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Adenosine A_1 and A_2 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, A_1 and A_2. 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 A_1 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 strueture 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

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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, A_1 receptors inhibit adenylate cyclase activity whereas A_2 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 A_1 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 A_1 receptors are promiscuous in that they will couple to a variety of effector systems, including adenylate cyclase, guanylate cyclase, potassium channels, calcium channels, phospholipase A_2 and C, and the sodium–calcium exchange system (Table I).[6] The coupling of A_1 receptors to G proteins has been explored in detail. Studying purified receptors in reconstituted vesicles, Freissmuth *et al.* found that A_1 receptors can couple to $G_{i1–3}$ and to G_0.[16] In contrast, the only known effector system to which the A_2 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 A_1 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, latertermed A_{2b}, was found in human fibroblasts[20] 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 A_1 adenosine agonists, and may be associated with a vasodilatory effect.[22] The intracellular concentrations of variousions are also modulated by adenosine. Adenosine A_1 agonists cause a decrease in the intracellular concentration of Ca^{2+}, via entry through voltage-sensitive N-type calcium channels in presynaptic terminals in the hippocampus.[23] 2-Chloroadenosine, an agonist, inhibits the p otassium-dependent uptake of $^{45}Ca^{2+}$ into cortical synaptosomes[24] 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 A_1-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 sarcoplasmatic reticulum,[27] does not appear to involve adenosine receptors. An A_3 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 A_3 receptor occurs in the brain and constitutes a site of intermediate affinity (1–10 nM) for the A_1-selective antagonist CPX[29]

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 A_1 receptors,[32] and a pertussis toxin-sensitive G protein appears to be involved.[31] These receptors may also couple to adenylate cyclase.[32] A_1 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 A_1 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 effects[37] that are blocked by xanthines. Hollingsworth *et al.* found that adenosine agonists, presumably acting through A_1 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 slices[39] and in an RCCT cell line derived from kidney cortical collecting tubules.[40] Conversely, adenosine agonists inhibit the generation of inositol phosphates in pituitary GH_3 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 A_1 receptors. Our laboratory has used an *in vivo* rat adipocyte model in which rats are infused with the A_1-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 A_1 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 PGE_1 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 A_1 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 A_1 receptors could not explain either the decreased sensitivity to PGE_1 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_{i1} and α_{12}, two forms of the G_i protein, were decreased by 59%, whereas α_{i3}, 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 A_1 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 DDT_1 MF-2 cell line we have been able to document that the A_1 receptor is, in fact, a substrate for phosphorylation, suggesting a further potential mechFlnism involved in the desensitizing process.[42] Much less information is available on the desensitization of A_2 receptors, but studies in the DDT_1 MF-2 cell lines suggest that its mechanism for desensitization is quite disparate from that seen in the A_1 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 A_2-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 A_1 and A_2 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 N^6- and C2-positions, yielding a wealth of useful, subtype-selective agonists. The N^6-substituted adenosine, derivatives CHA (N^6-cyclohexyladenosine, **3**), CPA (N^6-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 N^6- 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 N^6 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 A_1-selective. Studies of the dependence of potency on the size of the ring in N^6-cycloalkyl substituted analogs identified CHA and CPA as particularly potent and A_1-selective (280- and 780-fold, respectively).[47] Subsequent studies showed that N^6-bicycloalkyladenosines are even more A_1 selective, with the 1*R*, 2*S*, 4*S* isomer of N^6-(2-*endo*-norbomyl)adenosine(S-ENBA, **4**) being 4700-fold selective, while its 5′-Cl analog is even 16,000-fold selective for the A_1 receptor.[48] The N^6-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 N^6,[49] but they may be accommodated when present at a considerable distance from the N^6-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]

A_2 selectivity may be achieved by modifications of the 2-position. Small 2-substitutions, such as 2-CADO (**22**) tend towards relative nonselectivity or modest A_2 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 A_2 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 A_2 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. [^{3}HJCGS 21680 has been developed as a selective A_2 radioligand, and was instrumental in identifying the cloned A_2 receptor.[56] In contrast, a related amine derivative, APEC (**34**) was centrally active as a locomotor depressant[57] 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 A_2 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; A_2: 22nM; A,: 1580 nM),[60] 2-aralkoxy-adenosine derivatives,[61] such as MPEA (**28**; A_2: 11 nM; A_1: 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 A_2 agonists (K_i values 4 and 12 nM) with 36- and 17-fold selectivity, respectively, for the A_2 receptor in binding assays.[62]

A variety of A_2-selective agonists have been found in *in vivo* testing to be much more A_2-seiective than predicted in binding assays. In an assay of coronary vasodilation in the guinea pig, CHEA has an EC_{50} value of 1 nM at the A_2 receptor, resulting in an *8,700-fold* selectivity for the A_2 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 A_1 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-Ethy1carboxamidoadenosine (NECA, **32**) is nonselective but relatively potent (K_i= 10 nM at both A_1 and A_2 receptors[19]). NECA has been extensively used to define tissue responses mediated by A_2 receptor activation[7,65] and as a radioligand which labels A_2 receptors in rat striatum.[19] The A_1 component of the binding profile of [^{3}H]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-anomer[64] or the L-enantiomer,[20] nor the compounds in which the stereochemistry of the 2′-OH (9-β-B-arabinofuranosyladenine) or 3′-OH (9-β-D-xylofuranosyladenine) group is inversed,[20] is active. Significantly, the receptor does not discriminate between the α- and β-anomers of the weak antagonist 2′,3′-dideoxy-N^6-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 A_1 and A_2 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-N^6-cyclopentyladenosine is a potent and highly A_1-selective (8,200-fold) agonist.[67]

