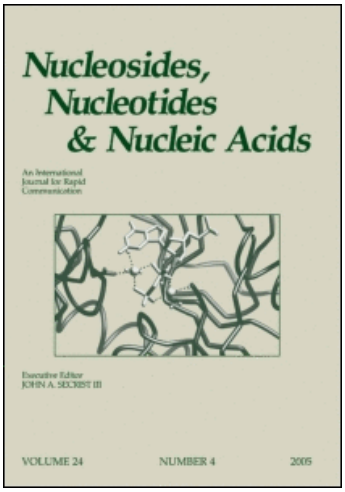

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Adenosine Receptor Classification: *Quo Vadimus*?

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ADENOSINE RECEPTOR CLASSIFICATION: *QUO VADIMUS?*

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CONTENTS

1. Introduction

Considerable progress has been made in our understanding of the diversity of adenosine receptors during the last decade, with the cloning of the orphan receptors RDC7 and RDC8 (1), and their subsequent characterisation as canine A_1 and A_2 receptors respectively (2,3), in the late 1980s. The principal objective of this review is to produce an integrated view of adenosine receptor classification, using the important observations from studies of molecular biology, receptor binding characteristics and functional pharmacology.

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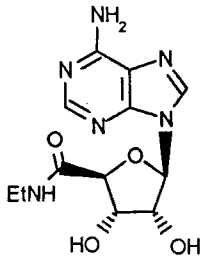
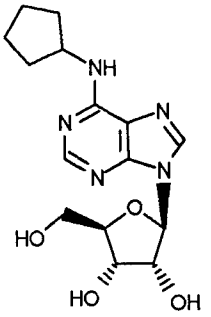
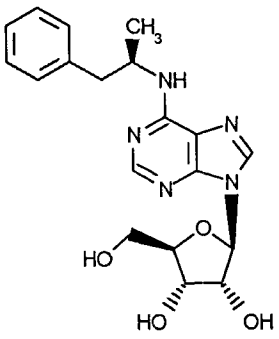


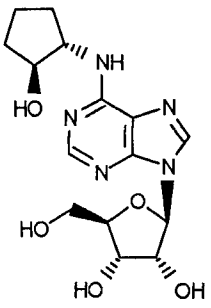
NECA



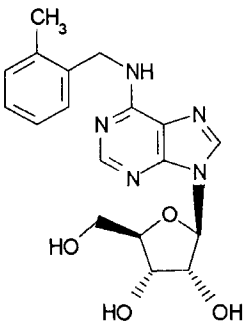
CPA



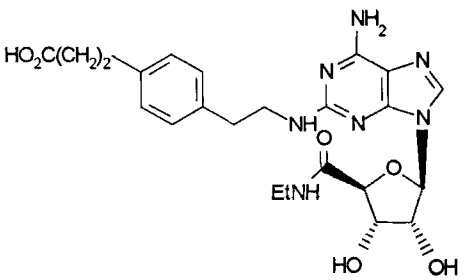
R-PIA



GR79236



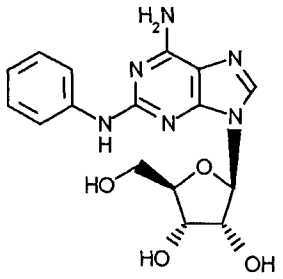
Metrifudil



CGS21680

(p.t.o)

Figure 1: Adenosine receptor agonists used in the characterisation of adenosine receptors.



CV1808

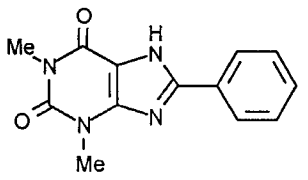
Abbreviations: NECA = 5'N-ethylcarboxamidoadenosine; CPA = cyclopentlyadenosine; PIA = phenylisopropyladenosine; GR79236 = N[(1S,*trans*)-2-hydroxycyclopentyl]adenosine; Metrifudil = N-[(2-methylphenyl)methyl]adenosine; CGS21680 = [[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-N-ethylcarboxamidoadenosine: CV1808 = 2-(phenylamino)adenosine

Figure 1. Continued

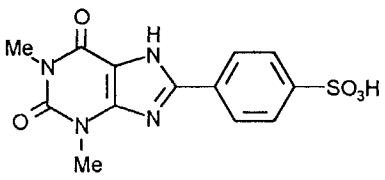
The structures of key compounds used in the functional classification of adenosine receptors, together with the abbreviations used throughout this review, are shown in FIG 1 and 2.

2. *Historical Perspective*

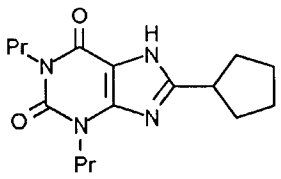
Although the biological actions of adenine nucleosides and nucleotides were first described by Drury and Szent Gyorgi (4), it was Burnstock (5) who developed the concept that specific receptors for these substances existed. From an extensive review of the literature, Burnstock (5) proposed that two types of receptor, which he termed P_1 and P_2, could be distinguished functionally using three criteria: (i) rank order of agonist potency, (ii) effects of antagonists and (iii) effects on adenylate cyclase activity and prostaglandin formation. Thus, P_1 receptors were characterised by the rank order adenosine > AMP > ADP > ATP, whilst for P_2 receptors the rank order was proposed to be ATP > ADP > AMP > adenosine. Furthermore, P_1 receptors were reported to be blocked by xanthines such as caffeine and theophylline whilst P_2 receptors were not. Finally, P_1 receptor activation was suggested to modulate adenylate cyclase activity, whilst P_2 receptor activation was linked to prostaglandin formation. Whilst the first two criteria have proved valuable for receptor classification, it is now clear that P_1 receptor activation is not always linked to adenylate cyclase (see below) and that P_2 receptor activation does not always result in prostaglandin formation. This concept has now been extended by the suggestion that a novel prejunctional purinoceptor exists which is activated by both adenosine and ATP: this receptor has been termed "P_3" (6,7)



