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Molecular Characterization of A_1 and A_{2a} Adenosine Receptors

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Abstract

Detailed amino acid sequence analyses of A_1 and A_{2a} adenosine receptors were assembled by analogy to other G-protein-coupled receptors and correlated with pharmacological observations. Sites for phosphorylation, palmitoylation, and sodium binding have been proposed. Striatal A_{2a} receptors from human and other species were photoaffinity-labeled using the selective, radioiodinated agonist PAPA-APEC. Selective chemical affinity labels for A_1 and A_{2a} receptors have been introduced. For example, an isothiocyanate, *p*-DITC-APEC (100 nM), irreversibly diminished the B_{max} for [^{3}H]CGS 21680 (2-[4-[(2-carboxyethyl) phenyl] ethylamino]-5'-N-ethylcarboxamidoadenosine) binding in rabbit striatal membranes by 71% (K_d unaffected), suggesting a direct modification of the ligand binding site. Novel trifunctional affinity labels have been designed. Rabbit and human A_{2a} receptors were characterized using [^{3}H]XAC binding in the presence of *50* or 25 nM CPX (8-cyclopentyl-l,3-dipropylxanthine), respectively. The inhibition of A_2 radioligand binding by the histidyl-modifying reagent diethylpyrocarbonate suggested the involvement of His residues in interactions with adenosine agonists and antagonists. Properties of transiently expressed mutants of bovine A_1 receptors in which either His^{251} or His^{278} residues have been substituted with Leu suggest that both histidines are important in binding.

Keywords

affinity labeling; sequence analysis; xanthines; chemical modification; mutagenesis

INTRODUCTION

Adenosine acts as a neuromodulator through at least two receptor subtypes [reviewed in Jacobson et al., 1992a; van Galen et al., 1992; Stiles, 1992; Linden, 19911, A_1 and A_2. A_2 receptors have been further divided into A_{2a} (high agonist affinity, in striatum) and A_{2b} (low agonist affinity, in fibroblasts) receptors. A number of effector mechanisms (cAMP, inositol phosphates, ion channels) are activated by adenosine, the best known being adenylate cyclase (inhibited by A_1 and activated by A_2). Several developments within the past few years have enabled a rigorous examination of the molecular structure and regulation of adenosine receptors: (1) the synthesis of adenosine derivatives, as agonists, and xanthine and non-xanthine antagonists with receptor subtype selectivity; and (2) the cloning of both A_1 and A_{2a} adenosine receptors [Maenhaut et al., 1990; Libert et al., 1991; Mahan et al., 1991; Reppert et al., 1991; Olah et al., 1992]. The cloning of both A_1 and A_2 adenosine receptors was a fortuitous achievement. The identity of the sequences RDC7 and RDC8, cloned from

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a dog thyroid cDNA library using the PCR technique through homology to other G-protein-linked receptors, was initially unknown and later found to resemble A_1 and A_{2a} receptors, respectively, in radioligand binding and effects on adenylate cyclase. Canine A_1 and A_{2a} receptors bear a 51% degree of homology to one another within transmembrane domains. Our goal is to define the parameters of the binding and receptor activation by purines using a variety of means, including synthetic, genetic, spectroscopic, and computational methodology.

Molecular Probes for Adenosine Receptors

We have synthesized new adenosine receptor ligands using a "functionalized congener approach" [Jacobson and Daly, 1991], by which potential positions for attachment of chains on a pharmacophore are empirically probed. The site of attachment must correspond to a region of relaxed steric requirements at or near the receptor binding site. This strategy has allowed us to target accessory sites of favorable interaction on the receptor, and actually enhance the affinity of the ligands. We have used such congeners to make fluorescent probes, superpotent lipid conjugates, affinity labels, etc. Molecular weights in the range of 34,000–40,000 Daltons have been measured for A_1 receptors [Stiles, 1992; Linden, 1991]. A_{2a} receptors have been labeled using the iodinated agonist PAPA-APEC (*p*-aminophenylacetyl conjugate of 2-[(2-aminoethylamino)carbonylethylphenylethylamino]-5'-N-ethylcarboxamidoadenosine) (Fig. 1) [Barrington et al., 1990; Nanoff et al., 1991], suggesting a molecular weight of 45,000 Daltons (bovine striatum). The measured molecular weights of the receptor proteins are in good agreement with the values calculated from sequences (36 K and 45 K for A_1- and A_{2a} -receptors, respectively). The rabbit (striatum), rat (PC12 cells), and frog (erythrocyte) A_{2a} receptors are of molecular weight 44–47K, and hamster (DDT_1-MF2 cells) A_{2a} receptors appear to be 40K. In membranes containing the rabbit A_{2a} receptors, cleavage of a 7,000 molecular weight fragment was observed in the absence of inhibitors of enzymatic proteolysis. This segment most likely corresponds to the long C-terminal tail found in the A_{2a} but not in the A_1 receptor. The receptor 38K fragment still binds ligands appropriately; however, the ability of guanine nucleotides to modulate the coupling to G-protein is enhanced following cleavage.

