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AGONIST ACTIVITY OF 2- AND 5′-SUBSTITUTED ADENOSINE ANALOGS AND THEIR N^6-CYCLOALKYL DERIVATIVES AT A_1- AND A_2-ADENOSINE RECEPTORS COUPLED TO ADENYLATE CYCLASE

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Abstract—The activity of N^6-cycloalkyl derivatives of adenosine, 2-chloroadenosine, 5′-chloroadenosine and *N*-ethylcarboximidoadenosine (NECA) and of 2-fluoroadenosine and 5-methylthioadenosines were compared at the A_1-adenosine receptor inhibitory to adenylate cyclase in rat fat cell membranes and at the A_{2A}-adenosine receptors stimulatory to adenylate cyclase in rat PC12 cell membranes. The N^6-cycloalkyl derivatives in all cases were more potent (4- to 23-fold) than the parent compound at the A_1 receptor, and were less potent (1.6- to 11-fold) than the parent compound at the A_{2A} receptor. N^6-Cyclopentyl-5′-chloroadenosine was the most selective agonist (900-fold) for the A_1 receptor, while 2-fluoroadenosine was the only agonist with some selectivity (4.8-fold) for the A_{2A} receptor. 5′-Methylthioadenosine was a weak agonist at both adenosine receptors. A 2-fluoro derivative of 5′-methylthioadenosine was somewhat more potent. Affinities of these analogs for inhibition of binding of radioligands to rat brain A_1 and A_{2A} receptors are presented.

Structure–activity relationships for adenosine analogs reveal both similarities and marked differences for interactions with the major classes of adenosine receptors. Presently, two major classes of adenosine receptors are proposed, the A_1-receptor that can inhibit adenylate cyclase and the A_2-receptor that activates adenylate cyclase [1]. Binding studies in brain cortical membrane with an appropriate ligand (e.g. [^{3}H]*R*-PIA† and [^{3}H]CHA) detect a high density of A_1-receptors [2, 3] which, however, may not all be interactive with adenylate cyclase [4]. Membranes of fat cells provide a model system for A_1-receptor-mediated inhibition of adenylate cyclase [5]. A division of A_2-receptors stimulatory to adenylate cyclase into two subtypes, the high-affinity A_{2A} receptor and the low-affinity A_{2B} receptor, has been proposed [6, 7]. Binding studies in brain striatal membranes with an appropriate ligand ([^{3}H]NECA, [^{3}H]CGS 21860) detect a high density of A_{2A}-receptors [8, 9]. Membranes of pheochromocytoma PC12 cells or human platelets provide model systems for A_{2A}-receptor-mediated stimulation of adenylate cyclase [10], while fibroblast cells [9] or brain slices [6] are two model systems for A_{2B}-receptor-mediated stimulation of adenylate cyclase. Relaxation of smooth muscle appears due to the interaction of adenosine and its analogs with A_2-receptors, but whether adenylate cyclase is involved remains uncertain [11].

Agonists selective for A_1 receptors have been developed through N^6-substitution, in particular with cyclopentyl and cyclohexyl moieties to yield N^6-cyclopentyladenosine [12] and N^6-cyclohexyladenosine [2]. Conversely, 2-substitution has led to analogs such as 2-phenylaminoadenosine (CV 1808) [13, 14], CGS 21680 and related 2-arylalkylamino NECAs [15, 16], 2-alkoxy and 2-aralkoxy adenosines [17, 18], and 2-alkynyladenosines [19], that are selective for A_{2A}-receptors. Few alterations of the ribose moiety of adenosine are tolerated. But the 5′-position can be modified and replacement of the —CH_2OH of adenosine with a —$CONHC_2H_5$ moiety yields NECA, an agonist with high potency and efficacy at A_1-, A_{2A}- and A_{2B}-receptors [13]. CGS 21680 represents a 2-substituted NECA. Other 5′-modified adenosines include 5′-deoxyadenosine, a weak partial agonist of A_{2B}-receptors [9, 20], and 5′-methylthioadenosine, a rather unique agent in being one of the few adenosine analogs that act as a competitive antagonist at A_{2B}-receptors of fibroblasts [9]. Many ribose-modified adenosine analogs, for example 2′,5′-dideoxyadenosine, while relatively inactive at adenosine receptors, noncompetitively inhibit adenylate cyclases via a so-called P-site [21, 22]. 5′-Methylthioadenosine is not active at the P-site [22] although it, like 2′,5′-dideoxyadenosine, does prevent the inhibitory effect of 2-fluoroadenosine on ADP-induced platelet aggregation [23]. The effect of 5′-methylthioadenosine, unlike that of 2′,5′-dideoxyadenosine, is *specific* for blockade of adenosine receptor-elicited responses [23].

