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Demonstration of Both A_1 and A_2 Adenosine Receptors in DDT_1 MF-2 Smooth Muscle Cells

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SUMMARY

Adenosine receptors of the A_1 and A_2 subtypes were characterized in membranes from DDT_1 MF-2 smooth muscle cells. These cells possess a high density of A_1 adenosine receptors (B_{max} = 0.8–0.9 pmol/mg of protein), as measured by both agonist and antagonist radioligands. Agonists compete for [^{125}I]N^6-[2-(4-amino-3-iodophenyl)ethyl]-adenosine (A_1 receptor-selective radioligand) binding with the following potency series: (*R*)-phenylisopropyladenosine [(*R*)-PIA] > 5′-*N*-ethylcarboxamide adenosine (NECA) > (*S*)-PIA, indicative of their interaction with A_1 adenosine receptors. Agonist competition for [^{3}H]8-{4-[{{[(2-aminoethyl)amino]carbonyl}methyl}oxy]phenyl}-1,3-dipropylxanthine ([^{3}H]XAC) (an antagonist radioligand for the A_1 adenosine receptor) was described by a two-state model of 1.3 nM (high affinity state, K_K) and 370 nM (low affinity state, K_L), with 70% of the receptors in the high affinity state (R_H). Addition of guanosine 5′-[β, α-imido]triphosphate (100 μM) shifted the (*R*)-PIA competition curves to the right to lower affinities. Photoaffinity labeling with the agonist photoprobe [^{125}I]N^6-[2-(4-amino-3-iodophenyl)ethyl]adenosine indicates that the A_1 adenosine receptor binding subunit is a M_r 38,000 protein. Adenosine receptor agonists [(*R*)-PIA, NECA, and (*S*)-PIA] inhibited isoproterenol-stimulated adenylate cyclase activity in DDT_1 MF-2 cell membranes with IC_{50} values of 62, 538, and 750 nM, respectively. Inhibition of adenylate cyclase by (*R*)-PIA was attenuated by the A_1 receptor antagonist XAC and following inactivation of G_i with pertussis toxin (100 ng/ml). Using a recently developed A_2 adenosine receptor agonist radioligand 2-[4-(2-{[4-aminophenyl]methylcarbonyl}ethyl)phenyl]ethylamino-5′-*N*-ethylcarboxamido adenosine ([^{125}I-PAPA-APEC), we have demonstrated the presence of A_2 adenosine receptors in this cell line. Saturation curves with ^{125}I-PAPA-APEC indicated the B_{max} and K_d values to be 0.21 pmol/mg of protein and 4.0 nM, respectively. In competition experiments, NECA was more potent at inhibiting ^{125}I-PAPA-APEC binding than (*R*)-PIA, with their respective IC_{50} values being 5.6 and 351 nM. The photolabeled A_2 adenosine receptor migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an M_r of 42,000. Finally, adenosine receptor agonists stimulated adenylate cyclase activity by ~2–3-fold with the following potency series: PAPA-APEC ≥ NECA > (*R*)-PIA, indicative of their interaction at A_2 receptors. These data represent the first demonstration of the presence of both A_1 and A_2 receptors in a single cell line, DDT_1 MF-2 smooth muscle cells.

Adenosine receptors comprise a group of cell surface receptors that mediate the physiological effects of adenosine. These receptors were classified by Burnstock (1) as P_1 or P_2 purinergic receptors, depending on their preferential interaction with adenosine (P_1) or ATP (P_2). The P_1 sites are further subdivided into A_1 and A_2 adenosine receptors, on the basis of their differential selectivity for a series of adenosine analogs (2, 3). The A_1 adenosine receptor potency series is (*R*)-PIA > NECA > (*S*)-PIA. At the A_2 receptors, the potency series is NECA > (*R*)-PIA > (*S*)-PIA.

Both A_1 and A_2 adenosine receptors are widely distributed in the central nervous system and peripheral tissues (4). Although the availability of agonist and antagonist radioligands has enabled detailed characterization of the A_1 adenosine receptor in various tissues (5–8), a similar characterization of the A_2 adenosine receptor has been lacking. Until recently, no truly useful radioligand for the A_2 adenosine receptor was available.

Demonstration of adenosine receptors in smooth muscle has been made primarily by functional assays. For example, adenosine can stimulate adenylate cyclase activity via A_2 adenosine receptors in vascular smooth muscle cells in culture (9, 10).

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ABBREVIATIONS: PIA, phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamide adenosine, APNEA, N^6-[2-(4-amino-3-iodphenyl)ethyl]adenosine; AZPNEA, N^6-[2-(4-azido-3-iodophenyl)ethyl]adenosine; XAC, 8-{4-[{{[(2-aminoethyl)amino]carbonyl}methyl}oxyl]phenyl}-1,3-dipropylxanthine; PAPA-APEC, 2-[4-(2-{[4-aminophenyl]methylcarbonyl}ethyl)-phenyl]ethylamino-5′-*N*-ethylcarboxamido adenosine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propansulfonate; Gpp(NH)p, guanosine 5′-[β,α-imido]triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G_i protein, inhibitory GTP-binding protein.

Whether this action mediates the vasorelaxant action of the nucleoside is still unclear, because concentrations of adenosine required to elevate cAMP are considerably higher than those required for full vasorelaxation (11, 12). In rat vas deferens, adenosine inhibits electrically induced contraction by interacting with A_1 adenosine receptors (13). A_1 adenosine receptors have also been observed in vascular smooth muscle, based on the greater potency of cyclohexyladenosine (a selective A_1 receptor agonist) over NECA to activate particulate guanylate cyclase (14). Because cGMP mediates vasodilatation in smooth muscle (15, 16), activation of guanylate cyclase might account for vasodilatation by A_1 agonists. To date, however, there has been no solid confirmation by radioligand binding or photoaffinity labeling that A_1 adenosine receptors are present in smooth muscle.

