), the original term, DPCPX has found more widespread use and will be adopted in this chapter.

penetration of the blood–brain barrier of most receptor ligands prevents their reliable use as activators of central receptors. Further, the pharmacokinetics of the various adenosinergic drugs are unknown. It is therefore not practicable to use, for example, a comparison between the effects of NECA and R-PIA on central nervous function after peripheral administration since the distributions of these two chemically distinct ligands are unknown and could be quite different. Indeed, application of R-PIA peripherally is a very inefficient route since most of the ligand accumulates in blood vessels and does not penetrate into the brain (Brodie *et al.*, 1987).

A further problem encountered with the potent but rather lipophilic N^6 -substituted A_1 receptor agonists is their restricted range of action. Thus, experiments in which R-PIA was applied intraventricularly showed it to affect evoked potentials of hippocampal neurones *in situ* only in the direct vicinity of the site of application (Brodie *et al.*, 1987). Diffusion within the cerebral ventricles is therefore severely restricted.

Careful consideration should also be given to the manner of ligand administration in *in vitro* and *in vivo* models employing direct application to neurones since this can influence interpretation of relative efficacies of different substances. Some controversy has been generated about the A_1 or A_2 nature of the receptors mediating the depressive actions of adenosine derivatives on neurones when R-PIA and NECA are applied either by iontophoresis *in vivo* (Phillis, 1982) or by bath application in an *in vitro* slice preparation (Reddington *et al.*, 1982; Dunwiddie and Fredholm, 1984). After iontophoretic application *in vivo*, NECA proved to be more effective than R-PIA as an inhibitor of neuronal activity, suggesting mediation via A_2 receptors. The pattern obtained after bath application *in vitro*, on the other hand, shows R-PIA to be more effective than NECA, consistent with the activation of A_1 receptors. This difference could be explained by the fact that the apparent relative potencies of such analogues depends upon how quickly they reach equilibrium in the tissue (Dunwiddie and Fredholm, 1984). Since NECA reaches equilibrium more rapidly than the more hydrophobic R-PIA or CHA, measurement of the effects of these substances at short time intervals shows NECA to be more potent than R-PIA and CHA. The reverse is, however, true when the substances are allowed to reach equilibrium before taking measurements. Direct comparison of the effects of *in vivo* application of adenosine derivatives to cerebellum by iontophoresis and bath application to cerebellar slices has confirmed this conclusion (Dunwiddie *et al.*, 1984). This demonstrates the importance of methodology in approaching the classification of adenosine receptors by pharmacological means and suggests that local and bath application of agonists gives information about different aspects of receptor–ligand interaction. This will be considered further in the following section.

The most reliable method for identifying receptor subtypes is the use of subtype-specific antagonists. At present, however, it is not possible to distinguish A₁ and A₂ receptors unequivocally in this way. Although antagonists such as DPCPX show a good selectivity for A₁ receptors, antagonists specific for A₂ receptors are unavailable. Currently, therefore, most approaches to receptor classification have employed agonists as described above. The sometimes anomalous behaviour of agonists demonstrates the importance of local factors in determining their pharmacological properties and illustrates the perils of using agonist dose-response relationships for receptor classification. It is therefore perhaps unsurprising that some effects of adenosine derivatives have been described that do not fit entirely into the A₁/A₂ classification scheme. For instance, the presynaptic action of adenosine derivatives on transmitter release at the neuromuscular junction (Ribeiro and Sebastião, 1986) and the interactions of adenosinergic drugs with histamine receptors to modulate the phosphatidylinositol second messenger pathway (Alexander *et al.*, 1989) differ from the original dose-response relationships described for adenylate cyclase activation or inhibition. This has led to the postulate of a third receptor type, designated A₃ (Ribeiro and Sebastião, 1986). However, in the absence of clearly specific antagonists it would seem premature to revise the current, albeit no longer completely adequate classification scheme by addition of another receptor subclass. Experience with other receptors such as those for β -adrenergic, muscarinic or dopaminergic drugs, has shown that more detailed knowledge of receptor structure obtained by molecular cloning techniques reveals distinct molecular receptor subspecies (see Venter *et al.*, 1988 for a review of some aspects of this field). It is to be hoped that recent progress in the purification of the A₁ adenosine receptor as outlined in Chapter 5 will soon lead to the establishment of the primary structure of this receptor and the development of probes for related receptor molecules. In our opinion, the molecular characterization of adenosine receptor subtypes should be awaited before a revision of the present receptor classification scheme is attempted.

Radioligand binding methods and physiological receptors

Progress in the study of receptors in recent years has been stimulated by the development of radioligand binding assays. In this method, the direct binding of radioactively labelled drugs is studied to cell membranes, cells in culture or tissue sections. The biological material is incubated with radioactively labelled drugs and the amount of saturable binding measured at the end of the incubation period after separating free and bound ligand. An estimate is also always made of the amount of radioligand non-specifically bound, which is by definition unsaturable. However, although saturability is a prerequisite for the identification of binding sites as biological receptors, it is in itself

insufficient as a criterion. For this reason, the relationships between ligand-binding sites and physiological receptors must be carefully established before ligand binding can be used with confidence as a tool for studying a specific receptor type.

The most common means of characterizing receptors is in terms of the relative affinities of agonists and antagonists. Comparisons are therefore generally made between the concentrations required to induce half maximal biological responses with the dissociation constants of radioligands in binding assays. In the case of the A_1 adenosine receptor, good correlations have been observed between the equilibrium binding properties of agonist and antagonist radioligands such as R-PIA, CHA or DPCPX, and the relative activities of such ligands as inhibitors of adenylate cyclase or modulators of evoked potentials in hippocampal slices. In electrophysiological experiments with slice preparations, drugs are usually added to the incubation medium and applied until a steady state is reached before taking measurements. Thus the relative activities of various agonist ligands are measured under conditions corresponding to those used in binding studies for estimating equilibrium binding properties. This is important in the light of the different apparent relative affinities of R-PIA and NECA found by direct application described above. This difference in apparent properties might therefore reflect the difference between a true affinity measured under equilibrium conditions and a rate of activation found after short-term application by iontophoresis or pressure injection. Methodological factors thus affect the experimental outcome and must be carefully considered when interpreting results in terms of receptor types.

The good correlation between the relative affinities of various A_1 agonists and antagonists in binding assays and their electrophysiological activities indicates the usefulness of such radioligands for studying the receptors responsible for the modulatory actions of adenosine on neuronal activity. Further support for this has been obtained in the rat hippocampus where a good correlation is found between the potency of adenosine in various hippocampal subregions and the number of binding sites for [3H]CHA (Lee *et al.*, 1983a, b). Generally, therefore, radiolabelled A_1 receptor ligands provide useful tools for studying electrophysiologically relevant receptors. The situation is more complex in the case of the A_2 receptors and will be considered further below.

Radioactive ligands for studying A_1 receptors

Several agonist and antagonist ligands with good specificity and high affinity are available for studying the A_1 adenosine receptors. For accurate quantitative studies as well as for clear autoradiograms it is desirable that the non-specific binding components be as small as possible. In general, the tritiated ligands

have proved to more useful since they show lower non-specific binding than iodinated ligands (Lohse *et al.*, 1987). For this reason, the tritiated ligands are most commonly used.

A second consideration is the agonist or antagonist nature of the radioligands available. It is a well-known fact that agonist binding can be influenced by several factors, such as guanine nucleotides and cations. This can yield important information about receptor regulation and has provided evidence for the heterogeneity of A₁ receptor-G protein complexes (Fastbom and Fredholm, 1987). However, it is often preferable to use an antagonist radioligand which allows, for instance, detailed analysis of multiple affinity states of agonists to be performed by displacement without having to take into account the binding of the radioligand itself to multiple sites. A frequently used ligand is [³H]DPCPX. This antagonist has a high affinity for A₁ receptors and shows only one affinity state. It has a low degree of non-specific binding and is therefore highly suitable for detailed studies of receptor properties.

For autoradiographic studies it is necessary to use ligands having a high specific radioactivity and low K_d to reduce exposure times and hinder dissociation of the radioligand from its receptor when the sections are washed free of unbound ligand. A low degree of non-specific binding is also desirable to give a clear receptor distribution. All these criteria are fulfilled by the tritiated A₁ receptor ligands, in particular by [³H]DPCPX which can at this time be considered the ligand of choice for studying A₁ receptor distribution by autoradiography, particularly if quantitative evaluation of the autoradiographs is required. Nevertheless, tissue sections labelled with tritiated ligands must be exposed to tritium-sensitive film for several weeks to obtain clear autoradiograms. Shorter exposure times can be obtained by using iodinated ligands such as [¹²⁵I]HPIA due to their higher specific radioactivities, but the higher background due to non-specific binding leads to a lower resolution than found with tritiated ligands (Weber *et al.*, 1988). However, when embarking upon a study with a new tissue it is often useful to perform preliminary experiments with an iodinated ligand to obtain a general impression of A₁ receptor distribution within a few days. More detailed experiments using tritium-labelled ligands can then be planned.

Radioactive ligands for studying A₂ receptors

The availability of specific ligands for studying the A₁ adenosine receptor has led to rapid progress in the characterization of this receptor class. In contrast, the A₂ receptor field has lagged behind in this respect. Until recently, the only ligand that could be used for studying the A₂ receptor was NECA, which has a higher affinity for A₂ receptors than the typical A₁ agonists, but nevertheless binds with a relatively high absolute affinity to the latter class.

Therefore in ligand binding experiments [^3H]NECA binds to both A_1 and A_2 receptors. To circumvent this problem, two strategies have been employed to eliminate [^3H]NECA binding to A_1 receptors: (1) the inclusion of a saturating amount of an unlabelled high-affinity A_1 ligand such as N^6 -cyclopentyladenosine (Bruns *et al.*, 1986) or (2) pre-incubation of membrane preparations with the alkylating agent, *N*-ethylmaleimide, which destroys the binding of agonists to the A_1 receptor (Yeung and Green, 1983, 1984).

In the first strategy, a small amount of non-radioactive A_1 agonist or antagonist is included in the reaction mixture to displace [^3H]NECA from A_1 sites, and the degree of non-specific binding is determined with a high concentration of an A_1/A_2 ligand such as R-PIA. The binding to A_2 receptors is then given by the difference in [^3H]NECA binding between a low and high concentration of non-radioactive displacer. This strategy has been applied successfully to membrane fractions from striatum where these sites have a pharmacological profile characteristic of an A_2 receptor, with NECA being a more effective displacer of [^3H]NECA binding than R-PIA (Bruns *et al.*, 1986). This method has, however, proved to be unsuccessful for the characterization of A_2 receptor sites in cryostat sections using autoradiography. In this case [^3H]NECA binds to three components (Lee and Reddington, 1986a, b; Reddington *et al.*, 1986): (1) the A_1 receptor – this binding can be displaced with low concentrations of A_1 ligands such as R-PIA or DPCPX as in membrane fractions; (2) the A_2 sites, which are further displaced by higher concentrations of R-PIA; and (3) a further class of saturable [^3H]NECA-binding sites that are displaced by unlabelled NECA but not by even millimolar concentrations of R-PIA. In membrane fractions, these non- A_1 /non- A_2 [^3H]NECA-binding sites are normally part of the 'non-specific' binding remaining in the presence of high concentrations of N^6 -substituted A_1 ligands; they increase the apparent non-specific binding level but do not interfere with the pharmacological analysis of [^3H]NECA binding to A_2 sites. The situation in autoradiography is more complicated since these sites have proved to have a specific distribution in the brain, and occur in regions lacking specific A_2 binding (see next section). The nature of these [^3H]NECA-binding sites is unknown, but their presence makes it impossible to pick out easily the areas containing specific A_2 sites without detailed quantitative autoradiographic analysis.

The problem of the non- A_1 /non- A_2 [^3H]NECA-binding sites can fortunately be overcome by use of the second strategy mentioned above. Pre-incubation of membrane fractions or cryostat sections with *N*-ethylmaleimide (NEM) inactivates binding not only to the A_1 receptors but also to this non-adenosine receptor site (Reddington *et al.*, 1986). The remaining [^3H]NECA-binding sites have the pharmacological properties of A_2 receptors with NECA being more effective as a displacer than R-PIA. In addition, the amount of

non-specific binding is low. This technique allowed the first accurate localization of these sites to be defined using autoradiography.

More recently, a new ligand has been introduced, [^3H]CGS 21680 that shows a high selectivity for A_2 receptor sites (Jarvis *et al.*, 1989b). Autoradiographic studies using this compound, which does not bind to the non- A_1 /non- A_2 sites described above, have essentially confirmed the earlier A_2 receptor distribution found using [^3H]NECA after NEM pretreatment (Jarvis and Williams, 1989). CGS 21680 will certainly prove to be an important substance for further analysis of A_2 receptors by ligand binding methods.

The classification of A_2 receptors into A_{2a} and A_{2b} subclasses should be borne in mind when considering ligand binding to A_2 receptors. As will be evident from the highly restricted localization of A_2 -binding sites, which corresponds to that of adenosine-stimulated adenylate cyclase in isolated membrane preparations, no binding is observed to areas such as the cerebral cortex that show a strong stimulation of cyclic AMP accumulation to adenosine in slice preparations. Therefore, for reasons that are unclear, only the A_{2a} subclass can at this time be approached using ligand binding studies.

Distribution of receptors

Information concerning the distribution of adenosine receptors has been derived from two general types of investigations. First, studies of the biochemical and physiological actions of adenosine have been undertaken in tissues taken from various sites within the nervous system. These include studies using cellular and broken cell preparations which have provided clues pertaining to the regional, cellular and subcellular localization of adenosine receptors. Radioligand binding studies provide a second source of information regarding the distribution of adenosine receptors. In these studies, the regional distribution of binding sites for adenosine receptor ligands has been elucidated using autoradiographic and regional dissection techniques. The next two sections will examine the distribution of A_1 and A_2 receptors in the central nervous system and will discuss current evidence regarding the subcellular localization of these receptors.

A_1 Receptors

The availability of specific agonist and antagonist ligands has greatly facilitated studies of the distribution of A_1 adenosine receptors (see above). Autoradiographic studies examining the binding of radiolabelled CHA, R-PIA, hydroxyPIA

and DPCPX provide a consistent picture of the regional distribution of A_1 receptors in the rat (Lewis *et al.*, 1981; Goodman and Snyder, 1982; Lee, 1985; Lee and Reddington, 1986a, b; Tetzlaff *et al.*, 1987; Weber *et al.*, 1988; Fastbom and Fredholm, 1990). High concentrations of A_1 sites are observed in the following regions: (i) molecular layer of the cerebellum, (ii) dendritic zones of the hippocampus proper, (iii) medial geniculate and, (iv) superficial layers of the superior colliculus (Fig. 1). Moderate levels of A_1 sites are observed in the following areas: (i) cerebellar granular layer, (ii) layers I, IV, V and VI of the neocortex, (iii) lateral septal nucleus, (iv) mammillary bodies, (v) caudate/putamen, (vi) molecular layer of the dentate gyrus, (vii) nucleus accumbens, (viii) substantia nigra, (ix) substantia gelatinosa of the spinal cord, and (x) several thalamic nuclei. In general, areas of white matter exhibit very low levels of binding except for a band of low-moderate binding in the middle portion of the corpus callosum. The pattern of distribution of A_1 receptors appears to be conserved across a variety of species in some regions of the brain (e.g. hippocampus: Fastbom *et al.*, 1977; Lee *et al.*, 1986) but not in other regions (e.g. cerebellum: Fastbom *et al.*, 1977).

The precise distribution of A_1 receptors can be quite complex in certain areas of the CNS. An example of the intricacy of this distribution pattern is found in the CA1 region of the hippocampus. High concentrations of A_1 sites are observed in the strata radiatum and oriens while only low-moderate levels are observed in the strata pyramidale and lacunosum/moleculare. Thus, a laminar pattern of A_1 sites is observed within the dendritic arbor of CA1 pyramidal cells. In addition to this laminar pattern of distribution, two gradients of receptor density are observed in the CA1 region in different aspects of the hippocampus. Higher levels of binding to A_1 sites are observed in the CA1 region of the septal aspect of the hippocampus than are observed in the temporal aspect of the hippocampus. This reflects a greater number of receptors (B_{\max}) in the septal aspect with no difference in the apparent affinity (K_d) of the binding sites between the two aspects. A second gradient of A_1 sites within the CA1 region is observed across the transverse axis of the hippocampus; the CA1a subregion exhibits greater binding than the CA1b subregion. Subtle differences in the density of receptors may be of crucial physiological importance. Electrophysiological studies have shown that the strength of adenosine's neuromodulatory action within the CA1 region is correlated with the local density of receptors (Lee *et al.*, 1983a, b). Thus, the concentration of A_1 receptors varies widely among, and within, regions of the CNS, and this appears to be one of the critical factors determining the potency of adenosine neuromodulation.

Adenosine receptors of the A_1 category are found on multiple cell types and in some cases have been shown to be restricted to specific structural elements on a given cell type. The early studies of Van Calker *et al.* (1979)

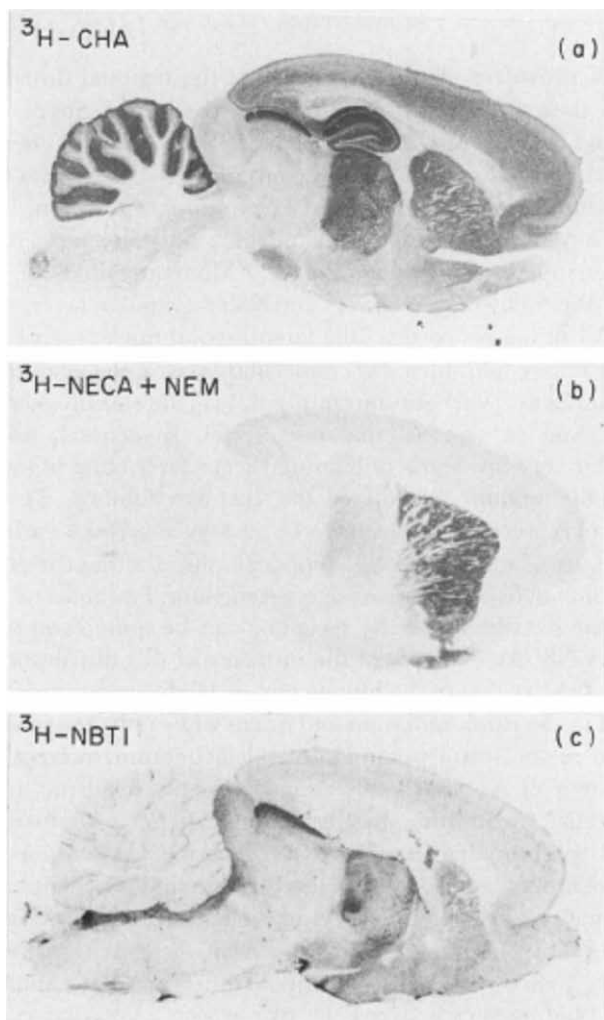


Figure 1 The distribution of: (a) A_1 receptors ($[^3H]$ CHA binding), (b) a subset of A_2 receptors (A_{2a} sites; $[^3H]$ NECA binding with NEM pretreatment) and (c) a subset of adenosine uptake sites ($[^3H]$ NBTI binding). Sagittal sections of the rat brain, cut with a cryostat, were incubated on microscope slides in the presence of the above radioligands. 3H -Sensitive film was placed against the slides to create an autoradiographic image of the binding sites (dark areas are positive for binding). In (a) $[^3H]$ CHA binds to A_1 receptors with highest density in the hippocampus, cerebellum and superior colliculus. In (b) the binding of $[^3H]$ NECA in the presence of NEM labels A_{2a} sites with highest densities in the striatum, nucleus accumbens and olfactory tubercle (see also Fig. 2b). In (c) $[^3H]$ NBTI binding to a subset of adenosine uptake sites is shown. In this plane of section, binding is observed in the superior colliculus, Purkinje cell layer of the cerebellum, inferior colliculus, striatum, thalamus, hypothalamus and the dorsal aspect of the brainstem.

utilized cell cultures of 'glial character' to derive the classification system of A_1 vs. A_2 receptors. This observation suggests that adenosine receptors of both A_1 and A_2 types can be found on neuroglia in brain tissue. The following discussion will focus primarily on the neuronal localization of adenosine receptors. This emphasis is a reflection of the limited number of studies examining adenosine function in glia cells rather than the relative functional importance of adenosine receptors on glial sites.

Electrophysiological studies have shown that adenosine inhibits neuronal activity in a wide range of brain regions (e.g. Kostopoulos and Phillis, 1977). Neuronal discharge rates and synaptic responses are attenuated by adenosine and analogues of adenosine in a dose-dependent manner and these effects are blocked by adenosine receptor antagonists (e.g. Reddington *et al.*, 1982). Data from studies of this type suggest that adenosine receptors are located on neurones in regions found across the neuroaxis.

Considerable direct and indirect evidence exists for a presynaptic locus of adenosine A_1 receptors on neurones. Adenosine inhibits the release of a variety of neurotransmitters including glutamate, GABA, noradrenaline (norepinephrine), acetylcholine, serotonin and dopamine. This effect is mediated by A_1 -type receptors in those cases in which the issue of receptor identity has been examined (Jakisch *et al.*, 1985; Fredholm and Dunwiddie, 1988; Brown *et al.*, 1990). Evidence demonstrating that adenosine inhibits neurotransmitter output in synaptosomal preparations (e.g. Michaelis *et al.*, 1979) strongly suggests that this effect is mediated by receptors located on presynaptic elements. Electrophysiological studies also indicate that adenosine exerts an effect on presynaptic release characteristics in the peripheral and central nervous systems (Ginsborg and Hirst, 1972; Dunwiddie and Haas, 1985). Taken together, these findings provide strong evidence for a presynaptic site of adenosine A_1 receptors and that activation of these receptors results in a reduction in the output of neurotransmitter.

Another type of evidence supporting the presynaptic localization of A_1 sites is derived from autoradiographic studies examining mutant animals in which specific cellular lesions occur spontaneously. Studies of this type suggest that cerebellar parallel fibres are A_1 receptor-positive. This is consistent with electrophysiological data demonstrating that the activity of cerebellar Purkinje cells is inhibited by adenosine, an effect mediated by A_1 receptors (Wojcik and Neff, 1983a; Dunwiddie *et al.*, 1984). These two sets of data suggest that adenosine inhibits neuronal activity in Purkinje cells by inhibiting the release of excitatory neurotransmitter from parallel fibres. Inhibition of the release of excitatory transmitters may represent a common mechanism underlying the inhibitory actions of adenosine at several sites in the nervous system, including the release of: (a) acetylcholine from intrastriatal neurones, and (b) glutamate from Schaffer collateral and commissural inputs to the hippocampus.

Evidence for both presynaptic and postsynaptic localization of A₁ receptors has been derived from radioligand binding studies examining the effects of electrolytic, mechanical and chemical lesions. Lesions of certain afferent systems to the hippocampus, striatum and superior colliculus result in reductions in the density of binding to A₁ sites in the target regions of the afferents (Goodman *et al.*, 1983; Schubert *et al.*, 1985, 1986; Geiger, 1986; Deckert and Jorgensen, 1988; Dragunow *et al.*, 1988; Onodera and Kogure, 1988; Alexander and Reddington, 1989). In contrast, lesions of other afferents to the hippocampus, spinal cord and striatum do not appear to reduce the amount of binding in the target structures (Murray and Cheney, 1982; Geiger *et al.*, 1984; Lloyd and Stone, 1985; Alexander and Reddington, 1989). These data suggest that A₁ sites are associated with presynaptic elements on some, but not all, afferent systems and that a given structure (e.g. hippocampus) can possess afferent systems that are A₁-positive and other afferents that are not. Studies of neuronal lesions also offer evidence for a postsynaptic localization of A₁ sites. Selective lesions of CA1 pyramidal cells result in a substantial reduction in A₁ binding sites in the dendritic fields of these cells (Onodera *et al.*, 1987). This finding suggests that A₁ receptors are located on the membranes of apical and basal dendrites of CA1 pyramidal cells.

Numerous problems exist, however, for the interpretation of lesion studies that attempt to establish the subcellular locus of a binding site. Assuming that a lesion is accurate, specific and complete, it is often unclear what the secondary effects of the lesion are on the associated or surrounding tissue. A good example of such problems is found in lesion studies involving afferents to the dentate gyrus of the hippocampus. Lesions of the entorhinal cortex or the perforant path (i.e. a major efferent system of the entorhinal cortex) denervate dentate gyrus granule cells. Electron microscopic studies of the molecular layer of the dentate gyrus, the synaptic target of the perforant path, have demonstrated a massive degeneration of synaptic boutons following lesions of the perforant path (Matthews *et al.*, 1976; Lee *et al.*, 1977). Lesions of this type also result in a substantial loss of [³H]CHA binding in the molecular layer of the dentate gyrus (Schubert *et al.*, 1986; Dragunow *et al.*, 1988). Lesion studies of the latter type have been interpreted as evidence for a presynaptic localization of adenosine A₁ receptors on terminals of the perforant path. The interpretation of these data, however, is complicated by several issues. Lesions of the perforant path result in the loss of not only presynaptic boutons of the perforant path but also their targets, the postsynaptic spines on dentate gyrus granule cells (Matthews *et al.*, 1976; Lee *et al.*, 1977). Trans-synaptic degeneration of this type is not unique to synapses in the hippocampal formation and may be a common event in denervation studies (see also Hattori and Fibiger, 1982). Consequently, the reduction of binding in the dentate gyrus molecular layer could reflect a loss of presynaptic and/or postsynaptic sites. It is noteworthy

in this context that lesions of postsynaptic granule cells also result in a substantial loss of [^3H]CHA binding in the molecular layer (Dragunow, pers. commun.; Lee, unpublished data). 'Selective' presynaptic or postsynaptic lesions may therefore have a significant impact on their associated synaptic elements (e.g. trans-synaptic degeneration). Moreover, lesions of this type result in substantial modifications of the microglial and astroglial populations in the region of denervation. A change in the density of a binding site could therefore reflect the modification or degeneration of one or more structural elements in a region. This renders the interpretation of lesion-induced alterations in binding sites equivocal in many cases.

A final consideration when examining the results of lesion studies is whether the experimental technique is actually capable of resolving the magnitude of change that occurs with deafferentation. If binding to a given afferent system comprises less than 5% of the total binding in a region, then it may not be feasible to observe a significant change in total binding following the selective removal of this system. For instance, adenosine is known to inhibit the release of noradrenaline in the hippocampus, an effect mediated by A_1 receptors (Fredholm and Dunwiddie, 1988). However, lesions of the noradrenergic afferents to the hippocampus do not alter binding to A_1 sites (Murray and Cheney, 1982). Several interpretations are possible for the negative results of this lesion study, only two of which will be considered here. First, it is possible that the noradrenergic terminals, which comprise a relatively small percentage of the neuropil in the hippocampus, possess insufficient numbers of A_1 receptors to be detected against the background for total A_1 binding in the hippocampus. Second, it is possible that terminal sprouting of another group of A_1 -positive afferents occurred and replaced the noradrenergic terminals. In this case, A_1 -positive, noradrenergic terminals could have been lost but replaced subsequently by the proliferation of another class of A_1 -positive terminal. While not a comprehensive discussion of the problems associated with lesion studies, the foregoing issues illustrate the difficulty of assigning a binding site to a specific cellular locus when utilizing data from lesion studies.

Electron microscopic studies of the ultrastructural distribution of iodohydroxy-PIA binding sites indicate that A_1 receptors are associated with multiple cellular elements in the CA1 region of the hippocampus (Tetzlaff *et al.*, 1987). This approach is unable to discriminate between presynaptic and postsynaptic localization at synaptic sites but it is estimated that *ca.* 30% of the binding is associated with synaptic complexes while little or no binding is found on the preterminal axons. In addition, a substantial amount (at least 20–30%) of binding occurs on postsynaptic, dendritic membranes along the extrasynaptic portions of the dendrites. These observations are consistent with electrophysiological studies demonstrating both synaptic and extrasynaptic actions of adenosine mediated by A_1 receptors in the CA1 region (Reddington *et al.*,

1982; Dunwiddie and Fredholm, 1984; Lee *et al.*, 1984; Dunwiddie and Fredholm, 1989).

Taken together, the above evidence indicates that adenosine A₁ receptors are distributed in a heterogeneous manner in the CNS and can be located on multiple cellular and subcellular elements. Even within a single region, A₁ receptors may be found at presynaptic, postsynaptic and perhaps glial sites. The preceding discussion points out that many studies, while suggesting a specific cellular localization, may ultimately provide less than compelling evidence as to the precise cellular site of a receptor. In this context, one more caveat to any study examining ligand binding sites must be reiterated. The presence of a binding site, even one that fits all the pharmacological criteria of a specific receptor category, does not establish the receptor nature of this site. Correlative physiological and/or biochemical studies are essential to authenticate the functional (i.e. receptor) character of any binding site.

A₂ Receptors

The characterization of A₂ receptors has proceeded somewhat more slowly due to the lack of specific ligands. Nonetheless, radioligand binding assays have been developed (see above) and compounds with increasing specificity for A₂ sites are becoming available (Jarvis *et al.*, 1989b). Recent evidence from radioligand binding studies, in conjunction with regional studies examining adenosine-stimulated cAMP formation, have provided a general picture of the distribution of A₂ receptors.

Biochemical studies examining the regional distribution of adenosine-sensitive adenylate cyclase in broken cell preparations have revealed a rather restricted distribution of what are now termed A_{2a} receptors (Premont *et al.*, 1979). Adenosine-stimulated adenylate cyclase was demonstrated in the striatum, nucleus accumbens, globus pallidus, olfactory tubercle and posterior portions of the cerebellar cortex in membrane samples prepared from these regions. A virtual absence of adenosine-stimulated adenylate cyclase was observed in a number of other CNS structures including the cerebral cortex, hippocampus and hypothalamus. In contrast to observations in broken cell preparations, adenosine-stimulated adenylate cyclase shows a much more widespread distribution when *in vitro* slices of the CNS are examined. These A₂ receptors (perhaps A_{2b} sites and A_{2a} sites combined) are found in numerous regions including the cerebral cortex, hippocampus, striatum and cerebellum (Daly, 1975).

Radioligand binding studies have met with some success in characterizing the distribution of A₂ receptors. As described above, [³H]NECA binds to both A₁ and A₂ receptors but the A₁ component of NECA binding can be blocked by inclusion of the alkylating agent, NEM, in the incubation mixture

(Yeung and Green, 1983, 1984). The autoradiographic distribution of [^3H]NECA binding in the presence of NEM is shown in Figs 1b and 2a. High levels of binding are seen in the striatum, nucleus accumbens and olfactory tubercle. These are the same areas exhibiting adenosine-stimulated adenylate cyclase in broken membrane preparations (Premont *et al.*, 1979),

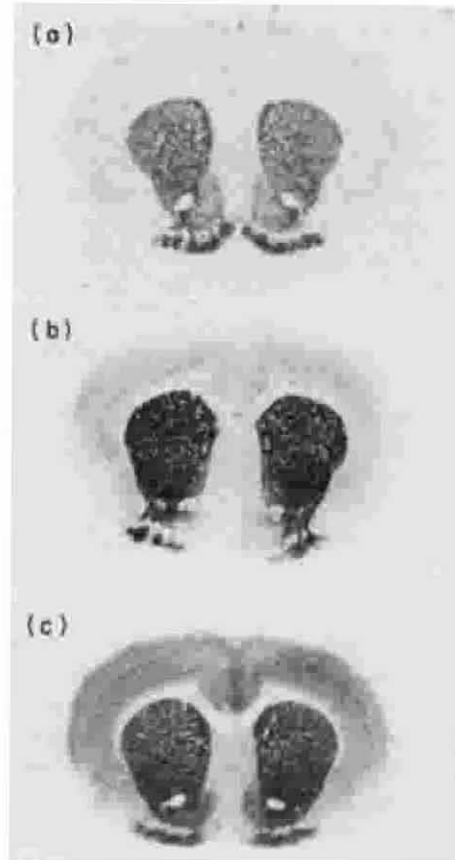


Figure 2 Autoradiographs of coronal sections from rat brain showing binding to A_2 receptors and non- A_1 , non- A_2 sites. (a) The binding of [^3H]NECA in the presence of NEM is shown. This reflects binding to A_{2a} sites and is concentrated in the striatum, nucleus accumbens and olfactory tubercle (Reddington *et al.*, 1986). (b) The binding of [^3H]CGS-21680 to A_{2a} sites shows a similar distribution to that seen in (a). (c) The pattern of [^3H]NECA binding in the presence of unlabelled R-PIA. The same sites are labelled as in (a) and (b); however, additional sites are labelled which do not resemble A_1 or A_2 sites. In this autoradiograph, increased levels of binding are seen throughout the neocortex with highest densities found in layer IV.

suggesting that these [^3H]NECA-binding sites are associated with A_{2a} receptors (see also Bruns *et al.*, 1986). Recently, an almost identical distribution of binding sites has been demonstrated for [^3H]CGS-21680, a ligand that binds rather specifically to A_{2a} sites (Fig. 2b; Jarvis and Williams, 1989). These findings indicate that A_{2a} receptors are highly concentrated in the striatum, nucleus accumbens and olfactory tubercle.

Another approach to the localization of A_2 sites has examined [^3H]NECA binding in the presence of unlabelled, A_1 -specific agonist or antagonist (Bruns *et al.*, 1986; Lee and Reddington, 1986a, b; Jarvis *et al.*, 1989a). Studies performed on membrane preparations have shown that [^3H]NECA binding in the presence of unlabelled CPA is highest in the striatum with lower levels found in the hippocampus, cortex and thalamus. Similar observations were obtained when the autoradiographic distribution of [^3H]NECA binding was studied in the presence of R-PIA or DPCPX (Fig. 2c, see also above). In addition to A_{2a} sites, the autoradiographic studies detected another class of non- A_1 , non- A_{2a} binding sites. These additional sites exhibit a rather specific pattern of distribution with high concentrations located in the medial geniculate, stratum lucidum of the CA3 region of the hippocampus (i.e. the terminal field of the mossy fibre system), several thalamic nuclei and parts of the amygdala (Lee and Reddington, 1986b). The nature of these non- A_1 , non- A_{2a} sites remains unknown but their unique distribution and saturability are suggestive of functional significance.

As mentioned previously, the designation of A_2 receptors was derived from studies on cells of 'glial character' (Van Calker *et al.*, 1979), suggesting that neuroglia can express A_2 receptors. In addition to their association with glial cells, the presence of A_2 receptors on neurones is supported by several lines of evidence. Cell culture studies examining primary cultures of striatal neurones have demonstrated the presence of adenosine-stimulated adenylate cyclase activity, indicating a neuronal localization of A_2 sites (Premont *et al.*, 1983). In addition, *in situ* lesions of intrinsic striatal neurones with kainic acid result in substantial reductions in both adenosine-stimulated adenylate cyclase (Wojcik and Neff, 1983b) and A_{2a} -binding sites (Alexander and Reddington, 1989); these findings are consistent with the presence of A_2 sites on striatal neurones. In this context it is also of interest that A_2 receptors have been demonstrated in highly purified cholinergic nerve endings from the rat striatum (Brown *et al.*, 1990). These receptors are coupled to adenylate cyclase and stimulate the depolarization-induced release of acetylcholine.

Other lesion studies, in which selective deafferentation of the striatum was examined, have provided some insights into the cellular locus of A_2 sites. Removal of dopaminergic nigral afferents to the striatum does not appear to alter adenosine-stimulated adenylate cyclase in broken cell preparations (Wojcik and Neff, 1983b) or change binding to A_{2a} sites (Alexander and

Reddington, 1989). This suggests that A_{2a} sites are not associated with the nigro-striatal pathway. Removal of cortical afferents to the striatum, however, reduces the levels of adenosine-stimulated adenylate cyclase (Wojcik and Neff, 1983b) but does not alter binding to A_{2a} sites in autoradiographic studies (Alexander and Reddington, 1989). The apparent contradiction between these studies could be the result of differences in the extent of the cortical lesions (Alexander and Reddington, 1989); however, the general limitations of lesion studies must also be considered when interpreting these data (Hattori and Fibiger, 1982). Thus, A_{2a} sites in the striatum: (a) appear to be associated with intrinsic striatal neurones, (b) may or may not be associated with the cortico-striatal pathway, and (c) do not appear to be associated with the nigro-striatal pathway.

In summary, A_2 receptors are associated with both glial and neuronal sites, as is the case for A_1 receptors. Less is known about the precise cellular and subcellular localization of A_2 sites but further information will no doubt be forthcoming as specific ligands become available.

The adenosinergic system

One of the major aims of a morphological approach to neurotransmitter and neuromodulator distribution is to define the anatomical systems which use specific chemical messengers in order to understand the functional significance of such chemical agents in the nervous system. In addition, knowledge of the distribution of chemically distinct pathways in the brain is of the utmost relevance for the development of drugs that affect specific neurological and behavioural functions. It is therefore worth considering the various approaches that are currently used to define such pathways in terms of their chemical messengers.

Several components of chemical transmitter systems have proved to be amenable to anatomical study. These are (i) enzymes involved in transmitter biosynthesis, (ii) the neurotransmitters themselves, (iii) their receptors, (iv) enzymes involved in transmitter inactivation, and (v) re-uptake mechanisms. When approaching the pathways that use adenosine, however, serious problems have been encountered. This is due to the fact that purines play such a central role in cellular metabolism so that many of the components involved in adenosine metabolism may not be directly related to its neuromodulatory function. In addition, there is some uncertainty about the details of many of the processes relevant to adenosine metabolism and function. Although many of these aspects are dealt with in detail elsewhere in this book,

some of the general difficulties involved in anatomically defining an adenosinergic system will be considered here.

Biosynthetic enzymes have proved to be invaluable for the identification of some neurotransmitter systems, since they are localized to the cells using the given transmitter, and can be visualized readily using immunohistochemical techniques. A good example is the staining of choline acetyltransferase for the identification of cholinergic neurones. In the case of adenosine, however, there is uncertainty about the biochemical pathways responsible for its production in the brain and whether it is released as adenosine itself or as a nucleotide precursor. Its central role in the metabolism of all cells also raises the question of whether adenosine is only released from neurones, or if glial cells might also serve as a source of the nucleoside (Lewin and Black, 1979). The most readily accessible enzyme involved in adenosine biosynthesis for anatomical studies is the ecto-enzyme, 5'-nucleotidase (Kreutzberg *et al.*, 1986). This enzyme hydrolyses various nucleoside monophosphates, including adenosine 5'-monophosphate, to the corresponding nucleosides. Although several 5'-nucleotidases have been described, the one that is most prominent in the brain is a membrane-bound ecto-enzyme, i.e. its active site is oriented towards the extracellular space. According to the scheme involving this enzyme, adenine derivatives are released from neurones in the form of ATP, which is a well-known constituent of cholinergic and adrenergic vesicles and is released together with the primary neurotransmitter. ATP is then broken down in the extracellular space ultimately to form adenosine. Although evidence has been obtained for a role for ecto-5'-nucleotidase in the production of adenosine in purified cholinergic nerve endings from rat striatum (Richardson *et al.*, 1987), the relative contributions of intracellular and extracellular production of the nucleoside are unknown. The association of the ecto-5'-nucleotidase with an adenosinergic system is therefore not necessarily a prerequisite. A further complication with use of this enzyme as an adenosinergic marker is its strong association with glial cells (Kreutzberg *et al.*, 1986). In addition, comparison between the distributions of 5'-nucleotidase and the electrophysiologically important A₁ adenosine receptors in the brains of various mammalian species showed no direct association between these two components (Lee *et al.*, 1986). Although the distributions of the A₁ receptors in the hippocampus of different mammals were similar, those of 5'-nucleotidase activity differed widely from species to species. This phylogenetic inconsistency, together with the demonstrable association of 5'-nucleotidase with glial cells makes this enzyme unsuitable as a marker for neuronal pathways utilizing adenosine.

A second component that might be useful as an anatomical marker for a chemical messenger is the transmitter itself. This has been useful in the case of the localization of pathways using catecholamines. In the case of adenosine, immunocytochemistry using anti-adenosine antibodies has revealed adenosine-

like staining in quite discrete sites in the central nervous system (Braas *et al.*, 1986). An underlying assumption in the use of adenosine-like immunoreactivity as a purinergic marker is that high endogenous concentrations of adenosine necessarily reflect a functional role for the nucleoside. However, the central role played by purines in energy metabolism makes it difficult to substantiate this. In addition, the sensitivity of adenosine concentrations to hypoxic conditions makes it difficult to rule out completely that high levels of adenosine immunoreactivity might be associated with cells that are particularly vulnerable to low oxygen levels. The possible glial source of adenosine mentioned above is also a complicating factor in this case. Therefore, although the immunochemical distribution of adenosine provides an interesting parameter indicating the degree of adenosine production, its use as a marker for adenosinergic neurones must be approached with care.

The action of a humoural substance must be terminated after it has fulfilled its messenger function. In the case of adenosine, this can be accomplished by a combination of re-uptake from the extracellular space via nucleoside transporters and subsequent intracellular metabolism, i.e. the intracellular breakdown to inosine by adenosine deaminase or re-incorporation into the nucleotide pool by phosphorylation catalysed by adenosine kinase. Some evidence also exists for an ecto-adenosine deaminase that could directly inactivate adenosine in the extracellular space (e.g. Meghji *et al.*, 1988). Nucleoside transporters have been extensively studied using radioactively labelled nucleoside derivatives such as nitrobenzylthioinosine (NBTI). An example of the distribution of [^3H]NBTI-binding sites is shown in Fig. 1. Although some overlap is observed between these binding sites and those corresponding to A_1 and A_{2a} receptors, the differences, for instance in the cerebral cortex and cerebellum, are more obvious than the similarities. It is important to note, however, that several nucleoside transport mechanisms are known to exist and that these are present on both neurones and glial cells (Bender and Hertz, 1986; Jarvis, 1987). Further, just as the ecto-5'-nucleotidase can hydrolyse a broad range of purine and pyrimidine monophosphates, the nucleoside transporters are not specifically involved in adenosine uptake but can interact with other nucleosides. This heterogeneity of function and localization is a serious complication for the use of nucleoside transporters as markers for adenosinergic neurones.

Finally, immunohistochemical studies of the distribution of adenosine deaminase in the brain have shown this enzyme to be localized quite specifically, and it has been suggested that this distribution might reflect the presence of adenosinergic neurones (Nagy *et al.*, 1990). However, the low amounts of adenosine deaminase immunoreactivity in areas such as the hippocampus and cerebellum, both of which are rich in adenosine receptors, together with the marked species differences that have been reported in

immunoreactive neuronal systems (see Meghji and Newby, 1990) make it at present unclear to what extent adenosine deaminase can be used as a marker for adenosinergic neurones.

The discrepancies and uncertainties involved in the distributions of the factors involved in adenosine metabolism make these unsuitable for general use in defining neuronal pathways using adenosine as a neuromodulator. Although some mismatch between the distributions of, for instance, a transmitter-synthesizing enzyme and the receptors on the target cells, is to be expected, the central role played by purines in the metabolism of both neurones and other cells makes it unlikely that such components can give more than a hint of a special role for adenosine. Identification of sites relevant for the important neuromodulatory function of adenosine is currently most reliable in terms of the distribution of the adenosinergic target molecules, the adenosine receptors. Autoradiographic techniques for studying the distributions of adenosine receptor types as described in this chapter will therefore continue to play a major part in defining those areas in which adenosine plays an important part in the modulation of neuronal function.

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CHAPTER 5

THE ADENOSINE RECEPTOR MOLECULE

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Characterization of adenosine receptors has advanced rapidly during the past decade. Our understanding of the A₁ receptor in particular has benefited from the development of a high-affinity agonist and antagonist ligands and the recognition that most A₁-mediated responses are inhibited in tissues treated with *Bordetella pertussis* toxin which catalyses the ADP-ribosylation of GTP-binding protein α -subunits. A₁ Receptors have been characterized in a number of tissues by radioligand binding, autoradiography, photoaffinity labelling and target size analysis. The receptor has been purified to homogeneity from brain and other tissues. Differences in structure-activity relationships for various A₁ receptor-mediated responses suggest that A₁ receptor subtypes may exist, but this has not yet been firmly established. A₂ adenosine receptors have been pharmacologically subdivided into two subtypes designated A_{2a}, which bind adenosine with relatively high affinity, and A_{2b} which bind adenosine with relatively low affinity. The A_{2a} receptor of striatum has been photoaffinity-labelled (Barrington *et al.*, 1989) but not yet purified. Specific high-affinity ligands for A_{2b} receptors are lacking, and relatively little is known about this subfamily. In this chapter we will summarize what has been learned about A₁ receptors with emphasis on emerging evidence that suggests that the same or similar A₁ receptors can couple to multiple GTP-binding proteins (G

proteins). The nature of the G proteins coupled to adenosine receptors may play a key role in determining the response to adenosine in a given tissue, and this receptor-G protein coupling may be subject to regulation.