Summarizing, adenosine agonists of high affinity and selectivity for the A_1 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 Ca^{2+}-independent phosphodiesterases (IC_{50}: 16μM).[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 A_1 and A_2 receptors.[73] Larger hydrophobic substituents may also be accommodated by the receptor, as illustrated by the relatively high potency of 1,3-dibenzylxanthine (**42**, A_1: 2 μM; A_2: 14 μM).[73] Bulk tolerance at the 3-position is more limited in the A_2 receptor than in the A_1 receptor. This is evident with the highly potent and 1740-fold selective compound J-BW-A844U (**63**, I-propyl-3-(2-iodo-3-aminophenethyl)-8-cyclopentylxanthine). Its parent compound CPX (**62**, 1,3-dipropyl-8-cyclopentylxanthine) is equipotent, and is 740-fold A_1 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 A_1 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 A_1 and A_2 receptors. Methyl substitution at N7 appears to be less unfavorable for interaction with A_2 receptors than it is for A_1 receptors, whereas larger 1-alkyl substituents enhance A_2 affinity more than A_1 affinity. On the other hand, substitutions at the 3-position may be more favorable for A_1 affinity.[73] This has allowed the development of some moderately A_2-selective xanthine derivatives, albeit of modest potency, e.g., DMPX, 1-propargyl-3,7-dimethylxanthine (**47**,A_1:45 μM; A_2: 16 μM).[73] The lack of potent and selective A_2 receptor antagonists still remains a major obstacle in the characterization of adenosine A_2 receptor pharmacology.

The greatest boost in affinity comes with substitutions at the 8-position. 8-PhenyltheophylJine (8-PT, **52**) is 100- and 30-fold more potent at A_1 and A_2 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-dipropy1-8-(2-amino-4-chloro)phenylxanthine) has optimal A_1 affinity and moderate A_1 selectivity (A_1:2.5 nM; A_2: 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 A_1

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 μM; 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 condudve to A_1 selectivity, prindpally 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 receptors[83], KFM 19 (**65**), a potent A_1-selective compound with sufficient aqueous solubility to display good bioavailability, currently under development as a potential cognition enhancer[84]; 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 group[20]) 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 sufficent 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 A_1 and A_2 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 dasses 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 bicydic or 6:6:5-fused tricydic 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 A_1 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 N^6-substituted 9-methyladenines[93,94] and the 2-phenyl-7-deazaadenines.[95] For the 9-methyladenines, SAR at the N^6 position parallels the SAR for similarly N^6-substituted adenosines to a large degree, suggestive of similar binding modes for 9-methyladenines and adenosine-derived agonists.[3] The recently described N-0861 [(±)N^6-endo-norbornyl-9-methyladenine; **69**) is potent and highly A_1 selective (A_1: 10 nM; A_2: 6100 nM in bovine brain).[96] A related compound, N^6-butyl-8-phenyladenine (**77**; A_1: 170 nM),[97] might be interpreted as an intermediate between an N^6-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 A_1-selective 7-deazaadenine, ADPEP (**74**; A_1: 4.7 nM, A_2: 3710 nM), is 35-fold more potent than its *S*-isomer. The analogy with the high stereoseiectivity of agonists substituted at the N^6-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 A_1-selective CP 68,247, **72**, and the moderately A_2-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 A_2-selective, substitution at the exocyclic amino group (R_1) may impart both high affinity at A_1 receptors and A_1 selectivity—reminiscent of N^6-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 imidazoqumoline[102] series, an exocyclic aromatic or cycloalkyl substituent (R_2) 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

triazoloquinazolines[101] and triazoloquinoxaiines,[98,99] highest affinity is associated with a chlaro substituent (R_3) at the same position of the benzene ring (similar substitutions have not been explored for the imidazoquinolines).

Finally, apart from N^6-butyl-8-phenyladenine, two other potentially interesting nonxanthine antagonists have emanated from a broad screening program for aden9sine 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 (A_1: 23 nM, A_2: 35 nM). HTQZ (76, A_1: 3100 nM, A_2: 120 nM) is 26-fold selective for A_2 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 usefu1 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 spedfic 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, MOPAe (AM1) calculations of the rotational freedom around the glycosidic bonds of adenosine and xanthine-7-ribosides[104] 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 agonists[105] and novel antagonists,[91,102,106] as well as to quantitatively predict the affinity of compounds in a series of agonists.[107]

N^6 substituents may greatly influence the affinity and subtype selectivity of adenosine-derived agonists. Although the majority of the N^6 substituents imparts A_1 selectivity, there are certain subdomains where the A_2 receptor appears to have more bulk tolerance than the A_1 receptor, as becomes evident from a model developed by Ortwine *et al.,* illustrated in Fig. 2(a).[105] These subtle differences between the N^6-regions of A_1 and A_2 receptors were exploited by researchers at Parke-Davis to develop the first A_2-selective agonists.[108] A similar model of the N^6-region of the A_1 receptor, based on the preferred conformations of 26 N^6-substituted agonists, was described by van Galen *el al.*[107] The original model of the N^6-region of Kusachi, based on the respective contributions of the various parts of the N^6-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 N^6 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 sterk hindrance) by 7-methyl-substituted 8-phenylxanthines and 8-cycloalkylxanthines (e.g. **60**), which readily explains the unexpectedly low affinity[112] of these compounds.