We have characterized the human A_{2a} receptor through radioligand binding and photoaffinity labeling [Ji et al., 1992]. A truly selective A_{2a} antagonist radioligand is lacking; however, one may use [^{3}H] XAC (8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]-oxy]phenyl]-1,3-dipropylxanthine) for certain species, such as primates and rabbit in which the A_{2a} receptors have particularly high affinity for 8-phenyl xanthines. Although A_1-selective in the rat brain, [^{3}H]XAC binds to human striatal A_{2a} receptors with high affinity (K_d = 2.98 nM). Twenty-five nM CPX (8-cyclopentyl-1,3-dipropylxanthine), an A_1-selective antagonist, in the incubation medium effectively eliminated 91% of [^{3}H]XAC (1 nM) binding to human A_1 receptors, yet preserved 90% of binding to A_2 receptors. [^{3}H]XAC exhibited saturable, binding to A_{2a} sites with a K_d of 2.98 nM and a B_{max} of 0.71 pmol/mg protein (25°C, nonspecific binding defined with 0.1 mM NECA (5'-N-ethylcarboxamidoadenosine)). The potency order for antagonists against 1 nM [^{3}H]XAC was CGS15943A > XAC ≈ PD115,199 > PAPA-XAC > CPX > HTQZ ≈ XCC ≈ CP-66,713 > theophylline ≈ caffeine, indicative of A_{2a} receptors. A_{2a} adenosine receptors were detected in the human cortex, albeit at a much lower density (~5%) than in the striaturn. Photoaffinity labeling using ^{125}I-PAPA-APEC revealed a molecular weight of 45K with 43K and 37K proteolytic fragments also observed. In the absence of proteolytic inhibitors, the 37K fragment, which still bound ^{125}I-PAPA-APEC, was predominant.

Isothiocyanate derivatives of adenosine have been developed as selective affinity labels for A_1 and A_2 adenosine receptors [Jacobson et al., 1992b]. An amine functionalized congener,

APEC (2-[(2-aminoethylamino) carbonylethylphenylethylamino]-5'-N-ethylcarboxamido-adenosine), is a selective agonist at A_{2a} adenosine receptors [Hide et al., 1992]. As a means of covalently inhibiting this receptor binding site, APEC was coupled to *m*- and *p*-phenylene-di-isothiocyanate (DITC). The resulting isothiocyanate derivatives in preincubation with rabbit or bovine striatal membranes irreversibly inhibited radioligand binding at A_{2a} but not A_1 receptors. Inhibition was prevented by theophylline (antagonist) or NECA (agonist). *p*-DITC-APEC (100 nM) diminished the B_{max} for [^{3}H]CGS 21680 binding by 71% (K_d unaffected), suggesting a direct modification of the ligand binding site. Bromoacetyl-APEC was ineffective as an affinity label. Selective inhibitors are potentially of interest in studies of the physiological role of adenosine receptors.

