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Adenosine A₁ and A₂ Receptors: Structure-Function Relationships

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I. INTRODUCTION

A. Scope of this Review

Adenosine is a neuromodulator that plays a pivotal role in maintaining adequate oxygen and energy supply throughout the body.¹ The actions of adenosine are mediated through specific cell-surface receptors, of which at least two subtypes are known, A₁ and A₂. Due to its potent actions on many organs and systems, adenosine is an obvious target for the development of new drugs,² and in the past decade adenosine receptors have become a subject of intense investigation. Potential therapeutic applications for agonists include, for instance, the prevention of reperfusion injury after cardiac ischemia or stroke, and the treatment of hypertension and epilepsy.³ Adenosine itself has recently been approved for the treatment of paroxysmal supra-ventricular tachycardia.⁴ Adenosine antagonists might be effective in, e.g., renal failure and as cognition enhancers.³ There are also recent indications that selective A₁ antagonists might be beneficial in cystic fibrosis.⁵

After an introduction on adenosine receptor subtypes, transduction mechanisms, and adenosine receptor regulation, this review will focus on the structure of adenosine receptor ligands, and on the structural information contained in the deduced amino acid sequences of the recently cloned adenosine receptor cDNAs. Many tools for the delineation of receptor physiology and pharmacology, as well as some potential therapeutic agents, have become available in recent years. The structure–activity relationships (SARs) of these compounds will be discussed, with some emphasis on the insights that have been gained using molecular modeling techniques. In addition, information about the structure of the receptor gathered with the aid of receptor labeling agents will be discussed, and a detailed analysis of

functional and structural domains of the receptor deduced from the amino acid sequences will be presented. The physiology, pharmacology, and therapeutic potential of adenosine receptors have been the subject of a number of recent reviews^{3,6-8} and will not be discussed in any detail in the present article.

B. Adenosine Receptor Transduction Mechanisms

1. Receptor–Effector Coupling and Subtypes—The most extensively studied effector system coupled to adenosine receptors is the adenylate cyclase system.⁹ In all tissues studied thus far, A₁ receptors inhibit adenylate cyclase activity whereas A₂ receptors stimulate the activity of this enzyme. The adenosine receptors regulate the adenylate cyclase indirectly by activating guanine nucleotide regulatory proteins (G proteins).^{10,11} The G proteins represent an ever-burgeoning family of coupling proteins.^{12,13} This diverse family includes the G_s protein known to stimulate adenylate cyclase and to interact with calcium channels directly; the G_i proteins, which now number three and are derived from distinct genes, and are capable of both inhibiting adenylate cyclase and opening K⁺ channels; the G_z protein, whose function likely involves activation of phospholipase C; and the G_o protein, which is found in great abundance in the brain and may well regulate calcium and/or other ion channels. G proteins are heterotrimeric, consisting of α -, β -, and γ -subunits. The α -subunits show considerable structural diversity. The β - and γ -subunits, which show less structural diversity, are tightly associated and may couple with various types of α -subunits. Although there is a great deal of information available now on the structure and number of G proteins, especially the α -subunits, much less information is available on the specificity and selectivity of many G proteins in terms of which receptors and effectors they couple to. It is clear, however, that the activation of G proteins by receptors is dependent on the presence of GTP and leads to the activation or inhibition of the effector system such as adenylate cyclase or phospholipases.

It is now known that there are multiple regulatory steps in the process of receptor-G protein coupling and activation. Those processes can be studied by radioligand binding, adenylate cyclase assays, and functional studies of G proteins such as their GTPase activity and the actual binding of GTP to the α -subunit.^{6,11} It has been known for a long time, for example, that GTP can decrease the affinity of agonists for the receptor and that magnesium ion is necessary for the induction of the agonist-specific high-affinity state.¹¹ In addition, in inhibitory receptor systems such as the A₁ receptor system, sodium is known to be important for the full inhibition of adenylate cyclase.

This effect of sodium is now thought to be derived from a specific sodium–aspartate interaction in the second transmembrane domain of the receptor.¹⁴ This type of regulation has recently been reviewed and will not be recapitulated here.¹⁵

As described above, recent work from a number of laboratories has documented that A₁ receptors are promiscuous in that they will couple to a variety of effector systems, including adenylate cyclase, guanylate cyclase, potassium channels, calcium channels, phospholipase A₂ and C, and the sodium–calcium exchange system (Table I).⁶ The coupling of A₁ receptors to G proteins has been explored in detail. Studying purified receptors in reconstituted vesicles, Freissmuth *et al.* found that A₁ receptors can couple to G_{i1-3} and to G_o.¹⁶ In contrast, the only known effector system to which the A₂ receptor couples is adenylate cyclase, which appears to be uniquely through G_s.

The classical system in which the inhibitory effects on adenylate cyclase of A₁ receptor stimulation have been observed is the adipocytes of white adipose tissue.¹⁷ Platelets contain a stimulatory, “high-affinity” A_{2a} receptor,¹⁸ which is also present in striatum.¹⁹ Another stimulatory receptor, later termed A_{2b}, was found in human fibroblasts²⁰ and is associated

with lower potency of adenosine agonists than at the A_{2a} receptor. A_{2b} receptors are found distributed throughout the brain, and although they have never been detected in radioligand binding assays, the resulting stimulatory adenylate cyclase effects (in regions such as the cortex which do not contain appreciable densities of A_{2a} receptors) can be studied in brain slices.²¹

Guanylate cyclase in the vasculature has been reported to be activated by A₁ adenosine agonists, and may be associated with a vasodilatory effect.²² The intracellular concentrations of various ions are also modulated by adenosine. Adenosine A₁ agonists cause a decrease in the intracellular concentration of Ca²⁺, via entry through voltage-sensitive N-type calcium channels in presynaptic terminals in the hippocampus.²³ 2-Chloroadenosine, an agonist, inhibits the potassium-dependent uptake of ⁴⁵Ca²⁺ into cortical synaptosomes²⁴ and inhibits calcium currents in rat dorsal root ganglion cells.²⁵ The calcium effect, which is blocked by xanthines, is likely related to the effect of adenosine in depressing the release of stimulatory chemical signals at the synapse.²⁶ This inhibition of calcium channels by adenosine agonists is associated with an A₁-mediated decrease in adenylate cyclase.^{24,25} Another effect of certain xanthines, such as caffeine, on the release of calcium from intracellular stores in the sarcoplasmic reticulum,²⁷ does not appear to involve adenosine receptors. An A₃ adenosine receptor has been tentatively classified by Ribeiro and co-workers,²⁸ and is associated with the actions of adenosine, presumably with calcium as a second messenger, at the neuromuscular junction. It has been proposed that the A₃ receptor occurs in the brain and constitutes a site of intermediate affinity (1–10 nM) for the A₁-selective antagonist CPX²⁹

In the heart^{30,31} and brain (hippocampal neurons),²³ adenosine increases K⁺ conductance, and this effect is reversed by xanthines. The SAR for effects in atrial membranes linked to potassium channels resemble that of A₁ receptors,³² and a pertussis toxin-sensitive G protein appears to be involved.³¹ These receptors may also couple to adenylate cyclase.³² A₁ receptors are also coupled to another type of potassium channel that is activated upon a drop in the intracellular ATP concentration.³³

Chloride channels have been found to be activated by adenosine in the hippocampus.³⁴ In CFPAC cells, originating in the pancreas of a cystic fibrosis patient, the selective A₁ adenosine antagonist CPX (see below) was found to activate chloride efflux.⁵

More recently, the connection between adenosine receptors and the metabolism of phosphoinositides has come to light. There are both inhibitory^{35,36} and stimulatory effects³⁷ that are blocked by xanthines. Hollingsworth *et al.* found that adenosine agonists, presumably acting through A₁ receptors in guinea pig brain slices,³⁸ augment histamine-evoked stimulation of phosphoinositide breakdown. In mouse brain, however, adenosine agonists inhibit histamine-induced phosphoinositide turnover. Adenosine stimulates phospholipase C in kidney slices³⁹ and in an RCCT cell line derived from kidney cortical collecting tubules.⁴⁰ Conversely, adenosine agonists inhibit the generation of inositol phosphates in pituitary GH₃ cells.³⁵

2. Receptor Regulation—The type of receptor regulation most extensively studied in adenosine receptors is that of agonist-induced desensitization. Desensitization or tachyphylaxis refers to a state of refractoriness of a cell to normally activating stimuli following chronic exposure to agonists. A general theme that has developed is that exposure to agonists leads to the uncoupling of the receptor from the effector system, a process that probably involves multiple pathways. These include downregulation of receptors and covalent modification of the receptors, such as phosphorylation, which may functionally uncouple the receptor from its G protein. In addition, it has been documented that alterations

in the quantity of the G proteins themselves can follow pathophysiologic interventions. It is now clear that although this is a general scheme, each receptor system in each cell does not undergo all of the above changes and there appear to be cell-specific mechanisms. What determines each of these pathways and their applicability in a given cell remains unknown.

Studies on the regulation of adenosine receptors have really only become available within the past 5–10 years. Several groups have now begun to probe the phenomenon of desensitization of A₁ receptors. Our laboratory has used an *in vivo* rat adipocyte model in which rats are infused with the A₁-selective adenosine analog *R*-PIA for periods of up to six days and adipocytes are then isolated and receptor membrane mediated events are studied. Studies in this system have documented that desensitization of A₁ receptor occurs in that the ability of *R*-PIA to inhibit adenylate cyclase is diminished by ca. 50%.⁴¹ This desensitization appears to be heterologous in nature in that the ability of PGE₁ to inhibit adenylate cyclase by its own distinct receptor is also diminished following treatment with *R*-PIA. Quite unexpectedly, it was found that stimulatory agents such as isoproterenol acting via β -adrenergic receptors, sodium fluoride acting via G proteins, and forskolin acting via the catalytic unit, all demonstrate an enhancement in their ability to stimulate cAMP accumulation. These findings suggested that in addition to possible receptor changes, additional alterations in the transmembrane signaling apparatus must be present. Quantitation of β -adrenergic receptor number and their coupling to G_s was found to be unchanged.⁴¹ It was found that A₁ receptors were decreased in number and partially uncoupled from G_i.⁴¹ Although these results might explain the decreased sensitivity to *R*-PIA, the changes in A₁ receptors could not explain either the decreased sensitivity to PGE₁ or the enhanced stimulation of adenylate cyclase by a variety of effectors. We were able to document that changes occurred in the quantity of the G proteins present in the membrane. Over a period of six days it was found that the α_{11} and α_{12} , two forms of the G_i protein, were decreased by 59%, whereas α_{13} , a third form of the G_i protein, was unchanged. In addition, it was observed that the quantity of the α_5 subunit of G_s increased over a period of time with maximums occurring by day 4 and remaining steady after that. The time courses found for changes in the G proteins paralleled the effects observed for adenylate cyclase, wherein the stimulatory agents demonstrated a significant enhancement by day 4 while the decrement in the ability of the inhibitory agonist to inhibit does not occur until day 6.

Although there were significant changes in G protein throughout the six-day treatment there were, however, no changes in the mRNA for any of the G proteins. This suggests that the alterations in G proteins relate more directly to either alterations in translation of the message or the stability of the G proteins within the membrane. Thus, the inhibitory A₁ adenylate cyclase system of rat adipocytes can be desensitized by *in vivo* exposure to adenosine agonists. In this model a heterologous pattern of desensitization is apparent and regulation of multiple components of the system occurs. Recently, in a cell culture model using the DDT₁ MF-2 cell line we have been able to document that the A₁ receptor is, in fact, a substrate for phosphorylation, suggesting a further potential mechanism involved in the desensitizing process.⁴² Much less information is available on the desensitization of A₂ receptors, but studies in the DDT₁ MF-2 cell lines suggest that its mechanism for desensitization is quite disparate from that seen in the A₁ receptor in that desensitization is much more rapid and appears to occur without downregulation or uncoupling. It should be noted, however, that these studies are done with an agonist radioligand since no A₂-selective antagonist radioligand is currently available. Much additional work needs to be done on the mechanisms of desensitization, and with the recent cloning of the A₁ and A₂ receptors we can now begin to probe whether transcriptional regulation of the receptor may occur.

