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# FUNCTIONAL CHARACTERIZATION OF THE $A_{2b}$ ADENOSINE RECEPTOR IN NIH 3T3 FIBROBLASTS

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**Abstract**—The adenosine (ADO) receptor in NIH 3T3 fibroblasts was characterized using a series of adenosine agonists and selected xanthine and non-xanthine antagonists. The ADO receptor elicited accumulations of cyclic AMP in intact NIH 3T3 fibroblasts and caused activation of adenylate cyclase in membrane preparations. The receptor had characteristics of the $A_{2b}$ subtype of adenosine receptor. ADO analogs had relatively high $EC_{50}$ values at the receptor and were antagonized competitively by xanthines. The rank order of potency for adenosine analogs in NIH 3T3 fibroblasts for cyclic AMP accumulation was: NECA > 2-ClADO > *R*-PIA ≫ CV1808, CGS 21680. The $EC_{50}$ for 2-ClADO was 4.3 $\mu$M in intact cells and 15 $\mu$M in membrane preparations. All ADO analogs were more potent at the $A_{2a}$ receptor of pheochromocytoma PC12 membranes than at the $A_{2b}$ receptor of fibroblast NIH 3T3 membranes. Structure–activity relationships suggested that the regions of interaction with 5′- and $N^6$-substituents of ADO were similar for both the PC12 $A_{2a}$ and NIH 3T3 $A_{2b}$ receptor. However, ADO analogs with large substituents in the 2′-position, such as 2-cyclohexylethoxyADO and CGS 21680, were highly selective for the $A_{2a}$ receptor. All ADO analogs tested were stimulatory to adenylate cyclase at the NIH 3T3 $A_{2b}$ receptor, including 5′-methylthioADO, which was a weak partial agonist. A series of xanthine antagonists were not selective for the NIH 3T3 $A_{2b}$ versus the PC12 $A_{2a}$ receptor. In all cases, xanthines were more potent as antagonists in the intact NIH 3T3 cells than in NIH 3T3 membranes. In a series of non-xanthine antagonists, most compounds were equipotent or slightly more potent at the $A_{2a}$ receptor except for alloxazine, which was approximately 9-fold selective for the $A_{2b}$ receptor.

*Key words:* adenosine receptors; xanthines; adenylate cyclase; pheochromocytoma cells; fibroblasts; cyclic AMP

There are at least two major classes of ADO‡ receptors—namely, an $A_1$ ADO receptor, inhibitory to adenylate cyclase, and an $A_2$ ADO receptor, stimulatory to adenylate cyclase [1–3]. Each type of ADO receptor has a characteristic rank order of potency for ADO analogs, and each receptor is competitively antagonized by xanthines. Two distinct subtypes of $A_2$ ADO receptors coupled to adenylate cyclase have been demonstrated in rat brain [4]. One receptor has a high affinity for ADO ($EC_{50}$ 0.1 to 1 $\mu$M) and can be functionally detected in rat striatal membranes but not in rat cerebral cortical membranes. The other receptor has a low affinity for ADO ($EC_{50}$ 5–10 $\mu$M) and can be functionally detected only in brain slices, including those from rat cerebral cortex and striatum. Another low affinity $A_2$ receptor, which is coupled to adenylate cyclase, has been reported in intact human VA13 fibroblasts [5], but this receptor, unlike the low-affinity brain $A_2$ receptor, is functionally detectable in membranes [6] as well as intact cells [5]. In 1986, the ADO analog CV1674 was shown to be 10,000 times more potent at the high-affinity receptor in rat striatum than at the low-affinity receptor in human VA13 fibroblasts and the two receptors were designated $A_{2a}$ and $A_{2b}$, respectively [7]. The recent cloning of two different $A_2$ ADO receptors confirms the existence of distinct $A_{2a}$ and $A_{2b}$ subtypes of $A_2$ receptors [8–11].

Structure–activity relationships are well known for the $A_{2a}$ receptor, largely due to the development of a highly potent and specific agonist, CGS 21680 [12, 13], which, in radioactive form, serves as a ligand for $A_{2a}$ receptors [14]. The $A_{2a}$ receptor is highly localized in the caudate, putamen, nucleus accumbens, and olfactory tubercle of the brain [15–

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‡ Abbreviations: ADO, adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; 5′-ClADO, 5′-chloroadenosine; MTA, 5′-methylthioadenosine; 2-ClADO, 2-chloroadenosine; 2-FADO, 2-fluoroadenosine; CV1808, 2-phenylaminoadenosine; CGS 21680, 2-[*p*-(2-carboxyethyl)-phenylethylamino] - 5′ - *N* - ethylcarboxamidoadenosine; CHA, $N^6$-cyclohexyladenosine; *R*-PIA, $N^6$-*R*-phenylisopropyladenosine; *R*-PI-NECA, $N^6$-*R*-phenylisopropyl-5′-*N*-ethylcarboxamidoadenosine; *S*-PIA, $N^6$-*S*-phenylisopropyladenosine; CV1674, 2-(*p*-methoxyphenyl)-adenosine; 8pSPT, 8-(*p*-sulfophenyl)theophylline; HPPI, 4 - hydroxy - 9 - phenyl - 9*H* - pyrimido[4,5 - b]indole; PD115,199, 1,3-dipropyl-8-{4-[*N*-methyl-*N*-(2-dimethylaminoethyl)sulfonamido]}xanthine; HTQZ, 3-(3-hydroxyphenyl)5*H*-thiazolo[2,3-b]quinazoline; CP-66,713, 4-amino - 8 - chloro - 1 - phenyl - [1,2,4]triazolo[4,3-a]quinoxaline; CGS 15943A, 9-chloro-2-(2-furyl)-[1,2,4]triazolo-[1,5-c]quinazolin-5-amine; DMEM, Dulbecco's Modified Eagle's Medium; and XAC, xanthine amine cogener.

17]. The $A_{2a}$ receptor is also present in rat PC12 cells [18] and human platelets [19]. The $A_{2a}$ receptor is also coexpressed with the $D_2$ dopamine receptor in rat striatum [8, 20].

Due to the lack of specific agonists or antagonists, less is known about the $A_{2b}$ receptor. Binding assays have not been developed for $A_{2b}$ receptors. The $A_{2b}$ receptor has been shown, based on biochemical assays, to be present throughout the rat brain [4] and in fibroblast (WI38 and VA13) [5, 6] and T-cell Jurkat cell lines [21]. A recently cloned $A_2$ receptor from rat brain appears to correspond to the $A_{2b}$ receptor in that CGS 21680 is very weakly active at the receptor [9, 11]. Although $A_{2b}$ receptor functional activity is manifest in intact cell systems, it has been difficult to demonstrate a functional $A_{2b}$ receptor in membrane preparations from brain [4]. A functional $A_{2b}$ receptor can be demonstrated in VA13 fibroblast membranes [6]. The reason that the $A_{2b}$ receptor has not been demonstrable in brain membranes is unknown. It is possible that it is not identical with the fibroblast $A_{2b}$ receptor. The receptor from fibroblasts has not been cloned.

In the present study, we characterized the ADO receptor in the NIH 3T3 fibroblast cell line as an $A_{2b}$ receptor and showed this receptor to be functional in membrane preparations. The NIH 3T3 receptor shared many features with the previously described VA13 fibroblast $A_{2b}$ receptor, although some differences did exist. Comparison of the potency of several ADO analogs at the $A_{2a}$ receptor in PC12 cells and the $A_{2b}$ receptor in NIH 3T3 fibroblasts confirmed that ADO analogs with large substituents in the 2-position are highly selective for the $A_{2a}$ subtype. Finally, examination of a series of xanthine and non-xanthine ADO receptor antagonists showed certain antagonists to have some selectivity for either the $A_{2a}$ or the $A_{2b}$ receptor.