A new radioiodinated xanthine (adenosine antagonist) that binds covalently to A_1 adenosine receptors was prepared via a general "trifunctional approach" to receptor ligands and used as a receptor probe [Jacobson et al., 1992c]. BH-DITC-XAC was synthesized via reaction of XAC with a trifunctional aryl diisothiocyanate crosslinker, containing the p-hydroxyphenylpropionyl group ("BH", Bolton-Hunter reagent) for radioiodination. Following reaction, a remaining isothiocyanate group reacts with the receptor protein. ^{125}I-BH-DlTC-XAC, prepared directly by the chloramine T method and purified by HPLC, bound specifically to A_1 receptors. This binding was inhibited in the presence of the adenosine agonists *R*-PIA, *S*-PIA, and NECA in a dose-dependent manner and with the order of potency characteristic of bovine A_1 receptors. Incubation of affinity-purified bovine A_1 receptors (prepared using a XAC-Sepharose affinity column) with ^{125}1-BH-DITC-XAC (0.8 nM) for 2 hours resulted in the specific and clean labeling of a polypeptide band corresponding to MW 36,000, identical to that previously found for the A_1 receptor.

Sequence Analysis of Adenosine Receptors and Correlation With Pharmacological Observations

Detailed models of A_1 and A_{2a} receptors (Fig. 2) consistent with labeling experiments have been assembled [van Galen et al., 1992] from an amino acid sequence analysis and by analogy to other G-protein-coupled receptors. These models conform with the seven transmembrane domain topology commonly found for receptors linked to G-proteins.

Glycosy lation—Rat A_1 receptors are N-glycosylated with a complex carbohydrate chain, and glycosylation of the A_2 receptor is heterogeneous in nature, containing carbohydrate chains of both the complex and the high-mannose types [Barrington et al., 1990]. Asparagine residues that are potential glycosylation sites are located on E-II in both the A_1 and A_2 sequences. Asn^{159} is a potential substrate for glycosylation in the A_1 receptor. For A_2 receptors, two *extracellular* glycosylation consensus sites were identified (Asn^{145} and Asn^{154} on E-II), but glycosylation likely occurs only at Asn^{154}.

Receptor phosphorylation—Desensitization of a number of G-protein-linked receptors is associated with phosphorylation by: (1) second-messenger-activated kinases like protein kinase A (CAMP-dependent protein kinase; PKA) and protein kinase C (PKC) (leading to heterologous desensitization) or (2) receptor-"specific" kinases, such as β-adrenoceptor receptor kinase (βARK) and rhodopsin kinase, that phosphorylate their substrates only in the agonist-occupied state of the receptor (implicated in homologous desensitization). The A_1 receptor in DDT$_1$MF-2 cells is phosphorylated in response to agonist [Ramkumar et al., 1991], but A_2 phosphorylation has not yet been studied.

The A_1 and A_2 sequences display a number of consensus patterns for phosphorylation by PKA, PKC or by casein kinase II (CK2), that has so far not been implicated in the

phosphorylation of G-protein-coupled receptors. The carboxyl terminus of the A_2 receptor is rich in serine and threonine, suggestive of phosphorylation by βARK.

Acylation—Many G-protein-linked receptors contain a cysteine residue in the C-terminal tail close to H-VII, that is palmitoylated and essential for G-protein coupling in adrenergic receptors and in rhodopsin, but not the m2-muscarinic receptor [Van Koppen and Nathanson, 1991]. The palmitoyl group would, in effect, create a fourth cytoplasmic loop by anchoring the cytoplasmic tail to the membrane. The role of an analogous cysteine residue present in the carboxy tail of the A_1 receptor (Cys^{309}), but not the A_{2a} receptor, is yet to be determined.

Disulfide bonds—Disulfide bonds between extracellular cysteines are probably involved in maintaining the integrity of the ligand binding site of muscarinic [Savarese et al., 1992] and other G-protein-linked receptors, 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. The analogous cysteines in the adenosine receptor sequences are Cys^{80} - Cys^{169} (A_1) and Cys^{77} - Cys^{166} (A_2). Non-intracellular 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 thereof (A_2). A disulfide bridge between two cysteines four residues apart would likely stabilize the β-turns predicted for E-II (A_2), E-III (A_1 and A_2) and which might also occur in E-I(A_2).

We have shown that [^{3}H]CGS 21680 binding to A_2 receptors is disrupted following treatment with the disulfide-reactive reagents mercaptoethanol (> 50 mM) or DTT or dithionite (> 10 mM) [Jacobson et al., 1992b]. This suggests that disulfide linkages indeed are involved in maintaining the structural integrity of the A_2 receptor.