II. STRUCTURE–ACTIVITY RELATIONSHIPS

A. Adenosine Agonists and Antagonists with Receptor Subtype Selectivity

1. Adenosine Derivatives as Adenosine Agonists—All known adenosine agonists are closely related to adenosine (**1**) itself, and very few modifications to its basic structure are allowed. This is especially true for the ribose moiety, where only the 5′-position is amenable to substitution. Modifications of the purine moiety are usually limited to substitutions at the N⁶- and C2-positions, yielding a wealth of useful, subtype-selective agonists. The N⁶-substituted adenosine, derivatives CHA (N⁶-cyclohexyladenosine, **3**), CPA (N⁶-cyclopentyladenosine, **2**), and *R*-PIA (*R*-phenylisopropyladenosine, **6**), the 2-substituted analog 2-CADO (2-chloroadenosine, **22**), the 5′-substituted analog NECA (5′-N-ethylcarboxamidoadenosine, **32**) and the 2,5′-disubstituted CGS 21680 (2-[[4-(2-carboxyethyl)phenethyl]-amino]adenosine-5′-N-ethylcarboxamide, **33**) have all been instrumental in defining receptor pharmacology and classification. Structures and affinities of representative N⁶- and C2/5′-substituted adenosine derivatives are shown in Tables II and III.

The presence of the amino group at position 6 of adenosine is essential for agonist activity. Replacement with, e.g., methyl, chlorine, or oxygen (resulting in inosine) yields compounds that are virtually inactive.²⁰ Disubstitution at N⁶ is also detrimental to activity, presumably because one hydrogen atom acts as a hydrogen bond donor,²⁰ but monosubstitution may yield highly potent agonists, often A₁-selective. Studies of the dependence of potency on the size of the ring in N⁶-cycloalkyl substituted analogs identified CHA and CPA as particularly potent and A₁-selective (280- and 780-fold, respectively).⁴⁷ Subsequent studies showed that N⁶-bicycloalkyladenosines are even more A₁ selective, with the 1*R*, 2*S*, 4*S* isomer of N⁶-(2-*endo*-norbornyl)adenosine(S-ENBA, **4**) being 4700-fold selective, while its 5′-Cl analog is even 16,000-fold selective for the A₁ receptor.⁴⁸ The N⁶-region is essentially hydrophobic in nature, and may accommodate very large substituents. Hydrophilic substituents are not well tolerated when the hydrophilic group is close to N⁶,⁴⁹ but they may be accommodated when present at a considerable distance from the N⁶-region.^{50,51} A functionalized congener approach to the design of adenosine agonists identified ADAC (**18**) as a high-affinity agonist and synthetic intermediate for larger biologically active conjugates, including biotin- and fluorescein-labeled probes (compounds **19–21**).⁵²

A₂ selectivity may be achieved by modifications of the 2-position. Small 2-substitutions, such as 2-CADO (**22**) tend towards relative nonselectivity or modest A₂ selectivity. CV 1808 (2-phenylaminoadenosine, **24**),⁵³ introduced as a potential hypotensive agent by the Takeda Co., was found to be of moderate potency (K_i = 100 nM) at A₂ receptors and was the first ligand with any selectivity (5-fold) at that subtype.¹⁹ Evaluation of a large number of alkylamino and arylamino modifications at the 2-position of NECA focused on 2-(2-phenylethyl)amino substituents. The carboxylic acid derivative CGS 21680 (**33**) was found to be 74-fold selective for the A₂ receptor, with a K_i value of 19 nM.⁵⁴ This carboxylate displayed greatly diminished penetration of the blood–brain barrier,⁵⁵ a desirable characteristic for a peripheral hypotensive agent. [³H]CGS 21680 has been developed as a selective A₂ radioligand, and was instrumental in identifying the cloned A₂ receptor.⁵⁶ In contrast, a related amine derivative, APEC (**34**) was centrally active as a locomotor depressant⁵⁷ and, moreover, served as an intermediate for the preparation of molecular probes (see below). Two compounds that are related (but lack the 5′-substituent), the 2-cyclohexylethylamino analog of adenosine, CGS 22492 (**25**) and the 2-cyclohexenylamino analog, CGS 22989 (**26**), are 530-fold and 210-fold selective, respectively, for the A₂ receptor with K_i values of 22 and 13 nM.⁵⁸

Further structural modifications at the 2-position led to development of 2-alkoxyadenosines,⁵⁹ e.g., CHEA (27; A₂: 22nM; A₁: 1580 nM),⁶⁰ 2-alkoxy-adenosine derivatives,⁶¹ such as MPEA (**28**; A₂: 11 nM; A₁: 49 nM),⁶⁰ as well as 2-alkynyladenosines.⁶² For example, the 2-alkynyl derivatives 2-hexynyladenosine (2-HNA, **29**) and 2-octynyladenosine (2-ONA, **30**) are potent A₂ agonists (K_f values 4 and 12 nM) with 36- and 17-fold selectivity, respectively, for the A₂ receptor in binding assays.⁶²

A variety of A₂-selective agonists have been found in *in vivo* testing to be much more A₂-selective than predicted in binding assays. In an assay of coronary vasodilation in the guinea pig, CHEA has an EC₅₀ value of 1 nM at the A₂ receptor, resulting in an 8,700-fold selectivity for the A₂ receptor.⁶⁰ MPEA is 39,000-fold selective in this functional assay, compared to only 5-fold in binding assays in rat brain.⁶⁰ The source of this discrepancy apparently lies not in species differences,⁶⁰ but more likely in pharmacokinetic and pharmacodynamic differences (e.g., penetration, receptor-effector coupling, spare receptors). Furthermore, agonists that are clearly A₁ selective in binding assays, such as CPA, appear to have little selectivity in such cardiovascular functional assays.⁵⁹ Obviously, caution is warranted in comparing binding data with data from functional assays.

Structural requirements for the ribose moiety are very strict. Ring opening or enlargement of the pentose ring to a hexose ring are detrimental for affinity, as is inversion of the stereochemistry at the C1'—C4' bond.⁶³ The presence of a hydroxyl group at the 2'-position is essential for both affinity and intrinsic activity. On the other hand, removal of the 3'- or 5'-hydroxyl group results in partial agonists that still have considerable affinity.⁶³ The 2',3'-dideoxy analog of CHA has been reported to act as a weak antagonist.⁶⁴ One notable exception to the generalization concerning the ribose moiety is the 5'-position. 5'-N-Ethylcarboxamidoadenosine (NECA, **32**) is nonselective but relatively potent (K_f = 10 nM at both A₁ and A₂ receptors¹⁹). NECA has been extensively used to define tissue responses mediated by A₂ receptor activation^{7,65} and as a radioligand which labels A₂ receptors in rat striatum.¹⁹ The A₁ component of the binding profile of [³H]NECA can be eliminated by the addition of 50 nM CPA. Several other 5'-modifications, including 5'-halo and 5'-methylthio, are also tolerated.⁶³

Of the stereoisomers of adenosine, only 9-β-D-ribofuranosyladenine is active. Neither the α-anomer⁶⁴ or the L-enantiomer,²⁰ nor the compounds in which the stereochemistry of the 2'-OH (9-β-D-arabinofuranosyladenine) or 3'-OH (9-β-D-xylofuranosyladenine) group is inverted,²⁰ is active. Significantly, the receptor does not discriminate between the α- and β-anomers of the weak antagonist 2',3'-dideoxy-N⁶-cyclohexyladenosine, in which both hydroxyl groups have been removed. On the basis of this observation, it has been argued by Lohse *et al.* that the 2'-OH and 3'-OH groups are the prime determinants for receptor binding/activation in the ribose moiety.⁶⁴

Few endocyclic modifications of the purine ring are allowed. In a series of deazaadenosines, tested for activity at both A₁ and A₂ receptors, 7-deazaadenosine is inactive and the potency of 3-deazaadenosine is quite low. 1-Deazaadenosine derivatives, however, are only slightly less potent than the parent compounds.⁶⁶ 1-Deaza-2-chloro-N⁶-cyclopentyladenosine is a potent and highly A₁-selective (8,200-fold) agonist.⁶⁷

Summarizing, adenosine agonists of high affinity and selectivity for the A₁ and A_{2a} subtypes have been developed over the past decade, and use of such selective agents in physiological studies is still a useful method of classification of adenosine receptor subtypes. Agonists that are selective for the A_{2b} subtype of the receptor are not yet available, however.

2. Xanthilles as Adenosine Antagonists—The first compounds that were identified as adenosine receptor antagonists were the naturally occurring xanthines, caffeine and theophylline,⁶⁸ and xanthines still constitute the class of antagonists that has been studied most extensively, both in terms of *in vitro* SAR and *in vivo* pharmacology. As adenosine antagonists, the simple xanthines are weak and nonselective and, in fact, they may also act biochemically through other mechanisms, such as phosphodiesterase inhibition or calcium mobilization.⁶⁹ For example, IBMX (3-isobutyl-1-methylxanthine, **43**) is more potent as an inhibitor of calcium-independent phosphodiesterases than as an adenosine antagonist. It has been proposed that IBMX is a locomotor depressant, rather than a stimulant, as is caffeine, because of its potency as an inhibitor of Ca²⁺-independent phosphodiesterases (IC₅₀: 16 μM).⁷⁰

Affinities for representative xanthines are given in Table IV. Whereas the prototypical xanthines, caffeine (**45**, 1,3,7-trimethylxanthine) and theophylline (**38**, 1,3-dimethylxanthine), have only moderate potency and are nonselective, combined substitutions at the 1-, 3-, and 8-positions can dramatically increase affinity, as well as impart subtype selectivity. Xanthine itself has very low affinity, which stresses the importance of the alkyl substituents at positions 1 and 3.^{71,72} Affinity increases with increasing chain length, the rank order of potency being methyl < ethyl > *n*-propyl < *i*-butyl (compounds **38–41**) at both A₁ and A₂ receptors.⁷³ Larger hydrophobic substituents may also be accommodated by the receptor, as illustrated by the relatively high potency of 1,3-dibenzylxanthine (**42**, A₁: 2 μM; A₂: 14 μM).⁷³ Bulk tolerance at the 3-position is more limited in the A₂ receptor than in the A₁ receptor. This is evident with the highly potent and 1740-fold selective compound J-BW-A844U (**63**, 1-propyl-3-(2-iodo-3-aminophenethyl)-8-cyclopentylxanthine). Its parent compound CPX (**62**, 1,3-dipropyl-8-cyclopentylxanthine) is equipotent, and is 740-fold A₁ selective.⁸

Substitution at the 7-position usually decreases affinity. Caffeine is threefold less potent than theophylline, and more bulky 7-substituents in most cases result in considerably decreased affinity.^{71,74} One exception is 7-benzyltheophylline (**48**), which is twofold more potent than theophylline at A₁ receptors.⁷⁵ Also, replacement of N7 of 8-phenyltheophylline by sulfur (resulting in 4,6-dimethyl-2-phenyl-5,7-dioxothiazolo[4,5-*d*]pyrimidine) drastically reduces affinity.⁷⁶

Modifications at the 1-, 3-, and 7-positions differentiate to some extent between A₁ and A₂ receptors. Methyl substitution at N7 appears to be less unfavorable for interaction with A₂ receptors than it is for A₁ receptors, whereas larger 1-alkyl substituents enhance A₂ affinity more than A₁ affinity. On the other hand, substitutions at the 3-position may be more favorable for A₁ affinity.⁷³ This has allowed the development of some moderately A₂-selective xanthine derivatives, albeit of modest potency, e.g., DMPX, 1-propargyl-3,7-dimethylxanthine (**47**, A₁: 45 μM; A₂: 16 μM).⁷³ The lack of potent and selective A₂ receptor antagonists still remains a major obstacle in the characterization of adenosine A₂ receptor pharmacology.