## MATERIALS AND METHODS

*Compounds.* Compounds were obtained from the following sources: 5′-*N*-ethylcarboxamidoadenosine (NECA) hydrate, 2-chloroadenosine (2-ClADO), $N^6$-*R*-phenylisopropyladenosine (*R*-PIA), $N^6$-*S*-phenylisopropyladenosine (*S*-PIA), $N^6$-cyclohexyladenosine (CHA), CV1808, 8-(*p*-sulfophenyl)-theophylline (8-pSPT), 8-cyclopentyl-1,3-dipropylxanthine, xanthine amine congener (XAC), 8-phenyltheophylline, and 1,7-dimethylxanthine from Research Biochemicals, Inc., Natick, MA; 5′-chloroadenosine (5′-ClADO), 2-(2-phenylethoxy)-adenosine, 2-(2-cyclohexylethoxy)adenosine, 2-fluoroadenosine (2-FADO), $N^6$-*R*-phenylisopropyl-5′-*N*-ethylcarboxamidoadenosine (*R*-PI-NECA), 2-(2-phenylethoxy)-9-methyladenine, 9-methyladenine and $N^6$-cyclohexyl-9-methyladenine from Dr. R. A. Olsson, University of South Florida College of Medicine, Tampa, FL; caffeine and 5′-methylthioadenosine (MTA) from the Sigma Chemical Co., St. Louis, MO; CGS 21680 and CGS 15943A from the Ciba-Geigy Corp., Summit, NJ; theophylline from Calbiochem, La Jolla, CA; 3-isobutyl-1-methylxanthine, alloxazine and 7-(β-chloroethyl)-theophylline from the Aldrich Chemical Co., Inc., Milwaukee, WI; CP-66,713 from Pfizer, Inc., Groton, CT; tracazolate from ICI Americas, Wilmington, DE; 1,3-dipropylxanthine from G. D. Searle, Chicago, IL; HTQZ and PD115,199 from the Warner-Lambert Co., Ann Arbor, MI; rolipram from Schering AG, Berlin, Germany; HPPI [22], 1-propargyl-3,7-dimethylxanthine [23] and 1,3-dipropyl-8-cyclohexyl-7-methylxanthine [24] were synthesized as described. Structures of agonists and antagonists are shown in Figs. 1 and 2.

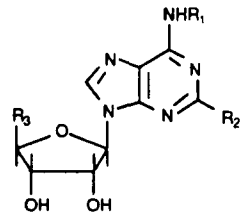
[α-$^{32}$P]ATP (800 Ci/mmol) and [$^3$H]cyclic AMP were from NEN/Dupont, Wilmington, DE; adenosine deaminase (calf intestine) was from Boehringer Mannheim, Indianapolis, IN; creatinine phosphokinase (Type I) and phosphocreatine were from the Sigma Chemical Co. All other materials were obtained from standard sources.

*Cell culture.* NIH 3T3 fibroblasts, derived from the Swiss mouse embryo, were provided by Dr. F. Gusovsky (National Institutes of Health, Bethesda, MD). Cells were cultured in DMEM (GIBCO, Grand Island, NY), containing 20% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown to confluence and split every 6–9 days in a ratio of 1:20 by treatment with 0.25% trypsin/1 mM EDTA for 5 min.

Rat PC12 cells, derived from a rat pheochromocytoma, were provided by Dr. G. Guroff (National Institutes of Health, Bethesda, MD). Cells were cultured in DMEM containing 6% horse serum, 6% fetal bovine serum and antibiotics. The cells were split every 7 days in a ratio of 1:7 by vigorously shaking the flasks to dislodge the cells. NIH 3T3 and PC12 cells were kept at 37° in a humidified atmosphere enriched in $CO_2$.

*Determination of cyclic AMP generation in intact NIH 3T3 fibroblasts.* NIH 3T3 cells were plated at a density of 1–3 × $10^4$ cells/well on 24-well culture plates and used 2 days later, when the cells had reached confluence. The cells were first washed with HEPES buffer containing the following: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 0.5 mM EDTA, 1.5 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, pH 7.4. The cells, maintained on a warm plate at 37°, were then preincubated with the HEPES buffer containing 30 μM rolipram and 3 U adenosine deaminase/mL for 10 min. Agonists were added and incubations were stopped after 10 min by removal of the buffer and addition of 1.0 mL of 0.1 N HCl. After 30 min, the acid was removed and neutralized with 5.0 N NaOH, and the amount of cyclic AMP was determined with a commercially available kit (Amersham, Arlington Heights, IL). When antagonists were tested, the cells were treated with the appropriate concentration of antagonist during the 10-min preincubation period. Each experiment was done in duplicate, and there was less than 10% deviation for the duplicates.

*Preparation of cell membranes.* NIH 3T3 cells were trypsinized and resuspended in DMEM, and PC12 cells were removed from flasks by vigorous shaking. Treatment of NIH 3T3 fibroblasts with trypsin had no effect on receptor-mediated adenylate cyclase activity (data not shown). The remainder of the membrane preparation was identical for both cell types. Cells were washed twice with 50 mM Tris–




| Analog | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| NECA | H | H | $CONHC_2H_5$ |
| 5'ClADO | H | H | $CH_2Cl$ |
| MTA | H | H | $CH_2SCH_3$ |
| 2-ClADO | H | Cl | $CH_2OH$ |
| 2-FADO | H | F | $CH_2OH$ |
| 2-PhenylaminoADO | H | —NH— | $CH_2OH$ |
| 2-PhenylethoxyADO | H | $-O-(CH_2)_2$ | $CH_2OH$ |
| 2-CyclohexylethoxyADO | H | $-O-(CH_2)_2$ | $CH_2OH$ |
| CGS 21680 | H | $-NH-(CH_2)_2$ — $-(CH_2)_2COOH$ | $CONHC_2H_5$ |
| CHA | | H | $CH_2OH$ |
| RPIA | | H | $CH_2OH$ |
| RPI-NECA | | H | $CONHC_2H_5$ |
| SPIA | | H | $CH_2OH$ |

Fig. 1. Structures of adenosine analogs.

HCl buffer (pH 7.4) containing 1 mM EDTA and 150 mM NaCl and then homogenized using a Polytron (setting 5) for 10 sec in 5 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged at 1000 *g* for 10 min, and the resulting supernatant was centrifuged at 48,000 *g* for 20 min. The pellet was then resuspended and recentrifuged at 48,000 *g* for 20 min. The final pellet was resuspended in 50 $\mu$M Tris–HCl (pH 7.4) and stored at −70° until used in adenylate cyclase assays.

*Determination of adenylate cyclase activity.* The incubation mixture for the adenylate cyclase assay contained the following: 0.1 mM [$\alpha$-$^{32}$P]ATP (0.9 $\mu$Ci/tube for PC12 cell membranes and 2.7 $\mu$Ci/tube for NIH 3T3 fibroblast membranes), 10 $\mu$M GTP, 5 mM $MgCl_2$, 0.1 mM cyclic AMP, 0.02 mg/mL adenosine deaminase, 0.1 mM rolipram, 0.2 mM EGTA, 5 U/tube creatine phosphokinase, 2.6 mM phosphocreatine, 30 $\mu$g/tube bovine serum albumin, and 50 mM Tris–HCl (pH 7.4) in a total volume of 250 $\mu$L. Agonists and antagonists were added from stock solutions in water or DMSO. When DMSO was used, the total concentration in the incubation mixture was 4%. This concentration of DMSO had no effect on adenylate cyclase in PC12 membranes. In NIH 3T3 membranes, the activity of adenylate cyclase increased by ~20% with 4% DMSO. A 4%