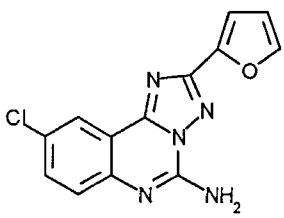
8-PT



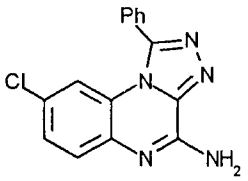
8-SPT



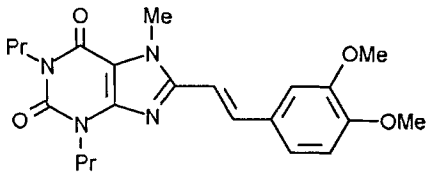
DPCPX



CGS15943A



CP66713



KF17837

Abbreviations: 8-PT = 8-phenyltheophylline; 8-SPT = 8-sulphophenyltheophylline; DPCPX = 1,3-dipropyl-8-cyclopentyl xanthine; CGS15943A = 9-fluoro-2-(2-furyl)-5,6-dihydro [1,2,4]triazolo{1,5-c}-quinazin-5-imine; CP66713 = 4-amino-8-chloro-1-phenyl[1,2,4]triazolo[4,3-αquinoxaline; KF17837 = (*E*)-1,3dipropyl-7-methyl-8(3,4-dimethoxyphenyl)xanthine.

Figure 2 Adenosine receptor blocking drugs used in the characterisation of A_1 receptors.

Shortly after the publication of this work, two groups independently provided evidence for the existence of two subtypes of adenosine receptor. Van Calker *et al.* (8) studied the effects of a range of adenosine analogues on cyclic AMP formation in cultured glial cells from the mouse. N^6-substituted adenosine analogues, such as PIA (FIG 1), were found to be more potent than adenosine as inhibitors of cyclic AMP formation, whereas the converse was true for stimulation of cyclic AMP formation. These workers therefore proposed that the opposing effects were mediated by different receptors, suggesting that the receptor mediating inhibition be termed A_1, and that mediating stimulation, A_2. Further

evidence for the existence of two receptors was provided by the work of Londos *et al.* (9). Here the effects of adenosine, PIA and another analogue, NECA (FIG 1), on adenylate cyclase activity in rat liver cells, I-10 Leydig cells and rat adipocytes were studied. Londos *et al.* also examined the effects of these compounds as stimulators of steroidogenesis in I-10 cells and as inhibitors of lipolysis in adipocytes. Two patterns of activity were apparent. A rank order of agonist potency of NECA > PIA > adenosine was obtained for stimulation of adenylate cyclase activity in liver and I-10 cells and of steroidogenesis in I-10 cells, and the receptors mediating these responses were termed R_a (to indicate activation of adenylate cyclase). Conversely, the order of potency for inhibition of adenylate cyclase and of catecholamine-induced lipolysis was PIA > NECA > adenosine, and the receptor mediating this effect was termed R_i (to indicate inhibition of adenylate cyclase) It is now generally accepted that R_i corresponds to the A_1 receptor, and R_a corresponds to the A_2 receptor. The A_1/A_2 nomenclature is presently the accepted convention, since it carries no implication with respect to second messenger (see 10).

Another study supporting the existence of two types of adenosine receptors was that of Smellie *et al.* (11). Here the R-enantiomer of PIA was found to be more potent than the S-enantiomer as an inhibitor of excitatory junction potentials in rat hippocampus, whereas there was a much smaller difference in potency between the two enantiomers as stimulants of cyclic AMP formation in this preparation.

These studies led to a semiquantitative definition of A_1 and A_2 receptors using rank orders of agonist potency as follows:

A_1 receptors: R-PIA > NECA > S-PIA & adenosine; R-PIA > S-PIA

A_2 receptors. NECA >R-PIA ≥ S-PIA & adenosine; R-PIA ≥ S-PIA

R-PIA was therefore classified as a selective A_1 receptor agonist. In addition, some workers considered NECA to be a selective A_2 receptor agonist. However, Londos *et al.* (9) did not claim this, and subsequent work (12, 13), has shown that the compound does not differentiate between A_1 and A_2 receptors.

The identification of adenosine receptor antagonists which are useful in the classification of adenosine receptors has proceeded much more slowly than that of agonists, and consequently advances in adenosine receptor classification derived from binding and functional studies initially largely depended upon studies with selective agonists. However, in recent years progress has been made in the development of selective antagonists. The prototype adenosine antagonists were the methylxanthines caffeine and theophylline (5), compounds which have a low affinity for adenosine receptors (pA_2 about 5), and demonstrate a wide range of unrelated actions, notably inhibition of cyclic nucleotide phosphodiesterases. Smellie *et al.*(14) were the first to demonstrate that substitution in the

8-position, notably in the case of 8-PT (FIG 2), could enhance potency at adenosine receptors and selectivity versus inhibition of phosphodiesterase(s). This compound has been widely used as an adenosine antagonist, although it does not differentiate between A_1 and A_2 receptors (15). Subsequently a number of other 8-substituted alkylxanthines have been shown to be selective for A_1 receptors, notably DPCPX (see below; FIG 1).

The cloning of the canine A_1 and A_2 receptor cDNAs was a major advance in our ability to investigate molecular diversity among adenosine receptors, and was achieved by a degenerate PCR approach using oligonucleotides based on six G-protein-coupled receptor sequences available at that time (1). Clones RDC7 and RDC8, identified from canine thyroid gland RNA, represented a novel class of 7 transmembrane spanning receptors, possessing very short amino terminal extracellular domains devoid of potential N-linked glycosylation sites. In addition, an aspartate residue, which is conserved in TM III of receptors for biogenic amines and is critical for ligand binding, was missing (1, 16). Subsequent studies of the distribution of the mRNA for these receptors and pharmacological analyses of the cloned receptors, expressed in a variety of cells, confirmed their identity as adenosine A_1 and A_2 receptor genes respectively. (3, 17). Furthermore, the A_1 and A_2 genes have been localised to the long arms of human chromosomes 22 and 11 respectively by *in situ* hybridisation (18). Since this clear demonstration that A_1 and A_2 subtypes are encoded by independent genes, having very different patterns of tissue specific expression, similar degenerate PCR-based and homology cloning approaches have been used to isolate cDNAs for these, and further related subtypes, from other species (discussed in more detail below: summarised in TABLE 1).