The greatest boost in affinity comes with substitutions at the 8-position. 8-Phenyltheophylline (8-PT, **52**) is 100- and 30-fold more potent at A₁ and A₂ receptors, respectively, than theophylline.⁷² This effect is additive with the effects of 1- and 3-substitution. *Meta* substituents and large *ortho* substituents on the phenyl ring are not well tolerated, but smaller electron-donating *ortho* substituents and *para* substituents may further enhance affinity. In a series of 8-phenylxanthines, PACPX (**53**, 1,3-dipropyl-8-(2-amino-4-chloro)phenylxanthine) has optimal A₁ affinity and moderate A₁ selectivity (A₁: 2.5 nM; A₂: 92 nM).^{19,72} Even very large *para* substituents are accepted by the receptor,⁷⁷ but charged groups (e.g., sulfonate, carboxylate) considerably reduce affinity and may abolish A₁

selectivity.⁷⁸ Thus 8-*para*-sulfophenyltheophylline (8-PST, **56**), which is often used as a peripheral-only antagonist, has relatively low affinity (A_1 : 4.5 μ M; A_2 : 6.3 μ M).⁷⁸ If separated by a considerable distance from the ring through a chain, the problem may be overcome, as exemplified by XAC (**57**), the prototypical xanthine functionalized congener.⁷⁷ XAC has led to a large number of molecular probes for the labeling of A_1 adenosine receptors (see below).

Affinity and selectivity for the A_1 receptor is enhanced even further by 8-cycloalkyl substitution.^{79–83} As mentioned, CPX (**62**)—which combines 1,3-dipropyl substituents with an 8-cyclopentyl group—is both highly potent and 740-fold selective (A_1 : 340 nM).⁸ The 8-cycloalkyl substituents that are conducive to A_1 selectivity, principally 8-cyclopentyl and 8-cyclohexyl, are limited in the substitutions possible that still retain nanomolar A_1 affinity. Examples of substituted cycloalkyl compounds that are potent and selective are KF15372 (**64**), which is even more potent and selective than CPX at guinea pig A_1 receptors⁸³, KFM 19 (**65**), a potent A_1 -selective compound with sufficient aqueous solubility to display good bioavailability, currently under development as a potential cognition enhancer⁸⁴, and KW 3902 (**66**, A_1 : 1.3 nM; A_2 : 380 nM), which is being developed for human use as a diuretic and renal protective agent.⁸⁵ On the other hand, 8-[*trans*-(acetamidomethyl)cyclohexyl]-1,3-dipropylxanthine (**67**) has surprisingly high potency at A_2 receptors (K_i for antagonism of adenylate cyclase activity in human platelets is 20 nM) versus A_1 receptors in rat adipocytes (K_i : 8 nM).⁸²

Few data on substitutions at other positions are available. Sulfur substitution of O^6 decreases affinity, whereas substitution of O^2 has little effect,⁸⁶ suggesting that the latter carbonyl oxygen is probably not involved in a hydrogen bond with the receptor, since sulfur is a very weak hydrogen bond acceptor. Data on 9-substitution are scarce, but suggest that this is probably detrimental for affinity.^{71,87}

More extensive alterations of the basic xanthine structure that have been studied include the following. Enlargement of the six-membered pyrimidinedione ring to a seven-membered diazepinedione ring leads to compounds that are weaker adenosine antagonists than the corresponding xanthines, possibly because of the lack of planarity of this ring system.⁷⁵ Of the several classes of mesoionic derivatives of xanthines that have been synthesized, none seems to include any particularly potent compounds.^{76,88} The insertion of an extra benzene ring between the five- and six-membered rings of xanthines leads to benzo-separated xanthines, some of which have appreciable affinity.⁸⁷

Xanthine-7-ribosides may be regarded as hybrids of xanthines and adenosine. They are slightly less active than the corresponding xanthines. Despite the presence of the ribose moiety that is essential for agonist activity, these compounds act as antagonists, not agonists.⁸⁹ Slight modifications of the ribose group of adenosine (e.g., replacement of the 5'-hydroxyl group by a methyloxycarbonyl group²⁰) or an N^6 -substituted adenosine [e.g., N^6 -cyclohexyl-2',3'-dideoxyadenosine (ddCHA)⁶⁴], may also result in (weak) antagonists, stressing the importance of an intact ribose moiety for potent activity as adenosine agonists.

Solubility of antagonists (both xanthines and nonxanthines) is a major point of concern. Whereas hydrophobic substituents may greatly enhance affinity, they also decrease water solubility dramatically, and the derivatives that have good solubility, like 8-PST, are not particularly potent. Bruns, in developing a ratio concept relating solubility to receptor affinity, has proposed that the greater the ratio, the more optimal the compound for *in vivo* studies.⁹⁰ Ratios 1000 have been suggested as sufficient for *in vivo* activity.

3. Nonxanthine Heterocycles as Adenosine Antagonists—In addition to the hundreds of xanthines that have been synthesized and studied as antagonists at A₁ and A₂ receptors, numerous nonxanthine antagonists have been identified in the last decade. In sharp contrast to adenosine agonists, for which structural requirements are very strict, adenosine antagonist activity is found in numerous, rather diverse chemical classes. A comprehensive list of classes of adenosine antagonists has been published elsewhere.³ Structures and affinities for some representative compounds are shown in Table V. For the most part, the SAR of these classes have not been exploited by medicinal chemists for the optimization of potency, selectivity, and physical properties. Also, *in vivo* data for most of these classes of nonxanthine antagonists are lacking.

In general, adenosine antagonists are planar, aromatic (or having a high π -electron density), nitrogen-containing heterocycles.⁹¹ The most potent representatives are 6:5-fused bicyclic or 6:6:5-fused tricyclic heterocycles. An interesting exception to this general rule has recently been published.⁹² The naturally occurring benzo[*b*]furan (**68**) contains an O-rather than an N-6:5-fused heterocycle, yet maintains considerable potency in binding to bovine A₁ receptors (*K_i*: 17 nM). This compound may prove to be a lead to other nonxanthine, non-nitrogen containing adenosine receptor antagonists. Hydrophobic substituents are essential for high affinity in nonxanthine (as well as xanthine antagonists), which makes them quite insoluble in water. This constitutes a major problem for testing them *in vivo*, as discussed in Sec. II.A.2.

Adenine serves as a template for two classes of nonxanthine antagonists of interest: the N⁶-substituted 9-methyladenines^{93,94} and the 2-phenyl-7-deazaadenines.⁹⁵ For the 9-methyladenines, SAR at the N⁶ position parallels the SAR for similarly N⁶-substituted adenosines to a large degree, suggestive of similar binding modes for 9-methyladenines and adenosine-derived agonists.³ The recently described N-0861 [(±)N⁶-endo-norbornyl-9-methyladenine; **69**] is potent and highly A₁ selective (A₁: 10 nM; A₂: 6100 nM in bovine brain).⁹⁶ A related compound, N⁶-butyl-8-phenyladenine (**77**; A₁: 170 nM),⁹⁷ might be interpreted as an intermediate between an N⁶-substituted 9-methyladenine and 8-phenyltheophylline, giving rise to speculation about similar binding modes for agonists and xanthine antagonists.

With 7-deazaadenines,⁹⁵ a 2-phenyl substituent enhances affinity considerably, and the influence on affinity of a 9-substituent is highly dependent on its stereochemistry. Thus the *R*-isomer of the A₁-selective 7-deazaadenine, ADPEP (**74**; A₁: 4.7 nM, A₂: 3710 nM), is 35-fold more potent than its *S*-isomer. The analogy with the high stereoselectivity of agonists substituted at the N⁶-position again suggests that the 9-substituent of 7-deazaadenines might occupy the same receptor region.⁹⁵

Tricyclic nonxanthine antagonists with potencies in the lower nanomolar range include the triazoloquinazolines (e.g., CGS 15943, **70**),^{98,99} the triazoloquinoxalines (e.g., the over-3000-fold A₁-selective CP 68,247, **72**, and the moderately A₂-selective CP 66,713, **71**)^{100,101} and the imidazoquinolines (e.g., CPPIQA, **73**).¹⁰² Both the structures and the SAR profiles of these three classes show some interesting similarities. Whereas the basic structures (Fig. 1) in all three classes appear to be nonselective or slightly A₂-selective, substitution at the exocyclic amino group (R₁) may impart both high affinity at A₁ receptors and A₁ selectivity—reminiscent of N⁶-substitution in agonists—in both triazoloquinoxalines^{100,101} and imidazoquinolines.¹⁰² Surprisingly, this does not seem to hold for the triazoloquinazolines.⁹⁹ In the triazolo-quinazoline^{100,101} and the imidazoquinoline¹⁰² series, an exocyclic aromatic or cycloalkyl substituent (R₂) may also contribute to high affinity, similar to the 8-position of xanthines. Analogous substitution for triazoloquinoxalines is not possible without quaternization of the ring system. In both

triazoloquinazolines¹⁰¹ and triazoloquinoxalines,^{98,99} highest affinity is associated with a chloro substituent (R₃) at the same position of the benzene ring (similar substitutions have not been explored for the imidazoquinolines).

Finally, apart from N⁶-butyl-8-phenyladenine, two other potentially interesting nonxanthine antagonists have emanated from a broad screening program for adenosine antagonists at Parke-Davis.⁹⁷ APPP (75), a 7-deaza-8-azaadenine derivative that is closely related to the 2-phenyl-7-deazaadenines described above, has high potency at both subtypes (A₁: 23 nM, A₂: 35 nM). HTQZ (76, A₁: 3100 nM, A₂: 120 nM) is 26-fold selective for A₂ receptors in rat binding assays; but disappointingly, this selectivity is much less in other species and *in vivo*. Because of their poor solubility, the SAR of neither compound has been further investigated.

B. Molecular Modeling Approaches to Receptor and Ligand Structure

Molecular modeling and computational chemistry are becoming increasingly useful tools in determining the active conformation of known receptor ligands and in the prediction of novel, active compounds. In principle, two roads can be taken. When the structure of the target protein and the position of the active site are known, it is theoretically possible to rationally design compounds that will specifically interact with the receptor. This approach has been taken in the case of enzymes that can be purified to such an extent that structure elucidation through x-ray crystallography or NMR methods becomes feasible. This goal has not yet been reached for any G-protein-coupled receptor. However, structural similarities between the G-protein-coupled receptor family and bacteriorhodopsin, of which the structure has been resolved, combined with data on the genetic engineering of specific amino acid residues, have permitted the building of convincing models of the ligand binding site of a number of receptors.¹⁰³ This should also be feasible for adenosine receptors now that their amino acid sequence has been elucidated (see below).

When little is known about the structure of the receptor protein, much information can still be gleaned from studying the active conformations of receptor ligands, both agonists and antagonists. For instance, MOPAC (AM1) calculations of the rotational freedom around the glycosidic bonds of adenosine and xanthine-7-ribosides¹⁰⁴ have confirmed that adenosine binds to the receptor in the *anti* confirmation.²⁰ Molecular modeling studies have been effectively used to make both qualitative predictions for the synthesis of subtype-selective agonists¹⁰⁵ and novel antagonists,^{91,102,106} as well as to quantitatively predict the affinity of compounds in a series of agonists.¹⁰⁷

N⁶ substituents may greatly influence the affinity and subtype selectivity of adenosine-derived agonists. Although the majority of the N⁶ substituents imparts A₁ selectivity, there are certain subdomains where the A₂ receptor appears to have more bulk tolerance than the A₁ receptor, as becomes evident from a model developed by Ortwine *et al.*, illustrated in Fig. 2(a).¹⁰⁵ These subtle differences between the N⁶-regions of A₁ and A₂ receptors were exploited by researchers at Parke-Davis to develop the first A₂-selective agonists.¹⁰⁸ A similar model of the N⁶-region of the A₁ receptor, based on the preferred conformations of 26 N⁶-substituted agonists, was described by van Galen *et al.*¹⁰⁷ The original model of the N⁶-region of Kusachi, based on the respective contributions of the various parts of the N⁶-phenylisopropyl substituent of R-PIA,^{49,109} was extended with a number of new subdomains [Fig. 2(b)]. The contribution (either positive or negative) to affinity of each of these subdomains could be determined by comparing pairs of agonists that differ in the occupation of only one subdomain. Thus it was possible to accurately predict the affinity of other agonists that were not included in the basis set. It has been suggested by Olsson that N⁶ and C2 substituents of adenosine derivatives may (partly) occupy the same receptor domain.¹¹⁰ Molecular modeling would be the technique of choice to evaluate this hypothesis.

Similar methods have been used to explore the C8-region of xanthine antagonists.¹¹¹ One of the striking results of this work indicates that a phenyl substituent at C8, in contrast to the earlier assumption that this substituent would be coplanar with the xanthine ring,^{72,98} actually forms a dihedral angle with the heterocycle of 220 degrees. This angle cannot be attained (because of steric hindrance) by 7-methyl-substituted 8-phenylxanthines and 8-cycloalkylxanthines (e.g. **60**), which readily explains the unexpectedly low affinity¹¹² of these compounds.