Two different models for the antagonist binding site of the A_1 receptor have been proposed.[91,102,106] The first[91] 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 hvo 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,S-*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 A_1 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 orientation[106] of adenosine with respect to the xanthines. On the basis of the remarkable parallels between N^6 substitution in agonists and C8 substitution in antagonists,[19] it was suggested that N^6 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 the-ophylline-9-riboside,[104] and a sterk fit that is superior to the N^6/C8 model. The parallels between the N^6 and C8 5ubstituents, including the preferred orientations of these substituents,[114] argue in favor of the second model. One argument against the N^6/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. N^6 substituents of A_1-selective agonists, C2 substituents of A_2-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 xanthines[3] and the 2- and 4-positions in imidazo[4,S-*c*]quinolinamines.[102] Furthermore, according to this model, the ribose moiety should bind with significantly different orientations to the A_1 and A_2 receptors, respectively, despite close analogies between the SAR profiles for ribose substitutions at A_1 and A_2 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 coronory vasodilator and platelet aggregation inhibitor. Its effects may at least be partly due to inhibition of adenosine reuptake, thus enhancing its actions on coronary artery[118] or platelet[119] A_2 receptors. Newer uptake inhibitors include mioflazine, 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] Mioflazine (**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 benzodíazepines on GABA receptors.[123] Several 2-amino-3-benzoylthiophenes stimulate binding of the agonist radioligand [^{3}H]CHA, but not of the antagonist [^{3}H]CPX, to A_1 receptors from rat brain, indicating that this action is specific for the agonist conformation.[124] SAR studies[125] 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 preferntial enhancing activity. Thus PD 81,723 (**81**, 2-amino-4,5-dimethyl-3-thienyl-[3-thienyl-[3-(trifluoromethyl)-phenyl]methanone) showed the most favorable enhancementlantagonism ratio in a series of benzoylthiophenes. Allosteric enhancers for the A_2 receptor have also been described, as exemplified by PD 120,918 (**82**)[123]

AICA riboside (83, 5-aminoirnidazole-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 myocardial[126] or cerebral[127] 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 methothrexate on AICA riboside transformylase. [129]

The ADA inhibitor, deoxycoformycin (**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 A_1 and A_2 receptors have been affinity labeled using agonist and antagonist molecular probes, containing either ^3H or high-specific activity, carrier-free^{125} I 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 photoafiinity crosslinking, in which a radiolabeled ligand containing a chemically reactive group, such as an aryl amine, is first hound to the receptor, and then exposed to a bifunctional crosslin king 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 A_1 receptor was labeled by photoaffinity crosslinking by Stiles *et al.*[133] using the ligand [^{125}I]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 ^{125}I and detected as a typically wide band on SDS gel electrophoresis. Choca *el al.*[136] used the azide derived from iodo-N^6-aminobenzyladenosine (^{125}I-ABA, 10) for photoaffinity labeling of the receptor. Klotz *et al.*[136] utilized 2-azido-N^6-*p*-hydroxyphenylisopropyl-adenosine (^{125}I-AHPIA, **31**) for this purpose. Lohse *et al.*,[137] in a study that revealed spare A_1 receptors in adipocytes, showed that AHPIA caused a persistent, irreversible activation of the receptor and a resultant decrease in cyclic AMP Levels. ^{125}I-AHPIA also irreversibly blocks A_2 receptors, but at higher concentrations.[138]

Xanthine functionalized congeners have also been used for the photoaffinity labeling of adenosine receptors.[139,140] The A_1 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 A_1 receptors using another xanthine aryl amino derivative, ^{125}I-azido-BW-A844U.

It appears that both agonist and antagonist photoaffinity labeling of A_1 receptors occurs in the same region of the peptide sequence of the receptor. Barrington *et al.*[142] conducted photoaffinity labeling of bovine brain A_1 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 A_2 receptor was accomplished using PAPA-APEC (**36**), an A_2-selective agonist, by Barrington *et al*[140] PAPA-APEC, derived from the A_2-selective adenosine agonist CGS21680, contains a long chain at the purine 2-position. ^{125}PAPA-APEC binds reversibly and in a saturable fashion to bovine brain A_2 receptors with a K_d value of 1.5 nM. In a subsequent step, ^{125}I-PAPA-APEC was cross-linked to the receptor, either: through photoaffinity cross-linking using SANPAH[140] or by conversion of the amine to an azide.[143] The A_2 binding site in bovine brain striatal membranes was found to be a single glycoprotein of molecular weight 45,000. The appropriate A_2 pharmacological properties were observed both for the reversible binding of ^{125}I-PAPA-APEC and for the protein labeling. Similarly, the molecular weights of A_2 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 A_2 receptors was observed. Treatment with glycosidases revealed a heterogeneous mixture of high mannose type and complex type carbohydrates to be present among bovine A_2 receptors.[146] In contrast, A_1 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 isomers[50] 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 A_1 receptors in the guinea pig AV node. Analogous APEC derivatives, including the *p*-DITC conjugate (**37**), appear to inhibit A_2 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 Nakata[153] (with rat brain) and by Olah *et al.* [154] (with bovine brain) for the complete purification of cortical A_1 receptors after solubilization.