Recently, 5′-methylthioadenosine was studied with regard to effects on three adenosine receptor

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† Abbreviations: [^{3}H]*R*-PIA, [^{3}H]-(*R*)-N^6-(phenylisopropyl)adenosine; [^{3}H]CHA, N^6-cyclohexyl[^{3}H]adenosine; [^{3}H]NECA, [^{3}H]-5′-(*N*-ethylcarboxamido)adenosine; and [^{3}H]CGS 21680, [^{3}H]-2-[*p*-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine.

systems, namely (i) A_1-receptors inhibitory to adenylate cyclase; (ii) A_2-receptors stimulatory to adenylate cyclase in neuroblastoma cell membranes; and (iii) A_2-receptors causing relaxation of smooth muscle [11]. 5'-Methylthioadenosine proved to be a weak agonist at the A_1-receptor, a competitive antagonist at the A_2-receptor of neuroblastoma cell membranes, and a weak smooth muscle relaxant. However, the lack of a potent inhibition of the smooth muscle relaxant effects of 5'-methylthioadenosine by xanthines [11, 24] suggests that it does not cause relaxation via the xanthine-sensitive A_2 receptor of smooth muscle. 5'-Methylthioadenosine does not antagonize the relaxant effects of NECA, whereas xanthines do antagonize the NECA response [24].

To further explore the effects of modifications of adenosine at the N^6-, 2- and 5'-positions with regard to affinity and agonist/antagonist activity, a set of 2-substituted (2-fluoro, 2-chloro) and 5'-modified (5'-methylthio, 5'-chloro, 5'-ethylcarboxamido) adenosines and, where available, the corresponding N^6-cycloalkyl derivative were assayed in binding and adenylate cyclase paradigms.

MATERIALS AND METHODS

Adenosine, N^6-cyclopentyladenosine, 2-chloroadenosine, 5'-methylthioadenosine, and NECA were from the Sigma Chemical Co. (St. Louis, MO) and Research Biochemicals Inc. (Natick, MA). N^6-Cyclopentyl-2-chloroadenosine, 2-fluoroadenosine, 5'-chloroadenosine and N^6-cyclohexylNECA were provided by Dr. R. A. Olsson (University of South Florida, Tampa, FL). 2-Fluoro-5'-methylthioadenosine was from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). N^6-Cyclopentyl-5'-chloroadenosine was provided by the Warner–Lambert Co., Parke–Davis Pharmaceutical Research (Ann Arbor, MI). [^{3}H]*R*-PIA and [^{3}H]NECA were from New England Nuclear (Boston, MA) and [α-^{32}P]ATP from Amersham (Arlington Heights, IL).

Inhibition of binding of [^{3}H]*R*-PIA to A_1-receptors in rat brain cerebral cortical membranes [25] and inhibition of binding of [^{3}H]NECA to A_{2A}-receptors in rat brain striatal membranes [9] were assayed as described. Theophylline (5 mM) was used to define nonspecific binding and 50 nM N^6-cyclopentyladenosine was present to block A_1-receptors in the A_2 binding assay. K_i values were calculated from IC_{50} values with the Cheng–Prusoff equation [26] with a K_D for [^{3}H]*R*-PIA of 1.0 nM and a K_D for [^{3}H]NECA of 8.5 nM.

Stimulation of adenylate cyclase activity via A_{2A}-receptors in rat pheochromocytoma PC12 cell membranes was assayed as described [10, 13]. Inhibition of isoproterenol-stimulated adenylate cyclase via A_1-receptors in rat adipocyte membranes was assayed as described [10].

