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PHARMACOLOGY AND BIOCHEMISTRY OF ADENOSINE RECEPTORS

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ABSTRACT

Adenosine modulates a variety of physiological functions via membrane-bound receptors. These receptors couple via G proteins to adenylate cyclase and K^+-channels. The A_1 subtype mediates an inhibition of adenylate cyclase and an opening of K^+-channels, and the A_2 subtype a stimulation of adenylate cyclase. Both subtypes have been characterized by radioligand binding. This has facilitated the development of agonists and antagonists with more than 1000-fold A_1 selectivity. A_1-selective photoaffinity labels have been used for the biochemical characterization of A_1 receptors and the study of their coupling to adenylate cyclase. Such selective ligands allow the analysis of the involvement of adenosine receptors in physiological functions. Selective interference with adenosine receptors provides new pharmacological tools and eventually new therapeutic approaches to a number of pathophysiological states.

INTRODUCTION

Biological effects of adenosine were described in the late 1920s which included coronary vasodilation and negative chronotropic and dromotropic effects (ref. 1). Interest in the role of adenosine as a physiological regulator was stimulated in the 1960s by three separate observations. The first was the hypothesis that adenosine serves as a feed-back signal, coupling increased cardiac load to coronary vasodilation and hence increased O_2-supply (ref. 2). The second was the observation that certain compounds such as dipyridamole enhanced the effects of exogenously applied adenosine; this effect was found to be due to inhibition of adenosine uptake (refs. 3, 4). And the third was the postulate of specific receptors for adenosine which are coupled to the production of cAMP in rat brain (ref. 5). Methylxanthines appeared to antagonize the effects of adenosine at these receptors, and this antagonism was

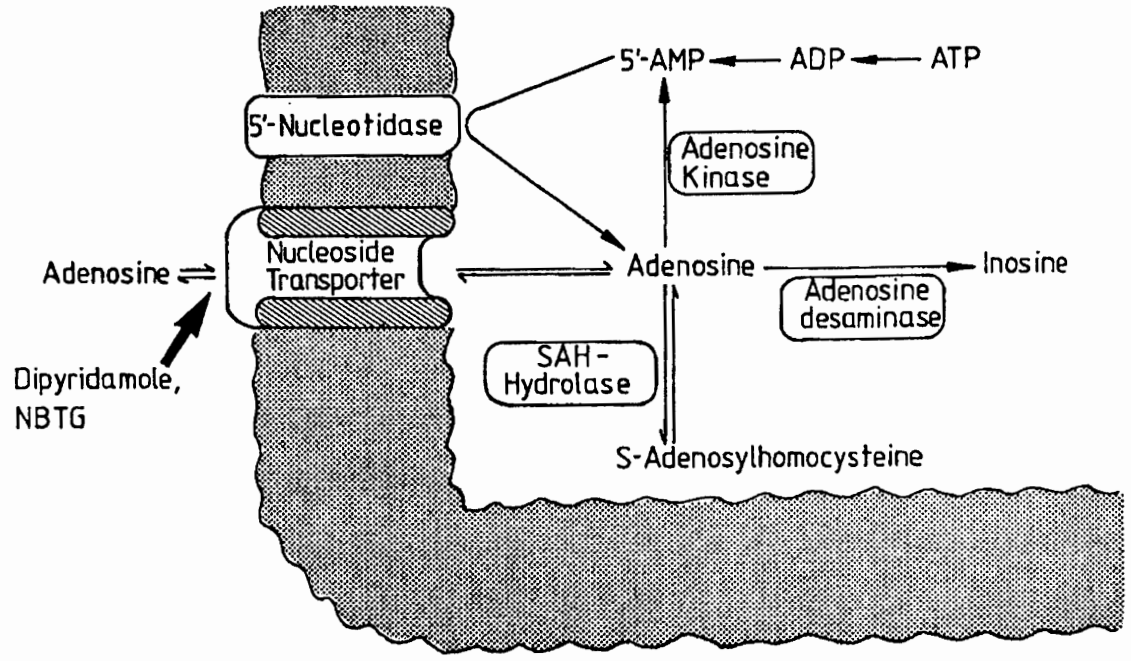

Fig. 1: Metabolism of adenosine

later postulated to represent the mechanism of the therapeutic effects of theophylline. These findings were the starting point for an intensive study of the physiological role of adenosine and its specific receptors.