Detailed studies on the regulation of A_1 and A_2 adenosine receptors have been hindered by the lack of a cell culture model containing both A_1 and A_2 receptors. To alleviate this problem, we have screened a number of cell lines to find an appropriate model. The DDT_1 MF-2 smooth muscle cell line was derived from a steroid-induced leiomyosarcoma tumor of the vas deferens of an adult Syrian hamster (17). These cells express α_1- (18) and β_2-adrenergic receptors (19) and glucocorticoid receptors (20) and possess a very responsive adenylate cyclase system (19). In the present study we have demonstrated the existence of both A_1 and A_2 adenosine receptors in membranes from DDT_1 MF-2 smooth muscle cells by radioligand binding and photoaffinity labeling using selective probes for these receptors and by adenylate cyclase assays. This study, we believe, represents the first detailed characterization of both types of P_1 adenosine receptors in a cultured cell line and should facilitate future studies on the structure, function, and regulation of adenosine receptors.

Experimental Procedures

Materials

Chloramine T, cAMP, dATP, GTP, ATP, creatinine phosphokinase, HEPES, phosphocreatine, Tris·HCl, and isoproterenol were from Sigma Chemical Co. (St. Louis, MO). (*R*)-PIA, (*S*)-PIA, and adenosine deaminase were from Boehringer-Mannheim (Indianapolis, IN). Electrophoresis reagents were from Bio-Rad Laboratories (Campbell, CA). Dulbecco's modified Eagle medium (high glucose), fetal bovine serum, and penicillin/streptomycin were from GIBCO Laboratories (Grand Island, NY). [^{3}H]XAC (160 Ci/mmol) and [α-^{32}P]ATP were from Dupont-New England Nuclear (Boston, MA). Na^{125}I (carrier-free) was from Amersham Corp. (Arlington Heights, IL). NECA was a generous gift from Dr. Ray Olsson (University of South Florida, Tampa, FL). All other reagents were of the highest available grade and were purchased from standard sources.

Cell Culture

DDT_1 MF-2 cells were grown in suspension in Dulbecco's modified Eagle medium (high glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown (in the absence of adenosine deaminase) at 37 ° in the presence of 20% O_2 and 5% CO_2. The cells were supplemented with fresh medium 12 hr before experiments were performed. Under these conditions, the addition of adenosine deaminase (0.3 units/ml) did not appear to alter the (*R*)-PIA mediated inhibition of adenylate cyclase or the density and affinity of A_1 adenosine receptors, when compared with cells not treated with adenosine deaminase. Experiments were performed on cells grown to a density of ~1 × 10^6 cells/ml.

Membrane Preparation

Cells were pelleted by centrifugation at 800 × *g* for 3 min and washed twice with Krebs phosphate buffer (128 mM NaCl, 1.4 mM $MgCl_2$ 5.2 mM KCl, 10 mM Na_2HPO_4, pH 7.4). The cells were then washed in 50 mM Tris·HCl buffer (pH 7.4), Dounce homogenized (15 up and down strokes), and centrifuged at 800 × *g* for 5 min. The supernatant was then centrifuged at 43,000 × *g* for 15 min and the resulting pellet was resuspended in 50 mM Tris·HCl buffer containing 10 mM $MgCl_2$ and 1 mM EDTA (termed 50/10/1 buffer, hereafter). Endogenously released adenosine was degraded using adenosine deaminase (~3 units/ml) and incubating the mixture for 10 min at 37 °. Membranes were then pelleted again by centrifugation at 43,000 × *g* for 10 min. The resulting pellet used for binding experiments was resuspended in 50/10/1 buffer containing ~3 units/ml adenosine deaminase. For ^{125}I-PAPA-APEC and ^{125}I-azido-PAPA-APEC binding, membranes were resuspended in 50 mM HEPES buffer (pH 7.2) containing 10 mM $MgCl_2$ (termed 50/10, hereafter).

Membranes used for adenylate cyclase were prepared by centrifuging the Dounce-homogenized cells (as above) at 43,000 × *g* for 15 min and resuspending the pellet in 75 mM Tris buffer (containing 200 mM NaCl, 12.5 mM $MgCl_2$, and 1 mM dithiothreitol, pH 8.12 at 5 °. Membranes were incubated with adenosine deaminase (~3 units/ml) for 15 min at 30 ° to eliminate endogenous adenosine before adenylate cyclase assays were performed.

Synthesis of [^{125}I]APNEA and [^{125}I]AZPNEA

These agonist radioligands were synthesized as described previously (5, 21).

Radioiodination of PAPA-APEC

PAPA-APEC was iodinated by the chloramine T method described previously (5, 22). Briefly, 10 μl of PAPA-APEC (0.1 mg/ml) were placed in a microfuge tube and dried completely under a stream of nitrogen. The residue was then dissolved in 30 μl of 0.5 M Na_2HPO_4 (pH 7.35), 1.5 mCi of Na^{125} (15 μl) was added, and the solution was mixed well. The reaction was initiated by the addition of 10 μl of chloramine T (1 mg/ml) and the mixture was incubated for 4 min at room temperature with constant agitation. Iodination was terminated by addition of 10 μl of sodium metabisulfite (2 mg/ml). ^{125}I-PAPA-APEC was then separated by high pressure liquid chromatography using a C_{18} μ-Bondapak column and a gradient protocol. The mobile phase was initially composed of a 60:40 mixture (v/v) of methanol and 20 mM ammonium formate (pH 7.8) and this was changed incrementally to a 50:50 mixture by 10 min. ^{125}I-PAPA-APEC emerged as the radioactive peak at 7 min and was collected and used for binding assays or lyophylized and resuspended in 3 N acetic acid for use in the synthesis of ^{125}I-azido-PAPA-APEC. The identity of the ^{125}I-PAPA-APEC was confirmed by thin layer chromatography and was the only fraction shown to bind a receptor possessing the appropriate A_2 receptor pharmacology.