Receptor affinity states

Adenosine receptors interact with G proteins to activate a variety of effector systems. As is the case with other G protein interactive receptors, the interaction of agonist-occupied receptors with G proteins apparently results in a decrease in the affinity of the G protein for bound GDP. The GDP then dissociates from the G protein resulting in a receptor-G protein complex without bound guanine nucleotide. In this 'blank' state, the receptor binds agonists with high affinity (Fig. 1). Receptor-G protein complexes are sometimes referred to as 'coupled' receptors, as opposed to 'uncoupled' receptors which are not associated with G proteins and do not bind agonists with high affinity. The high-affinity binding site is not observed in intact cells (Martens *et al.*, 1987) probably because endogenous GTP quickly occupies the guanine nucleotide-binding site which exists on the α -subunit of G proteins, resulting in activation of the G protein and rapid conversion of the receptor into a low-affinity state for agonists.

Trace concentrations of Mg^{2+} or other divalent cations have been found to enhance high-affinity binding of agonist ligands to A_1 receptors inasmuch as high-affinity binding of agonists is lost if membranes are pretreated with high concentrations of chelators, and such binding is restored upon the addition of $MgCl_2$ (Linden *et al.*, 1987). Mg^{2+} has also been shown to be required for the activation of the G protein which stimulates adenylyl cyclase (Birnbaumer, 1990), and agonists may act in general by reducing the concentration of



Figure 1 Schematic representation of interactions between A_1 adenosine receptors and GTP-binding proteins. The binding of adenosine (ADO) or other agonists to uncoupled receptors (R) results in a binary complex (ADO-R) with a low affinity for agonists. The binary complex interacts with inactive G protein (G-GDP). In the resulting ternary complex, the affinity of the G protein for GDP is reduced, so GDP dissociates. In the absence of bound guanine nucleotide the receptor changes conformation (R') to a state in which it binds agonists with high affinity. This ternary complex is represented in brackets since in intact cells it is transient due to the rapid binding of GTP which activates the G protein (G-GTP), dissociates the receptor-G protein complex, and converts the receptor back to a low-affinity state for adenosine.

Mg²⁺ ion necessary to drive G protein activating reactions. Since membranes are variably contaminated with GTP, some of the effect of Mg²⁺ to enhance high-affinity agonist binding to membranes may be the result of activating Mg²⁺-sensitive GTPase activities, thus causing depletion of membrane-associated GTP. Solubilization of coupled receptors results in a marked diminution in the effect of removing Mg²⁺ to attenuate high-affinity agonist binding (Gavish *et al.*, 1982; Linden *et al.*, 1987; Stroher *et al.*, 1989). This may be due in part to the fact that solubilized receptors have less contaminating endogenous guanine nucleotides than membranes.

The effect of divalent cations such as Mg²⁺ to enhance agonist binding to A₁ receptors is counteracted by monovalent cations such as Na⁺, which decreases high-affinity agonist binding to A₁ receptors (Gavish *et al.*, 1982; Green, 1984; Patel and Linden, 1988). NaCl acts in the physiological concentration range in part by promoting the dissociation of receptor-G protein complexes, and in part by decreasing the affinity of both coupled and uncoupled receptors for agonists. The histidine alkylating agent, diethylpyrocarbonate (DEP), also decreases the affinity of the agonist N⁶-phenylisopropyladenosine (PIA) for the A₁ receptor of bovine brain (Garritsen *et al.*, 1990b). Since the presence of 145 mM NaCl augments the effect of DEP to inhibit PIA binding, it appears that a NaCl-induced change in receptor conformation may facilitate exposure of histidyl residues to DEP. It is notable that NaCl has sometimes been reported to be required for adenosine agonists to produce inhibition of adenylyl cyclase activity (Londos *et al.*, 1981). This apparent requirement for NaCl could result in part because the monovalent cation diminishes the affinity of receptors for endogenous adenosine, allowing for the more effective removal of prebound endogenous adenosine and hence better expression of receptor-mediated responses.

The diuretic amiloride competes for both agonist and antagonist binding to bovine brain A₁ receptors with a *K_i* in the low micromolar range (Garritsen *et al.*, 1990a). NaCl and protons reduce the potency of amiloride at concentrations which have little effect on the binding of PIA or the binding of the antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). These data suggest that the amiloride-binding site may overlap with, but not coincide with the ligand binding domain. Although the potency order of amiloride analogues suggests that the site of interaction between A₁ receptors and amiloride is not on a Na⁺/Ca²⁺ exchanger (Garritsen *et al.*, 1990a), it has been reported that adenosine, acting via an A₁ receptor can activate Na⁺/Ca²⁺ exchange in ewe heart by a pertussis toxin-sensitive mechanism (Brechler *et al.*, 1990).

At very high concentrations (> 500 mM) NaCl has been found to markedly enhance the binding of acidic but not uncharged antagonists (Linden *et al.*, 1988). Also, the carboxyl-alkylating agent N,N'-dicyclohexylcarbodiimide

reduces the affinity of [^3H]DPCPX for A_1 receptors (Garritsen *et al.*, 1990b). These data suggest that a carboxyl group is located in the vicinity of the adenosine recognition site (Garritsen *et al.*, 1990b). High NaCl may mask repulsion between acidic groups on ligands and on the receptor binding domain (Linden *et al.*, 1988).

Cryptic binding sites

Several investigators have found that agents such as guanine nucleotides and *N*-ethylmaleimide (NEM), which uncouple A_1 receptors from G proteins, produce reciprocal effects on agonist and antagonist radioligand binding (Yeung and Green, 1983; Green, 1984). There are several potential mechanisms by which uncoupling of receptors from G proteins might increase antagonist binding. One possibility is that antagonists bind preferentially to uncoupled receptors as suggested by Green and co-workers (Leung and Green, 1989; Leung *et al.*, 1990), who noted that the binding of antagonists to solubilized receptors appeared to increase the fraction of small, presumably uncoupled receptors detected by gel-permeation chromatography. It is possible, however, that the effect of antagonists in these experiments was to displace prebound endogenous adenosine which stabilizes solubilized receptor–G protein complexes. Binding of antagonists to solubilized receptors is known to stabilize solubilized receptors (Helmke and Cooper, 1989; Munshi and Linden, 1989) and might produce an apparent shift in the fraction of uncoupled receptors without necessarily binding preferentially to the uncoupled state. Our experience with purified bovine brain receptor–G protein complexes is that uncoupling agents such as GTP and NEM reduce high-affinity agonist binding but have little effect on antagonist binding to receptor–G protein complexes (Prater *et al.*, 1991). If antagonists do bind preferentially to uncoupled receptors they might produce ‘inverse agonist’ effects, i.e. produce effects opposite to agonists even when no agonists are present.

Uncoupling receptors occupied by prebound endogenous adenosine would result in an apparent increase in the number of antagonist radioligand binding sites. To remove endogenous adenosine, receptors are routinely incubated with the 36-kDa enzyme adenosine deaminase to convert endogenous adenosine to inosine. It is generally not appreciated that adenosine deaminase is a reversible enzyme (Zielke and Suelter, 1971), and the relationship between [adenosine] and [inosine] at pH 7 is given by:

$$K_{\text{eq}} = ([\text{inosine}][\text{NH}_3])/([\text{adenosine}][\text{H}_2\text{O}]) = 38$$

However, since ammonia is over 99% ionized at neutral pH, the steady state [adenosine] will not exceed nanomolar levels unless initial adenosine levels exceed 1 mM. It is possible, however, that millimolar concentrations of adenine nucleotides which can break down to form adenosine may be present in crude membranes which contain ATP-rich synaptic granules. In most instances, however, significant endogenous adenosine is most likely to reside only in membrane vesicles inaccessible to adenosine deaminase.

Klotz *et al.* (1990) have noted that GTP can increase antagonist binding to CHAPS solubilized receptors. Solubilized receptors are defined as those remaining in a $100\,000 \times g$ supernatant of detergent-treated membranes after centrifugation for 0.5–1 h. We have found that similar CHAPS supernatants are turbid and contain small ($< 1\ \mu\text{m}$ diameter) vesicles which do not pellet under these circumstances and which can be detected by electron microscopy (R. Saponja and J. Linden, unpublished data). Hence even 'solubilized' receptors may not be entirely free of vesicles.

The effect of GTP to reduce the binding affinity of agonists is associated with a marked increase in the dissociation rate of prebound agonist. This is a predictable manifestation of the change in affinity since the radioligand $K_d = K_{-1}/K_1$, where K_{-1} is the dissociation rate constant. The association rate constant K_1 , is limited by diffusion and changes little in response to conformation changes in the receptor. If GTP increased the affinity of receptors for antagonists, it is predictable that this would be accompanied by a decrease in the dissociation rate of antagonists. On the other hand, if GTP increases antagonist binding by displacing endogenous agonist, no change in the dissociation rate of antagonists would be anticipated. Experimentally, the latter has been observed, and GTP has no effect on the dissociation rate of prebound antagonist (Klotz *et al.*, 1990). Weber *et al.* (1990) found that whereas GTP has little effect on [^3H]DPCPX binding to rat brain membranes, addition of GTP enhances binding of the radioligand to tissue sections fivefold. These investigators concluded that endogenous adenosine has an even bigger influence on radioligand binding to tissue sections than to membranes, and they suggested using GTP to unmask cryptic binding sites and improve autoradiographic visualization of A_1 receptors by antagonist radioligands. In another study, autoradiographic evidence indicated that the effects of guanine nucleotides on radioligand binding differ in various brain regions (Fastbom and Fredholm, 1990). This could be due to regional variations in levels of endogenous adenosine or in the nature of G proteins coupled to A_1 receptors.

Partial activation of receptors by endogenous adenosine might also account for the fact that adenosine receptor antagonists have been found to stimulate adenyl cyclase activity in adipocyte membranes (Ramkumar and Stiles, 1988). The authors suggested that adenosine receptor antagonists activate adenyl cyclase as a consequence of binding to G proteins. However, in

cardiac membranes treated to permeabilize vesicles, A₁ antagonists did not increase adenylyl cyclase activity (Liang, 1989). These data suggest that adenosine receptor antagonists do not interact with G proteins, but act by blocking the effects of residual endogenous adenosine or other endogenous receptor agonists to disinhibit adenylyl cyclase (Linden, 1989).

Photoaffinity labelling and target size analysis

The first compounds used to photoaffinity-label adenosine receptors were A₁ selective agonists, [¹²⁵I]N⁶-azidobenzyladenosine (Choca *et al.*, 1985; Patel and Linden, 1988) and [¹²⁵I]C²-azido-2-hydroxyphenylisopropyladenosine (Klotz *et al.*, 1985). Using these probes, the molecular mass of brain receptors of several species, and rat fat receptors, were found to be 34–35 kDa. In some subsequent studies the molecular mass of A₁ receptors has been reported to be as high as 38–40 kDa. These variable molecular mass determinations may reflect slight differences in receptor motility depending on the conditions of sodium dodecylsulphate polyacrylamide electrophoresis.

Photoaffinity labelling of adenosine receptors by agonists was found to be inhibited by GTP analogues as expected on the basis of the fact that agonists bind selectively to coupled receptors. Since the β -subunits of G proteins have molecular masses of 35–36 kDa, there was some concern that the photoaffinity-labelled polypeptide might be a component of a G protein tightly associated with coupled receptors. However, Klotz and Lohse (1986) showed that the receptor can be deglycosylated, resulting in a decrease in apparent molecular mass to 32 kDa. Furthermore, A₁ receptors uncoupled from G proteins can be labelled by antagonist photoaffinity probes: an 8-phenylxanthine (Linden *et al.*, 1987; Earl *et al.*, 1988) and an 8-cyclopentylxanthine (Patel *et al.*, 1988). These results clearly indicate that photoaffinity-labelled polypeptides are not constituents of G proteins tightly associated with A₁ receptors.

The apparent molecular mass of 34–35 kDa for A₁ receptors is large enough to include the receptor among the family of G protein interactive receptors which contain seven 20–26 amino acid stretches of generally hydrophobic transmembrane segments. It is possible that photoaffinity labelling might detect only a subunit or proteolytic fragment of the receptor. That this might be the case was suggested by initial estimates of the size of the brain receptor based on radiation inactivation analysis of the binding of agonist radioligands from which the receptor size was calculated to be 63–80 kDa (Reddington *et al.*, 1987; Frame *et al.*, 1988). More recently Reddington *et al.* (1989) found that the site labelled by the antagonist [³H]DPCPX has a radiation inactivation size of 58 kDa in the absence of guanine nucleotide, and this size

is reduced to 33 kDa in the presence of guanine nucleotides. These investigators also examined the effect of radiation to destroy previously photoaffinity-labelled receptors and determined an inactivation size of 35 kDa. These data are consistent with a receptor size of 34–35 kDa and with the notions that high-affinity agonist binding occurs selectively to coupled receptors, and that antagonists can bind to either coupled or uncoupled receptors. The 58–80 kDa size of coupled receptors is in the right size range to reflect a receptor–G protein α -subunit complex (about 75 kDa).

A₁ Adenosine receptors have purportedly been detected with anti-idiotypic antibodies which label a 36-kDa protein on Western gels (Ku *et al.*, 1987). Although this is close in size to the photoaffinity-labelled receptor, on non-reducing gels the antigen increases in size to 62 kDa. In contrast, no change in the apparent size of the photolabelled or silver-stained purified receptors is seen in gels run under reducing or non-reducing conditions. Moreover, in its glycosylated form the receptor appears as a broad 34–35-kDa polypeptide, presumably due to microheterogeneity in the degree of glycosylation, whereas the 36-kDa anti-idiotypic antigen appears as a sharp band more typical of non-deglycosylated polypeptides. It appears quite likely, therefore, that anti-idiotypic antibodies do not detect A₁ receptors.

Affinity chromatography of A₁ receptors

The first successful affinity purification of A₁ receptors involved the use of an agonist affinity column, N⁶-aminobenzyladenosine–agarose (ABA–agarose), to purify coupled A₁ receptors of bovine brain (Munshi and Linden, 1989). When CHAPS-solubilized receptors were passed through this column, coupled but not uncoupled receptors bound (Fig. 2). When the pass through of this column was examined using various radioligands it was found that 40–50% of antagonist binding and over 90% of high-affinity agonist binding sites had adhered to the affinity column. This is consistent with the observation that 40–50% of receptors solubilized from bovine brain remain coupled, i.e. bind agonists with high affinity. A disadvantage of agonist affinity chromatography is that only coupled receptors adhere to the affinity column. A major advantage is that receptors can be rapidly and specifically eluted by uncoupling agents such as GTP or NEM. It is notable that pertussis toxin fails to elute receptors which are bound to the affinity column (R. Munshi and J. Linden, unpublished data). This probably indicates that G proteins in ternary complexes with agonists and receptors are not substrates for pertussis-catalysed ADP-ribosylation.

Following elution of receptors from affinity columns, 39- and 41-kDa G protein α -subunits can be detected by ADP-ribosylation catalysed by pertussis

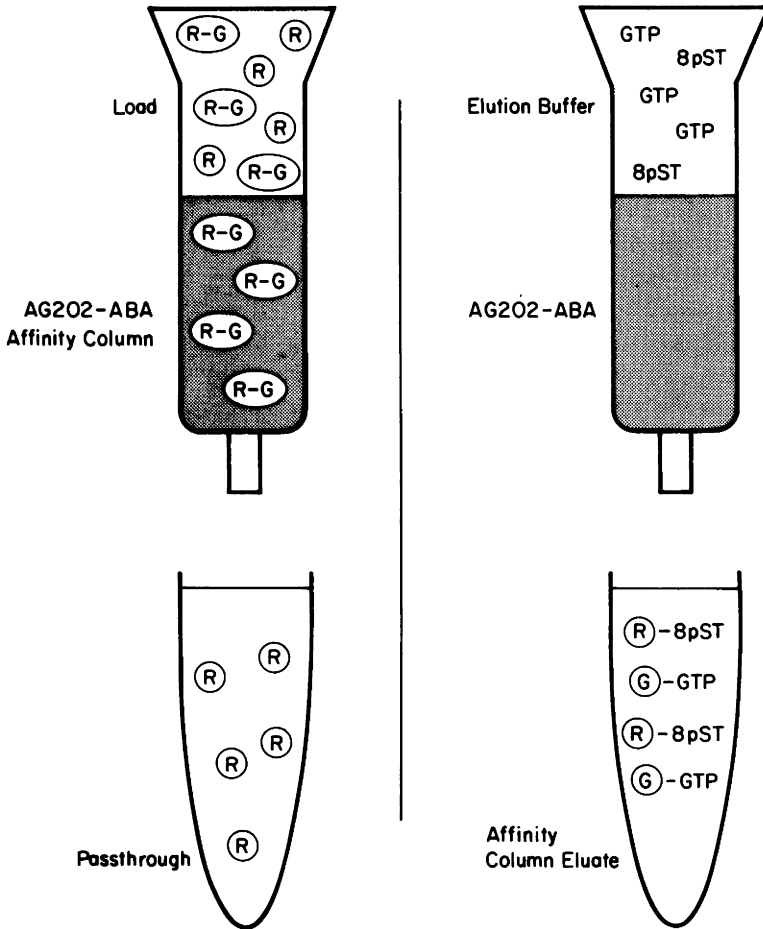


Figure 2 Scheme used for agonist affinity chromatography of A₁ adenosine receptor-G protein complexes. Left: Detergent-solubilized receptors containing coupled (R-G) and uncoupled (R) receptors are passed over an agonist affinity column (AG202-ABA). Coupled receptors which have a high affinity for agonists are selectively retained. Right: Receptors are eluted upon the addition of GTP which uncouples R-G complexes. Receptor elution can be accelerated by the addition of an antagonist radioligand such as 8-*p*-sulphophenyltheophylline (8pST).

toxin (Munshi and Linden, 1989). We have used specific antisera to characterize the nature of G proteins which are co-purified along with bovine brain A₁ adenosine receptors. The α -subunits associated with A₁ receptors consist predominantly of G_{o α} and G_{ia1} (Linden *et al.*, 1990). Both 35- and 36-kDa forms of G proteins β -subunits also are present (Fig. 3). We have

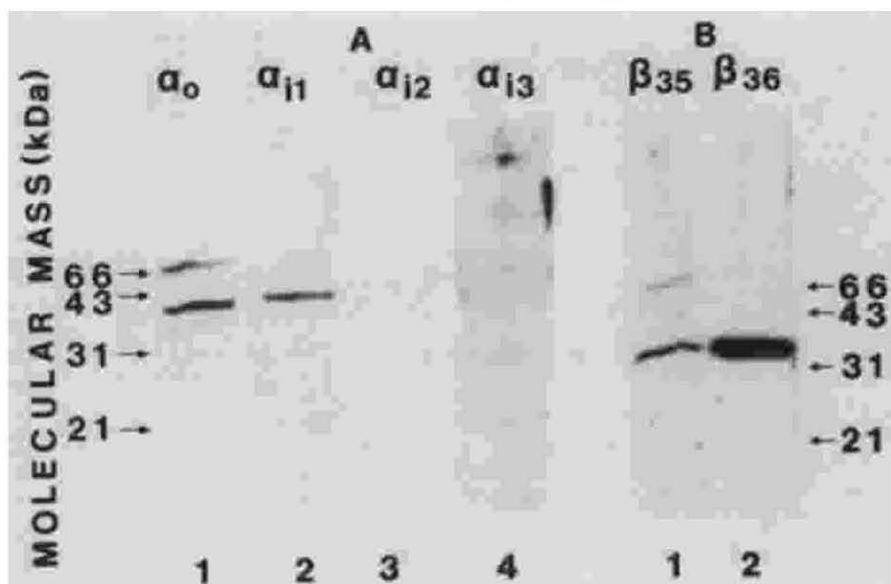


Figure 3 Identification of G protein subunits co-purified with bovine brain A_1 receptors. Polypeptides in affinity-purified receptor–G protein complexes were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. The membranes were incubated with G protein subtype-selective rabbit antisera provided by S. Mumby, followed by ^{125}I -labelled goat anti-rabbit IgG. Labelled peptides were detected by autoradiography. (A) G protein α -subunits. (B) G protein β -subunits.

taken steps to show that only those G proteins associated with receptors adhere to the affinity column. If solubilized receptors are pretreated with the agonist PIA, then neither receptors nor G proteins adhere to the affinity column. It could be argued, however, that PIA prevents the non-specific binding of G proteins to affinity columns. Hence we have used another means to demonstrate that G proteins do not non-specifically adhere to affinity columns. Solubilized receptors were adsorbed to an antagonist column consisting of 1,3-dipropyl-8-(4-acrylate)phenylxanthine (BWA-1433U) coupled to agarose. As shown in Fig. 4, passing solubilized receptors over the antagonist affinity column prior to agonist affinity chromatography over an ABA–agarose column, resulted in a comparable decrease in the number of purified receptors and G proteins. This provides strong evidence that G proteins which adhere to agonist affinity columns are associated with receptors.

Attempts to purify bovine brain A_1 receptors on antagonist columns have resulted in only partial purification of receptors (Olah *et al.*, 1989), possibly because the very high affinity of the bovine brain receptor for such compounds

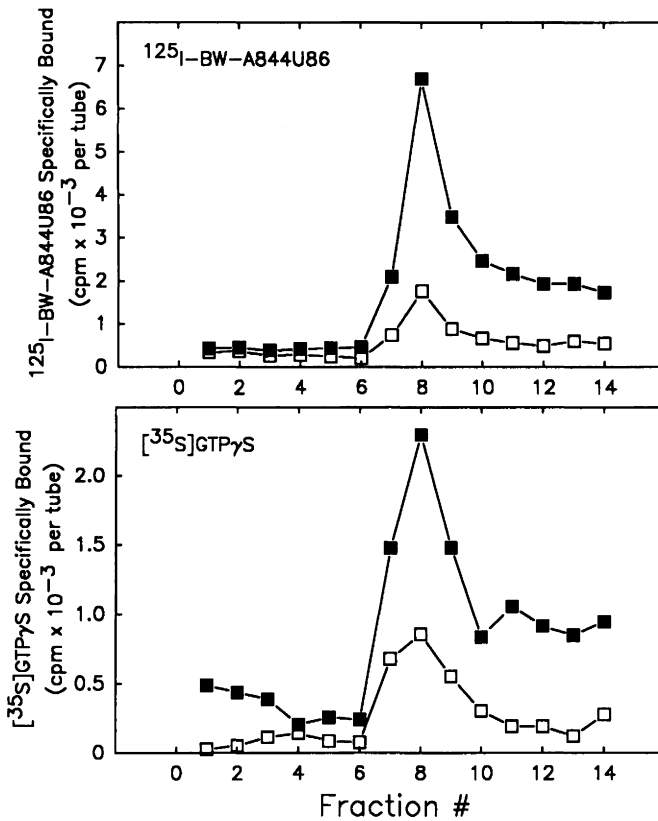


Figure 4 Effects of depleting A_1 receptors on agonist affinity chromatography of receptors and G proteins. CHAPS solubilized bovine brain receptors were passed over columns containing either no ligand (solid squares) or the A_1 receptor antagonist BWA-1433U (open squares) coupled to agarose. The antagonist affinity column retained 68% of applied receptors as measured by binding of the radioligand [^{125}I]-BW-A844U86. The eluates of both columns were applied to parallel agonist affinity columns and eluted with GTP (see Fig. 2). Following removal of GTP receptors (top) and G proteins (bottom) were measured in the elution fractions.

combined with non-specific hydrophobic interactions precludes efficient elution of receptors. However, Nakata (1989) successfully purified to homogeneity a 34-kDa rat brain receptor (rat receptors bind most xanthines with 5–10-fold lower affinity than bovine receptors) over an antagonist column composed of xanthine amino cogener (XAC) coupled to agarose. The same procedure was used to purify the A_1 receptor of rat testes (Nakata, 1990). As expected, receptors purified by antagonist affinity chromatography are not coupled to

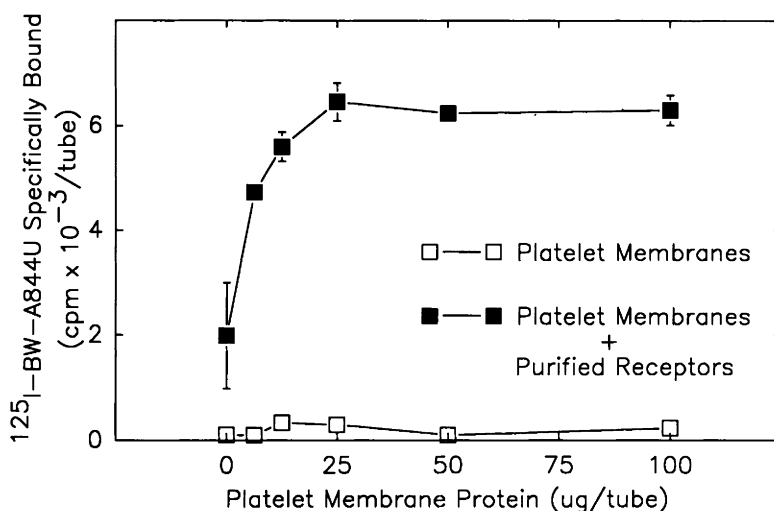


Figure 5 Increased detection of purified A_1 receptors by the addition of platelet membranes. Receptors were affinity purified as described (Munshi and Linden, 1989) and assayed for A_1 receptors with and without the addition of various amounts of human platelet membranes.

G proteins. This is consistent with the idea that unlike agonists, binding of antagonists does not stabilize receptor-G protein complexes.

In order to detect radioligand binding to affinity-purified A_1 adenosine receptors it is essential to include phospholipid in the elution buffer (Munshi and Linden, 1989). The effect of phospholipid may be to improve retention of the solubilized receptors onto polyethyleneimine-treated glassfibre filters used to trap solubilized receptors. As was the case for receptors purified by agonist affinity chromatography, radioligand binding could not be detected to the antagonist affinity-purified receptors without the addition of a source of phospholipid. Nakata (1989) utilized heated (80°C) affinity column pass through in order to restore detectable radioligand binding. We have recently found that binding to affinity-purified A_1 receptors can also be enhanced by the addition of small amounts of platelet membranes, which lack A_1 receptors (Fig. 5).

Reconstitution of brain A_1 receptors and G proteins

Receptors purified by agonist affinity chromatography are eluted from affinity columns by the addition of GTP which, by binding to associated G proteins,

convert the receptor to a low-affinity state. If GTP is removed from affinity co-purified receptors and G proteins in the presence of phospholipid, some (50%) of the receptors and G proteins spontaneously recouple to form high-affinity agonist binding sites (Fig. 6). The purified receptors can also couple to other sources of G proteins. For instance, if purified receptors are reconstituted with human platelet membranes, some of the receptors spontaneously couple to the platelet G proteins (Munshi and Linden, 1990). The property of bovine receptors to bind various ligands with very high affinity is preserved following reconstitution into human platelet membranes. This suggests that species differences in receptor affinities are due to the nature of the receptor and not to the nature of associated G proteins or the composition of the membranes in which the receptors reside.

Although platelet membranes contain some G proteins which cannot be ADP-ribosylated by pertussis toxin, almost all high-affinity agonist binding to brain A_1 receptors reconstituted into platelet membranes is prevented by

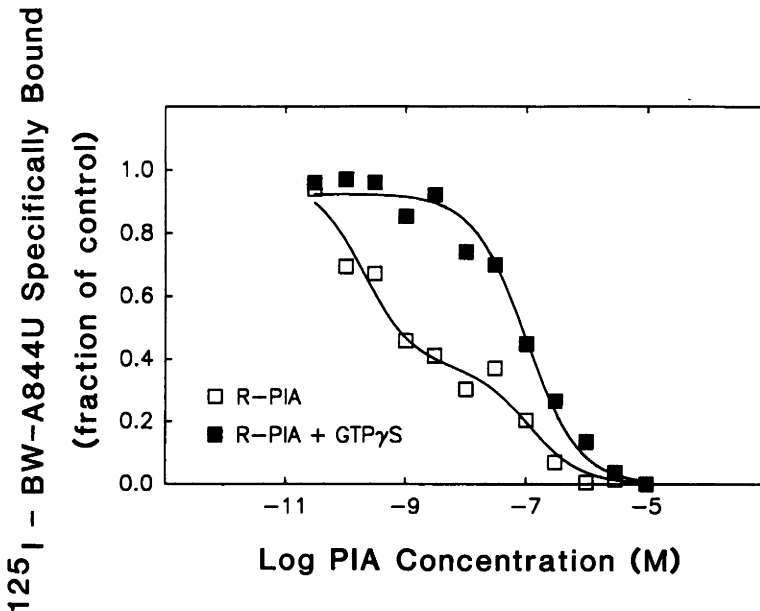


Figure 6 Evidence for coupling of affinity co-purified A_1 receptors and G proteins. Receptors and G proteins were co-purified by agonist affinity chromatography (Fig. 2). The purified material, containing 0.01% asolectin, was passed over Dowex AG1 \times 8 to remove GTP. To demonstrate coupling between purified receptors and G proteins, competition by the agonist, PIA, for antagonist radioligand binding sites was assessed in the absence and presence of 10 μ M GTP γ S.

pertussis toxin-catalysed ADP-ribosylation (Munshi and Linden, 1990). This suggests that although A_1 receptors appear to be capable of coupling to a number of G proteins, most if not all of these may belong to the pertussis-sensitive subfamily. In the rat hippocampus, A_1 receptors and 5-HT_{1A} receptors appear to be co-localized on the same pyramidal cells and coupled to a common pool of pertussis toxin-sensitive G proteins (Zgombick *et al.*, 1989). Fredholm and co-workers have noted that *in vivo* pertussis toxin treatment attenuates some, but not all, A_1 effects in slices of the rat hippocampus. It is not clear, however, if the variable effect of pertussis toxin occurs because some A_1 responses are resistant to the toxin or because some receptor-G protein complexes have lower sensitivity or accessibility to the toxin. There are examples of variability in the sensitivity of A_1 -mediated responses to inactivation by pertussis toxin. For instance, A_1 receptors inhibit cyclic AMP accumulation and the breakdown of inositol phospholipids in brown adipose tissue and in GH₃ pituitary cells. In both instances more toxin is required to prevent adenosine from inhibiting inositol phospholipid breakdown than to prevent adenosine from inhibiting adenylyl cyclase (Linden and Delahunty, 1989). Thus it is possible, but not proven, that all A_1 receptor-mediated responses are mediated by pertussis-sensitive G proteins.

Regulation of A_1 receptor coupling to G proteins

A_1 Adenosine receptors can be downregulated and have been shown to undergo homologous desensitization in the CNS and in other tissues. The number of A_1 receptors coupled to G proteins is reflected by high-affinity agonist binding sites measured in membranes devoid of guanine nucleotides. It is these high-affinity binding sites which may be critical for producing adenosine-mediated responses, since all known adenosine responses are mediated by G proteins. A_1 Receptors coupled to G proteins have been detected associated with clathrin-coated vesicles in bovine brain (Gonzalez-Calero *et al.*, 1990). Thus it is possible that endocytosis via coated vesicles during downregulation responses precedes uncoupling of receptor-G protein complexes.

Prolonged treatment of adipose tissue with PIA not only reduces the number of A_1 receptors, but also decreases detectable levels of all three forms of $G_{i\alpha}$ (Green *et al.* 1990). The downregulation of adipocyte A_1 receptors during chronic exposure to PIA was also associated with changes in the amounts of G_s , but not with changes in mRNA for G proteins (Longabaugh *et al.*, 1989). Thus it appears that expression of G protein α -subunits is influenced by adenosine receptor-mediated changes in second messengers or modified coupling to A_1 receptors.

Injection of PIA onto the chorioallantoic membrane of embryonic chicks results in a dose-dependent decrease in the number of high-affinity agonist radioligand binding sites on myocardial membranes and a decrease in the efficacy of PIA as a negative inotropic agent (Shryock *et al.*, 1989). In cultured atrial cardiomyocytes, exposure to PIA also reduced receptor number ($[^3\text{H}]\text{DPCPX}$ binding sites) and inhibited the ability of PIA to inhibit adenylyl cyclase and contractility (Liang and Donovan, 1990). An interesting aspect of this study is that the adenylyl cyclase response was completely desensitized when the total number of receptors measured by antagonist radioligand binding was reduced by $<40\%$. However, no high-affinity binding sites remained, i.e. all of the receptors had become uncoupled. Leid *et al.* (1988) have provided evidence that solubilized porcine atrial A_1 receptors are coupled to more than 1 population of G proteins, thus different responses may desensitize at different rates. Modified coupling of A_1 receptors to G proteins could play a role in producing an unusually high sensitivity of adipocytes from obese Zucker rats to A_1 agonists (Vannucci *et al.*, 1989). Taken together, a general conclusion from all of these studies is that uncoupling of receptors from G proteins, rather than downregulation of receptor number *per se*, may play the most important role in the desensitization responses of brain, adipose tissue and heart.

Repeated dosing of rats with theophylline (Murray, 1982; Szot *et al.*, 1987) or caffeine (Green and Stiles, 1986; Boulenger and Marangos, 1989) results in an increase in the number of high-affinity agonists radioligand binding sites and increased adenosine receptor mediated inhibition of adenylyl cyclase in the brain, possibly as a result of increased coupling of A_1 receptors to G proteins. Pretreatment with theophylline also produced a significant increase in seizure threshold for convulsant agents (Murray, 1982). Treatment of guinea pigs with theophylline produced a 30% increase in the number of high-affinity agonist binding sites on cardiomyocytes within 2 days (Wu *et al.*, 1989). This was accompanied by a sixfold increase in the potency of adenosine to inhibit slow inward Ca^{2+} current. The effect of theophylline to apparently increase the sensitivity of the CNS and of the myocardium to adenosine is notable because it suggests that endogenous levels of adenosine are high enough to activate A_1 receptors even in normoxic tissues.

Conclusions

The A_1 adenosine receptor in the CNS is a 34–35-kDa glycoprotein. It is not yet clear if subtypes of A_1 receptors exist, but regardless of whether there are single or multiple related receptors, the receptor(s) can interact with a number

of different pertussis toxin-sensitive G proteins to activate several different effector systems. Diversity in tissue responses to A₁ adenosine receptor activation, responses to chronic exposure to agonists or antagonists and perhaps genetic variability in the response to adenosine may be regulated in part by the nature of the G proteins to which receptors are tightly coupled. Alterations in the coupling between A₁ receptors and G proteins appear to play an important role in modulating receptor-mediated responses. The factors which regulate these interactions between adenosine receptors and various G proteins are not yet understood.

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CHAPTER 6

POST-RECEPTOR MECHANISMS

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Introduction

Intracellular events following stimulation of adenosine receptors have been extensively studied and many of the intracellular consequences of such stimulation are becoming clear, especially as information is integrated from diverse disciplines such as pharmacology, oncology and genetics. When considering post-receptor mechanisms for adenosine as well as other neurotransmitters it is important to bear in mind the very probabilistic and complex nature of the systems involved. Intracellular responses to agonist stimulation of adenosine, or any other, receptors will vary according to the cell type,

antecedent, current and post-stimulation events. It is probably best to view the aftermath of receptor stimulation as a cascade of intracellular events with each point in the cascade being highly probabilistic and with many negative and positive feedback loops governing the flow and direction of information transfer.

It is important to stress that receptor stimulation does not command the cell to initiate a set, invariant, chain of events. Rather it merely provides a piece of information which is integrated with other cellular information in determining cellular actions. Within this frame of reference the post-receptor mechanisms following adenosine receptor stimulation are discussed. Logistically this is presented as starting from receptor stimulation and tracing consequential events to intracellular targets and the genome, although it should be borne in mind that a considerable amount of information is probably transferred in the opposite direction as well.

Intermediatory G proteins

Adenosine A₁ and A₂ receptors were initially characterized on the basis of their effect on cyclic adenosine monophosphate (cAMP) production, with A₁ receptors inhibiting synthesis and A₂ receptors stimulating synthesis (Van Calcar *et al.*, 1979; Londos *et al.*, 1980; see Chapter 4). These receptors do not act directly on adenylyl cyclase but rather act via intermediatory guanine nucleotide-binding proteins (G proteins) which bind either guanine triphosphate (GTP) or guanine diphosphate (GDP). G Proteins are not a single protein but consist of a heterotrimeric association of alpha, beta and gamma subunits. The α -subunit binds GTP and directly regulates adenylyl cyclase and other enzymes and effectors. The β/γ -subunit complex is required for efficient coupling of the α -subunit to receptors, and influences the rate of GDP dissociation from the GTP/GDP-binding site (Johnson, 1990). A number of different subunit species with different functional attributes have been discovered and consequently there are a number of distinct species of G protein heterotrimers. These include a species that stimulates adenylyl cyclase (G_s) and one that inhibits the cyclase (G_i) (Birnbaumer, 1990; Birnbaumer *et al.*, 1990). Many neurotransmitter receptors are thought to act via G protein intermediators including adenosine receptors (Caron, 1989; Jackson, 1990).

When G_s is activated by adenosine A₂ receptors it preferentially binds GTP and in this state undergoes dissociation into an α -subunit and a β/γ -heterodimer. Free α -GTP subunit (which has low affinity for the β/γ -heterodimer) regulates the activity of adenylyl cyclase. The α -subunit has an autocatalytic GTPase function (Borne *et al.*, 1989) which catalyses the

hydrolysis of α -GTP to α -GDP (which has a high affinity for the β/γ -heterodimer). In this hydrolysed form the α -subunit reassociates to the β/γ -heterodimer and re-establishes the trimeric form of the G_s holoenzyme (i.e. to its pre-stimulated G_s -GDP complex formation).

Adenosine A_1 receptors interact with G_i in an analogous way except that G_i inhibits adenylyl cyclase either directly, or indirectly by reducing the effectiveness of G_s (Birnbaumer, 1990; Birnbaumer *et al.*, 1990).

Second messenger production

Second messengers are crucial in mediating the effects of neurotransmitters and represent the internalization of the extracellular signal. Typically, second messengers also represent a considerable amplification of the original (neurotransmitter) signal. The first second messenger system to be discovered, that involving the production of cAMP, is also one of the best characterized (Schramm and Selinger, 1984; Worley *et al.*, 1987). This second messenger can be increased and decreased by the stimulation of adenosine A_2 and A_1 receptors respectively (Van Calcar *et al.*, 1979; Londos *et al.*, 1980), and appears to be the primary second messenger mediating many of the effects of adenosinergic compounds acting at adenosine receptors (Phillis and Wu, 1981; Stone, 1981; Dunwiddie, 1985; Snyder, 1985). Other second messenger systems have been discovered, notably those involving phosphatidylinositol (PI) turnover (Berridge, 1988, 1989; Berridge and Irvine, 1989) and calcium (Berridge, 1990; Rink and Merritt, 1990). Compounds acting at adenosine receptors can modulate a number of these non-cAMP second messengers, although it is not clear to what extent many of these actions may be secondary to changes in cAMP levels, since the second messenger systems do not act independently and there are considerable interactions ('crosstalk') between these systems at many levels. Nevertheless, many of the actions of adenosinergic compounds do not appear to be primarily mediated via a modulation of cAMP, suggesting that non-cAMP second messengers may be coupled to adenosine receptors (Stone, 1984; Hamprecht and Van Calcar, 1985; Fredholm and Dunwiddie, 1988).

Cyclic adenosine monophosphate

The effects of adenosine on cAMP have been well-characterized since the initial discovery that adenosine is a potent activator of cAMP accumulation in brain slices (Sattin and Rall, 1970). Adenosine has a concentration-dependent biphasic effect on cAMP accumulation. In low micromolar

concentrations ($1-25 \times 10^{-6} \text{ M}$) adenosine stimulates adenylyl cyclase, and in higher micromolar concentrations ($> 10^{-4} \text{ M}$) adenosine inhibits adenylyl cyclase (Haslam and Lynham, 1972). This biphasic action of adenosine has been demonstrated in many different tissue types including brain (Premont *et al.*, 1977a, b) and is attributable to actions at adenosine A_2 receptors (acting via G_s to produce an increase in cAMP) and adenosine A_1 receptors (acting via G_i to produce a decrease in cAMP) (Fig. 1). Caffeine, and other methylxanthines which are adenosine A_1 and A_2 receptor antagonists, block both the stimulatory and inhibitory actions of adenosine on adenylyl cyclase (Sattin and Rall, 1970; Daly, 1985).

Many other neurotransmitters, including noradrenaline, dopamine and 5-hydroxytryptamine, also modulate the activity of adenylyl cyclase via intermediary G proteins. Also, the compound forskolin can enhance agonist stimulation of adenylyl cyclase at low concentrations (*ca.* 10^{-9} M), possibly by an action at G_s , and can apparently directly stimulate adenylyl cyclase in higher concentrations (*ca.* 10^{-6} M) (Seamon and Daly, 1985). A

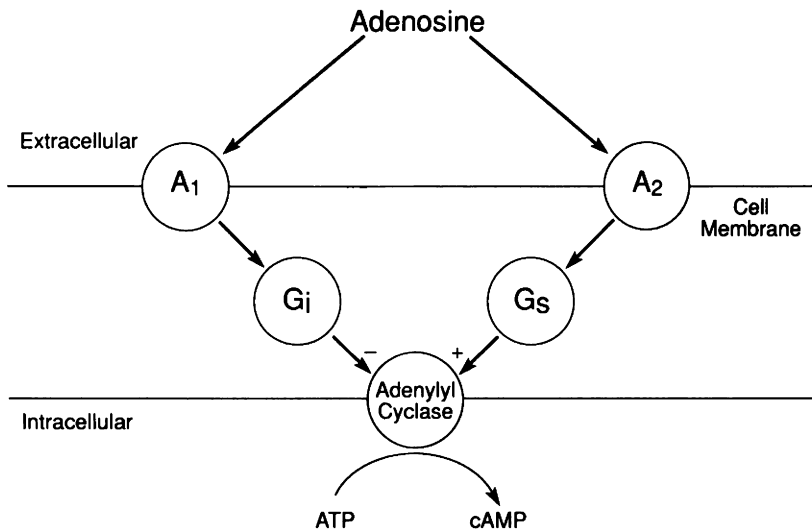


Figure 1 Regulation of adenylyl cyclase by adenosine receptor stimulation. Adenosine A_2 receptor agonists stimulate the rate of ATP metabolism to cAMP by increasing the activity of adenylyl cyclase, an action mediated via the stimulatory G protein, G_s . Adenosine A_2 receptor antagonists block this action. Conversely, adenosine A_1 receptor agonists inhibit the rate of ATP metabolism to cAMP by decreasing the activity of adenylyl cyclase, an action mediated via the inhibitory G protein, G_i . Adenosine A_1 receptor antagonists block this action.

tritiated form of this compound, which has a high affinity for adenylyl cyclase, has been successfully used to map the regional distribution of adenylyl cyclase (Worley *et al.*, 1986).

Autoradiograms reveal dense labelling of [^3H] forskolin to adenylyl cyclase in the caudate and olfactory tubercle (Worley *et al.*, 1986), structures in which the vast majority of adenosine A_2 receptors are visualized by autoradiography (Lee and Reddington, 1986; Jarvis *et al.*, 1989). The highly discrete distribution of adenylyl cyclase, as mapped by [^3H] forskolin (Worley *et al.*, 1986), can also be detected in many of the brain structures in which adenosine A_1 receptors have been identified such as the caudate, hippocampus, substantia nigra, cerebellum and cerebral cortex (Deckert *et al.*, 1988). In fact, the regional distribution and density of [^3H] forskolin-binding sites is strikingly similar to the regional distribution of adenosine A_1 and A_2 receptors labelled with the A_1/A_2 ligand [^3H] *N*-ethyl carboxyamidoadenosine (NECA) (Jarvis *et al.*, 1989).

Activity of cAMP is terminated by the action of a number of distinct cyclic nucleotide phosphodiesterases (Thompson and Appleman, 1971). Many methylxanthines, including caffeine, theophylline and isobutylmethylxanthine (IBMX), are relatively potent (IC_{50} 1–1000 $\times 10^{-6}$ M), non-selective, phosphodiesterase inhibitors (Weishaar *et al.*, 1985). These methylxanthines therefore not only modulate cAMP levels by actions directly at adenosine receptors, but also can affect levels of cAMP by retarding its catabolism as a result of inhibition of cyclic nucleotide phosphodiesterases.

Diacylglycerol / phosphatidylinositol

Inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) are prominent second messengers that mediate many of the actions of a large number of neurotransmitters including acetylcholine (Fisher *et al.*, 1983; Fisher and Bartus, 1985), 5-hydroxytryptamine (Conn and Sanders-Bush, 1985; Kendall and Nahorski, 1985) and noradrenaline (Exton, 1985; Kemp and Downes, 1986). IP_3 and DAG are simultaneously formed from the hydrolytic breakdown of the phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP_2) by the action of the enzyme phospholipase C (Worley *et al.*, 1987). Since the components of this second messenger system are recycled this system is often referred to as phosphatidylinositol (PI) turnover. As with adenylyl cyclase, phospholipase C is coupled to neurotransmitter receptors via G proteins (Drummond, 1983; Downes, 1989; Exton, 1990).