3. *Adenosine A_1 receptors*

Characterisation.

Following the work of Van Calker and co-workers and Londos *et al.* (8, 9), efforts focused on the search for ligands which could be used to characterise and better define receptor sub-type pharmacology. The majority of these studies utilised tissues derived from rats or guinea-pigs. Using this approach, many workers have reported that N^6-substituted derivatives of adenosine, such as CPA (FIG 1) and the recently described GR79236 (FIG 1), behave as agonists at adenosine A_1 receptors and show significant and useful selectivity of action with respect to adenosine A_2 receptors (13, 19, 20). Overall, these data are largely consistent with the suggestion that the rank order of agonist potency which characterises A_1 receptors is: CPA ≥ R-PIA ≥ NECA > S-PIA >CGS21680, CV1808 (FIG 1; see Section 4 for a full discussion of the properties of CGS21680 and CV1808). It should be noted that, at most, CPA is only 10-fold more potent than NECA. It is interesting that the rank orders of potency of compounds in functional pharmacological studies and their rank order of affinity in binding studies are usually in good agreement (for example, see 21), suggesting that compound to compound variations in the intrinsic efficacy of these widely-

Table 1: Summary of Cloned Adenosine Receptor cDNAs

Subtype	Main sites of mRNA localisation (rat)	Size of protein (rat)	Species cloned from	Tissue source of RNA from which cloned	Cells in which expressed	References
A1	brain, spinal cord, testis, white adipose tissue	326aa	canine	thyroid	CHO, Cos 7	1, 2
			rat	brain/striatum	Cos 6M, NIH 3T3	26, 113
			human	hippocampus	CHO	24, 25
			bovine	brain	Cos 1, Cos 7, CHO	33, 40
			rabbit	kidney		41
A2a	striatum, heart, lung, thymus, spleen, white adipose tissue	412aa	canine	thyroid	Y1 adrenal cells dog thyrocytes Cos 7 Xenopus oocytes	1, 17
			rat	striatum neuroblastoma	Cos 6M	77, 78
			human	hippocampus	HEK 293	79
A2b	pars tuberalis, large intestine, bladder	332aa	rat	hypothalamus	Cos 6M	82, 83
			human	hippocampus	CHO	84
A3	testis	320aa	rat	striatum cerebellum	CHO	61, 62
			human	striatum	CHO	111
			sheep	pars tuberalis		110

used agonists are of limited importance in determining their relative potency in functional studies.

Although most of the literature on the characterisation of adenosine A_1 receptors involves studies with agonists, for this purpose antagonists are usually much more useful. It was consequently a major advance when DPCPX (PD116,948; FIG 2) was described in the literature (19, 22). In both binding and functional pharmacological studies, the compound was reported to behave as a selective adenosine A_1 receptor blocking drug, showing up to 700-fold selectivity for adenosine A_1 over A_2 receptors. In these early studies using rat tissues DPCPX was reported to have an affinity for A_1 receptors of $\sim 10^{-9}$M, compared with $\sim 3 \times 10^{-7}$M for A_2 receptors. Subsequent studies using adipocytes confirmed that DPCPX also had nanomolar affinity for human A_1 receptors (23).

Human, rat, bovine and rabbit A_1 receptors have recently been cloned and expressed (24 - 26). The pharmacological characterisation of these receptors remains somewhat rudimentary at present, largely depending upon the demonstration that the receptors had affinities for DPCPX in the nanomolar range, and that the rank order of potency for displacement of ligand binding by limited selections of agonists was as predicted. Each group also showed that activation of the expressed receptor inhibited forskolin-stimulated cyclic AMP generation in the cells in which they were expressed.

Species variants

For some time the literature has contained hints which suggest that there may be some inter-species differences in the pharmacological characteristics of A_1 receptors. The vast majority of studies investigating this area of pharmacology have used tissues derived from rat or guinea-pig. Both by comparison of data obtained from different studies, and sometimes from within the same study, we are aware of no convincing data using agonists which suggests that receptors in rat, guinea-pig or human tissues are likely to be different. Although studies with xanthine antagonists, most notably 8-phenyltheophylline and DPCPX, have periodically hinted that the compounds may have higher affinities in rat than guinea-pig tissues, there is no consistent agreement that this is so (see 27 for a review). This issue may only be resolved directly by a systematic comparison of the properties of cloned, expressed receptors from the two species.

However, functional pharmacological and receptor binding studies have suggested that species variants of the A_1 receptor do exist. The most comprehensively documented example is the bovine (brain) A_1 receptor. Early binding studies showed that many compounds had higher affinities for receptors in membranes prepared from bovine brain than from other species; for example, the K_D value for [^{3}H]1,3-dipropyl-8-phenylxanthine was 68nM in rat brain membranes and 5.5nM in bovine tissues (28). Subsequent work clearly demonstrated a very unusual rank order of affinity for key agonists when studying displacement of R-[^{3}H]-PIA binding in bovine brain membranes, namely: CPA = R-PIA > S-PIA > NECA (29 - 31) These peculiarities were found to be conserved when solubilised receptors were reconstituted into human platelet membranes and studied using ligand binding (32). This story has recently been elegantly completed with the reports that cloned, expressed bovine A_1 receptors possess the distinctive ligand binding characteristics of the native and reconstituted receptors (33).