Synthesis of ^{125}I-Azido-PAPA-APEC

The parent compound PAPA-APEC was iodinated as described above. The conversion of the aryl amine to the aryl azide was performed by reacting 20 μl of ice-cold sodium nitrite (20 mg/ml) and 20 μl of the ^{125}I-PAPA-APEC (from above) on ice for 10 min. This was followed by the addition of 10 μl of ice-cold sodium azide (5 mg/ml) and the mixture was allowed to react for 5 min on ice. An additional 10 μl of sodium azide were added and the mixture was incubated for 5 min at room temperature. The reaction was terminated by alkalinization with 8 μl of ammonium hydroxide and the ^{125}I-azido-PAPA-APEC was separated by high pressure liquid chromatography using an isocratic protocol with a mobile phase consisting of a 75:25 mixture (v/v) of methanol and 20 mM ammonium formate (pH 7.8). ^{125}I-Azido-PAPA-APEC emerged as a radioactive peak at 6 min. On thin layer chromotography (chloroform/methanol/acetic acid in a ratio of 85:10:5), R_F values for PAPA-APEC, ^{125}I-PAPA-APEC, and ^{125}I-azido-PAPA-APEC were

0.11, 0.22, and 0.34, respectively. Each of the purified radioligands was assumed to have a specific activity of 2200 Ci/mmol.

Radioligand Binding in DDT$_1$ MF2 Cell Membranes

[^{3}H]XAC binding. Membranes (~ 20–40 μg/assay tube) were incubated for 1 hr at 37 ° with six to eight concentrations of [^{3}H]XAC (0.2–6 nM) in a total volume of 250 μl of 50/10/1 buffer. This incubation time was optimal to ensure steady state binding as determined from [^{3}H]XAC association curves (data not shown). (*R*)-PIA (10 μM) was used to define nonspecific binding, which normally averaged from 30 to 50% of the total binding. Following incubation, membranes were rapidly filtered over 25-mm glass fiber filters (No. 32; Schleicher & Schuell) by vacuum and washed three times with 3 ml of 50/10/1 buffer containing 0.03% CHAPS. Filters were allowed to extract for at least 6 hr in toluene-based scintillation fluid before counting.

(*R*)-PIA competition curves were performed by incubating membranes with a single concentration of [^{3}H]XAC (~1.5 nM) and increasing concentrations of (*R*)-PIA, with or without Gpp(NH)p (100 μM).

[^{125}I]APNEA Binding. Binding experiments were performed, as described above, using 0.2–6 nM [^{125}I]APNEA and ~20–40 μg of membrane protein/assay tube. Incubations were for 1 hr at 37 °. Agonist competition curves were performed using a single concentration of the radioligand (~1 nM) and different concentrations of the competitors [(*R*)-PIA, NECA, (*S*)-PIA].

^{125}I-PAPA-APEC binding. Membranes (~60 μg of protein) were incubated for 1 hr with ^{125}I-PAPA-APEC (0.2–6 nM) in a total volume of 250 μl of 50/10 buffer. Nonspecific binding was defined with 10 mM theophylline and averaged ~50% of total binding at concentrations of the radioligand at the K_d. This concentration of theophylline was optimal and defined a level of nonspecific binding similar to that defined by NECA (100 μM). Agonist competition experiments were performed using a single concentration of ^{125}I-PAPA-APEC (~1.5 nM) and various concentrations of NECA or (*R*)-PIA. Separation of the bound radioligands was performed as described above.

Photoaffinity Labeling

[^{125}I]AZPNEA. DDT$_1$ MF-2 cell membranes (~450 μg of protein/ml), prepared as described above, were suspended in a final volume of 3 ml of 50/10/1 buffer (21). To separate aliquots of membranes was added either H_2O, (*R*)-PIA, (*S*)-PIA, or XAC (drugs added in a final concentration of 10 nM). [^{125}I]AZPNEA (5 nM) was then added to all aliquots and the mixtures were incubated at 37 ° for 45 min. The samples were washed with 40 ml of ice-cold 50/10/1 buffer and centrifuged at 45,000 × *g* for 5 min. This wash was repeated once and the resulting pellet was resuspended in 10 ml of the same buffer and exposed to UV light for 4 min to initiate incorporation. Membranes were then pelleted by centrifugation and solubilized in SDS-glycerol buffer for SDS-PAGE.

^{125}I-Azido-PAPA-APEC. Labeling of A_2 adenosine receptors was performed by incubating membranes (~250–300 μg of protein) with 0.8 nM ^{125}I-azido-PAPA-APEC, in the absence or presence of competitors, in polypropylene tubes that were individually wrapped with aluminum foil. Incubations were for 45 min at 37 ° in a final volume of 1 ml of 50/10 buffer. Following incubation, membranes were centrifuged and washed twice with 50/10 buffer containing 0.01% CHAPS, centrifuged at 43,000 × *g* for 5 min, and resuspended in 5 ml of buffer. Photoincorporation of ^{125}I-azido-PAPA-APEC was performed by exposing the membranes on ice to UV light for 4 min and at a distance of 1 cm. The resulting mixture was washed twice with 50/10 buffer containing 0.01% CHAPS. The membrane pellets were solubilized in SDS-glycerol buffer before SDS-PAGE was performed.