Two different models for the antagonist binding site of the A₁ receptor have been proposed.^{91,102,106} The first⁹¹ was constructed by optimizing the overlap in molecular electrostatic potential (EP) between a number of adenosine antagonists (Fig. 3). In all antagonists investigated, one central Y-shaped area of negative EP can be discerned, as well as two domains of positive EP. Further-more, all antagonists share a nitrogen atom capable of accepting an H bond at position 7 and two domains where hydrophobic substitution in general enhances affinity. This model has successfully predicted the potency of imidazo-[4,5-c]quinolin-4-amines as adenosine antagonists. For example, CPPIQA (**73**), which conforms with the required EP distribution and which has two hydrophobic substituents at the appropriate sites (a 2-phenyl and a 4-cyclopentyl group), has an affinity of 1.5 nM in binding to A₁ receptors in calf brain and of 10 nM in rat brain.¹⁰² It is likely that agonists and antagonists bind to the same site of the receptor.¹¹³ According to the EP distribution described in this first model, adenosine and xanthine antagonists would be expected to bind upside down with respect to each other, i.e., N1, N3, N7, and N9 in adenosine would be superimposable with C2, C6, N9, and N7, respectively, in xanthines. Peet *et al.* have proposed a different orientation¹⁰⁶ of adenosine with respect to the xanthines. On the basis of the remarkable parallels between N⁶ substitution in agonists and C8 substitution in antagonists,¹⁹ it was suggested that N⁶ and C8 might occupy the same receptor domain. This model correctly predicts the stereoselectivity of xanthines with C8-phenylisopropyl substituents, similar to *R*- and *S*-PIA (compounds **50** and **51**). It is not yet clear which of the two ways of fitting adenosine and xanthines is more likely to reflect reality. Arguments in favor of the first (flipped) model are the affinity of theophylline-7-riboside, but not theophylline-9-riboside,¹⁰⁴ and a steric fit that is superior to the N⁶/C8 model. The parallels between the N⁶ and C8 substituents, including the preferred orientations of these substituents,¹¹⁴ argue in favor of the second model. One argument against the N⁶/C8 model is the contrasting effects on affinity observed with lipid conjugates of ADAC and XAC, the prototypical agonist and antagonist functionalized congeners for adenosine receptors.¹¹⁵

A third model that integrates the respective orientations of agonists and antagonists has also been proposed.¹¹⁶ In this model, it is assumed that there are three principal binding domains: one for the heterocycle, a ribose domain, and a single hydrophobic domain. N⁶ substituents of A₁-selective agonists, C2 substituents of A₂-selective agents, and C8 substituents of xanthines would then all bind to this same hydrophobic domain. This, however, is at odds with the fact that there clearly are several distinct regions in the receptor where hydrophobic substituents may independently enhance affinity, e.g., the 1-, 3-, and 8-positions in the xanthines³ and the 2- and 4-positions in imidazo[4,5-c]quinolinamines.¹⁰² Furthermore, according to this model, the ribose moiety should bind with significantly different orientations to the A₁ and A₂ receptors, respectively, despite close analogies between the SAR profiles for ribose substitutions at A₁ and A₂ receptors.^{20,63} Therefore new compounds predicted on the basis of this model should first be synthesized and biologically evaluated to lend it credibility.

C. Agents Acting Indirectly on Adenosine Receptors

Several classes of agents that stimulate adenosine receptors indirectly, causing the actions of locally released adenosine to be enhanced, have been described. These include nucleoside transport inhibitors, allosteric enhancers, the “site and event specific” potentiator AICA riboside, and inhibitors of adenosine deaminase (ADA). The potential therapeutic advantage of such an approach is that effects will be limited to sites where adenosine levels are elevated, i.e., in tissues with high metabolic rates or inadequate oxygen supply. Thus the beneficial effects of adenosine receptor stimulation may be exerted without unwanted adenosine effects elsewhere. Potential applications include stroke and myocardial ischemia. The structures of some representative compounds are shown in Fig. 4.

Nucleoside transport inhibitors prolong the actions of locally released adenosine by interfering with its reuptake into the cell. Although adenosine is the preferred substrate, many other nucleosides may also be transported, including uridine and inosine.¹¹⁷ A classic example of a transport inhibitor is dipyridamole (**78**), a broadly used coronary vasodilator and platelet aggregation inhibitor. Its effects may at least be partly due to inhibition of adenosine reuptake, thus enhancing its actions on coronary artery¹¹⁸ or platelet¹¹⁹ A₂ receptors. Newer uptake inhibitors include miflazine, lidoflazine, and R75231, developed by Janssen Pharmaceuticals.^{120,121} Of these compounds, R75231 (**79**) has a very interesting pharmacological profile in that it combines very good biological availability with a potent and long duration of action *in vivo*. It shows considerable promise as a cardioprotective agent.¹²¹ Miflazine (**80**), which, unlike R75231, can cross the blood–brain barrier, has been shown to be an effective hypnotic in the dog.¹²² Some inosine derivatives are also highly potent nucleoside transport inhibitors. Nitrobenzylthioinosine (NBI) is often used as a radioligand and pharmacological tool (K_d : 0.65 nM in binding to uptake sites in calf lung).¹²⁰

A newer approach is allosteric enhancement, i.e., facilitation of binding of adenosine to the receptor through an allosteric mechanism, much like the actions of benzodiazepines on GABA receptors.¹²³ Several 2-amino-3-benzoylthiophenes stimulate binding of the agonist radioligand [³H]CHA, but not of the antagonist [³H]CPX, to A₁ receptors from rat brain, indicating that this action is specific for the agonist conformation.¹²⁴ SAR studies¹²⁵ suggest that an intramolecular hydrogen bond between one of the amino hydrogens and the carbonyl oxygen is essential for activity. Substitutions at the 3- and 4-positions may increase activity. These compounds were originally developed from a series of nonxanthine adenosine antagonists, and they all have some residual antagonist activity. Since the SAR profiles for allosteric enhancement and antagonism differ considerably, it has been possible to develop compounds with preferential enhancing activity. Thus PD 81,723 (**81**, 2-amino-4,5-dimethyl-3-thienyl-[3-thienyl-[3-(trifluoromethyl)-phenyl]methanone) showed the most favorable enhancement/antagonism ratio in a series of benzoylthiophenes. Allosteric enhancers for the A₂ receptor have also been described, as exemplified by PD 120,918 (**82**).¹²³

AICA riboside (**83**, 5-aminoimidazole-4-carboxamide riboside) is an intermediate in the biosynthesis of purines which greatly increases adenosine release from energy-deprived tissue. It has been shown to reduce the damage after myocardial¹²⁶ or cerebral¹²⁷ ischemia. The mechanism through which adenosine is generated in tissues with a high metabolic rate is still unclear.¹²⁸ The immunosuppressant actions of the anticancer agent methotrexate have also been ascribed to elevated adenosine levels due to an increase in AICA riboside, resulting from the inhibitory action of methotrexate on AICA riboside transformylase.¹²⁹

The ADA inhibitor, deoxycytidine (**84**), has been reported to elevate adenosine levels in ischemic brain, but a neuroprotective effect has not yet been unequivocally shown.^{130,131}

Thus the therapeutic potential of ADA inhibitors is questionable, all the more so because such treatment may prove to be hazardous in view of the fatal dysfunction of the immune system associated with congenital ADA deficiency.¹³²

III. RECEPTOR STRUCTURE: AFFINITY LABELING AND CHEMICAL MODIFICATION STUDIES

The A₁ and A₂ receptors have been affinity labeled using agonist and antagonist molecular probes, containing either ³H or high-specific activity, carrier-free¹²⁵I for detection of the labeled receptor on electrophoretic gels. Two photochemical approaches have been used to form a covalent bond between the ligand and the adenosine receptor: (i) indirect photoaffinity crosslinking, in which a radiolabeled ligand containing a chemically reactive group, such as an aryl amine, is first bound to the receptor, and then exposed to a bifunctional crosslinking reagent, such as SANPAH [N-succinimidyl-6-(4-azido-2-nitrophenylamino)hexanoate]. SANPAH is a chemical (through acylation of amines) and photochemical (through the generation of a reactive nitrene) crosslinker; (ii) direct photoaffinity labeling, in which the ligand is preactivated for photolysis (e.g., with an aryl azido group), leading to reaction with the receptor. Direct photoaffinity labeling generally affords a 5- to 10-fold higher percentage of available receptor being labeled, compared to photoaffinity crosslinking, which typically results in labeling of only 1-2% of the receptor.

The A₁ receptor was labeled by photoaffinity crosslinking by Stiles *et al.*¹³³ using the ligand [¹²⁵I]APNEA (**12**) in combination with SANPAH. In addition, the amine of iodo-APNEA has been converted to an azido group via diazotization and treatment with sodium azide for direct photoaffinity labeling.¹³⁴ The resulting azido-iodo-APNEA was then bound to the receptor and photolyzed, resulting in covalent attachment. In each case, a protein of molecular weight 36,000 was labeled with ¹²⁵I and detected as a typically wide band on SDS gel electrophoresis. Choca *et al.*¹³⁶ used the azide derived from iodo-N⁶-aminobenzyladenosine (¹²⁵I-ABA, **10**) for photoaffinity labeling of the receptor. Klotz *et al.*¹³⁶ utilized 2-azido-N⁶-*p*-hydroxyphenylisopropyl-adenosine (¹²⁵I-AHPIA, **31**) for this purpose. Lohse *et al.*,¹³⁷ in a study that revealed spare A₁ receptors in adipocytes, showed that AHPIA caused a persistent, irreversible activation of the receptor and a resultant decrease in cyclic AMP Levels. ¹²⁵I-AHPIA also irreversibly blocks A₂ receptors, but at higher concentrations.¹³⁸

Xanthine functionalized congeners have also been used for the photoaffinity labeling of adenosine receptors.^{139,140} The A₁ receptor was labeled using an antagonist ligand, PAPA-XAC (**58**), by Shies and Jacobson.¹³⁹ XAC (xanthine amine congener, **57**) was coupled to a prosthetic group, the *para*-aminophenylacetyl (PAPA) group, for radioiodination. This group contained an aryl amine designed for the dual purpose of photoaffinity cross-linking and photoaffinity labeling via the corresponding azide. Patel *et al.*¹⁴¹ photoaffinity labeled A₁ receptors using another xanthine aryl amino derivative, ¹²⁵I-azido-BW-A844U.

It appears that both agonist and antagonist photoaffinity labeling of A₁ receptors occurs in the same region of the peptide sequence of the receptor. Barrington *et al.*¹⁴² conducted photoaffinity labeling of bovine brain A₁ receptors using azido-derivatized agonists (AZPNEA) and antagonists (preformed PAPA-XAC-SANPAH and azido-PAP-XAC) in parallel, followed by partial peptide mapping using proteolytic enzymes. Peptide fragments of identical molecular weights were observed when the proteolysis was carried out following photolabeling and denaturation. Alternately, when the agonist and antagonist ligands were first bound to the receptor in membranes, followed by limited proteolysis and then irradiation, distinct and different peptide fragments were obtained. Thus there is biochemical evidence for different conformational states for the agonist-occupied compared

to the antagonist-occupied **A₁** receptors. Agonists and antagonists incorporate into the same subunit of the receptor protein, yet the agonist- and antagonist-occupied receptor conformations have different accessibility to proteolytic enzymes at specific cleavage sites. The difference in conformation likely relates to the ability of an agonist to initiate a transmembrane signal, whereas an antagonist occupies the receptor but does not produce a physiological effect.