RESULTS

The affinities of adenosine analogs for A_1-receptors in rat brain cortical membranes and A_{2A}-receptors in rat brain striatal membranes are presented in Table 1. The N^6-cycloalkyl derivatives had higher affinities at A_1-receptors than the parent compound, while having lower affinities than the parent compound at A_{2A}-receptors. The potencies (and efficacies) in inhibiting isoproterenol-stimulated adenylate cyclase of rat fat cell membranes via an A_1-receptor and in stimulating adenylate cyclase of rat pheochromocytoma PC12 cell membranes also are provided in Table 1. Again the N^6-cycloalkyl compounds were more potent at the A_1-receptor than the parent compound and less potent at the A_{2A}-receptor than the parent compound. All analogs were full agonists at the inhibitory A_1-receptor of fat cell membranes (data not shown) and all were agonists at the stimulatory A_{2A}-receptor of PC12 cell membranes. Representative concentration–response curves for activity at the A_1- and A_{2A}-receptors are shown in Figs. 1 and 2. Most analogs were somewhat less efficacious than 2-chloroadenosine, NECA and N^6-cyclohexylNECA at the A_{2A}-receptor. In certain cases, such as 5'-methylthioadenosine, the response in PC12 cell membranes was not fully maximal at the highest concentration (300 μM) tested (Fig. 1) and in these cases the EC_{50} represents the concentration that causes a response 50% of that caused by 300 μM. In the case of 2-fluoroadenosine, the effects on adenylate cyclase in rat PC12 cell membranes were biphasic with the stimulation followed at high concentrations with an inhibition, resulting in a bell-shaped curve (Fig. 1). 5'-Methylthioadenosine and 5'-chloroadenosine were partial agonists at the A_{2A}-receptor of PC12 cell membranes, an unexpected finding in view of the competitive antagonist activity of 5'-methylthioadenosine and other analogs with lipophilic 5'-moieties at the A_{2B}-receptor of human fibroblast VC13 cells [9].

DISCUSSION

N^6-Cycloalkyl derivatives of adenosine and of 2-chloroadenosine are potent and selective agonists at A_1-receptors [13, 27]. The present results extend this observation to N^6-cyclopentyl-5'-chloroadenosine and N^6-cyclohexylNECA (Table 1). This and other N^6-substituted NECAs previously were reported [28] to be selective for A_1-receptors, based on a comparison of A_1-receptor binding data with A_{2A}-receptor adenylate cyclase data. 2-Chloroadenosine, like adenosine, was relatively nonselective for A_1- and A_{2A}-receptors, while 2-fluoroadenosine was nearly 5-fold selective for the A_{2A}-receptor of PC12 cell membranes compared to the A_1 receptor of fat cell membranes (Table 1). It was not selective for the A_2-receptor when rat brain binding data for A_1- and A_2-receptors were compared (Table 1). 2-Fluoroadenosine was unique among the agents tested in showing a bell-shaped response curve in PC12 cell membranes (Fig. 1). It appears likely that this is due to the P-site inhibition of adenylate cyclase by 2-fluoroadenosine. 2-Fluoroadenosine is nearly 9-fold more potent than adenosine at the P-site [22].

The most remarkable finding of the present study was the agonist activity at the A_{2A}-receptor of 5'-methylthioadenosine, an analog previously reported to be a competitive antagonist (K_i 8.2 μM) at A_{2B}-

Table 1. Activity of adenosine and adenosine analogs at A_1- and A_{2A}-receptors

Adenosine (Ado) and analogs	K_i or EC_{50}^* (nM) A_1-Receptor rat brain binding vs [^{3}H]*R*-PIA	A_1-Receptor rat fat cell inhibition of adenylate cyclase	A_{2A}-Receptor rat striatum binding vs [^{3}H]NECA	A_2-Receptor rat PC12 cell stimulation of adenylate cyclase
Ado	—†	73 ± 13	—†	150 ± 10 (0.70)
N^6-CyclopentylAdo	0.32 ± 0.03	19 ± 1	510 ± 120	3240 ± 320 (0.83)
2-ChloroAdo	6.7 ± 1.0	127 ± 19	76 ± 12	460 ± 50 (0.93)
N^6-Cyclopentyl-2-chloroAdo	0.6 ± 0.1	3.0 ± 0.5	950 ± 90	730 ± 110 (0.70)
2-FluoroAdo	5.9 ± 0.4	2170 ± 320	28 ± 6	440 ± 150 (0.68)
5'-MethylthioAdo	243 ± 3	3700 ± 530	1180 ± 160	8900 ± 620 (0.50)
2-Fluoro-5'-methylthioAdo	84 ± 3	680 ± 60	950 ± 80	1830 ± 330 (0.60)
5'-ChloroAdo	20 ± 1	140 ± 20	140 ± 18	860 ± 340 (0.62)
N^6-Cyclopentyl-5'-chloroAdo	0.63 ± 0.07	6.0 ± 0.6	1360 ± 280	5400 ± 930 (0.59)
NECA	5.1 ± 0.3	104 ± 12	9.7 ± 1.3	130 ± 10 (1.0)
N^6-CyclohexylNECA	0.43 ± 0.03	0.92 ± 0.16	170 ± 50	160 ± 30 (1.0)

* Values are means ± SEM (N = 3). Values in parentheses are maximal efficacies compared to NECA set equal to 1.0 as a full agonist.