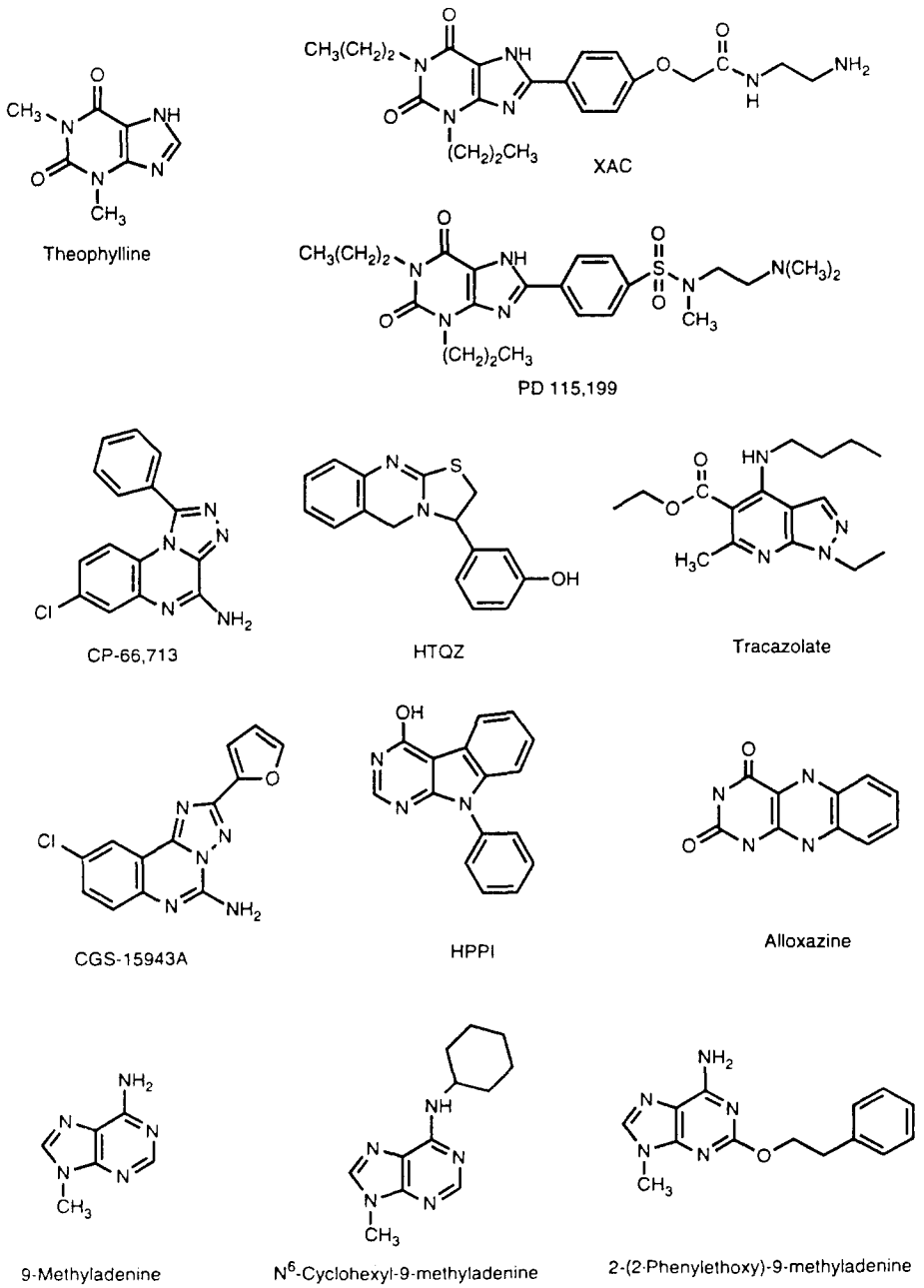



Fig. 2. Structures of theophylline, xanthine amine congener (XAC), PD115,199 and non-xanthine antagonists.

DMSO control was included in each experiment using those compounds requiring DMSO as a solvent.

Incubations were initiated by the addition of membrane protein (~10 μg for PC12 and ~10–300 μg for NIH 3T3 cells) and were conducted for 10 min at 37°. The reaction was stopped by the addition of 0.5 mL of 10% trichloroacetic acid, and 1 mM cyclic AMP containing [$^3$H]cyclic AMP was added to each tube. Cyclic AMP was isolated by a two-step chromatographic procedure using Dowex (200–400 mesh, Bio-Rad, Richmond, CA) and alumina columns essentially as described [25]. The recovery of [$^3$H]cyclic AMP was used as a correction factor in the determination of the amount of [$^{32}$P]-cyclic AMP formed. Each experiment was done in triplicate, and there was less than 10% deviation for the triplicates.

*Analysis of data.* The $EC_{50}$ values were obtained from concentration–response curves from at least three experiments. The Kaleidagraph computer program (Synergy Software, Reading, PA) was used for intact cells and the GraphPAD (GraphPAD

Software, Inc., San Diego, CA) program was used for membranes. Antagonist potency was calculated using the Schild equation for cell membranes or from determination by a Schild plot for intact cells [26]. Efficacy is defined as the maximal response to analog divided by the maximal response to NECA.

## RESULTS

*Cyclic AMP generation in intact NIH 3T3 fibroblasts.* Basal levels of cyclic AMP in NIH 3T3 fibroblasts were generally less than 20 pmol/well/10 min, and this increased to approximately 500 pmol/well/10 min with maximal stimulation with NECA. All ADO analogs tested increased intracellular cyclic AMP and this effect was antagonized by 8pSPT (Fig. 3A–E, see also Fig. 6A). The rank order of potency of NECA > 2ClADO > *R*-PIA > *S*-PIA was characteristic of an $A_2$ ADO receptor (Table 1). CV1808 had very low activity (Fig. 3C) as would be expected for an $A_{2b}$ receptor [19]. The $EC_{50}$ was 51 $\mu$M. The $EC_{50}$ for the most potent analog, NECA, was 0.46 ± 0.04 $\mu$M (Table 1). $EC_{50}$ Values for 2-phenylethoxyADO and 2-cycloethylethoxyADO could not be determined due to their weak activity and poor solubility. However, each of these compounds did show a concentration-dependent increase in cyclic AMP, which was antagonized by 8pSPT. CGS 21680 had no activity up to 3 $\mu$M (Fig. 3D). At 30 $\mu$M, CGS 21680 caused only a marginal increase in cyclic AMP, 0.04 and 0.08 in two experiments relative to a maximal response to NECA set equal to 1.0. The low activity of CGS 21680 is characteristic of that reported for $A_{2b}$ receptors [17]. Schild analysis showed an antagonist potency of 8pSPT of 1.0 to 2.6 $\mu$M versus ADO agonists. CGS 21680 was nearly inactive, and the limited activity and solubility of 2-phenylethoxyADO and 2-cyclohexylADO precluded a reliable Schild analysis. In Table 2, the present results with the NIH 3T3 fibroblast cells are compared with values reported for VA13 fibroblast cells [5, 29]. In all cases, the adenosine analogs were more potent in NIH 3T3 cells.

*Stimulation of adenylate cyclase in NIH 3T3 fibroblast membranes.* The basal activity of adenylate cyclase in NIH 3T3 membranes was 10 pmol/mg/min, and this activity increased 4-fold upon maximal stimulation by NECA and 14-fold with 10 $\mu$M forskolin. Most analogs were about 2-fold less potent in membrane preparations than in intact NIH 3T3 fibroblasts; NECA was about 4-fold less potent (Table 1). The rank order of potency of compounds substituted in the 5′-position in NIH 3T3 cell membranes was NECA > 5′-ClADO > MTA (Table 1, Fig. 4A). The relative maximal response of these compounds paralleled the order of potency with NECA > 5′-ClADO > MTA (Table 1). For the 5′-substituted compounds, both the order of potency and relative maximal response were the same in NIH 3T3 membranes and PC12 cells (Fig. 4, A and B). The rank order of potency for the 2-substituted analogs in NIH 3T3 membranes was 2-ClADO > 2-FADO > 2 - phenylethoxyADO > 2 - cyclohexylethoxyADO. 2-PhenylaminoADO and CGS 21680 were even less active (Table 1). At 100 $\mu$M, CGS 21680 reached only 12% of the maximal stimulation evoked by NECA. The rank order of potency for $N^6$-substituted adenosines in NIH 3T3 membranes was *R*-PIA > CHA ≃ *S*-PIA. The diastereomer *S*-PIA was about 3 times less potent than *R*-PIA. Structural modification of *R*-PIA by adding the 5′-*N*-ethylcarboxamido group increased the activity of *R*-PIA, resulting in a compound (*R*-PI-NECA) with intermediate potency between NECA and *R*-PIA (Fig. 5). *R*-PI-NECA was the most potent $N^6$-substituted compound tested in the NIH 3T3 membranes and was approximately equipotent to 5′-ClADO. All of the ADO analogs had a maximal response lower than that of NECA (Table 1). Two of the analogs, 2-cyclohexylethoxyADO and MTA, had very low relative maximal responses of 0.35 and 0.23, respectively, relative to NECA set equal to 1.0.