SDS-PAGE

Electrophoresis was performed according to the method of Laemmli (23) using homogeneous gels, with the stacking gel containing 3% acrylamide and the separating gel 12–15% acrylamide. Electrophoresis was performed at a constant current of 10 mA. Premixed SDS-PAGE standards from Pharmacia were iodinated using the chloramine T method and include albumin (M_r = 67,000), ovalbumin (M_r = 45,000), carbonic anhydrase (M_r = 29,000), trypsinogen (M_r 24,000), and soybean trypsin inhibitor (M_r = 20,000). Following electrophoresis, gels were dried using a Bio-Rad gel dryer and used for autoradiography at 80 °, using X-ray film (XAR-5) and Cronex Lightening Plus intensifying screens.

Adenylate Cyclase Assay

Adenylate cyclase activity in DDT$_1$ MF-2 cell membranes (pretreated with adenosine deaminase) was determined as described previously (24). Briefly, 20 μl of membranes (~50 μg of protein) were incubated with 20 μl of reaction mixture (0.14 mM ATP, 5 mM phosphocreatine, 1 μM cAMP, 30 units/ml creatine phosphokinase, 5 μM GTP, ~1.5 μCi of [α-^{32}P]ATP) and 10 μl of H_2O or drugs. Papaverine (0.1 mM) was included in all experiments to provide adequate inhibition of the low K_m cyclic AMP phosphodiesterase. Assay tubes were incubated for 10 min at 30 ° and terminated by addition of 1 ml of ice-cold stop solution containing ~15,000 cpm of [^{3}H]cAMP, 0.3 mM cAMP, and 0.4 mM ATP. Cyclic AMP is isolated by the method of Salomon *et al.* (25).

Data Analysis and Protein Determination

Saturation and competition curves were analyzed by a computer-assisted curve-fitting program (26, 27) equipped with a statistical package. Other statistical analyses were performed using the Student *t* test (two-tailed) with an α probability of 0.05. Error bars shown in the text and in the figures are standard errors. Protein concentrations were determined by the method of Bradford (28), using bovine serum albumin as standard.

Results

Quantitation of A_1 adenosine receptors in DDT$_1$ MF-2 cell membranes. Fig. 1 shows a saturation curve of [^{3}H]XAC, an A_1 adenosine receptor-selective radioligand, in DDT$_1$ MF-2 cell membranes. Radioligand binding experiments were carried out for 1 hr at 37 °, which was adequate to ensure steady state binding (data now shown). [^{3}H]XAC binding was saturable, with a B_{max} of 0.81 ± 0.09 pmol/mg (mean ± SE) and equilibrium dissociation constant (K_d) of 2.7 ± 0.5 nM.

Agonist [(*R*)-PIA] competition curves versus [^{3}H]XAC were shallow, as evident from the Hill coefficients that were significantly less than unity ($p < 0.05$). These competition curves

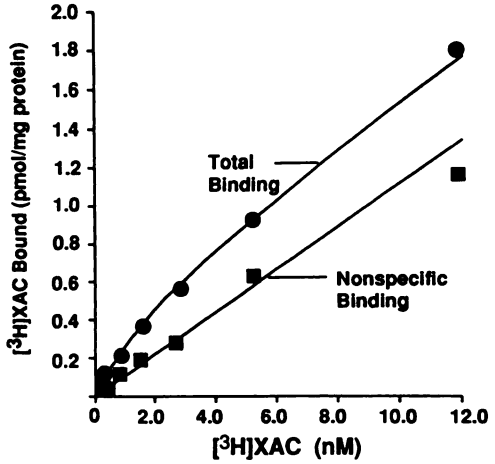

Fig. 1. [^{3}H]XAC saturation curve in DDT$_1$ MF-2 smooth muscle cell membranes. Experiments were performed by incubating [^{3}H]XAC with membranes (20–40 μg/assay tube) in the absence (specific binding) and presence (nonspecific binding) of 10 μM(*R*)-PIA. This is representative of five similar experiments performed in duplicate.

were more appropriately described by a two-state model (26, 27), with K_H and K_L being 1.3 ± 0.3 and 370 ± 175 nM, respectively, and ~70% of the receptors in the high affinity (R_H) state (Table 1, Fig. 2). A two-state model was statistically favored over a one-state model ($p < 0.05$). Addition of Gpp(NH)p (100 μM) to the incubation mixture shifted the K_H to a novel high affinity state of 7.3 nM without changing K_L or without significantly affecting the Hill slopes. In addition, this treatment decreased the percentage of receptors in the high affinity state to 57%. However, these curves were still most appropriately described by a two-state model. These results are consistent with those observed for the A_1 receptor in other tissues; guanine nucleotides are unable to shift all of the receptors to a low affinity state (7).

An agonist radioligand ([^{125}I]APNEA) was also used to study the properties of the A_1 adenosine receptor. Fig. 3 and Table 2 demonstrate that this ligand labels a population of receptors equivalent to that labeled by the antagonist [^{3}H]XAC. Fig. 4 demonstrates that the receptors labeled by [^{125}I]APNEA are of the A_1 adenosine receptor subtype, as evidenced from the agonist potency series in competition binding experiments [(*R*)-PIA > NECA > (*S*)-PIA]. K_i values for these competitors were 0.9 ± 0.3, 6.8 ± 1.8, and 57.9 ± 22.2 nM, respectively.

Photoaffinity labeling of the A_1 adenosine receptors.

TABLE 1

(*R*)-PIA competition curves in the absence and presence of 100 μM Gpp(NH)p

Treatment	Hill coefficient	K_H[a]	K_L	R_H[b]	p[c]
		nM		%	
Control	0.56 ± 0.09[d]	1.3 ± 0.3	370 ± 175	70 ± 4	<0.05
Gpp(NH)p	0.59 ± 0.12[d]	7.3 ± 2.3[e]	279 ± 132	57 ± 10	<0.05

[a] K_H and K_L, high and low dissociation constants, respectively, calculated assuming a two-state model.
[b] % R_H, percentage of total receptors in the high affinity state.
[c] *p*, *F* statistics derived from comparing the applicability of a two-state model over a one-state model in analyzing the data.
[d] For Hill coefficient, statistically significantly different from unity ($p < 0.05$).
[e] Statistically significantly different from control ($p < 0.05$). Similar increases in K_H in the presence of Gpp(NH)p have been described (36, 37).