IV. RECEPTOR STRUCTURE: MOLECULAR BIOLOGY APPROACHES

A. Cloning of A_1 and A_2 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 A_2 receptor sequence. When expressed in COS7 cells, the RDC8 protein specifically binds [^{3}H]CGS21680, indicative of the A_{2a} subtype.[56] A second eDNA

fragment, RDC7, with a high degree of homology to RDC8, was found to code for the A_1 adenosine receptor.[156] PCR methods have enabled the subsequent cloning of the rat[157,158] and bovine A_1 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 add 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 fonowing irradiation. Bacteriorhodopsin is present in the membrane of *Halobacterium hnlobium* in a two-dimensional crystallike 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 indicted 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 ratiom;le 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 fu ture adjustments may be warranted. [168] Since it is not likely that solid (x-ray crystallographic or 20 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–Fasman[169] and Garnier–Osguthorpe–Robson[170] 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 A_2 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 helicesindeed 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 Val^{87}, Phe^{185}, and Trp^{188} (A_1 numbering) face the inside. These residues are homologous to Asp^{113}, Ser^{204}, and Ser^{207}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 detennined 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 [^{3}H]CPX has yielded a value of 58 kDa for the A_1 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 A_1 receptor vary between 34–38 kDa between troups[136,147,150,153,173]; the A_2 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 molecule 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 A_1 and A_2 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 induded 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 A_1 sequence also from a very short carboxy tail. The A_2 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 A_1 receptor is uncharacteristic of receptors inhibitory to this enzyme.[162]

2. Receptor Glycosylatiol1—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 A_1 and A_2 receptors are N-glycoslyated.[143,147,148] Asparagine residues that are potential glycosylation sites are located on E II in both the A_1 and A_2 sequences (Table VII). Asn^{159} is a potential substrate for glycosylation in the A_1 receptor. The rat A_1 receptor was reported to contain a single carbohydrate chain of the complex type.[147,148] For the A_2 receptor, four glycosylation consensus sites were identified, of which only two are presumably on the extracellular side of the membrane and accessible for glycosylation (Asn^{145} and Asn^{154} on E II), the other two being located on the intracellular side (Asn^{39} and Asn^{348}). Glycosylation of the A_2 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 Asn^{145} and Asn^{154}, respectively, However, Barrington *et al.* [143] have argued against glycosylation at two different sites, since treatment with endoglycosidase F—which cleaves the aspargine–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 Asn^{145} to glycoslyation 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 (βARK) 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 A_1 and A_2 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 A_2 receptor is rich in serine and threonine, typical for phosphorylation by βARK. This raises the question whether phosphorylation plays a role in adenosine receptor regulation. It has recently been shown that incubation of DDT_1MF-2 cells with the adenosine agonist *R*-PIA results in desensitization of A_1-mediated adenylate cyclase activity and in a concomitant **3–4**-fold increase in incorporation of [^{32}P]orthophosphate in the 36-kDa band of the A_1 receptor protein.[42] In the same cell type, treatment with *R*-PIA also resulted in desensitization of A_2

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 βARK 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 cif adenosine receptors has not been demonstrated.

5. Disufide Bonds—Disulfide bonds between extracellular cysteines are probably involved in maintaining the integrity of the ligand binding site of the β_2 adrenoceptor[182] 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 ^{125}I-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 (Cys^{106} and Cys^{190}) 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 Cys^{80}-Cys^{169} (A_1) and Cys^{77}-Cys^{166} (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 Cys^{260}-Cys^{263} (A_1) and Cys^{71}-Cys_{74}, Cys^{146}-Cys^{159} and Cys^{259}-Cys^{262}, or any combination there-of (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 [^{3}HJCGS 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, aTe 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 N^6-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 A_1 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 A_2 receptors.[152]

In keeping with these observations, we have suggested that the two conserved histidine residues in H VI and H VII (His^{251} and His^{278} in the A_1 receptor and His^{250} and His^{278} in the A_2 receptor, respectively) are potential candidates for this involvement in the binding site. Interestingly; His_{278} is homologous to Lys_{296} 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 site[187] 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 A_2 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 A_1 receptor, 15 out of the 17 cprresponding 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 hall 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 (Pro_{138}) has also been implicated in G protein interaction.[189,190] This residue-is highly conserved in related receptors, including A_1 (Pro^{112}) and A_2 (Pro^{109}), 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 A_2 receptor in interacting with the G protein. Partial proteolysis at a single site has been demonstrated for A_2 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 A_2 receptor likely plays a part in interacting with the G protein. The involvement of the N-terminal part of the carboxy tail of the A_1 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. Asp^{79} in the corresponding sequence in α_2 receptors is important in the allosteric modulation of agonist binding by Na^+.[14] Since agonist binding to A_1[192,193] and A_2[145] receptors is similarly regulated by Na^+, by analogy the homologous residues (Asp^{55} and Asp^{52}, respectively) may be expected to serve the same function.

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202. Note added in proof: A homologue of this receptor (termed A_3, but distinct from the A_3 receptor proposed by Ribeiro[28]) 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.

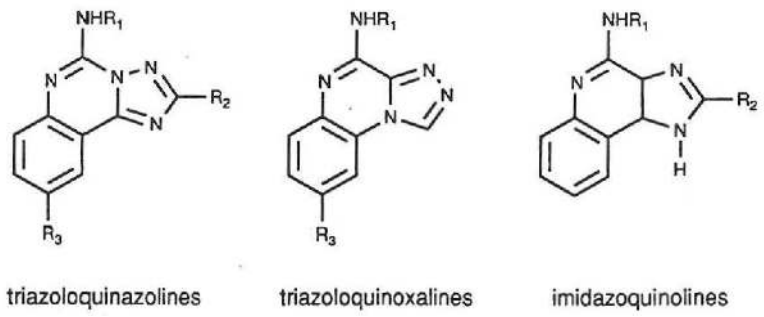


Figure 1.
Basic structures of tricyclic nonxanthine antagonists.