SOURCES AND FUNCTIONS OF ADENOSINE

Adenosine exists in the intracellular space mainly in its phosphorylated forms, i.e. AMP, ADP, and most importantly ATP. Several enzymes keep the concentrations of intracellular adenosine in the range of 1 µM or below (see Fig. 1). These include the phosphorylation of adenosine to AMP by adenosine kinase, the deamination by adenosine deaminase, and the coupling to S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase. However, under circumstances of enhanced O_2-demand such as increased load of the contractile myocardium, or a reduced O_2-supply, increased amounts of adenosine are formed by the action of 5'-nucleotidase. Adenosine can pass the cell membrane by facilitated diffusion, which is a concentration-dependent bidirectional process (ref. 6). From the extracellular space adenosine can activate specific membrane-bound receptors. Inactivation of adenosine occurs mainly via uptake into cells by the nucleoside carrier and conversion as outlined above and

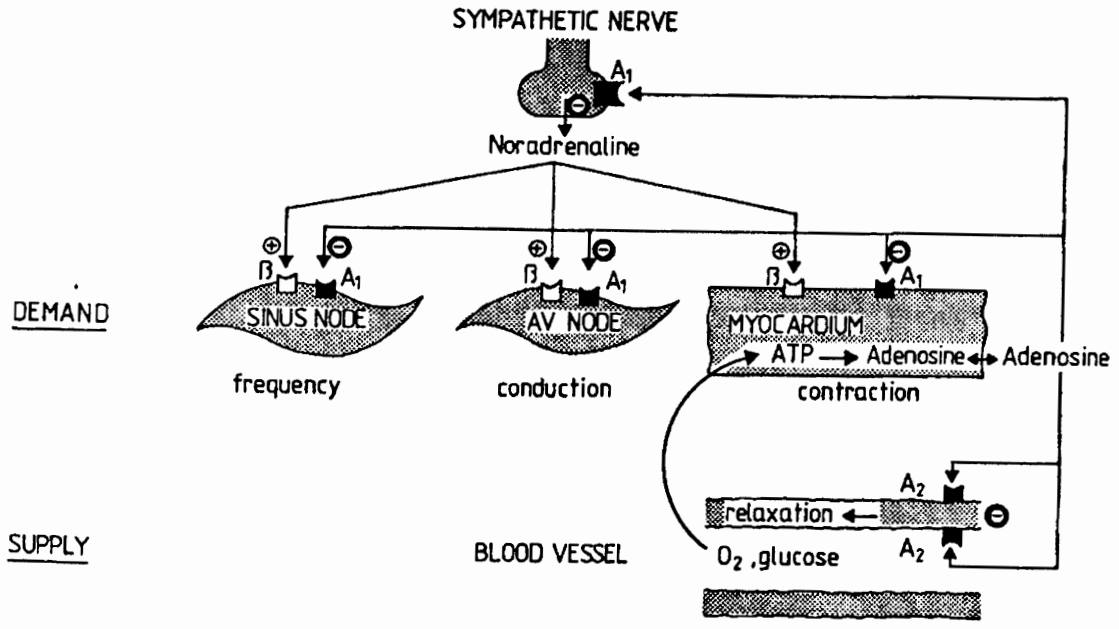

Fig. 2: Negative feed-back by adenosine in the heart

secondly via deamination to the biologically largely inactive derivative inosine.

The interaction of adenosine with its receptors causes a number of effects in a variety of organs. These effects constitute an inhibitory feed-back in many organs. This is nicely demonstrated by regarding the effects of adenosine in the heart (see Fig. 2): Adenosine production and release increase with excessive ATP-consumption. Adenosine reduces the heart rate by a direct effect on the sinus node, reduces AV-conduction, and it has negative inotropic effects both on the atrium and - at least in the presence of adrenergic stimulation - on the ventricle. In addition, it reduces the sympathetic drive on the heart by inhibiting the release of noradrenaline from the nerve endings. Likewise, adenosine inhibits the action of the central nervous system: postsynaptic hyperpolarisation and presynaptic inhibition of the release of a number of transmitters in combination result in the sedative and anticonvulsant properties of adenosine (ref. 7). Adenosine receptor antagonists, such as the methylxanthines, reverse these effects. Consequently, theophylline and coffeine increase heart rate and contractility, and stimulate the central nervous system.