The first labeling of the **A₂** receptor was accomplished using PAPA-APEC (**36**), an **A₂**-selective agonist, by Barrington *et al.*¹⁴⁰ PAPA-APEC, derived from the **A₂**-selective adenosine agonist CGS21680, contains a long chain at the purine 2-position. ¹²⁵I-PAPA-APEC binds reversibly and in a saturable fashion to bovine brain **A₂** receptors with a *K_d* value of 1.5 nM. In a subsequent step, ¹²⁵I-PAPA-APEC was cross-linked to the receptor, either: through photoaffinity cross-linking using SANPAH¹⁴⁰ or by conversion of the amine to an azide.¹⁴³ The **A₂** binding site in bovine brain striatal membranes was found to be a single glycoprotein of molecular weight 45,000. The appropriate **A₂** pharmacological properties were observed both for the reversible binding of ¹²⁵I-PAPA-APEC and for the protein labeling. Similarly, the molecular weights of **A₂** receptors in rabbit striatum (MW 47,000) and in human striatum, rat PC12 cells, and frog erythrocytes (all MW 45,000) were determined.^{144,145} Proteolytic cleavage of the rabbit and human **A₂** receptors was observed. Treatment with glycosidases revealed a heterogeneous mixture of high mannose type and complex type carbohydrates to be present among bovine **A₂** receptors.¹⁴⁶ In contrast, **A₁** receptors are glycosylated only with complex type carbohydrates.^{147,148}

Chemical affinity labeling occurs when a covalent bond between a reactive ligand and a specific nucleophile on the receptor protein occurs spontaneously. Purine functionalized congeners have been coupled to bifunctional alkylating and acylating cross-linking reagents for this purpose.¹⁴⁹ ADAC, APEC, and XAC were condensed with bifunctional alkylating and acylating cross-linking reagents, such as *m*- or *p*-phenylene diisothiocyanate (DITC), to provide a chemically reactive site (e.g., an isothiocyanate group, NCS) on the ligand. The *p*-DITC conjugates of ADAC (e.g., **20**) and XAC,¹⁴⁹ and the corresponding *meta* isomers, selectively inactivated adenosine receptors during incubations of brain membranes with submicromolar concentrations of the purine. Tritiated DITC-XAC isomers⁵⁰ specifically labeled the receptor protein (MW 38,000), as detected by 5DS gel electrophoresis. *m*-DITC-XAC (**59**) was used by Dennis *et al.*¹⁵¹ to detect spare **A₁** receptors in the guinea pig AV node. Analogous APEC derivatives, including the *p*-DITC conjugate (**37**), appear to inhibit **A₂** receptors irreversibly.¹⁵²

Among the goals in the design of purine functionalized congeners has been the isolation of receptors by affinity chromatography.⁷⁷ Since the primary amino group of XAC is located at an insensitive site on the molecule, at a distance from the pharmacophore, it is possible to couple the xanthine to large molecules, including insoluble polymeric matrices, without losing the ability to bind to the receptor. XAC has been utilized by Nakata¹⁵³ (with rat brain) and by Olah *et al.*¹⁵⁴ (with bovine brain) for the complete purification of cortical **A₁** receptors after solubilization.

IV. RECEPTOR STRUCTURE: MOLECULAR BIOLOGY APPROACHES

A. Cloning of **A₁** and **A₂** Adenosine Receptors

1. Introduction—Recently, RDCS, a cDNA fragment from canine thyroid previously amplified via the polymerase chain reaction (PCR) and detected by virtue of sequence homology to other G-protein-linked receptors, but of unknown identity,¹⁵⁵ was identified as that coding for the **A₂** receptor sequence. When expressed in COS7 cells, the RDC8 protein specifically binds [³H]CGS21680, indicative of the **A_{2a}** subtype.⁵⁶ A second cDNA

fragment, RDC7, with a high degree of homology to RDC8, was found to code for the A₁ adenosine receptor.¹⁵⁶ PCR methods have enabled the subsequent cloning of the rat^{157,158} and bovine A₁ receptors (Stiles, unpublished). Notwithstanding the substantial species differences in SAR for both agonists and antagonists, the actual differences in sequence are rather subtle, and the interpretation of the sequence data is virtually identical for all three species. In the following discussion, we will refer to the canine receptors, unless otherwise noted. Interestingly, the sequence of another G-protein-linked receptor apparently closely related to the adenosine receptors was recently reported.¹⁵⁹ It is presently unknown whether this receptor is activated by adenosine.²⁰²

The analysis of these adenosine receptor sequences may benefit from the substantial body of information on the secondary structures and the functions of specific receptor domains of other G-protein-linked receptors that have been sequenced earlier (Refs. 160–164 and references therein). These receptors include the α - and β -adrenergic receptors, the muscarinic receptors and the visual receptor rhodopsin. Other representatives of this class include 5-HT and dopamine receptors and a series of membrane proteins that is thought to represent a large class of olfactory receptors.¹⁶⁵

The amino acid sequences of all these receptors display a typical pattern of seven stretches of 20–28 hydrophobic residues, which are thought to traverse the cell membrane as right-handed α -helices. This structure assignment was first proposed on the basis of the hydropathy profiles of G-protein-linked receptor sequences, which are very similar to the profile of bacteriorhodopsin.¹⁶⁴ Bacteriorhodopsin is a proton pump that is not linked to a G protein, but resembles rhodopsin inasmuch as it is also activated by the isomerization of retinal following irradiation. Bacteriorhodopsin is present in the membrane of *Halobacterium halobium* in a two-dimensional crystalline lattice, which has allowed a detailed study of its tertiary structure with the aid of electron diffraction techniques.¹⁶⁶ Evidence for the seven transmembrane domain structure assignment of the G-protein-coupled receptors has been obtained from various methods, including binding of antibodies raised against putative extracellular domains, proteolysis studies, and affinity labeling.¹⁶⁴

2. Alignment with other G-Protein-Coupled Receptors and Secondary

Structure—An alignment of both adenosine receptor subtypes and some other G-protein-linked receptors is shown in Fig. 5. The seven helical domains, as determined by the Kyte–Doolittle method,¹⁶⁷ are indicated by horizontal bars (H I to H VII). They are connected by three extracellular loops (E I to E III) and three cytoplasmic loops (C I to C III; refer to Table VI for numbering). The N terminus is located on the extracellular side and the C terminus on the cytoplasmic side.

This alignment is slightly different from the one presented by Libert *et al.*,¹⁵⁵ because we did not allow for gaps within the transmembrane domains (differences occur in H I, H IV, H V and H VII). The rationale behind this limitation is that those residues that are conserved throughout a family, and certainly between closely related receptors like both adenosine receptor subtypes, are likely to serve similar functions. The introduction of only a one-residue gap would already cause a major spatial reorientation (a 100° shift) of the residues to follow. It should be noted that the Kyte–Doolittle algorithm can at best yield an approximation of the transmembrane domains, so future adjustments may be warranted.¹⁶⁸ Since it is not likely that solid (x-ray crystallographic or 2D NMR) structural data on G-protein-coupled receptors will become available within the next few years, indirect evidence, e.g., from proteolysis studies or studies with antibodies directed against putative extracellular domains, will be needed to assess the correctness of these assignments.

The prediction of the secondary structure of the extracellular and cytoplasmic domains may be even more elusive. Two often-used algorithms, the Chou–Fasman¹⁶⁹ and Garnier–Osguthorpe–Robson¹⁷⁰ methods, give dissimilar results in many cases (Table VI), but they agree on the β -sheet nature of C II, the occurrence of turns in E III and the (partially) α -helical nature of C III (both subtypes). Furthermore, there is agreement on the occurrence of turns in E II of the A₂ receptor.

With respect to the orientation of the seven helices, it has been noted in many other receptors that the transmembrane domains are often amphipathic in nature, i.e., hydrophobic and hydrophilic amino acids line up on opposite parts of the helix. It is assumed that the hydrophobic face is directed toward the lipophilic cell membrane, whereas the more hydrophilic parts face each other, forming a relatively hydrophilic center.¹⁶⁰ A so-called helical wheel representation of both subtypes (modeled as right-handed α helices) is shown in Fig. 6. The seven helices are placed in the same circular orientation as the one that has been crystallographically determined for bacteriorhodopsin.¹⁶⁶ Most of the helices indeed show dear hydrophilic and hydrophobic faces, and our orientation of these helices is based on maximum interaction of the hydrophobic face with the membrane. This does not apply for H III and H V. These residues were oriented so that Val⁸⁷, Phe¹⁸⁵, and Trp¹⁸⁸ (A₁ numbering) face the inside. These residues are homologous to Asp¹¹³, Ser²⁰⁴, and Ser²⁰⁷ in the β_2 receptor, which are considered essential for ligand binding.¹⁶¹ A model for the transmembrane topology of both subtypes, based on the above observations, is shown in Fig. 7.

B. Receptor Characteristics and Posttranslational Modifications

1. Receptor Size—The apparent molecular weight of adenosine receptors has been experimentally determined by various methods. Hydrodynamic analysis points to a mass of ca. 250–280 kDa.^{171,172} This likely represents a receptor–G–protein–detergent complex, although formation of receptor aggregates cannot be ruled out. Radiation inactivation analysis with the antagonist radioligand [³H]CPX has yielded a value of 58 kDa for the A₁ receptor, decreasing to 33 kDa in the presence of guanine nucleotide. The lower value probably reflects the receptor protein, whereas the higher value also includes part of the G protein.¹⁷² Various groups have determined apparent molecular weights with the aid of SDS-polyacrylamide gel electrophoresis, using photoaffinity or irreversibly acylating agents. Reported values for the A₁ receptor vary between 34–38 kDa between groups^{136,147,150,153,173}; the A₂ receptor has been reported to be 45–47 kDa.^{140,144} In our opinion, these variations reflect experimental margins of error, differences in technique, and/or differences in glycosylation rather than in receptor structure. Interspecies differences do not seem to account for variations in molecular weight.¹⁷⁴ Also, the apparent molecular weight is the same in the presence or absence of disulfide-reducing agents,¹⁷⁴ which argues against the existence of (disulfide-linked) receptor aggregates. Experiments like these are usually performed in the presence of protease inhibitors, which makes it less likely that the labeled peptides are products of partial proteolysis of the receptor protein.

The observed molecular weights are quite low in comparison to other G-protein-linked receptors, which in general have masses around 60 kDa. This has given rise to an ongoing dispute within the adenosine receptor field, either questioning whether these values truly represent the binding subunit or whether adenosine receptors might not belong to the same general class of G-protein-linked receptors with seven transmembrane sections. However, the masses calculated on the basis of the published sequences (36399 and 45060 Da for A₁ and A₂ receptors, respectively) are in excellent agreement with the experimentally obtained ones (although it should be realized that the contributions of any posttranslational modifications are not included in the calculated values). The low masses—compared to

related receptors—result from a very short N terminus and a short third cytoplasmic loop, and in the case of the A₁ sequence also from a very short carboxy tail. The A₂ sequence shares its short C III loop and long carboxy tail with many other receptors that stimulate adenylate cyclase. However, the short C III loop of the A₁ receptor is uncharacteristic of receptors inhibitory to this enzyme.¹⁶²

2. Receptor Glycosylation—As reported by Libert *et al.*,¹⁵⁵ no potential glycosylation sites are present near the N terminus, in marked contrast to the other members of the G-protein-linked receptor family hitherto sequenced. Yet it is well established that both A₁ and A₂ receptors are N-glycosylated.^{143,147,148} Asparagine residues that are potential glycosylation sites are located on E II in both the A₁ and A₂ sequences (Table VII). Asn¹⁵⁹ is a potential substrate for glycosylation in the A₁ receptor. The rat A₁ receptor was reported to contain a single carbohydrate chain of the complex type.^{147,148} For the A₂ receptor, four glycosylation consensus sites were identified, of which only two are presumably on the extracellular side of the membrane and accessible for glycosylation (Asn¹⁴⁵ and Asn¹⁵⁴ on E II), the other two being located on the intracellular side (Asn³⁹ and Asn³⁴⁸). Glycosylation of the A₂ receptor is heterogeneous in nature, containing carbohydrate chains of both the complex and the highmannose types.¹⁴³ It is conceivable that this heterogeneity results from differential glycosylation of Asn¹⁴⁵ and Asn¹⁵⁴, respectively. However, Barrington *et al.*¹⁴³ have argued against glycosylation at two different sites, since treatment with endoglycosidase F—which cleaves the asparagine–glycan bond—results in a single cleavage product on SDS-PAGE, without intermediate bands. In addition, the effects of α -mannosidase and neuraminidase are not additive, further suggesting that the two types of carbohydrate chains do not reside on the same receptor. This contrasts with findings for the β_2 receptor, which appears to contain two distinct types of glycosylation.¹⁷⁴ A speculative explanation for there only being a single chain might be that the proximity of the two asparagine sites makes the addition of a second chain sterically hindered, once a bulky glycan group has been attached to the first asparagine. Also, the accessibility of Asn¹⁴⁵ to glycosylation may be influenced by the putative involvement of the adjacent cysteine residue in a disulfide bond (see below). Site-directed mutagenesis studies will have to show whether the proposed asparagine residues indeed are glycosylated.