† Adenosine cannot be assayed in binding paradigms because of the presence of adenosine deaminase.

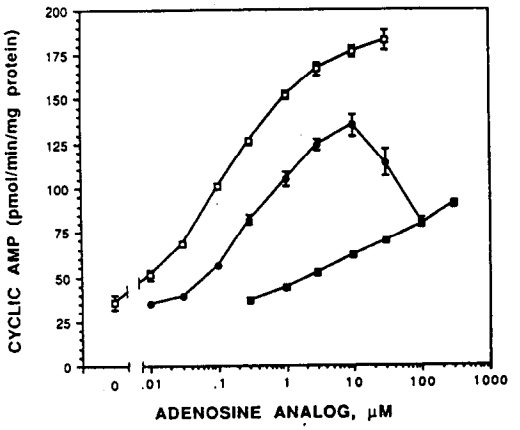

Fig. 1. Stimulation of adenylate cyclase by adenosine and analogs in rat PC12 cell membranes. Adenylate cyclase activity was determined after incubation with various concentrations of NECA (□), 2-fluoroadenosine (●), or 5'-methylthioadenosine (■) for 10 min at 37°. Values are means ± SEM (N = 3). Where error bars are not shown, they were smaller than the size of the symbol.

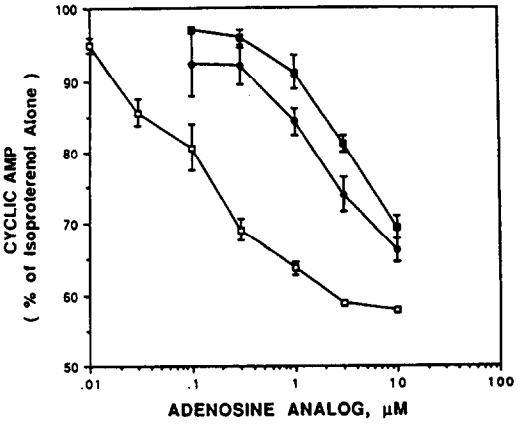

Fig. 2. Inhibition of isoproterenol-stimulated adenylate cyclase by adenosine analogs in rat fat cell membranes. Adenylate cyclase activity was determined after incubation with 10 μM isoproterenol and various concentrations of NECA (□), 2-fluoroadenosine (●), or 5'-methylthioadenosine (■) for 10 min at 37°. Values are means ± SEM (N = 3). Control levels of adenylate cyclase activity were 100 ± 20 pmol cAMP/min/mg protein. Isoproterenol caused a 2.0 ± 0.2 fold stimulation of activity.

receptors of human fibroblasts [9]. We had seen an antagonism by 5'-methylthioadenosine of the activation of cyclic AMP accumulation by 2-chloroadenosine via an A_{2B}-receptor in guinea pig brain cerebral cortical slices (unpublished data). Munshi *et al.* [11] reported antagonist activity of 5'-methylthioadenosine (K_i 8.2 μM) at an A_2-receptor of undefined subtype in mouse neuroblastoma 2a cell membranes. 5'-Methylthioadenosine also is a potent agonist (EC_{50} 0.09 μM) at an A_1-receptor inhibitory to adenylate cyclase in rat brain cerebellar membranes [11], a value much lower than that found for the inhibitory A_1-receptor of rat fat cell membranes (EC_{50} 3.7 μM, Table 1). 5'-Methylthioadenosine was a weak agonist (EC_{50} 8.9 μM) with an efficacy about 50% of that of NECA at the A_{2A}-receptor stimulatory to adenylate cyclase in rat PC12 cell membranes (Table 1, Fig. 1). It is possible that 5'-methylthioadenosine has much lower efficacy at other A_2-receptor systems and, therefore, appeared to be an antagonist when tested in consort with an adenosine receptor agonist.