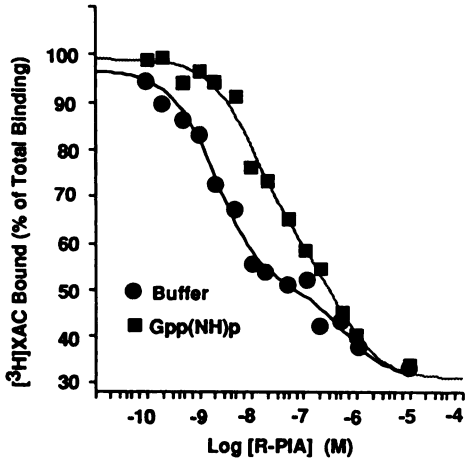

Fig. 2. (*R*)-PIA competition curves versus [^{3}H]XAC. Membranes were incubated with [^{3}H]XAC (1.5 nM) and various concentrations of (*R*)-PIA in the absence or presence of Gpp(NH)p (100 μM). Specific [^{3}H]XAC binding was 0.23 ± 0.04 and 0.31 ± 0.06 pmol/mg of protein for control and Gpp(NH)p-treated membranes, respectively. Results are presented as the averaged data from five independent experiments of each treatment group. Competition curves were analyzed by a curve-fitting program (Scafit) according to a two-state model (27, 28).

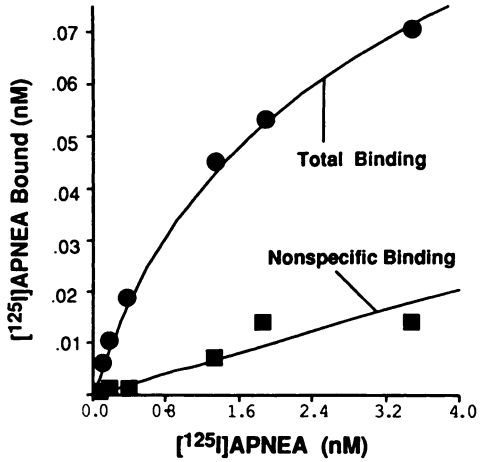

Fig. 3. [^{125}I]APNEA saturation curve. Experiments were performed using 20–40 μg of DDT$_1$ MF-2 cell membranes incubated with [^{125}I]APNEA in the absence (total binding) and presence (nonspecific binding) of 10 μM (*R*)-PIA. This is a representative of three similar experiments, each performed in duplicate. B_{max} and K_d values for this representative experiment were 0.90 pmol/mg of protein and 1.4 nM, respectively.

TABLE 2

Measurement of A_1 adenosine receptor density and affinity in DDT$_1$ MF-2 cell membranes

Ligand	B_{max}	K_d	n[a]
	pmol/mg of protein	nM	
[^{3}H]XAC	0.81 ± 0.09	2.7 ± 0.5	5
[^{125}I]APNEA	0.90 ± 0.11	1.8 ± 0.5	3

[a] Number of experiments.

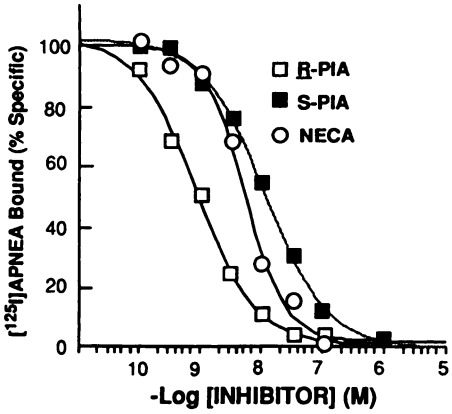

Fig. 4. Agonist competition curves versus [^{125}I]APNEA. Competition curves were derived by incubating [^{125}I]APNEA (~0.5 nM) with membranes (as in Fig. 3) and different concentrations of the competitors. Specific binding in the absence of competitors averaged ~0.21 pmol/assay tube. This figure is representative of three similar experiments. The curves were fitted by a curve-fitting program (27, 28).

The agonist photoaffinity probe [^{125}I]AZPNEA has been used previously to characterize the A_1 adenosine receptors in several tissues (21). The photolabeled receptor migrates with an M_r ~38,000 (Fig. 5) and is similar to A_1 adenosine receptors described previously in other tissues (21). Labeling was completely inhibited by 10 nM (*R*)-PIA and XAC and less completely by 10 nM (*S*)-PIA, as expected for agents competing stereospecifically at A_1 adenosine receptors.

A_1 adenosine receptor-mediated inhibition of adenylate cyclase activity. A_1 adenosine receptors in several

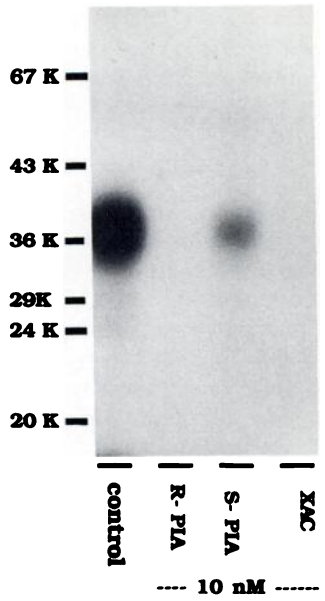

Fig. 5. Photoaffinity labeling of A_1 adenosine receptors in DDT_1 MF-2 cell membranes. Cell membranes were incubated with [^{125}I]AZPNEA (0.5 nM) in the absence (control) or presence of (*R*)-PIA, (*S*)-PIA, or XAC. Incubations were for 1 hr at 37 °, following which membranes were centrifuged, washed, and exposed to UV light. The photolabeled receptor was resolved on 12% acrylamide SDS-PAGE and subjected to autoradiography. The migration profile of iodinated molecular weight standards is indicated on the *left*.