CLASSIFICATION OF ADENOSINE RECEPTORS

The first evidence for the existence of different subtypes of adenosine receptors was presented by van Calker et al. (ref. 8) who demonstrated an

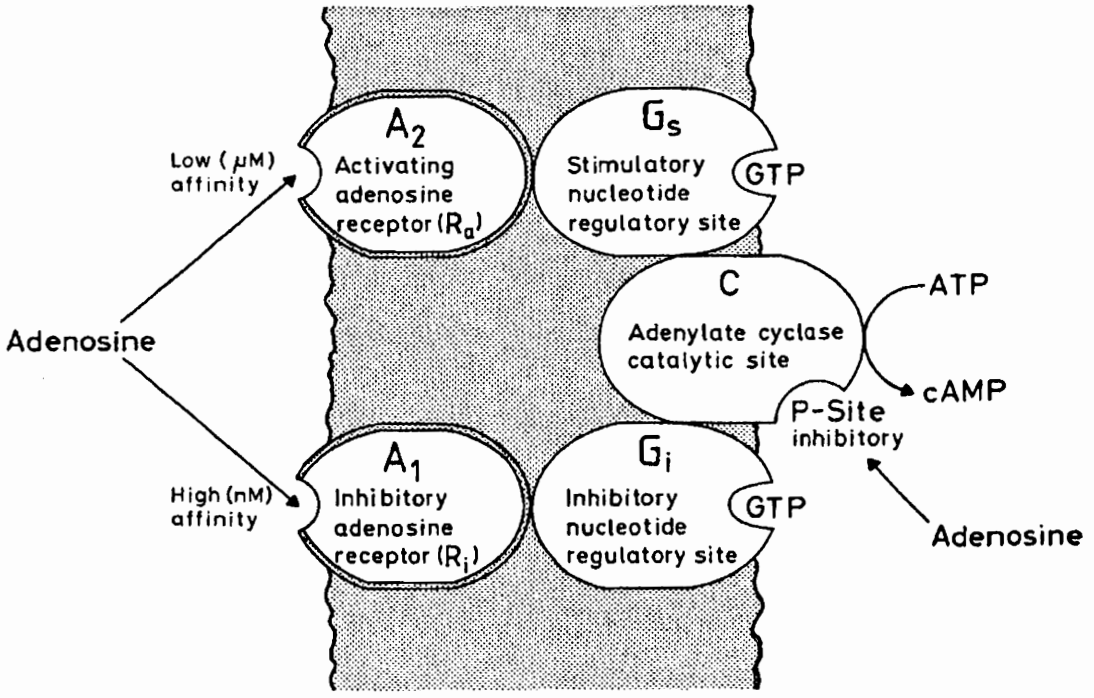

Fig. 3. Adenosine receptors

inhibitory effect of adenosine on cAMP-production in primary neuronal cultures. These authors proposed to term this inhibitory receptor A_1 in contrast to the A_2 receptor which mediates the stimulation of cAMP-production initially described by Sattin and Rall (ref. 5). A similar subdivision was proposed by Londos et al. (ref. 9), now with a terminology of R_i and R_a receptors, of which the R_i receptor inhibited adenylate cyclase in fat cells, and the R_a receptor stimulated the adenylate cyclase of liver and Leydig tumour cells. Londos et al. (ref. 9) also showed that the order of potency of adenosine analogs was different at the two receptor subtypes: whereas N^6-substituted derivatives such as R-PIA (R-N^6-phenylisopropyl-adenosine) was more potent than the 5'-substituted analogue NECA (5'-N-ethyl-carboxamidoadenosine) at the R_i receptor, the reverse was the case at the R_a receptor. In addition, the R_i receptor showed a marked stereoselectivity for the two isomers of PIA which was less pronounced at the R_a receptor. These two characteristics have served as the basis for the classification of the physiological effects of adenosine, although the A_1/A_2 terminology is now generally preferred.

