Characterization of adenosine A_1 receptor in a cell line (28A) derived from rabbit collecting tubule

WILLIAM S. SPIELMAN, KARL-NORBERT KLOTZ, LOIS J. AREND, BARBARA A. OLSON, DAVID G. LEVIER, AND ULRICH SCHWABE
Departments of Physiology and Biochemistry, Michigan State University, East Lansing, Michigan 48824; and Pharmakologishes Institut der Universität Heidelberg, D-6900 Heidelberg, Germany

Spielman, William S., Karl-Norbert Klotz, Lois J. Arend, Barbara A. Olson, David G. LeVier, and Ulrich Schwabe. Characterization of adenosine A_1 receptor in a cell line (28A) derived from rabbit collecting tubule. *Am. J. Physiol.* 263 (*Cell Physiol.* 32): C502-C508, 1992.—We have previously reported that in several renal cell types, adenosine receptor agonists inhibit adenylyl cyclase and activate phospholipase C via a pertussis toxin-sensitive G protein. In the present study, in 28A cells, both of these adenosine receptor-mediated responses were inhibited by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a highly selective A_1 adenosine receptor antagonist. The binding characteristics of the adenosine A_1 receptor in the 28A renal cell line were studied using the radiolabeled antagonist [^{3}H]DPCPX to determine whether two separate binding sites could account for these responses. Saturation binding of [^{3}H]DPCPX to 28A cell membranes revealed a single class of A_1 binding sites with an apparent K_d value of 1.4 nM and maximal binding capacity of 64 fmol/mg protein. Competition experiments with a variety of adenosine agonists gave biphasic displacement curves with a pharmacological profile characteristic of A_1 receptors. Comparison of [^{3}H]DPCPX competition binding data from 28A cell membranes with rabbit brain membranes, a tissue with well-characterized A_1 receptors, reveals that the A_1 receptor population in 28A cells has similar agonist binding affinities to the receptor population in brain but has a considerably lower density. Addition of guanosine 5′-triphosphate (100 μM) to 28A cell membranes caused the competition curves to shift from biphasic to monophasic, indicating that the A_1 receptors exist in two interconvertible affinity states because of their coupling to G proteins. In the absence of evidence for subpopulations of the A_1 receptor, it appears that in 28A cells, a single A_1 receptor population, as defined by ligand binding characteristics, couples via one or more pertussis toxin-sensitive guanine nucleotide binding proteins to two different biological signaling mechanisms.

calcium; phosphoinositides; adenosine 3′,5′-cyclic monophosphate; receptor binding; signal transduction; G proteins

ADENOSINE is a ubiquitous compound that, among other actions, alters hemodynamics, inhibits neurotransmission, platelet aggregation, and lipolysis, and stimulates glucose oxidation (1, 14, 16). Previous research has demonstrated the existence of two extracellular receptors for adenosine through which changes in adenylyl cyclase activity and adenosine 3′,5′-cyclic monophosphate (cAMP) production are mediated (11, 13, 24, 25). These receptors are denoted A_1 and A_2, and their binding to agonist results in the inhibition and stimulation of cAMP production, respectively. Recent cloning of these two receptors (21, 26) shows them to be members of the large class of hormone receptors that, like the visual pigment rhodopsin, are coupled to their intracellular effector systems via guanine nucleotide binding proteins (G proteins).

Radiolabeled binding studies have revealed the presence of both of these receptor types in the renal cortex and medulla in a variety of species (6, 10, 28, 30, 34), although in most of these studies no distinction can be made as to the particular renal cell types or nephron segments that might possess the receptors, and the post-receptor signaling mechanism cannot be ascertained. Recent reports from this laboratory have demonstrated the presence of A_1- and A_2-like effects of adenosine analogues on cAMP accumulation in primary cultures of rabbit cortical collecting tubule (RCCT) cells (4) and rabbit thick ascending limb of the loop of Henle (mTAL) cells (9).