All agonists were much less potent in NIH 3T3 membranes than in PC12 membranes (see below).

*Stimulation of adenylate cyclase in PC12 membranes.* Basal adenylate cyclase activity in PC12 membranes was ~60 pmol cyclic AMP/mg protein/min, and this activity increased ~5-fold with maximal NECA stimulation (Fig. 4B). The activity profile for ADO analogs was consistent with that of an $A_2$ receptor (Table 1). The rank order of potency was: NECA > 2-ClADO > *R*-PIA = 5′-ClADO ≥ 2-FADO > CHA > *S*-PIA > MTA. Selectivity ratios of $A_{2a}/A_{2b}$ ranged from 670 to greater than 1000 for 2-phenethoxyADO, 2-cyclohexylethoxyADO, and CGS 21680. These 2-substituted analogs, thus, are highly selective for the $A_{2a}$ receptors. 5′-ClADO and MTA were the least selective, being only 5-fold more potent at $A_{2a}$ receptors. NECA was about 15-fold more potent at $A_{2a}$ receptors and the remaining 2-substituted and $N^6$-substituted analogs were 15- to 29-fold more potent in PC12 membranes than in NIH 3T3 membranes.

*MTA as a partial agonist in NIH 3T3 cells.* MTA has been reported to be an antagonist of the $A_{2b}$ receptor in VA13 fibroblasts [5]. MTA was an agonist at the NIH 3T3 fibroblast receptor in both intact cells and membrane preparations, and this activity was antagonized competitively by 8pSPT (Table 1, Fig. 6A). Since MTA was found to have a low maximal response compared with NECA, we performed experiments to determine if MTA was a partial agonist. In the presence of NECA, MTA shifted the concentration–response curve to the right in a concentration-dependent manner in both NIH 3T3 membranes and intact cells (Fig. 6, B and C). Thus, MTA is a partial agonist at the NIH 3T3 fibroblast receptor.

*Antagonism of adenosine receptor-elicited stimulation of adenylate cyclase.* All xanthines tested inhibited NECA-stimulated adenylate cyclase in NIH 3T3 cell membranes. The rank orders of potency for xanthine antagonists (8-phenyltheophylline > 8-pSPT > theophylline) were similar for NIH 3T3 and PC12 cell membranes (Table 3). Most of the xanthines had comparable potencies at the $A_{2a}$ and $A_{2b}$ receptors in membrane preparations. The xanthines also were assessed for antagonist potency in intact NIH 3T3 cells (Table 3). In all cases, the

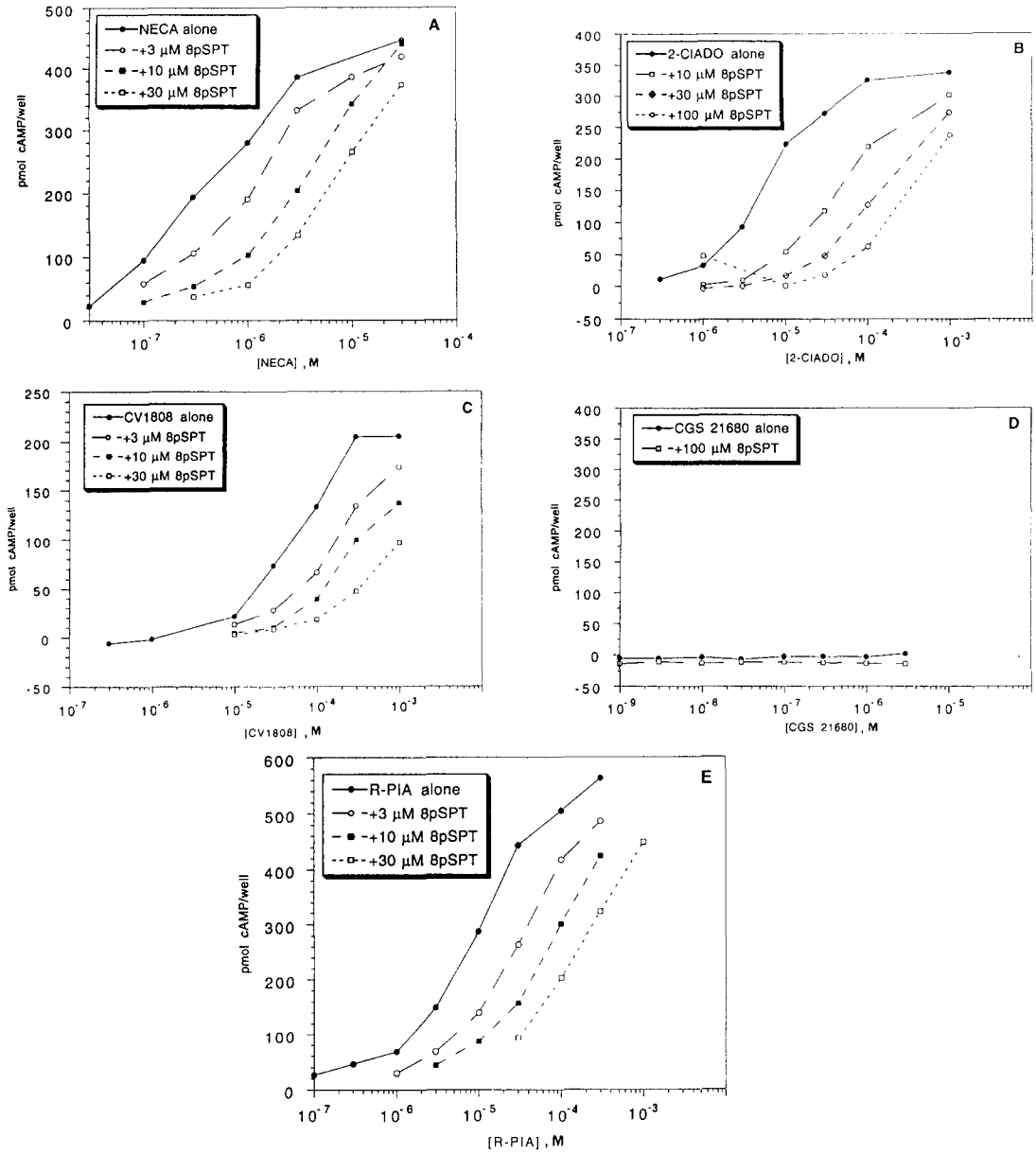



Fig. 3. Adenosine analog-elicited accumulation of cAMP in intact NIH 3T3 fibroblasts and antagonism by 8-*p*-sulfophenyltheophylline (8pSPT). Confluent NIH 3T3 fibroblasts were incubated with various concentrations of agonist for 10 min. To examine the effect of the antagonist, 8pSPT was added to the cells 10 min before the agonist. Levels of cAMP were measured as described in Materials and Methods. Each graph shown (A–E) represents one of three similar experiments with values being means of duplicates. (A) NECA; (B) 2-ClADO; (C) CV1808; (D) CGS 21680; and (E) *R*-PIA.

xanthines appeared several-fold more potent as an antagonist in intact cells than in membranes. In Table 4, the present results with the NIH 3T3 fibroblasts are compared with values reported for VA13 fibroblast cells [7, 30]. In all cases, the $K_B$ values were comparable in magnitude.

A series of non-xanthines were compared for potency as antagonists of the $A_{2b}$ receptor of NIH 3T3 membranes and the $A_{2a}$ receptor of PC12 membranes (Table 5). Most were several-fold more potent at the $A_{2a}$ receptor. An exception was alloxazine, which appeared to be about 9-fold more potent at the $A_{2b}$ receptor. The non-xanthine antagonists have been studied previously, primarily on $A_{2a}$ and $A_1$ receptors as follows: CP 66,713 [32], HTQZ [33], tracazolate [7, 31], CGS 15943A [31, 34], HPPI [22], alloxazine [7, 31], and 9-methyladenines [31, 35, 36]. Tracazolate, alloxazine and 9-methyladenine were reported as antagonists at $A_{2b}$ receptors of VA13 fibroblast cells [5, 7]. CGS 15943A [34] and analogs of tracazolate [37] were reported as antagonists at $A_{2b}$ receptors in brain preparations.