In addition to the classical effects of adenosine receptors on adenylate cyclase, new coupling modes appeared to be emerging from the study of different effector systems. The classical scheme is that the adenosine receptors -

like many other adenylate cyclase coupled receptors - are coupled to adenylate cyclase via guanine nucleotide binding proteins (see Fig. 3). Thus, the A_1 receptor inhibits adenylate cyclase via the inhibitory protein G_i (or N_i), and the A_2 receptor stimulates adenylate cyclase via the stimulatory protein G_s (or N_s). However, several observations suggest that A_1 adenosine receptors may mediate effects independently from cAMP. First, adenosine receptors have been postulated to be directly coupled to ionic channels via guanine nucleotide binding proteins. There is now good evidence that an adenosine receptor with the pharmacological profile of an A_1 subtype leads to opening of a K^+-channel in the atrium (ref. 10). A direct inhibition of calcium currents by adenosine analogues has been postulated by Dolphin and coworkers (ref. 11). Secondly, adenosine has effects on the glucose transporter of fat cells which appear to be independent of cAMP and occur with a pharmacological profile of A_1 receptors, and it has been assumed that these receptors can also couple via a G protein to the glucose transporter (ref. 12).

A subdivision of A_2 receptors has been proposed from the comparison of a number of adenosine receptor ligands in enhancing cAMP-levels in fibroblasts and in competing for [^{3}H]NECA binding to membranes form corpus striatum (see below; ref. 13). An A_{2a} subtype with high affinity for NECA and other agonists is thought to be present in corpus striatum and possibly human platelets, and an A_{2b} receptor with lower affinity for NECA in other parts of the brain and in peripheral tissues. This proposal will need additional confirmation by studies of different tissues.

RADIOLIGAND BINDING STUDIES

Radioligand binding studies of the A_1 receptor were reported by a number of groups in 1980 (refs. 14-16). These studies have extended our knowledge of adenosine receptors and have largely confirmed the subclassification proposed from adenylate cyclase studies. In contrast to the large number of ligands available for the A_1 subtype, the development of radioligands for the A_2 receptor has been largely unsuccessful.

Radioligand binding studies of A_1 adenosine receptors have mainly relied on agonists, such as the N^6-substituted adenosine analogues [^{3}H]N^6-cyclohexyl-adenosine (ref. 14) and [^{3}H]R-N^6-phenylisopropyladenosine (ref. 15). Detection of A_1 receptors in tissues with low densities has become possible with the synthesis of radioiodinated agonists (ref. 17). Using these ligands, A_1 receptors have been demonstrated in numerous tissues, for example brain (refs. 14-16), fat cells (ref. 18), and heart (ref. 19). Apart from the simple demonstration of the presence of A_1 receptors, these radioligands have also allowed studies of their functional regulation. Thus, agonist binding was observed to be regulated by guanine nucleotides and divalent cations

(ref. 20), an observation that has been made for numerous G protein-coupled receptors (ref. 21). It is assumed that A_1 receptors per se have a low affinity for agonists, which is markedly increased upon coupling of the receptor to the G_i protein (ref. 22). The coupling between A_1 receptors and G_i appears to be particularly tight, since these two proteins remain associated even after solubilization with detergents (refs. 23,24).

The only antagonist radioligand for A_1 receptors available until recently was [^{3}H]1,3-diethyl-8-phenylxanthine ([^{3}H]DPX; ref. 14). Although this radioligand has the advantage of recognizing the receptor alone and receptor-G_i complexes with similar affinity (ref. 22), its low affinity has precluded its more general use. With the synthesis of selective high affinity antagonists this problem has been overcome (see below).

Binding studies to A_2 receptors have been more difficult. [^{3}H]NECA can be used as a radioligand for membranes of corpus striatum, when the A_1 component of [^{3}H]NECA binding is eliminated either by SH-modification of G_i (ref. 25) or with A_1-selective ligands (ref. 13). The residual binding has the pharmacology of the A_2 receptor.

The new antagonist [^{3}H]PD 115,499 appears to be another and probably more useful radioligand for the proposed A_{2a} subtype (ref. 26). This ligand does not appear to recognize A_{2b} receptors, and little binding was detected in tissues other than corpus striatum.

Similar approaches have not been successful in peripheral A_2 receptor-containing tissues. [^{3}H]NECA binding in liver (ref. 27) and human platelet (ref. 28) membranes appeared to occur largely to non-receptor sites. More recently, an A_1-selective xanthine amine congener 8-(4-[([([(2-aminoethyl)amino)carbonyl]methyl)oxy]phenyl)-1,3-dipropylxanthine ([^{3}H]XAC) has been used as a radioligand for A_2 receptors in human platelets (ref. 29). Although the binding showed the appropriate pharmacology of A_2 receptors, non-specific binding was still unacceptably high.