Sodium regulatory site—The sequence [SN]-L-A-x-[AT]-D occurs near the cytoplasmic end of H-II in both A_1 and A_2 receptors [Van Galen et al., 1992] and in many other G-protein-linked receptors. In α_2-adrenergic receptors, the Asp residue of the analogous sequence is important in the allosteric modulation of agonist binding by Na^+. Since agonist binding to both adenosine receptors is similarly regulated by Na^+, the Asp^{55} (A_1) and Asp^{52} (A_2) may constitute the site of sodium binding.

Ligand binding site—The binding sites for structurally diverse nonpeptidic ligands, such as rhodopsin, norepinephrine, and acetylcholine, that bind to G-protein-coupled receptors is thought to occur roughly midway along the transmembrane helices, near the center of the cavity. A negatively charged aspartate residue on H-III, 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 of these ligands. Unlike the biogenic amines, adenosine is uncharged at physiological pH, and an anionic residue at this position would offer no electrostatic advantage in binding. The specific aspartate residue corresponds to an uncharged (Val^{87}) residue in A_1 receptors.

The inhibitory effects of diethylpyrocarbonate (DEP, a His-modifying reagent) in pharmacological studies with wild type receptors have implicated two histidines in interactions with adenosine agonists and antagonists (shown for both A_1 [Klotz et al., 1988] and A_{2a} [Jacobson et al., 1992b] receptors). Exposure of membranes to DEP (2.5 mM) followed by washing was found to inhibit the binding of [^{3}H]CGS 21680 and [^{3}H]XAC to A_{2a} receptors, by 86% and 30%, respectively. NECA protected against inhibition of [^{3}H]CGS 21680 binding better than did theophylline, and the converse was found for

[^{3}H]XAC binding. There are two conserved histidine residues in transmembrane domains VI and VII (His^{251} and His^{278} in A_1 receptors) that are likely the putative histidine residues affected by DEP.

These two histidyl residues have each been replaced with leucyl residues by site-specific mutation of bovine A_1 receptors [Olah et al., 1992] and expression in COS7 cells. Leu^{278} decreased both agonist and antagonist binding by > 90%. In contrast, Leu^{251} decreased antagonist but not agonist affinity, and a decreased receptor number was found for both agonist and antagonist binding.

In collaboration with Dr. Ad IJzerman, we have recently devised a preliminary molecular model for the binding of adenosine to the A_1 receptor [IJzerman et al., 1992], that is consistent with the observed pharmacology, structure activity relationships, and calculated ligand conformation. This model may be used to predict sites for interaction between specific amino acid residues of the receptor and its ligands, and may be tested in studies of mutagenesis of the receptor. Eventually the objective will be to synthesize improved ligands, including chemical affinity labels and possibly ligands of high selectivity for other subtypes (e.g., A_{2b}) [Stehle et al., 1992], consistent with the model.

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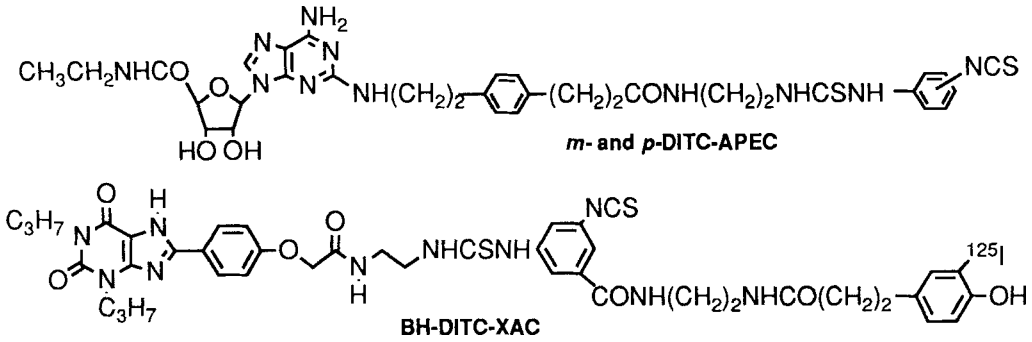

Figure 1.

Structures of isothiocyanate-bearing affinity labels for adenosine receptors. BH-DITC-XAC and the DITC-APEC isomers are selective for A_1- and A_2-receptors, respectively.