Although adenosine and its active analogues have been reported to stimulate PIP_2 hydrolysis in peripheral organs (Narang *et al.*, 1990) and in cultured cells (Ali *et al.*, 1990), it has been reported that in rat striatal slices adenosine agonists have no direct effect on PIP_2 hydrolysis (el-Etr *et al.*, 1989a). However,

adenosine agonists have been reported to dose-dependently potentiate carbachol- or noradrenaline-induced accumulation of inositol phosphates (el-Etr *et al.*, 1989a, b), an action that is also reliably observed in peripheral tissues (Lerner *et al.*, 1988; Okajima *et al.*, 1989; Ali *et al.*, 1990; Narang *et al.*, 1990), although the mechanism for this action is not clear. Adenosine and several analogues have been reported to inhibit phosphatidylinositol kinases both in brain (Endemann *et al.*, 1987) and other tissues (Doctrow and Lowenstein, 1987; Whitman *et al.*, 1987). Phosphatidylinositol 4-kinase has been purified to near homogeneity. Adenosine has been reported to inhibit this enzyme which phosphorylates inositol in the 4-position, i.e. catalyses the conversion of PI to phosphatidylinositol-4-phosphate (PIP) which is the precursor to PIP₂ (Walker *et al.*, 1988) and may therefore affect PI turnover. However, concentrations of adenosine required for these effects are high (IC₅₀ = 25–100 μ M) and the complexity of PI turnover cannot be understated. Considerably more studies are required to determine the role of adenosine in PI turnover. The potentiation of carbachol- or noradrenaline-induced PI turnover by adenosine may also be an action via cAMP since considerable 'crosstalk' occurs between these two second messenger systems (Worley *et al.*, 1987; Bouvier, 1990) and cAMP can regulate PI turnover, at least in murine T cells (Lerner *et al.*, 1988).

Whereas DAG is highly lipophilic and therefore interacts with membrane-bound target molecules, the other second messenger breakdown product of PIP₂, IP₃, is highly hydrophobic. IP₃ therefore migrates to the cytosol where it binds to specific intracellular receptors which gate the intracellular release of the second messenger, Ca²⁺ (Streb *et al.*, 1983; Burgess *et al.*, 1984).

Calcium

Calcium plays an essential and necessary role in many intracellular processes, not least of which is as a necessary requirement for neurotransmitter release (Blaustein, 1971). Adenosine has been demonstrated to inhibit the release of many neurotransmitters, including acetylcholine (Spignoli *et al.*, 1984; Pedata *et al.*, 1986), dopamine (Ebstein and Daly, 1982; Wood *et al.*, 1989), noradrenaline (Harms *et al.*, 1978; Ebstein and Daly, 1982), and GABA (Hollins and Stone, 1980; Peris and Dunwiddie, 1985/86) and a number of reports suggest that these actions are independent of altered cAMP levels (reviewed in Dunwiddie, 1985; Ribeiro and Sebastião, 1986).

In view of the large number of neurotransmitters, the release of which can be blocked by adenosine and its analogues, and the essential role of calcium in exocytosis (Blaustein, 1971), it is likely that adenosine affects the intracellular mobilization or effects of calcium. It has been proposed that a novel non-A₁ or A₂ adenosine receptor (the 'A₃' receptor) may gate the flux of calcium

into neurones and thereby alter the intracellular concentration of calcium (Ribeiro and Sebastião, 1986). It has been reported that adenosine and its analogues can inhibit the potassium-evoked calcium current into neurones (Ribeiro *et al.*, 1979; Wu *et al.*, 1982) although others have failed to replicate this effect (Barr *et al.*, 1985; Michaelis *et al.*, 1988; Garritsen *et al.*, 1989). Other studies suggest that adenosine may increase K^+ efflux from nerve terminals (Michaelis and Michaelis, 1981; Trussell and Jackson, 1987) which may indirectly alter intracellular Ca^{2+} levels by virtue of a K^+ -mediated change in membrane potential (Michaelis *et al.*, 1988). Whether such an effect of adenosine on K^+ channels is a direct action or a consequence of cAMP modulation requires careful investigation. It has been reported that cAMP can modulate K^+ channels in a number of brain regions including the locus coeruleus (Andrade and Aghajanian, 1985; Aghajanian and Wang, 1986) and CA1 pyramidal neurones of the hippocampus (Madison and Nicoll, 1982, 1986).

Second messenger targets

Second messengers act at specific intracellular target proteins. All major target proteins thus far discovered are protein kinases, which catalyse the addition of a phosphate group (phosphorylation) to substrate proteins. Typically, second messenger target proteins phosphorylate a large number of substrate proteins. Phosphorylated substrate proteins can be visualized by autoradiography following incubation with an activated kinase and γ -[^{32}P]ATP and subsequent separation of proteins by gel electrophoresis. Interestingly, many of the substrate proteins for second messenger kinases are themselves protein kinases or phosphorylases which catalyse the removal of a phosphate group (dephosphorylation). Second messenger target proteins have been intensely investigated, not only because of their role in mediating the effect of second messengers, but also because many viral oncogenic transforming proteins are homologues of components of second messenger transduction mechanisms (Curran *et al.*, 1988; Schonthal, 1990) and several tumour-promoting compounds bind to second messenger targets such as protein kinase C (Blackshear, 1988; Martelly and Castagna, 1989; Jaken, 1990).

cAMP-dependent protein kinase (A-kinase)

This target protein for cAMP is one of the smallest, simplest and best-understood protein kinases. The A-kinase holoenzyme is a tetramer consisting of two catalytic subunits coupled to two regulatory subunits which inactivate

the catalytic subunits. Binding of cAMP to the regulatory subunit promotes dissociation of the tetramer into a regulatory dimer and two free catalytic monomers (Taylor *et al.*, 1990). When dissociated, the A-kinase catalytic subunits are no longer subject to the inhibitory influences of the regulatory subunits. In this free (activated) state the A-kinase catalytic subunits phosphorylate a large number of proteins including tyrosine hydroxylase (Joh *et al.*, 1978), the nicotinic acetylcholine receptor (Huganir and Greengard, 1983), voltage-dependent sodium channels (Costa *et al.*, 1982), voltage-sensitive calcium channels (Curtis and Caterall, 1985), and synapsin I (which may play a role in neurotransmitter release) (Browning *et al.*, 1985). Many of these actions will directly affect neuronal excitability via altered ion conductance, receptor sensitivity, receptor-effector coupling and exocytosis and probably underlie many of the acute inhibitory actions of adenosine. Activation of the catalytic subunit of A-kinase may also result in long-term changes in cell functioning by actions within the nucleus, since A-kinase can phosphorylate transcription factor substrates which regulate gene expression (Grove *et al.*, 1987; Montminy *et al.*, 1990).

Few pharmacological studies of A-kinase have employed adenosinergic compounds to alter cAMP levels and thereby modulate the activity of A-kinase. Typically, cAMP or more stable analogues of cAMP (such as 8-bromo-cAMP) or compounds which stimulate adenylyl cyclase directly such as forskolin have been used to investigate A-kinase function. Nevertheless, 2-chloroadenosine has been reported to increase the rate of phosphorylation of tyrosine hydroxylase (Berry-Kravis *et al.*, 1988). Also, the effects of 2-chloroadenosine on neurotransmitter release at motor nerve terminals can be blocked by the A-kinase inhibitor H₇ (1-(5-isoquinolinyI-sulphonyl)2-methyl piperazine) (Chen *et al.*, 1989), although this kinase inhibitor will also block other protein kinases such as protein kinase C (Garland *et al.*, 1987; Chen *et al.*, 1989).

Mechanisms of adenosinergic signal transduction to the nucleus

Of all of the post-receptor mechanisms involved in adenosine signal transduction least is known about the translocation of signals from the cytoplasmic compartment to the nucleoplasmic compartment, which must involve translocation across the nucleocytoplasmic membrane. In the case of the cAMP-mediated pathway, there is considerable controversy concerning the mechanism whereby A-kinase regulates gene transcription and in particular if the A-kinase catalytic subunit regulates transcription factors directly or if intermediary phosphoproteins mediate the action of A-kinase (see Nigg, 1990). Immunological evidence suggests that when cAMP causes the catalytic subunit monomers of A-kinase to dissociate from the regulatory subunit dimer, the catalytic subunits

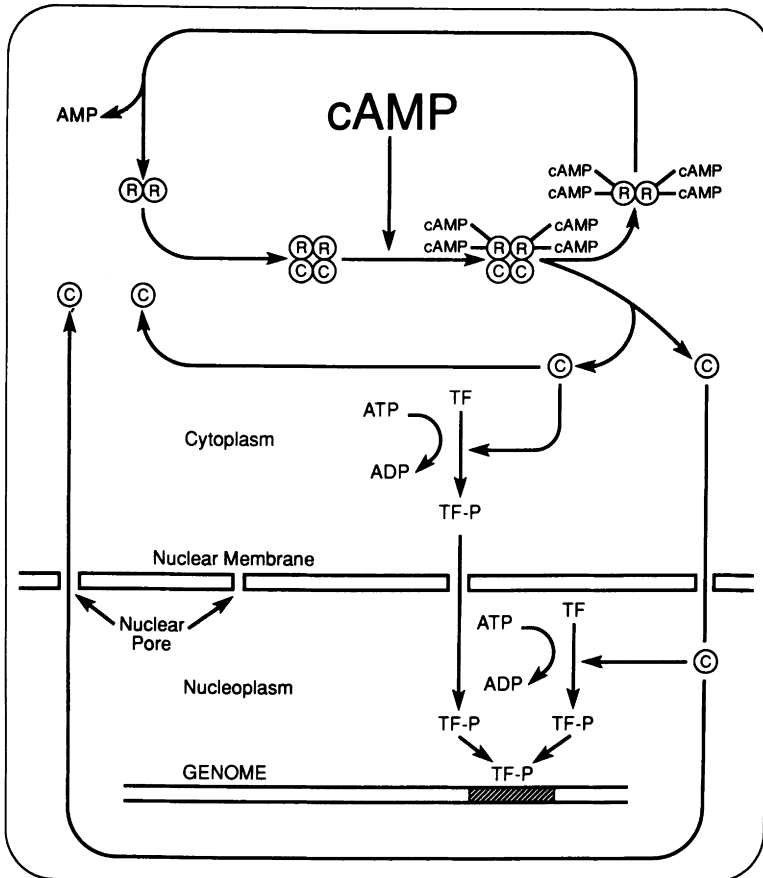


Figure 2 Regulation of gene transcription by cAMP via activation of A-kinase and transcription factors. In its less active form, A-kinase exists as a heterotetramer consisting of two regulatory (R) subunits and two catalytic (C) subunits. The R subunits inhibit considerably the catalytic efficacy of the C subunits. Binding of cAMP to the R subunits induces dissociation of the heterotetramer and thereby removes the negative regulatory control on the activity of the C subunits. In the free state, the C subunits phosphorylate many substrate proteins. Prominent substrate proteins are transcription factors (TF) which are readily and heavily phosphorylated. The addition of a phosphate group (P) to transcription factors (denoted by TF-P) may occur either in the cytoplasm or the nucleus, since C, TF and TF-P all traverse the nuclear membrane, although most evidence suggests this is predominantly a nuclear event. Binding of TF-P to specific enhancer sequences of nucleotides in the genome induces the transcription of cAMP-regulated genes. Catalytic activity of C is inhibited by the reassociation of C subunits with cAMP-free R subunit dimer.

translocate to the nucleus (Nigg, 1988). Translocation of the catalytic subunit from the cytoplasm to the nucleoplasm is probably via nuclear pores in the nuclear membrane. Nuclear pores are known to transport proteins containing a recognition sequence motif of amino acids (which act as a nuclear location signal) via an active or facilitated transport mechanism (Goldfarb, 1989).

Translocation of the A-kinase catalytic subunit may also involve phosphorylation, since the transportation of proteins into the nucleus is postulated to be dependent upon the phosphorylation of sequences close to recognition sites (Rihs and Peters, 1989). Within the nucleus, the catalytic subunit of A-kinase is thought to regulate gene transcription by phosphorylating transcription factors, although transcription factors may be phosphorylated outside of the nucleus, and then these get transported into the nucleus, or both mechanisms may occur since they are not mutually exclusive (see Fig. 2).

Other kinases

The transnuclear membrane signal transduction mechanisms of other protein kinases which may partially mediate the actions of adenosine are not well-understood. Activated protein kinase C can induce a large number of genes by phosphorylation of transcription factors (Verma and Sassone-Corsi, 1987). When activated by second messengers, protein kinase C translocates from the cell membrane to the cytoplasm (Huang, 1990). It has been suggested that activated (i.e. soluble) protein kinase C may be transported to the nucleus in an analogous way to A-kinase (Kikkawa and Nishizuka, 1986; Fields *et al.*, 1988; Kiss *et al.*, 1988) although evidence is much more equivocal than the evidence for nuclear translocation of A-kinase (see Nigg, 1990).

Genomic effects of adenosine

The genomic consequences of adenosine modulation of cAMP may be extensive. cAMP can transcriptionally regulate many genes including those coding for peptide neurotransmitters such as somatostatin, vasoactive intestinal polypeptide, and enkephalin, as well as genes for transcription factors such as c-fos (Montminy *et al.*, 1990). Induction of transcription factors such as c-fos by cAMP will result in considerably greater gene regulation, since transcription factors regulate gene expression by binding directly to nucleotide sequences of DNA and are known to enhance transcription of large numbers of genes (Morgan and Curran, 1989a, b).

The cAMP response element (CRE)

A common characteristic of many cAMP-inducible genes is that they are associated with very similar enhancement regions of DNA located at a distance from the cAMP-inducible gene and functioning to stimulate transcription of the cAMP-inducible gene. This enhancer region, which includes a highly conserved, palindromic core motif consisting of the sequence 5'-TGACGTCA-3' has been termed the cAMP response element (CRE) (Montminy *et al.*, 1986b). The CRE has been reported to be critical for cAMP-responsiveness of genes as well as for the activity of oncogene products to control transcription (Lee and Green, 1987). Genes not regulated by cAMP can be made to be responsive to cAMP by experimentally inserting DNA containing the CRE nucleotide sequence upstream of the target gene, indicating that the CRE alone can confer cAMP inducibility to a target gene (Montminy *et al.*, 1986b).

The cAMP response element binding protein (CREB)

Intensive investigations to reveal factors that regulate transcription in response to increased cAMP have concentrated on ligands that bind to the CRE. Such studies have discovered that CRE binding activity of nuclear extracts of brain tissue is due to a 43-kDa protein termed CRE-binding protein (CREB) (Montminy and Bilezikjian, 1987; Yamamoto *et al.*, 1988). CREB has been purified to homogeneity and demonstrated to stimulate the *in vitro* transcription of the somatostatin gene in nuclear extracts containing the CRE, but not in mutations lacking functional CRE sequences (Montminy *et al.*, 1986a; Montminy and Bilezikjian, 1987).

Since all of the known cellular effects of cAMP have been attributed to an action on the catalytic (C) subunit of A-kinase (Montminy *et al.*, 1986b) it is important to determine if CREB is a substrate for the C subunit of A-kinase. Empirical studies reveal that not only is CREB efficiently phosphorylated by the C subunit of A-kinase *in vitro*, but that phosphorylation of nuclear extract of brain tissue with the C subunit of A-kinase induced a 10–15-fold increase in CRE-dependent transcription (Montminy and Bilezikjian, 1987).

Like many other transcription factors, CREB binds to its primary genomic recognition site (cognate sequence) in the form of a dimer. Phosphorylation of CREB by the catalytic subunit of A-kinase does not affect either the rate at which CREB associates into, or dissociates from, its dimeric state. Nor does the extent of phosphorylation significantly affect the ability of the CREB dimer to bind to the CRE (Montminy *et al.*, 1990). Since A-kinase does regulate the transcriptional activity of CREB, it is assumed that phosphorylation of CREB alters its transcriptional-enhancing properties. This is thought to

involve the exposure of negatively charged residues in the CREB molecules which interact electrostatically with positively charged residues of proteins involved with the initiation of transcription of cAMP-inducible genes. A hypothetical schematic representation of CREB enhancement of transcription of a cAMP-regulated gene is depicted in Fig. 3.

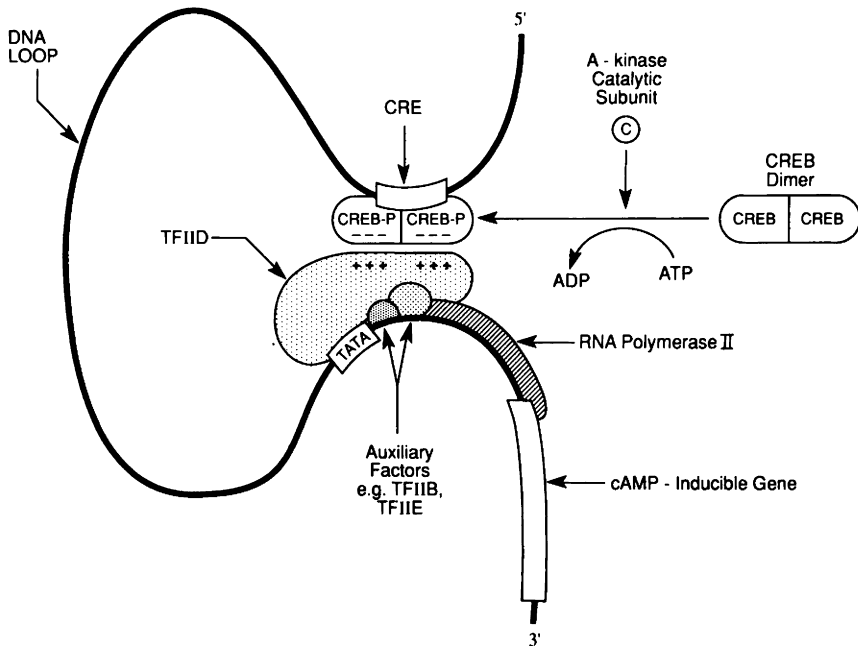


Figure 3 CREB enhancement of transcription of a cAMP-regulated gene. The transcription factor CREB is known to bind to the CRE-binding sites of the genome. When the dimer consists of phosphorylated CREB (CREB-P) negatively charged amino acid groups (so called 'acid blobs' or 'negative noodle motifs') are postulated to align and interact with positively charged groups in a protein or proteins (shaded elements in the figure) involved in initiation of transcription of a cAMP-inducible gene. These electrostatic interactions are postulated to be the basis for the transcription enhancing ability of CREB. The example illustrated is based upon a hypothetical gene which is regulated by the ubiquitous TATA box promoter and involves a pre-initiation complex consisting of RNA polymerase II, the TATA box factor TFIID and auxiliary factors TFIIB and TFIIIE which are commonly associated with this promoter. The illustration depicts CREB transactivating the cAMP-regulated gene by forming a DNA loop in order to explain the ability of the CRE to act over large distances in an orientation-independent manner. In view of the large number of genes which are enhanced by CREB, many alternate mechanisms are likely.

CREB-related transcription factors

CREB is one of an extended family of related proteins which act as transcription factors. Included in this family of proteins are closely related proteins such as CRE-binding protein-1 (CRE-BP1) and CRE-binding protein-2 (CRE-BP2), which, like CREB, bind to the CRE DNA motif (Maekawa *et al.*, 1989; Ivashkiv *et al.*, 1990; Kara *et al.*, 1990). CRE-BP1 has been cloned (from a human foetal brain cDNA expression library) and sequenced to reveal that CRE-BP1 is a close relative of CREB, as judged by the close homology between the primary structure of CRE-BP1 and CREB (Maekawa *et al.*, 1989; Kara *et al.*, 1990). The murine protein CRE-BP2 also exhibits considerable homology to both CREB and CRE-BP1 (Ivashkiv *et al.*, 1990).

The transcription factors Jun and Fos

Much more distantly related to CREB are the transcription factors Fos and Jun which have many similarities to CREB. Levels of the proteins Fos and Jun as well as c-fos and c-jun mRNA are rapidly expressed in response to increased cAMP (Berkowitz *et al.*, 1989; Fisch *et al.*, 1989; Muller *et al.*, 1989; Wu *et al.*, 1989) as well as in response to other second messengers (Kacich *et al.*, 1988; Condorelli *et al.*, 1989; Morgan and Curran, 1989a, b; see also Fig. 4).

Comparison of the structure of Fos and Jun with that of CREB reveals many homologies in important functional domains of these proteins. CREB, Fos and Jun all contain a basic residue-rich region of approximately 30 amino acids followed by a region of 4–5 leucines equally spaced at 7-residue intervals. This structure, dubbed the 'leucine zipper' (Landschulz *et al.*, 1988), is thought to be the basis for dimerization between these proteins which can form both homodimers (apparently with the exception of Fos) and more importantly Fos–Jun, and Jun–CREB heterodimers (Cohen and Curran, 1990). Interestingly, the CREB-related transcription factors CRE-BP1 and CRE-BP2 can form heterodimers with c-jun but not c-fos (Ivashkiv *et al.*, 1990; MacGregor *et al.*, 1990).

Although Fos and Jun are thought to primarily enhance transcription via the associated protein-1 (AP-1) DNA-binding site (Morgan and Curran, 1989a; Abate *et al.*, 1990), the core sequence of this site (viz. 5'-TGACTCA-3') differs from the core sequence of the CRE-binding site (viz. 5'-TGACGTCA-3') by only a single nucleotide. It is then not surprising that the Fos:Jun dimer binds to the CRE, albeit with reduced affinity compared to its affinity to the AP-1 DNA-binding site (Sassone-Corsi *et al.*, 1990). The ability of CREB, Fos and Jun to form heterodimers with each other strongly suggests that increases in adenosinergic regulation of cAMP is likely to elicit an

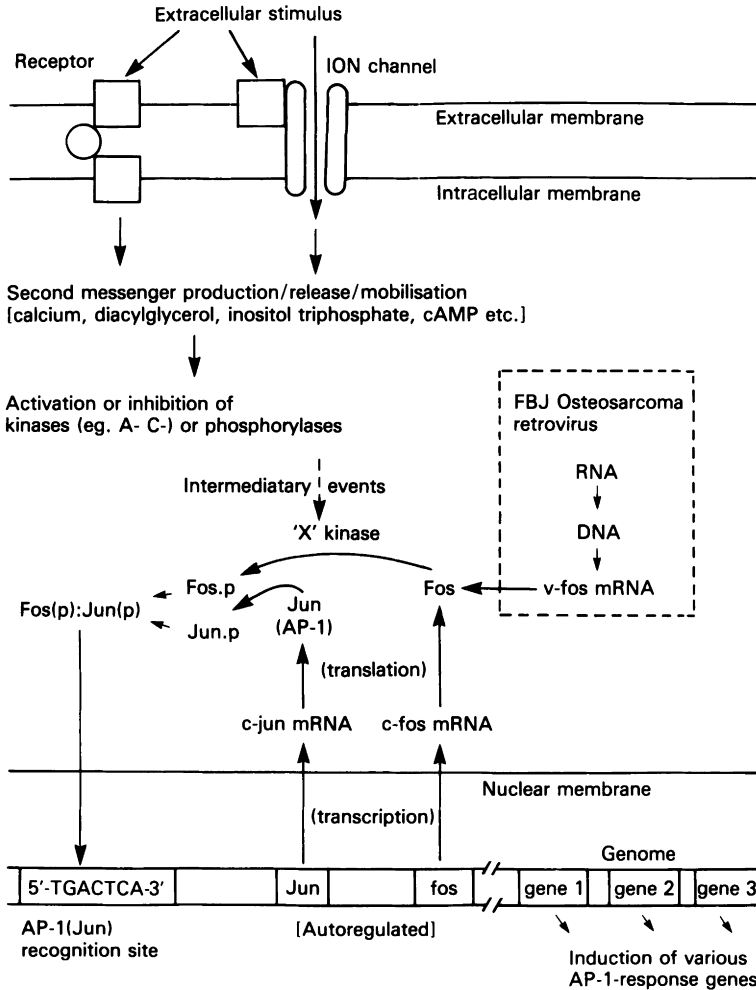


Figure 4 The role of transcription factors Fos and Jun in signal transduction. Expression of c-fos and c-jun mRNA as well as the protein products c-Fos and c-Jun (also known as AP-1) are rapidly and transiently increased in response to changes in a number of second messengers, including cAMP. The mechanism of this induction has yet to be elucidated, although both Fos and Jun are heavily phosphorylated (indicated as Fos-P and Jun-P) and it is assumed, by analogy to similar transcription factors such as CREB, that phosphorylation (by a number of kinases including A- and C-kinases) increases the transcriptional enhancing properties of these proteins. Both Fos and Jun are oncogene products of tumour-producing viruses, and the viral derivation of Fos is outlined in the rectangle within the figure. The Fos-P:Jun-P heterodimer binds to the AP-1 (also known as Jun)-recognition sites in the genome and thereby enhances the transcription of a large number of genes including the jun and fos genes (i.e. positive autoregulation occurs).

orchestrated response of transcription factor heterodimers which alter the expression of cAMP-regulated genes via genomic enhancer binding sites such as CRE and AP-1.

Conclusions

In the preceding discussion of adenosinergic post-receptor mechanisms much emphasis has been placed on the cAMP/A-kinase/CRE axis. In part this is because this signal transduction mechanism does appear to play a major role in the post-receptor actions of adenosine. In part it is also because there is insufficient data to determine with any degree of confidence the role of other transduction mechanisms (e.g. involving Ca^{2+} or PI turnover) in the post-receptor actions of adenosine. When it is not even clear as to how many distinct adenosine receptor subtypes are extant, this is surely to be expected.

The one very salient feature of adenosinergic post-receptor mechanisms is the extremely dynamic and interrelated nature of the post-receptor cellular events. All known second messengers partake in considerable 'crosstalk' and many second messengers, including cAMP, appear to share common 'third messengers' such as the transcription factors Jun and Fos. In such a scheme, replete with positive and negative feedback loops, the consequences of adenosine receptor stimulation are surely far reaching and branch considerably beyond the cAMP/A-kinase/CRE axis.

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CHAPTER 7

ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS

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Introduction

As the role of adenosine as a factor in a multitude of disease processes has become evident, the potential for adenosine receptors as bona fide targets for drug discovery has become increasing compelling (Stone, 1989; Williams, 1990a), although few entities have made their way to the marketplace (Williams, 1990b, 1991a).

Adenosine receptors have been classified into three major subclasses based on their pharmacological properties (Hamprecht and Van Calker, 1985; Bruns *et al.*, 1986): the A₁, A_{2a} and A_{2b} subtypes. In addition, evidence has been proposed for the potential existence of A_{1a} and A_{1b} (Gustafsson *et al.*, 1990) and A₃ (Ribeiro and Sebastião, 1986) subtypes. Well-documented species differences in adenosine receptors (Murphy and Snyder, 1982; Schwabe *et al.*, 1985; Ferkany *et al.*, 1986; Ukena *et al.*, 1986a; Stone *et al.*, 1988) have also been used as a basis to (re)define adenosine receptor subtypes (Wiener *et al.*, 1990).

Ligands for adenosine receptors, like those for most receptors, can be classified into agonists and antagonists (Williams and Sills, 1990) as well as allosteric modulators (Bruns and Fergus, 1989a) and metabolic potentiators (Engler, 1987; Grueber *et al.*, 1989). The structure–efficacy relationships of agonists for either adenosine receptor have not been extensively studied although Bazil and Minneman (1986) have defined such activities in studies related to adenylate cyclase activation. Pirovano *et al.* (1989) have directed focus to the 3'- and 5'-positions of the ribose moiety in regard to partial agonist activity.

Historical perspective

Adenosine itself has been extensively used as a probe for the study of adenosine systems in mammalian tissues since the initial reports on the cardiovascular actions of the purine some 60 years ago (Drury and Szent-Gyorgyi, 1929). Due to the metabolic lability of the purine nucleoside, various modified analogues were synthesized with chemical focus primarily on the 2-, N⁶- and

5'-positions. The potential utility of such agents was for use in hypertension. One of the first compounds to come from these synthetic efforts was 2-chloroadenosine (2-CADO; Fig. 1). Anecdotal evidence suggests that 2-CADO was evaluated as an antihypertensive agent in Australia in the early 1950s with observed effects on blood pressure that led the clinicians to conclude that adenosine agonists were not viable therapeutic entities, a viewpoint also expressed in 1986 following bolus administration of adenosine to normal subjects (Sylvén *et al.*, 1986). Despite such findings, efforts continued to identify more stable agonists with 5'-*N*-ethylcarboxamidoadenosine (NECA; Stein and Prasad, 1979; Fig. 1) and *N*⁶-cyclohexyladenosine (CHA; Bruns *et al.*, 1980; Fig. 1). The availability of these newer agents, while yet to lead to a bona fide drug, provided the tools by which to define subclasses of the adenosine receptor.

On the antagonist front, the seminal observation by Sattin and Rall (1970) that the methylxanthines, caffeine and theophylline, were adenosine antagonists,

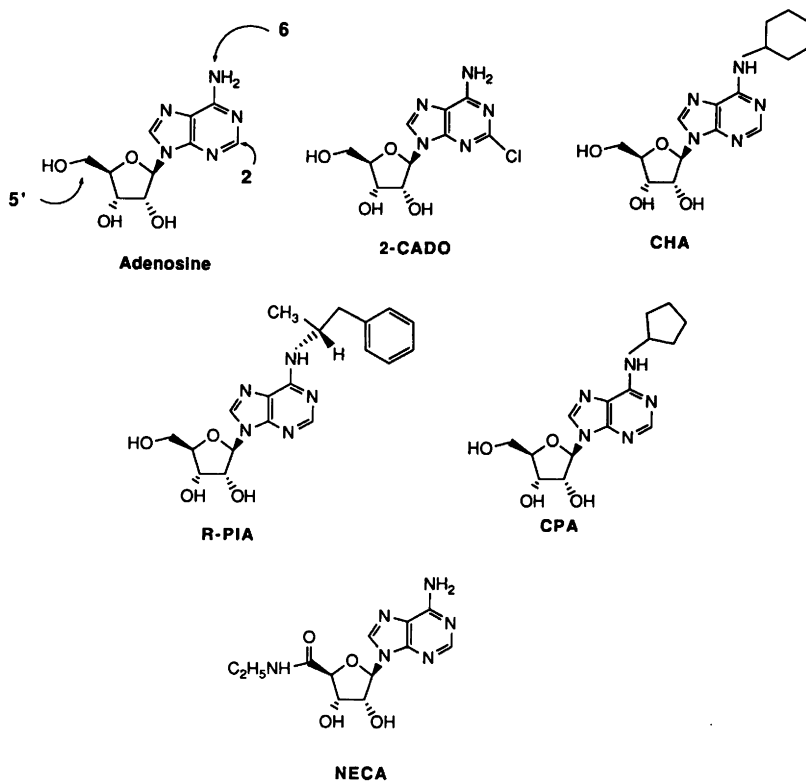


Figure 1 Structures of 'classical' adenosine agonists.

led to a reappraisal of the molecular mechanisms of this class of compound that had been used for over a century for the treatment of asthma (Persson, 1985). The methylxanthines were well-characterized as phosphodiesterase inhibitors and there still remains some controversy as to whether their use in asthma is due to antagonism of the actions of adenosine released from mast cells (Persson, 1985; Griffiths and Holgate, 1990). The development of binding assays for both the A₁ (Bruns *et al.*, 1980) and A_{2a} (Bruns *et al.*, 1986; Jarvis *et al.*, 1989) receptors provided a convenient means by which to screen other heterocycles for activity as adenosine antagonists (Williams, 1989).

Since NECA can recognize both the A_{2a} and A_{2b} receptor subclasses (Dunwiddie *et al.*, 1990), the data reviewed below is referred to in the nomenclature used in the original reports. In the majority of instances, the A₂ receptor described is probably the A_{2a} receptor.

Adenosine agonists

Known agonists for the adenosine receptor are analogues of adenosine modified in either the purine or ribose portions of the molecule. The ribose and N⁶-amino groups are essential for agonist activity (Bruns, 1980; Muller *et al.*, 1990). Substitutions in the 2-, N⁶- and 5'-ribose positions confer favourable activity on analogues while 7-, 8- and 9-substituted analogues generally have diminished binding activity (Bruns, 1980; Daly, 1985; Jacobson, 1988; Van Galen, 1990; Trivedi *et al.*, 1990; Ueeda *et al.*, 1991a, b). Modifications in, or removal of, the ribose ring have similarly resulted in reduced agonist activity with the emergence of antagonist activity (Taylor *et al.*, 1986; Peel *et al.*, 1990). Substitution of a hexose for the pentose ring decreases activity (Taylor *et al.*, 1986) while the 2'-hydroxyl group is essential for affinity and efficacy, and removal of the hydroxyl groups at the 3'- or 5'-positions reduces efficacy resulting in partial agonists. 2'-3'-Dideoxy-CHA has been reported as a weak antagonist (Lohse *et al.*, 1988a). The 9-methyladenines (Ukena *et al.*, 1987; Peel *et al.*, 1990) are also antagonists. Carbocyclic and thio-ribose analogues (Bruns, 1980; Dunham and Vince, 1986; Chen *et al.*, 1988) retain significant agonist activity. 5'-Substituted adenosines such as NECA also maintain receptor activity while modifying receptor selectivity (Stein and Prasad, 1979).

Considerably more is known about the structure-activity relationships for the A₁ receptor than the A₂ because of the earlier development of the binding assay for the former and a paucity of ligands for the latter receptor. For many adenosine analogues synthesized before the A₂ assays were in place, often only A₁ activity was determined. Nonetheless, because of the restricted number of pharmacophores for the adenosine receptor, knowledge of the parameters

necessary for favourable receptor–ligand interactions is limited. This is further compounded in the case of defining efficacy parameters when knowledge of the receptor structure has yet to be reported.

Structure–activity relationships

The A₁ receptor

CHA, 2-CADO and *R*-*N*⁶-phenylisopropyladenosine (R-PIA; Fig. 1) represent the prototypic ligands found to be active in A₁ binding assays and functional systems. The *S*-isomer of PIA has also been useful in determining the stereospecificity for the *N*⁶-region in the ligand–receptor interaction and was at one time used to distinguish A₁- from A₂-mediated responses (Stone, 1985; Stone *et al.*, 1988).

Alkyl substitutions in the hydrophobic *N*⁶-position are consistent with an increase in A₁ activity with the *N*⁶-isopropyl derivative having greater A₁ activity than either the ethyl or *n*-propyl derivatives (Trivedi *et al.*, 1990). Hydroxypropyl substitution at either the 2- or 3-position can diminish A₂ activity, thus increasing A₁ selectivity.

Overall, the *N*⁶-region of the A₁ receptor is more tolerant of large rigid and semi-rigid substituents than the A₂ receptor (Moos *et al.*, 1985). Aryl *N*⁶-substituted adenosines are A₁ selective with *N*⁶-phenyladenosine being 140-fold selective with a *K_i* value of 5 nM (Table 1). *N*⁶-Benzyladenosine is a weaker entity (*K_i* = 120 nM) being only twofold selective. Compounds related to *N*⁶-(2-phenethylamino)adenosine have been extensively used to model the *N*⁶-binding pocket of the A₁ receptor (Kusachi *et al.*, 1985; Daly *et al.*, 1986a; Van Galen *et al.*, 1987, 1989, 1990a, b; Pirovano *et al.*, 1989). Using a 'functionalized congener' approach involving amino acid and biotin substitutions in the *para* position of the phenyl ring, Jacobson *et al.* (1985a, b) have explored the steric requirements of an A₁ hydrophobic region. *Meta* and *para* substitutions on *N*⁶-phenyladenosine increase A₁ activity while modifications of *N*⁶-(2-phenethyl)adenosine which has 13-fold selectivity with a *K_i* value of 12 nM can alter A₁ affinity over a 4000-fold range (0.3 nM–1 μM; Trivedi *et al.*, 1990). *N*⁶-Diaryl-substituted analogues substituted in the α-position, i.e. *N*⁶-(diphenylmethyl)adenosine (Daly *et al.*, 1986a) are associated with a loss of A₁ activity while the 2,2-diphenylethyl analogue (Cl-936; Bridges *et al.*, 1987; Fig. 2) has nanomolar affinity at the A₂ receptor. Branching at the α-carbon reduces A₂ receptor activity (Trivedi *et al.*, 1990).

*N*⁶-2-Indanyladeniosine, an *N*⁶-benzocycloalkyladenosine, shows reasonable A₁ activity with 70-fold selectivity (Trivedi *et al.*, 1989). The *N*⁶-cycloalkyladenosines, CHA and its cyclopentyl analogue, CPA (Fig. 1) are potent and highly selective A₁ agonists (Moos *et al.*, 1985; Table 1) that have

Table 1 Receptor selectivity of adenosine agonists

Compound	Affinity (nM)		A ₂ /A ₁
	A ₁	A ₂	
CHA	1.2	500	416
CPA	0.6	460	767
NECA	11	15	1.4
(S)-ENBA	0.3	1390	4700
CCPA	0.4	3900	9800
CI 936	6.8	25	3.6
DPMA	142	4.4	32
CV 1808	800	110	0.14
CGS 21680	1408	19	0.03
PAPA-APEC	> 1000	1.5	0.002
CHEA	8700	1	0.0012 ^a
MPEA	8360	0.190	0.00003 ^a

^a Data derived from functional assays (Ueeda *et al.*, 1991a, b).

See text for abbreviations and data sources.

been extensively used to define A₁ receptor functionality. Both CHA and CPA are available as radioligands for the A₁ receptor (Williams and Jacobson, 1990). *N*⁶-Bicycloalkyladenosines are even more A₁ selective with *N*⁶-(2-*endo*-norbornyl)adenosine (*S*-ENBA; Trivedi *et al.*, 1989; Fig. 2) being 4700-selective for the A₁ receptor. *N*⁶-1-Adamantyladenosine, where the bicyclic ring size is increased, results in a dramatic decrease in receptor affinity.

The *N*⁶-aryladenosines have also been bisubstituted with additional modifications in both the 2- and 5'-positions. 2-Chloro-CPA (CCPA; Fig. 2) is 10 000-fold A₁ selective (Lohse *et al.*, 1988b) while 2-amino-CPA is 2000-fold A₁-selective (Trivedi, 1988). 2-Phenylamino-CPA, while retaining A₁ activity, is only 12-fold selective for the A₁ receptor, suggesting a negative interaction between aromatic substituents in the *N*⁶- and 2-positions (Trivedi and Bruns, 1989). Replacement of the 5'-hydroxyl group on *N*⁶-substituted adenosine analogues leads to an enhancement in A₁ selectivity while 5'-uronamides have increased A₂ selectivity. 5'-Chloro-5'-deoxy-CPA is threefold more selective than CPA as an A₁ agonist (Taylor *et al.*, 1986). 5'-Chloro-5'-deoxy-*S*-ENBA has a *K*_i of 0.4 nM at the A₁ receptor and is nearly 5000-fold selective (Trivedi *et al.*, 1990).

In general, compounds substituted in the 2-position have not been extensively documented, possibly due to difficulties in their synthesis (Ueeda *et al.*, 1991a). Derivatives such as 2-CADO with small groups in the 2-position

Adenosine receptor agonists and antagonists

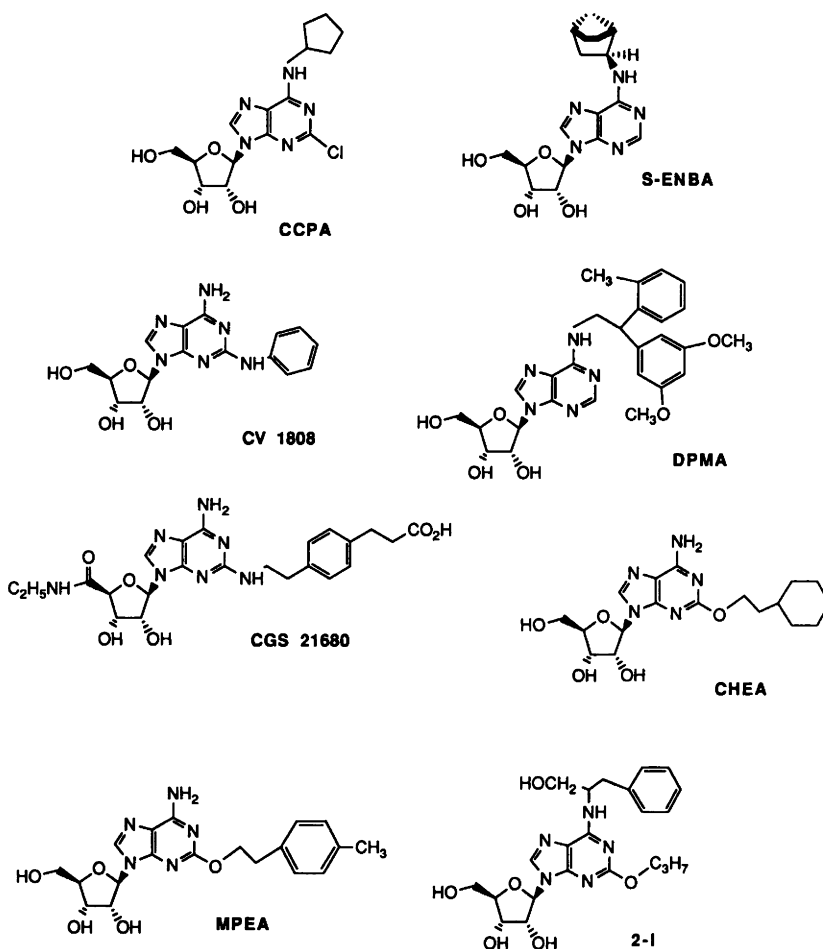


Figure 2 Structures of more recent chemically modified adenosine agonists.

are equi-active at both A_1 and A_2 receptors (Bruns *et al.*, 1986). 2-Alkylamino derivatives like the 2-phenylamino-substituted adenosines (Marumoto *et al.*, 1975, 1985; Kawazoe *et al.*, 1980) are A_2 receptor selective and include CV 1808 (Fig. 2). As noted by Van Galen (1990), 2-halo-, 2-thio-, 2-amino- and 2-oxo-adenosine derivatives are potent coronary vasodilators, an A_2 receptor-mediated event, and inhibitors of platelet aggregation, their effects being mediated via adenosine receptor activation. Such compounds are generally A_2 selective.

1-Substituted adenosine analogues include the hypotensive agent, doridosine (1-methylisoguanosine), a product derived from marine sources (Baird-Lambert *et al.*, 1980). 1-Deaza-adenosine is a reasonably effective agonist (Cristalli *et al.*, 1985).

The A₂ receptor

The original classification of A₁ and A₂ receptors in terms of alterations in adenylate cyclase activity (Van Calker *et al.*, 1979; Londos *et al.*, 1980) as well as the delineation of the distinct effects of adenosine on cardiac conduction (A₁ receptor-mediated) and coronary vasodilation (A₂ receptor-mediated) focused efforts to identify ligands selective for either the A₁ or the A₂ receptor. For a period of time, the 5'-substituted adenosine analogue, NECA (Stein and Prasad, 1979) was used to define tissue responses mediated by A₂ receptor activation. This ligand was, however, non-selective in its interactions with adenosine receptors, being approximately equipotent ($K_i = 10\text{--}15\text{ nM}$) at both A₁ and A₂ receptors (Bruns *et al.*, 1986). Thus the attribution of effects elicited by NECA to A₂ receptor-mediated processes was only valid if such effects were not seen with equivalent doses/concentrations of A₁-selective ligands such as CHA or CPA. For the development of the seminal A₂ receptor binding assay developed by Bruns and co-workers (Bruns *et al.*, 1986), the A₁ component of the binding profile of NECA was eliminated by the use of 50 nM CPA.

The 2-alkylamino adenosine analogue, CV 1808 (Marumoto *et al.*, 1975; Fig. 2) is a weak ($K_i \sim 100\text{ nM}$) ligand at A₂ receptors with a modest fivefold selectivity versus the A₁ receptor (Table 1). *N*⁶-1-Naphthylmethyl-adenosine (Kusachi *et al.*, 1985) was among the first moderately A₂-selective (threefold) but potent ($K_i = 10\text{ nM}$), ligands. *N*⁶-(*RS*)-2-(3,5-Dimethoxyphenyl)-2-phenylethyladenosine has a K_i value of 6 nM for the A₂ receptor with a fivefold selectivity (Bristol *et al.*, 1988). A series of 2,3-diphenylpropyl *N*⁶-substituted adenosine analogues including CI 936 (Bridges *et al.*, 1987) were found to have potent A₂ receptor activity ($K_i = 20\text{ nM}$) but were still A₁-selective. DPMA (PD 125 944; *N*⁶-[2]-(3,5-dimethoxyphenyl)-2-(2-methylphenyl-ethyladenosine); Fig. 2) is 39-fold selective for the A₂ receptor with a K_i value of 3 nM (Bridges *et al.*, 1988). Further evaluation of 2-substituents on NECA led to the identification of CGS 21577 (2-phenethyl-amino NECA; Hutchison *et al.*, 1989), and CGS 21680C (2-[*p*]-2-carboxy-ethyl)phenethylamino NECA; Fig. 2), the latter a potent ligand at the A₂ receptor ($K_i = 14\text{ nM}$) with 70–140-fold selectivity for this receptor. Interestingly, CGS 21680 has approximately the same affinity for the A₂

receptor as the parent compound, NECA, while the extended sidechain at the 2-position acts to decrease the A_1 affinity of these ligands. Consequently, the CGS 21577 is more active at the A_1 receptor than the corresponding carboxyethyl analogue, CGS 21680. A limited structure–activity relationship for these 2-arylalkylamino adenosine-5'-uronamides and related compounds has been reported (Hutchison *et al.*, 1990). CGS 21680 has been derivatized as PAPA-APEC and used as a probe to explore the A_{2a} receptor (Barrington *et al.*, 1989). Bisubstitution with groups favouring A_2 selectivity in the N^6 - and C2-positions results in a loss in potency with retention of receptor selectivity (Trivedi and Bruns, 1989).