One possibility to overcome this problem is the separation of A_2 receptors from non-receptor binding sites. Such a separation can be obtained by solubilization and gel filtration of human platelet membranes. This allows studies of [^{3}H]NECA binding to A_2 receptors with acceptable non-specific binding (30%).

DEVELOPMENT OF SELECTIVE LIGANDS

During the past years considerable progress has been made in the development of A_1-selective ligands. On the other hand, there are still no compounds with appreciable A_2-selectivity; 2-phenylaminoadenosine has an affinity of about 100 nM for A_2 receptors in human platelet and striatal membranes and a 5- to 10-fold selectivity.

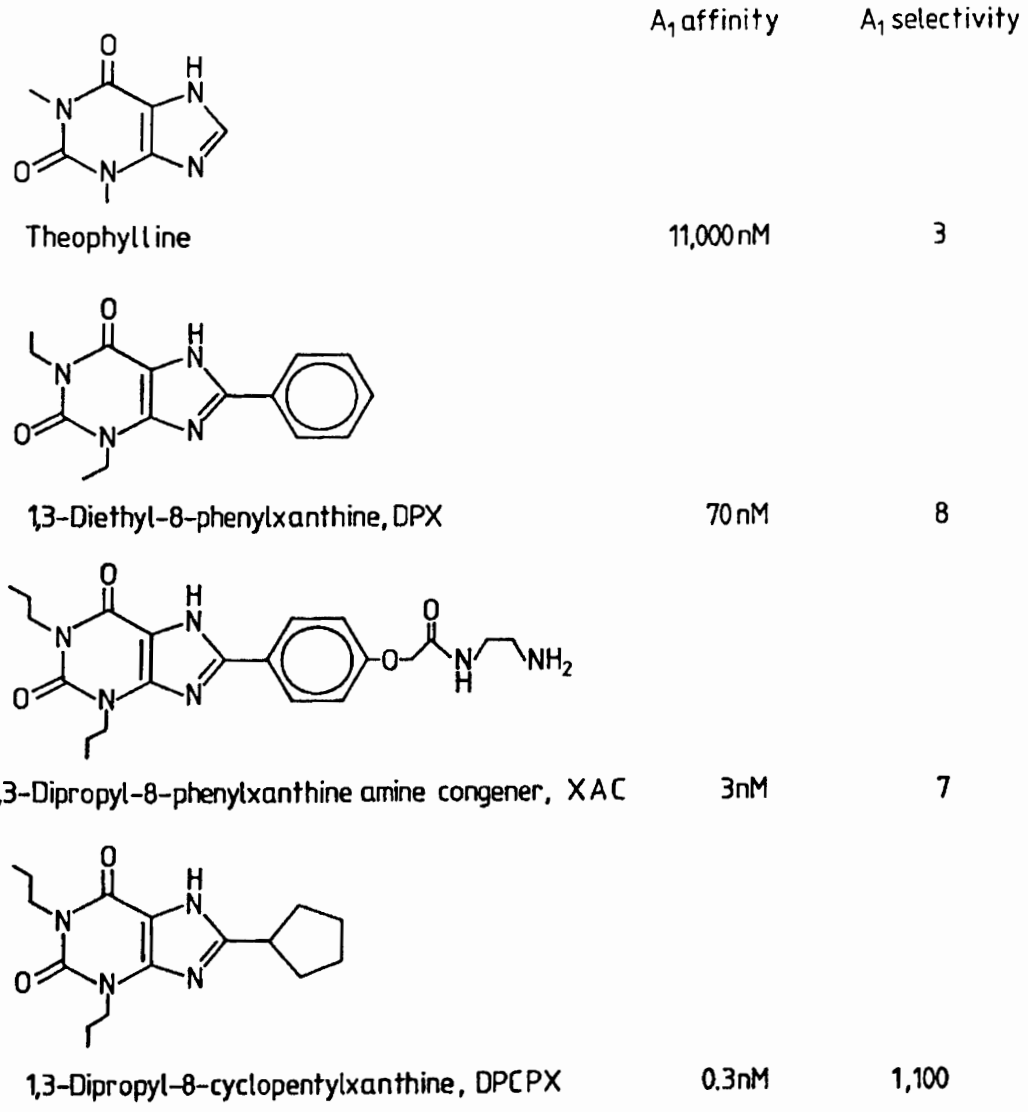

Fig. 4. A_1 receptor-selective antagonists .
K_i-values for the A_1 receptor were determined from [^{3}H]PIA binding to to rat brain membranes, K_i-values for the A_2 receptor from [^{3}H]NECA binding to rat striatal membranes (Data from ref. 34).