Furthermore, a number of workers have noted that the pharmacological characteristics of responses in dog tissues which would be expected to be mediated via activation of adenosine A_1 receptors (negative inotropic and dromotropic effects in the heart, inhibition of lipolysis in isolated adipocytes) were unusual, not least in that DPCPX apparently had a somewhat lower affinity than would have been expected by precedent.

Thus Martin (34) recently reported that DPCPX could antagonise the negative inotropic activity of NECA in dog isolated left atrium, but the pA_2 was found to be 7.3, approximately 1.5-2 log orders lower than might have been expected. We recently reported that $3x10^{-7}M$ DPCPX had no effect on the antilipolytic effects of NECA in dog adipocytes, in marked contrast to rat cells (27). Furthermore, the rank orders of agonist potency for bradycardic responses in anaesthetised dogs were unusual, being: NECA > R-PIA > S-PIA (35, 36). The dog adenosine A_1 receptor was the first to be cloned and expressed, subsequently being found to show 91% amino acid sequence homology with the rat receptor. The limited pharmacological data of Libert *et al.* (3) show that [^{3}H]CHA was displaced by NECA and CPA with similar affinity (3-4nM). Hence although it must remain a possibility that the responses referred to above may be mediated by another receptor mechanism (unrelated to A_1 receptors), it does seem more likely that there is a species difference in the functional characteristics of canine A_1 receptors when compared with those from rat, guinea-pig and man. A definitive comparison of the properties of this receptor with that in canine tissues and with A_1 receptors from other species really is impossible until further data on key compounds, particularly DPCPX, R-PIA, NECA and S-PIA, are published, although the reported affinity of CPA for the expressed receptor (3-4nM) is somewhat lower than values typical of rat, human and guinea-pig receptors (23, 37, 38).

Molecular cloning of A_1 receptor cDNAs (canine, rat, human, bovine and rabbit: TABLE 1) has revealed various shared structural features. Despite the absence of N-linked glycosylation sites in the amino terminal domain, one or two potential sites are present in the second extracellular (E2) loop. The third cytoplasmic loop of all four species homologues is very short for receptors which couple to Gi, being only 34 amino acids (39). A conserved cysteine residue in the carboxyl terminal tail is a potential site for palmitoylation and two histidine residues, identified as being important for ligand binding (40), are conserved in the sixth and seventh transmembrane domains in all four receptors. The protein sequences are very similar, having > 92% identity within the putative transmembrane region between species homologues. The amino acids of transmembrane regions I and II and the first intracellular loop are perfectly conserved, most of the differences being located in the E2 loop and the C-terminus (41). However, although site directed mutagenesis on the bovine receptor has shown the importance of residues His-251 and His-278 for ligand binding (40), the approach has not been used to investigate the importance of non-conserved residues for the apparent species-specific pharmacological properties discussed above.

Sub-types

The first attempt to sub-type adenosine A_1 receptors was proposed by Ribeiro and Sebastiao (42). The hypothesis was that A_1 receptors could be subdivided, so that those coupled to inhibition of adenylate cyclase would retain the term A_1. However, those in excitable tissues (nerve and heart) were envisaged not to inhibit adenylate cyclase activity

when activated, and this second group were termed A_3 receptors. The pharmacological basis of this sub-division depended upon the contention that different rank orders of agonist potency applied to the two sets of tissues, in particular the observation of Londos *et al.* (9) that R-PIA was some 100-fold more potent than NECA in rat adipocytes. Neither we, nor other workers, could confirm this observation, and we have recently suggested that this hypothesis, as originally conceived, should be regarded as untenable (20).

However, the observation that adenosine A_1 receptors can couple to transducing mechanisms other than Gi-dependent inhibition of adenylate cyclase is interesting. The other mechanisms discussed include effects on potassium channel activity and calcium flux. Several groups have shown that stimulation of A_1 receptors activates a potassium channel in right atria, so producing the characteristic decrease in beat frequency (43 - 45). In addition, a number of groups have published data, both from experiments using whole tissues (46, 47) and cells in culture (48), which suggests that adenosine A_1 receptor activation can modulate PI turnover. These studies on second messenger systems have been paralleled by the appearance of a growing body of evidence which shows that activation of A_1 receptors can initiate "excitatory" as well as "inhibitory" responses. The first such evidence came from Stoggall and Shaw (49) who suggested that the guinea-pig aorta contains a small population of A_1 receptors which mediate a contractile response. Subsequently A_1 receptors have been shown to mediate contractile responses in guinea-pig myometrium and rat colonic muscularis mucosae and spleen (50 - 52). Interestingly, it has further been suggested that the second messenger system(s) activated by A_1 receptors may be under hormonal control with the receptor coupling depending upon hormonal status (53). The possibility that heterogeneity of transduction mechanisms may mean multiple receptor sub-types has been discussed elsewhere (54), but it is our view that the model of Fredholm and Dunwiddie (55) which envisages one receptor type eliciting different second message signals by interacting with different G-proteins remains perfectly viable.

The most recent attempt to sub-classify adenosine A_1 receptors was made by Gustaffson *et al.* (56, 57) and Wiklund *et al.* (58). These authors suggested that A_1 receptors could be sub-divided into two sub-types, namely A_{1a} and A_{1b}. The proposal is based upon the observation that 2-(methoxyphenyl)adenosine (CV 1674) did not inhibit the twitch response in electrically stimulated guinea-pig ileum, yet has been reported to have modest affinity for A_1 receptors in rat brain (59). This evidence should be treated with caution for three reasons: firstly, the data compare agonist potency with ligand affinity; secondly, the reported affinity in rat brain is modest; and thirdly, the hypothesis compares data across species, which may generate a spurious view. At present the lack of commercial availability of CV 1674 makes it difficult for other laboratories to investigate its potential value in receptor classification studies.