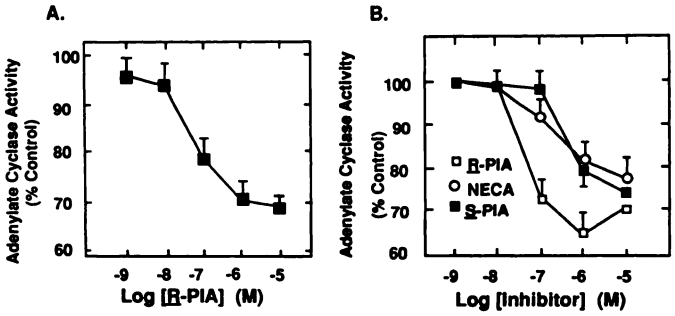

Fig. 6. Inhibition of adenylate cyclase activity in DDT_1 MF-2 cell membranes by adenosine analogs. A, Adenylate cyclase assays were performed as described in Experimental Procedures using 10 μM isoproterenol and various concentrations of (*R*)-PIA. Isoproterenol-stimulated adenylate cyclase in the absence of inhibitor (control) was 32.8 ± 6.1 pmol/min/mg of protein, compared with basal activity of 3.7 ± 0.5 pmol/min/mg of protein. Results are expressed as the averaged data from eight independent experiments. B, Adenylate cyclase activity was assayed in the presence of isoproterenol (10 μM) and various concentrations of inhibitors. The IC_{50} values (nM) for (*R*)-PIA, NECA, and (*S*)-PIA for inhibiting adenylate cyclase were 62 ± 10, 538 ± 189, and 750 ± 250, respectively (mean ± SE of four independent experiments).

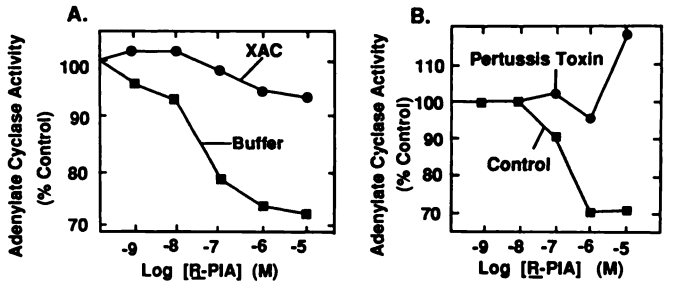

Fig. 7. Attentuation of (*R*)-PIA mediated inhibition of adenylate cyclase by XAC and pertussis toxin. A, Adenylate cyclase assays were performed in the presence of isoproterenol (10 μM) and either reaction buffer or XAC (1 μM) diluted in buffer. Adenylate cyclase activity in absence of (*R*)-PIA (control) was 32.1 and 34.5 pmol/min/mg of protein for buffer and XAC, respectively. B, DDT_1 MF-2 cells in culture were treated with Dulbecco's modified Eagle's medium or Dulbecco's modified Eagle's medium plus pertussis toxin (100 ng/ml) for 18 hr. Control adenylate cyclase activity [in the absence of (*R*)-PIA] averaged 23.7 and 24.6 pmol/min/mg of protein.

tissues are coupled to the inhibition of adenylate cyclase (3, 4, 24). We, therefore, tested the ability of the agonist (*R*)-PIA to inhibit isoproterenol-stimulated adenylate cyclase in membranes prepared from these cells. Nanomolar concentrations of (*R*)-PIA had no effect on basal adenylate cyclase in these cells but stimulated the enzyme at higher micromolar concentrations (see below). Inhibition of adenylate cyclase by (*R*)-PIA could be elicited following the activation of the enzyme by a stimulatory agent (e.g., isoproterenol). Isoproterenol (10 μM) was, therefore, used routinely to demonstrate the inhibitory effects of various adenosine analogs on adenylate cyclase activity. This concentration of isoproterenol resulted in a 8–12-fold stimulation of adenylate cyclase, which was a submaximal response; maximal response was attained with 100 μM isoproterenol (data not shown). Adenylate cyclase activity in the presence of isoproterenol was linear with time to 15 min (data not shown). All adenylate cyclase assays were terminated after 10 min of incubation.

(*R*)-PIA, in a dose-dependent manner, inhibited adenylate cyclase by ~30–40% with an IC_{50} of 66 nM (Fig. 6). Fig. 6B shows that the order of potency of the analogs for inhibiting isoproterenol-stimulated adenylate cyclase activity in these cells was typical of an A_1 adenosine receptor-mediated response. Inhibition of adenylate cyclase was effectively attenuated by XAC (a selective A_1 receptor antagonist) and by pertussis toxin (which ADP ribosylates and inactivates the G_i coupling protein) (Fig. 7). The identity of the G_i subtype(s) that couples A_1 adenosine receptor to adenylate cyclase in these cells is not known at present.

Quantitation of A_2 adenosine receptors in DDT_1 MF-2 cell membranes. We have recently developed ^{125}I-PAPA-APEC, an agonist radioligand that interacts specifically with A_2 adenosine receptors (22). Using this radioligand, we can clearly show saturable and high affinity binding in DDT_1 MF-2 membranes (Fig. 8). B_{max} and K_d values were 0.21 ± 0.03 pmol/mg of protein and 4.0 ± 1.3 nM, respectively. These values are similar in magnitude to those previously observed in bovine striatum (22). Both NECA and (*R*)-PIA competed for ^{125}I-PAPA-APEC binding sites with IC_{50} of 5.6 ± 3.4 and 351 ± 117 nM, respectively (Fig. 9A). These values are lower than those observed in bovine striatum (22). This order of potency is indicative of their interaction at the A_2 adenosine receptor.