Further structural modification of the 2-substituted alkoxyadenosines originally described by the Takeda group (Marumoto *et al.*, 1975) led to a series of 24 full agonists with selectivity for the A_2 receptor (Ueeda *et al.*, 1991a). Characterized in functional assays in guinea-pig coronary tissues rather than binding assays, 2-(2-cyclohexylethoxy)adenosine (CHEA; Fig. 2) had an EC_{50} value of 1 nM at the A_2 receptor modulating coronary vasodilation resulting in an 8700-fold selectivity for the A_2 receptor. 2-(4-Methylpentyloxy)adenosine has an EC_{50} value of 3 nM at the coronary A_2 receptor and a selectivity of 6400-fold. A related series of 26 2-aralkoxyadenosines was evaluated in the same experimental paradigms (Ueeda *et al.*, 1991b). One of these, 2-[2-(4-methylphenyl)-ethoxy]adenosine (MPEA; Fig. 2) was 44 000-fold selective for the coronary A_2 receptor with an EC_{50} value of 190 μ M. An (*S*)- N^6 -substituted, 2-propoxy adenosine analogue (compound 2.1, Fig. 2) has been recently described by Peet *et al.* (1990) as being 43-fold selective for the A_1 receptor in binding assays with a K_i value of 44 nM. The corresponding *R*-diastereomer was less active at the A_1 receptor (K_i = 3.7 μ M and only fivefold selective).

Evaluation of the Olsen alkoxy compounds in binding assays using brain tissue and other species may alter this phenomenal selectivity ratio but will permit a better comparison with data generated on other adenosine analogue series as well as reflect structure activity as opposed to structure activity/efficacy parameters. This is especially important since the assessment of adenosine receptor selectivity via these functional paradigms indicates far greater selectivity for the 2-substituted adenosines than that seen using binding assays. For instance, DPMA is 13 000-fold selective and CGS 21680, 33 000-fold selective in functional assays as compared to 39-fold and 70–140-fold, respectively in binding assays. Furthermore, the A_1 -selective agonists, CPA and DPMA, were considerably less selective in these functional assays than has been seen by numerous laboratories using binding assays. 2-Alkoxy-NECA analogues have also been reported with selective A_2 receptor activity (Olsson, 1991).

Adenosine antagonists

The majority of antagonist ligands for the adenosine receptor have been xanthine pharmacophores based on the observation that theophylline and caffeine were, in addition to being phosphodiesterase inhibitors, antagonists of the biochemical actions of adenosine (Sattin and Rall, 1970). The finding that 8-phenyl substitution can markedly increase activity at the receptor while diminishing activity resulted in analogues such as 8-phenyltheophylline (8-PT, Fig. 3) that have nanomolar affinity at the A₁ receptor (Smellie *et al.*, 1979; Bruns, 1981; Bruns *et al.*, 1983; Daly *et al.*, 1986b, c; Williams, 1989).

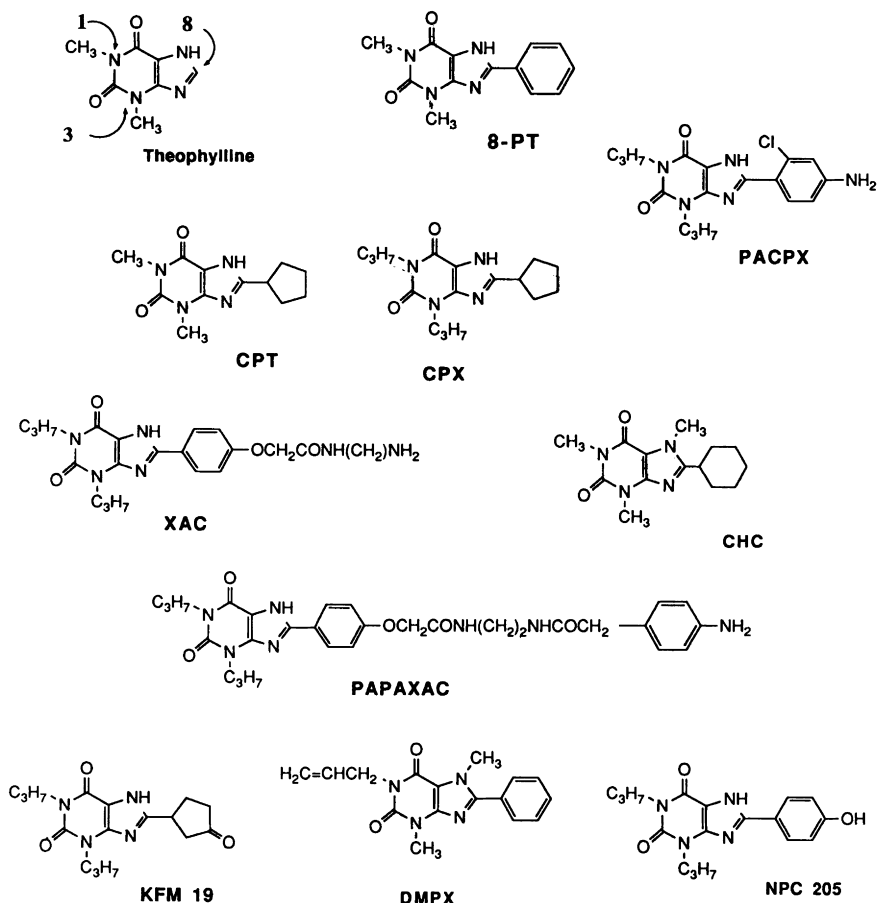


Figure 3 Structures of xanthine adenosine antagonists.

Alterations in the substituents at the 1- and 3-positions alter both the activity and pharmacological selectivity of the xanthines. The 1-methyl group appears essential for activity while large substituents in either the 1- or 3-positions enhance A_1 selectivity as in 1,3-dipropylxanthine which is 20-fold more active than theophylline ($K_i = 450$ nM; Table 2). The rank order of potency for 1,3-disubstituted xanthines is methyl < ethyl << propyl \leq isobutyl (Daly *et al.*, 1986b). Increases in affinity resulting from 1,3-dialkyl substitution

Table 2 Receptor selectivity of adenosine antagonists

Compound	Affinity (nM)		
	A_1	A_2	A_2/A_1
Theophylline	8500	25 000	3
1,3-Dipropylxanthine	450	5200	12
8-PT	86	850	10
PACPX	2.5	92	37
CPT	11	1400	130
CPX	0.5	340	740
XAC	0.9	27	30
PD 115 199	14	16	1.1
NPC 205	1.3	108	83 ^a
DMPX	45 000	1100	0.02
KFM-19	10	1512	151
CHC	28 000	190	0.01
Cartazolate	360	2200	6
FR 113453	17	10 900	407
CGS 15943A	22	3	0.14
CP 41 475	27 000	42 000	1.2
CP 66 713	0.3	0.02	0.07
CP 68 247	28	> 100 000	> 3570
CPQ	7.3	1000	137
HTQZ	3100	120	0.04
ADQZ	610	330	0.6
IQA	1600	1400	0.9
CPIQA	43	290	6.7
CPPIQA	10	450	45
CPMA	540	4900	9.1
Carbamazepine	31 000	200 000	6.5
Amiloride	11 000	17 000	1.6
MBA	70	—	—

^a Data derived from functional assays (Kaplita *et al.*, 1990).
See text for abbreviations and data sources.

and addition of an 8-phenyl group are additive in nature (Bruns *et al.*, 1983). Concomitant with the increase in receptor selectivity, these substitutions cause a dramatic decrease in solubility (Bruns *et al.*, 1988).

Substitution in the 7-position has varying effects on receptor affinity but can abolish the enhanced affinity seen with 8-phenyl-modified xanthines, suggesting some degree of steric hinderance at the receptor (Bruns, 1981; Ukena *et al.*, 1986a; Francis *et al.*, 1989).

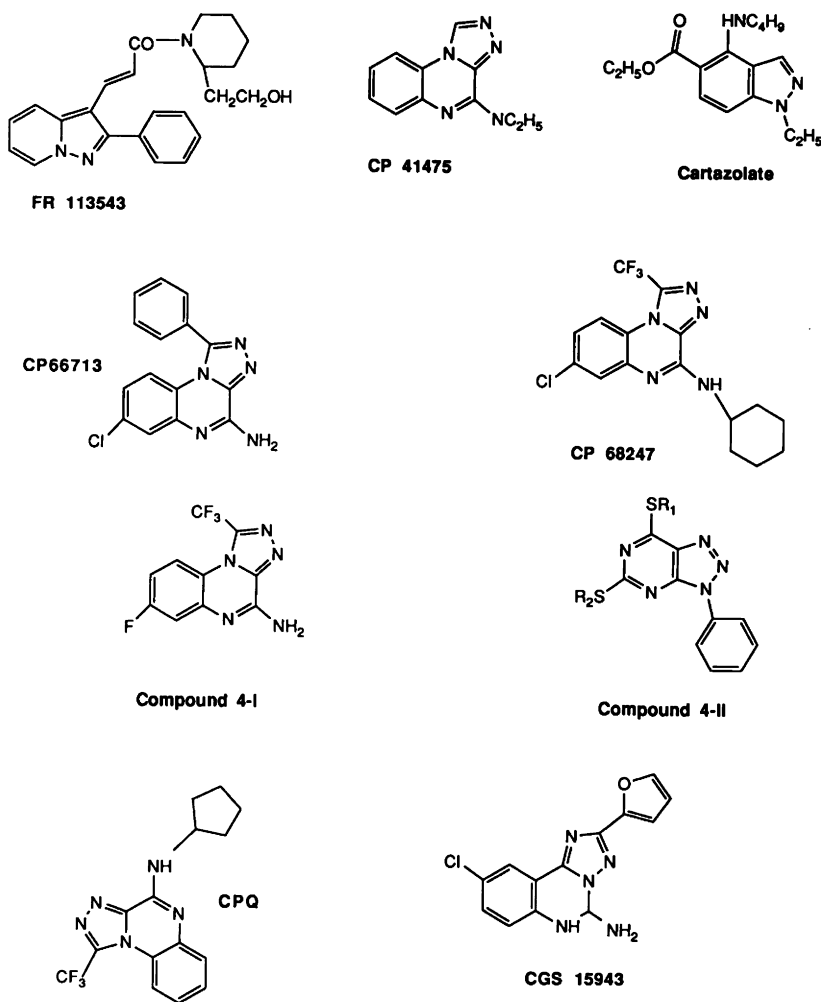


Figure 4 Structures of heterocyclic adenosine receptor antagonists.

Modification of 8-phenyl-substituted xanthines has become a major growth industry in the area of adenosine medicinal chemistry (Williams, 1989) with considerable success in developing potent and selective antagonists for the A_1 receptor. The A_2 receptor has been less amenable to targeting using the 8-phenylxanthine pharmacophore.

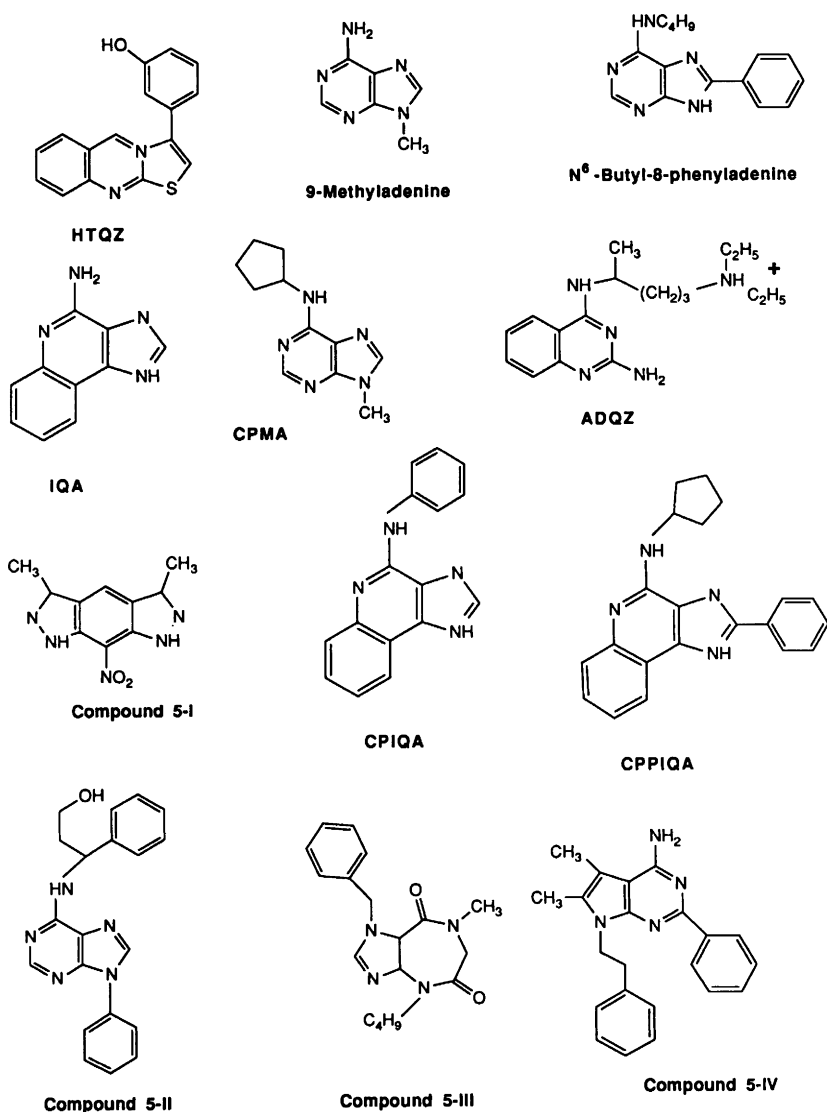


Figure 5 Structures of heterocyclic adenosine receptor antagonists.

A number of other planar nitrogen-containing heterocyclic adenosine antagonists have also been identified as antagonists of adenosine receptors. CGS 15943 (Fig. 4; Williams *et al.*, 1987; Francis *et al.*, 1989), CP 66 718 (Fig. 4; Sarges *et al.*, 1990a, b) and ADQZ (Fig. 5; Bruns *et al.*, 1988) representing some of the more interesting entities.

Xanthine antagonists

The A₁ receptor

The majority of 8-phenyl-substituted xanthines evidence A₁ selectivity. 8-PT shows a 100-fold increase in A₁ activity compared with theophylline and a 30-fold increase in A₂ activity (Bruns *et al.*, 1986). 1,3-Diethyl-8-phenylxanthine (DPX; Fig. 3; Table 2) is a potent A₁ antagonist ($K_i = 10$ nM; Bruns *et al.*, 1986) with 18-fold selectivity. The cyclopentyl xanthine, 8-cyclopentyl-1,3-dipropylxanthine (CPX; PD 116 948, DPCPX; Bruns *et al.*, 1987a) has subnanomolar affinity ($K_i = 0.5$ nM) for the A₁ receptor and is 740-fold selective. Cyclopentyltheophylline (CPT; Fig. 3; Bruns *et al.*, 1987b) is 130-fold selective for the A₁ receptor with a K_i value of 11 nM. PACPX (1,3-dipropyl-8-(2-amino-4-chloro)-phenylxanthine; Fig. 3; Table 2) has high potency ($K_i = 3$ nM) and selectivity (37-fold) for the A₁ receptor (Bruns *et al.*, 1983). This antagonist appears to be non-competitive in its actions at the A₂ receptor (Williams *et al.*, 1987; Leung and Green, 1989) while its receptor affinity is highly species-dependent (Stone *et al.*, 1988). Substitutions on the 8-phenyl group have varying effects. *Para* substitutions increase A₁ affinity while substitutions at the *meta* and *ortho* positions reduce activity (Bruns *et al.*, 1983) the latter via steric interference at the N⁷-position (Bruns, 1981). The 2-hydroxy-4-methoxyphenyl analogue of PACPX is also highly active at the A₁ receptor (Daly *et al.*, 1986c).

NPC 205 (Fig. 3; Kaplita *et al.*, 1990) the 4-hydroxy analogue of DPX is 2–3 times more active than PACPX at the A₁ receptor in rat and guinea-pig brain. Based on functional activity in blocking adenosine effects on A₁ (adenylate cyclase inhibition, rat cortex) and A₂ (adenylate cyclase stimulation, PC 12 cell) receptor function, NPC 205 was approximately 100-fold A₁-selective. KFM 19, a recently reported analogue of CPT (Schingnitz *et al.*, 1991) is 150-fold selective for the A₁ receptor and has been reported active in animal models of cognition impairment. 8-Cyclohexyl (*trans*-4-acetamido)-1,3-dipropylxanthine (Katsushima *et al.*, 1990) is some 800-fold selective for the A₁ receptor with a K_i value of 12 nM.

Jacobson *et al.* (1985a, b, 1986, 1987) substituted various functional groups to 8-phenylxanthines as part of their functionalized congener approach to adenosine antagonists. The amine congener, XAC (xanthine amine congener; Fig. 3) is approximately 30-fold selective for the A_1 receptor with a K_i value of 0.9 nM, while the corresponding carboxylate congener, XCC is some 60-fold less active at the A_1 receptor. A variety of other functional groups including biotin, spin labels and fluorescent probes have been introduced at the *para* position of the 8-phenyl substituent and have been used to explore the function and structure of the A_1 receptor (Jacobson, 1988; Jacobson *et al.*, 1991a). PD 113 297 has a hydrophilic tail at the *p*-position and is 12-fold A_1 -selective with a K_i value of 6 nM (Hamilton *et al.*, 1985). The acylating *m*-phenylene diisothiocyanate congener of XAC (*m*-DITC-XAC) has been used as irreversible inhibitor of the A_1 receptor (Stiles and Jacobson, 1988; Boring *et al.*, 1991). PAPAXAC (*p*-aminophenylacetyl-XAC; Fig. 3) represents an iodlatable congener that is used to label the A_1 receptor and can also be used as a photoaffinity label (Stiles and Jacobson, 1987).

BW A827 (1-propyl-3-[4-amino-3-iodophenyl]ethyl)-8-*p*-[(carboxymethoxy)phenyl]xanthine is an A_1 -selective ligand with an affinity of 1 nM (Linden *et al.*, 1988). BW A947U the corresponding aryl azido analogue has been used as photoaffinity label for the A_1 receptor (Earl *et al.*, 1988) while BW A844U, a cycloalkyl analogue of BW A827 is a potent antagonist ($K_i = 0.2$ nM) of the A_1 receptor.

The A_2 receptor

Few of the xanthines synthesized to date have had any significant selectivity or affinity for A_{2a} receptors. 3,7-Dimethyl-1-propargylxanthine (DMPX, Ukena *et al.*, 1986b; Fig. 3; Table 2) is approximately fourfold selective for the A_2 receptor *in vitro*. While a relatively weak antagonist DMPX has been used as a tool to explore A_2 receptor function *in vivo* (Seale *et al.*, 1988).

PD 155 119, a sulphonamide congener of DPX (Hamilton *et al.*, 1985) that is equi-active at A_1 and A_2 receptors (K_i , $A_1 = 14$ nM; K_i , $A_2 = 16$ nM), has been used as an A_2 antagonist radioligand in a manner analogous to NECA with CPX being used to block the A_1 portion of specific binding (Bruns *et al.*, 1987a). 1,3-Dipropyl-8-*trans*-4-((acetamidomethyl)cyclohexyl)xanthine, while approximately threefold A_1 -selective, was a potent ligand ($K_i = 20$ nM) at the A_2 receptor having approximately the same activity as PD 115 199 (Katsushima *et al.*, 1990). Cyclohexylcaffeine (CHC; Fig. 3) is 140-fold selective for the A_2 receptor with a K_i value of 190 nM (Shamim *et al.*, 1989). The selectivity of CHC appears, however, to be highly dependent on the assay system used for compound evaluation. A series of xanthine-7-ribosides have provided further evidence for adenosine binding to the A_1

receptor in an anticonfiguration (Van Galen *et al.*, 1990b). Of these, dibutylxanthine-7-riboside has a K_i value of 450 nM.

Xanthine solubility

As noted above, while 1-, 3- and 8-substitutions to the xanthine pharmacophore increase activity at the adenosine receptor, this is accompanied by a dramatic loss in solubility (Bruns *et al.*, 1988; Bruns and Fergus, 1989b). Thus while PACPX is a very potent antagonist, its solubility (210 nM) precludes its use as either a radioligand or research tool. XAC and PD 115 199 (solubility, 90 and 35 μ M, respectively) represent approaches to improving solubility and the usefulness of these adenosine antagonists as research tools.

Non-xanthine antagonists

Pyrazolopyrimidines

A number of 4-thio- (Davies *et al.*, 1983) and 4-amino- (Trivedi *et al.*, 1990) pyrazolo(3,4*d*)pyrimidines have been reported as adenosine antagonists. The most potent of the latter (A_1 K_i = 23 nM; A_2 K_i = 35 nM) was the 1,6-diphenyl analogue (Bruns and Hamilton, 1987). Structure–activity studies for a series of 21 5-aryl-7-oxopyrazolo(4,3*d*)pyrimidines at the A_1 receptor (Hamilton *et al.*, 1987) provided evidence that the 6-membered ring of this pharmacophore may bind to the same receptor domain as the corresponding 5-membered ring of the xanthine pharmacophore. The most active compound in this series, the 2-amino-4-chloro pyrazolo(4,3*d*)pyrimidine analogue (K_i = 176 nM) is related to PACPX and showed a corresponding increase in activity over the corresponding phenyl analogue (K_i = 580 nM). Sulphonamide substitution in the *p*-position, like that seen with PD 113 297 and PD 115 199, increased both solubility and activity (K_i = 68 nM). Takeda (Naka and Nagaoka, 1990) have claimed five pyrazolopyrimidines as putative cognition enhancers. 1-Benzyl-6-(4-methoxyphenyl)-3-propyl-1,2,3,4-tetrahydro-5*H*-imidazo(2'*l*:5,1)pyrazolo(3,4*d*)pyrimidine-2,4-dione is A_1 -selective.

Pyrazolopyridines

The (3,4*b*)pyrazolopyridines represent A_1 -selective adenosine antagonists. Cartazolate (Fig. 4) is sixfold A_1 -selective with a K_i value of 360 nM (Bruns *et al.*, 1986) originally identified as a putative anxiolytic agent interacting with the central benzodiazepine receptor and subsequently identified as an

adenosine antagonist by Psychoyos *et al.* (1982). FR 113 453 (Fig. 4) is a recently described A_1 receptor selective pyrazolopyridine adenosine antagonist (Terai *et al.*, 1990; Shiokawa *et al.*, 1990). The compound is a potent renal vasodilator producing diuretic and saliuretic actions through effects on the renal tubule. A newer series of pyrazolo(1,5a)pyridines have been claimed recently as cognition enhancers (Ohnashi *et al.*, 1989). Their interactions with CNS adenosine systems have yet to be determined.

Triazoloquinazolines

The pyrazoloquinoline benzodiazepine receptor inverse agonist, CGS 8216, was identified as an adenosine antagonist (Czernik *et al.*, 1982). Modification of this compound led to the identification of the triazoloquinazoline, CGS 15943A (Williams *et al.*, 1987; Francis *et al.*, 1989; Fig. 4; Table 2) a potent (A_1 $K_i = 33$ nM; A_2 $K_i = 3$ nM) adenosine receptor antagonist with slight *in vitro* selectivity for the A_2 receptor although this has not been confirmed in other studies (Daly *et al.*, 1988a, b; Sarges *et al.*, 1990a). The structure–activity relationship for this series has been reported and discussed in relation to the antagonist binding site of the adenosine receptor and is discussed in more detail below. CGS 15943A has been developed as a radioligand (Jarvis *et al.*, 1991).

Triazoloquinoxalinamines

The triazoloquinoxalinamines have a similar 6:5 fused-ring system to adenine and were identified as potential antidepressants in empirical preclinical evaluation (Sarges *et al.*, 1990a). The initial lead, CP 41 475 (4-(diethylamino)-(1,2,4) triazolo(4,3a) quinoxaline; Fig. 4) was a weak antagonist with approximately equal activity in A_1 and A_2 receptor binding assays (IC_{50} , $A_1 = 27$ μ M; IC_{50} , $A_2 = 42$ μ M). Evaluation of a series of 138 analogues of CP 41 475 also led to the identification of CP 68 247 (8-chloro-4(cyclohexylamino)-1-(trifluoromethyl)(1,2,4) triazolo(4,3a) quinoxaline; Fig. 4) which had over 3000-fold selectivity for the A_1 receptor with an IC_{50} value of 28 nM. The corresponding cyclopentyl analogue of CP 68 247 was the most potent A_1 antagonist identified, with an IC_{50} value of 6 nM, similar to that observed by these workers for CGS 15943. CP 66 713 (4-amino-8-chloro-1-phenyl(1,2,4)-triazolo(4,3a)quinoxaline; Fig. 4), in which a phenyl group replaced the trifluoromethyl, was found to be 13-fold selective for the A_2 receptor with an IC_{50} value of 21 nM. In a previous report (Sarges *et al.*, 1990b), CP 66 713 had been found to be greater than 450-fold selective for the A_2 receptor. This discrepancy appeared to be due to the solubility of this compound and its inability to interact at high concentrations (~ 1 μ M) with the A_1 receptor. The

lower selectivity of CP 66 713 for the A₂ receptor has been independently confirmed (Sarges *et al.*, 1990a). In an independent initiative, Trivedi and Bruns (1988) also identified the triazoloquinoxalinamines as adenosine receptor antagonists, characterizing CPQ (*N*-cyclopentyl-1-(trifluoromethyl)-(1,2,4) triazolo(4,3a)quinoxalin-4-amine; Fig. 4) as a potent ($K_i = 7$ nM) and selective (140-fold) A₁ receptor antagonist. Activity at the A₁ receptor was favoured by CF₃, C₂F₅ or ethyl substitutions in the 1-position and secondary amines in the 4-position. Hydrophobic substituents at the exocyclic amine group (cyclohexyl, cyclopropyl) also enhanced A₁ activity. Affinity at the A₂ receptor was dependent on an amine at the 4-position while phenyl, ethyl or CF₃ substitutions in the 1-position enhanced activity as did 7- or 8-halo groups or a 7-methoxy addition.

The trifluoromethyl substituent in the triazoloquinoxalinamines, i.e. CP 68 247 and CPQ, has been suggested to increase adenosine receptor activity because of an inductive effect on the amine proton resulting from the presence of the CF₃ group causing it to become more acidic for hydrogen bonding to the receptor(s) (Trivedi and Bruns, 1988).

Despite the extensive structure-activity relationship reported for the Pfizer triazoloquinoxalinamines (Sarges *et al.*, 1990a), it is not clear how their selective interactions with adenosine receptors reflect their observed preclinical effects as putative antidepressants. For instance, CP 41 475 which is essentially non-selective in its interactions with either A₁ or A₂ receptors and is active in the micromolar concentration range *in vitro*, has a minimum effective dose (MED) in eliciting putative antidepressant activity of 10 mg/kg p.o. CP 68 247, the most selective A₁ ligand with an IC₅₀ value of 28 nM has an MED of > 32 mg/kg p.o. A similar MED is observed for the A₂ selective triazoloquinoxalinamine, CP 66 713. The MED of 1–3.2 mg/kg p.o. for 4-amino-8-fluoro-1-trifluoromethyl(1,2,4) triazolo(4,3a)quinoxaline (compound 4-I, Fig. 4) was some 3–10 greater than CP 41 475 which is threefold A₂-selective (A₁, IC₅₀ = 290 nM; A₂, IC₅₀ = 100 nM; Sarges *et al.*, 1990a) raising the possibility that the *in vivo* antidepressant activity may reflect antagonism at both A₁ and A₂ receptors in the CNS.

Triazolopyrimidines

A series of dithiotriazolopyrimidines have been reported by Quinn *et al.* (1990) which are A₁-selective. Their generalized structure is shown in Fig. 4 (compound 4-II).

Thiazoloquinazolines

Assessment of a number of heterocyclic structures in adenosine binding assays resulted in the identification of the thiazoloquinazoline, HTQZ (3-(3-

hydroxyphenyl)-5*H*-thiazolo(2,3*b*)quinazoline; Fig. 5). The compound had reasonably high affinity ($K_i = 120$ nM) for the A_2 receptor (Bruns *et al.*, 1988) with 25-fold selectivity. The activity seen with HTQZ in rat brain tissue appears, like that seen with PACPX (Bruns *et al.*, 1983; Stone *et al.*, 1988) to be highly species dependent (Trivedi *et al.*, 1990).

Quinazolines

Further examination of the quinazoline pharmacophore in binding studies led to the identification of ADQZ (2-amino-4-{[4-(diethylamino)-1-methylbutyl]amino}quinazoline; Fig. 5) which is twofold A_2 -receptor selective (Table 3; Bruns and Coughenour, 1987) with a K_i value of 330 nM. ADQZ is considered of additional interest because it contains an aminoalkyl sidechain instead of the aryl or cycloalkyl groups found in the majority of adenosine receptor antagonists (Bruns *et al.*, 1988).

Benzodipyrzoles

A series of benzo(1,2*c*:5,4)dipyrzoles also function as weak adenosine A_1 receptor antagonists (Peet *et al.*, 1988). A nitro analogue (compound 5-I, Fig. 5) was sevenfold A_1 -selective with a K_i value of 1.9 μ M.

Imidazoquinolinamines

The imidazoquinolinamines may represent the first reported adenosine antagonists whose interaction with the A_1 receptor was based on the modelling of the antagonist binding site of the A_1 receptor (Van Galen *et al.*, 1990a). These compounds are structurally related to the triazoloquinazolinamines and the triazoloquinoxalin-4-amines and are based on the model of the A_1 antagonist binding site developed by Van Galen *et al.* (1990a) which, like that suggested for PACPX (Bruns *et al.*, 1986), involves a 'backward binding' or 'flipped orientation' in relation to adenosine agonists. While the nitrogen positions differ from those in the 6-, 7- and 8-positions, based on modelling of the triazoloquinazolinamines and the triazoloquinoxalin-4-amines, they appear to correspond to the positions described for the latter in this working model. The unsubstituted compound, 1*H*-imidazo(4,5*c*)quinolin-4-amine (Fig. 5; IQA) was essentially non-selective (A_1 , $K_i = 1600$ nM; A_2 , $K_i = 1400$ nM). The N^6 -cyclopentyl analogue (CPIQA) was sevenfold A_1 receptor selective with a K_i value of 43 nM (Fig. 5) while the N^6 -cyclopentyl/phenyl-substituted compound (CPPIQA) was 45-fold A_1 -selective with a K_i value of 10 nM (Fig. 5). Phenyl substitutions in both positions reduce A_1 activity. Favourable substitutions in the N - and 2-positions are not additive, a phenomenon similar to that seen for N^6 /C2-substituted adenosine analogues

(Trivedi, 1988; Trivedi and Bruns, 1989; Trivedi *et al.*, 1990). Thus as with the majority of heterocycles examined to date, hydrophobic substituents at an exocyclic amino group enhance activity at A_1 receptors. A 2-phenyl substitution also enhances A_1 activity.

9-Methyladenines

The adenosine antagonist activity, albeit weak, of 9-methyladenine (Fig. 5) was initially described by Bruns (1981). Investigation of the structure–activity relationship of this pharmacophore by Ukena *et al.* (1987) showed that N^6 -substitution increased activity at the A_1 receptor with N^6 -cyclopentyl-9-methyladenine (CPMA; Fig. 5) having a K_i value of 540 nM. Since the *R*- and *S*-PIA derivatives were fourfold different in activity, paralleling similar observations for the corresponding purine nucleosides, it was suggested that the 9-methyladenines bind to the same N^6 -receptor domain as adenosine (Ukena *et al.*, 1987; Bruns, 1990). A series of 9-methyladenine analogues have been described by Peet and co-workers (1990). The analogue of compound 2-I (Fig. 2), where a phenyl group replaces the ribose (compound 5-II, Fig. 5) is some 100-fold less active at the A_1 receptor with one-fourth of the selectivity of the ribose-containing analogue. Compound 5-II is probably antagonist in nature.

Van Galen (1990) has suggested that the N^6 -butyl-8-phenyladenine (A_1 K_i = 170 nM; Bruns *et al.*, 1988) may represent a structure intermediate between 9-methyladenine and 8-phenyltheophylline.

Imidazodiazepinediones

The imidazodiazepinediones were synthesized by Daly *et al.* (1990) from hypoxanthine. The most active compound in this series (compound 5-III, Fig. 5), was 12-fold selective for the A_1 receptor with a K_i value of 11.3 μ M.

7-Deaza-2-phenyladenines

From a series of 7-deaza-9-phenyladenines with weak adenosine antagonist activity (Daly *et al.*, 1988a,b) a series of 7-deaza-2-phenyladenines with greater activity have been developed (Muller *et al.*, 1990). The most potent of these (K_i = 4.7 nM; compound 5-IV, Fig. 5) was approximately 800-fold selective for the A_1 receptor. The *meta*-chloro (*rac*)-1-phenylethyl analogue while sixfold less active (K_i = 28 nM), was over 2000-fold selective. Chloro-substitution, while decreasing affinity, increased ligand solubility. Lipophilic substitutions in the 9-position favour affinity and were suggested to bind to the same region on the receptor as N^6 -substituents in adenosine.

Miscellaneous agents active at the adenosine receptor

Several miscellaneous compounds have been described as having activity at adenosine receptors. Alloxazine (Bruns, 1981) is twofold A_2 -selective with activity in the micromolar range. The anticonvulsant, carbamazepine (Fig. 6) has been repeatedly described (Skerritt *et al.*, 1982; Weir *et al.*, 1984, 1990)

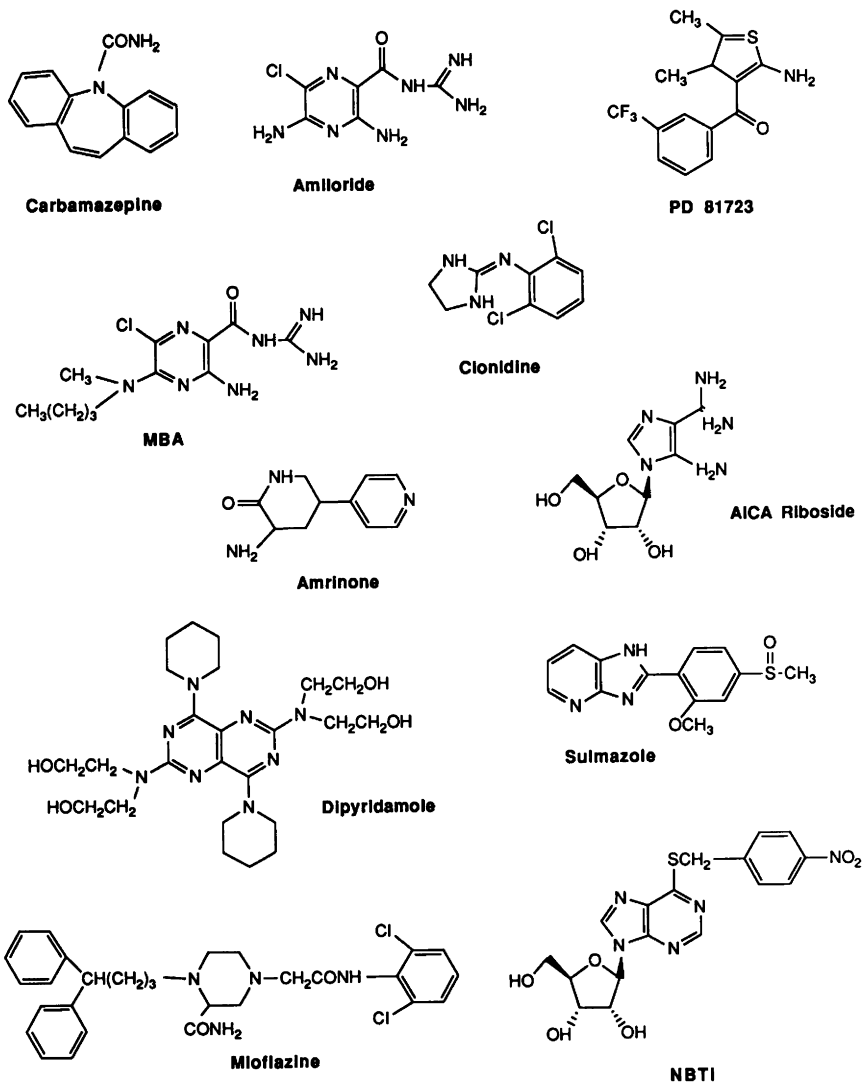


Figure 6 Allosteric modifiers, uptake blockers.

as having the potential to act as an A_1 receptor antagonist at concentrations within its therapeutically effective dose although evaluation of a series of 12 analogues (Marangos *et al.*, 1983) showed no clear relationship between their adenosine binding activity and anticonvulsant activity. Interactions with central A_2 receptors are also very weak (Williams and Jarvis, 1988), although chronic carbamazepine has been reported to downregulate A_2 receptors (Elphick *et al.*, 1990). The diuretic, amiloride (Fig. 6) can also competitively interact with adenosine A_1 receptors (Garritsen *et al.*, 1990) with a K_i value of 2 μM . This effect may be allosteric in nature and it is not clear at this time how interactions with A_1 receptors are reflective of the diuretic action of this class of compound. The 5-(*N*-methyl-*N*-butyl) analogue of amiloride, MBA (Fig. 6) has a K_i value of 70–90 nM at A_1 binding sites in calf brain. The diuretic actions of FR 113 453 (Terai *et al.*, 1990) may be of interest in regard to the observed interactions of the loop diuretics on adenosine systems.

The α_2 -receptor agonist, clonidine (Fig. 6) also has reported activity as an adenosine receptor antagonist (Watanabe *et al.*, 1983; Lai *et al.*, 1984) although the nature of this interaction is somewhat complex (Williams, 1987). The cardiotonic actions of sulmazole and amrinone (Fig. 6) have also been ascribed to adenosine antagonism (Parsons *et al.*, 1988).

Solubility issues

The majority of the non-xanthine adenosine antagonists described have yet to prove useful as therapeutic entities. For many of these, issues related to solubility (Bruns *et al.*, 1988) have been a determining factor although efficacy problems and lack of receptor selectivity in the *in vivo* setting, especially in regard to tissue systems (Williams, 1991a) are equally valid. Both CGS 15943 and CP 66 713 were withdrawn *en route* or during clinical trials. The status of other adenosine antagonists is unknown.

Allosteric modulators

Recent findings in molecular pharmacology have confounded the basic precepts of receptor theory with the identification of neurotransmitter receptor complexes (Williams and Sills, 1990). The first such complex was that for the benzodiazepines and GABA (Williams and Olsen, 1988), although similar complexes have been described for the *N*-methyl-D-aspartate receptor (Lehman *et al.*, 1987; Young and Fagg, 1990). Based on the evaluation of a series of 2-amino-3-benzoyl-thiophenes which allosterically enhance A_1 receptor binding and A_1 receptor functionality, Bruns (Bruns and Fergus, 1989a) has suggested

that there may be allosteric sites linked to the A_1 receptor, ligand interactions with which may alter adenosine-mediated responses. While PD 81 723 (Fig. 6) produced a maximal 40% enhancement of A_1 binding at approximately 30 μM , Bruns has emphasized that this is a 'first generation' entity and that more potent analogues will be required to more extensively evaluate the allosteric site hypothesis. Interestingly, however, the effects of amiloride and clonidine on adenosine receptor binding may be reflective of similar modes of interaction.

Receptor–ligand interactions

Despite the considerable effort expended in deriving novel adenosine agonists and a variety of heterocyclic antagonists, little has been reported to date regarding modelling of the A_1 and A_2 receptors, where data on the various modified adenosine analogues and the xanthine and heterocyclic antagonists have been used to develop an integrated, ligand-based model of the receptor. The Leiden group (Van Galen *et al.*, 1990a) has been the most objective in developing such a model for the A_1 antagonist site.

Agonist binding

Daly (1985), in summarizing available information, described distinct purine- and ribose-binding domains on the adenosine receptor based on the anti-configuration of the purine ribose observed from the seminal structure–activity studies of Bruns (1980, 1981). As previously noted, the ribose group is essential for agonist activity, modifications in the 2'- and 3'-positions decreasing activity (Taylor *et al.*, 1986) while the presence of a non-hydrogen donor in the 5'-position, as in 5'-methylthioadenosine, results in a loss in efficacy.

The importance of hydrogen bonding to the ribose ring has been discussed previously, this involving the 2', 3'- and 5'-positions. Similarly the importance of substituents in the N^6 , C2- and 5'-positions to both affinity and receptor selectivity has been noted. Emphasis on $N^6/5'$, $N^6/C2$ and $5'/C2$ bisubstituted adenosine analogues has led to important new research tools including CGS 21680 and DPMA which have furthered understanding of receptor structure–activity. To date, there have been no reports of trisubstituted $N^6/C2/5'$ entities.

A model of the A_1 receptor involving four hydrophobic aliphatic sites in the N^6 region termed S-1–S-4 was introduced by Olsson's group (Kusachi *et al.*, 1985; Olsson, 1984; Paton *et al.*, 1986) based on the functional assessment of over 140 N^6 -substituted adenosine analogues in cardiovascular test systems. The S-1 region was proposed to accommodate three carbon atoms, the S-2

region defined the stereochemistry of the N^6 'pocket' favouring the *R*-configuration. The proposed S-3 region was important in defining the receptor selectivity of N^6 -substituted analogues, while the S-4 region was a sterically constrained hydrogen-bonding region. This model was further refined (Daly *et al.*, 1986a) to include S-1^A and S-3^A subregions to account for branching at the C1- and C3-positions of the N^6 sidechain.

Van Galen *et al.* (1989) at Leiden and Ortwine *et al.* (1990) at Parke-Davis have both used computer-assisted modelling techniques to further refine the Olsson model. The Leiden group from data on 26 N^6 -substituted analogues have proposed five subregions of the N^6 -binding domain. Subregion *C* represents a cycloalkyl-favouring area and subregion *B* a bulk tolerant region. Subregions *F-1*–*F-3* have been termed 'forbidden areas', favourable interaction with which reduces A_1 receptor affinity. Using the modelling program SYBYL, the Parke-Davis group (Ortwine *et al.*, 1990) have modelled the N^6 region in terms of interactions of ligands selective for the A_{2a} receptor. Based on the binding activity of N^6 -diaryladenosines such as CI 936 and DPMA, a two-site model has been proposed. A primary binding site favouring an aromatic ring that is either electronically neutral or electron donating that is sterically restricted to favour the 4–6 region and an accessory binding site which had no electronic contribution to binding. In the former binding site, 2(6)-substituted groups contributed to A_2 selectivity while in the latter, substitutions at the 3- and 5-positions determined the extent of A_2 receptor selectivity. This model also considered the lack of additivity of A_2 favouring substituents in the N^6 - and C2-positions and their lack of additivity in terms of steric interference (Trivedi and Bruns, 1989).

A model of the C2-binding region of the A_2 receptor has been proposed by Olssen and co-workers (Ueeda *et al.*, 1991a) based on the activity of CGS 21680 and the 2-alkoxy and 2-aralkoxyadenosine analogues described above. Incorporating the Ortwine/Trivedi model, Ueeda *et al.* envisage a hydrophobic C2 pocket accessible to aralkyl substituents at both the N^6 - and C2-positions. This region was proposed to be divided into three subregions: *X* accommodates the C2 linker to the purine which in the case of the compounds studied was oxygen but could be extended to -NH-, -S- and -CH₂-; a hydrophobic subregion that can accommodate a cyclohexane; and an alkyl subregion that can accommodate an ethyl group. This latter subregion reflects the potency of the 2-alkoxyadenosines studied but has limited bulk tolerance, with cyclopentyl being more active than cyclohexyl. Agonist potency was considered to be reflected in the number of methylene or methyl residues interacting with the hydrophobic subregion. Lack of suitable substituents available for interaction with the hydrophobic region results in a negative influence of the alkyl subregion on ligand potency. A C2 region, different from that proposed for the A_2 receptor, may exist for the A_1 receptor consisting of the *X* and

alkyl subregions. This model was further refined via evaluation of the 2-alkoxyadenosines (Ueeda *et al.*, 1991b).