Furthermore, there is little suggestion of the existence of A_1 receptor subtypes from molecular biology studies in the literature. Published cloning strategies have yielded only species homologues of the canine A_1 receptor, RDC7. The A_1 gene has been localised to human chromosome 22 but no other major regions of hybridisation were seen with this probe, suggesting that there is only one human gene within the limits of homology detectable by this technique (3). We are aware of no published low stringency Southern hybridisation studies which demonstrate (within the limits of detection of this technique) that other genes related to A_1 receptors exist in the mammalian genome. Our own studies have not revealed any unexplained bands that may represent genes for novel, related, receptor subtypes (unpublished data).

Published degenerate PCR cloning studies have mostly used RNA isolated from brain tissues (TABLE 1). Similar studies on other tissues or genomic DNA may reveal further variants generated by differences in RNA processing of the already cloned genes, or perhaps identify less-related genes. Knowledge of the genomic organisation of the receptor genes is important in assessing the likelihood of finding subtypes that may arise by alternative splicing events. The human A_1 receptor gene has been reported to contain at least 3 introns (60) and the rabbit gene has at least one intron, within the second intracellular loop (41).

A novel member of the family of adenosine receptors has however been cloned by two groups using degenerate PCR on rat brain RNA (61, 62). Although it shares 58% and 57% homology to the rat A_1 and A_{2a} receptor proteins within the putative transmembrane domains it is unusual in that it does not bind xanthine antagonists. This receptor, termed the A_3 receptor, is discussed more fully in Section 5.

Summary

Considerable advances have been made recently in the pharmacological and molecular characterisation of adenosine A_1 receptors. We believe that the evidence currently available demonstrates that the bovine receptor, and probably indicates that the canine receptor, constitute species variants with their own, discrete properties with respect to synthetic ligands. None the less, specific site-directed mutagenesis to investigate whether the modest species-to-species variations in receptor structure are responsible for the different functional properties will be required for formal evaluation of this hypothesis. However, if receptor sub-types are defined as two, or more, receptor proteins expressed by a single species which have related but different characteristics with respect to ligands, our belief is that there is currently no compelling evidence that receptor sub-types of the A_1 receptor exist. Furthermore, it is clear that the original concept that the activation of adenosine A_1 receptors invariably affects tissue function through an inhibition of adenylate cyclase is an over-simplification.

4. *Adenosine A_2 receptors*

Characterisation

Initial attempts to investigate the pharmacology of adenosine A_2 receptors were hampered by the lack of suitably selective agonists and antagonists. Thus adenosine A_2 receptors were defined in terms of the relative potencies of the non-selective agonist NECA and A_1 selective agonists such as R-PIA, CHA and CPA. In addition, as previously discussed (see Section 2) A_2 receptors exhibited little stereo selectivity for the isomers of PIA.

Whilst these criteria could be used in many instances to define adenosine A_2 receptors, it became increasingly difficult to reconcile the results from a number of functional pharmacological studies with the existence of A_1 and A_2 receptors as originally defined. For example, in the bovine coronary artery Mustafa and Askar (63) reported that NECA was only 5 times more potent than R-PIA at relaxing this blood vessel and there was a 30-fold difference in the potency of R- and S-PIA. Similarly, Edvinsson and Fredholm (64), studying relaxation of cat cerebral arteries, found a 10-fold difference in the potencies of NECA and R-PIA and again a 30-fold difference in the potencies of R- and S-PIA. In both of these studies the authors concluded that the responses studied were mediated by A_2 receptors although it is clear that the relative potencies of the agonists studied do not support this view. Others workers however suggested that their data were inconsistent with the existence of either A_1 or A_2 receptors. For example, Foster *et al.* (65) reported that the ratio of the potencies of NECA, R-PIA, and S-PIA for inhibition of aggregation in the human platelet were inconsistent with the existence of either A_1 or A_2 receptors. A similar conclusion was reached by Bhalla *et al.* (66) who studied adenosine receptor mediated relaxation of the dog coronary artery. Indeed, it was even suggested that adenosine-induced reduction in coronary artery tone in the dog may be mediated by a hybrid receptor with characteristics of both A_2 and A_1 receptors (67).

Initial radioligand binding studies in the rat brain used [^{3}H]NECA. This compound, however, has been shown to bind to both A_1 and A_2 receptors and to a third site that is an A_2-like binding protein of unknown function (59, 68). In order to eliminate A_1-binding in the rat striatum, pretreatment with the sulphydryl alkylating agent N-ethylmaleimide has been used with the resulting binding providing SAR consistent with the presence of A_2 receptors (68). Bruns *et al.* (59) further characterised these A_2 receptor binding sites using [^{3}H]NECA as the ligand, in the presence of unlabelled CPA to block A_1 receptors. In this study, NECA was more potent than R-PIA which in turn was 15 times more potent than S-PIA at inhibiting [^{3}H]NECA binding. More importantly however, when affinity ratios could be calculated between [^{3}H]CHA binding at A_1 sites and [^{3}H]NECA binding at striatal A_2 receptors, so allowing compounds with A_1 or A_2 selectivity to be identified.

The development of antagonists, in particular xanthines, which exhibit selectivity for A_2 over A_1 receptors has also proved rather difficult. Certain non-xanthines such as methyladenines, pyrazolo pyridines, alloxazines and quinazolines have been shown to be adenosine receptor antagonists (see 69). In general their binding affinities are low and they are unsuitable as pharmacological tools. However, amongst these non-xanthines the triazoloquinazoline, CGS15943 (FIG 2), was initially characterised in both pharmacological and biochemical studies to be a selective A_2 receptor antagonist with lower affinity for A_1 receptors (70, 71).

Receptor sub-types

As discussed above, many results reported in the literature were inconsistent with pharmacological characteristics proposed for A_2 receptors. Comparison of the structure activity relationships (SAR) for inhibition of [^{3}H]NECA binding in the rat striatum with the potency of agonists for stimulation of cyclic AMP accumulation in the human fibroblast yielded two distinct orders of potency. These authors proposed that the receptor in the striatum be termed an A_{2a} site whilst that in the fibroblast was of the A_{2b} subtype. Furthermore, CV1808 was selective for the A_{2a} site. It was also noted by Bruns *et al.*, (59) that the R- to S-PIA ratio was high at A_1 receptors, low at A_{2b} receptors and intermediate at the A_{2a} site. This finding may go some way to explaining difficulties previously encountered when trying to use this criterion to classify adenosine receptors.