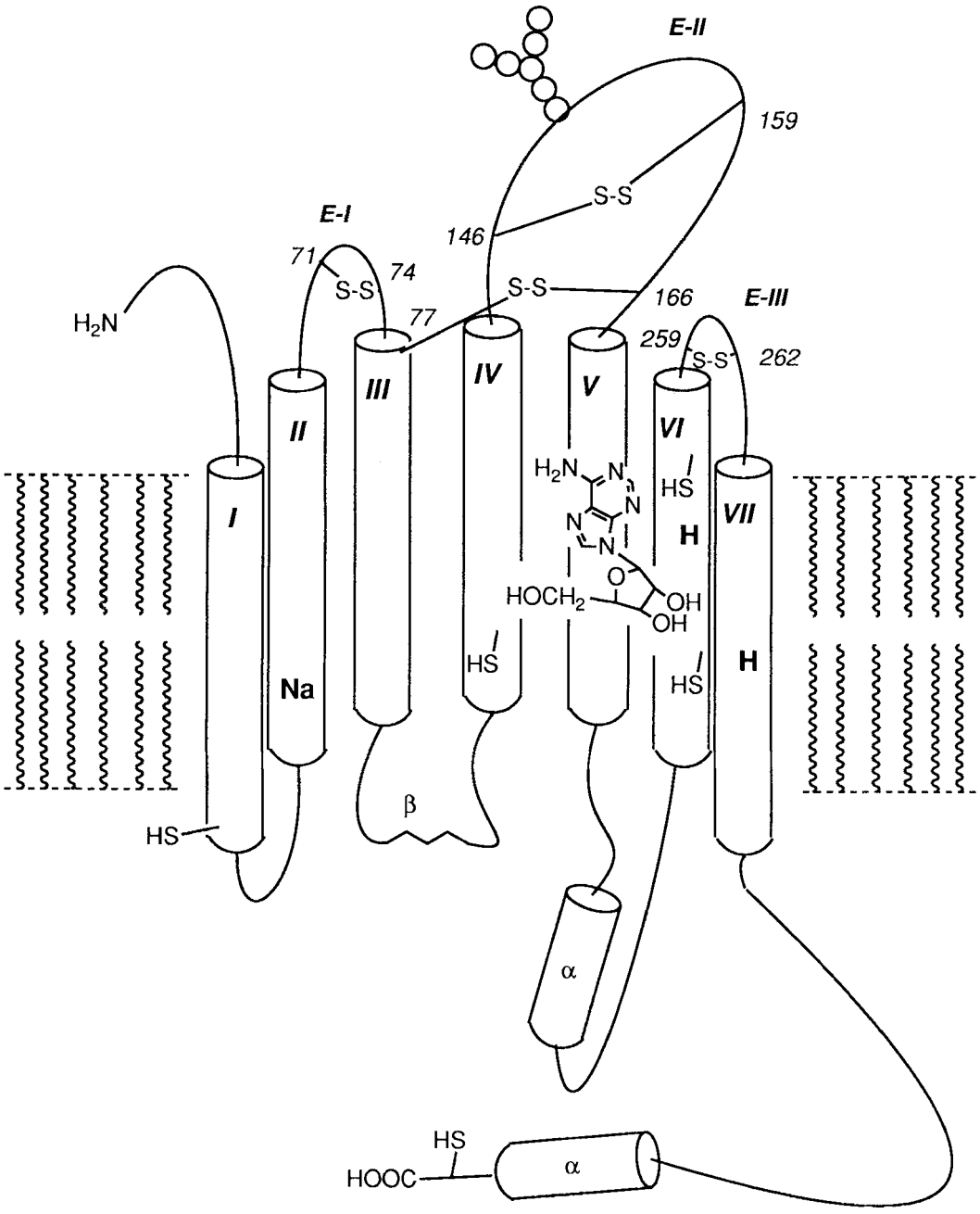

Figure 2.

A proposed model for canine A_{2a} receptors deduced from the primary sequence, including seven transmembrane helices (*I–VII*), three extracellular loops (*E-I–III*), two histidinyl residues possibly involved in ligand binding (H), a sodium binding site (Na), and hypothetical disulfide bonds (S-S, residue numbers in italics) and thiol groups. Thiol of Cys^{82} is not shown.