Theoretical models defining the relative contributions of substituents in both the N^6 - and C2-positions have yet to be proposed and it is important to note that the N^6/A_1 model from Ortwine *et al.* restricted movement in all regions of the adenosine molecule except the N^6 region during modelling. Such approaches are common to the current 'state of the art' in computer-assisted molecular modelling. Nonetheless, in the absence of corresponding data in regard to the sequence and conformation of the receptor, such models must be viewed as approximations. The C2-substituted alkoxy and 2-aryloxy NECA analogues have yet to be examined for modelling the A_2 receptor.

Antagonist binding

The information used to derive theoretical models of antagonist binding to either A_1 or A_2 receptors differs from that used for agonists in that data from a more diverse group of potent structures is available and that data, almost exclusively, is derived from binding studies permitting a more comparative basis for the determination of structure–activity relationships.

In the xanthines, the majority of adenosine receptor-selective 8-phenyl-substituted entities are A_1 in nature. These include CPX, PACPX, CPT and XAC. CHC, an 8-substituted xanthine is A_2 -selective as is the 7-substituted xanthine, DMPX.

The xanthines have been proposed to bind to the A_1 receptor in a diametrically opposed manner to the purine nucleosides (Bruns *et al.*, 1986; Jacobson, 1988), the C4/C5 purine dipole being rotated 180° . In this model, the N^7 of theophylline would correspond to the N^9 region of adenosine while the 7-methyl group of caffeine would occupy a similar position to ribose C1. The 8-phenyl substituent in the xanthines would then be considered to occupy a similar hydrophobic pocket to the N^6 region of adenosine. The latter would account for the increase in activity seen with a number of antagonist pharmacophores when a cyclopentyl or cyclohexyl group is introduced in a position corresponding to the 8-position of the xanthine pharmacophore. However, in the case of CHC (Shamim *et al.*, 1989), the cyclohexyl group imparts different receptor selectivity than the corresponding group on adenosine, possibly reflecting the additional nitrogen group in the purine.

Comparison of the triazoloquinazoline, CGS 15943 with the 8-phenylxanthines led to a model (Francis *et al.*, 1989) where the furyl moiety of the CGS compound was overlapped with the 8-phenyl of the substituted xanthines. Furthermore, the triazolopyrimidine portion of CGS 15943 was proposed to overlap with the imidazopyrimidine portion of the xanthine. It was alternatively suggested (Francis *et al.*, 1989) that overlapping of the benzene ring of the

CGS compound with the xanthine 8-phenyl group would permit the furyl group of the former to assume a position adjacent to the xanthine benzene ring. The N^7 group on the xanthine could then be viewed as proximal to the 4-position of the triazoloquinazoline pharmacophore.

In their working model of the A_1 antagonist site, Van Galen *et al.* (1990a) describe 'a flat π -electron-rich fused heterocycle with a well defined molecular electrostatic potential (MEP) pattern' being common to the antagonist classes studied. The MEP pattern was further resolved into a Y-shaped negative potential area with two areas of positive electrostatic potential with an essential nitrogen atom at position 7 acting as a hydrogen bond acceptor. In finding that substitutions in the N - and C2-positions of the imidazoquinolinamines, like those in adenosine, were not additive in their effects on receptor affinity, Van Galen *et al.* (1991), using the MEP model were unable to support the proposed concurrence of the N^6 and C8 regions proposed by Francis *et al.* noting that the triazoloquinazoline model was based on 'a very limited number of compounds'. Rather, the Leiden group argued that the N^6 and C8 regions, as represented in bisubstituted imidazoquinolinamines such as CPPIQA (Fig. 5), are distinct sites.

In the benzodipyrzole series (Peet *et al.*, 1988), comparisons have also been made with the xanthines. The C5 methyl group in the former series is suggested to bind to the same region as the xanthine N^1 substituent while the N^7 of the benzodipyrzole pharmacophore may be analogous to the carbonyl oxygen at position 2 in the xanthines.

Modelling adenosine receptors on the basis of structure-activity relationships for rather limited numbers of compounds and structural series represents but the first step towards the more sophisticated models that currently exist for the β -adrenoceptor (Kobilka *et al.*, 1987; Lohse *et al.*, 1990) where the structural requirements for both affinity and efficacy, at both the receptor and ligand levels are being defined in molecular terms.

The data currently available for A_1 and A_2/A_{2a} receptors are complex and not always comparable. These then limit the accuracy of the models proposed. Such factors include: data from different species; data using different ligands; comparison of binding with functional data; the limitations of data on key analogues that would permit broader comparison across different pharmacophore series especially in the antagonist area; and ultimately, lack of knowledge of receptor structure.

With the continued advances in receptor cloning, sequencing and expression, preliminary reports on the isolation and/or molecular sizing of A_1 and A_{2a} receptors (Barrington *et al.*, 1989; Cooper and Caldwell, 1990), it will not be too long until information comparable to that for the adrenoceptors is available for adenosine receptors. Sequencing and expression of the human receptors from different tissues will then permit the design of ligands selective for chosen therapeutic targets.

Ligand interactions with non-receptor sites

In addition to extracellular receptor sites, adenosine analogues have the potential to interact with nucleoside transporter sites as well as to function as adenosine potentiators (Engler, 1987). Xanthines, as well as the other heterocyclic adenosine antagonists described, can inhibit phosphodiesterase activity (Daly, 1982; Sarges *et al.*, 1990a; Kaplita *et al.*, 1990). Therefore, in the assessment of the potential physiological role of adenosine and the role of adenosine antagonists as therapeutic agents, due caution should be given in interpreting the role of such agents only in terms of their interactions with the cell surface adenosine receptor.

Selective compounds for the nucleoside transporter and the 'potentiator' site have been described. Dipyrindamole and nitrobenzothioinosine (NBTI; Fig. 6) are uptake inhibitors with different modes of action at the molecular level (Marangos *et al.*, 1987). While CV 1808 has activity as a transport inhibitor as well as receptor ligand (Taylor and Williams, 1982), more recent receptor agonists such as CGS 21680C (Balwierczak *et al.*, 1989) lack this dual activity.

Newer transport inhibitors such as mioflazine (Fig. 6) and related structures interact with the adenosine transporters in a manner different to NBTI such that pseudo-Hill coefficients are significantly greater than unity (IJzerman *et al.*, 1989). This has led to a model of the transporter site where mioflazine and related piperazine acetamides can interact with more than one NBTI-binding site.

AICA riboside (Fig. 6; Grueber *et al.*, 1989) represents another unusual molecule in terms of its pharmacological actions as the prototypic adenosine potentiator. While the mode of action of this compound is still unclear, it is currently in clinical trials for use in diabetes and reperfusion injury.

Efficacy issues

While binding assays offer a simple, high-throughput means by which to delineate the interaction of a ligand with a receptor (Williams, 1991b), the intrinsic efficacy of such ligands is more difficult to determine. Both biochemical and *in vitro* and *in vivo* functional assays are confounded by a number of intrinsic issues. As noted, tissue and species variability can confound attempts to compare compound activity across systems while problems with compound solubility add an additional layer of complexity to evaluating the structure-activity/structure-efficacy relationships for compounds interacting with receptors. An additional problem, originally noted by Linden (1989) in a brief communication, relates to the use of adenosine deaminase (ADA) in studying

adenosine ligand binding. Almost without exception, the level of radioactivity bound *in vitro* is dramatically reduced unless endogenous adenosine is removed by ADA treatment or co-incubation. This issue has also been cogently addressed by Schiemann *et al.* (1990) in a study of antagonist binding in guinea-pig myometrium. These authors found that antagonist binding was increased by approximately 80% in the presence of guanine nucleotides, an effect that they ascribed to the existence of a vesicular pool of endogenous adenosine that was inaccessible to ADA actions. The physiological significance of such reservoirs of adenosine and the factors that determine (i) their availability to the receptor and (ii) their effect on receptor binding and ligand interactions remain to be determined, especially in the CNS where adenosine levels are high. Based on the work of Schiemann and co-workers, the existence of receptors 'hidden' by endogenous adenosine may have important consequences for issues related to receptor reserve, for the ability of 'allosteric' modulators to increase ligand binding and for a potential role in pathophysiologies involving adenosine and ATP. In addition, the potential for conversion to ATP may be of additional significance in relation to P₂ receptor functionality as well as inactivation of responses involving adenosine receptor activation (Meghji and Newby, 1990; Nagy *et al.*, 1990).

Few of the adenosine agonists described to date have been systematically examined in tissues or *in vivo* following their *in vitro* characterization. Examination of the A₂-selective ligand, CGS 21680C, in *in vitro* binding assays and in a perfused working heart preparation (Hutchison *et al.*, 1989) showed that the selectivity of this agonist increased as the pharmacological system under evaluation became more complex. In binding assays, CGS 21680 was approximately 140-fold selective in the original publication, with an IC₅₀ of 22 nM. In the isolated perfused working heart model this selectivity was increased to >1500-fold. Ueeda *et al.* (1991a) in their guinea-pig model found a selectivity ratio of 33 000-fold for CGS 21680, suggesting an additional complication involving species differences. These workers reported similar differences in the *in vitro* and *in vivo* profiles of the 2-alkoxyadenosines which they ascribe to a number of factors. They suggested that the reduced number of A₁ receptors in cardiac tissue required full occupancy for efficacy while the more abundant A₂ receptors might require occupancy of only a few receptors to elicit a maximal response. Receptor accessibility was also considered as a factor in functional selectivity. Similarly, studies on the A₂-selective xanthine antagonist, DMPX (Seale *et al.*, 1988) show that the fourfold selectivity assessed by biochemical evaluation is reflected as a 57-fold separation when assessed in terms of agonist-induced hypothermia in the intact animal.

Whatever the reason, it appears crucial to the development of adenosine agonists as therapeutic agents, that a better understanding of both the efficacy of such molecules and the coupling relationships in various tissues is obtained.

The possibility that A_2/A_{2a} receptors are more efficiently coupled to their effector mechanisms than A_1 receptors must be considered, especially in light of the fact that A_1 receptor-mediated inhibition of cyclase activity is usually determined in an artificial system primed with forskolin. The existence of second messenger pathways in addition to cAMP must also be borne in mind when evaluating biochemical data for functional efficacy (Cooper and Caldwell, 1990; Potter, 1990).

Adenosine ligands as therapeutic entities

Despite the 60 years or so since Drury and Szent-Gyorgyi (1929) first reported on the biological actions of adenosine, only adenosine has been approved for human use (Jacobson *et al.*, 1991). Significant effort has been expended within the pharmaceutical industry to identify selective agents. And while many of these represent important research tools, none has progressed very far in terms of clinical trials (Williams, 1991a).

Surveying the adenosine patent literature over the past 5 years indicates a very high level of interest from Japanese pharmaceutical companies, with many of the proprietary agents described having undergone testing in animal models of inflammation, cognition, affective disorders and hypertension. Given the wealth of evidence documenting the pharmacological and physiological actions of adenosine and the increasing number of novel agonist and antagonist molecules being described, it cannot be too long before other molecules acting at the adenosine receptor join the parent compound in the clinical setting.

Note added in proof

Both the canine A_1 and A_2 receptors have been cloned as members of the G-protein coupled-receptor family and were subsequently identified as adenosine receptors (Libert *et al.*, 1989; Maenhaut *et al.*, 1990). Both receptors have the seven transmembrane helices common to G-protein associated receptors. Efforts are now under way to model the receptor to identify critical ligand binding sites in the transmembrane helices (Jacobson *et al.*, 1991b).

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CHAPTER 8

RELEASE OF ADENOSINE AND ATP FROM NERVOUS TISSUE

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Introduction

There is much evidence that exogenously administered ATP, adenosine and their analogues act at specific purinoceptors to elicit various responses in the central and peripheral nervous systems. However, the idea that endogenous purines are released from cells has met considerable opposition, particularly in the case of ATP which performs numerous essential intracellular functions. Nevertheless, there is good evidence that ATP is co-stored and secreted with 5-hydroxytryptamine (5-HT) from platelets (Detwiler and Feinman, 1973) and with catecholamines from adrenal chromaffin cells (White *et al.*, 1987). By analogy with chromaffin cells, it has generally been thought that ATP is co-stored with noradrenaline within sympathetic noradrenergic nerve vesicles and co-secreted with noradrenaline when action potentials invade the varicosities. However, the amounts of ATP stored within noradrenergic

sympathetic vesicles may be quite small (Fried *et al.*, 1978). ATP is also co-stored with acetylcholine (ACh) within cholinergic synaptic vesicles (Dowdall *et al.*, 1974) and its release from motor nerves has been demonstrated (Silinsky, 1975). There is mounting evidence, reviewed below, that ATP is released to function as a co-transmitter with noradrenaline or ACh in various peripheral tissues.

There is no corresponding evidence that adenosine is stored in synaptic vesicles and secreted from nerves, so that it is unlikely that it functions as a neurotransmitter. Nevertheless, endogenous extracellular adenosine appears to be an important neuromodulator. In some cases, the adenosine appears to be derived from released nucleotide(s). A series of ecto-enzymes capable of converting extracellular nucleotides to adenosine exists on many cells. Once released from nerves, ATP can be converted to ADP, 5'-AMP and ultimately adenosine by these ecto-enzymes. Ecto-protein kinases (Ehrlich *et al.*, 1986) should also convert released ATP to ADP, which would then be metabolized by the ectonucleotidases to adenosine. Finally, ecto-cyclic AMP phosphodiesterase has been described in some tissues (Rosberg *et al.*, 1975; Selstam and Rosberg, 1976; Rosenberg and Dichter, 1989). This enzyme could convert cyclic AMP to 5'-AMP, which could then be metabolized to adenosine by ecto-5'-nucleotidase. Adenosine itself can also exit many cells, including nerves, on the bidirectional, facilitated-diffusional nucleoside transporter (Wu and Phillis, 1984; Paterson *et al.*, 1985; Deckert *et al.*, 1988) when the intracellular concentration exceeds the extracellular concentration. This could occur when nerves are depolarized, or during periods of increased metabolic demand such as seizures, ischaemia, hypoxia and hypoglycaemia, where intracellular adenosine levels become elevated. There is evidence that a Na^+ -dependent transport system for adenosine, which can actively accumulate adenosine, may exist in central neurones (Johnston and Geiger, 1989). When the Na^+ -electrochemical gradient is reversed, this transporter could also provide a means of releasing adenosine from nerves.

Both adenosine and ATP may also be released from supposedly non-secretory cells such as the *Torpedo* electric organ (Israel *et al.*, 1976), striated muscle (Abood *et al.*, 1962), cardiac myocytes (Forrester and Williams, 1977; Darius *et al.*, 1987) and vascular endothelial cells (Pearson and Gordon, 1979; Sedaa *et al.*, 1990). In addition to being released from vascular sympathetic nerves when they are stimulated, relatively large amounts of ATP appear to be released postsynaptically following activation of α -adrenoceptors located on the vascular endothelium and smooth muscle (Sedaa *et al.*, 1990). The mechanism by which ATP is released from these supposedly non-secretory cells has not been established, but the observation that these cells apparently release ATP reinforces the need to evaluate the possibility that systems capable of transporting nucleotides exist in the membranes of certain cells.

In this chapter, we will review the evidence that adenosine and ATP are released in the central and peripheral nervous systems. Where possible, functions for the released purines will be discussed but the reader is referred to this volume and other reviews (Dunwiddie, 1985; White, 1988; Stone, 1989; Olsson and Pearson, 1990; White, 1991) for specific details concerning the actions of extracellular purines.

Release of purines in the central nervous system

Release of adenosine

Besides being an important inhibitory neuromodulator in the CNS, neuronally released adenosine may also affect cerebral blood flow through its actions at A₂ purinoceptors located on the vascular smooth muscle. There have been many studies of the release of adenosine evoked from various CNS preparations by several stimuli, with earlier studies detecting the release of radiolabelled purines and more recent studies detecting the release of endogenous, unlabelled adenosine. The findings from some of the studies, with their appropriate references, are summarized in Table 1.

Depolarization with K⁺, veratridine or electrical stimulation has been shown to release adenosine both *in vivo* and *in vitro* from slices and synaptosomes from various brain regions. Some of the adenosine is derived from the extracellular metabolism of released nucleotide. However, much of the release arises from intracellular adenosine exiting cells on the bidirectional nucleoside transporter (Fredholm *et al.*, 1980, 1983, 1988; Jonzon and Fredholm, 1985; White and MacDonald, 1991). Depolarization may increase the energy demand on the cells, thus increasing intracellular adenosine and favouring the efflux of adenosine on the bidirectional, facilitated-diffusional transporter. On the other hand, K⁺-evoked release of adenosine from cortical slices appears to be partly mediated indirectly by the release of an excitatory amino acid (EAA) which, in turn, acts at *N*-methyl-D-aspartate (NMDA) receptors to promote adenosine release (Hoehn and White, 1990a). In spreading depression, the extraneuronal levels of K⁺ become elevated; if these K⁺ levels are sufficient to release adenosine, the adenosine could act at inhibitory P₁ purinoceptors and play some role in the decreased neuronal activity observed during spreading depression. Depolarization releases adenosine from purified cholinergic synaptosomal preparations (Richardson and Brown, 1987). Moreover, electrical stimulation of cortical slices appears to release radiolabelled purines from both cholinergic and noradrenergic sources (Pedata *et al.*, 1989). These studies provide strong evidence that adenosine is released from neurones in the CNS.

Table 1 Summary of adenosine or radiolabelled purine release in the CNS. (Superscripts refer to specific references in reference column).

Releasing stimulus/ preparation	Region	Comments	References
K ⁺			
<i>In vivo</i>	Cortex ¹ , striatum ²⁻⁴	Primarily adenosine <i>per se</i> ²	Jhamandas and Dumbrille, 1980 ¹ ; Barberis <i>et al.</i> , 1984 ² ; Van Wylen <i>et al.</i> , 1986 ³ ; Ballarin <i>et al.</i> , 1987 ⁴
Slices	Cortex ⁵⁻⁸ , striatum ⁹	Primarily adenosine <i>per se</i> ⁸ ; cortical release partially mediated by released EAA acting at NMDA receptor ⁸	Shimizu <i>et al.</i> , 1970 ⁵ ; Hollins and Stone, 1980 ⁶ ; Hoehn and White, 1989 ⁷ , 1990a ⁸ ; Wojcik and Neff, 1983 ⁹
Synaptosomes	Cortex ¹⁰⁻¹⁴ , hypothalamus ¹⁵ , striatal ¹⁶ , whole brain ¹⁷ , spinal cord ¹⁸ , other ¹⁹	Release from striatal interneurons ⁹ Primarily adenosine <i>per se</i> ¹²⁻¹⁷ ; possibly via nucleoside transporter ¹⁴⁻¹⁷ Purified cholinergic nerve terminals ¹⁸ Dorsal > ventral ¹⁸ Amygdala > cortex > striatum > hippocampus > thalamus > hypothalamus > cerebellum > pons/medulla ¹⁹	Kuroda and McIlwain, 1974 ¹⁰ ; Daval <i>et al.</i> , 1980 ¹¹ ; Daval and Barberis, 1981 ¹² ; Bender <i>et al.</i> , 1981 ¹³ ; Hoehn and White, 1990b ¹⁴ ; Fredholm and Vernet, 1979 ¹⁵ ; Richardson and Brown, 1987 ¹⁶ ; Macdonald and White, 1985 ¹⁷ ; Sweeney <i>et al.</i> , 1987a ¹⁸ ; White and Macdonald, 1990 ¹⁹
<i>In vitro</i>	Retina ^{20,21}	Primarily release of radiolabelled nucleosides and bases ²⁰ ; probably via nucleoside transporter ²⁰ ; light flashes also induce purine release ²¹	Perez <i>et al.</i> , 1986 ²⁰ , 1988 ²¹

Veratridine <i>In vivo</i>	Cortex ¹ , striatum ²	Primarily adenosine <i>per se</i> ²	Jhamandas and Dumbrille, 1980 ¹ ; Barberis <i>et al.</i> , 1984 ²
Slices	Cortex ^{5,22} , hypothalamus ²³	Possibly via nucleoside transporter ²³	Shimizu <i>et al.</i> , 1970 ⁵ ; Fredholm and Vernet, 1978 ²² ; Fredholm and Jonzon, 1981 ²³
Synaptosomes	Cortex ^{10,12} , hypothalamus ¹⁵ , striatum ¹⁶ , whole brain ¹⁷ , spinal cord ¹⁸ , other ¹⁹	Primarily adenosine <i>per se</i> ¹² Purified cholinergic nerve terminals release both ATP and nucleoside; release of nucleoside probably via nucleoside transporter ¹⁶ Ca ²⁺ -independent ¹⁷ About 50% released as nucleotides ^{17,18} . dorsal > ventral ¹⁸ Amygdala > cortex > thalamus > cerebellum > striatum > hippocampus > hypothalamus > pons/medulla ¹⁹	Kuroda and McIlwain, 1974 ¹⁰ ; Daval and Barberis, 1981 ¹² ; Fredholm and Vernet, 1979 ¹⁵ ; Richardson and Brown, 1987 ¹⁶ ; MacDonald and White, 1985 ¹⁷ ; Sweeney <i>et al.</i> , 1987a ¹⁸ ; White and MacDonald, 1991 ¹⁹
Electrical stimulation <i>In vivo</i>	Cortex ²⁴ , hippocampus ²⁵	Evidence for transneuronal transfer of labelled purines during perforant path stimulation ²⁵	Sulakhe and Phillis, 1975 ²⁴ ; Schubert <i>et al.</i> , 1976 ²⁵
Slices	Cortex ²⁶⁻³¹ , hippocampus ^{32,33} , hypothalamus	Decreased radiolabelled purine release following cholinergic and noradrenergic denervation ³¹ <i>In vitro</i> release following <i>in vivo</i> loading ³²	Pull and McIlwain, 1972a ²⁶ , b ²⁷ , 1975 ²⁸ ; Stone, 1981 ²⁹ ; Pedata <i>et al.</i> , 1988 ³⁰ , 1989 ³¹ ; Lee <i>et al.</i> , 1982 ³² ; Jonzon and Fredholm, 1985 ³³ ; Fredholm and Jonzon, 1981 ²³
Synaptosomes	Cortex ^{10,12} , hypothalamus ¹⁵		Kuroda and McIlwain, 1974 ¹⁰ ; Daval and Barberis, 1981 ¹² ; Fredholm and Vernet, 1979 ¹⁵
Cultures	Glial ³⁴		Cacchiagli <i>et al.</i> , 1988 ³⁴

Table 1 *continued*

Releasing stimulus/ preparation	Region	Comments	References
EAA			
<i>In vivo</i>	Cortex ^{1,35} , hippocampus ³⁶ , striatum ²	Cortical release evoked by glutamate aspartate, NMDA, kainate, quisqualate, and quinolinate ^{1,35} ; hippocampal release evoked by kainate ³⁶ ; striatal release evoked by glutamate ²	Jhamandas and Dumbrille, 1980 ¹ ; Perkins and Stone, 1983 ³⁵ ; Lehmann <i>et al.</i> , 1987 ³⁶ ; Barberis <i>et al.</i> , 1984 ²
Slices	Cortex ^{28,7,8,37,38}	Glutamate releases primarily adenosine <i>per se</i> ⁸ ; NMDA, kainate and quisqualate all release ³⁷ ; spare receptors for NMDA-evoked adenosine release ³⁸	Pull and McIlwain, 1975 ²⁸ ; Hoehn and White, 1989 ⁷ , 1990a ⁸ ; Hoehn <i>et al.</i> , 1990 ³⁸
Synaptosomes	Cortex ¹⁴	Glutamate-evoked adenosine release from synaptosomes due to transport of glutamate into synaptosomes, not receptors ¹⁴ ; adenosine possibly derived from released AMP or ADP ¹⁴	Hoehn and White, 1990b ¹⁴
<i>In vitro</i>	Retina ³⁹	Glutamate, aspartate, NMDA, kainate and quisqualate release ³⁹	Perez and Ehinger, 1989 ³⁹
Opioids			
<i>In vivo</i>	Cortex ^{40,41} , spinal cord ⁴²	Efflux of radiolabel from cortex is primarily nucleotide ⁴⁰ Adenosine released by morphine likely originates from primary afferent nerve terminals in spinal cord ⁴³	Phillis <i>et al.</i> , 1980a ⁴⁰ , b ⁴¹ ; Sweeney <i>et al.</i> , 1987b ⁴² ; Sawynok and Sweeney, 1989 ⁴³

Slices	Cortex ^{22,29,44} , hippocampus ⁴⁵	Morphine potentiates depolarization- and hypoxia-evoked release of purines ^{22,29,44,45} ; met-enkephalin also releases ²⁹	Fredholm and Vermet, 1978 ²² ; Stone, 1981 ²⁹ ; Wu <i>et al.</i> , 1982 ⁴⁴ ; Fredholm <i>et al.</i> , 1987 ⁴⁵
Synaptosomes	Spinal cord ^{18,46}	Released primarily as adenosine <i>per se</i> ¹⁸ ; possible primary afferent origin ⁴⁶	Sweeney <i>et al.</i> , 1987a ¹⁸ , 1989 ⁴⁶
Noradrenaline Synaptosomes	Hypothalamus ¹⁵ , spinal cord ¹⁸	Released primarily as a nucleotide ¹⁸ ; equal release from dorsal and ventral spinal cord ¹⁸	Fredholm and Vernet, 1979 ¹⁵ ; Sweeney <i>et al.</i> , 1987a ¹⁸
Cultures	Cortical ⁴⁷	Adenosine derived from released cyclic AMP, probably from glia ⁴⁷	Rosenberg and Dichter, 1989 ⁴⁷
Histamine Slices	Cortex ²⁸	Small but consistent effect of 100 μ M histamine ²⁸	Pull and McIlwain, 1975 ²⁸
Muscarinic Slices	Cortex ³⁰	Oxotremorine potentiates electrically stimulated release from cortex ³⁰	Pedata <i>et al.</i> , 1988 ³⁰
<i>In vitro</i>	Retina ³⁹		Perez and Ehinger, 1989 ³⁹
5-HT <i>In vivo</i> Synaptosomes	Spinal cord ⁴⁸ Spinal cord ⁴⁹	Releases a nucleotide from primary afferents which is metabolized to adenosine ⁴⁹	Sweeney <i>et al.</i> , 1990 ⁴⁸ Sweeney <i>et al.</i> , 1988 ⁴⁹
Ethanol Synaptosomes	Cerebellar ⁵⁰	Pharmacologically relevant concentrations release adenosine; may mediate ethanol-induced motor disturbances ⁵⁰	Clark and Dar, 1989 ⁵⁰

Table 1 *continued*

Releasing stimulus/ preparation	Region	Comments	References
Hypoxia/ischaemia/hypotension <i>In vivo</i>	CSF ³¹ , cortex ^{52,53} , striatum ^{3,54-6}	Mediated in part by NMDA receptors ⁵⁵	Berne <i>et al.</i> , 1974 ⁵¹ ; Phillis <i>et al.</i> , 1988 ⁵² ; Park <i>et al.</i> , 1988 ⁵³ ; Van Wylen <i>et al.</i> , 1986 ³ ; Zetterström <i>et al.</i> , 1982 ⁵⁴ ; Hagberg <i>et al.</i> , 1986 ⁵⁵ , 1982 ⁵⁶
Slices	Cortex ²⁶ , hippocampus ⁴⁵	Anoxia-induced release potentiated by morphine ⁴⁵	Pull and McIlwain, 1972a ²⁶ ; Fredholm <i>et al.</i> , 1987 ⁴⁵
Hypoglycaemia <i>In vivo</i>	Striatum ⁵⁷	Mediated in part by NMDA receptors ⁵⁷	Butcher <i>et al.</i> , 1987 ⁵⁷
Slices	Cortex ²⁶		Pull and McIlwain, 1972a ²⁶

Glutamate and specific agonists for receptors such as NMDA, kainate, quisqualate and quinolinate, also release adenosine both *in vivo* and from various *in vitro* preparations. EAA receptor-mediated release appears to occur primarily as adenosine *per se* rather than from the extracellular conversion of released nucleotide (Hoehn and White, 1990a). Studies with uncompetitive antagonists such as Mg^{2+} and MK-801 suggest that only a small fraction of the available NMDA receptors must be activated for adenosine release from cortical slices to be maximal (Hoehn *et al.*, 1991); in other words, there appear to be spare receptors for NMDA-evoked adenosine release. This contrasts with NMDA-evoked noradrenaline release for which there do not appear to be spare receptors. Significantly, NMDA-evoked noradrenaline release appears to be mediated by the generation of propagated action potentials, whereas adenosine release is largely independent of action potentials (Hoehn *et al.*, 1991). The latter finding suggests that adenosine is released from a site quite close to the NMDA receptor. The presence of spare receptors for NMDA-evoked adenosine release effectively shifts the dose/response curve to the left, so that NMDA is 33-times more potent at releasing adenosine than at releasing noradrenaline. These observations have important functional implications. It seems unlikely that adenosine, released when NMDA receptors are activated, would function as an endogenous protectant against excessive stimulation (Dragunow and Faull, 1988) because adenosine release is maximal at levels of NMDA receptor activation which produce little generation of action potentials (as indicated by noradrenaline release). However, adenosine released during low levels of NMDA receptor activation could inhibit the further release of glutamate (Dolphin and Archer, 1983; Corradetti *et al.*, 1984; Fastbom and Fredholm, 1985; Burke and Nadler, 1988) and/or diminish postsynaptic responses (Segal, 1982; Proctor and Dunwiddie, 1987). Thus released adenosine could provide an inhibitory threshold which must be overcome in order for NMDA-mediated neurotransmission to proceed maximally in the cortex. Released adenosine may serve to maintain the selectivity of NMDA-mediated processes in the cortex such as learning, memory and synaptic plasticity.

Adenosine is also released from cortical synaptosomes when L-glutamate is taken up by the Na^{+} -dependent, high-affinity transporter located on glutamatergic nerve terminals (Hoehn and White, 1990b). In this case, the adenosine is derived from a released nucleotide, possibly ADP or 5'-AMP. This process occurs in the immediate vicinity of presynaptic A_1 purinoceptors located on glutamatergic nerve terminals and could function to modulate the further release of glutamate.

Pathological conditions such as hypoxia, ischaemia and hypoglycaemia have all been shown to release adenosine from various brain regions. Significantly, part of the release appears to be mediated indirectly by a released

EAA acting at NMDA receptors (Hagberg *et al.*, 1986; Butcher *et al.*, 1987). The remainder may occur as a result of the intracellular accumulation of adenosine, due to decreased oxidative phosphorylation and glycolysis, and its efflux on the nucleoside transporter.

Activation of opioid receptors has been shown to release adenosine in various brain regions. Morphine-evoked release of radiolabelled purines from cortex *in vivo* appears primarily as nucleotide (Phillis *et al.*, 1980a) whereas release from spinal cord *in vivo* occurs as adenosine *per se* (Sweeney *et al.*, 1987a). In the spinal cord, part of the analgesic effects of morphine seem to be mediated indirectly by the release of adenosine (reviewed by Sawynok and Sweeney, 1989). Release of adenosine appears to originate from capsaicin-sensitive primary afferent terminals located in the dorsal spinal cord. Opioids, including morphine and met-enkephalin, potentiate depolarization and hypoxia-evoked release of adenosine from cortical and hippocampal slices (Fredholm and Vernet, 1978; Stone, 1981; Wu *et al.*, 1982; Fredholm *et al.*, 1987). A possible role for adenosine in the actions of opioids at higher brain centres has not yet been established.

Adenosine is also released by several other putative neurotransmitters in the CNS. Noradrenaline releases nucleotides which are subsequently converted to adenosine in cortical cultures and in spinal cord synaptosomes. In cortical cultures, the adenosine appears to be derived from cyclic AMP released from glia (Rosenberg and Dichter, 1989). In the spinal cord, the nucleotide is not released specifically from capsaicin-sensitive primary afferents (Sawynok and Sweeney, 1989). 5-HT also releases a nucleotide which is subsequently metabolized to adenosine in the spinal cord (Sweeney *et al.*, 1988; Sawynok and Sweeney, 1989). In contrast to noradrenaline, 5-HT-evoked release occurs from capsaicin-sensitive primary afferent sources. The spinal antinociceptive effects of 5-HT, but not noradrenaline, appear to be mediated by released adenosine. Relatively large concentrations of histamine release adenosine (Pull and McIlwain, 1972a, b), and oxotremorine potentiates electrically evoked release of purines (Pedata *et al.*, 1988) from cortical slices, suggesting possible roles for histamine and muscarinic receptors in adenosine release.

Pharmacologically relevant concentrations of ethanol potentiate depolarization-evoked release of adenosine from cerebellar synaptosomes (Clark and Dar, 1989). The authors have suggested that cerebellar adenosine may mediate some ethanol-induced motor disturbances.

K⁺, EAAs and light flashes release radiolabelled purines in the retina (Perez *et al.*, 1986, 1988; Perez and Ehinger, 1989). K⁺ appears to release adenosine via the nucleoside transporter.

Finally, it should be borne in mind that, although there is clear evidence that adenosine is released from nerves, it may also be released from glia. Adenosine is apparently released when glial cell cultures are stimulated

electrically (Caciagli *et al.*, 1988). Moreover, exposure of glial cultures to metabolic poisons releases adenosine; release occurs via the nucleoside transporter (Meghji *et al.*, 1989). This contrasts with the noradrenaline-evoked release of adenosine from cortical cultures, which apparently arises from glia but is derived from released cyclic AMP (Rosenberg and Dichter, 1989).

Release of ATP

Released ATP could provide an important source of extracellular adenosine in the CNS. In addition, ATP has been shown to exert excitatory actions in the CNS which are not mediated by P_1 purinoceptors (Phillis and Wu, 1981). Extracellular ATP might also be a substrate for ecto-protein kinases (Ehrlich *et al.*, 1986), or active phospholipase C (Gebicke-Haerter *et al.*, 1988; Pearse *et al.*, 1989) in the CNS. Detection of ATP release is hampered by the rapid degradation of the nucleotide by ecto-enzymes. Nevertheless, several studies have demonstrated that ATP is released when various preparations are depolarized with K^+ , veratridine or electrical stimulation. The results of these studies and their references are shown in Table 2.

Elevated K^+ releases ATP from synaptosomes prepared from whole brain and various regions. Release is Ca^{2+} -dependent and thus resembles the release of classical transmitters. Synaptosomal ATP release is not distributed uniformly throughout the brain, suggesting that it may function in specific regions. Affinity-purified cholinergic synaptosomes release ATP, suggesting that ATP may be co-released with ACh.

Veratridine also releases ATP. However, release is not Ca^{2+} -dependent and therefore does not resemble the release of classical transmitters. The regional profile for veratridine-evoked release of ATP from synaptosomes differs from that obtained with K^+ , suggesting that it may occur from different sources. Veratridine releases ATP from purified cholinergic synaptosomes. In the striatum, adenosine derived from released ATP may provide a negative feedback to modulate ACh release (Richardson and Brown, 1987).

Both K^+ and veratridine release 2–3 times more ATP from dorsal than ventral spinal cord synaptosomes, findings which are consistent with the possibility that ATP might function as a sensory transmitter in low-threshold, primary afferent inputs to the dorsal horn (Fyffe and Perl, 1984; Salter and Henry, 1985). ATP is also released by K^+ and veratridine from primary cultures of striatum, where there is evidence that it might function as a substrate for an ecto-protein kinase that phosphorylates specific membrane proteins (Zhang *et al.*, 1988).

Direct electrical stimulation of the sensorimotor cortex releases ATP into cortical cups *in vivo*. In addition, ATP appears to be released presynaptically during electrical stimulation of the Schaffer collaterals in rat hippocampal

Table 2 Summary of ATP release in the CNS

Releasing stimulus/ preparation	Region	Comments	References
K ⁺ Synaptosomes	Whole brain	Ca ²⁺ -dependent release resembles classical neurotransmitters	White, 1977, 1978
	Spinal cord	2–3 times more release from dorsal than ventral spinal cord	White <i>et al.</i> , 1985
	Striatal	Release from purified cholinergic nerve terminals	Richardson and Brown, 1987
	Other	Release from striatum > cortex > medulla > hypothalamus > cerebellum	Potter and White, 1980
Cultures	Striatum	Evidence that released ATP is substrate for an ecto-protein kinase	Zhang <i>et al.</i> , 1988

Veratridine	Synaptosomes	Whole brain	Not Ca^{2+} -dependent 2–3 times more release from dorsal than ventral spinal cord	White, 1977, 1978 White <i>et al.</i> , 1985
		Spinal cord		
	Striatum		Release from purified cholinergic nerve terminals; evidence that cholinergic nerve terminals in striatum possess ectophosphohydrolases producing adenosine for feedback inhibition of ACh release; cholinergic nerve terminals in cortex lack ectophosphohydrolases	Richardson and Brown, 1987
	Other		Release from medulla > striatum > hypothalamus > cortex > cerebellum	Potter and White, 1980
Electrical stimulation	Cultures	Striatum	Evidence that released ATP is substrate for an ecto-protein kinase	Zhang <i>et al.</i> , 1988
	<i>In vivo</i>	Cortex	Direct stimulation of sensorimotor cortex	Wu and Phillis, 1978
	Slices	Hippocampus	Release by high-frequency stimulation of Schaffer collaterals; release appears to originate pre-synaptically; released ATP may regulate synaptic efficacy (but see Stone and Cusack, 1989)	Wieraszko <i>et al.</i> , 1989; Wieraszko and Seyfried, 1989

slices (Wieraszko *et al.*, 1989). This and the finding that ATP potentiates synaptic transmission in pyramidal CA1 neurones (Wieraszko and Seyfried, 1989) are consistent with the possibility that ATP might function as a neurotransmitter and/or modulator in specific pathways in the CNS. However, Stone and Cusack (1989) were unable to observe any P_2 -mediated effects of stable analogues of ATP on CA1 neurones, so that the issue of a possible function for ATP in the hippocampus remains unresolved.

Release of purines in the peripheral nervous system

Release of purines at motor endplates

Studies demonstrating the release of purines in the peripheral nervous system are summarized in Table 3. In 1962, Abood *et al.* demonstrated that ATP was released when a frog nerve-muscle preparation was stimulated electrically. Release of ATP appeared to be related to depolarization of these tissues and they postulated that the efflux of ATP might be secondary to excitation or perform some primary function in the excitatory event. Subsequently, the co-release of ATP with ACh as a consequence of propagated action potentials in rat phrenic nerve innervating the diaphragm was demonstrated (Silinsky, 1975). ATP is also released when cholinergic synaptosomes from the *Torpedo* electric organ are depolarized (Morel and Meunier, 1981). Significantly, botulinum toxin A inhibits the release of ACh without decreasing the release of ATP from *Torpedo* synaptosomes (Marsal *et al.*, 1989), suggesting that ATP may not be co-stored and secreted from the same vesicles as ACh. Large amounts of ATP are also released postsynaptically from the *Torpedo* electric organ during stimulation of the motor nerve as a result of nicotinic receptor activation (Israel *et al.*, 1976); this ATP could provide a source of adenosine to modulate secretion from the motor nerve.

Applied ATP potentiates the action of ACh in the rat hemidiaphragm (Ewald, 1976) and activates a size-selective conductance to Na^+ , K^+ and Cl^- in chick muscle (Hume and Thomas, 1988). ATP interacts with a receptor distinct from the nicotinic receptor in chick muscle (Hume and Honig, 1986), suggesting that it may function as a co-transmitter with ACh in this case. On the other hand, ATP appears to interact more directly with nicotinic receptors at frog skeletal endplates (Akasu *et al.*, 1981) and *Xenopus* myotomal muscle cells (Igusa, 1988), suggesting that it may function as a modulator of cholinergic transmission in these tissues.

Activation of presynaptic P_1 purinoceptors decreases ACh release and consequently inhibits motor nerve function (Ribeiro and Sebastião, 1987). Adenosine, derived from neuronally released ATP or released postsynaptically from the muscle, could act presynaptically to inhibit further transmitter release.

Table 3 Summary of release of ATP in the peripheral nervous system

Nerve type	Tissue	Comments	References
Motor	Frog nerve-muscle	Direct depolarization, possibly not physiological	Abood <i>et al.</i> , 1962
	Rat phrenic nerve	Neuronal	Silinsky, 1975
	<i>Torpedo</i> synaptosomes	Presynaptic ¹ but not from cholinergic vesicles ²	Morel and Meunier, 1981 ¹ ; Marsal <i>et al.</i> , 1989 ²
	<i>Torpedo</i> electric organ	Large amounts released following stimulation of motor nerve; due to activation of nicotinic receptors on the electric organ	Israel <i>et al.</i> , 1976
Sympathetic	Guinea-pig vas deferens	Release from noradrenergic nerves ^{3,4} but probably not from adrenergic vesicles ⁵	Lew and White, 1987 ³ ; Kirkpatrick and Burnstock, 1987 ⁴ ; Ellis and Burnstock, 1989 ⁵
	Rat tail artery	Large amounts released post-synaptically from endothelium and smooth muscle; smaller amount from adrenergic nerves	Sedaa <i>et al.</i> , 1990
	Myenteric terminals	Released by 5-HT, ACh, K ⁺ , veratridine ^{6,7} ; may not be released from noradrenergic vesicles ⁸	White, 1988 ⁶ , 1991 ⁷ ; Hammond <i>et al.</i> , 1988 ⁸
Parasympathetic	Bladder detrusor	Released from guinea-pig ⁹ but not rabbit ¹⁰	Burnstock <i>et al.</i> , 1978 ⁹ ; Chaudhry <i>et al.</i> , 1984 ¹⁰
Enteric	Taenia coli	Due to propagated action potentials in nerves in one study ⁹ but not in another ¹¹	Burnstock <i>et al.</i> , 1978 ⁹ ; White <i>et al.</i> , 1981 ¹¹

Note: Numerous studies have demonstrated the release of radiolabelled purines from tissues. Usually adenosine is detected. The adenosine could be derived from released ATP, or released as such postsynaptically from the target tissues. Superscripts refer to specific references in reference column.

Release of purines from sympathetic nerves

The evidence that ATP functions as a co-transmitter with noradrenaline in the sympathetic nerves innervating the vas deferens is quite convincing (reviewed by White, 1988, 1991). The second, slow contraction observed following sympathetic nerve stimulation in the guinea-pig vas deferens is mediated by α -adrenergic receptors. However, the initial generation of ejps which summate to generate an action potential and initiate the fast twitch response are non-adrenergic. ATP activates relatively non-specific cation currents in vas smooth muscle and mimics the neurogenic twitch response. Application of the P_{2x} antagonist arylazidoaminopropionyl ATP (ANAPP₃) or desensitization of P_{2x} purinoceptors with α,β -methylene ATP block both the responses to exogenously administered ATP and the initial twitch response evoked by sympathetic nerve stimulation.

Release of ATP has been detected when the sympathetic nerves innervating the vas deferens are stimulated electrically (Lew and White, 1987). Release is tetrodotoxin-sensitive and persists when the postsynaptic responses on the muscle are blocked by prazosin and α,β -methylene ATP, indicating that it occurs from the sympathetic varicosities. Release is abolished following chemical sympathectomy with 6-hydroxydopamine but is unaffected when the nor-adrenergic stores are depleted by reserpine pretreatment (Kirkpatrick and Burnstock, 1987). These findings provide strong evidence that ATP is released and functions as a co-transmitter with noradrenaline in the sympathetic nerves innervating the vas deferens.

Fredholm *et al.* (1982) concluded from their study of the nerve-mediated release of [³H]purines in the rat vas deferens that most of the released adenosine originated from the smooth muscle rather than from sympathetic nerves. They proposed that released adenosine could exert a negative feedback control on the release of transmitters from the sympathetic nerves in the vas deferens. However, a study with the P_1 antagonist 8-phenyltheophylline failed to provide evidence that endogenous extracellular adenosine modulates sympathetic nerve activity in the guinea-pig vas deferens (Sneddon *et al.*, 1984).

There is evidence that ATP functions as an excitatory co-transmitter with noradrenaline in the sympathetic nerves innervating blood vessels (reviewed by White, 1988, 1991). As in the vas deferens, the ejps and initial twitch response to sympathetic nerve stimulation in blood vessels are non-adrenergic; they are mimicked by ATP and antagonized by appropriate P_{2x} antagonists. Evidence that ATP is released from sympathetic nerves has been difficult to obtain. However, Sedaa *et al.* (1990) have recently shown that sympathetic stimulation of the rat tail artery promotes ATP release. Most of the ATP

release occurs as a consequence of the activation of α -adrenoceptors on the endothelial cells, some from α -adrenoceptor activation on the vascular smooth muscle, and a smaller amount from the sympathetic nerves themselves. Nevertheless, the authors concluded that the amount of ATP released from the nerves could be sufficient to act as a co-transmitter with noradrenaline. It is uncertain what functions, if any, the ATP that is released from the vascular smooth muscle and endothelium might have. In the latter case, ATP might act at P_{2Y} purinoceptors located on the endothelium, thereby releasing NO which relaxes the smooth muscle. In this sense, ATP released from the endothelium might function as an autostimulator of endothelium-mediated vascular relaxations.