Several of the antagonists decreased basal levels of adenylate cyclase in both NIH 3T3 and PC12 cell

Table 1. Effects of adenosine analogs on adenylate cyclase activity in PC12 and NIH 3T3 membranes and cyclic AMP generation in intact NIH 3T3 fibroblasts

| Adenosine analogs | PC12 membranes* Adenylate cyclase $EC_{50}$ ($\mu$M) efficacy | NIH 3T3 membranes Adenylate cyclase $EC_{50}$ ($\mu$M) efficacy | NIH 3T3 intact cells Cyclic AMP generation $EC_{50}$ ($\mu$M) |
|---|---|---|---|
| NECA | 0.13 ± 0.02 (8)<br>1.0 | 1.9 ± 0.1 (19)<br>1.0 | 0.46 ± 0.04 (8) |
| 5'-ClADO | 0.79 ± 0.03 (4)<br>0.51 | 4.1 ± 0.4 (3)<br>0.65 | ND† |
| MTA | 10.2 ± 1.4 (4)<br>0.31 | 51 ± 3 (4)<br>0.23 | 24 ± 4 (3) |
| 2-ClADO | 0.46 ± 0.05 (3)<br>0.95 | 15 ± 4 (4)<br>0.65 | 4.3 ± 1.2 (3) |
| 2-FADO | 0.99 ± 0.15 (4)<br>0.68 | 27 ± 3 (3)<br>0.58 | ND |
| 2-PhenylaminoADO (CV1808) | 0.50 ± 0.10 (3)<br>0.93 | —‡<br>0.67 (1 mM) | 51 ± 4 (3) |
| 2-PhenylethoxyADO | 0.045 ± 0.004 (3) | 30 ± 3 (3)<br>0.56 | —‡<br>(100 $\mu$M) |
| 2-CyclohexylethoxyADO | 0.054 ± 0.019 (3) | 56 ± 7 (3)<br>0.35 | —†<br>(100 $\mu$M) |
| CGS 21680 | 0.074 ± 0.005 (3)<br>0.84 | —‡<br>0.12 (100 $\mu$M) | —‡<br>0.00 (3 $\mu$M) |
| CHA | 1.8 ± 0.3 (3)<br>0.91 | 53 ± 3 (3)<br>0.77 | 30 ± 7 (3) |
| *R*-PIA | 0.76 ± 0.31 (3)<br>0.77 | 19 ± 3 (3)<br>0.82 | 9.3 ± 3.1 (3) |
| *R*-PI-NECA | 0.34 ± 0.30 (3)<br>0.87 | 6.0 ± 0.8 (3)<br>0.82 | ND |
| *S*-PIA | 4.2 ± 1.0 (3)<br>0.83 | 65 ± 9 (3)<br>0.52 | 47 ± 7 (3) |

Values are means ± SEM, with the number of experiments indicated in parentheses. Efficacy = maximal response/maximal response to NECA.

* Certain values (2-ClADO, 2-phenylaminoADO, 2-phenylethoxyADO, 2-cyclohexylethoxyADO, CGS 21680, CHA, *R*-PI-NECA, *S*-PIA) are from prior publications [18, 19, 27, 28].

† ND = not determined.

‡ Did not reach a maximum at the highest concentration tested, and an $EC_{50}$ could not be calculated.

membranes at the concentrations used. Compounds that decreased adenylate cyclase in PC12 cells were: CP 66,713 (31% at 1 $\mu$M), HTQZ (29% at 3 $\mu$M) and HPPI (27% at 3 $\mu$M). Of the antagonists tested, six decreased basal adenylate cyclase by 15% or greater in NIH 3T3 membranes at the concentrations used. These were: CP 66,713 (15% at 0.3 $\mu$M), HTQZ (23% at 3 $\mu$M), HPPI (18% at 10 $\mu$M), 9-methyladenine (21% at 100 $\mu$M), $N^6$-cyclohexyl-9-methyladenine (16% at 100 $\mu$M), and 1,3-dipropyl-8-cyclohexyl-7-methylxanthine (21% at 10 $\mu$M). For each of these compounds, the $K_B$ value was calculated with the effect on basal adenylate cyclase taken into account.

Table 2. Comparison of effects of adenosine analogs on cyclic AMP generation in intact VA13 and NIH 3T3 fibroblasts

| Adenosine analogs | Cyclic AMP generation $EC_{50}$ ($\mu$M) VA13 cells* | NIH 3T3 cells |
|---|---|---|
| NECA | 2.6 ± 0.7 (3) | 0.46 ± 0.04 (8) |
| MTA | $K_B$ 8.2 ± 0.9 (3) | 24 ± 4 (3) |
| 2-ClADO | 24 | 4.3 ± 1.2 (3) |
| CHA | 160 | 30 ± 7 (3) |
| *R*-PIA | 150 | 9.3 ± 3.1 (3) |
| *S*-PIA | 750 | 47 ± 7 (3) |

Values are means ± SEM with the number of experiments given in parentheses.

* Values from the literature [5, 29].

## DISCUSSION

The ADO receptor in NIH 3T3 fibroblasts exhibited properties expected of an $A_{2b}$ type of receptor. The potency and the rank order of potency of ADO agonists were consistent with the $A_{2b}$ receptor. The low affinity for 2-ClADO (4–15 $\mu$M) was similar to that in rat cortical slices (~20 $\mu$M) [4] or fibroblast VA13 cells (24 $\mu$M) [5]. Stimulation of adenylate cyclase was antagonized by 8pSPT. The NIH 3T3 fibroblast $A_{2b}$ receptor was functional in

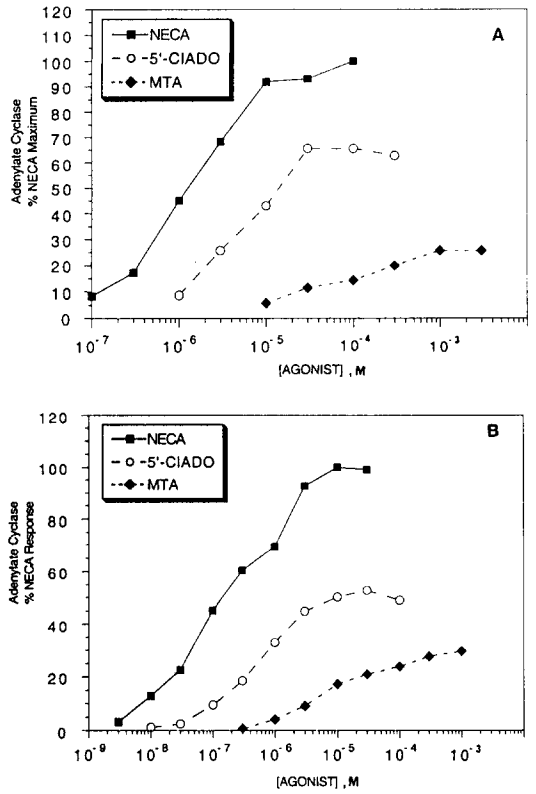



Fig. 4. Potencies of adenosine analogs with 5'-position modifications. Membrane preparations from NIH 3T3 fibroblasts (A) and PC12 cells (B) were incubated with various concentrations of NECA, 5'-ClADO, or MTA, and adenylate cyclase activity was measured as described in Materials and Methods. Maximal NECA response was defined as the response to 100 $\mu$M NECA for NIH 3T3 fibroblast membranes and to 10 $\mu$M NECA for PC12 membranes. Each graph represents one of three similar experiments with values being means of triplicates.