Other binding studies also showed that agonists with 2-amino substituents such as CGS21680 (FIG 1) have marked activity at the rat striatal receptor (72-74). In order to determine the applicability of the A_{2a} and A_{2b} classification in peripheral tissues we recently undertook a study of the SAR of adenosine agonists in a range of preparations believed to contain A_2 receptors (13). In this study we found that the order of agonist potency for relaxation of the dog coronary artery, inhibition of human platelet aggregation and inhibition of fMLP-induced superoxide anion (O_2^-) generation in the human neutrophil was CV1808, CGS21680>=NECA>CPA. However, for relaxation of the guinea-pig aorta the rank order was NECA>CPA>CV1808>=CGS21680. These results suggest that the receptors in the dog coronary artery, human platelet and human neutrophil are similar if not identical to the A_{2a} receptors in the rat striatum whilst those in the aorta may be of the A_{2b} subtype. Another interesting finding to emerge from this study was that the agonist, metrifudil (FIG 1), a compound that has been shown to be a vasodilator in man (75), was modestly selective for A_2 over A_1 receptors but could not be used to differentiate between A_2 receptor subtypes. Further heterogeneity may exist, a possibility raised by the recent finding that in human brain two A_2-like binding sites were detected, one which corresponds to the A_{2a} and one which has intermediate A_1 and A_2 characteristics (76).

Since cloning of the canine "A_2" receptor cDNA (1), further species homologues have been isolated from rat (77, 78) and man (79). These genes are now thought to represent the A_{2a} subtype, on the basis of the pharmacological characteristics of the expressed receptors and localisation of their transcripts. Conserved structural features include the presence of two potential N-linked glycosylation sites in the second extracellular loops (77). Unusually, the C3 loops are fairly equivalent in size to that of the negatively coupled A_1 receptor but the carboxyl terminus of the A_{2a} receptors is very long. The receptor proteins exhibit 84 - 93% identity over their entire length. As is the case for A_1 receptors, the majority of the species differences are located within the carboxyl terminus and E2 loops.

Extensive mRNA localisation studies in all three species have shown that A_{2a} receptor transcripts in the brain are confined exclusively to medium-sized striatal neurones (77, 80, 81). One study further localises them to a subset of these neurones that express enkephalin but not substance P mRNA (81). Northern analysis of A2a mRNA in rat peripheral tissues showed high levels of expression in the heart, lung, spleen, thymus, oesophagus and epididymal fat but no expression in kidney, gastrointestinal tract, or bladder (82).

The existence of an A_{2b} receptor was confirmed by the isolation of a cDNA from rat (RFL9 - 82) which, when expressed, is positively coupled to adenylyl cyclase but does not bind [^{3}H]CGS-21680. mRNA for this receptor is expressed by VA13 fibroblasts, the cell line used by Bruns to define A_{2b} receptors pharmacologically (59) which strongly suggests that RFL9 represents this receptor (83). A cDNA for a human A_{2b} receptor that has 86% homology to the RFL9 product has also been cloned (84). The rat A_{2b} receptor has 73% and 62% identity respectively with rat A_{2a} and A_1 receptors within the putative transmembrane regions. The corresponding figures for the human A_{2a} receptor are 73% and 59%.

Interestingly, although both A_{2a} and A_{2b} receptors are positively coupled to cyclase, the A_{2b} carboxyl terminal tail is considerably shorter (41 residues) than that of A_{2b} (100 residues). This region from both subtypes is rich in potential phosphorylation sites, unlike the A_1 receptor which only has one such residue (85). The mRNA distribution is also quite different to that of the A_{2a} gene. RFL9 transcripts have been predominantly identified in the large intestine, caecum and bladder and are only moderately expressed in the central nervous system. *In situ* hybridisation has revealed very low levels of expression in the rat brain with the exception of a small, strong area of hybridisation to the hypophyseal pars tuberalis (83). We are aware of no similar studies on distribution of the human receptor in the literature. As is the situation for A_1 receptors, there is little published molecular biology data to support the existence of further A_2-like subtypes. During mapping of the A_{2a} gene (to human chromosome 11) a second, minor hybridising site was also identified on the long arm of

chromosome 10 which is most likely to represent the A_{2b} subtype (18) but no further loci were identified that could represent further closely related subtypes.

Thus evidence supporting the proposition that A_2 receptors are heterogeneous was provided initially by functional studies with agonists, and subsequently confirmed using molecular approaches. However, data with receptor blocking drugs which supports the concept of A_{2a} and A_{2b} receptors remains scarce. The antagonist CGS15943, which was originally characterised as a selective A_2 receptor antagonist (70) versus A_1 receptors, has been investigated further. For example, Jarvis *et al.* (73) reported pKi values of 7.7 and 8.5 for A_1 ([^{3}H]CHA in rat cortex) and A2a (either [^{3}H]CGS21680 or [^{3}H]NECA in the presence of CPA in rat striatum) receptors respectively. Furthermore, Jarvis *et al* (86) subsequently reported that [^{3}H]CGS15943 labelled an A_1-site in rat cortex showing an apparent affinity of 4nM, a value consistent with a pKi of approximately 8.5. These studies are supported by our own work in which the antagonist has been found to displace [^{3}H]DPCPX from A_1 receptors in rat brain membranes, with a pKi value of 7.6 (unpublished observations). Overall these later studies suggest that CGS15943 shows a modest, i.e. <10-fold, selectivity for the A_{2a} versus the A_1 receptor, and have apparently failed to determine its affinity for A_{2b} sites. Surprisingly, there are very few studies reported which have attempted to characterise CGS15943 using functional responses apart from the original study of Ghai *et al.* (70). An exception is that of Croning *et al.*, (87) who were able to demonstrate that CGS15943 had approximately 50-fold selectivity for A_{2a} (dog coronary artery) versus A_{2b} (guinea pig aorta) receptors. Further characterisation of the interaction of CGS15943 with adenosine receptors, particularly receptors cloned and expressed in a cell line, is clearly necessary in order to clarify its value as a pharmacological tool.