For the A_1 receptor, both selective agonists and antagonists have been developed. The synthesis of antagonists was based on two observations: A_1-selectivity of xanthines can be enhanced by alkyl-substituents in the positions 1 and 3 and by ring substituents in position 8. 1,3-Diethyl-8-phenylxanthine (DPX) was the first of such compounds (ref. 14) with a moderate affinity and A_1-selectivity (see Fig. 4). 8-(2-amino-4-chlorophenyl)-1,3-

TABLE 1
Properties of antagonist radioligands for A_1 receptors (Data from ref. 34).

Radioligand	Rat brain K_D(nM)	Nonspecific binding at K_D	Specific activity Ci/mmol
[^{3}H]DPX	68	40 %	13
[^{3}H]XAC	1.2	20 %	103
[^{3}H]DPCPX	0.18	1.3 %	105

dipropylxanthine (PACPX; ref. 30) had a much higher affinity and selectivity, but its lipophilicity limited a more general use. The same approach has been used for the synthesis of the "xanthine amine congener" XAC (ref. 31). Finally, a 8-cyclopentyl substituent leads to 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX; refs. 32-34). This compound combines a high affinity for A_1 receptors with an approximately 1000-fold selectivity (ref. 34). Its affinity for A_2 receptors is similarly low in platelet and striatal membranes.

Both XAC and DPCPX have been tritiated and used as radioligands for A_1 receptors in a variety of tissues (refs. 31, 33, 34). Table 1 demonstrates that of the 3 antagonist radioligands for A_1 receptors, [^{3}H]DPCPX has the most desirable properties: high affinity, selectivity and specific radioactivity together with low nonspecific binding. These properties allow radioligand binding studies not only with membranes but also with intact cells (see below).

A_1-selective agonists have been available relatively early. They are all N^6-substituted adenosine analogues. N^6-cyclohexyladenosine (CHA) and R-N^6-phenylisopropyladenosine (R-PIA) were the first radioligands successfully used for the identification of A_1 receptors (refs. 14-15). Both show already a marked A_1-selectivity as evaluated from binding studies (Table 2). Interestingly, as for the 8-position of xanthines, a cyclopentyl-substituent in the N^6-position leads to a very marked A_1-selectivity (ref. 35). Tritiated N^6-cyclopentyladenosine (CPA) has also been used as a radioligand for A_1 receptors (ref. 36). Derivatives of CPA appear to have an even higher A_1-selectivity.

PHOTOAFFINITY LABELLING OF A_1 RECEPTORS

Photoaffinity labels are ligands containing a photoreactive group, which upon UV-irradiation forms a covalent bond between the ligand and the protein to which the ligand was attached. Photoaffinity labelling has allowed the bio-

TABLE 2

A_1 receptor-selective agonists. Values were determined as in Fig. 4.

Adenosine analogue	A_1 affinity	A_1 selectivity
5'-N-ethylcarboxamidoadenosine	8.2 nM	3.3
R-N^6-phenylisopropyladenosine	1.3 nM	375
N^6-cyclohexyladenosine	1.4 nM	535
N^6-cyclopentyladenosine	0.8 nM	1400

chemical characterization of a number of membrane-bound receptors. Starting from A_1-selective N^6-substituted adenosine analogues, photoaffinity labels have been synthetized for the A_1 receptor (refs. 37-38). R-2-azido-N^6-hydroxy-phenylisopropyladenosine (R-AHPIA) can be incorporated with 30-40% yield into A_1 receptors of brain membranes. The compound can easily be radioiodinated to ^{125}I-AHPIA, which is a high affinity (2 nM) photoreactive radioligand for the A_1 receptor. Labelling of rat brain membranes with ^{125}I-AHPIA followed by SDS-polyacrylamide gel electrophoresis gives a specifically labelled band with an apparent molecular weight of 35,000. Labelling can be inhibited by several compounds with a pharmacological profile typical for the A_1 receptor, and is modulated by guanine nucleotides. Reducing agents such as DTT do not alter the electrophoretic pattern. These data indicate that the binding subunit of the A_1 receptor is a monomeric protein with an apparent molecular weight of 35,000, which may represent either a part of or the whole A_1 receptor.