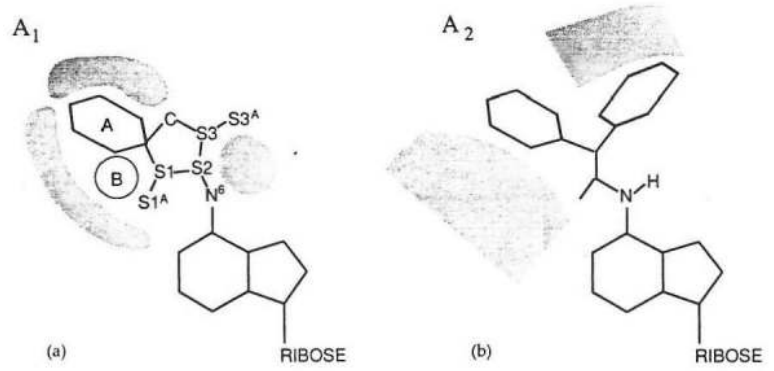


Figure 2.

Computer-generated models of the N^6 region of (a) A_1[91] and (b) A_2[105] receptors. Shaded areas indicate presumed receptor boundaries. The N^6 region of the A_1 receptor is divided into subdomains (S1, S2, S3, A, B, C) where hydrophobic substitution may enhance affinity.

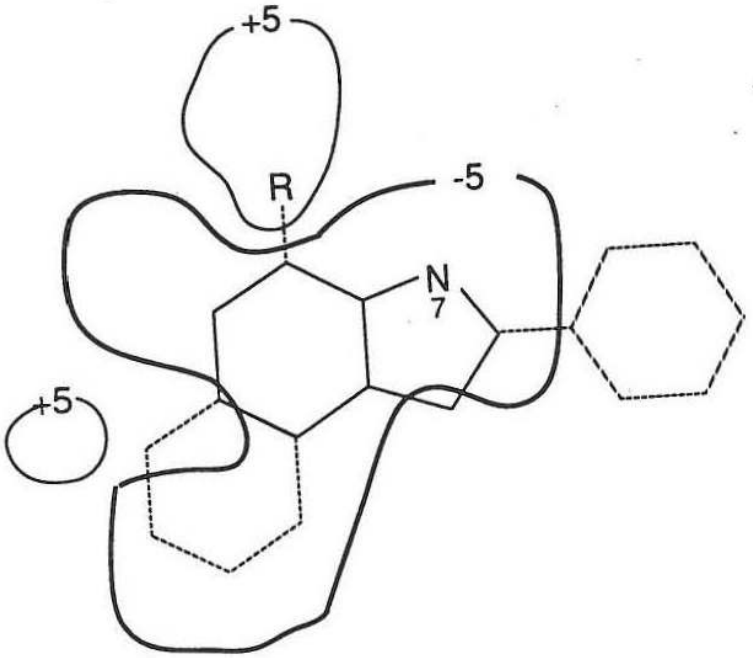


Figure 3.

Model for the antagonist binding site of the A_1 receptor. Indicated are areas of preferred electrostatic potential (+5 and -5 kcal/mol boundaries); a mtrogen atom at position 7 that is thought to act as a hydrogen bond acceptor; and areas where hydrophqbic substituents may enhance affinity.

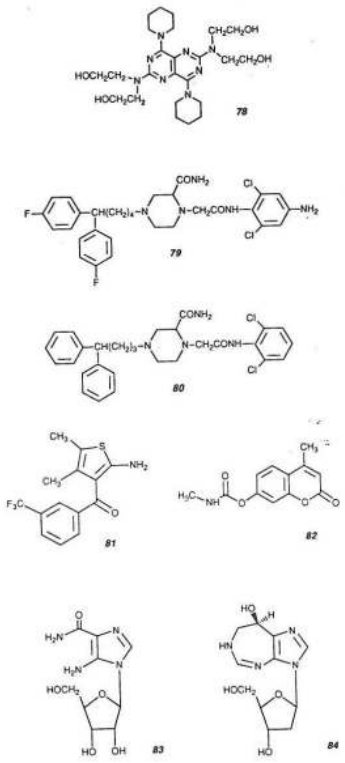


Figure 4.
Structures of some representative compounds (**78–84**) that enhance the actions of adenosine in an indirect manner.