Extracellular adenosine relaxes most blood vessels by acting at A_2 purinoceptors on the vascular smooth muscle. In addition, it could act at presynaptic A_1 purinoceptors to inhibit the release of transmitters from the sympathetic nerves. There is little evidence that adenosine is released *per se* from sympathetic nerves but it could arise from the extracellular degradation of released ATP and postsynaptically from the vascular smooth muscle. In the case of coronary vessels, much of the extracellular adenosine probably arises from the myocardium or cardiac endothelial cells. Activation of cardiac β -receptors may mediate the release of adenosine in the ischaemic guinea-pig heart (Wangler *et al.*, 1989), whereas α_1 -adrenoceptor activation promotes adenosine release in the ischaemic dog myocardium (Kitakaze *et al.*, 1987). There is evidence that extracellular nucleotides may contribute significantly to the increased plasma adenosine levels obtained in the coronary circulation of the rat heart during hypoxia (Moser *et al.*, 1989; Headrick and Willis, 1989).

ATP is released from noradrenergic nerve varicosities (synaptosomes) isolated from ileal myenteric plexus when they are depolarized with K^+ or veratridine or stimulated by nicotinic and serotonergic agonists (reviewed by White, 1988, 1991). ATP does not appear to be the non-adrenergic, non-cholinergic (NANC) neurotransmitter at ileal smooth muscle, but it depolarizes and hyperpolarizes certain nerves intrinsic to the myenteric plexus (Katayama and Morita, 1989), suggesting that it might function as a neurotransmitter within the myenteric plexus.

Several studies have failed to demonstrate strictly parallel presynaptic modulation of ATP and noradrenaline release from sympathetic nerves, as would be expected if ATP and noradrenaline are stored together in the same vesicles. Clonidine, acting at presynaptic α_2 -adrenoceptors, decreases the release of [3H]noradrenaline from noradrenergic myenteric nerve varicosities without affecting the release of ATP (Hammond *et al.*, 1988). Angiotensin III inhibits the release of ATP while enhancing the release of [3H]noradrenaline in the guinea-pig vas deferens (Ellis and Burnstock, 1989). These and other

findings reviewed elsewhere (White and MacDonald, 1991; White, 1991) raise the possibility that ATP is released from a population of sympathetic vesicles separate from those which contain noradrenaline.

Release of purines from parasympathetic nerves

There is considerable evidence, including mimicry of responses by ATP and antagonism of responses to both ATP and nerve stimulation by P_{2x} purinoceptor antagonists, that ATP might be the non-cholinergic excitatory transmitter released from the parasympathetic pelvic nerves innervating the bladder (reviewed by White, 1988, 1991). Neurogenic release of ATP has been detected when guinea-pig (Burnstock *et al.*, 1978), but not rabbit (Chaudhry *et al.*, 1984), bladder detrusor strips are electrically stimulated transmurally.

Release of purines from enteric nerves

ATP may be a NANC transmitter in the guinea-pig taenia coli, and in the colon and rectum of other species (reviewed by White, 1988, 1991). Release of ATP resulting from propagated action potentials in enteric nerves of the guinea-pig taenia coli has been reported in one study (Burnstock *et al.*, 1978), but was not confirmed subsequently (White *et al.*, 1981). Although it was thought that ATP might be a NANC transmitter in the duodenum, the results of a recent study suggest that this is not the case in the rat (Serio *et al.*, 1990). NO might be the NANC transmitter that mediates relaxations in the canine ileocolonic junction (Bult *et al.*, 1990). However, ATP releases NO from vascular endothelial cells. The possibility that ATP is released from NANC nerves and then promotes the release of NO to relax the smooth muscle should be considered. There is evidence, reviewed elsewhere (White, 1988, 1991), against ATP being a NANC inhibitory transmitter in stomach and ileum.

Conclusions

It is clear that there are numerous means whereby extracellular purines can arise in the nervous system. Release of an EAA and the subsequent activation of NMDA receptors appears to mediate, in part, the release of adenosine produced by hypoxia, ischaemia and hypoglycaemia in the CNS. Even K⁺-evoked adenosine release appears to be mediated partly by the activation of NMDA receptors, suggesting that released EAAs may play pivotal roles in

evoking adenosine release in the CNS. Only a small fraction of the available NMDA receptors must be activated in order for adenosine release to be maximal. Consequently, it seems unlikely that the adenosine released during NMDA receptor activation will provide much protection against NMDA-mediated excitotoxicity in the CNS. Of course, during periods of hypoxia, ischaemia or hypoglycaemia, intracellular adenosine might accumulate and be released on the bidirectional nucleoside transporter independent of NMDA receptor activation. This adenosine, by decreasing both the release of EAAs and postsynaptic neuronal activity, and/or increasing cerebral blood flow through its actions at A₂ purinoceptors located on vascular smooth muscle, might protect against EAA-mediated excitotoxicity. However, the observation that exogenously administered adenosine and its analogues protect against ischaemic/hypoxic (Evans *et al.*, 1987; Goldberg *et al.*, 1988) and NMDA-mediated (Connick and Stone, 1989) damage in the CNS suggests that the levels of endogenous extracellular adenosine achieved during these conditions are insufficient to provide much protection. The use of adenosine agonists or drugs that increase the levels of endogenous extracellular adenosine may provide new therapies for the prevention of cerebral damage due to hypoxia, ischaemia, hypoglycaemia or seizures.

There is some evidence that ATP may function as a neurotransmitter in the CNS, particularly the spinal cord. There is much better evidence that ATP is released as a co-transmitter with noradrenaline from the sympathetic nerves innervating the vas deferens and certain blood vessels. ATP may also be released as a co-transmitter with ACh from parasympathetic nerves innervating the bladder and from motor nerves innervating striated muscle. Although it has previously been thought that ATP is co-stored with either noradrenaline or ACh within their respective synaptic vesicles, the results of recent studies indicate that ATP is not likely to be secreted from noradrenergic or cholinergic vesicles but from other sites, possibly separate populations of synaptic vesicles located within adrenergic or cholinergic nerves. The observation that the release of ATP can be modulated independently from the release of classical transmitters such as noradrenaline and ACh raises the possibility that new therapies may be developed to differentially affect purinergic versus adrenergic or cholinergic nerve-mediated responses.

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CHAPTER 9

ELECTROPHARMACOLOGY OF ADENOSINE

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Single neurone firing

Following the biochemical studies of Sattin and Rall (1970) demonstrating that adenosine could have a receptor-mediated effect on cyclic AMP levels in the central nervous system, independent of any change in the supply of intracellular purines, one of the first functional studies of adenosine in a physiological system was on single neurone firing. Phillis *et al.* (1974) applied a wide variety of purine and pyrimidine compounds to single neurones in the cerebral cortex of anaesthetized rats and observed that adenosine and related analogues were able to produce a suppression in the activity of spontaneously firing cells. Most prominent amongst the results was that adenosine itself and several nucleotides including adenosine 5'-monophosphate (AMP) and adenosine

5'-triphosphate (ATP) inhibited virtually all the cells tested. Subsequent work revealed that a similar depressant action could be detected in several regions of the central nervous system, such as the olfactory bulb, the striatum, hippocampus and thalamus (Kostopoulos and Phillis, 1977). Adenosine responses were shown to be enhanced by uptake inhibitors such as papaverine and hydroxynitrobenzylthioinosine (HNBTI), as well as by inhibition of adenosine deaminase. Compounds used to produce inhibition of the latter enzyme such as 2'-deoxycytosine or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) were themselves able to produce clear inhibition of cell firing.

The ability of ATP and the more stable analogue β,γ -methylene ATP (APPCP) to depress neurones and the ability of methylxanthines to block this effect has been confirmed by others (Stone and Perkins, 1981) (Fig. 1). The α,β -methylene ATP analogue (APCPP) has a very much weaker action consistent with the view that the nucleotides in general require to be metabolized to AMP or to adenosine in order to exert their depressant effects.

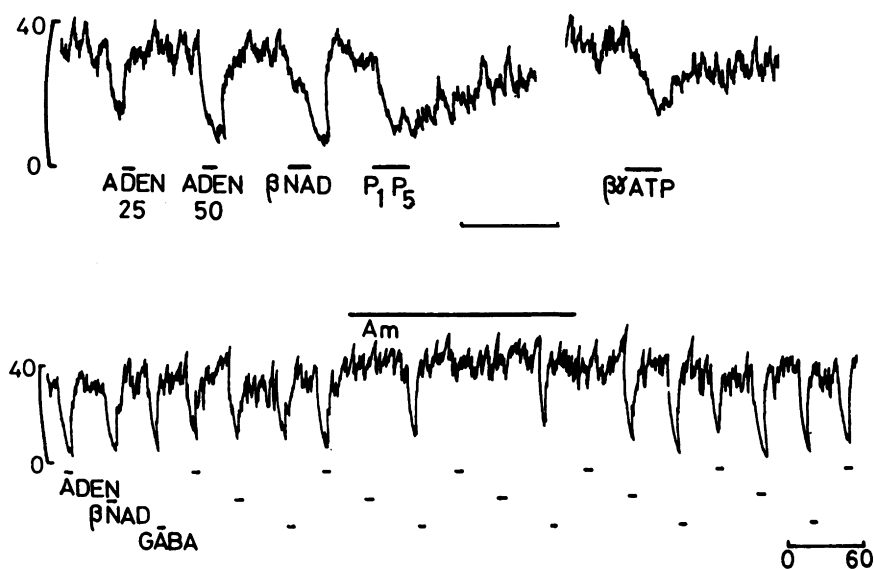


Figure 1 (A) Ratemeter record of the firing rate of a cortical neurone, showing the depression of firing induced by the microiontophoresis of adenosine, 25 or 50 nA (ADEN), β -NAD, 60 nA, P_1P_5 -diadenosine pentaphosphate, 82 nA (P_1P_5) and $\beta\gamma$ -methylene ATP, 50 nA. Time bar is 1 min. (B) Ratemeter record of the firing rate of a cortical neurone depressed by the iontophoresis of adenosine, 49 nA (ADEN), β -NAD, 58 nA, and GABA, 40 nA. The application of aminophylline, -10 nA (Am) completely blocked responses to adenosine and NAD while GABA responses were unchanged. Ordinate is in spikes/s; time bar 1 min. (From Stone and Perkins, 1981.)

Receptor subtypes

The nature of the receptor mediating these changes of spontaneous firing rate remains controversial. The failure of APCPP as well as a range of dinucleotides to induce methylxanthine-resistant changes of cell firing, and the ability of methylxanthines to block adenosine effects are consistent with the involvement of a P_1 (nucleoside) rather than a P_2 (nucleotide) receptor (Burnstock, 1978; Stone, 1989).

In two simultaneous reports (Stone, 1982; Phillis, 1982) neurones in the cerebral cortex of anaesthetized rats were tested using several analogues of adenosine. Both authors reported that *R*-phenylisopropyladenosine (*R*-PIA) was a relatively weak inhibitor of cell firing, high ejection currents being required to produce an effect and the responses being generally smaller and far more delayed and prolonged than responses to adenosine or AMP. In addition, although Stone (1982) observed only weak effects with 5'-*N*-ethylcarboxamide adenosine (NECA), Phillis (1982) was able to obtain consistent and reproducible depressions of firing with this compound. Both workers concluded that the receptor mediating the inhibition of cell firing might be of the A_2 subtype. It has since been suggested, however, that the relative activities of PIA and NECA when ejected from a point microiontophoretic source *in vivo* may be distorted by the high lipid solubility and thus limited penetration of *R*-PIA (Dunwiddie, 1984). In contrast if experiments are performed on brain slices and perfusion with agonists allowed to continue to equilibrium, the reverse profile is obtained leading to the conclusion that the A_1 subtype is more likely to be involved at least in the hippocampus and cerebellum.

The most recent additional work on this subject has been the demonstration by Phillis (1990) that the relatively selective A_2 agonist CGS 21680 is a powerful inhibitor of cell firing *in vivo*, thus supporting the original contention. The question is of more than academic interest since it is clear that presynaptic effects, to be discussed below, are mediated usually by an A_1 inhibitory receptor whereas the postsynaptic site has never been fully characterized in central neurones. It is clearly important to the development of centrally acting, functionally specific drugs to ascertain the nature of postsynaptic receptors and the extent of any differentiation from presynaptic sites.

Interactions with drugs

Evidence from electrophysiological studies has contributed to the concept that endogenous adenosine may be involved in the actions of several classes of

centrally acting drugs. These include demonstrations that the inhibitory effects of opiates upon neuronal activity in the striatum are blocked by theophylline in parallel with responses to adenosine at a time when responses to the inhibitory amino acid γ -aminobutyrate (GABA) are unaffected (Stone and Perkins, 1979). This was but one element of a substantial amount of work indicating that adenosine, released from neuronal tissue by opiates, may contribute to some of their neuronal depressant, and perhaps analgesic properties. These studies have been reviewed in detail (Stone, 1989; Sawynok and Sweeney, 1989).

Similarly, the benzodiazepines and many other CNS depressants have been shown to inhibit the neuronal uptake of adenosine (Phillis and Wu, 1982). There has ensued much debate as to whether this effect, which results in a relatively small percentage reduction of uptake at clinically relevant concentrations, is likely to be a major factor in the pharmacology of these drugs. Nevertheless, several benzodiazepines, antidepressants and anticonvulsants are able to enhance neuronal depression by adenosine even when administered systemically (Phillis, 1984a, b) and it remains a very real possibility that adenosine could be involved either in the clinically desired effects, or the undesirable 'side-effects' of some of these compounds.

Nucleotides

The absence of a nucleotide receptor in most regions of the central nervous system appears likely from the similarity of responses to ATP and adenosine on most neurones. Nevertheless, excitatory effects of adenine nucleotides have been observed in certain restricted brain regions, including the dorsal horn of the spinal cord, where Jahr and Jessell (1983) demonstrated that about 27% of the neurones tested could be depolarized by this nucleotide even in the absence of synaptic activity. Adenosine and AMP were inactive on these cells, although rigid analogues of ATP such as APPCP were as active as the parent compound. The effects were not blocked by xanthines, supporting the view that a P_2 receptor might be involved.

Excitatory effects to ATP have also been seen in the rat trigeminal nucleus, where Salt and Hill (1983) reported excitatory responses to ATP on nociceptive neurones. Interestingly, Salter and Henry (1985) observed that ATP could apparently discriminate between neurones in the cat dorsal horn which received nociceptive and non-nociceptive inputs. The latter group of cells showed a predominantly excitatory response to the nucleotide, whereas

non-nociceptive wide dynamic range cells were usually inhibited or showed biphasic responses. Only 14% of these cells revealed a pure excitatory effect, which was not blocked by theophylline. This work, in fact, led the authors to propose that ATP could be a transmitter or co-transmitter of low-threshold afferents into the dorsal horn, a conclusion similar to that drawn by Fyffe and Perl (1984) in the cat substantia gelatinosa, where it was observed that the cells excited by iontophoretically applied ATP were more likely to be those stimulated by unmyelinated primary afferents from the skin. It is interesting in this context to note that ATP can also excite neurones in sensory ganglia. Krishtal *et al.* (1983) demonstrated such an effect together with the discovery that the depolarization was mediated by a cation-carried inward current which was rapidly activated but showed rapid desensitization. Depolarization in bullfrog spinal ganglia has also been seen with ATP as well as the ability of the nucleotide to enhance depolarization mediated by GABA (Morita *et al.*, 1984).

In the light of these nucleotide effects and the availability of analogues of ATP which were unable to produce adenosine on hydrolysis, a recent attempt was made to re-examine the pharmacology of neurones in the hippocampus. Stone and Cusack (1989) applied the L-isomer of APPCP (L-AMPPCP) as well as the β,γ -difluoromethylene analogue of 2-methylthioATP to slices of rat hippocampus *in vitro*. Neither of these compounds had any significant effect on spontaneous cell firing or on evoked potentials, confirming the absence of any P_{2X} or P_{2Y} receptors in this preparation, for which the agonists used are respectively selective agonists.

In spite of this result it has been reported that submicromolar concentrations of ATP can produce a long-lasting excitation of neurones in the hippocampal slice (Wieraszko and Seyfried, 1989). However, the conditions of those experiments were rather unusual: a static bath system was used rather than the perfusion system normally employed, and slices were used from mouse brain rather than rat or guinea-pig. Nevertheless, attempts to replicate those results in the present author's laboratory have failed (unpublished), leading to the speculation that the explanation of this difference may be a purely strain-dependent one. In fact it appears that the strain selected by Wieraszko and Seyfried (1989) is a strain which is genetically sensitive to epileptic seizures. It is possible, therefore, that the excitatory effects of ATP are not a general property of hippocampal slices but may be peculiar to an increased excitability state present in this particular mouse strain. As a further complication to this picture, however, Nishimura *et al.* (1990) have now reported an excitatory action of adenosine itself in guinea-pig hippocampus, at submicromolar concentrations. It will clearly be important to explore the reproducibility of such effects on other systems, and in other species and strains.

Presynaptic effects

Specifically presynaptic effects of adenosine have been demonstrated on a wide variety of nerve terminals throughout the peripheral and central nervous systems. The list of research reports on this subject is immense (Stone, 1981a, 1989; White, 1988) but it is clear that adenosine will inhibit the release of acetylcholine (Ginsborg and Hirst, 1972; Sawynok and Jhamandas, 1976; Katsuragi *et al.*, 1985; Christofi and Cook, 1986), noradrenaline (Hedqvist and Fredholm, 1976; Clanachan *et al.*, 1977) and some non-adrenergic, non-cholinergic transmitters (Dahlen and Hedqvist, 1980) in the peripheral nervous system and the release of dopamine (Michaelis *et al.*, 1979), glutamate (Fastbom and Fredholm, 1985; Burke and Nadler, 1988), acetylcholine (Corrieri *et al.*, 1981; Pedata *et al.*, 1983) and serotonin (Feuerstein *et al.*, 1988) in the central nervous system. There are also reports that adenosine will suppress the release of noradrenaline in the CNS (Fredholm *et al.*, 1983; Jonzon and Fredholm, 1984; Jackisch *et al.*, 1985) but this is a very weak action even with normally potent analogues such as R-PIA, and may not be of any physiological significance. The release of GABA is similarly weak and seen only at very high concentrations of adenosine (Hollins and Stone, 1980). It is largely this presynaptic inhibitory effect of adenosine which is responsible for the ability to depress synaptically evoked potentials both peripherally, for example at the neuromuscular junction, as well as centrally, as in the hippocampus. The ability of adenosine to suppress evoked potentials in the latter region has been shown to correlate well with the density of adenosine receptors revealed by binding studies (Lee *et al.*, 1983).

Presynaptic mechanisms

The means by which adenosine inhibits transmitter release has been the subject of much debate. Early work (Ribeiro *et al.*, 1979) demonstrated a reduction of calcium influx into synaptosomal preparations of the CNS but these results have been difficult to reproduce (Barr *et al.*, 1985) and some results from electrophysiological preparations are inconsistent with such a phenomenon (Dunwiddie, 1984). It is interesting to note, however, that adenosine has been reported to modify the calcium N channel activity in postsynaptic sites (Macdonald *et al.*, 1986; Madison *et al.*, 1987) and it is this channel which is thought to mediate the calcium influx causing transmitter release. It is not clear whether the presynaptic effects of adenosine are therefore mediated via the N channel or whether such an effect could contribute to a wider spectrum of activity. Recent evidence has cast doubt on the involvement of presynaptic

N channels since these do not seem to be affected by adenosine on chick brain synaptosomal membranes (Lundy *et al.*, 1990).

It is also possible that adenosine may modify potassium currents in presynaptic terminals since an increase in potassium permeability similar to that which has been observed postsynaptically (see below) would result in a shortening of the presynaptic action potential and thus of calcium influx and transmitter release. In support of this possibility, it has been demonstrated that blockers of potassium channels such as 4-aminopyridine are able to prevent the inhibitory effects of adenosine on transmitter release both in peripheral sympathetic nerves (Stone, 1981b) as well as in glutamate-releasing fibres in the hippocampus (Scholfield and Steel, 1988).

Partly because of the difficulty in demonstrating a clear-cut inhibition of calcium movements into nerve terminals, Silinsky (1984) has proposed an alternative hypothesis in which the interaction of adenosine or its analogues with an externally directed receptor produces a transmembrane change in the access or availability of intraterminal calcium to the secretion/release process (Silinsky *et al.*, 1987). This hypothesis has been developed partly on the basis of adenosine's inhibition of release induced by calcium-filled liposomes in which calcium entry to the nerve terminal is independent of transmembrane flux. There may, however, be multiple mechanisms involved in this inhibitory action of adenosine since this parent nucleoside has been reported to have inhibitory effects on both evoked and spontaneous transmitter release at the neuromuscular junction, whereas 2-chloroadenosine has an inhibitory effect only on evoked release (Silinsky, 1984).

Strong evidence in favour of an effect of adenosine on calcium fluxes has been provided by Schubert's group using calcium-sensitive electrodes and recording extracellular levels of calcium in the region of nerve terminals of hippocampal slices. Here adenosine and R-PIA are able to prevent the small decrement in extracellular calcium concentration which occurs with the invasion of synaptic terminals by action potentials (Schubert *et al.*, 1986). This is clearly a powerful method for investigating the movements of calcium in a more physiological system than the use of severely disrupted and distorted synaptosomal preparations. However, it has to be realized that there are many compartments into which calcium can move, including both neuronal and glial compartments, and it would be of great value to repeat studies on hippocampal preparations using intracellular indicators of calcium concentration which can reveal the cellular site of the calcium movements. Early indications from the use of fura 2 in single hippocampal cells have indeed confirmed that the extracellular application of adenosine can produce a substantial fall in intracellular calcium activities of neuronal cells (Y. Kudo, pers. commun.). It remains unclear whether this effect, observed in postsynaptic cells, also occurs in presynaptic nerve terminals.

It is not clear whether this possible multiplicity of presynaptic purine effects is also related to the nature of the adenosine receptors. Thus at both the neuromuscular junction and in striatal synaptosomes it has been suggested that A₁ receptors may have an inhibitory effect on transmitter release, whereas A₂ sites may promote release. A similar dual regulation of acetylcholine release in the neocortex has been reported by Spignoli *et al.* (1984). Occasionally presynaptic receptors with characteristics of A₂ sites have been reported (Kennedy and Burnstock, 1984).

An appreciation of the mechanism of adenosine's presynaptic effects is complicated by the fact that both in electrophysiological studies and in studies using potential-sensitive dyes it appears that adenosine does not change the membrane potential of nerve terminals (Creveling *et al.*, 1980; Stone, 1980).

Postsynaptic mechanisms

Studies from several groups have led to the conclusion that the postsynaptic effects of adenosine are mediated by potassium channels. Most groups have observed a hyperpolarizing response to adenosine though with variable changes in membrane resistance. Segal (1982), however, observed a consistent decrease in membrane input resistance consistent with an increase of potassium conductance. Similarly studies on cultured neurones from mouse striatum have revealed an outward current induced by adenosine which was highly voltage-dependent (Trussell and Jackson, 1985).

Patch-clamp studies also demonstrated that the reversal potential for the current depended on external potassium ion concentrations. This voltage dependency may determine the size of the membrane potential response to adenosine which will depend critically on the excitability state and synaptic input to the cell being recorded. The involvement of a voltage-dependent potassium channel, therefore, may account for some of the variability observed by previous groups.

On hippocampal neurones Greene and Haas (1985) have confirmed that adenosine is able to hyperpolarize but this group reported a modulation of specific calcium-independent conductances responsible for the after-hyperpolarization which follows spike activity. At low concentrations (10 μM) adenosine appeared to enhance this after-hyperpolarization whether following individual action potentials, bursts of spikes or calcium-mediated spikes. At higher concentrations (50 μM) the after-hyperpolarization was diminished and a direct hyperpolarization was induced (confirming the work of Siggins and Schubert, 1981). The same group (Haas and Greene, 1984) have also reported that adenosine enhances accommodation of hippocampal neurones during the

application of depolarizing pulses, probably due to its increasing a calcium-dependent potassium channel conductance. In contrast to the striatal results quoted above, there is evidence that the changes of hippocampal pyramidal cell excitability involve potassium channels which are not voltage-sensitive (Gerber *et al.*, 1989).

Studies from preparations of sympathetic or parasympathetic ganglia have contributed to knowledge of the membrane mechanism involved in adenosine responses. In the rat superior cervical ganglion Henon and McAfee (1983) showed that adenosine depressed the hyperpolarizing after-potential and rate of rise of calcium-mediated spikes which were recorded after treating cells with a combination of tetrodotoxin and the potassium channel blocker tetraethylammonium. These effects of adenosine were mimicked by other analogues and potentiated by dipyrindamole and blocked by theophylline. An extracellular P_1 site was therefore clearly implicated, the authors concluding that the mechanism was inhibition of a voltage-dependent calcium current which reduced the calcium-activated potassium conductance normally responsible for the after-hyperpolarization. This conclusion is clearly consistent with the reduction of after-hyperpolarization observed by Dunwiddie and Proctor (1987). Other studies by MacDonald *et al.* (1986) and Madison *et al.* (1987) have also confirmed that adenosine can suppress calcium-dependent potassium conductances.

A study on cat parasympathetic ganglia revealed a slow hyperpolarization of the ganglionic neurones which appeared to be mediated by adenosine since it could be blocked by methylxanthines or adenosine deaminase (Akasu *et al.*, 1984). The hyperpolarization was varied in a predictable manner by potassium concentrations and appeared to involve an increase in potassium conductance, again a finding consistent with the studies on postsynaptic cells in the central nervous system.

On mouse dorsal root ganglion neurones in culture adenosine produced up to a 28% shortening of the duration of calcium action potentials at a concentration of 1 mM (Macdonald *et al.*, 1986). Voltage-clamp studies revealed that adenosine could reduce the depolarization-evoked calcium conductance with no effect on membrane conductance in the presence of cadmium (to block calcium channels). Adenosine also reduced a current-voltage slope relating to small step commands interposed upon a large voltage step which maximally activated the voltage-dependent calcium current. These findings suggest that adenosine may produce a direct suppression of calcium movements rather than an indirect effect mediated by a primary change of potassium current. Similar conclusions have been drawn by Dolphin *et al.* (1986) and by Henon and McAfee (1983).

Whilst A_2 effects of adenosine on membrane potential have not been detected in the mammal, interesting results have been obtained with the

Xenopus oocyte. Here adenosine produces a hyperpolarizing response due to increasing potassium conductance. This is sometimes preceded by a transient depolarization due to a change of chloride conductance, but since this is mimicked by ATP and is insensitive to theophylline it is possible that this indicates a P_2 receptor (Lotan *et al.*, 1982, 1986). The hyperpolarizing P_1 response is enhanced by forskolin and mimicked by the intracellular administration of cyclic AMP. Theophylline is able to block the response when applied outside the cell, as expected from its ability to block the adenosine receptor, but when administered intracellularly this xanthine enhances the response to adenosine as might be expected from its ability to inhibit phosphodiesterase. At present the structure–activity relationships for this response have not been studied in sufficient detail to confirm or refute the possibility that the elevation of cyclic AMP and the increase of potassium current are mediated by an A_2 receptor comparable with that seen in mammals.

Second messengers

The fact that most of the inhibitory effect of adenosine on transmitter release is mediated by A_1 receptors has been interpreted by some groups to imply the mediation by a reduction of adenylate cyclase activity. The point has now been made several times, however, that the original definition of an A_1 site as mediating inhibition of cyclase may represent only one action of adenosine and does not necessarily imply that this second messenger system mediates all effects of A_1 receptors defined in terms of structure–activity relationships for agonists or antagonists (Stone, 1985, 1989). Arguing against the involvement of cyclase inhibition is the fact that cyclic AMP analogues do not prevent the inhibitory effects of adenosine in the central nervous system (Dunwiddie and Proctor, 1987). A possible coupling of adenosine receptors to adenylate cyclase was supported by the inhibitory effect of pertussis toxin on adenosine responses. The consensus view that cyclic AMP levels are not involved in the presynaptic effects of adenosine led Fredholm and Lindgren (1987) to propose a resolution to this apparent paradox, namely the involvement of a pertussis toxin-sensitive G protein which was dissimilar from the G_i and G_o proteins defined in other systems. Indeed it is now clear that pertussis toxin will block several G proteins, some of whose functions remain unclear.

The inhibitory effects of adenosine at A_1 receptors can also be diminished by *N*-ethylmaleimide (Yeung and Green, 1983; Fredholm *et al.*, 1985) which is thus a useful tool for uncovering and studying in isolation A_2 receptors and their potential characteristics.

Patch-clamp studies have indicated the involvement of a G protein in the response to adenosine. If cells were perfused intracellularly using the patch-clamp technique adenosine responses tended to diminish with time unless GTP was included in the intracellular perfusion medium (Trussell and Jackson, 1987). The adenosine responses observed in the presence of GTP could then be inhibited by treatment with pertussis toxin, indicating the probable involvement of one or other of the GTP-dependent proteins within the membrane. It is interesting that in this patch-clamp system neither dibutyryl cyclic AMP nor the adenylate cyclase activator forskolin were able to modify adenosine responses, implying that the adenosine receptor may be coupled via an individual G protein directly to an ion channel without any requirement for the intermediate modulation of adenylate cyclase. This conclusion would be entirely consistent with the presynaptic results and postulate of Fredholm's group noted above.

Axonal effects

Ribeiro and Sebastião (1984a, b) have demonstrated that millimolar concentrations of adenosine and a number of analogues at high concentrations can increase the efficacy of a partial tetrodotoxin block of frog sciatic nerve. It is not yet clear whether this action is mediated by an increase of potassium conductance and the relationship of these purine effects to purine actions on central neuronal cells is uncertain since the effects do not appear to be prevented by xanthines. ATP and related nucleotides have the opposite effects, reversing a tetrodotoxin blockade, but internal ATP appears to have complex effects on the voltage-dependent gating of potassium channels. It is therefore possible that some intracellular penetration of applied purines could contribute to effects seen on the axonal tissues.

Adenosine as a neuromodulator

Interactions with noradrenaline

Adenosine is known to potentiate the stimulatory effect of noradrenaline upon adenylate cyclase activity in slices of guinea-pig neocortex (Sattin and Rall, 1970). Indeed this was one of the first indications of an extracellular action of adenosine in the central nervous system. In later electrophysiological studies the co-administration of adenosine and noradrenaline by microiontophoresis

to single cells in the cerebral cortex was also found to yield a small but significant enhancement in the depression of cell firing (Stone and Taylor, 1978). Similar potentiative interactions both in terms of the amplitude of response and the time course of responses have been reported in peripheral tissues (Hedqvist and Fredholm, 1976) and this interaction may therefore represent one physiological role of adenosine released as a result of tissue activity.

Interaction with acetylcholine

In 1976 Ewald reported that high concentrations of ATP perfused at the frog neuromuscular junction could potentiate substantially the depolarization induced by acetylcholine. Subsequent work by Akasu *et al.* (1981) using voltage-clamp techniques revealed that the mechanism was of an increased postjunctional membrane conductance. This group concluded that the interaction between ATP and the nicotinic receptor was a very selective one in which the nucleotide was able to increase either the number of ionic channels activated by acetylcholine or the unit conductance for each nicotinic-activated channel. In other words there appeared to be no direct evidence for an interaction between ATP and acetylcholine directly at the receptor site.

In the *Xenopus* oocyte preparation it has been found that acetylcholine will reduce the hyperpolarization induced in this cell by adenosine (Dascal *et al.*, 1985; Stinnakre and van Rhensterghem, 1986). This interaction involves a muscarinic receptor for acetylcholine and the mechanism is being elucidated by the research group. There appear to be concomitant changes in the phosphatidylinositol (PI) turnover system and protein kinase activity which may explain the interaction. Indeed it is now known that in a variety of peripheral mammalian tissues there are also interactions between acetylcholine and adenosine at muscarinic receptors which may involve either a mutual potentiation or antagonism of effect, the nature of the interaction apparently depending on the tissue being considered.

In the central nervous system acetylcholine produces a powerful excitation of neurones in the hippocampus via muscarinic receptors. This effect is probably mediated by a suppression of the M current potassium channels. When adenosine is perfused over brain slices at concentrations of 10 μM or greater, the muscarinic excitation can be markedly suppressed (Fig. 2) (Brooks and Stone, 1988). In comparison there is very little modification of excitatory responses to dicarboxylic amino acids such as glutamate and aspartate or their stable and more potent analogues such as kainate, quisqualate and *N*-methyl-D-aspartate (NMDA). The mechanism of this interaction has yet to be explored in detail but again may be accounted for by an increase of potassium current produced by adenosine which cancels out directly or

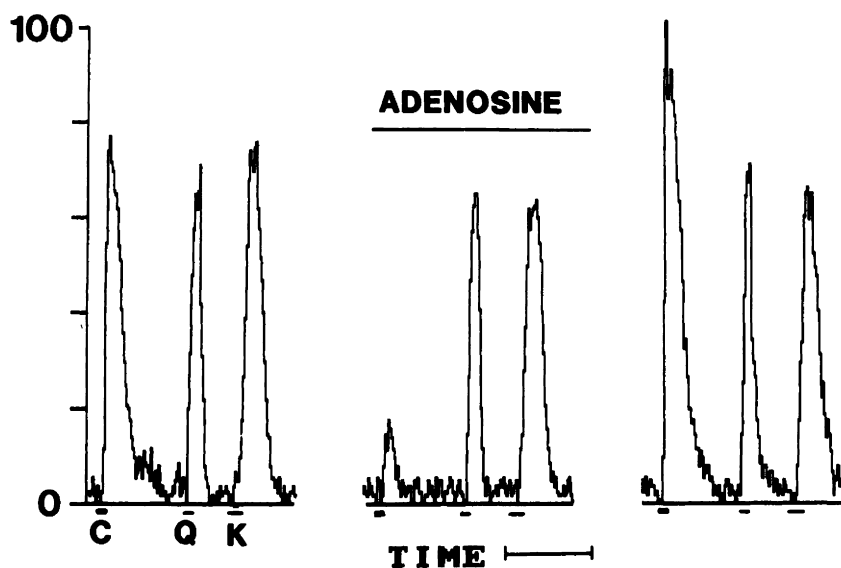


Figure 2 Ratemeter records of single cell firing rate in response to the microiontophoretic application of carbachol (C), quisqualate (Q), and kainate (K) before, during and after superfusion with adenosine, $10\ \mu\text{M}$. The middle panel was recorded 3 min after beginning the adenosine superfusion and the third, recovery sequence, was obtained 6 min after ending the adenosine superfusion. Time scale: 1 min. (From Brooks and Stone, 1988.)

indirectly the suppression of M current by acetylcholine. It is not clear whether the interaction involves a mutual displacement from receptor sites or is mediated by a common post-receptor site of action related to the M channels.

The converse phenomenon has also been demonstrated, namely that cholinomimetics will diminish the presynaptic activity of adenosine (Worley *et al.*, 1987). This interaction appears to involve the PI system, and cholinomimetic activity is mimicked by phorbol esters.

Interactions with amino acids

Although the interaction just described between adenosine and muscarinic receptors occurs at concentrations of adenosine which have little effect on direct excitatory amino acid responses, it may be that other interactions with the dicarboxylic amino acid receptors may be of importance under different circumstances. In the neocortical slice, for example, adenosine can increase the DC potential produced by the application of NMDA (Mally *et al.*, 1990), a response which reflects the mass depolarization of a large number of neurones

projecting to subcortical white matter. Although this enhancement of NMDA responses would superficially suggest an excitatory effect of adenosine it is likely that it would involve the inhibition of inhibitory interneurons which would also be activated by a non-specific dicarboxylic amino acid stimulus. The effects of adenosine are certainly mediated by a P_1 type of receptor since they can be prevented by theophylline and indeed the *in vitro* potentiation of NMDA responses can be prevented by the chronic administration of this xanthine (Mally *et al.*, 1990).

Recent evidence suggests that the excitatory amino acids may actually mediate a change of adenosine sensitivity. The observation was made by Bartrup and Stone (1988) that the presynaptic inhibitory effects of adenosine are dependent on magnesium ion concentrations in the medium bathing hippocampal slices. In magnesium-free medium adenosine responses are substantially reduced, whereas they can be enhanced by increasing magnesium concentrations to 4 or 8 mM. This effect appeared to be independent of any activation of NMDA receptors by zero magnesium media, even though it is now widely recognized that NMDA responses are dependent on voltage-sensitive blockade by magnesium of membrane ionic channels (Stone and Burton, 1988). It was discovered, however, that this independence of NMDA receptors and adenosine receptors was more apparent than real and was seen only if 2-amino-5-phosphonopentanoic acid (2AP5), the NMDA antagonist, was applied *after* the removal of magnesium ions from the medium. It was subsequently realized that if 2AP5 was present *before* removing magnesium, thus preventing any transient activation of NMDA receptors, then adenosine responses would remain unaffected (Bartrup and Stone, 1990a). The implication of this work is that NMDA itself can suppress responses to adenosine; this was confirmed directly by superfusing the slices with NMDA and demonstrating a loss of adenosine sensitivity. It was also supported by the fact that the channel-blocking compound dizocilpine (MK-801) could restore responsiveness to adenosine in magnesium-free medium (Fig. 3). Increasing neuronal excitability non-selectively by raising the potassium content of the bathing medium, even in the presence of 2AP5, caused no change in adenosine responses. Removing 2AP5 in the presence of potassium resulted in a loss of adenosine sensitivity, presumably because of the increased activation of NMDA receptors in the presence of raised potassium levels, which would cause some relief of the magnesium-dependent blockade of NMDA receptor-associated ion channels.

This phenomenon, of an NMDA receptor-mediated suppression of adenosine sensitivity, is of potentially great importance in studies of the mechanisms controlling neuronal excitability. Recent work has implicated NMDA receptors in the production of epileptiform activity both in brain slices and *in vivo* (reflected in the anticonvulsant activity of NMDA antagonists) and in

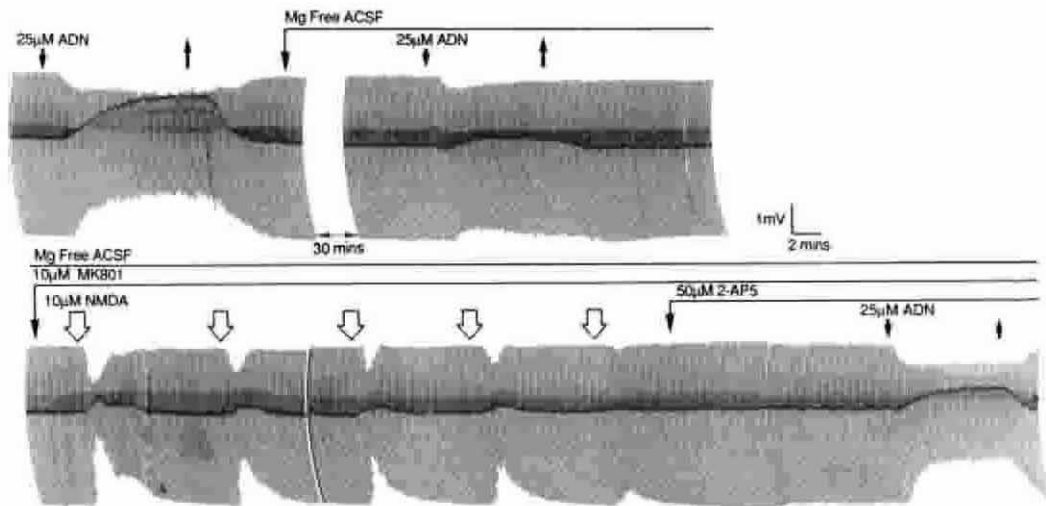


Figure 3 Consecutive records of the evoked population spike potential and its inhibition by a submaximal concentration of adenosine, $25\mu\text{M}$ (ADN). This response was lost after perfusing with magnesium-free medium for 30 min. In the lower continuation, MK-801 ($10\mu\text{M}$) was superfused and pulses of NMDA added into the medium to confirm the use-dependent blockade of the NMDA-associated channels. Even though the preparation was still superfused with magnesium-free solution, the presence of MK-801 restored sensitivity to adenosine. This occurred whether 2AP5 was also present, as in this case, or was absent. Calibrations: 1 mV, 2 min. (From Bartrup and Stone, 1990a.)

long-term changes of neuronal excitability as seen in long-term potentiation, a phenomenon encountered in the hippocampus and other regions of the central nervous system and which may be important to an understanding of the processes underlying learning and memory.

The mechanism of the NMDA/adenosine interaction remains unclear. Much of what has been said in this chapter concerns presynaptic effects of adenosine and, while the evidence is not strong, occasional claims have been made for the existence of NMDA receptors upon presynaptic terminals. It is possible that the observed interaction could therefore be mediated by direct interplay between these receptors on presynaptic terminals. Alternatively it is conceivable that activation of postsynaptic NMDA receptors may cause the release of a neuromodulator which is then able to modify the inhibitory effects of adenosine on presynaptic terminals.

Interactions with dihydropyridines

In view of the association between adenosine and calcium movements mooted above it is interesting to consider the possible relationship between adenosine receptors and binding sites for ligands which modify calcium channel function. Most notably there have been several reports of some form of relationship between adenosine receptors and binding sites for the dihydropyridine group of calcium channel activators and blockers. Thus several of the dihydropyridines can reduce the binding of adenosine and its analogues to membranes from the central nervous system while having little effect on a variety of other neuromodulators (Morgan *et al.*, 1987; Cheung *et al.*, 1987; Hu *et al.*, 1987).

Equally, binding studies and the direct examination of adenosine uptake have revealed that some dihydropyridines are moderately effective inhibitors of the nucleoside transporter (Marangos *et al.*, 1984; Phillis *et al.*, 1984). In a recent study on hippocampal slices an attempt was therefore made to determine whether these interactions could be demonstrated in a more physiological system. As reported by Bartrup and Stone (1990b) the dihydropyridine calcium channel blocker nifedipine was able to enhance responses to adenosine consistent with the diminution of uptake. In addition it was able to reduce sensitivity to adenosine analogues which are not substrates for the nucleoside transporter (Fig. 4). This latter action may thus indicate an antagonistic action at adenosine receptors which could be the functional expression of the binding displacement alluded to above.

Of additional interest was the finding that the dihydropyridine calcium channel activator BAYK 8644 showed the same activity as nifedipine while the calcium channel blockers nimodipine and nitrendipine had relatively little activity. The dihydropyridine site involved in modulating purine activity can therefore be dissociated from the classical calcium channel site.

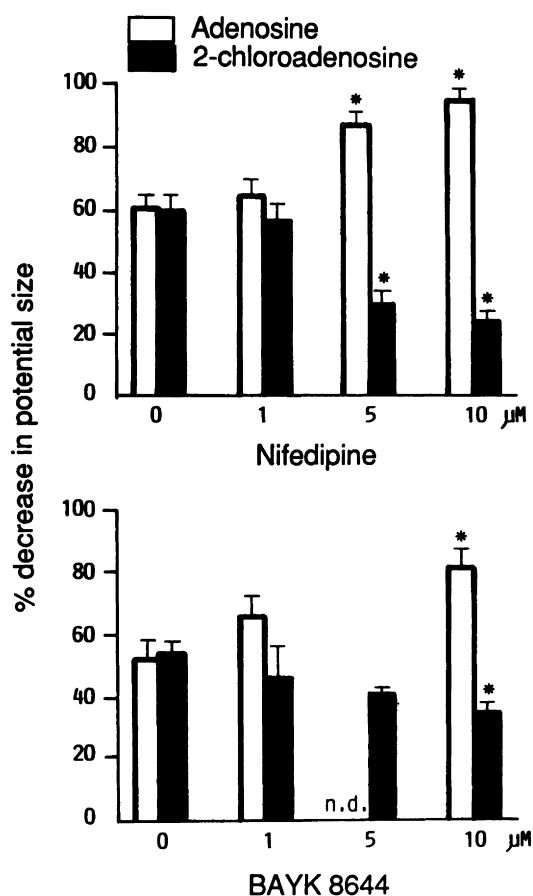


Figure 4 Histogram of the change in population potential size induced by adenosine ($25 \mu\text{M}$) (open columns) or 2-chloroadenosine ($0.5 \mu\text{M}$) (shaded columns) in the absence of dihydropyridines or in the presence of 1, 5 or $10 \mu\text{M}$ nifedipine or BAYK 8644. The columns show the mean \pm 1 SEM for $n = 6$. * $P < 0.05$ compared with responses in control medium. (From Bartrup and Stone, 1990c.)

Conclusions

In summary, adenosine has a number of actions on neuronal tissue both centrally and peripherally. Many of these effects are directed towards achieving a reduction of neuronal or tissue activity, and include the direct inhibition of neuronal firing, the suppression of release of neurotransmitters and the reduction of sensitivity to acetylcholine. Recent work is beginning to

reveal novel mechanisms by which adenosine receptor function is itself modified, as by activation of excitatory amino acid receptors, magnesium ions, or the use of dihydropyridine compounds. These modifiers of purine activity may prove to be of value in understanding the role of purine receptors in the normal and abnormal functioning of the nervous system.