Treatment of the photoaffinity labelled A_1 receptor with neuraminidase leads to an increase of its electrophoretic mobility, indicating the presence of carbohydrate residues with terminal sialic acids (ref. 39). Total deglycosylation can be otained with enzymatic (ref. 40) or chemical methods (ref. 39); this gives a core protein with a molecular weight of 32,000. Although the native receptors from different tissues have different apparent molecular weights, this difference disappears after total deglycosylation. These data indicate that the A_1 receptor contains complex-type carbohydrate chains, which may vary from tissue to tissue.

ADENOSINE RECEPTOR-ADENYLATE CYCLASE COUPLING

Two techniques have recently enlarged our knowledge of the coupling of adenosine receptors: the first was the use of agonist photoaffinity labelling for functional studies (ref. 41) and the second the development and use of radioligands for binding studies in intact cells (ref. 34,41). These methods

TABLE 3
Binding and effects of the A_1 agonist R-PIA in intact cells.

Cell type	Receptor number	Binding	Effect
	B_{max} fmol/mg membrane protein	[^{3}H]DPCPX inhibition K_i (nM)	cAMP inhibition IC_{50} (nM)
Cardiomyocytes	20	60	60
Fat cells	600	73	1.2

have allowed a comparison of receptor occupancy with cAMP-responses.

The covalent incorporation of the photoaffinity agonist R-AHPIA into A_1 receptors leads to their persistent activation (ref. 41). This can be seen by a persistent reduction of cAMP-levels of isolated fat cells (which contain a high number of A_1 receptors) or a persistent inhibition of adenylate cyclase in membranes. The fact that a covalently bound agonist produces constant activation is a direct demonstration of the validity of the occupancy theory of receptor activation as developed by Clark (ref. 42). This theory predicts that a receptor is activated as long as it is occupied by an agonist, whereas the rate theory (ref. 43) assumes that receptor activation occurs only at the very moment of agonist binding to the receptor. Interestingly, only a small proportion of receptors needs to be occupied in order to produce an effect. For example, occupation of 5% of the A_1 receptors of isolated fat cells with R-AHPIA reduces the cAMP-levels by 50%.

This receptor reserve can also be observed by comparing agonist effects on intracellular cAMP with radioligand binding to intact cells. [^{3}H]DPCPX and - with some limitations - [^{3}H]PIA can be used to label A_1 receptors in intact cells such as fat cells or cardiomyocytes. Antagonists compete for this binding with the same affinity as in membranes. Agonists, however, have low affinities for A_1 receptors in intact cells, and these affinities agree well with those of the low affinity state in membranes, which probably represent the A_1 receptor uncoupled from the G_i protein. In cells with low receptor densities, such as cardiomyocytes, half maximal inhibition of binding and half maximal effect occur at the same concentration (Table 3). In cells with high receptor densities, such as fat cells, effects occur at much lower concentrations than binding. This shows a receptor reserve in tissues with high receptor numbers.

Using similar techniques, a receptor reserve can also be demonstrated for

A_2 receptors of human platelets. Different receptor reserves in different A_1 and A_2 receptor-containing tissues mean that effects mediated by these receptors can occur at markedly different concentrations of agonists. Consequently, the selectivity of an agonist as determined from its binding affinity may markedly differ from its selectivity in intact tissues.

PHYSIOLOGICAL ROLE OF ADENOSINE RECEPTORS

The development of subtype-selective ligands - even with the cautions mentioned above - allows the investigation of the involvement of adenosine receptors in physiological functions.