Table 2. Comparison of potencies of adenosine and adenosine analogs at A_{2A}-receptors of rat pheochromocytoma cell membranes with potencies at A_{2B}-receptors of human fibroblast cells*

Adenosine (Ado) and analogs	A_{2A}-receptor rat PC12 cell membranes EC_{50} (μM)	A_{2B}-Receptor human VC13 cells EC_{50} (μM)	K_i (μM)
Ado	0.15 ± 0.01	15.4 ± 0.6	
N^6-PentylAdos	3.2 ± 0.3†	140‡	
2-ChloroAdo	0.46 ± 0.05	24	
2-FluoroAdo	0.44 ± 0.15	25	
5'-MethylthioAdo	8.9 ± 0.6		8.2 ± 0.9
NECA	0.13 ± 0.01	2.6	

* Values for A_{2A}-receptors are from Table 1. Values for A_{2B}-receptors are from Refs. 7 or 9.
† N^6-CyclopentylAdo.
‡ N^6-*n*-PentylAdo.

The presence of a 2-fluoro substituent increased the potency of 5'-methylthioadenosine as an agonist by several-fold at both the fat cell A_1-receptor and the PC12 cell A_{2A}-receptor. Such an increase did not pertain in a comparison of adenosine and 2-fluoroadenosine (Table 1). As in the fat cell, the 2-fluoro substituent increased affinity of 5'-methyladenosine several-fold for the rat brain A_1-receptor. However, the 2-fluoro substituent did not increase affinity of 5'-methylthioadenosine at the rat striatal A_{2A}-receptor. A 2-fluoro substituent greatly increases the potency of adenosine, adenine arabinoside and adenine xylofuranoside as P-site inhibitors [22]. But such an enhancing effort at the P-site is not relevant to the present results since 5'-methylthioadenosine does not have P-site activity even at 1 mM [22].

The results with 5'-chloroadenosine paralleled those with 5'-methylthioadenosine. Both analogs lack the hydrogen donor group (CH_2OH or CONHR) thought to be associated with agonist activity at adenosine receptors, yet both are agonists at A_1- and A_{2A}-receptors. Indeed, the potency of 5'-chloroadenosine was only 2-fold less than adenosine at the A_1-receptor of fat cell membranes, while being 6-fold less than adenosine at the A_{2A}-receptor of PC12 cell membranes (Table 1). To our knowledge, agonist effects of 5'-chloroadenosine on an A_{2B}-receptor have not been reported. Data for 5'-methylthioadenosine, 5'-chloroadenosine, and N^6-cyclopentyl-5'-chloroadenosine versus [^{3}H]ligand binding to brain A_1- and A_{2A}-receptors have been reported [7, 29, 30] and are in essential agreement with the present results. In isolated rat hearts both 5'-methylthioadenosine and 5'-chloroadenosine had very low activity in reducing the heart rate, an A_1-receptor-mediated response, while both have moderate activity in enhancing coronary flow, an A_2-receptor-mediated response, being about 25- and 4-fold less potent, respectively, than 2-chloroadenosine [30]. Both 5'-methylthioadenosine and 5'-chloroadenosine cause relaxation of guinea pig trachea with the latter many-fold more potent than the former [24]. The relaxation elicited by 5'-methylthioadenosine is not antagonized by a xanthine, while that elicited by 5'-chloroadenosine is "partially" antagonized. A N^6-cycloalkyl substituent markedly increased potency of 5'-chloroadenosine at A_1-receptors, while decreasing potency at the A_{2A}-receptors, as also was the case for adenosine, 2-chloroadenosine and NECA (Table 1).

The present results provide an example of a difference between A_{2A}- and A_{2B}-receptors, besides the former being a "high-affinity" and the latter a "low-affinity" receptor [6, 7]. A comparison of A_{2A}- and A_{2B}-receptors for those compounds of the present series for which data on an A_{2B}-receptor-mediated enhancement of cyclic AMP accumulation are available is presented in Table 2. The agonist (A_{2A}) versus antagonist (A_{2B}) activity for 5'-methylthioadenosine was most remarkable. For the classical agonists, namely adenosine, 2-chloroadenosine, 2-fluoroadenosine, the N^6-pentyladenosines, and NECA, the potency at the "high-affinity" A_{2A}-receptor was 20- to 100-fold higher than at the "low-affinity" A_{2B} receptor (Table 2). It is remarkable and anomalous that 5'-methylthioadenosine is equipotent at both the "high-affinity" A_{2A}-receptor and the "low-affinity" A_{2B}-receptor, where it is a partial agonist at the former and a competitive antagonist at the latter. It should be noted that a potent and A_{2A}-selective 2-substituted NECA, namely CGS-21680, is virtually inactive at A_{2B}-receptors [31], a further indication of marked differences between these two subtypes of A_2-receptors.

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