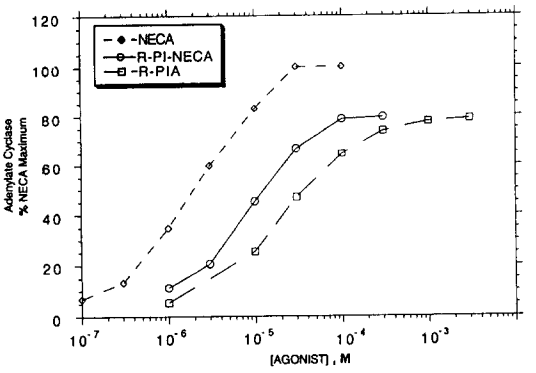



Fig. 5. Potencies of adenosine analogs with substituents at the $N^6$-position. Membranes from NIH 3T3 fibroblasts were incubated with various concentrations of NECA, *R*-PI-NECA or *R*-PIA, and adenylate cyclase activity was measured as described in Materials and Methods. Maximal NECA response was defined as the response to NECA at 100 $\mu$M. The graph represents one of three similar experiments with values being means of triplicates.

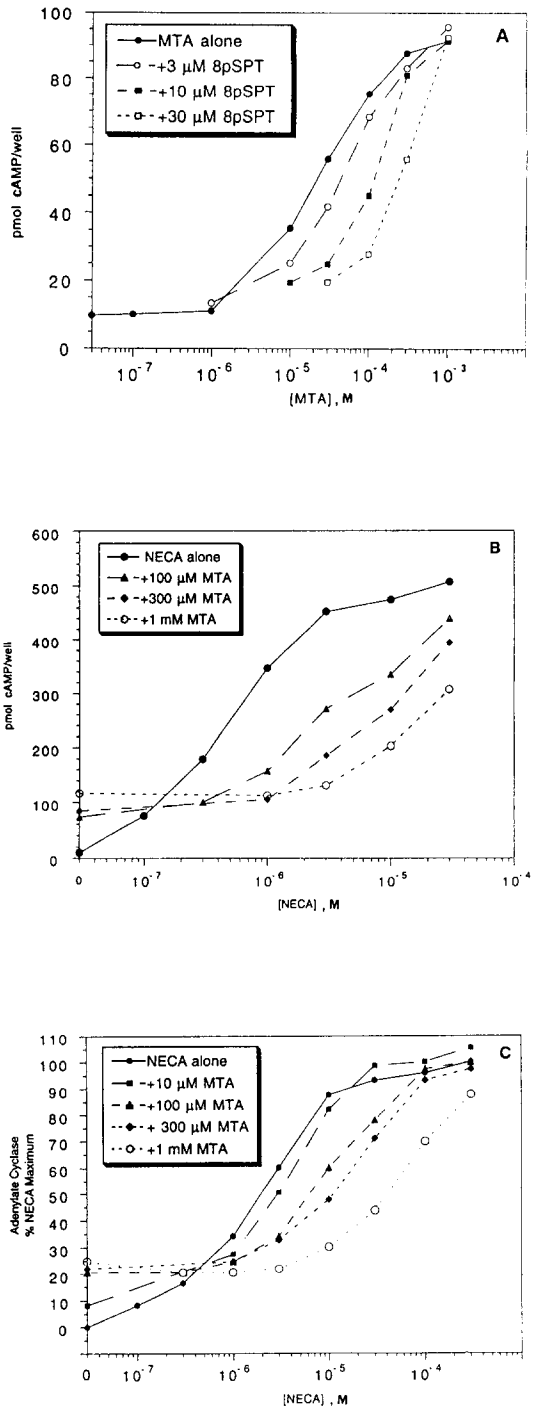



Fig. 6. MTA as a partial agonist in intact NIH 3T3 fibroblasts and NIH 3T3 fibroblast membranes. Confluent NIH 3T3 fibroblasts were incubated with various concentrations of MTA (A) or NECA (B) for 10 min. To examine the effect of agents as antagonists, 8pSPT (A) or MTA (B) was added 10 min before the agonist. cAMP generation was measured as described in Materials and Methods. NIH 3T3 membranes (C) were incubated with various concentrations of NECA alone or in the presence of MTA, and adenylate cyclase activity was measured as described in Materials and Methods. NECA maximum is defined as the response to NECA at 300 $\mu$M (C). Each graph represents one of three similar experiments with values being means of triplicates.

Table 3. Potencies of xanthines as antagonists of NECA-elicited stimulation of adenylate cyclase in PC12 and NIH 3T3 membranes

| Xanthine antagonists | $A_{2A}$ | $A_{2b}$ | | |
|---|---|---|---|---|
| | PC12 membranes Adenylate cyclase $K_B$ ($\mu$M) | NIH 3T3 membranes Adenylate cyclase $K_B$ ($\mu$M) | NIH 3T3 intact cells Cyclic AMP generation $K_i$ ($\mu$M) | Ratio membrane $A_{2b}/A_{2a}$ |
| Caffeine | 36 ± 4 (8)* | 30 ± 5 (3) | 13 ± 6 (3) | 0.83 |
| Theophylline | 14 ± 0.4 (3)* | 32 ± 7 (3) | 4.9 ± 0.4 (3) | 2.3 |
| 8-Phenyltheophylline | 0.49 ± 0.08 (5) | 0.96 ± 0.20 (3) | 0.096 ± 0.006 | 2.0 |
| 8-pSPT | 3.9 ± 1.0 (3)* | 4.5 ± 1.3 (3) | 1.8 ± 0.3 (5) | 1.2 |
| 1,3-Dipropylxanthine | 5.4 (4.0–7.3) (3)* | 3.4 ± 0.1 (4) | 0.68 ± 0.28 (3) | 0.62 |
| 8-Cyclopentyl-1,3-dipropylxanthine | 0.25 (0.1–0.59) (3)* | 0.36 ± 0.08 (3) | 0.017 ± 0.003 (3) | 1.4 |
| 8-Cyclohexyl-1,3-dipropyl-7-methylxanthine | 7.1 ± 1.4 (3) | 12 ± 3 (4) | 2.7 ± 0.1 (3) | 1.7 |
| 3,7-Dimethyl-1-propargylxanthine | 8.6 ± 0.4 (3)* | 11 ± 2 (3) | 2.5 ± 0.6 (3) | 1.3 |
| 7-(2-Chloroethyl)-theophylline | 6.3 (3.5–11) (3)* | 7.1 ± 0.3 (3) | 1.4 ± 0.4 (3) | 1.1 |
| 3-Isobutyl-7-methylxanthine | 4.9 ± 0.8 (3) | 12 ± 1 (3) | 1.6 ± 0.2 (3) | 2.4 |
| 1,7-Dimethylxanthine | 20 ± 3 (3) | 39 ± 3 (3) | 3.5 ± 0.7 (3) | 2.0 |
| Xanthine amine congener | 0.019 ± 0.001 (3)* | 0.30 ± 0.001 (3) | 0.0064 ± 0.0017 (3) | 16 |
| PD115,199 | 0.031 ± 0.03 (3) | 0.16 ± 0.02 (3) | ND† | 5.2 |

Values are means ± SEM (or 95% confidence limits) with the number of experiments indicated in parentheses.
* Values are from prior publications [18, 24, 28].
† ND, not determined.

Table 4. Comparison of potencies of xanthines as antagonists of NECA-elicited accumulation of cyclic AMP in intact VA13 and NIH 3T3 fibroblast cells

| Xanthine antagonists | $K_B$ (μM) | |
|---|---|---|
| | VA13 cells* | NIH 3T3 cells |
| Caffeine | 13 ± 2 (2) | 13 ± 6 (13) |
| Theophylline | 4.8 ± 0.8 (4) | 4.9 ± 0.4 (3) |
| 8-Phenyltheophylline | 0.18 ± 0.02 (3) | 0.096 ± 0.006 |
| 8pSPT | 1.2 | 1.8 ± 0.3 (5) |
| 1,3-Dipropylxanthine | 0.68 ± 0.03 (2) | 0.68 ± 0.28 (3) |
| 7-(2-Chloroethyl)theophylline | 0.98 ± 0.22 (2) | 1.4 ± 0.4 (3) |
| 3-Isobutyl-7-methylxanthine | 3.5 ± 1.0 (2) | 1.6 ± 0.2 (3) |
| 1,7-Dimethylxanthine | 4.5 | 3.5 ± 0.7 (3) |

Values are means ± SEM, with the number of experiments indicated in parentheses.