3. Receptor Phosphorylation—Receptor phosphorylation has been implicated in the mechanism of both homologous and heterologous desensitization of a number of G-protein-linked receptors.¹⁷⁵ There are two types of kinases involved in these processes. Second-messenger activated kinases like protein kinase A (cAMP-dependent protein kinase; PKA) and protein kinase C (PKC) have been shown to phosphorylate, among others, α - and β -adrenergic receptors.¹⁷⁶ They seem to be involved in heterologous desensitization. Receptor-“specific” kinases, like β -adrenoceptor receptor kinase (β ARK) and rhodopsin kinase, phosphorylate their substrates only in the agonist-occupied state of the receptor and may mediate homologous desensitization.¹⁷⁷ Casein kinase II (CK2) phosphorylates a multitude of substrates,¹⁷⁸ among others the insulin receptor, but has so far not been implicated in the phosphorylation of G-protein-coupled receptors.

The A₁ and A₂ sequences display a number of consensus patterns for phosphorylation by PKA, PKC, or CK2 (Table VII). Furthermore, in common with many other receptors stimulatory to adenylate cyclase, the carboxyl terminus of the A₂ receptor is rich in serine and threonine, typical for phosphorylation by β ARK. This raises the question whether phosphorylation plays a role in adenosine receptor regulation. It has recently been shown that incubation of DDT₁MF-2 cells with the adenosine agonist *R*-PIA results in desensitization of A₁-mediated adenylate cyclase activity and in a concomitant 3–4-fold increase in incorporation of [³²P]orthophosphate in the 36-kDa band of the A₁ receptor protein.⁴² In the same cell type, treatment with *R*-PIA also resulted in desensitization of A₂

receptors, but this did not seem to be linked to PKA or PKC.⁴² The identification of potential phosphorylation sites for PKC, PKA, CK2, and β ARK warrants further study of the occurrence, mechanism, and role of adenosine receptor phosphorylation.

4. Acylation—In many G-protein-linked receptors, a cytosolic cysteine is situated at a distance of ca. 10–15 residues from the C terminus of H VII. For rhodopsin,¹⁷⁹ the β_2 receptor,¹⁸⁰ and the α_{2A} receptor,¹⁸¹ it has been shown that this residue is palmitoylated. It is thought that the palmitoyl group anchors part of the C terminal cytoplasmic tail to the membrane, thus creating a fourth cytoplasmic loop. In the human β_2 receptor sequence, replacement of this cysteine with a serine leads to a nonpalmitoylated receptor, which is impaired in its coupling to G_s . It was therefore speculated that the presence of the putative fourth cytoplasmic loop is important for interaction with the G protein.¹⁸⁰ A similar cysteine residue is present in the carboxy tail of the A_1 receptor (Cys309), but not the A_2 receptor. To date, posttranslational acylation of adenosine receptors has not been demonstrated.

5. Disulfide Bonds—Disulfide bonds between extracellular cysteines are probably involved in maintaining the integrity of the ligand binding site of the β_2 adrenoceptor¹⁸² and rhodopsin,¹⁸³ although these cysteines may not be part of the binding site itself. This is suggested by the susceptibility of ligand binding to dithiothreitol (DTT), a disulfide reducing agent, and by site-directed mutagenesis studies. In the β_2 receptor, the affinity for ¹²⁵I-CYP (cyanopindolol) is drastically reduced when any of the four extracellular cysteines, but not the three transmembrane cysteines, is replaced by a valine residue.¹⁸² Two of these residues (Cys¹⁰⁶ and Cys¹⁹⁰) have been shown to form a disulfide bond, and they are highly conserved throughout the G-protein-coupled receptor family (Fig. 5), suggestive of a similar role in all receptors. The analogous cysteines in the adenosine receptor sequences are Cys⁸⁰-Cys¹⁶⁹ (A_1) and Cys⁷⁷-Cys¹⁶⁶ (A_2), respectively. Extracellular cysteines are abundant in A_1 and especially A_2 receptors (7 and 11 cysteines, respectively). Other potential candidates for the formation of disulfide bonds include Cys²⁶⁰-Cys²⁶³ (A_1) and Cys⁷¹-Cys⁷⁴, Cys¹⁴⁶-Cys¹⁵⁹ and Cys²⁵⁹-Cys²⁶², or any combination thereof (A_2). A disulfide bridge between two cysteines four residues apart would likely stabilize the turns predicted for E II (A_2), E III (A_1 and A_2), and which might also occur in E I (A_2) (Table VI).

It has recently been shown that binding of the agonist [³H]JCGS 21680 and the antagonist [³]XAC to A_2 receptors of rabbit striatum is susceptible to treatment with the disulfide reactive reagents DTT, dithionite, and mercaptoethanol.¹⁵² This suggests that disulfide linkages indeed are involved in maintaining the structural integrity of the A_2 receptor.

Treatment of A_1 receptors at room temperature with N-ethyl maleimide (NEM), a free sulfhydryl modifying agent, does not affect antagonist binding,¹⁸⁴ (The influence of NEM on agonist binding is generally ascribed to an interaction with G_i , not the ligand binding site.) Hence, it is less likely that cysteines that are accessible to NEM and are involved in ligand binding, either direct or indirect, are present in the reduced state. Therefore these residues likely occur as disulfides or acylated, as discussed above.

C. Function of Specific Receptor Domains

1. Ligand Binding Site—As is evident from Fig. 5, the largest homology between the respective sequences (indicated by the shaded areas) occurs in the lower half of the transmembrane domains (i.e., near the cytoplasmic side) and in those parts of the cytoplasmic domains that are close to the membrane. These areas are generally thought to be important for interaction with G proteins, the feature that all these receptors have in common. On the other hand, the respective ligands for the various receptors differ greatly, and much less homology occurs in the upper half of the helices and in the extracellular

domains. Thus these may well be the areas where ligand binding occurs. Indeed, the binding site for ligands as structurally diverse as light-activated retinal (rhodopsin), (nor)epinephrine (adrenergic receptors), and acetylcholine (muscarinic receptors) has been shown to be buried in the membrane (roughly halfway), in the center of the cavity formed by the seven transmembrane domains. A negatively charged aspartate residue that is conserved in all receptors that are activated by biogenic amines is thought to act as the counter-ion for the positively charged amine in the ligand.¹⁶³ Unlike these biogenic amines, adenosine is uncharged at physiological pH, and this specific aspartate residue is substituted by valine in adenosine receptors.

The affinity profile of a homologous series of N⁶-alkyl-, -alkylamine-, and -alkyladenosine-substituted adenosines somewhat suggests that the adenosine binding site may also be located at a considerable distance from the membrane surface.¹⁸⁵ Klotz *et al.*¹⁸⁴ have shown for the A₁ receptor that at least one and possibly two histidine residues in the binding pocket may be involved in interactions with adenosine agonists and antagonists. Similar observations have been made for A₂ receptors.¹⁵²

In keeping with these observations, we have suggested that the two conserved histidine residues in H VI and H VII (His²⁵¹ and His²⁷⁸ in the A₁ receptor and His²⁵⁰ and His²⁷⁸ in the A₂ receptor, respectively) are potential candidates for this involvement in the binding site. Interestingly; His₂₇₈ is homologous to Lys₂₉₆ in rhodopsin, the residue that is thought to form a Schiff base with its ligand, retinal.¹⁸⁶ It is also of interest to note that the adenosine-receptorlike sequence reported by Meyerhof *et al.*¹⁵⁹ does contain the histidine residue in H VII, but not in H VI. It is not known whether this receptor is capable of binding adenosine.

Inspection of the helical wheel projections of the adenosine receptors (Fig. 6) suggests that two distinct regions are present in the upper half (i.e., proximal to the extracellular surface) of the receptor cavity (Fig. 8). The histidine residue on H VI is surrounded by predominantly hydrophobic residues, whereas the histidine on H VII is present in a largely hydrophilic environment. This hydrophilic region extends over the full length of the helices, not unlike an ion channel. Although G-protein-coupled receptors do not act as true ion channels, it has been suggested that ion movement could play an important role in receptor activation.¹⁶⁰ A potential site for interaction with sodium is also located in this hydrophilic region (see below).

There is ample evidence that the binding site itself must be of a hydrophobic nature, since hydrophobic substituents may greatly enhance affinity, in both agonists and antagonists, but hydrophilic substituents are seldom tolerated.^{91,107} The putative involvement of these two histidine residues in ligand binding should of course be confirmed, e.g., with ligands that bind irreversibly to the binding site¹⁸⁷ or with the aid of site-directed mutagenesis.²⁰³

2. G Protein Interactions—As is apparent from studies with related receptors, all domains at the cytoplasmic surface of the cell membrane are involved to some degree in interacting with G proteins.¹⁶² In the A₂ sequence, a consensus pattern for interaction with G proteins is present in the C-terminal side of C-I/cytoplasmic side of H II (Table VII). (Note: Although no such pattern is detected in the A₁ receptor, 15 out of the 17 corresponding amino acids do actually comply with the consensus, which seems to indicate that the definition of the consensus pattern needs some minor adjustment.) It should be noted that, given the relatively high degree of homology within the G-protein-linked receptor family in the lower half of the transmembrane sections and the cytoplasmic domains close to the membrane (see above), the described pattern may not be the sole indicator of interaction of the receptor with a G protein. Nevertheless, the pattern is common in G-protein-linked

receptors but is not found in other proteins,¹⁸⁸ and hence it is very likely that this receptor domain is important for interaction with G proteins. However, mutations of C I in the β_2 receptor remained inconclusive in this respect, because they invariably led to receptors that were expressed very poorly.^{189,190}

A proline residue in C II of the β_2 receptor (Pro₁₃₈) has also been implicated in G protein interaction.^{189,190} This residue is highly conserved in related receptors, including A₁ (Pro¹¹²) and A₂ (Pro¹⁰⁹), and thus might serve a similar function there.

Studies with chimeric adrenergic receptors indicate that C III and the adjacent helices V and VI are the main determinants for recognition of the G protein.¹⁶⁴ This has been shown using recombinant DNA techniques, where the C III domain of the α_2 receptor has been replaced by the corresponding domain of the β_2 receptor. The resulting chimera can mediate stimulation of adenylate cyclase, rather than the inhibition seen with the native receptor.¹⁹¹

There is indirect evidence for the involvement of the carboxy tail of the A₂ receptor in interacting with the G protein. Partial proteolysis at a single site has been demonstrated for A₂ receptors of rabbit striatum. Omission of protease inhibitors during the membrane preparation leads to an increase on SDS-PAGE in a specifically labeled peptide of 38 kDa molecular weight, rather than the 47-kDa peptide that is predominantly labeled when proteolysis is inhibited.¹⁴⁴ The resulting 38 kDa can still bind agonist ligands, but it is much less tightly associated with the G protein than is the native 47-kDa protein. The carboxy tail is the only place where deletion of such a large 9-kDa fragment is conceivable without a complete disruption of the helical domains that constitute the binding site. Hence, the carboxy tail of the A₂ receptor likely plays a part in interacting with the G protein. The involvement of the N-terminal part of the carboxy tail of the A₁ receptor, and especially its potential palmitoylation, has already been discussed.

Site-directed mutagenesis studies and chimeric receptors will be needed to further clarify the involvement of the various cytoplasmic domains of the adenosine receptors in interaction with the G protein.

3. Sodium Regulatory Site—The sequence [SN]-L-A-x-[AT]-D near the cytoplasmic end of H II is highly conserved in all G-protein-linked receptors, including both adenosine receptor subtypes. Asp⁷⁹ in the corresponding sequence in α_2 receptors is important in the allosteric modulation of agonist binding by Na⁺.¹⁴ Since agonist binding to A₁^{192,193} and A₂¹⁴⁵ receptors is similarly regulated by Na⁺, by analogy the homologous residues (Asp⁵⁵ and Asp⁵², respectively) may be expected to serve the same function.