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CHAPTER 10

POTENTIAL THERAPEUTIC ROLES FOR ADENOSINE IN NEUROLOGIC DISEASE

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Although the cardiovascular effects of adenosine were first observed in the 1920s (for review see Dunwiddie, 1985), it has only been recently that the physiologic regulator properties of the purine have begun to be exploited by pharmacologists. The effects of adenosine on central nervous system (CNS) functions has only been appreciated since the early 1920s (for reviews see Williams and Cusack, 1990), and the exploitation of these effects is now open for investigation. It is clear that the pharmacologic applications of adenosine as a potentially beneficial therapeutic agent in cardiovascular or CNS medicine represent a marked departure from the cytotoxic application of adenosine-derived anti-metabolites in cancer chemotherapy. However, in order to formulate workable adenosine-based therapeutic strategies for the modulation of cellular physiology it is critical that the potential cytotoxic effects of adenosine derivatives (potential for incorporating into DNA or RNA) be kept in mind.

CNS physiology of adenosine

Adenosine has a multitude of effects on many different organs (see Stone and Simmonds, 1991). Its cardiovascular effects include bradycardia and hypotension (Dunwiddie, 1985). In the kidney it constricts the renal artery thereby inhibiting renal filtration. In the immune system the purine inhibits the activation of numerous subtypes of immune cells and has the general effect of immunosuppression (Newby, 1984). The focus of the current discussion will, however, be the CNS and here adenosine also has profound effects. The major behavioural effects of adenosine include: sedation, anti-seizure activity (Snyder, 1985), and the more recently realized neuroprotective effects (Dragunow and Faull, 1988). The potential therapeutic applications of such effects are obvious and include indications such as sleep disorder, epilepsy and stroke. These and other potential uses of purinergic ligands are reviewed elsewhere in greater detail than possible here (Stone and Simmonds, 1991), but the major areas in which clinical interest is being expressed will be briefly reviewed, with emphasis on the recently discovered neuroprotective effects of purines in excitotoxicity and cerebral ischaemia.

Adenosine is now widely accepted to be a major non-peptide neuromodulator that acts through specific ectocellular receptors (Williams and Cusack, 1990). There are apparently at least two subtypes of the adenosine receptor as judged by both pharmacologic and biochemical criteria (Snyder, 1985; Chapter 4). The A_1 receptor can mediate the adenosine-induced reduction in cyclic adenosine monophosphate (cAMP) levels. A_2 Receptor stimulation by adenosine can result in increases in cAMP levels (Snyder, 1985). Pharmacologically A_1 receptors respond with high potency to adenosine derivatives substituted at the N^6 -position such as N^6 -cyclohexyladenosine (CHA) and N^6 -cyclopentyladenosine (CPA). A_2 Receptors are most potently affected by various 5'-substituted adenosine derivatives such as N -ethylcarboxamide adenosine (NECA) and CGS-21680 (Chapter 7). On autoradiographic analysis, A_1 and A_2 receptors also have different anatomical distributions in the brain (Williams and Cusack, 1990). A_1 Receptors predominate in the hippocampus, cerebral cortex and cerebellum whereas A_2 receptors are localized in the striatum (Chapter 4).

Initially it was thought that the cAMP perturbations resulting from adenosine receptor occupancy were of importance regarding the physiological functions of the system in neural tissue. Recently several observations have begun to change this perception. First, it has now become clear that adenosine probably exerts the majority of its physiologic effects via a rather ubiquitous inhibition of calcium-dependent neurotransmitter release (Fastbom and Fredholm, 1985; Burke and Nadler, 1988; Fredholm and Dunwiddie, 1988). The presynaptic effects of adenosine therefore appear to be of major

importance. In this regard a substantial body of literature now argues for an effect of adenosine (mediated probably by A_1 receptors) on either presynaptic calcium channels (N-type) and/or presynaptic potassium channels (Michaelis *et al.*, 1988; Gross *et al.*, 1989). The rationale is that adenosine inhibits neurotransmitter release either by opening presynaptic potassium channels or by blocking presynaptic calcium channels. The potassium channel effect would result in neural hyperpolarization and inhibit neurotransmitter release indirectly, whereas the calcium channel blocking effect would directly inhibit release. Further work is required to clarify which site is the more important component of adenosine's functions. The emerging picture of the mechanism of adenosine action involves the adenosine receptor residing in proximity with either presynaptic calcium and/or potassium channels and communicating with them by some membrane-associated mediator, such as a GTP-binding protein (Fredholm *et al.*, 1989).

Sedation

Less controversy exists regarding the profound behavioural actions of adenosine and adenosine agonists. The purine and its metabolically stable, and therefore longer acting, analogues have profound sedative/hypnotic and anticonvulsant properties (Marangos and Boulenger, 1985). Very low doses of adenosine A_1 agonists cause a sedation that is rather unique in that it very much resembles natural sleep (Radulovacki *et al.*, 1982). A physiological role in sleep is supported by reports that adenosine concentrations increase in animals deprived of REM sleep, a procedure which also causes an upregulation of adenosine receptor number in the CNS. Adenosine has, in fact, been referred to as the brain's natural sedative, sleep-facilitating agent and for this reason has stirred some interest as a therapeutic target for the development of new drugs to treat sleep disorders (Wauquier *et al.*, 1987). Directly acting agonists may not be the most appropriate drugs to use, as discussed below, because of the non-selective nature of their actions. Greater interest centres on the use of agents such as 2'-deoxycoformycin (an adenosine deaminase inhibitor) and transport blockers such as soluffazine. Both these compounds increase the amount and duration of sleep, including REM sleep (Ashton *et al.*, 1987; Radulovacki *et al.*, 1983). There remain some questions as to the specificity and site of action of these drugs, however, since another deaminase inhibitor, 9-[2-hydroxy-3-nonyl]adenine (EHNA), seems to reduce electroencephalographically measured sleep duration, despite a reduction of locomotor activity (Mendelson *et al.*, 1983).

The role of endogenous adenosine as an overall suppressant of neuronal activity and behaviour may explain the ability of xanthines, now generally recognized as selective antagonists of adenosine at normal behaviourally effective doses, to promote increases in arousal, alertness and attention. Several authors have made impressive correlations between xanthine behavioural stimulation and adenosine receptor blockade to support this view (Snyder *et al.*, 1981; Coffin *et al.*, 1984).

Anticonvulsant effects

As regards seizure modulation, adenosine agents have received considerable attention (Marangos and Boulenger, 1985). Adenosine agonists have been shown to inhibit a variety of experimentally induced seizures in animals and the term 'endogenous anticonvulsant' has been coined by some to describe adenosine's role in brain function (Dragunow, 1988). The anticonvulsant properties of adenosine are less apparent against seizures induced by electrical stimuli than chemical stimuli, however, and until the significance of this is understood, and the relationship between different forms of experimental seizures and human epilepsy are better appreciated, there remain questions about the clinical utility of this effect. The efficacy of purines in preventing sound-induced (audiogenic) seizures in a population of genetically sensitive mice is generally regarded as an important indicator that purines would be of clinical value in man.

It is clear that the anticonvulsant activity is mediated by a central action of purine analogues, since they are still active after intraventricular administration. The anticonvulsant activity does not correlate with sedative and locomotor depression, indicating the probability of a distinct site and/or mechanism of action.

A relationship between endogenous adenosine activity and seizures is supported by the finding that the density of A₁ receptors falls in the cerebellum of rats following leptazol seizures. Conversely xanthine treatments are said to increase the duration of seizure-related electrical activity, increase the frequency of seizure episodes and induce a corresponding increase of adenosine receptor number. This would be consistent with a role for endogenous adenosine as a mediator involved in attempts to terminate seizure activity (Szot *et al.*, 1987).

It has been suggested that some clinically useful anti-epileptic agents may act at least partly by modifying the functional status of purine systems in the CNS. Thus, both phenobarbitone and carbamazepine can displace ligands from the adenosine receptor and may be able to act as antagonists at these sites. These paradoxical findings may find resolution if the administration of

these anticonvulsants causes an upregulation of adenosine receptor number to levels at which seizures are suppressed. The alternative view has been expressed that, since there is little correlation between the activity of several carbamazepine analogues at adenosine receptors and their anticonvulsant potencies (Marangos *et al.*, 1983), the apparent antagonism of adenosine may only be of relevance to the toxic and proconvulsant consequences of administering the drugs in high concentrations.

Despite the uncertainties of detail, the anticonvulsant properties of purines have generated considerable interest in the clinical area. It is important to point out that both the sedation and anticonvulsant behavioural properties of adenosine are easily rationalized by its proposed site of action as either a presynaptic calcium channel blocker or a potassium channel opener. Both of these effects would result in decreased neurotransmitter release and diminished neural firing.

Analgesics

In the search for novel pharmacological approaches to analgesia, any group of compounds producing a generalized suppression of neurotransmitter release and thus neuronal activity requires serious consideration. Purine agonists have been known to produce antinociception in animal models since 1975 (Vapaatalo *et al.*, 1975) and subsequent work has confirmed that a range of ligands are effective in some, but not all, antinociceptive test paradigms (Yarbrough and McGuffin-Clineschmidt, 1981; Holmgren *et al.*, 1983; Delander and Hopkins, 1987; Doi *et al.*, 1987).

The mechanism of analgesia is not understood, though the localization of purine receptors and uptake sites in regions of CNS associated with nociceptive pathways provides a morphological basis for the effect (see Sawynok and Sweeney, 1989). Opiates are known to induce a release of adenosine from brain slices, synaptosomes and the cortical surface *in vivo*, and some of the inhibitory effects of opiates on neuronal firing can be prevented by xanthines. It is thus conceivable that endogenous adenosine could mediate some effects of opiates, probably by the presynaptic suppression of release of excitatory afferent neurotransmitters (see Sawynok and Sweeney, 1989; Stone and Simmonds, 1991, for reviews).

While some workers have evidence for the involvement of an A₁ receptor in mediating antinociception, consistent with a presynaptic site involvement, others have reported that NECA is more active than A₁-selective ligands. There seems to be a consensus view therefore that A₂ receptors are also, perhaps even primarily, involved in at least some later phases of antinociceptive

behaviour in animals (Holmgren *et al.*, 1986; Sawynok *et al.*, 1986; Delander and Hopkins, 1987).

Neuroprotection

The ability of adenosine agonists to suppress calcium-dependent neurotransmitter release (Burke and Nadler, 1988) becomes particularly relevant when one considers the post-ischaemic or post-trauma processes that occur in nervous tissue. The prevailing view is that certain excitatory amino acid neurotransmitters (glutamate and aspartate) are toxic to nerve cells post-ischaemia (Cotman and Iversen, 1987; Swan *et al.*, 1989), particularly via *N*-methyl-D-aspartate (NMDA) receptors (Stone and Burton, 1988), and this process is thought to be responsible for a substantial portion of the neurodegeneration that ensues. A presynaptic modulator of glutamate and aspartate release may therefore be expected to have a beneficial effect on such a process.

During the past several years considerable evidence has been generated attesting to both the mechanistic rationale for, and the experimental demonstration of, the neuroprotective properties of adenosine (Goldberg *et al.*, 1988; Arvin *et al.*, 1989; Connick and Stone, 1989; Von Lubitz *et al.*, 1989). At the mechanistic level it has been amply demonstrated that adenosine inhibits excitatory amino acid release under a variety of experimental conditions (Burke and Nadler, 1988). Given the now established role of the amino acid neurotransmitters in post-ischaemic neurological damage, the rationale for the neuroprotective properties of adenosine becomes clear. In addition adenosine has other actions that suit it to a cytoprotective function. Specifically, the vasodilating effects of adenosine serve to enhance cerebral blood flow post-ischaemia which might be expected to afford a benefit as regards the degree of post-reperfusion injury. Also the inhibitory effect of adenosine on platelet aggregation and therefore blood clotting can be beneficial as regards the decreased incidence of future embolic events. The inhibitory effect of adenosine on immune system function also serves to ward off the immunologic processes that result in the tissue destruction and damaging free radical generation which results post-ischaemia. Adenosine and adenosine agonists therefore display numerous physiologic properties which can be viewed as beneficial as regards post-reperfusion neuroprotection.

The literature that has developed to date regarding adenosine and neuroprotection is extensive. Numerous laboratories have now generated positive results with various adenosine analogues in a variety of animal models (for review see Marangos, 1990), as well as cultured cells. Neuroprotective effects are observed in both global and focal ischaemia models with either pre-

ischaemic or post-ischaemic drug treatment regimens. It has also been shown that acute treatment with the adenosine receptor antagonist theophylline exacerbates the neural damage resulting from forebrain ischaemia (Rudolphi *et al.*, 1987). Such observations strongly suggest that endogenous post-ischaemic adenosine production acts as a natural neuroprotective agent and adds further mechanistic support to the studies which show neuroprotective effects with adenosine agonists.

Additional studies have involved the chronic administration of adenosine receptor antagonists which has been shown to increase the number of adenosine receptors in the brain (Marangos *et al.*, 1987). Animals so treated have been shown to be more resistant than controls to post-ischaemic neural injury (Rudolphi *et al.*, 1989). One interpretation of these results is that the upregulated adenosine receptor complement affords an increased adenosinergic tone and therefore serves to inhibit to a greater degree the excitotoxic processes (over-release of glutamate and aspartate) that result in neurodegeneration.

Collectively these studies point to the potential clinical use of adenosinergic strategies for the treatment of ischaemic disorders. Several issues are, however, potentially troubling as regards this strategy. First, and foremost, are the rather substantial peripheral side-effects of adenosine agonists. A₁ Receptors modulate heart rate and their stimulation by agonists results in profound bradycardia. A₂ Receptors are major mediators of smooth muscle tone with agonists resulting in blood vessel wall relaxation and marked hypotensive effects. Bradycardia and hypotension can be significant problems in neurologic patients and might mitigate against the use of adenosine agonists in stroke and head trauma patients. Indeed the systemic administration of *R*-phenylisopropyladenosine causes a hypotension which greatly exacerbates excitotoxin-induced neuronal damage (Connick and Stone, 1989).

The side-effect issue can possibly be dealt with by employing a strategy utilizing co-administration of adenosine receptor antagonists that do not enter the brain. Agents such as sulphophenyltheophylline (SPT) have been shown to block the peripheral effects of adenosine agonists while having no effect on the central protective effects (Von Lubitz and Marangos, 1990). The combined central agonist/peripheral antagonist therapeutic approach, although attractive conceptually, may, however, prove to be difficult to develop in the clinic.

An additional issue worthy of special consideration is the hypothermic effect of adenosine agonists. The neuroprotective effects of hypothermia are now well-documented (Busto *et al.*, 1989; Ikonomidou *et al.*, 1989). The question therefore arises as to whether the protective properties of adenosinergic agents result from hypothermia or from inhibitory effects on excitatory amino acid release. Certainly only those studies where strict temperature regulation was maintained can be rationalized on the basis of more specific presynaptic

neurochemical effects. It is clear that the studies involving chronic antagonist upregulation of the adenosine receptor (Rudolphi *et al.*, 1989) probably do not involve hypothermia, but nevertheless it will be important to do the necessary quantitative studies to establish the precise contribution of hypothermia to the neuroprotective effects of adenosinergic agents. The hypothermia question is also of importance as regards other therapeutic approaches to post-reperfusion injury. Core and brain temperatures are two variables that have in general not been adequately controlled in many pre-clinical ischaemia efficacy studies.

Strategies for adenosine therapeutics

There are essentially three ways of devising adenosine-based treatment for any particular indication, whether it be neurologic, psychiatric or peripheral in nature. The most direct and specific approach involves the use of adenosine receptor subtype specific agonists or antagonists. This is probably the most potent approach, but also carries with it the highest side-effect profile, since virtually all of the adenosine receptors being targeted are affected (i.e. peripheral and central). The second approach to adenosinergic therapy would involve the inhibition of adenosine transport using agents which specifically block purine uptake into cells (Deckert *et al.*, 1988). The recently described multiplicity of purine uptake sites in various cell types opens up potential new therapeutic possibilities. The rationale for this approach in ischaemic therapy is that the locally increased adenosine release that occurs at ischaemic tissue foci would be prolonged in the presence of adenosine uptake blockers. This effect should be relatively event- and site-specific, since adenosine release is generally very low in normal non-traumatized tissue. Efficacy in ischaemic paradigms has yet to be demonstrated using adenosine transport blockers, and remains one of the more interesting experiments to perform.

A third adenosine-based strategy for therapeutics shares some of the characteristics of the adenosine uptake blocker strategy and involves the inhibition of specific adenosine-metabolizing enzymes such as adenosine deaminase, adenosine kinase or AMP deaminase. This scenario will also serve to prolong the half-life of ischaemia- or seizure-induced adenosine production and therefore maximize the resultant therapeutic benefit. Recent results with the adenosine deaminase inhibitor 2'-deoxycoformycin (Phillis and O'Regan, 1989) showing a protective effect in the gerbil global ischaemia model indicate that such an approach is useful. Again this type of neuroprotective strategy would be expected to be somewhat less encumbered by the global side-effects seen with the direct adenosine agonist approach. The different adenosinergic

Table 1 Adenosine-based therapeutics

I. Direct global targeting of receptor subtypes
All receptors of a given subtype are affected
Substantial side-effects
Not event- or site-specific
II. Indirect effect on adenosine levels
Enzyme inhibitors and uptake blockers
Event- and site-specific
Increase half-life of endogenously generated adenosine

therapeutic strategies are summarized in Table 1. It would appear that the adenosine transport blocker approach is the most subtle since it is the least intrusive as regards potential side-effects. Inhibition of purine-metabolizing enzymes is a scenario that should be approached with caution.

Conclusion

Adenosine is indeed a fascinating molecule whose role in physiology is varied and profound. The general picture that emerges is that the purine serves a cytoprotective or perhaps a retaliatory (Newby, 1984) role. It seems to regulate cell and organ function to a level consistent with the supply of metabolic fuel. Adenosine is generated under conditions where ATP is utilized more rapidly than it can be generated and its effects result in a slowing of neuronal activity and thus of calcium-mediated excitotoxicity. The question then arises of whether these natural cytoprotective and anticonvulsant properties of adenosine can be further enhanced by pharmacologic manipulation? The data reviewed suggest that it can, but one wonders at what price. Some of the more subtle approaches, i.e. metabolic and uptake blockers, seem to be the most elegant. Adenosine uptake blockers would appear to be the most subtle in their action, since contrary to enzyme inhibitors they do not have direct effects on the functioning of key metabolic enzymes. The primary effect of adenosine uptake blockers is a rearrangement of adenosine from an intra- to extracellular location. The net result is that more adenosine is accessible to the receptor and that its half-life is increased since most of the adenosine-metabolizing enzymes are intracellular. Relatively little work has been done with adenosine transport blockers as regards CNS therapeutics. The recently described heterogeneity of adenosine transport sites (Deckert *et al.*, 1988) adds an additional rationale for therapeutic initiatives in the future.

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CHAPTER 11

ADENOSINE AND THE CENTRAL NERVOUS SYSTEM CONTROL OF AUTONOMIC FUNCTION

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Introduction

Adenosine has potent pre-, post- and non-synaptic effects in the nervous system (see, for example, Phillis and Wu, 1981; Stone, 1981). The functional expression of these effects appears to be predominantly inhibitory. Depression of locomotor activity, inhibition in behaviour and anticonvulsant actions are

examples of adenosine inhibitory actions in the central nervous system (CNS). All these effects can be antagonized by xanthines (e.g. theophylline, caffeine).

The present chapter deals with adenosine and CNS control of respiration and of the cardiovascular system. The effects of adenosine on the sensors for the chemoreceptor and baroreceptor reflexes will also be discussed as part of the system used by the nucleoside to regulate respiration and circulation.

Since adenosine is predominantly inhibitory in the CNS, one expects that its administration into or near the anatomic structures that centrally regulate respiration or circulation would cause depression in the respiration and cardiovascular systems. In fact, as will be reviewed in this chapter, adenosine and its analogues act at the CNS to inhibit pulmonary ventilation and decrease heart rate (HR) and blood pressure (BP).

Administration of adenosine or adenosine analogues intravenously (i.v.), intraperitoneally (i.p.), intra-arterially (intracarotid (i.c.), intra-aortic (i.a.)) into the right atrium cause stimulation of ventilation. However, if the adenosine analogue (e.g. R-PIA) can cross the blood-brain barrier, there is an initial increase in pulmonary ventilation followed, a few minutes later, by a depression, which mimics the effect of its administration into the CNS (Wessberg *et al.*, 1985; Monteiro and Ribeiro, 1987). This excitatory phase is not present in glomectomized (with the carotid sinus nerves cut) animals (Eldridge *et al.*, 1984; Monteiro and Ribeiro, 1987), suggesting that the increase in ventilation elicited by adenosine results from activation of carotid body structures. This effect on arterial chemoreceptors according to Biaggioni *et al.* (1987) might also be responsible for the increase in BP and HR observed in conscious man. It appears, therefore, that peripheral administration of adenosine is excitatory of both respiration and blood circulation and that central administration is inhibitory.

Adenosine and respiration

Adenosine is an essential component in adjusting oxygen supply to oxygen needs under different physiological and pathophysiological conditions. The rate of adenosine formation is greatly accelerated in conditions such as hypoxia or ischaemia (see, for example, Winn *et al.*, 1981; Zetterström *et al.*, 1982). Respiration is regulated to keep appropriate partial pressures of O₂ and CO₂ in the arterial blood. Decrease in PaO₂ and pH or increase in PaCO₂ in the blood stimulate arterial chemoreceptors, namely the carotid body chemoreceptors, and through activation of these sensors cause stimulation of ventilation (see, for example, McQueen, 1983). Hypoxia (decrease in PaO₂), a situation where adenosine formation is greatly enhanced (e.g. Winn *et al.*, 1981; Zetterström

et al., 1982), is the physiological stimulus for activation of arterial chemoreceptors and increased respiration. This increase in ventilation rapidly compensates for the decrease in PaO_2 and, therefore, protects the CNS from the deleterious effects of hypoxia or ischaemia (see Heymans and Neil, 1958). This could represent a key function of adenosine as a homeostatic substance.

If some of the arterial chemoreceptors are non-operative (e.g. animals with carotid sinus nerves cut), hypoxia (i.e. decrease in PaO_2 at the CNS) is associated with a marked depression in respiration (Millhorn *et al.*, 1984).

The hypoxic drive to respiration provided from the chemosensors of the carotid body reaches the brain via primary sensory neurones whose cell bodies are located in the petrosal ganglion. The inputs are organized in a restricted portion of the nucleus of the tractus solitarius (NTS) in the dorsal medulla, particularly the commissural nucleus and the medial subnucleus in close proximity to the obex (e.g. Donoghue *et al.*, 1984; Houslay *et al.*, 1987; Houslay and Sinclair, 1988). The NTS also possesses other respiratory neurones in the ventrolateral NTS, which seem not to be directly contacted by the peripheral chemoreceptor afferents (see Spyer *et al.*, 1990). One aspect of particular interest is that the NTS is a structure with a high density of adenosine uptake sites (Deckert *et al.*, 1987).

In the next sections, for the sake of simplicity, the following will be described separately: (1) adenosine, respiration and the peripheral arterial chemoreceptors, and (2) adenosine and the CNS control of respiration.

The carotid body chemoreceptors

Humans

In healthy human volunteers, intravenous boluses or intravenous infusions of adenosine cause marked, transient or for the period of the infusions, increases in respiration, mostly due to increased depth of respiration (Watt and Routledge, 1985; Fuller *et al.*, 1987). This effect of adenosine is mainly due to an increase in tidal volume, and is not mimicked by its metabolite, inosine (Reid *et al.*, 1987). It is more pronounced when the nucleoside is infused proximal to the carotid circulation, suggesting involvement of the carotid body chemoreceptors in the stimulation of respiration (Watt *et al.*, 1987c). This effect of adenosine was not significantly different in a group with a mean age of 25 years from that of an average age of 66 years old (Watt *et al.*, 1989).

Observations also made in healthy human volunteers by Maxwell *et al.* (1986) showed that adenosine increases resting ventilation, as well as the hypoxia ventilatory response, but does not alter the hypercapnic response, in

agreement with the idea that adenosine may stimulate respiration by a peripheral rather than a central action. This excitatory effect of adenosine is antagonized by theophylline but not by enprofylline, a xanthine almost devoid of adenosine receptor blocking properties (Maxwell *et al.*, 1987).

Studying the blood gases (PaO_2 and $PaCO_2$) and pH in human volunteers, Biaggioni *et al.* (1987) found that infusion of adenosine stimulates respiration with increases in PaO_2 and pH and decreases in $PaCO_2$.

In summary, considering the limitations of the studies performed in humans, it seems clear that adenosine given i.v., as bolus injections or infusions, excites ventilation. No direct evidence exists about the potential central depressing effect of adenosine in humans. However, some indirect evidence is provided by the finding that the adenosine antagonists caffeine and theophylline that act on the CNS stimulate respiration in humans.

Animal studies

Rats

In rats anaesthetized with pentobarbitone, adenosine injected into the common carotid artery (i.c.) before cutting the carotid sinus nerves increases both the amplitude and the frequency of the respiratory movements. The effect starts immediately after the end of the injections and lasts for about 30–40 s for the highest dose (100 nmol). After section of the carotid sinus nerves the excitatory effect of adenosine on ventilation disappears (Monteiro and Ribeiro, 1987). Endogenous adenosine appears to exert a 'tonic' excitation on ventilation mediated by carotid body chemoreceptors, since the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and the adenosine uptake inhibitor, dipyrindamole, both mimic the excitatory effect of adenosine. These effects of EHNA and dipyrindamole are antagonized by the adenosine receptor antagonist, 1,3-dipropyl-8(*p*-sulphophenyl)xanthine (DPSPX). DPSPX itself can produce two different effects on respiration. During intracarotid infusions of cumulative doses of DPSPX, depending on the dose, there is an inhibitory effect not present in glomectomized rats and an excitatory effect present in both glomectomized and in non-glomectomized animals (Monteiro and Ribeiro, 1989a). Another interesting aspect from this study is that in relation to the carotid body chemoreceptor activation by adenosine in rats (Monteiro and Ribeiro, 1989a), the nucleoside seems to be inactivated by both deamination and uptake. Also in cats, McQueen and Ribeiro (1983) found that the adenosine uptake blocker, dipyrindamole enhances the excitatory effect of adenosine on the carotid body chemosensory discharge.

Cats

In pentobarbitone-anaesthetized and curarized cats, adenosine i.c. causes a rapid and marked increase of spontaneous carotid body chemoreceptor

discharge, the intensity, duration and onset of which is dose-dependent. Infusion of adenosine also evokes an increase in discharge, which persists for the duration of the infusion, and returns to pre-infusion control levels within 30 s of stopping the infusion. Adenosine increases the average discharge and also the maximum discharge frequency (McQueen and Ribeiro, 1981). The overall increase in discharge caused by adenosine results more from a sustained increase throughout the response (McQueen and Ribeiro, 1981) than from a sudden transient increase, such as occurs with acetylcholine (ACh) (see McQueen, 1983).

Adenosine has potent vascular effects (e.g. Berne, 1980). To exclude that the chemoexcitation described by McQueen and Ribeiro (1981) was not a consequence of changes in BP or blood flow through structures of the carotid body, Runold *et al.* (1990a) performed elegant experiments using the isolated and superfused cat carotid body. They found that adenosine in concentrations equivalent to those used *in vivo*, also increases chemoreceptor discharge. These results suggest that the excitatory effect of adenosine on chemoreceptor discharge could be independent of the adenosine vascular effects.

Structure of the carotid body where adenosine acts

The general consensus concerning the mammalian carotid body is that it is composed of type I cells and sensory nerve endings enveloped by type II cells; all these components are contained in a rich vascular network. Two kinds of synapses have been proposed: one is efferent or motor, and another is afferent or sensory (for review see, for example, Eyzaguirre and Fidone, 1980).

It has been suggested that substances such as sodium cyanide (NaCN) or CO₂ act preferentially on carotid body type I cells, and substances such as ACh and dopamine act predominantly on the sensory nerve endings (see, for example, Fidone and Gonzalez, 1986). Chemoreceptor responses to NaCN- and CO₂-equilibrated Locke solutions were slightly and variably reduced after adenosine injections. This variability was further verified by dose-response curves to NaCN performed during infusions of adenosine. The chemoexcitatory responses to ACh and the chemoinhibitory responses evoked by dopamine are both increased after adenosine injections. These effects were confirmed by dose-response curves to both ACh and dopamine obtained during adenosine infusions (Ribeiro and McQueen, 1983). This sensitization by adenosine suggests that the nucleoside might have a direct action on the sensory nerve endings, which are postsynaptic in relation to type I cells. The possibility of adenosine decreasing the release of an inhibitory transmitter (e.g. dopamine) from the type I cells, that tonically inhibits chemoreceptor activity, cannot be excluded from the studies so far published (see Ribeiro *et al.*, 1988 for discussion). It may also be that the excitatory effect of adenosine on chemoreceptor activity results from a decrease in the release of a transmitter

(e.g. dopamine) that tonically inhibits chemosensory activity together with direct stimulation by adenosine of the chemosensory nerve endings.

Several studies have shown that adenosine is able to excite sensory nerve endings. In human patients with duodenal ulcer i.v. adenosine causes transient epigastric discomfort, indistinguishable from spontaneous pain. This effect is antagonized by aminophylline (Watt *et al.*, 1987b). Extensive studies by Sylvén *et al.* (1986) demonstrate that adenosine i.v. induces angina pectoris-like pain. In the human blister base preparation, Bleehen and Keele (1977) observed that adenosine causes pain.

Taking together this information on adenosine and sensory nerve terminals, one is tempted to speculate that in contrast with the consistent and predominant presynaptic inhibitory effect of adenosine at efferent terminals (e.g. motor nerve endings), at the sensory nerve terminals the nucleoside could be preferentially stimulatory providing excitatory inputs to the central nervous system. In this way adenosine may become part of the system used by the body to protect itself against insults (e.g. hypoxia, ischaemia) in which there is an increase in the formation of extracellular adenosine.

The aortic body chemoreceptors

Besides carotid body chemoreceptor activation by adenosine, other arterial chemoreceptors are involved in the regulation of respiration, as is the case with aortic body chemoreceptors. Using experimental protocols similar to those employed by McQueen and Ribeiro (1981) to establish the effects of adenosine on carotid body chemoreceptor activity, and the same animal species, the cat, Runold *et al.* (1990b) found that intra-aortic administration of adenosine causes a dose-dependent increase in chemoreceptor discharge recorded from the aortic nerve.

Adenosine and the vagal pulmonary afferents

Adenosine injected into the pulmonary circulation of adult cats via the right atrium causes an increase in breathing, an effect not modified by carotid sinus nerve and aortic nerve sections, but which can be prevented by cervical vagotomy (Runold, 1989). These authors also observed that in the recordings from single fibres of the vagus nerve both the tracheo-pulmonary C-fibres and low-threshold pulmonary stretch receptors are stimulated by adenosine.

Summary

In summary, chemoreceptors present in the carotid body, aortic body and pulmonary circulation can all be activated by adenosine (McQueen and

Ribeiro, 1981; Runold, 1989; Runold *et al.*, 1990b) and may participate in the final excitatory response to increasing adenosine in the arterial blood. The relative contribution of these different structures for the final adenosine excitatory response remains to be established, though See *et al.* (1983) described the non-chemical vagal respiratory afferents as being unable to maintain rhythmic ventilation. The chemical afferent inputs are essential for the generation of the respiratory rhythm, and at the periphery, the major role is committed to the carotid body followed by the least important aortic afferents.

The mechanism triggered by adenosine to cause chemoreceptor activation might occur at presynaptic (decreasing the release of an excitatory transmitter from the type I cell) and/or postsynaptic levels. Considering the postsynaptic levels, a possibility can be envisaged in the light of the results obtained with the rat sympathetic ganglia (Henon and McAfee, 1983a). In this preparation, 2-chloroadenosine (CADO), besides inhibiting excitatory postsynaptic potentials (EPSPs) probably through decreasing transmitter release per impulse as it does in most of the central and peripheral synapses (see Ribeiro and Sebastião, 1986), also possesses facilitatory effects on synaptic transmission during repetitive firing. In the presence of CADO more EPSPs exceed threshold and the number of action potentials increases markedly. Adenosine would enhance the transmission of high-frequency impulses, much like a 'high-pass filter' (see Henon and McAfee, 1983b).

Central effects of adenosine on respiration

Rats

In adult awake rats (normal and chemodenervated), Burr and Sinclair (1988) showed that the lipid-soluble adenosine stable analogue, R-PIA injected i.p. depresses respiration in the same manner as it does in anaesthetized rats (Hedner *et al.*, 1982; Mueller *et al.*, 1984; Wessberg *et al.*, 1985), cats (Eldridge *et al.*, 1984), and rabbits (Hedner *et al.*, 1984; Lagercrantz *et al.*, 1984). Also CADO administered intracerebroventricularly (i.c.v.) in hyperoxic halothane-anaesthetized rats depresses respiration in great part as a result of a decrease in tidal volume (Mueller *et al.*, 1984).

Central chemoreceptors are activated by changes in the hydrogen ion concentration in the interstitial fluid in the brain, and are mainly involved in the ventilatory and circulatory adjustments during hypercapnia and chronic alteration of acid-base balance (see, for example, O'Regan and Majcherczyk, 1982). The response to CO₂ increase (10% CO₂ + 90% O₂) is inhibited after i.c.v. R-PIA, suggesting that this effect of R-PIA might be exerted at the CNS (Hedner *et al.*, 1982).

Using halothane-anaesthetized rats, Wessberg *et al.* (1985) injected i.c.v. as well as i.p. CADO, R-PIA, CHA and NECA and found that all these adenosine analogues reduce respiratory frequency (f) and tidal volume (V_T) as well as minute ventilation (V_E). These effects were observed in both normal and vagotomized rats. NECA was more potent than R-PIA. These authors did not describe excitatory effects of the peripherally administered analogues. Barraco and Janusz (1989) also used NECA. They microinjected this adenosine analogue into respiratory-related regions (ventromedial region of the caudal NTS at the level of the caudal tip of the area postrema), of spontaneously breathing rats and found significant dose-related reductions in respiratory rate.

Theophylline but not enprofylline (devoid of adenosine receptor antagonistic properties) antagonized the respiratory depression induced by R-PIA. The finding that theophylline increases basal respiration suggests that adenosine might exert a tonic respiratory depressant influence (Wessberg *et al.*, 1985). According to Wessberg *et al.* (1985), adenosine regulates centrally the respiratory system by decreasing inspiratory neural drive and prolonging expiratory drive.

Cats

In adult cats vagotomized and glomectomized (with the carotid sinus nerves cut to avoid carotid body chemoreceptor influences) R-PIA i.v. or given into the third cerebral ventricle causes depression in respiration, involving decreases in both tidal volume and respiratory frequency (Eldridge *et al.*, 1984). These effects can be antagonized by theophylline (Eldridge *et al.*, 1985). Theophylline on its own increases ventilation, suggesting a central tonic inhibition in respiration by adenosine (Eldridge *et al.*, 1985).

Also, microinjections of R-PIA into the region of the nucleus paragigantocellularis lateralis of adult cats reduce ventilation (Runold, 1989).

Adenosine and respiration during development

Preterm, neonates

In contrast with adults, adenosine and its analogues administered peripherally in preterms and neonates depress respiration rather than causing excitatory effects on pulmonary ventilation. R-PIA i.p. in preterm rabbit neonates (25 days gestational age) causes marked respiratory depression and irregular breathing, which can be antagonized by theophylline (Hedner *et al.*, 1984). In unanaesthetized rabbits, the ventilatory inhibition caused by R-PIA i.p. was more pronounced in 1–3-day-old animals than in 8-day-old animals

(Runold *et al.*, 1986). R-PIA i.p. also depresses respiration in urethane-anaesthetized decerebrated pups. A similar effect is observed with R-PIA applied onto the exposed surface of the fourth ventricle (Lagercrantz *et al.*, 1984). Also, adenosine injected into rabbit pups, in a dose that in adult rabbits causes increase in ventilation, depresses ventilation (Watt *et al.*, 1987a). This effect is attenuated by aminophylline (Watt *et al.*, 1987a). Similar results were obtained with adenosine in kittens (Lagercrantz and Runold, 1985). In foetal sheep R-PIA i.v. causes complete cessation of foetal breathing movements (Smith *et al.*, 1986).

In experiments with rabbits, Runold *et al.* (1986) observed that the respiratory depression to hypoxia, a situation related to the increase in formation of extracellular adenosine, is more dramatic in newborns than in older subjects.

In foetal sheep, intracarotid infusion of adenosine decreases the incidence of breathing in a manner similar to that observed for hypoxia (Koos and Matsuda, 1990), and theophylline antagonizes the reduction in the incidence of breathing caused by severe hypoxia (Koos and Matsuda, 1990).

Adenosine hypoxia, ischaemia and respiration

Hypoxia has a biphasic effect on respiration, initially increasing ventilation followed by a decrease. This inhibitory effect is much more pronounced in the newborn than in older subjects and has been attributed to a central depression (see, for example, Lagercrantz *et al.*, 1986). Hypoxia causes rapid depression of ventilation in foetal sheep (Boddy *et al.*, 1974). Preterm infants become apnoeic even when exposed to moderate hypoxia (Rigatto *et al.*, 1975). Hypoxia also produces ventilatory depression in newborn infants after a transient stimulation (Cross and Warner, 1951). Animals glomectomized (i.e. with the carotid sinus nerves cut) behave like newborns in relation to hypoxia, which is very depressive of respiration. This depression is antagonized by theophylline and completely disappears 10 min after returning to the hyperoxic state. This suggests that adenosine might be involved in the respiratory depression induced by hypoxia (Millhorn *et al.*, 1984). The adenosine receptor antagonist, 8-phenyltheophylline reduces the responsiveness of carotid chemoreceptors to hypoxia. This could be considered an indication that adenosine released during hypoxia can be involved in chemosensory excitation (McQueen and Ribeiro, 1986).

In pentobarbitone-anaesthetized rats, the adenosine antagonist DPSPX i.c. decreases significantly the respiratory stimulation induced by bilateral common carotid occlusion, in consonance with the idea that the increase in ventilation produced by ischaemia at the carotid body level involves the release of adenosine (Monteiro and Ribeiro, 1989b).

Neurotransmitters and neuromodulators involved in the control of respiration

Besides dopamine, endorphins (including enkephalins), serotonin and substance P are neuromodulators involved in central respiratory regulation (Moss *et al.*, 1986). Experimental data reviewed in this chapter strongly suggest adenosine as an interesting candidate to modulate chemoreceptor reflexes. Experiments with kainic acid, a neurotoxin that acts via glutamate receptors, support the idea that glutamate may represent the neurotransmitter of the first central synapse of chemoreceptor afferents at the NTS (see Houslay and Sinclair, 1988; Brew *et al.*, 1990). Glutamate itself excites respiration (Brew *et al.*, 1990). It needs to be investigated if adenosine decreases the release of glutamate at the NTS and, if it does so, whether this effect contributes to the depression of respiration.

Besides the inhibitory effects induced by administration of adenosine into the NTS the nucleoside decreases the release of excitatory transmitters in almost all regions in the brain (see, for example, Ribeiro and Sebastião, 1986).

The NTS receives influences from the hypothalamus where noradrenaline is a neurotransmitter, from the suprapontine regions where glutamate, aspartate and acetylcholine are neurotransmitters, from the raphe nuclei where 5-hydroxytryptamine is the neurotransmitter (see, for example, Lagercrantz, 1987). It has been demonstrated that all these neurotransmitters can be inhibited by adenosine at the CNS (see, for example, Ribeiro and Sebastião, 1986). This complex situation indicates that adenosine could have potential effects in these regions, namely modulating the release of neurotransmitters; eventually this might contribute to the final depressive responses in respiration during hypoxia.

The type of adenosine receptor involved in respiration

At the carotid body chemoreceptors

Agonists

In both cat carotid body chemoreceptor studies (McQueen and Ribeiro, 1986) and rat respiration studies (Monteiro and Ribeiro, 1987), the rank order observed for the adenosine agonists was NECA > CADO > R-PIA, S-PIA, this relative position being compatible with an A₂ adenosine receptor (see Daly *et al.*, 1981) involved in the peripheral regulation of respiration by adenosine.

Cyclic AMP mimetics

Studies into the nature of the second messenger involved in this excitatory effect of adenosine showed that the cyclic AMP analogue, dibutyryl cyclic

AMP also excites respiration via activation of carotid body chemoreceptors (Ribeiro and McQueen, 1983). Low PaO_2 appears to augment cyclic AMP levels in the carotid body (Pérez-García *et al.*, 1990).

Antagonists

The use of xanthines to characterize the adenosine receptor involved in excitation of respiration revealed that 1,3-dipropyl-8-(4-carboxymethoxyphenyl)xanthine (XCC), benzenesulphonamide, *N*-(2-(dimethylamino)ethyl)-*N*-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) (PD115.199) and 1,3-dipropyl-8-(4-(2-aminoethylamino)carbonylmethoxyphenyl)xanthine (XAC) have much higher affinities than 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), a selective A_1 adenosine receptor antagonist; this being again compatible with an A_2 adenosine receptor subtype (Ribeiro and Monteiro, 1991).

At the central nervous system

Agonists and antagonists

The relative position of the adenosine agonists is NECA > CADO > R-PIA also compatible with an A_2 adenosine receptor subtype (Wessberg *et al.*, 1985). No studies have yet been carried out using the more selective antagonists in order to define a profile.

Cyclic AMP mimetics

Cyclic AMP analogues, such as bromocyclic AMP injected in the same region of the NTS where NECA depresses respiration, also decrease pulmonary ventilation by decreasing both frequency and minute volume (Barraco *et al.*, 1988).

Summarizing this point, it appears clear-cut that A_2 adenosine receptors are involved in excitation of respiration, though it might be that this is just the final response of activating different adenosine receptors (A_1 and A_2 and others, e.g. A_3 , see Ribeiro and Sebastião, 1986), since several structures should, in principle, be activated by the nucleoside, and therefore, will participate in the actions of adenosine. At the carotid body, besides the fenestrated capillaries, where no studies on adenosine receptors are available, there is also no information about the potential effect of adenosine on the cell that behaves as the chemosensor, the type I cell (very rich in neurotransmitters and modulators), or the type II cell that behaves like the glial cells.

At the CNS, the same sort of reservation should be taken into account during evaluation of the results obtained with the aim of characterizing the type of adenosine receptor involved in respiration.

Nevertheless, adenosine is, indeed, at least in hypoxic ischaemic situations, an important modulator of respiration and a strong candidate to join the

other mediators already proposed to regulate respiration such as endorphins, prostaglandins and substance P (see, for example, Lagercrantz, 1987).

Adenosine and the central nervous system control of the cardiovascular system

Recent studies have established that the anatomical structure that integrates most of the arterial chemosensory information, the NTS, is also a major relay nucleus for afferent inputs from baroreceptors (Brody *et al.*, 1986). Neuronal cell groups of the NTS regulate reflex cardiovascular activity through changes in sympathetic and parasympathetic tone (Palkowitz and Zaborszky, 1977). The automatic cardiovascular control is achieved in the medulla oblongata through integration of inputs from higher centres with afferent inputs from receptors in chest and neck structures (Reis *et al.*, 1984). These afferent inputs travel in the vagus and glossopharyngeal nerves and terminate in the NTS. NTS neurones have projections to many rostral and ventral sites, like the C₁ area in the ventral lateral medulla (Ross *et al.*, 1985). Stimulation of the NTS produces inhibition of sympathetic tone while stimulation of the C₁ area leads to an increase in sympathetic activation (Granata *et al.*, 1985). Interaction between the peripheral arterial chemoreflexes and the cardiovascular system (alterations in HR, cardiac output and peripheral vascular resistance) is a well-known phenomenon (see, for example, Daly, 1986).

The effects of adenosine and adenosine analogues

Central administration of adenosine and its analogues near the brain structures involved in respiration elicit important circulatory effects. Adenosine administered in microinjections stereotaxically into the NTS of rats causes decrease in HR and in systolic and diastolic BP. Maximal changes occur usually 90 s following the injections. Similar effects have also been obtained with injections of adenosine into the area postrema. These effects can be antagonized by the adenosine antagonist, DPSPX (Tseng *et al.*, 1988). Adenosine deaminase injected into NTS and the area postrema blocks the inhibitory effect of adenosine on HR and BP. When adenosine is injected into the C₁ area of the ventral lateral medulla it does not cause any pharmacologic effect on BP or HR, suggesting that the effect of adenosine is selective for the former areas (Tseng *et al.*, 1988).

Injection of the stable (non-hydrolysable) adenosine analogues, NECA and R-PIA, into the lateral cerebral ventricle of anaesthetized rats causes decrease in BP and HR, with R-PIA being more potent (Barraco *et al.*, 1986).

These effects are antagonized by caffeine, and can be dissociated from the adenosine locomotor depressing effects, since a decrease in locomotor activity can be obtained with doses of these analogues 10–100-fold lower than the doses that cause depression in BP or HR (Barraco *et al.*, 1984). The doses of CADO and R-PIA that when injected into the lateral ventricle of hyperoxic halothane-anaesthetized rats cause bradycardia and hypotension also produce decrease in respiration (Mueller *et al.*, 1984).