Classically, the evaluation of the type of adenosine receptor mediating a given effect has been performed by examing the order of potency of adenosine agonists as originally described for adenylate cyclase experiments (ref. 9). Thus, a high degree of stereospecificity plus a higher potency of R-PIA than NECA are taken as evidence for an A_1 receptor, and the reverse as evidence for an A_2 receptor. However, this approach has several drawbacks: first, the presence of spare receptors alters the concentrations of agonists required to elicit effects (see above); this is particularly true for A_2 receptors where most adenosine analogues are only partial agonists (ref. 37). Secondly, pharmacokinetic differences may obscure the order of potency at the site of action. Thus, it has often been observed that NECA was equipotent with R-PIA in apparently A_1-mediated effects. Consequently, the use of selective antagonists and of highly selective ligands is preferable to the classical use of NECA and the isomers of PIA. Proposals for the use of more selective ligands have, for example, been made by Bruns et al. (ref. 44).

Since selective ligands have been synthetized only for the A_1 receptor, A_2 receptor-mediated effects can be investigated only indirectly, i.e. by the absence of effects of A_1-selective ligands. For example, DPCPX antagonized the decrease in heart rate by R-PIA in isolated rat hearts, but did not alter the increase in coronary flow caused by R-PIA (ref. 45). This suggests that A_1 receptors mediate the reduction of heart rate, and A_2 receptors coronary vasodilation. However, R-PIA was almost equipotent in the two effects, underlining again the difficulty of receptor classification with agonists.

A_1 adenosine receptors can be visualized in the renal cortex by autoradiography with A_1-selective ligands. They are mainly associated with the periglomerular space (ref. 46). One of the most prominent effects of adenosine in the kidney is the regulation of renin release (ref. 47). CPA inhibits renin release at low concentrations, and DPCPX readily reverses this inhibition. This indicates that adenosine inhibits renin release via A_1 receptors.

Neurotransmitter release from many central and peripheral synapses is inhi-

TABLE 4
Physiological effects of adenosine

TISSUE	EFFECT	RECEPTOR
NERVOUS SYSTEM		
peripheral	transmitter release ↓	A_1
central	neuronal firing ↓	A_1
HEART	heart rate ↓	A_1
	AV-conduction ↓	A_1
	contractility ↓	A_1
KIDNEY	renin release ↓	A_1
	renin release ↑	A_2
	vasoconstriction	A_1
	vasodilatation	A_2
SMOOTH MUSCLE		
blood vessels	relaxation	A_2
trachea	relaxation	A_2
taenia coli	relaxation	A_2
PLATELETS	antiaggregatory	A_2
FAT CELLS	antilipolytic	A_1

bited via A_1 receptors (ref. 7). Such an inhibition of neural transmission can be measured electrophysiologically in the hippocampal slice (ref. 48). In this model the stratum radiatum efferents are electrically stimulated and the population spike of the CA_1 neurones is recorded. A_1-selective agonists cause a marked depression of the population spike amplitude, and at higher concentrations completely abolish the spike. DPCPX antagonizes this effect, and given alone causes an enhancement of the spike amplitude. This indicates that neural transmission in the hippocampus is under the tonic inhibition of A_1 receptors.

A number of physiological effects of adenosine have been attributed by these and similar experiments to one of the adenosine receptor subtypes. Table 4 gives an overview of the most important of these effects. Possible therapeutic effects of adenosine receptor ligands can be derived from the physiological effects mediated by adenosine receptors (Table 5). These include modification of cardiac, nervous and vascular functions. Adenosine receptor antagonists such as theophylline are already long-established drugs in the treatment of bronchial asthma and infant apnea. Whereas the site of action in the treatment of bronchial asthma is still uncertain (ref. 49), it is likely

TABLE 5

Possible therapeutic properties of adenosine receptor ligands

A_1 RECEPTOR	
Agonists	Antagonists
CNS: sedation	CNS: stimulation
Inhibition of noradrenaline release	Treatment of infant apnea
Inhibition of renin release	Renal vasodilatation
Treatment of hypertension	Treatment of renal failure
Inhibition of AV-conduction	Facilitation of AV-conduction
Treatment of supraventricular tachycardia	Treatment of AV-block
A_2 RECEPTOR	
Agonists	Antagonists
Vasodilatation	Vasoconstriction
Inhibition of platelet aggregation	

that the beneficial action of theophylline in infant apnea is due to a blockade of central A_1 receptors. The inhibition of AV-conduction by adenosine has already been used in the treatment of supraventricular tachycardia (ref. 50).

The evaluation of the therapeutic potential of adenosine receptor ligands for other conditions will require further studies. The development of subtpye-selective ligands has now opened the way for the investigation of these therapeutic approaches.

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