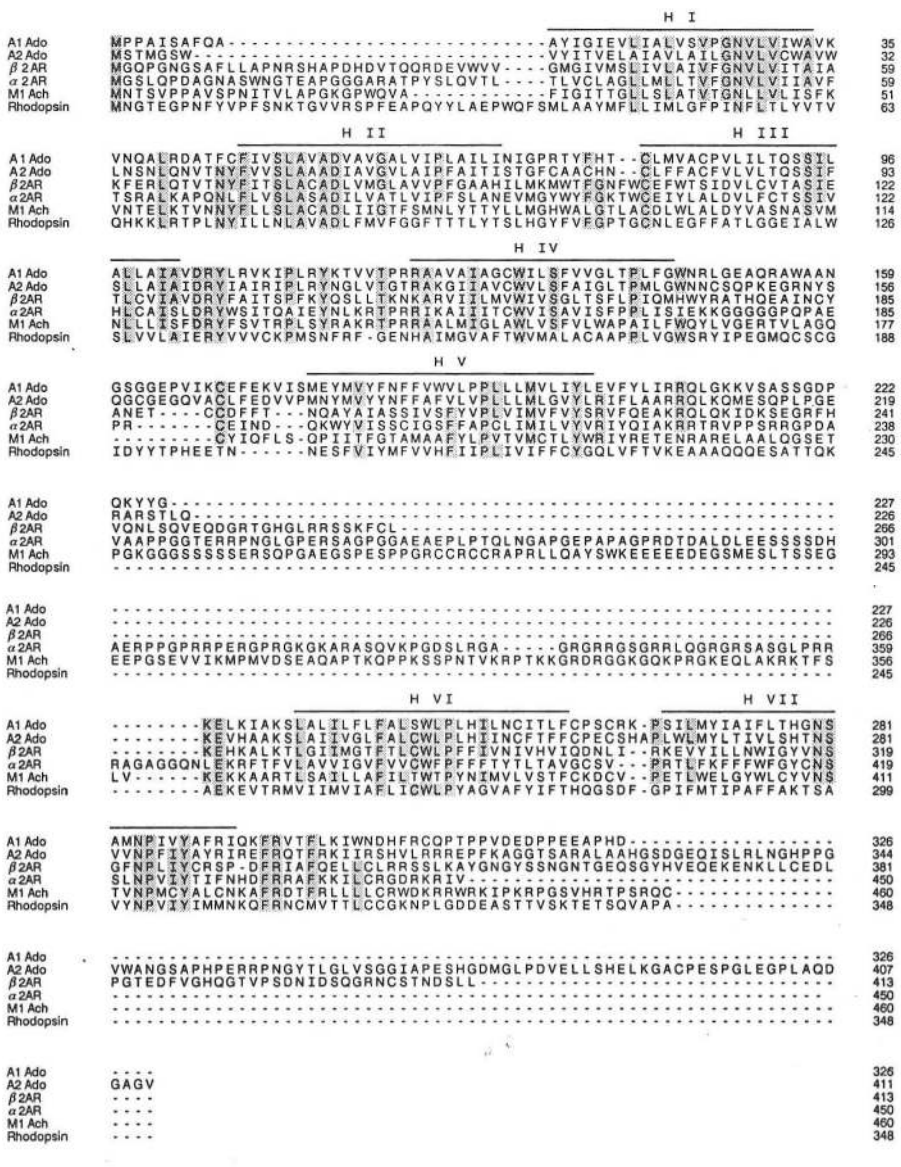


Figure 5.

(Overleaf) Alignment of the canine A_1 and A_2 receptors with the human β_2- and α_{2A}-adrenergic receptors, the rat Ml 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]

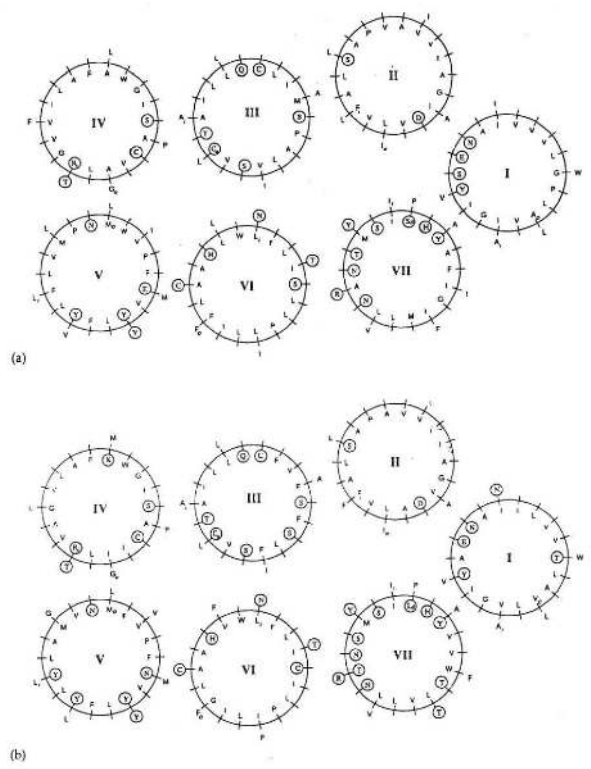


Figure 6.

Helical wheel representations of the (a) A_1 and (b) A_2 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 1 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 *0* (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.

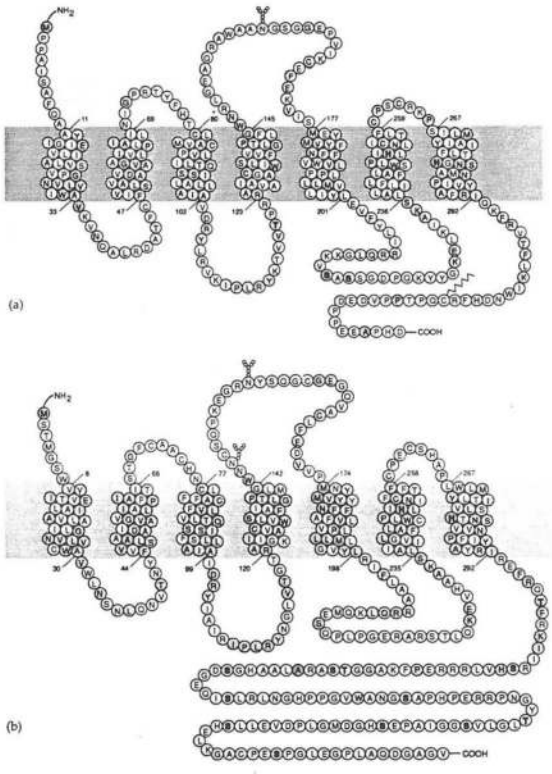


Figure 7.