When administered into the fourth ventricle, in particular the region of the area postrema, the doses of NECA or R-PIA used were 100–10 000 times lower than those required to inhibit BP to a similar magnitude through i.c.v. administration (Barraco *et al.*, 1987b) or after i.v. administration (Barraco *et al.*, 1987a). The fourth ventricle, in particular the region of the area postrema, appears to be the most sensitive site for the hypotensive actions of NECA, the third ventricle, posterior hypothalamus, medial preoptic area and NTS being somewhat less sensitive. For example, microinjections of NECA into the more rostral regions of the NTS are less effective in eliciting cardiovascular responses than injections into the caudal NTS, and microinjections into the dorsal brainstem at sites lateral and ventrolateral to the caudal NTS are virtually ineffective (Barraco and Janusz, 1989). This high sensitivity suggests that the bulbo-pontine central mechanisms may contribute to the blood pressure depressor and bradycardia effects of the adenosine analogues (Barraco *et al.*, 1986).

The peripheral (intraperitoneal) administration of the lipid-soluble adenosine stable analogue, R-PIA, which easily crosses the blood–brain barrier in pentobarbitone-anaesthetized rats, also decreases HR and BP in association with decreases in respiration (Hedner *et al.*, 1982).

Since adenosine excites peripheral arterial chemoreceptors and these sensors through a cardiovascular reflex influence the cardiovascular system (Daly, 1986), it is possible that some of the cardiovascular effects of adenosine result from interaction with the arterial chemoreceptor cardiovascular reflex.

As occurs with respiration, adenosine administered intravenously (bolus injections) in conscious normal volunteers is excitatory (Watt and Routledge, 1986). It initially increases systolic and diastolic arterial blood pressure, followed by a reduction in systolic and diastolic pressures. Adenosine infusions decrease HR and systolic pressure but decreases slightly diastolic pressure; these effects are mediated through reflex autonomic mechanisms (Robertson *et al.*, 1988). These haemodynamic effects and the excitatory respiratory effects are in part due to chemoreceptor activation, since adenosine is pressor when infused in the aortic arch near the origin of the carotid arteries and depressor when infused in the descending aorta (Robertson *et al.*, 1988). In conscious man, adenosine stimulates via activation of these chemoreflexes the sympatho-adrenal system with cardiovascular consequences: increase in HR and BP

(Biaggioni *et al.*, 1986). In support of this idea comes the interesting observation by Biaggioni *et al.* (1987) that the excitatory effects of i.v. bolus injections of adenosine on HR and BP are not present in patients with autonomic failure.

Baroreflexes

Adenosine injected i.v. in conscious dogs reduces baroreflex sensitivity resulting from reduction of the tachycardia that normally follows a fall in systemic BP. This effect of adenosine is direct on the sinoatrial node and not on the afferent, efferent or central integrative mechanisms that make up the baroreflexes. This effect is easier to recognize when the cholinergic influence to the heart is abolished with atropine (Hintze *et al.*, 1985).

Adenosine antagonists (e.g. DPSPX) injected into the NTS of rats inhibit the baroreflex sensitivity, suggesting that endogenous adenosine has a facilitatory role on the baroreflex (Mosqueda-Garcia *et al.*, 1989). Also, intracisternal administration of caffeine to conscious and anaesthetized animals causes inhibition in baroreflex activation (Mosqueda-Garcia *et al.*, 1989), further supporting the view that central endogenous adenosine is involved in the medullary regulation of BP.

The mechanism by which the adenosine antagonists inhibit baroreflex sensitivity is unclear. Although adenosine and glutamate have many different and almost opposite effects in various CNS regions, both substances given in the NTS decrease BP, HR and have similar effects on baroreflex activation (Mosqueda-Garcia *et al.*, 1989). Cardiovascular effects of adenosine can be inhibited by various glutamate antagonists (preliminary data from Mosqueda-Garcia *et al.*, 1989). This suggests that adenosine might act by enhancing the effect and/or facilitating the release of glutamate, which might activate the inhibitory influences. Glutamate may represent the neurotransmitter of the first central synapse of the baroreceptor afferent synapsing more rostrally in the NTS (Talman *et al.*, 1980). Support for this hypothesis is provided by the demonstration that glutamate concentrations are high in this region (Dietrich *et al.*, 1982) and increase as glutamate turnover does during hypoxia (see Brew *et al.*, 1990). Whether this increase in glutamate results or follows increases in adenosine needs investigation.

Adenosine and the hypotension induced by hypoxia

Adenosine levels are elevated in the NTS following brief exposure to hypoxia (Gallman and Millhorn, 1988). A central role for adenosine in the hypotension elicited by hypoxia in the anaesthetized but not in the non-anaesthetized rat has been proposed (Simpson *et al.*, 1989).

The finding that the depressing effect of adenosine on BP and HR is less pronounced in genetically hypertensive rats than in normotensive rats suggests that the purinergic receptors might be modified in the genetically hypertensive rats (Robertson *et al.*, 1988).

Concluding remarks

It is quite well established that endogenous adenosine is a modulator of cell activity inhibiting or stimulating the work of the cell according to the cell specific function. The respiratory and cardiovascular controls are complex mechanisms integrating several variables, chemo- and baroreflexes that interact, as well as a number of afferents that drive stimuli to the respiratory and cardiovascular centres. Adenosine could interfere with some or all of these components at pre-, post- or non-synaptic levels. The relative participation of each effect of the nucleoside for its final response, as well as the mechanisms involved (type of adenosine receptor; type of second messenger) in each effect that contributes to the final respiratory and circulatory adenosine responses, are aspects that need further investigation.

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CHAPTER 12

PURINE METABOLIC DISORDERS AND NEUROLOGICAL DYSFUNCTION

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Adenosine in the nervous system

Observations on the regulatory functions of adenosine predated the description of genetic diseases specific to the purine metabolic network by many years, the first purine disorder being recognized in 1954. Since then a number of

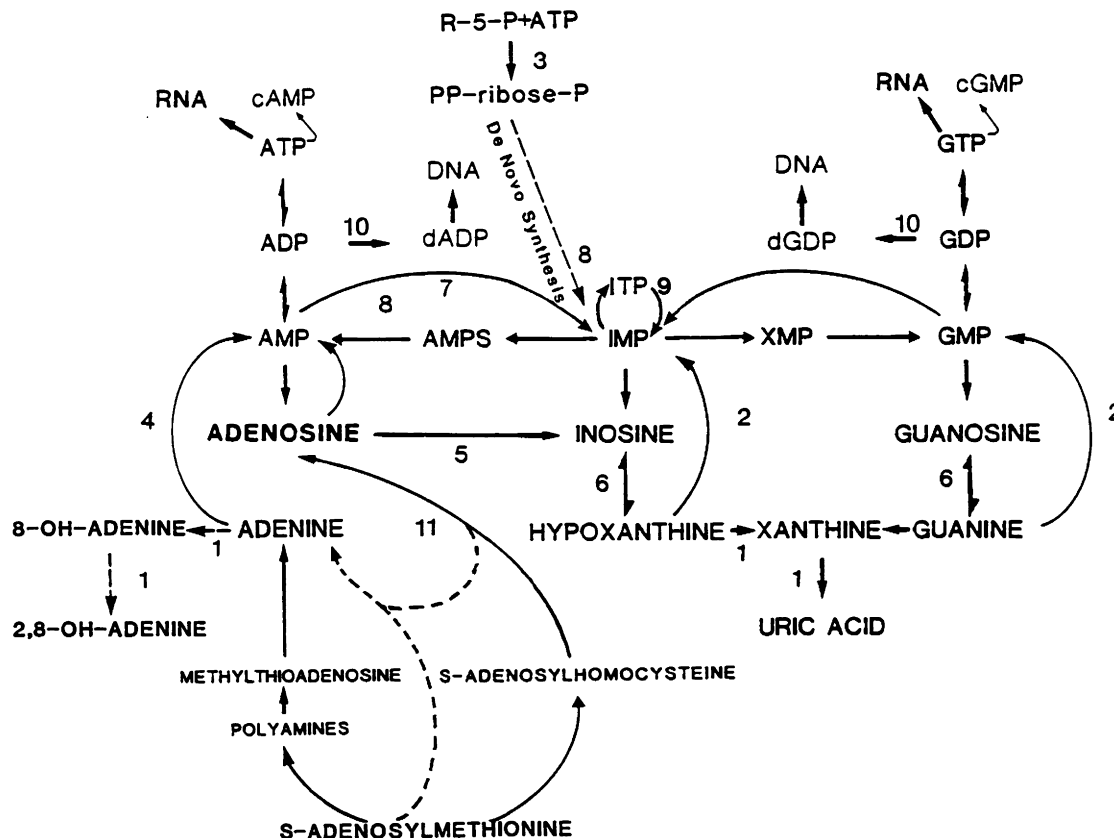


Figure 1 Purine metabolic pathways in man. The numbers refer to the following purine enzymes: 1: xanthine oxidase (XOD:EC 1.2.3.2); 2: hypoxanthine–guanine phosphoribosyltransferase (HGPRT:EC 2.4.2.8); 3: phosphoribosylpyrophosphate synthetase (PRPS:EC 2.7.6.1); 4: adenine phosphoribosyltransferase (APRT:EC 2.4.2.7); 5: adenosine deaminase (ADA:EC 3.5.4.4); 6: purine nucleoside phosphorylase (PNP:EC 2.4.2.1); 7: AMP deaminase (AMPDA:EC 3.5.4.6); 8: adenylosuccinate lyase (ASA:EC 4.3.2.2); 9: inosine triphosphate pyrophosphohydrolase (ITPase:EC 3.6.1.19); 10: ribonucleotide reductase (EC 1.17.4.1); 11: S-adenosylhomocysteine hydrolase (SAHH:EC 3.3.1.1). The dotted lines indicate the putative pathway involving SAHH, whereby SAM or adenosine could be converted to adenine and thence ATP.

defects with a proven genetic basis have been identified (Fig. 1), predominantly in the past two decades (reviewed in Simmonds, 1987, and various authors in Scriver *et al.*, 1989). The clinical consequences of these disorders may be life-threatening or benign and range from immunodeficiency, cerebral palsy and anaemia, to kidney stones and gout. The problem is mainly a paediatric one, but presentation in adult life is not uncommon.

This chapter focuses on those genetic purine enzyme defects associated with specific neurological deficits. The clinical manifestations may include generalized hypotonia, developmental retardation with compulsive self-mutilation (Watts *et al.*, 1982; Kelley and Wyngaarden, 1983), inherited nerve deafness (Simmonds, 1987; Becker *et al.*, 1988), muscle weakness (Fishbein, 1985; Sabina and Holmes, 1988), epilepsy and autistic behaviour (Jaeken and Van den Berghe, 1984), thereby underlining the importance of normal purine metabolism to motor development and function.

Disorders of purine salvage

Hypoxanthine–guanine phosphoribosyltransferase (HGPRT) deficiency

Hypoxanthine–guanine phosphoribosyltransferase (HGPRT) is an essential part of the so-called 'inosinate cycle' (Fig. 2a) and catalyses the transfer of the phosphoribosyl moiety of PP-ribose-P to hypoxanthine (or guanine) to form IMP, or GMP, respectively, with the highest enzyme activity being found in brain and testes (Kelley and Wyngaarden, 1983; Harkness *et al.*, 1988).

Two types of defect – complete or partial deficiency – exist: the complete deficiency, presenting in childhood as the Lesch–Nyhan syndrome, characterized by self-mutilation, choreoathetosis, spasticity and developmental retardation (Seegmiller *et al.*, 1967; Watts *et al.*, 1982), contrasts with the partial type generally presenting in adolescence with gout or urolithiasis (Kelley and Wyngaarden, 1983) due to the gross uric acid overproduction resulting from the enhanced purine synthesis when HGPRT (and consequently the inosinate cycle) is defective (Fig. 2b). Varying degrees of expression between these two extremes are now being recognized (Simmonds, 1987).

The molecular mechanisms in this defect have been the subject of detailed investigation. The HGPRT gene is located on the long arm of the X-chromosome and codes for a peptide containing 218 amino acids; the nucleotide sequence of the nine exons within a 44-kb stretch of genomic DNA has been determined. Cloning of mutant cDNA sequences from patients

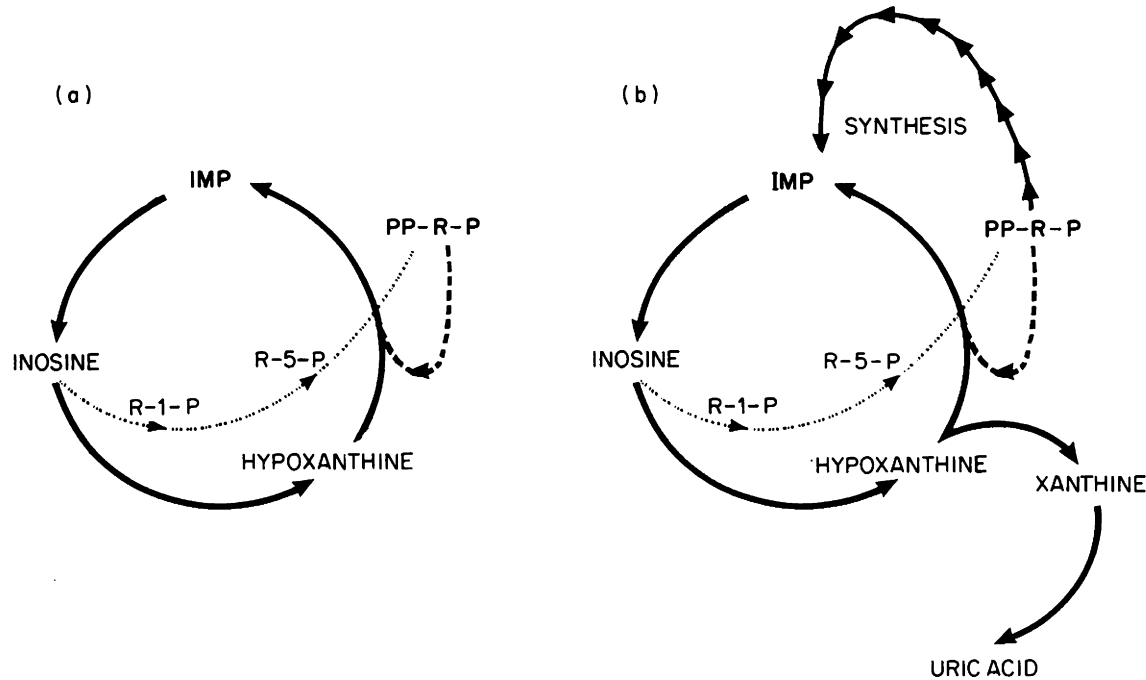


Figure 2 (a) The so-called 'inosinate cycle' involving the catabolism of IMP by 5'-nucleotidase to inosine which is then cleaved by PNP to ribose-1-phosphate and hypoxanthine. Hypoxanthine is subsequently salvaged by the enzyme HGPRT and recycled to IMP. The ribose-1-phosphate moiety is converted to ribose-5-phosphate which stimulates PPRP synthesis and is itself recycled. (b) The inosinate cycle and its role in the control of the ten-step *de novo* synthetic pathway, indicating the potential for uncontrolled purine synthesis and uric acid overproduction when one component of the cycle (HGPRT) is defective. Note that the ability to convert hypoxanthine to xanthine and uric acid is exclusive to the liver and intestinal mucosa in humans.

has succeeded in revealing point mutations, partial and complete gene deletions as well as an inversion and internal duplication (Davidson *et al.*, 1988; Caskey and Stout, 1989; Gibbs *et al.*, 1989).

Efforts to define the metabolic basis for the neurological findings have not met with similar success and treatment with a variety of medications has provided no sustained benefit (Watts *et al.*, 1982). A transgenic mouse model lacking HGPRT has proved disappointing in that it does not phenotypically reflect the Lesch–Nyhan syndrome, presumably because of an ability to utilize alternative pathways to sustain purine and neurotransmitter levels (Finger *et al.*, 1988). Brains of Lesch–Nyhan patients at autopsy have not revealed any specific morphological abnormalities, leading to the proposal that a reduced supply of ATP may be the central problem (Harkness *et al.*, 1988). Some workers have implicated a lower concentration of free amino acids, which could restrict the supply of precursors for protein or neurotransmitter synthesis (Rassin *et al.*, 1982; Harkness *et al.*, 1988). Others have speculated that the higher concentrations of purine nucleosides and bases found in Lesch–Nyhan brain might be sufficient to significantly affect benzodiazepine-modulated γ -aminobutyric acid (GABA) mechanisms (Marangos *et al.*, 1981; Kish *et al.*, 1985). Raised hypoxanthine CSF levels, suggesting local CNS production high enough to trigger seizures by interfering with diazepam receptor binding and GABA function, have also been proposed, as has abnormal monoamine metabolism, based on the altered levels of neurotransmitters in the CSF (Janovitch *et al.*, 1988; Scriver *et al.*, 1989).

The putative supersensitivity of the striatal dopamine D₁ receptor may be involved because the striatum normally contains HGPRT in high concentration. Impaired brain GTP synthesis – implied from the severe GTP depletion in the erythrocytes of Lesch–Nyhan patients (Simmonds *et al.*, 1988) and the fact that the erythrocyte, like the brain, is heavily dependent on salvage by HGPRT to sustain GTP levels (Allsop and Watts, 1985) – could severely restrict the supply of the GTP to the regulatory G proteins which normally interact with specific membrane receptors, such as the dopamine D₁ receptor and the A₁ adenosine receptor (Gilman, 1987; Stiles, 1988).

At present there is no successful treatment for the Lesch–Nyhan disorder. Bone marrow transplantation has been ineffective and the defect is a candidate for gene transfer experiments. Undoubtedly, the major difficulty will lie in ensuring expression of recombinant genes in the central nervous system (Ledley, 1987; Caskey and Stout, 1989).

Purine nucleoside phosphorylase (PNP) deficiency

PNP is the second enzyme of the 'inosinate cycle' discussed above (Fig. 2). It thus shares an equal importance with HGPRT in the overall control of

purine metabolism. Under physiological conditions PNP catalyses the degradation of the nucleosides inosine and guanosine and their deoxy-analogues to the corresponding bases, hypoxanthine and guanine respectively, which are then recycled by HGPRT. This deficiency is, in effect, a double disorder; although HGPRT activity is intact the enzyme cannot function without its substrates, hypoxanthine and guanine, normally provided by PNP (Simmonds, 1987).

In clinical terms this means the defect is associated directly with immunodeficiency and indirectly with CNS dysfunction. It is noteworthy that several years before PNP deficiency was identified in children with T cell immunodeficiency (Giblett, 1985), two kindreds were reported as having cerebral palsy coincidentally associated with defective cellular immunity (Hagberg *et al.*, 1970). Patients may present initially with head lag and excessive irritability at 3 months (Simmonds, 1987). They subsequently become susceptible to varicella, vaccinia or cytomegalovirus, and may die from such infections. The immunodeficiency is considered to relate to T cell-specific dGTP accumulation with subsequent inhibition of ribonucleotide reductase and DNA synthesis (Kredich and Hersfield, in Scriver *et al.*, 1989; Fairbanks *et al.*, 1990).

PNP deficiency resembles HGPRT deficiency in that the associated neurological abnormalities show a broad spectrum of presentation, the most severe occurring in patients with the complete enzyme deficiency (Simmonds, 1987). Severely deficient patients have remained extremely hypotonic and developmentally retarded and have manifest spastic tetraparesis. The neurological symptoms as well as gross purine overproduction – in this case in the

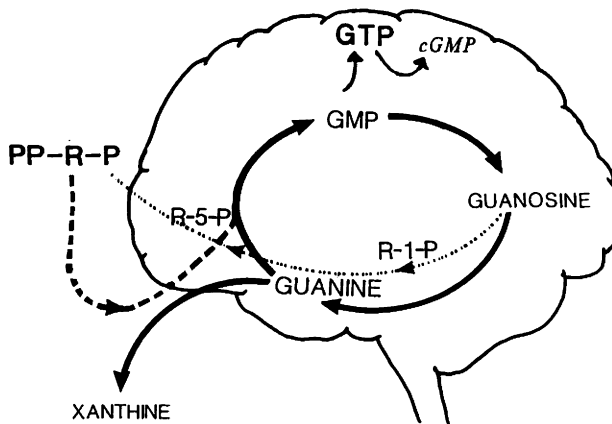


Figure 3 The proposed 'guanylate cycle' illustrating its putative importance to brain because of an unusual dependence on salvage – comparable to that in the human erythrocyte – for sustaining GTP at concentrations compatible with normal physiological function.

form of nucleosides inosine and guanosine and their deoxynucleoside analogues, with uric acid being absent (Stoop *et al.*, 1977) – are undoubtedly due to lack of a functional salvage cycle.

Severe erythrocyte GTP depletion, as in HGPRT deficiency, is also a facet of PNP deficiency and a similar mechanism has been proposed to account for the motor deficits in this disorder (Simmonds *et al.*, 1988). One interpretation of these combined results is that in addition to the 'inosinate cycle' an equally important cycle – the guanylate cycle – exists (Fig. 3), which is of particular significance to the human brain. Whether GTP depletion could also be a factor in the expression of the immunodeficiency in PNP deficiency is unknown, but the predominantly normal immune status of Lesch–Nyhan subjects would argue against this.

The purine nucleotide cycle

Myoadenylate deaminase (M-AMPDA) deficiency

Adenylate deaminase (AMPDA) catalyses the deamination of AMP to IMP (Fig. 4). The enzyme is normally subject to a variety of controls, being inhibited in some tissues by GTP and inorganic phosphate (P_i) and stimulated

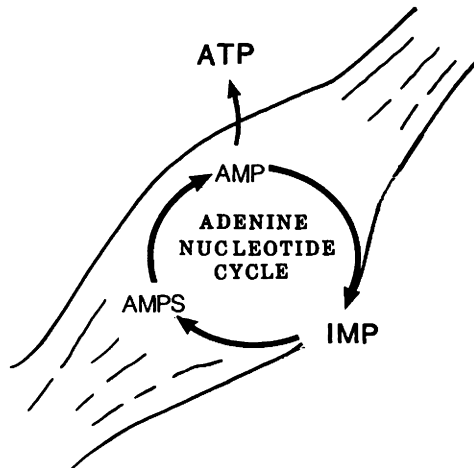


Figure 4 The adenine nucleotide cycle illustrating the importance to skeletal muscle of AMPDA acting in concert with AMPS synthetase and AMPS lyase; activity of the muscle-specific isoenzyme of AMPDA is an order of magnitude greater than in any other tissue.

by ATP (Lowenstein, 1972; Henderson, 1978). There are a number of tissue-specific isoenzymes of AMPDA (Ogasawara *et al.*, 1982), the heart isoenzyme having quite different controls from that in skeletal muscle (Purzycka-Preis and Zydowo, 1987).

In skeletal muscle, AMPDA activity is high and the enzyme is also considered to form part of an important cycle (Lowenstein, 1972) – the purine nucleotide cycle – involved in the interconversion of IMP and AMP (Fig. 4). In this cycle AMPDA acts in concert with adenylosuccinate synthetase (which requires GTP and aspartate), and adenylosuccinate lyase. During exercise, AMPDA activity increases, thereby augmenting local ammonia production and stimulating glycolysis. Fumarate produced in the second part of the cycle would likewise stimulate the citric acid cycle and energy production (Sabina and Holmes, 1988).

Studies of patients with muscle weakness or poor exercise tolerance have revealed AMPDA deficiency in up to 3% (Sabina and Holmes, 1988; Scriver *et al.*, 1989). The majority of these have presented with muscle cramps or myalgias following exercise and 67% had their first symptoms in childhood or adolescence (Fishbein, 1985). While muscle weakness occurred in 27%, muscle wasting did not, and hypotonia developed in only 8%. Discrepant findings may relate to the fact that the defect may be primary (inherited), or secondary to an associated neuromuscular disorder (Sabina and Holmes, 1988). The prognosis in most instances is good, with no evidence of progressive debilitation or structural damage. Ribose has been successful in some but not all patients (Zollner *et al.*, 1986).

The test used in diagnosis – the lactate/ammonia ratio during ischaemic exercise (Fishbein, 1985) – does not lead to NH_3 production, or the increase in plasma hypoxanthine and inosine (Sinkeler *et al.*, 1987) observed in normal subjects, and plasma adenosine has showed no significant changes. However, the adenosine content of end-exercise muscle samples was threefold higher in patients, a finding supported by studies showing adenosine production in muscle biopsies from AMPDA-deficient patients, but not controls (Swain *et al.*, 1984).

It is tempting to speculate that some of the clinical associates in this disorder relate not only to the diminished energy supply arising from the lack of a functional purine nucleotide cycle, but also the enforced degradation of AMP to adenosine (Fig. 1) when the normal catabolic route to IMP via AMPDA is deficient.

Adenylosuccinase deficiency

The enzyme involved in this instance is adenylosuccinate lyase, which catalyses the second step in the formation of AMP from IMP in the purine nucleotide

cycle (Fig. 4) and also the eighth step in the ten-step *de novo* synthetic pathway (Fig. 5). In the reaction involving the purine nucleotide cycle the substrate is adenylosuccinic acid (AMPS) and the product is AMP. The substrate for the *de novo* synthetic reaction is succinylaminoimidazole carboxamide ribotide (SAICAR); the product is aminoimidazole carboxamide ribotide (AICAR) with fumarate also being released in both instances.

This defect results in the accumulation of the two substrates for the enzyme in the form of their nucleoside derivatives – succinyladenosine (SAR) and SAICARiboside – in plasma, CSF and urine, where gross purine overproduction is evident (Jaeken and Van den Berghe, 1984). The defect has been demonstrated in liver, skeletal muscle and kidney, but not red cells or granulocytes, suggesting that, as with AMPDA deficiency, there may be several isoenzymes in human tissue. An autosomal recessive mode of inheritance has been suggested. Recent studies of the mutant human enzyme are consistent with a structural mutation in the adenylosuccinase gene (Barshop *et al.*, 1989).

Although the history at birth has been unremarkable, patients with this enzyme defect have presented in infancy with severe psychomotor retardation. Some have shown pronounced autistic features, with hypokinesia and inability to maintain eye contact. There was axial hypotonia with normal tendon reflexes and also some evidence of self-mutilation. Cerebellar hypoplasia was visible on CT scan. As yet, no treatment is available.

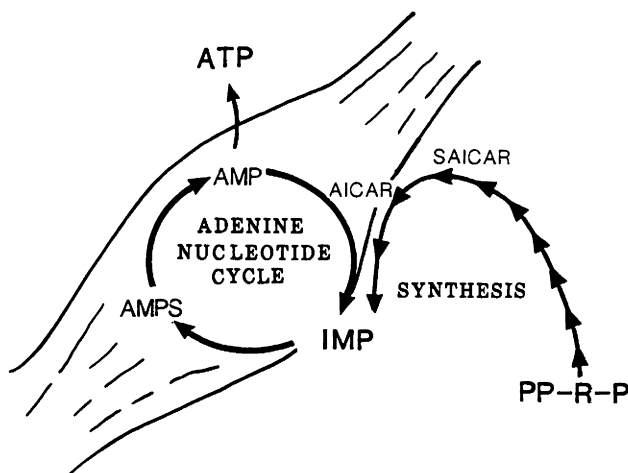


Figure 5 Relationship between the ten-step *de novo* synthetic pathway and the adenine nucleotide cycle, illustrating the potential for the accumulation of the seventh intermediate in this route, SAICAR as well as SAR, in AMPS-lyase deficiency and the eighth intermediate, AICAR, in the Lesch–Nyhan syndrome.

Recent investigations have failed to find any link between the psychomotor defects and possible interference by SAICAR and SAR with the cortical membrane-binding sites for adenosine (Vincent and Van den Berghe, 1989).

The *de novo* synthetic pathway

PP-Ribose-P synthetase (PRPS) superactivity

PP-Ribose-P synthetase (PRPS) catalyses the reaction of ribose-5-phosphate with ATP to form PP-ribose-P, an allosteric regulator of purine synthesis *de novo* (Fig. 1). The enzyme exists as a complex aggregate of up to 32 subunits, with only the 16 and 32 subunits having significant enzyme activity. A variety of molecular defects, both catalytic and regulatory, lead to reduced sensitivity to feedback inhibition, particularly by ADP and GDP, and overactivity under physiological conditions (Becker *et al.*, 1988) and increased purine synthesis (Fig. 2b).

The PRPS gene has recently been cloned and cDNA studies of the human synthetase gene have confirmed the existence of two distinct subunits: PRPS1 located in the Xq21-qter region of the X-chromosome just above the gene for HGPRT, and PRPS2 at a different region, namely Xpter-q21 (Taira *et al.*, 1989), together with two PRPS1-related genes, assigned to chromosomes 7 and 9 respectively.

PRPS superactivity was first recognized as the basis for the purine overproduction in adolescent males presenting with gout and urolithiasis (Sperling *et al.*, 1972). Affected individuals may be distinguished from HGPRT deficiency because HGPRT activity is normal. An association between the kinetic defect and the severity of the phenotypic expression, as in HGPRT deficiency, was suggested subsequently from the discovery of a small number of patients presenting neonatally with neurological deficits, including inherited deafness as well as uric acid overproduction (Simmonds, 1987) and a family (Becker *et al.*, 1988) with metabolic and neurologic abnormalities intermediate between these two extremes.

As with the Lesch–Nyhan syndrome, the metabolic basis for the neurological complications is not established. However, the findings of low erythrocyte GTP levels in patients with severe neurological deficits suggests a similar inability to sustain GTP at levels compatible with normal physiological function in the CNS. The additional finding of impaired conversion of nicotinamide to its mononucleotide may provide a metabolic basis for the low NAD levels in erythrocytes of severely affected cases (Micheli *et al.*, 1990).

There are several other enzyme deficiencies of the purine network, other than those involving the cycles discussed above, where the presence or absence of any neurological associates is of interest in the context of this chapter.

Xanthine oxidase deficiency

Xanthine oxidase (or xanthine dehydrogenase [XOD], since in human tissue NAD^+ is the electron acceptor) is a molybdenum-containing enzyme which normally catalyses the conversion of hypoxanthine to xanthine and xanthine to uric acid (Fig. 1). Activity is confined almost exclusively to the liver and intestinal mucosa in man (Simmonds, 1987), but the enzyme shows a widespread tissue distribution in other animal species. Xanthine, and to a lesser extent hypoxanthine, replace uric acid as the purine end-product in this defect. Up to 30% of patients with the single defect present with xanthine lithiasis. The remainder are generally asymptomatic, although myopathy has been reported in five cases.

Combined xanthine oxidase/sulphite oxidase deficiency

By contrast, combined XOD/sulphite oxidase (SO) deficiency is a potentially lethal disorder which generally presents as seizures in the neonatal period (Johnson *et al.*, 1988). In this combined defect xanthinuria is accompanied by the more severe clinical manifestations associated with SO deficiency, including ocular lens dislocation and neurological dysfunction involving developmental regression, ataxia and spastic paresis. The defect results from the absence of a common cofactor containing molybdenum and appears to relate to inability to synthesize the pteridyl moiety of the cofactor.

The recent description of a milder defect in a subject aged 17, with normal urinary levels of uric acid together with the raised xanthine and hypoxanthine levels, indicates genetic heterogeneity in the defect (Johnson *et al.*, 1988). Treatment from age 4 with a diet low in sulphur amino acids reduced the excretion of sulphur compounds, but although growth and mental development were satisfactory, motor impairment was not improved.

Dietary molybdenum restriction has led to an acquired enzyme deficiency, as evidenced by a report of the more serious neurological associates of the combined defect in a patient on total parenteral nutrition (Abumrad *et al.*, 1979).

Adenosine deaminase (ADA) deficiency

ADA catalyses the deamination of adenosine (Fig. 1) to inosine or 2'-deoxyinosine to 2'-deoxyadenosine (dAdo). The importance of ADA for

the catabolism of dAdo became evident with the identification of dAdo in body fluids (Simmonds *et al.*, 1978), and the finding of dATP in erythrocytes and lymphoid cells (Coleman *et al.*, 1978; Carson *et al.*, 1979). The lymphotoxicity is considered to relate to dATP accumulation in thymocytes and B cells, leading to inhibition of ribonucleotide reductase and DNA synthesis (Simmonds, 1987). Other mechanisms such as inactivation of vital cellular methylation reactions by dAdo directly, may be contributory (Hershfield, 1983, and in Scriver *et al.*, 1989).

ADA deficiency is responsible for 20–30% of cases with recessively inherited severe combined immunodeficiency (SCID). Affected infants are subject to recurrent infections and frequently present with diarrhoea, failure to thrive and candidiasis (Giblett, 1985). Severe cases present neonatally with no detectable lymphocytes in peripheral blood or bone marrow. However, the defect may not be suspected in later presenters where lymphocyte numbers may be normal (Morgan *et al.*, 1987).

This is an autosomal recessive disorder. The gene for ADA is encoded as a single 32-kb locus containing 12 exons (Wiginton *et al.*, 1986) on the long arm of chromosome 20 (20q 13.2-qter). Structural changes leading to an unstable ADA protein were considered to be responsible for some defects since mRNA was usually detectable in normal or supra-normal amounts. Specific point mutations and deletions have since been detected (Markert *et al.*, 1988).

Some patients have had associated bone and hair growth abnormalities. Neurological abnormalities have been present occasionally, but have been non-specific and attributed to a combination of malnutrition and viral infection. The accumulation of adenosine in plasma and urine in late presenters (Morgan *et al.*, 1987) suggests that chronic accumulation of this nucleoside has no effect on the regulatory functions attributed to adenosine *in vivo*.

2,8-Dihydroxyadenine (2,8-DHA) lithiasis and adenine phosphoribosyltransferase (APRT) deficiency

Adenine phosphoribosyltransferase (APRT) catalyses the salvage reaction in which adenine condenses with PP-ribose-P to form AMP (Fig. 1). Two types of defect, designated type I (found predominantly in Caucasians) and type II (found exclusively in Japan), have been identified (Simmonds, 1987, and in Scriver *et al.*, 1989). Type I stone-formers have no detectable enzyme activity in erythrocyte lysates, while type II have approximately 25% of normal APRT activity. Mutant APRT from subjects with the type I defect shows different mutations on each allele, while a single mutation appears to be responsible for the type II deficiency and results in a reduced affinity for PP-ribose-P (Hikada *et al.*, 1988; Kamatani *et al.*, 1989).

Both defects result in the inability to salvage adenine, which is then oxidized

by XOD to 2,8-dihydroxyadenine (2,8-DHA) (Fig. 1). Over-excretion of this extremely insoluble purine is generally associated with the formation of kidney stones. Acute renal failure has occurred in up to one-third of cases, resulting in permanent renal damage and progression to dialysis and transplantation (Simmonds, 1987). One such case presented in coma on several occasions and in addition to adenine and its metabolites, an unidentified adenosine-like compound was found in the urine (Greenwood *et al.*, 1982). However, specific association with the clinical symptoms was not proven.

Inosine triphosphate pyrophosphohydrolase (ITPase) deficiency

A deficiency of the enzyme ITPase has now been reported in North America, Sweden and the UK. It is associated with the accumulation of high levels of an unusual nucleotide, ITP in the erythrocytes (Duley *et al.*, 1990) and possibly other cell types. ITP accumulation in ITPase-deficient subjects assumes a cycle (Fig. 6), in which ITP is continuously synthesized and degraded at a relatively high rate. Although there is no evident physiological role for this seemingly futile cycle, its relationship with the inosinate cycle requires clarification. It has been suggested that the enzyme may be important for preventing the accumulation of unusual nucleotides such as dITP or XTP into DNA (Wang and Morris, 1974).

The complete deficiency does not appear to be lethal. An early report inferred that partial ITPase deficiency might be more frequent in mentally retarded populations, but this was not supported by larger population studies. A wide tissue distribution has been reported for ITPase in animal cells, with

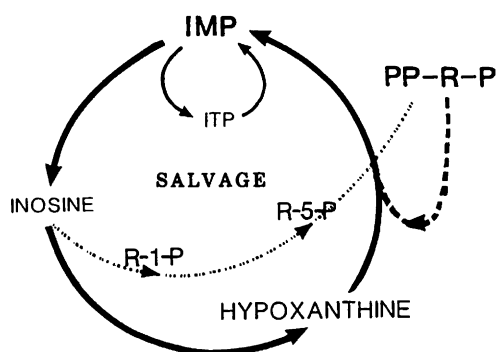


Figure 6 Diagram showing the inosinate cycle in the human erythrocyte, which lacks a functional adenine nucleotide cycle as well as the capacity to synthesize purines *de novo*, and depicting its relationship with the seemingly futile ITPase cycle which leads to the accumulation of high levels of ITP in ITPase deficiency.

the highest activity in brain and liver. Clinical significance for the complete deficiency, which apparently exists in all cell types, thus remains to be established.

S-Adenosylhomocysteine (SAH) hydrolase

SAH-hydrolase catalyses the hydrolysis of *S*-adenosylhomocysteine (SAH) which arises from the various vital cellular methylation reactions (Fig. 1) for which *S*-adenosylmethionine (SAM) is the methyl donor, SAH being a potent inhibitor of such reactions (Hershfield, 1983, and in Scriver *et al.*, 1989). A virtual lack of enzyme activity is found in erythrocytes in ADA deficiency. This is not a primary defect, but considered to be secondary to suicide inactivation of SAH-hydrolase by deoxyadenosine (Hershfield, 1983). A partial deficiency of erythrocyte SAH-hydrolase has also been found in PNP and in HGPRT deficiency and attributed to inactivation by inosine, either accumulating directly in PNP deficiency, or secondary to hypoxanthine accumulation in HGPRT deficiency. The gene coding for SAH-hydrolase is syntenic with ADA on the long arm of chromosome 20 (20cen-q13.1). To date no primary defect has been identified. However, the enzyme is of interest because of its central role in removing the products of cellular methylation reactions involving SAM.

Methionine adenosyltransferase (MAT) deficiency and the effects of SAM in depression and dementia

Reduced erythrocyte methionine adenosyltransferase (MAT) activity and low SAM levels have been reported in the CSF of severely depressed patients, including a group with Alzheimer's dementia (Bottiglieri *et al.*, 1991). The antidepressant effects of SAM have been reported by several groups. The beneficial effect has been attributed to an underlying disturbance of methylation associated with folate depletion within the nervous system. The mechanism of action on mood and behaviour is of interest in that it may aid in understanding the biological mechanisms involved in such disorders. Our own studies of SAM metabolism in the erythrocyte have demonstrated a beneficial effect of SAM in increasing ATP levels (Montero *et al.*, 1990) which, interestingly, occurs by a route not involving ADA or adenosine kinase. The mechanism appears to involve binding of this adenosine analogue to the adenosine-binding protein SAH-hydrolase (Fig. 1), with subsequent release of adenine and conversion to AMP by APRT. These results suggest that ATP depletion may be an additional, or alternative, factor in the associated clinical abnormalities and more work needs to be done in this area.

Conclusion

What can be learnt from these 'experiments of nature' in terms of understanding neurological abnormalities generally and the relationship of adenosine to this? The first interesting point is that apart from XOD/SO deficiency (which is really a disorder of sulphur metabolism), the genetic purine defects manifesting neurological deficits principally involve either the salvage and nucleotide interconversion cycles essential for sustaining intracellular ATP, cyclic AMP, GTP and cyclic GMP levels, or the *de novo* pathway essential for their synthesis (Figs 1 and 7).

The second is the curious fact that the one defect where adenosine has been demonstrated to accumulate (ADA deficiency) is not associated with neurological abnormalities. This would argue against any involvement of ADA in the normal regulatory functions of adenosine in the CNS, or alternatively that chronic adenosine accumulation blunts these responses.

The third interesting point is the low erythrocyte GTP levels in three separate disorders involved in the salvage cycle, which suggests that the ability to sustain GTP levels through guanine salvage may be peculiarly important to the human brain where, as in the erythrocyte, a heavy dependence on salvage results from a restricted ability to synthesize purines *de novo* (Fig. 7). Adenosine and other receptors interact with guanine nucleotide-binding proteins (G proteins) and recent data indicate that GTP influences the binding affinity of A₁ receptors for antagonists (Stiles, 1988). Lack of an effective salvage cycle in these genetic defects could thus restrict the supply of GTP to (a) membrane-bound G protein receptors that modulate vital signalling mechanisms; (b) several key enzymes controlling important purine pathways in the human CNS, such as the purine nucleotide cycle essential for ATP synthesis.

The fourth point is the involvement of the purine nucleotide cycle which has been reported to be particularly active in brain, as well as skeletal muscle (Lowenstein, 1972). In this regard it is interesting that accumulation of the abnormal nucleotide ZTP (formed from the *de novo* synthetic intermediate 5-amino-4-imidazole carboxamide riboside – AICAR) is, in bacterial systems, considered an 'alarmone' for folate deficiency consequent upon GTP depletion (Sidi and Mitchell, 1985). ZMP is itself an inhibitor of the second step of the conversion of IMP to AMP in the purine nucleotide cycle, the enzyme deficient in *S*-adenylosuccinase deficiency. Recent studies have demonstrated that AICAR enhances adenosine production during ATP catabolism (Gruber *et al.*, 1989). Both ZTP and AICAR accumulate in the Lesch–Nyhan syndrome (Fig. 5). Consequently, a cascade of secondary events could seriously compromise the CNS in a variety of ways not yet clearly understood.

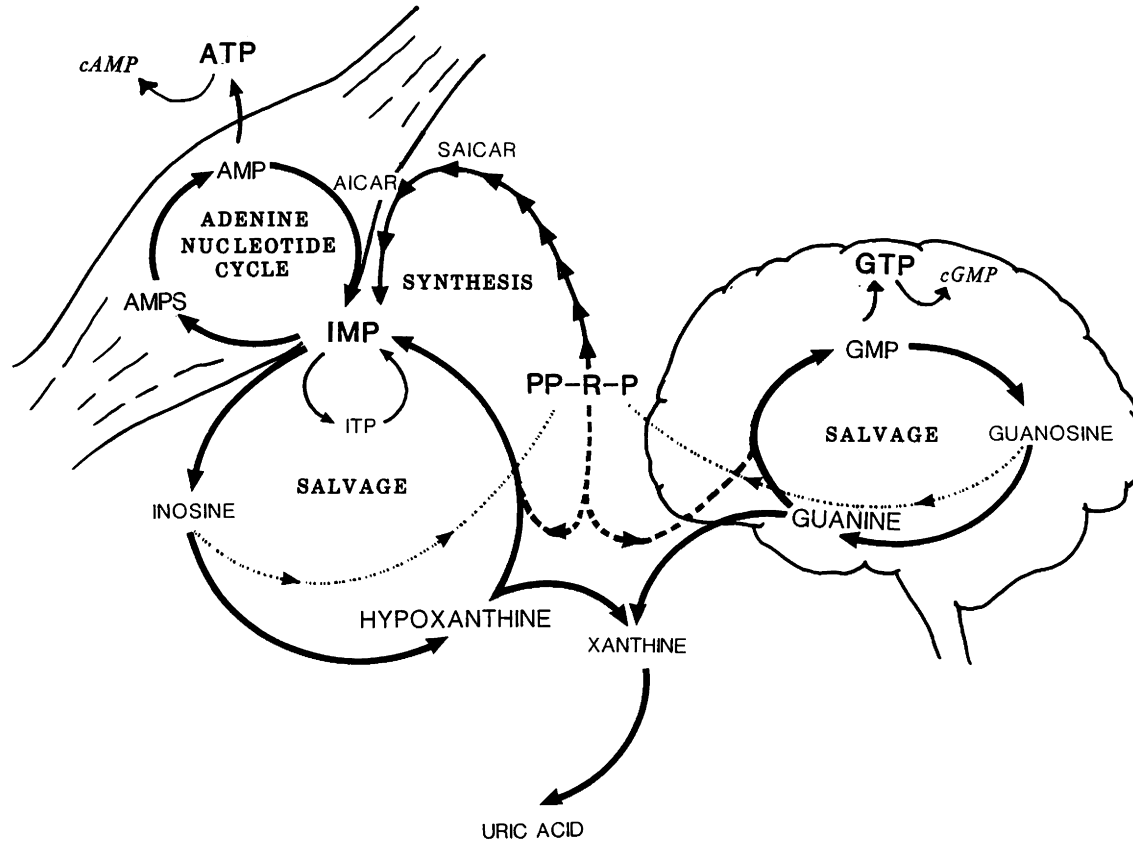


Figure 7 Model showing the interaction of the three routes involving purine synthesis, salvage and nucleotide interconversion and their suggested tissue-specific importance.

It is evident that functional purine metabolic pathways are of vital importance to the CNS. It is also obvious that our knowledge is scanty and much more needs to be done to establish the role of purines in the normal functioning of the nervous system in health as well as disease. More detailed study of these rare genetic metabolic diseases may be useful in achieving this objective.

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