Biography

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202. Note added in proof: A homologue of this receptor (termed A₃, but distinct from the A₃ receptor proposed by Ribeiro²⁸) has now been cloned and has a pharmacology R-PIA≈NECA>S-PIA, but does not recognize antagonist ligands such as XAC and IBMX. It is coupled to a PTX-sensitive G protein and inhibits adenylate cyclase. (Zhou Q-X, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O. *Proc. Natl. Acad. Sci. USA.* in press.
203. Note added in proof: It has now been shown through site-directed mutagenesis that the two proposed histidine residues are indeed involved in ligand binding (Olah ME, Ren H, Ostrowski J, Jacobson KA, Stiles GL. *J. Bioi. Chem.* in press.

Figure 1.
Basic structures of tricyclic nonxanthine antagonists.

Figure 2. Computer-generated models of the N⁶ region of (a) A₁⁹¹ and (b) A₂¹⁰⁵ receptors. Shaded areas indicate presumed receptor boundaries. The N⁶ region of the A₁ receptor is divided into subdomains (S1, S2, S3, A, B, C) where hydrophobic substitution may enhance affinity.

Figure 3.

Model for the antagonist binding site of the A₁ receptor. Indicated are areas of preferred electrostatic potential (+5 and -5 kcal/mol boundaries); a nitrogen atom at position 7 that is thought to act as a hydrogen bond acceptor; and areas where hydrophobic substituents may enhance affinity.

Figure 4.
Structures of some representative compounds (**78–84**) that enhance the actions of adenosine in an indirect manner.

		H I	
A1 Ado	MPPAISAFQA	AYIGIEVIAIVSVPGNVIVWVK	35
A2 Ado	MSTMGSW	VYITVELAIAVLAILGNVLCWVW	32
β 2AR	MGQPGNGSAFLAPNRSHAPDHVDVTOORDEVWV	GMGIVMSIVLAIVFGNVLVITIA	59
α 2AR	MGSLOPDAGNASWNGTEAPGGGARATPYSLOVTL	TLVCLAGLLMLLVFGNVLVITIAVF	59
M1 Ach	MNTSVPAYSPNITVLAPGKGWQVA	FIGITITGLSLATVGNLLVLSFK	51
Rhodopsin	MNGTEGPNFYVPSNKTGVVRSPEAPQYLAEPWQFSMLAAYMFLIMLGFPINFLTLYVTV		63
		H II	H III
A1 Ado	VNOAEDATFCFVSLAVADVAVGALVLIPLAILINIGPRTYHT	CLMVACPVLILTOSSTL	96
A2 Ado	LNSNCONVTNYFVVSAAADIAVGLVLIPIFAITISTGFCACHN	CLFFACFVLVLTOSSIF	93
β 2AR	KFERKOTVTNYFITSLACADLVMLAVVPFGAAHILMKMWTFGNFWCEFWTSIDVLCVTAIE		122
α 2AR	TSRAEKAPONLFLVLSASADILVATLVIPFSLANEVMGYWYFGKTWCEIYLALDVLFCSTSSIV		122
M1 Ach	VNTEKTVNNYFLLSLACADLIIGTFSMNLYTTLLMGHWALGTLACDLWALDVAASNASVM		114
Rhodopsin	QHKKEKRTPLNYLLNLAVADLFMVFGGFTTLLTSLHGYFVEGPTGCLNEGFFATLGGELIALW		126
		H IV	
A1 Ado	ALLATAVDRLRVKIPLRKYKTVTPRRAAVAIAGCWILSFVVGLTPLFGWNRLGEAQRAWAAN		159
A2 Ado	SLLATAIDRYIAIRIPLRYNGLVIGTRAKGIIAVCWVLSFAIGLTPMLGWNNSQPKERNYS		156
β 2AR	TLCVIAVDRYFAITSPFKYOSLLTKNKARVITIMVWTVSGLTSTFLIQMHVYRATHOEAINCY		185
α 2AR	HLCAISLDRYWSITQAIENLNKRTPRRIKAIITICWVISAVISFPPLISIEKKGSGGPGPAE		185
M1 Ach	NELLISFDRYFSVTRPLSYRAKRTPRRAALMIGLAWLVSFVLWAPAILFWOYLVGERTVLAGO		177
Rhodopsin	SLVVLAIERYVVCVKPMSNFRF-GENHAIMGVAFTWVMALACAAPPLVWGSRYIPEGMQSCSG		188
		H V	
A1 Ado	GSGGEPVIKCEFEKVISM EYMYVFNFFVWVLPPLLMVLIYLEVFLYLRRLQKKKVSASSGDP		222
A2 Ado	QCGGEGQVACLFEDEVPMNYMYVNFFAFVLVPLLLMLGVYLRIFLAARROLKQMESOPLPGE		219
β 2AR	ANET---CCDFFT---NQAYATASSIVSFYVPLVIMVYVSRVFOEAKRQLOKIDKSEGRFH		241
α 2AR	PR---CEIND---QKWWYISSCIGSEFFAPCLIMILYVRYVQIAKRRTRVPPSRGPD		238
M1 Ach	---CYIOFLS-QPITITFGTAMAAFYLPVTVMCTLYWRIYRETERARELAALOGSET		230
Rhodopsin	IDYYTPHEETN---NESFYIMFVVHFIIPILIVIFFCYGQLVFTVKEAAAOQESATTOK		245
A1 Ado	QKYYG		227
A2 Ado	RARSTLQ		226
β 2AR	VONLSOVEODGRTGHGLRRSSKFCFL		266
α 2AR	VAAPPGGTERRNGLGERSAGPGGAEPLPTOLNAPGEPAPAPQPRDOTALDLEESSSSDH		301
M1 Ach	PGKGGSSSSSSERSOPGAEGSPESPGRCCRCRAPRLQAYSWKEEEEDGSMESLTSSEG		293
Rhodopsin			245
A1 Ado			227
A2 Ado			226
β 2AR			266
α 2AR	AERPPGPRRPERGPRGKGRASQVKPGDSLGA	GRGRRGSGRRLLQGRGRSASGLP	359
M1 Ach	EEPGSEVVKMPMVDSEADQPTKQPKSSPNTVKRPTKKGRDRGGKQKPRGKEQLAKRKTFS		356
Rhodopsin			245
		H VI	H VII
A1 Ado	-----KELKIAKSLALTLFLFAESWLP LHLNLCITLFCPSCRK-PSIEMYIAIFLTHGNS		281
A2 Ado	-----KEVHAAKSLAIIIVGLFALCWLP LHLINCFITFFCPECSHAPLWIMYLTIVLSHTNS		281
β 2AR	-----KEHKALKTIGIIMGTETLCWLP FFEVNIHVHVDNLI--RKEVYILLNWIGYVNS		319
α 2AR	RAGAGGNLEKRFTEVLAVVIGVGVVQWFFFTTYTLTAVQCSV--PRTLFKFFFWGYCNS		419
M1 Ach	LV-----KEKKAARTLSAILLAFLITWTPRYNIMVLVSTFCKDCV--PETLWELGYWLCYVNS		411
Rhodopsin	-----AEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTHQGSDF-GPIFMITPAFFAKTSA		299
A1 Ado	AMNPVYAFRIQKFRVTEFLKIWNDFHRCOPTPPVDEDPPEEAPHD		326
A2 Ado	VVNPFIYAYRIREFROTFRKIIRSHVLRREPFKAGGTSARALAAHGSDDGEQISLRLNGHPPG		344
β 2AR	GFNPLIYCRSP-DFRIAFQELCLRRSSSLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDL		381
α 2AR	SLNPVLYTIFNHDERRAFKKILCRGDRKRIV		450
M1 Ach	TVNPMCYALCNKAFROTFRLLLCRWDKRRWKIPKPGSGVHRTSPSRCC		460
Rhodopsin	VYNPVIYIMMNKQERNCMVTTLCCKGNPLGDDASTTVSKTETTSQVAPA		348
A1 Ado	VWANGSAPHERRPNGYTLGLVSGGIAPESHGDMGLPDVELLSHELKGCAPESPGLEGPLAQD		326
A2 Ado	PGTEDFVGHQGTVPSDNIDSGGRNCSTNDSLL		407
β 2AR			413
α 2AR			450
M1 Ach			460
Rhodopsin			348
A1 Ado	GAGV		326
A2 Ado			411
β 2AR			413
α 2AR			450
M1 Ach			460
Rhodopsin			348

Figure 5. (Overleaf) Alignment of the canine A₁ and A₂ receptors with the human β ₂- and α _{2A}-adrenergic receptors, the rat M1 acetylcholine receptor, and bovine rhodopsin. Alignment was performed with the MACAW multiple sequence alignment program. Putative transmembrane sections (indicated by horizontal bars and roman numbers) were treated as blocks without allowing gaps. Nucleotide sequences were retrieved from the GenBank database; implied amino acid sequences were generated with the GeG Sequence Analysis Software Package.²⁰¹

Figure 6.

Helical wheel representations of the (a) A₁ and (b) A₂ receptors. Sequences are modeled as right-handed α -helices (Le., 3.6 residues per turn; thus consecutive residues appear at 100° intervals. Helices are viewed from the extracellular side; H I should be read clockwise, H II counterclockwise, and so on). The first and last residues of a helix are indicated by either *i* (in; Le., cytoplasmic) or *o* (out; Le., extracellular). The first 18 residues of a helix are indicated on the inside of the wheel, any remaining residues on the outside. The arrangement of the helices is based on the structure of bacteriorhodopsin, as determined by cryoelectron microscopy.¹⁶⁶ Hydrophilic residues are encircled and shaded.

Figure 7.

Membrane topology of the (a) A_1 and (b) A_2 receptors. The N terminus is located on the extracellular side, the C terminus on the cytosolic side. Residues that are conserved in both adenosine receptor subtypes are shaded. Histidines that are putatively involved in ligand binding, and serines/threonines that potentially can be phosphorylated are shown in boldface. Potential sites for asparagine glycosylation and cysteine palmitoylation are also indicated.

Figure 8.

A generalized map of the upper half of each transmembrane helix of the adenosine receptor, showing separate domains of hydrophobic and hydrophilic environment, and the location of the two histidine residues that are putatively involved in the ligand binding site. This model applies in both A₁ and A₂ receptors.

TABLE I

Effector Mechanisms Related to the Biological Effects of Adenosine

System	Action	Tissue/Cell	Subtype	Ref.
Adenylate cyclase	Inhibition	Adipocytes	A ₁	17
		Heart	A ₁	32
		Smooth muscle	A ₁	43
	Stimulation	Platelets	A _{2a}	18
		Striatum	A _{2a}	19
		Smooth muscle	A _{2b}	43
		Brain	A _{2b}	21
		Fibroblasts	A _{2b}	20
Guanylate cyclase	Activation	Smooth muscle	A ₁	22
Low K_m cAMP phosphodiesterase		Adipocytes	A ₁	44
		Brain	A ₁	45
Ion Channels				
Potassium efflux	Activation	Heart	A ₁	31
		Hippocampus	A ₁	23
ATP-sensitive K ⁺ channels	Activation	Heart	A ₁	33
Calcium influx	Inhibition	Brain	A ₁	23 – 25
	Inhibition	Neuromuscular junction	A ₃ ?	28
Phosphoinositide metabolism	Inhibition	GH ₃ cells	A ₁	35
	Stimulation	Kidney RCCT cells	A ₁	46
		Thyroid FRTL-5 cells	A ₁	46

Structure and Affinity of N⁶-Substituted Adenosine Derivatives at A₁ and A₂ Adenosine Receptors

TABLE II

Compound	R ₁	A ₁ (nM) ^a	A ₂ (nM) ^b	A ₂ /A ₁
1 adenosine	H	—	—	—
2 CPA	cydopentyl	0.59	462	780 ¹⁹
3 CHA	cydohexyl	1.3	514	400 ¹⁹
4 S-ENBA		0.3	1,390	4,700 ⁴⁸
5 C-ENBA		0.24	3,900	16,000 ⁴⁸
6 R-PIA		1.2	124	100 ¹⁹

Compound	R ₁	A ₁ (nM) ^a	A ₂ (nM) ^b	A ₂ /A ₁
7 IHPIA		0.94 ^c	—	— ¹⁹⁵
8 N ⁶ -phenylado	phenyl	4.62	663	140 ¹⁹
9 N ⁶ -benzylado	benzyl	12.0	285	2.37 ¹⁹
10 I-ABA		0.7	—	— ¹⁹⁹
11 N ⁶ -(2-phenethyl)ado	2-phenylethyl	12.7	161	13 ²⁰⁰
12 APNEA		2.0	—	— ¹³³
13 N ⁶ -(3-phenylpropyl)ado	3-phenylpropyl	25	—	— ⁴⁹
14 N ⁶ -sulfo(phenyl)propylado	3-(p-sulfo(phenyl)propyl)	610	—	— ^d
15 CI-936		6.8	25	3.7 ¹⁰⁸
16 DPMA		142	4.4	0.031 ¹⁰⁸
17 N ⁶ -fluorenylado		5.2	4.9	0.94 ¹⁰⁸
18 ADAC		0.85	210	250 ¹⁹⁶
19 Slearoul-ADAC		0.22	8,400	38,000 ¹⁹⁶
20 P-DITC-ADAC		0.47	191	410 ¹⁴⁹
21 FITC-ADAC		7.1	—	— ⁵²

^a K_i or IC₅₀ values in nM, displacement of [³H]PIA in rat brain membranes, unless indicated otherwise.