In a recent status report from the IUPHAR purinoceptor subcommittee (88) two compounds, CP-66713 and KF17837 (FIG 2), were proposed as selective A_{2a} receptor antagonists. Whilst this may prove to be the case, these compounds are at present poorly characterised and not widely available. We are presently only aware of single publications on each antagonist which characterise their interaction with adenosine receptors. Using ligand binding assays Sarges *et al.* (89) reported CP-66713 to have a pKi value of 6.6 at A_1 ([^{3}H]CHA in rat cortex) and 7.8 at A_{2a} ([^{3}H]NECA in the presence of CPA in rat striatum) receptors, whilst Shimada *et al.* (90) reported KF17837 to have a pKi value of 6.4 at A_1 and 8.1 at A_{2a} receptors using the same methodology. Whilst the selectivity of either or both of these compounds may subsequently be verified, it seems premature to recommend their use in receptor characterisation studies, not least because their affinities for A_{2b} receptors have not been reported. However, a recent publication has demonstrated that KF17837 can selectively block A_{2a} versus A_1 receptors in anaesthetised rats (91). The extent of this selectivity could not be investigated fully because of the compounds limited solubility in appropriate vehicles.

Characterisation of the A_{2b} site has been limited by the lack of any suitably selective ligands. An initial study reported that 1,3-diethyl-8-phenylxanthine (DPX) had a 22-fold higher affinity for A_{2b} receptors than for A_{2a} receptors (59) although this could not been confirmed in a subsequent study (87).

Adenosine A_2 receptors were originally described as mediating stimulation of adenylate cyclase and therefore it is not surprising that this transduction system has been widely studied. However, it has been suggested that some A_2 receptor mediated responses may not be linked to changes in cyclic AMP levels. For example, in the human neutrophil Cronstein and co-workers (92) reported that the adenosine agonist NECA did not elevate intracellular cyclic AMP levels although this agonist inhibited fMLP-induced superoxide anion (O_2^-) generation. Furthermore, whilst the addition of the phosphodiesterase inhibitor Ro20-1724 and NECA resulted in cyclic AMP elevation in the neutrophil it had no effect on the inhibition of O_2^- generation induced by this agonist.

Species variants

In contrast with A_1 receptors, species differences in SAR have not been widely reported for A_{2a} or A_{2b} receptors. A study specifically addressing this question (93) reported that the order of activity of adenosine agonists in radioligand binding experiments was similar at high affinity A_2 receptors (A_{2a}) from the rabbit, man, rat, mouse guinea-pig and calf. Interestingly, the same study also noted that there were distinct interspecies differences when antagonists were studied with higher affinities reported at rabbit calf and human A_2 receptors than those from guinea-pig, mouse and rat. The physiological significance of these findings is unclear at present.

Summary

In conclusion, adenosine A_2 receptors can be divided, using pharmacological methods, into at least two subtypes which have been termed A_{2a} and A_{2b} sites. Agonists and antagonist showing selectivity for the A_{2a} receptor have been described. Molecular biological techniques have led to the cloning of both A_2 receptor subtypes from rat and man.

5. *Further adenosine receptors*

An underlying criterion which emerged from Burnstock's early work on P_1 (adenosine) receptors was that responses mediated through their activation were blocked by methylxanthines. The general acceptance of this concept created an expectation that methylxanthines would have significant affinity for all adenosine receptors. However, there have been occasional indications in the literature of adenosine-mediated effects which are resistant to antagonism by standard alkyl-xanthines, such as theophylline and 8-PT.

In 1977 Londos and Wolff (94) demonstrated that adenosine can inhibit adenylate cyclase by interacting with an intracellular "P" site, which is located on the catalytic subunit

of adenylate cyclase. The site is resistant to blockade by theophylline and 8-phenyltheophylline and has relatively low affinity for adenosine (K_i 10-20μM; 95). The physiological role of this site is unclear.

More recently Church *et al.* (96) reported that adenosine and some of its derivatives stimulated antigen-induced 5-HT release from rat serosal mast cells. This response was accompanied by a potentiation of the increase in cyclic AMP concentrations caused by antigen, an effect characterised as being sensitive to 8-PT and resulting from the activation of A_2 receptors. However, the stimulation of 5-HT release was not blocked by this xanthine, and the rank order of agonist potency differed from that expected for an A_1- or A_2-receptor-mediated event. IgE-mediated histamine release from rat peritoneal mast cells was also enhanced by adenosine analogues through activation of a receptor which was not blocked by theophylline (97). Furthermore, in a rat tumour mast cell line (RBL-2H3) the xanthine-resistant receptor couples to phospholipase C (98, 99), its activation evoking a transient generation of inositol 1,4,5, triphosphate and an increase in cytosolic calcium, so enhancing the secretory response to antigen.

Adenosine has been reported to be able to modulate histamine-induced PI turnover in mammalian cerebral cortex (100, 101). The receptor presumed to mediate these responses displays a rank order of agonist potency characteristic of A_1 receptors, albeit with low absolute potencies. Although 8-PT demonstrates a typical affinity for this site, that of DPCPX is uncharacteristically low (K_D=8-24nM; 102).

In contrast, 8-PT has been used to detect xanthine-resistant sites in the guinea-pig aorta (103). In this study vasorelaxant responses to NECA were competitively antagonised, whereas those induced by a wide range of C^2-substituted adenosine analogues (purported to be selective A_2 agonists) were not. Thus, 30μM 8-PT produced the expected 20-fold rightward shift in the concentration-effect curve to NECA, but only a 2 - 6-fold shift in those of other agonists, implying that both A_{2b} and xanthine-resistant receptors are functional in the preparation.