* Values are from the literature [7, 30].

Table 5. Potencies of non-xanthines as antagonists of NECA-elicited stimulation of adenylate cyclase in PC12 and NIH 3T3 membranes

| Non-xanthine antagonist | $A_{2A}$ PC12 membranes* $K_B$ (μM) | $A_{2b}$ NIH 3T3 membranes $K_B$ (μM) | Ratio $A_{2b}/A_{2b}$ |
|---|---|---|---|
| CP 66,713 | 0.051 ± 0.002 (3) | 0.26 ± 0.01 (3) | 5.1 |
| HTQZ | 0.89 ± 0.19 (3) | 4.8 ± 0.5 (3) | 5.4 |
| Tracazolate | 2.4 (0.7–3.2) (3) | 5.4 ± 0.3 (3) | 2.3 |
| CGS 15943A | 0.0019 (0.0004–0.0087) (3) | 0.041 ± 0.004 (3) | 2.2 |
| HPPI | 2.5 ± 0.29 (3) | 3.4 ± 0.5 (3) | 1.4 |
| Alloxazine | 20 (7.4–56) (3) | 2.3 ± 0.2† (3) | 0.12 |
| 9-Methyladenine | 24 ± 5 (3) | 108 ± 9‡ (4) | 4.5 |
| $N^6$-Cyclohexyl-9-methyladenine | 21 (12–38) (3) | 121 ± 28 (4) | 5.8 |
| 2-(2-Phenylethoxy)-9-methyladenine | 0.31 ± 0.03 (3) | 1.5 ± 0.02 (3) | 4.8 |

* Values are from a prior publication [31] with the exception of CP 66,713, HTQZ, HPPI, 9-methyladenine, and 2-(2-phenylethoxy)-9-methyladenine. Either means ± SEM or means with 95% confidence limits are reported.

† Alloxazine had a $K_B$ value of 1.1 ± 0.04 (N = 2) in intact VA13 cells [30].

‡ 9-Methyladenine had a $K_B$ value of 55 ± 17 (N = 3) in intact VA13 cells [30].

both intact cells and membrane preparations. Although there were some differences, the NIH 3T3 and VA13 fibroblast receptors appeared very similar. The NIH 3T3 receptor exhibited very low activity in response to certain 2-substituted ADO analogs, namely CGS 21680, 2-phenylethoxyADO and 2-cyclohexylethoxyADO, which are known to be potent and selective for the $A_{2a}$ receptor [14, 17, 38, 39].

One of the features widely, but incorrectly, thought to be characteristic of the low affinity $A_{2b}$ receptors is the difficulty to detect a functional receptor in membrane preparations. The premise is based on the fact that the adenosine receptor-mediated stimulation of adenylate cyclase is readily detected in rat brain slices, but not in rat brain membranes [4]. In striatal membranes a functional $A_{2a}$ receptor rather than an $A_{2b}$ receptor is detected. Similarly, a functional $A_{2b}$ receptor can be detected in intact Jurkat T-cells, but not in membrane preparations [21]. There have been prior reports, however, of a functional low-affinity $A_{2b}$ receptor in membranes of VA13 fibroblasts [6], paralleling the functional $A_{2b}$ receptor found in intact VA13 cells [5]. Functional $A_{2b}$ receptor activity was shown in both intact cells and membranes of NIH 3T3 cells in the present study, but there was about a 2-fold lower potency for most ADO analogs in membranes versus intact cells and a 4-fold lower potency for NECA. In addition, the intact cells showed a 25-fold increase in cyclic AMP accumulation over baseline with NECA, while in membrane preparations, there was only a 4-fold increase in adenylate cyclase activity. Furthermore, the potency of xanthine antagonists versus NECA-stimulated adenylate cyclase was greater in intact cells than in

membranes (Table 3). These observations suggest that $A_{2b}$ receptor coupling to a functional response is somehow altered by preparation of membranes. In brain membranes, the loss of a functional response may be related to such alterations. It is also possible that the brain $A_{2b}$ receptor is different from the receptors in fibroblasts. Binding assays are not available for $A_{2b}$ receptors.

The higher potency of xanthines in the intact NIH 3T3 fibroblast cells is reminiscent of the apparent relatively high potency of xanthines as antagonists at the $A_{2b}$ receptor of intact VA13 fibroblasts compared with binding affinities for $A_{2a}$ receptors [7, 30]. Indeed, the $K_B$ values for NIH 3T3 and VA13 fibroblasts for the five xanthines tested in the present study were in each case, almost identical in the two fibroblast cell lines (Table 4). However, these five xanthines had larger $K_B$ values when tested in NIH 3T3 membranes (Table 3). Thus, the earlier findings of relatively high potency of antagonists appear due to differences in intact cells versus membrane preparations rather than actual differences in activity of xanthines at $A_{2a}$ versus $A_{2b}$ receptors. Certainly, in membrane preparations the activities of xanthines at $A_{2a}$ and $A_{2b}$ receptors were comparable (Table 3). The potencies of xanthines as antagonists of $A_{2a}$ receptors of intact PC12 cells compared with membranes require study.

Comparison of agonist potencies for the NIH 3T3 (present study) and the VA13 fibroblast [5] ADO receptor showed many similarities as well as some differences (Table 2). In intact NIH 3T3 fibroblasts, the ADO analogs had lower $EC_{50}$ values than in intact VA13 fibroblasts. But values for NECA, 2-ClADO and 2-FADO in NIH 3T3 fibroblast membranes were comparable with the values reported in intact VA13 fibroblasts. In both cell lines, the rank order of potency for ADO analogs was similar, with NECA being the most potent. All ADO analogs had much lower potencies in the NIH 3T3 and VA13 fibroblasts than in PC12 membranes, consistent with the low-affinity $A_{2b}$ receptor in the fibroblasts and the high-affinity $A_{2a}$ receptor in PC12 cells. The difference in potency between stereoisomers of PIA (3.4- to 5.1-fold) was in agreement with other $A_2$ systems [19]. Another similarity between the two fibroblast cell lines was the weak activity of ADO analogs with aryl rings in the 2-position. In the VA13 fibroblasts, CV1674 has an $EC_{50} > 1$ mM [37] and CV1808 is also only weakly active [7]. Although CV1674 was not tested in the present study on NIH 3T3 fibroblasts, due to unavailability of the compound, CV1808 was one of the weakest analogs, with an $EC_{50}$ of 51 $\mu$M in intact cells and an $EC_{50}$ estimated to be slightly less than 1 mM in membranes. Therefore, in both the NIH 3T3 and VA13 fibroblasts, the receptor was a low-affinity adenosine receptor, the rank orders of potency of ADO analogs were in general agreement, and analogs with aryl groups at the 2-position were very weak agonists.

One difference between the NIH 3T3 and VA13 fibroblast ADO receptor is that MTA has been reported to be an antagonist at the VA13 fibroblast receptor [5], but was a partial agonist at the NIH 3T3 fibroblast receptor (Fig. 6A). As a partial agonist in NIH 3T3 fibroblasts, MTA showed xanthine-sensitive stimulation of adenylate cyclase with a low maximal response relative to NECA (23% of NECA maximum, Table 1, Fig. 6A) and MTA antagonized the NECA response in a concentration-dependent manner (Fig. 6, B and C). The activity of MTA may reflect a true difference between the NIH 3T3 and VA13 ADO receptor, but it is also possible that the efficacy of MTA in VA13 cells was so low that the agonist activity could not be detected. MTA has been shown to be an antagonist at an $A_2$ receptor in mouse neuroblastoma 2a cells with a $K_i$ of 4.4 $\mu$M [40], which is in good agreement with the $K_B$ of 8.2 $\mu$M found in VA13 fibroblasts [5]. In addition, MTA has been stated to be an antagonist of the $A_{2b}$ receptor in guinea pig cerebral cortical slices [27]. MTA is an agonist at $A_{2a}$ receptors of PC12 cells ([27] and present study) with a potency similar to that reported for its antagonist potency in neuroblastoma [40] and VA13 fibroblasts [5]. MTA is also an agonist at $A_1$ receptors of rat cerebellar membranes [40] and rat adipocyte membranes [27]. Thus, the possibility exists that in VA13 fibroblasts, MTA antagonizes the $A_2$-mediated cyclic AMP accumulation by acting through the $A_1$ receptor. Indeed, the $A_1$ receptor has been shown to be present in the VA13 fibroblasts [41]. In fibroblasts, *R*-PIA and CHA caused inhibition of prostaglandin$_1$-stimulated cyclic AMP accumulation, and this effect was attenuated by xanthines and pertussis toxin pretreatment. In the NIH 3T3 fibroblasts, there was no significant inhibitory activity of *R*-PIA on isoproterenol-stimulated adenylate cyclase (data not shown), and others have reported the lack of $A_1$ receptors in NIH 3T3 fibroblasts [42]. The difference in MTA activity between the NIH 3T3 and VA13 fibroblast is apparent, but whether it reflects a true receptor difference, or just a difference in the efficacy of coupling in the two cell lines, is not known.