^b Displacement of [³H]NECA in rat striatal membranes (in the presence of 50 nM CPA), unless indicated otherwise.

^c K_D value.

^d M. Maillard *et al.*, to be published.

^e FITC: fluorescein isothiocyanate.

C2- and /or 5--Substituted Adenosine Analogs

TABLE III

Compound	R ₂	A ₁ (nM) ^a	A ₂ (nM) ^b	A ₂ /A ₁
R ₃ = CH ₂ OH; R ₁ = H (unless noted)				
22 2-CADO	Cl	9.3	63	6.8 ¹⁹
23 CCPA	Cl; R ₁ = cyclopropyl	0.6	950	1500 ¹⁹⁷
24 CV 1808	NHΦ	560	119	0.21 ¹⁹
25	NH(CH ₂) ₂ -C ₆ H ₁₁	12,000	22	0.0018 ⁵⁸
26 CGS 22989	NH(CH ₂) ₂ -C ₆ H ₉	2,700	13	0.0048 ⁵⁸
27 CHEA	O(CH ₂) ₂ -C ₆ H ₁₁	1,500	22	0.014 ⁶⁰
28 MPEA	O(CH ₂) ₂ -C ₆ H ₄ -CH ₃	48	11	0.22 ⁶⁰
29 2-HNA	C≡C-(CH ₂) ₃ CH ₃	147	4.1	0.028 ⁶²
30 2-ONA	C≡C-(CH ₂) ₅ CH ₃	211	12	0.057 ⁶²
31 I-AHPA		1.5	—	— ¹³⁶
32 NECA	H	6.3	10.3	1.6 ¹⁹
33 CGS 21680	NH(CH ₂) ₂ -C ₆ H ₄ -(CH ₂) ₂ COOH	2,600	15	0.0058 ⁵⁸

Compound	R ₂	A ₁ (nM) ^a	A ₂ (nM) ^b	A ₂ /A ₁
34 APEC		240	5.7	0.024 ¹⁹⁸
35 Biotinyl-APEC		14.3 ^d	>5,000	>350 ¹⁹⁸
36 Iodo-PAPA-APEC		—	1.5 ^c	— 140
37 <i>p</i> -DITC-APEC		280	7.1 ^d	0.025 ¹⁹⁸

^a *K_i* or IC₅₀ values in nM, displacement [³H]CHA in rat brain membranes, unless indicated otherwise.

^b Displacement of [³H]NECA in rat strial membranes (in the presence of 50 nM CPA), unless indicated otherwise.

^c *K_d* in bovine brain.

^d *V*_{versus} 125I-PAPA-APEC in bovine brain.

Xanthine Derivatives as Adenosine Antagonists

R₇ = H, unless noted

TABLE IV

Compound	R ₁	R ₃	R ₈	A ₁ (nM) ^a	A ₂ (nM) ^d	A ₂ /A ₁
38 theophylline	Me	Me	H	8,500	25,000	3.0 ¹⁹
39 1,3-diethylx	Et	Et	H	3,300	—	— ⁷³
40 1,3-dipropylx	Pr	Pr	H	700	6,600	9.4 ¹⁹
41 1,3-dibutylx	(CH ₂) ₃ CH ₃	(CH ₂) ₃ CH ₃	H	500	—	— ⁷³
42 1,3-dibenzylx	CH ₂ φ	CH ₂ φ	H	2,000	—	— ⁷³
43 IBMX	Me	CH ₂ CH(CH ₃) ₂	H	7,000	16,000	2.3 ¹⁹
44 enprofylline	H	Pr	H	55,000	137,000	2.5 ¹⁹
45 caffeine	Me	Me	H(R ₇ =ME)	29,000	48,000	1.7 ¹⁹
46 1-propyl-3,7-dimethylx	Pr	Me	H(R ₇ =Me)	38,000	48,000	1.3 ¹⁸⁵
47 DMPX	—CH ₂ C≡CH	Me	H(R ₇ =Me)	45,000	16,000	0.36 ¹⁸⁵
48 7-Bz-theophylline	Me	Me	H(R ₇ =CH ₂ φ)	6,000	46,000	7.7 ⁷⁵
49 1,3-dibutylx-7-ribose	<i>n</i> -Bu	<i>n</i> -Bu	H(R ₇ =ribose)	450 ^h	—	— ⁸⁹
50 8-substituted <i>R</i> -PIA analog	Pr	Pr		6.9	160	23 ¹⁰⁶
51 8-substituted <i>S</i> -PIA analog	Pr	Pr		61	850	14 ¹⁰⁶
52 8-PT	Me	Me	phenyl	86	850	9.9 ¹⁹

Compound	R ₁	R ₃	R ₈	A ₁ (mM) ^d	A ₂ (mM) ^d	A ₂ /A ₁
53 PACPX	Pr	Pr		2.5	92	37 ¹⁹
54 XCC	Pr	Pr		58 ⁷⁷	2,200 ¹⁹⁶	40
55 8-PST	Pr	Pr		50	795	6 ⁸
56 8-PST	Me	Me	<i>p</i> -(SO ₃ H)phenyl	4,500	6,300	1.4 ⁷⁸
57 XAC	Pr	Pr		1.2 ⁷⁷	63 ¹⁹⁶	53
58 I-PAPA-XAC	Pr	Pr		0.1 ^f	—	— 139
59 <i>m</i> -DITC-XAC	Pr	Pr		2.4	340	140 ¹⁴⁹
60 CHC	Me	Me	cyclohexyl (R ₇ = Me)	28,000	190 ^d	0.007 ^{112,4}
61 CPT	Me	Me	cyclopentyl	11	1400	130 ¹⁹
62 CPX	Pr	Pr	cyclopentyl	0.46	340	740 ⁸
63 I-BW-A844U	Pr	Pr		0.23	2000	8700 ⁸
64 KF15372	Pr	Pr		3 ^e	430	140 ⁸³
65 KFM19	Pr	Pr		10.5	1510	144 ⁸⁴

Compound	R ₁	R ₃	R ₈	A ₁ (nM) ^a	A ₂ (nM) ^d	A ₂ /A ₁
66 KW-3902	Pr	Pr		1.3	380	290 ⁸⁵
67 1,4-trans-cyclohexyl derivative	Pr	Pr		8 ^e	20 ^d	2.5 ⁸²

^a *K_I* or IC₅₀ values in nM, displacement of [³H]PIA or [³]CHA in rat brain cortical membranes, unless indicated otherwise.

^d Displacement of [³H]NECA in rat brain striatal membranes (in the presence of 50 nM CPA), unless indicated otherwise.

^c *K_I* for antagonism of adenylylate cyclase inhibition in rat adipocytes.

^d *K_I* for antagonism of adenylylate cyclase activation in human platelets.

^e *K_I* or IC₅₀ value for displacement of [³H]CHA in guinea pig forebrain membranes.

^f *K_d* in bovine brain.

^g In bovine brain.

^h [³H]CPX binding to bovine brain membranes.

ⁱ This compound is nonselective in other (e.g., adenylylate cyclase) assays and is not useful as an A₁-selective ligand. 112

TABLE V

Nonxanthine Adenosine Antagonists

Compound		A ₁ (nM) ^a	A ₂ (nM) ^b	A ₂ /A ₁
68 benzo(<i>b</i>)furan derivate		17 ^{c,92}	—	—
69 N-0861		10 ^c	6,100 ^c	610 ⁹⁶
70 CGS 15943		21	3.3	0.16 ⁹⁸
71 CP66713		270	21	0.078 ¹⁰²
72 Cp 68247		28	>100,000	>3,000 ¹⁰¹
73 CPPIQA		10	450	45 ¹⁰²
74 ADPEP		4.7	3,700	790 ⁹⁵

Compound		$A_1(\text{nM})^a$	$A_2(\text{nM})^b$	A_2/A_1
75 APPP		23	35	1.5 ⁹⁷
76 HTQZ		3,100	120	0.04 ⁹⁷
77 N ⁶ -butyl-8-phenyladenine		170	660	4 ⁹⁷

^a K_i or IC_{50} values in nM, displacement of [³H]PIA or [³H]CHA in rat brain cortical membranes, unless indicated otherwise.

^b Displacement of [³H]NECA in rat brain striatal membranes (in the presence of 50 nM CPA), unless indicated otherwise.

^c In bovine brain.

Secondary Structure for the Cytoplasmic and Extracellular Loops, as Predicated by teh Chou–Fasman¹⁶⁹ and Garnier-Osguthorpe-Robson¹⁷⁰ Methods. Calculations were Performed with the GCG Sequence Analysis Software Package¹⁷⁹ on a Convex C-240 Computer.

TABLE VI

A ₁ Receptor	Chou-Fasman			Garnier-Osguthorpe-Robson			Common
	Turn	α Helix	β Sheet	Turn	α Helix	β Sheet	
C I(34-46)	0	+++	+	0	0	+	
E I(70-79)	+	0	++	++	0	+	
C II(103-122)	0	0	+++	0	0	++	β Sheet
C III(202-235)	+	++	+	+	++	+	(α helix)
E III(260-266)	++	0	+	+++	0	0	turn

A ₂ Receptor	Chou-Fasman			Garnier-Osguthorpe-Robson			Common
	Turn	α Helix	β Sheet	Turn	α Helix	β Sheet	
C I(31-43)	+	0	++	+	0	+	
E I(67-76)	+	0	++	+++	0	0	
C II(100-119)	0	0	+++	+	+	++	β sheet
E II(143-173)	+++	+	0	+++	+	+	turn
C III(199-234)	+	+++	+	0	++	0	α helix
E III(259-266)	++	0	+	+++	0	0	turn

TABLE VIIConsensus Patterns as Described in the Prosite Database¹⁸⁸

Pattern for:	A ₁	A ₂
PKA phosphorylation	K ²¹³ KVS ^a ; R ²⁶⁴ KPS ^b	n.d. ^c
PKC phosphorylation	T ¹²⁰ PR; S ²⁶² CR	T ²⁹⁸ FR; S ³²⁰ AR; S ³³⁵ CR
CK2 phosphorylation	S ¹⁶¹ GCE; S ²¹⁸ SGD	S ³²⁹ DGE; S ³⁷⁴ HGD
Asn glycosylation	N ¹⁵⁹ GSG	N ³⁹ VTN ^c ; N ¹⁴⁵ CSQ; N ¹⁵⁴ YSQ; N ³⁴⁸ GSA
G protein interaction	n.d. ^e	L ³ QNVVTNYFVVSLLAAADI

^aThe residues that may be phosphorylated or glycosylated are printed in boldface.

^bS²⁶⁷, S²⁶², and S¹⁶¹ are located extracellularly and are thus not available for phosphorylation; the phosphorylation sites at S²⁶² and S²⁶⁷ are absent in the rat A₁ sequence.

^cn.d.: not detected.

^dN³⁹ and N³⁴⁸ are located intracellularly and are thus not available for glycosylation.

^eThe sequence that is complementary to the pattern detected in the A₂ receptor is L⁴⁰RDATFCFIVSLAVADV. All but the sixth and the seventh amino acid residues comply with the consensus pattern.