One of the C^2-analogues used in the above study, the A_{2a}-selective agonist CV-1808, also evokes xanthine-resistant relaxations in guinea-pig isolated trachea (104), and is key in a recent report which suggests that a novel site, termed "A_4", exists in rat brain (105). [^{3}H]CV-1808 was not displaced from this site by a range of standard compounds, including xanthine antagonists. The CNS "A_4" site has been claimed to have a functional counterpart in the porcine coronary artery, where CV1808, but not CGS21680, NECA or CPA, induced a relaxant response via activation of potassium channels. This suggests that the putative "A_4" receptor differs from another DPCPX-resistant site in pig coronary artery which has been characterised by Merkel et al (106). Here CPA is active as a vasorelaxant, apparently

once again via activation of potassium channels. Recently, a novel compound, RG14202 (N-5'-ethyl-N^6-(cyclopentyl)adenosine-5'-uronamide), has also been shown to be active at this site (107). These data imply that in addition to evoking relaxation of vascular smooth muscle through stimulation of adenylate cyclase, some agonists can induce a response by stimulating at least one form of a receptor which (i) is resistant to xanthines, and (ii) can activate potassium channels.

The adenosine A_3 receptor

Confirmation of the existence of at least one further receptor, which does not bind standard xanthine adenosine receptor blocking drugs, has recently been provided by molecular studies. Thus, a novel member of the adenosine receptor gene family has been isolated by two groups using degenerate PCR on rat brain RNA (61, 62). This receptor has 58% and 57% amino acid identity to the rat A_1 and A_{2a} receptors respectively within the putative transmembrane regions. In common with A_1, A_{2a} and A_{2b} receptors a potential glycosylation site is present in the second extracellular loop, although there are two further potential glycosylation sites within the amino terminal domain, which are not present in the other subtypes . The cloned receptor, expressed in Cos 7 and CHO cells, binds adenosine receptor agonists with a profile inconsistent with that of A_1, A_{2a} or A_{2b} receptors, does not bind adenosine antagonists and is negatively coupled to adenylyl cyclase. It therefore appears to be functionally more closely related to A_1 receptors than A_2 receptors and has been termed an A_3 receptor (62). Two larger A_3 receptor specific transcripts (2.0 and 3.5 kb) were identified in RBL-2H3 cells (108), a cell line previously reported to contain a xanthine-resistant receptor (see above). Recently two groups have cloned human homologues to this receptor. One cDNA was isolated from the promyelocytic leukaemic cell line HL60 (109), and the other from human heart (Genbank accession number L20463). Subsequently publications have appeared which describe the cloning of what appear to be the sheep (110) and human (111) homologues of this receptor. Interestingly, however, there are important functional differences, with the ability of sheep and human receptors to interact particularly with receptor blocking drugs being somewhat different from the rat receptor. Furthermore both the human and sheep receptors only show 72% identity with respect to the rat receptor, but 85% identity with each other.

Analysis of mRNA from a number of rat tissues has revealed expression of a 1.5kb A_3 transcript in the testis only, with no signal detectable in the bladder, liver, spleen, adrenal gland, kidney, lung, heart, brain or cerebellum (61). Low level expression has however been detected by RT-PCR in lung, kidney, heart and parts of the brain (62). Expression of this transcript in rat testis is first detectable at postnatal day 28 and increases during the second and third months after birth (61). Analysis of genomic clones for this A_3 receptor has revealed that there is at least one intron (>2kb) just outside the third transmembrane domain (62). In addition, Ramkumar *et al* (108) have reported that it is this adenosine receptor

which is expressed by RBL-2H3 cells. Interestingly, the pattern of expression of the cloned human receptor is apparently very different, with highest levels in the lungs and liver (111).

Already some limited data from in vivo experiments identifying effects which have might be mediated via the activation of A_3 receptors have been published. In particular, Fozard and co-workers have suggested that receptor activation induces a hypotensive effect in pithed rats (112).

Summary

It is clear that numerous reports had accumulated in the literature during the last decade which described responses which were apparently mediated via adenosine receptors which were clearly neither A_1- nor A_2-like. The cloning and expression of the A_3 receptor is undoubtedly, at the very least, a part of the answer. However, there are two important points to bear in mind at present:

(i) That the structural and functional diversity between the rat and the human/sheep receptors raises the possibility that these receptors may not be true homologues of one another, but rather represent subtypes of closely related receptors.

(ii) That it remains to be proven that there are not further adenosine receptor types, still to be identified, which are involved in the generation of these responses.

6. *Conclusions and future perspectives*

Adenosine receptor classification is now at a very interesting stage in its development. Compared with 7-TM receptors for some ligands, such as the burgeoning 5-HT family of receptors (113), there are still remarkably few adenosine receptors clearly defined and characterised. Furthermore, the classification of A_1 and A_2 adenosine receptor largely remains where it was left by the work of Bruns and colleagues in the mid-1980s. The classification scheme, principally established by comparing the profiles of receptor-selective agonists in primary tissues and membrane homogenates from tissues, has now been confirmed at the molecular level. Interestingly, and somewhat unusually given their generally accepted significance for such work, our understanding of adenosine receptor classification and heterogeneity has largely been established without input from studies using receptor-selective blocking drugs. Whether greater heterogeneity among A_1 and A_2 receptors exists remains to be established, although it does seem to be increasingly unlikely, especially since many laboratories must now have attempted homology cloning based on the gene structures of the known receptors.

In marked contrast, the newer receptors are likely to become an increasing focus of interest for workers in this area. A classification problem which remains to be resolved, of course, is whether the rat A_3 receptor in particular should be classified along with the xanthine-sensitive A_1 and A_2 receptors or not. If it is, then Burnstock's concept that receptors for **all**

adenosine (P_1) and nucleoside triphosphates (P_2) can be universally distinguished on the basis of sensitivity to xanthines in general will have to be abandoned.

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