Membrane topology of the (a) A_1 and (b) A_2 receptors. The N terminus is located on the extracellular side, the C tenninus 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 palmiloylation are also indicated.

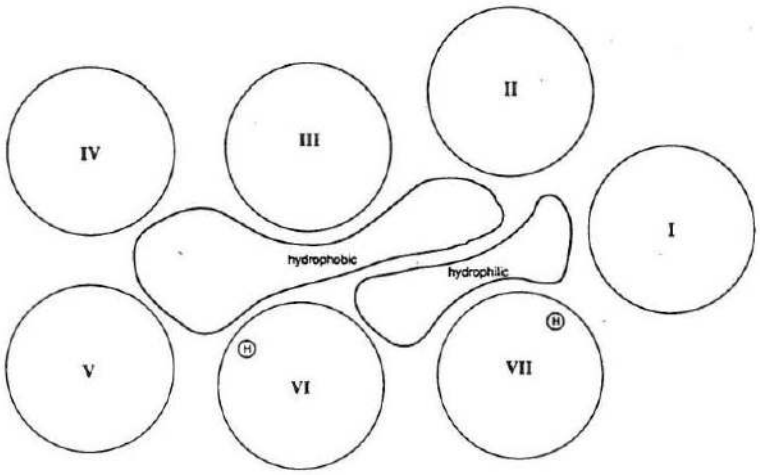


Figure 8.

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 A_1 and A_2 receptors.

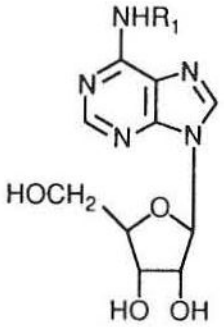
TABLE I

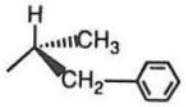
Effector Mechanisms Related to the Biological Effects of Adenosine

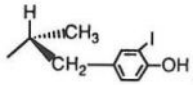
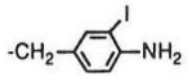
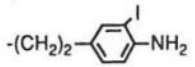
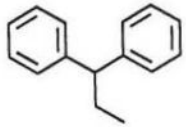
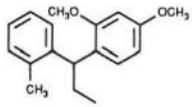
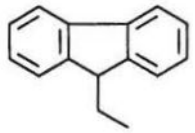
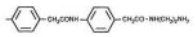
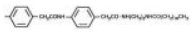
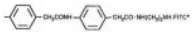
System	Action	Tissue/Cell	Subtype	Ref.
Adenylate cyclase	Inhibition	Adipocytes	A_1	17
		Heart	A_1	32
		Smooth muscle	A_1	43
	Stimulation	Platelets	A_{2a}	18
		Striatum	A_{2a}	19
		Smooth muscle	A_{2b}	43
		Brain	A_{2b}	21
		Fibroblasts	A_{2b}	20
Guanylate cyclase	Activation	Smooth muscle	A_1	22
Low K_m 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

TABLE II

Structure and Affinity of N^6-Substituted Adcnusine Derivatives at A_1 and A_2 Adenosine Receptors



Compoun	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		(5′-deoxy-5′-chloro derivative)	0.24	3,900	16,000[48]
6 *R*-PIA			1.2	124	100[19]

Compoun	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-phenethy)ado	2-phenylethyl	12.7	161	13[200]
12 APNEA		2.0	—	— [133]
13 N^6-(3-phenylpropy)ado	3-phenylpropyl	25	—	— [49]
14 N^6-sulfophenyl-propylado	3-(*p*-sulfo(phenyl)propy	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 IC_{50} values in nM, displacement of[^{3}H]PIA in rat brain membranes, unless indicated otherwise.

[b] Displacement of [^{3}H]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 isolhiocyanate.

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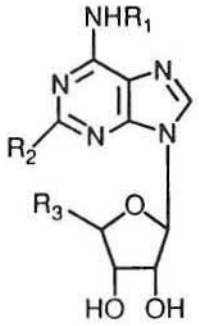
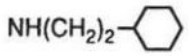
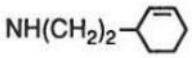
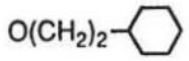
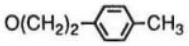
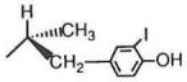
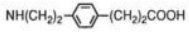
TABLE III

C2- and /or 5−-Substituted Adenosine Analogs



Compound	R_2	A_1(nM)[a]	A_2 (nM)[b]	A_2/A_1
	$R_3 = CH_2OH$; R_1 = H (unless noted)			
22 2-CADO	CI	9.3	63	6.8[19]
23 CCPA	CI;R_1 = cyclopetly	0.6	950	1500[197]
24 CV 1808	NHΦ	560	119	0.21[19]
25		12,000	22	0.0018[58]
26 CGS 22989		2,700	13	0.0048[58]
27 CHEA		1,500	22	0.014[60]
28 MPEA		48	11	0.22[60]
29 2-HNA	C≡C-$(CH_2)_3CH_3$	147	4.1	0.028[62]
30 2-ONA	C≡C-$(CH_2)_5CH_3$	211	12	0.057[62]
31 I-AHPIA		1.5	—	— [136]
32 NECA	H	6.3	10.3	1.6[19]
33 CGS 21680		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 *p*-DITC-APEC		280	7.1[d]	0.025[198]

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

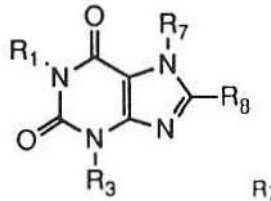
[b] Displacement of [^{3}H]NECA in rat strial membranes (in the presence of 50 nM CPA), unless indicated oterwise.