Photoaffinity labeling of the A_2 adenosine receptors. The apparent molecular weight of the A_2 adenosine receptor was determined with the photoaffinity probe ^{125}I-azido-PAPA-APEC, a derivative of PAPA-APEC shown to bind specifically to A_2 receptors.[1] The photolabeled receptor migrates with an M_r of ~42,000 (Fig. 9B), similar to the molecular weight determined for the bovine brain A_2 receptors (22). Labeling was inhibited to a greater degree by NECA (3.3 nM) than by (*R*)-PIA (3.3 nM), as expected from the order of potency of these

[1] Barrington, W. W., K. A. Jacobson, and G. L. Stiles. The glycoprotein nature of the A_2 adenosine receptor binding subunit. Submitted for publication.

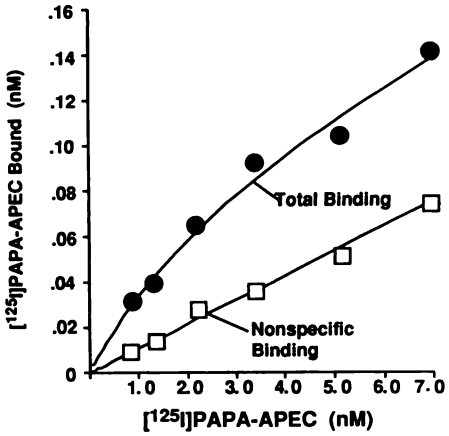

Fig. 8. ^{125}I-PAPA-APEC saturation curve in DDT_1 MF-2 cell membranes. Membranes (~60 μg) were incubated with different concentrations of the radioligand for 45 min at 37 °, in the absence (total binding) or presence (nonspecific binding) of theophylline (10 mM). This curve is representative of five similar experiments. Curves were modeled by a computer program, as previously described (27, 28). B_{max} and K_d values for this representative experiment were 0.17 pmol/mg of protein and 2.9 nM, respectively.

analogs at A_2 receptors. Furthermore, theophylline, a nonselective adenosine receptor antagonist, inhibited photoaffinity labeling of these receptors completely.

A_2 adenosine receptor-mediated activation of adenylate cyclase. A_2 adenosine receptors in DDT cells are functionally coupled to activation of adenylate cyclase. As observed in Fig. 10, PAPA-APEC is more efficacious at increasing adenylate cyclase activity (over basal) than either NECA or (*R*)-PIA at the highest concentration of each drug tested. Fold stimulation obtained with PAPA-APEC, NECA, and (*R*)-PIA were 2.8 ± 0.2, 2.1 ± 0.1, and 1.8 ± 0.1 at 10 μM concentrations of each drug tested (mean ± SE of three experiments). The potency series of these drugs for activating adenylate cyclase is as follows: PAPA-APEC > NECA > (*R*)-PIA (the EC_{50} values for activation of adenylate cyclase were 0.13 ± 0.06 and 0.38 ± 0.26 μM for PAPA-APEC and NECA, respectively). Because (*R*)-PIA did not produce a near maximal stimulation of adenylate cyclase at 10 μM, it is difficult to calculate an EC_{50} value for this agent. However, its potency is significantly less than that of NECA, as expected from its relative potency at A_2 adenosine receptors.

Discussion

The study of adenosine receptors has been limited by the unavailability of a suitable cell line that possesses these receptors. We (7) and others (29) have previously used rat adipocytes to demonstrate the existence and regulation of adenosine receptors in this tissue. Adipocytes, however, can only be used as a primary culture model and they lose receptor density and functional responses when maintained in culture for extended periods (30). In contrast, the DDT_1 MF-2 cells possess a high density of A_1 and a useful quantity of A_2 receptors, the levels of which were not affected by duration in culture or by frequency of passaging. Thus, the use of these cells should greatly accelerate the study of adenosine receptors *in vitro*.

The existence of A_1 adenosine receptors in DDT_1 MF-2 cells is supported by several pieces of evidence. Using both agonist ([^{125}I]APNEA) and antagonist ([^{3}H]XAC) radioligands, we detected a high density of receptor (0.8–0.9 pmol/mg of protein) with the characteristic A_1 receptor potency series [(*R*)-PIA > NECA > (*S*)-PIA]. The quantity of receptors detected by the agonist in this tissue was equivalent to that detected by the antagonist radioligand. Photoaffinity labeling of the receptor with the agonist probe ([^{125}I]AZPNEA) demonstrated a labeled protein migrating at M_r 38,000, which was identified as the A_1 adenosine receptor by its characteristic pharmacology and similarity in molecular weight of the adipocyte and brain receptor (21). Furthermore, this receptor is functionally coupled to the inhibition of isoproterenol-stimulated adenylate cyclase activity, a property ascribed to A_1 adenosine receptors (3, 4, 24, 29). ADP-ribosylation of G_i proteins by pertussis toxin abolished this inhibitory effect of (*R*)-PIA, implicating these proteins in the mediation of inhibition of adenylate cyclase.