Another difference between the NIH 3T3 and VA13 fibroblasts was in the structure–activity relationship of compounds substituted in the $N^6$-position. The difference in activity of CHA and *R*-PIA in the NIH 3T3 and PC12 cells was 2.4- to 3.2-fold (Table 1), whereas these analogs show virtually no difference in activity in the VA13 cells [5]. In addition, the difference in activity of *R*-PIA and 2-ClADO was 1.2- to 2.3-fold in both NIH 3T3 and PC12 cells (Table 1), but is 6.3-fold in VA13 cells [5]. These observations suggest that the region of the ADO receptor that interacts with the $N^6$-position of ADO analogs is more similar between the PC12 $A_{2a}$ and NIH 3T3 $A_{2b}$ receptor than between the NIH 3T3 and VA13 fibroblast $A_{2b}$ receptors.

Comparison of the $A_{2a}$ receptor in PC12 cells and the $A_{2b}$ receptor in NIH 3T3 cells showed that all analogs tested were more potent at the $A_{2a}$ receptor. Regarding the 5′-substituted compounds, NECA was 5–15 times more potent at the $A_{2a}$ receptor. Both the rank order of potency and magnitude of maximal response followed the order: NECA > 5ClADO > MTA. MTA is a weak partial agonist at both the $A_{2a}$ and $A_{2b}$ receptor (Table 1, Fig. 6). Thus, the two subtypes of receptors were similar in the region of interaction with the 5′-

substituent of ADO analogs. The $N^6$-substituted compounds were approximately 15–30 times more potent at the $A_{2a}$ receptor than at the $A_{2b}$ receptor. In both systems, addition of the $N^6$-group appears to decrease potency of the compound by an equivalent amount. For example, addition of the *R*-phenylisopropyl group in the $N^6$-position of NECA decreased the potency of the compound by about 3-fold at both the PC12 $A_{2a}$ and NIH 3T3 $A_{2b}$ receptor (Table 1, Fig. 5). This structural change also reduces the maximal response to the analog by an equivalent amount for both receptors (Table 1).

The receptor domain of interaction with the 2-position of ADO analogs varies greatly at the $A_{2a}$ and $A_{2b}$ receptors. ADO analogs with large chemical groups in the 2-position were developed initially to enhance potency and selectivity of the $A_2$ receptor over the $A_1$ receptor. However, these compounds proved to be highly selective for the $A_{2a}$ versus the $A_{2b}$ receptor. CV1808 is 5- to 20-fold selective for the $A_2$ versus the $A_1$ receptor [19]. It shows an even higher degree of selectivity for the $A_{2a}$ versus the $A_{2b}$ receptor, namely > 100-fold in the present study and about 1000-fold in the VA13 fibroblast [Table 1, and Ref. 7]. CV1674 is only about 2-fold selective for the $A_2$ versus the $A_1$ receptor, but is > 1600-fold selective for the $A_{2a}$ versus the $A_{2b}$ VA13 fibroblast receptor [7]. CGS 21680 is > 170-fold more potent at $A_{2a}$ receptors versus $A_1$ receptors in binding assays and > 1500-fold more potent in functional assays [12–14, 43]. CGS 21680 has very low activity at the NIH 3T3 $A_{2b}$ receptor and, thus, is highly selective for the $A_{2a}$ receptor (Table 1). In guinea pig heart preparations, the $A_2/A_1$ activity ratio was 8700 for 2-(2-cyclohexylethoxy)ADO and 8200 for 2-phenylethoxyADO [38, 39]. The present study showed 2-(2-cyclohexylethoxy)ADO to be about 1100-fold and 2-phenylethoxyADO to be about 700-fold selective for the $A_{2a}$ receptor versus the $A_{2b}$ receptor (Table 1). In the guinea pig heart preparations [38, 39], the $A_2$ receptor stimulates coronary blood flow, and the high activity of the 2-substituted ADO analogs suggests that an $A_{2a}$ receptor is involved in intact coronary vasculature. In contrast, CGS 21680 and other 2-substituted ADO analogs are very weak in relaxing guinea pig aorta [44], suggesting that $A_{2b}$ receptors are involved. CV1808 did not relax guinea pig trachea in a xanthine-sensitive manner [45], suggesting that the xanthine-sensitive component of NECA-elicited relaxations involves an $A_{2b}$ receptor.

Xanthine antagonists did not appear to be markedly selective for either the high-affinity $A_{2a}$ or low-affinity $A_{2b}$ receptor, suggesting perhaps that these heterocycles do not bind at the same site as adenosine analogs or that the ribose ring is the primary determinant for low or high affinity at the $A_2$ receptor subtypes. However, non-xanthine antagonists whose heterocyclic systems more closely resemble the adenine system (CP66,713, CGS 15953A, tracazolate and the 9-methyladenines) are all several-fold less potent at the "low-affinity" $A_{2b}$ receptor compared with the "high-affinity" $A_{2a}$ receptor. Thus, such antagonists may bind in a similar fashion to adenosine agonists at $A_2$ receptors. Of the ADO receptor antagonists tested, alloxazine was the only compound shown to be selective for the $A_{2b}$ receptor, with a selectivity of about 9-fold for the NIH 3T3 $A_{2b}$ receptor versus the PC12 $A_{2a}$ receptor. The most potent antagonist at the NIH 3T3 $A_{2b}$ receptor was CGS 15943A with a $K_B$ of $0.041 \pm 0.004\ \mu M$. Although CGS 15943A is also a potent antagonist at the $A_{2a}$ receptor, the potency of this compound at the $A_{2b}$ receptor suggests that it may be useful in binding studies of the $A_{2b}$ receptor. Many of the non-xanthine ADO antagonists reduced basal activity of adenylate cyclase in both PC12 and NIH 3T3 membranes.

In summary, a series of ADO analogs and xanthine and non-xanthine antagonists were used to characterize an ADO receptor in NIH 3T3 fibroblast cells. The results indicate that this cell contains an $A_{2b}$ receptor. Comparison of the $A_{2a}$ receptor in PC12 cells with the $A_{2b}$ receptor in NIH 3T3 fibroblasts showed differences expected in such a comparison; namely, lower potency of all analogs at the $A_{2b}$ receptor and the high degree of selectivity of several 2-phenyl and 2-(ar)alkyloxy ADO analogs at the $A_{2a}$ receptor. None of the adenosine analogs were selective for the $A_{2b}$ receptor, which would be expected for a "low-affinity" adenosine receptor. The potencies of antagonists at the $A_{2b}$ receptor versus the $A_{2a}$ receptor indicated xanthines to be generally non-selective, certain non-xanthine antagonists containing an adenine or adenine-like ring to be selective for the $A_{2a}$ receptor, and alloxazine to be selective for the $A_{2b}$ receptor. The $A_{2b}$ receptor in the NIH 3T3 fibroblast was similar to that in the VA13 fibroblast, but there were some differences. Whether these differences are indicative of further subtypes of $A_2$ receptors is not clear. The NIH 3T3 fibroblast assay for the $A_{2b}$ receptor will facilitate the rapid screening of new compounds, which may lead to the development of agonists and antagonists specific for such $A_{2b}$ receptors.

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