[c] K_d in bovine brain.

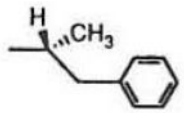
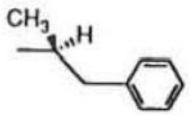
[d] Versus ^{125}I-PAPA-APEC in bovine brain.

TABLE IV

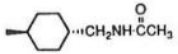
Xanthine Derivatives as Adenosine Antagonists



R_7 = H, unless noted

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	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	H	500	—	—[73]
42 1,3-dibenzylx	$CH_2\phi$	$CH_2\phi$	H	2,000	—	—[73]
43 IBMX	Me	$CH_2CH(CH_3)_2$	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	—$CH_2C{\equiv}CH$	Me	H(R_7 = Me)	45,000	16,000	0.36[185]
48 7-Bz-theophylline	Me	Me	H(R_7 = $CH_2\phi$)	6,000	46,000	7.7[75]
49 1,3-dibulylx-7-riboside	*n*-Bu	*n*-Bu	H(R_7 = ribose)	450[h]	—	—[89]
50 8-substituted *R*-PIA analog	Pr	Pr		6.9	160	23[106]
51 8-substituted *S*-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(nM)[a]	A_2(nM)[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	*p*-(SO_3H)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 *m*-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 IC_{50} values in nM, displacement of [^{3}H]PIA or [^{3}]CHA in rat brain cortical membranes, unless indicated otherwise.

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

[c] K_i for antagonism of adenylate cyclase inhibition in rat adipocytes.

[d] K_i for antagonism of adenylate cyclase activation in human platelets.

[e] K_i or IC_{50} value for displacement of [^{3}H]CHA in guinea pig forebrain membrances.

[f] K_d in bovine brain.

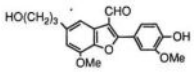
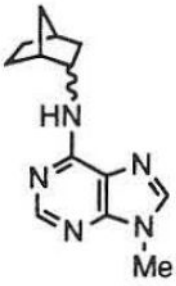
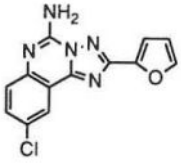
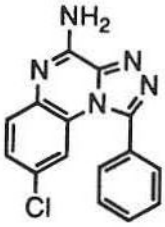
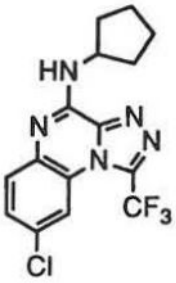
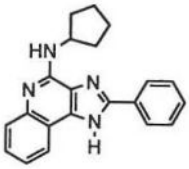
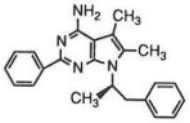
[g] In bovine brain.

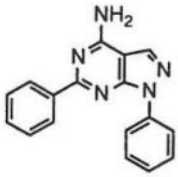
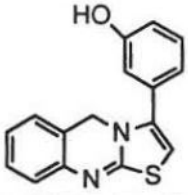
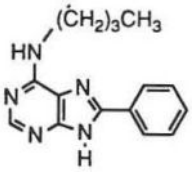
[h] [^{3}H]CPX binding to bovine brain membranes.

[i] This compound is nonselective in other (e.g., adenylaate cyclase) assays and is not useful as an A_1-selective ligand.[112]

TABLE V

Nonxanthine Adenosine Antagonists

Compound		A_1(nM)[a]	A_2(nM)[b]	A_2/A_1
68 benzo(*b*)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(nM)[a]	A_2(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 [^{3}H]PIA or [^{3}H]CHA in rat brain cortical membranes, unless indicated otherwise.

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

[c] In bovine brain.

TABLE VI

Secondary Structure for the Cytoplasmic and Extracellular Loops, as Predicated by teh Chou–Fasman[169] and Garnier-Osguthorpe-Robson[170] Methods. Calculations were Performed with the GCG Sequence Analysis Software Pakage[179] on a Convex C-240 Computer.

	Chou-Fasman			**Garmier-Osguthorpe-Robson**			
A_1 Receptor	**Turn**	**αHelix**	**βSheet**	**Turn**	**αHelix**	**β Sheet**	**Common**
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
	Chou-Fasman			Gamier-Osguthorpe-Robson			
A_2 Receptor	Turn	α Helix	β Sheet	Turn	α Helix	β Sheet	Common
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 VII

Consensus Patterns as Described in the Prosite Database[188]

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 interation	n.d. [e]	L^{3}QNVTNYFVVSLAAADI

[a]The residues that may be phosphorylated or glycosylated are printed in boldface.

[b]S^{267}, S^{262}, and S^{161} are located extracellularly and are thus not available for phosphorylation; the phosphorylation sites at S^{262} and S^{267} are absent in the rat A_1 sequence.

[c]n.d.: not detected.

[d]N^{39} and N^{348} are located intracellularly and are thus not available for glycosylation.

[e]The sequence that is complementary to the pattern detected in the A_2 receptor is L^{40}RDATFCFIVSLAVADV. All but the sixth and the seventh amino acid residues comply with the consensus pattern.