Demonstration of A_2 adenosine receptors in DDT_1 MF-2 cells was aided by the selective A_2 agonist radioligand ^{125}I-PAPA-APEC (22). The binding of ^{125}I-PAPA-APEC was sat-

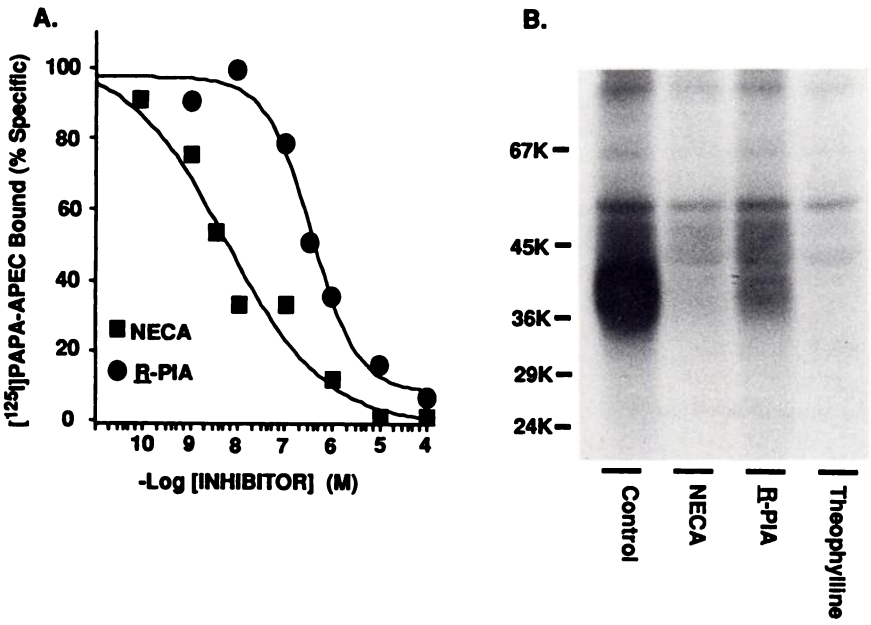

Fig. 9. Competition of various ligands with A_2 adenosine receptors. A, Membranes were incubated with 1.5 nM concentrations of the radioligand in the presence of various concentrations of NECA or (*R*)-PIA. The data presented were analyzed by computer modeling (27, 28) and represent the mean of five independent experiments with standard errors at each point of less than 10%. Specific ^{125}I-PAPA-APEC binding in the absence of inhibitors was 30.1 fmol/mg of protein. B, Labeling was performed as described in Experimental Procedures using ~0.75 nM ^{125}I-azido-PAPA-APEC, in absence or presence of NECA (3.3 nM), (*R*)-PIA (3.3 nM), or theophylline (10 mM). The labeled A_2 adenosine receptor migrates with M_r ~42,000 on a 14% polyacrylamide gel. The migration of labeled standards is indicated on the *left*. Experiments were repeated three times.

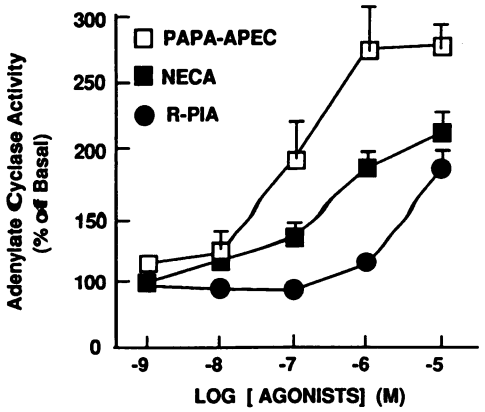

Fig. 10. Activation of adenylate cyclase by adenosine analogs in DDT_1 MF-2 membranes. Assays were performed as described in Experimental Procedures, for 10 min at 30 °. Basal adenylate cyclase activity averaged 3.0 ± 0.6 pmol/mg/min. Data are presented as the mean ± standard error of three independent experiments.

urable and of higher affinity than that observed for [^{3}H]NECA in vascular smooth muscle (31). NECA competed with 60-fold higher affinity for this site than (*R*)-PIA, indicative of the binding site being the A_2 adenosine receptor. Furthermore, the molecular weight of the labeled receptor (42,000) was similar to that observed in bovine brain (22) but different from the A_1 adenosine receptor (38,000). Labeling of this M_r 42,000 species was preferentially inhibited by NECA over (*R*)-PIA. Adenosine analogs activate smooth muscle adenylate cyclase with the order of potency being PAPA-APEC > NECA > (*R*)-PIA. This order of potency suggests that the site of action of these analogs is the A_2 adenosine receptor.

The specific role of each of these receptor subtypes on smooth muscle function is not clear at present. It has been proposed that A_2 adenosine receptor activation leads to smooth muscle relaxation via activation of adenylate cyclase (9, 10). In this respect, the finding that A_1 adenosine receptor activation leads to inhibition of adenylate cyclase is quite confusing. However, this property of adenosine analogs is evident only in the presence of an activator of adenylate cyclase (i.e., isoproterenol). In the absence of isoproterenol, adenosine analogs can themselves activate adenylate cyclase via A_2 adenosine receptors. Thus, assuming a role of cAMP in smooth muscle relaxation, one would expect adenosine and its analogs to relax vas deferens smooth muscle contracted by norepinephrine (in the absence of concurrent β_2-adrenergic agonists) by activating A_2 adenosine receptors. On the other hand, in the presence of β_2-adrenergic receptor activation, one might expect adenosine to produce smooth muscle contraction via A_1 adenosine receptors. Examples of A_1 adenosine receptors mediating smooth muscle contraction have been described (32, 33).

In addition to adenylate cyclase, the coupling of A_1 adenosine receptors to other effector systems has been described. For example, A_1 adenosine receptors can activate particulate guanylate cyclase (14) and inhibit phospholipase C (34) or Ca^{2+} channels (35), actions that might underlie the smooth muscle relaxant effect of adenosine in vas deferens (13).

In summary, our data clearly demonstrate the presence of both A_1 and A_2 adenosine receptors in DDT_1 MF-2 clonal cells. This model system should be extremely useful in studying the regulation of A_1 and A_2 adenosine receptors.

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