We recently demonstrated in primary cultures of RCCT cells (2) and in RCCT-28A cells (28A cells), a cloned cell line derived from RCCT cells (3), that in addition to the classical A_1 and A_2 receptors coupled to the inhibition and stimulation of adenylyl cyclase, respectively, adenosine receptor agonists also stimulate the turnover of membrane inositol phosphates and cause the elevation of cytosolic free calcium. Similar to the inhibition of cAMP, these responses are coupled to a pertussis toxin-sensitive G protein and are inhibited by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a xanthine derivative that blocks the A_1-mediated inhibition of cAMP but has no effect on the 5′-(*N*-ethylcarboxamido)adenosine (NECA)-induced (A_2) increase in cAMP (2). These observations suggest that the adenosine-induced acceleration of inositol phosphate production and elevation of cytosolic free calcium concentration ($[Ca^{2+}]_i$) may result from activation of A_1 adenosine receptors.

The presence of two different signaling mechanisms associated with adenosine A_1 receptor activation raises the question of whether two classes of A_1 receptors exist. One possibility is that both the inhibition of adenylyl cyclase and the increase in inositol phosphate production are evoked by activation of a single receptor population via divergent coupling mechanisms. Alternatively, the two responses may be the result of independent A_1 receptor subpopulations indistinguishable in their specificity for currently available agonist or antagonist ligands.

Although binding analysis of A_1 adenosine receptors has been reported in a variety of tissues and cells, it has not been reported for a cell type that exhibits multiple signaling mechanisms associated with activation of the A_1 receptor. The present study provides a detailed characterization of the adenosine A_1 receptor through the use of radiolabeled ligand binding analysis and concentration-effect inhibition studies using DPCPX, a relatively specific A_1 antagonist, and pertussis toxin in an attempt to determine whether a single population of A_1 receptors is coupled to divergent signaling pathways.

0363-6143/92 $2.00

METHODS

Culture of RCCT-28A cells. RCCT-28A cells were cultured as previously described (3). Briefly, the cells were grown to confluency for 7 days before use in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, glutamine (2 mM), and dexamethasone (1 μM) in a 37°C incubator with a water-saturated 7% CO_2 atmosphere.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ in 28A cells was measured as described previously (2, 18) using the fluorescent Ca^{2+} chelator, fura-2. The 28A cells were detached from culture dishes by brief trypsin treatment, and the cell suspensions ($\sim 10^7$ cells/ml) were treated with fura-2/AM (final concn 8 μM) in simplified saline solution (SSS) containing 2 U adenosine deaminase (ADA)/ml SSS for 30 min at 37°C in a shaking water bath. The cells were centrifuged and washed with SSS [composition in mM: 145 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES), pH 7.4]. The cells were diluted 1:100 with SSS, and fluorescence was measured with a SPEX dual-wavelength spectrofluorimeter, with 340- and 380-nm excitation wavelengths and 505-nm emission wavelength.

Measurement of total cyclic nucleotide accumulation. Treatments were done in triplicate using cells grown for 4 days in 24-well dishes. Culture medium was removed, and the cells were washed once with Krebs buffer (in mM: 118 NaCl, 25 $NaHCO_3$, 14 glucose, 4.7 KCl, 2.5 $CaCl_2$, 1.8 $MgSO_4$, and 1.8 KH_2PO_4, pH 7.4). The cells were pretreated for 1 h in Krebs solution containing 0.1 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), a phosphodiesterase inhibitor that is not an adenosine receptor antagonist, and 2 U of ADA/ml Krebs at 37°C. After pretreatment, the buffer was aspirated and the cells were treated with Krebs plus Ro 20-1724 and ADA and containing the various hormones at 37°C for 30 min. Treatment was terminated by adding 75 μl of 0.2 N HCl. The cells were frozen, thawed, allowed to stand at 4°C for 60 min, and neutralized with 200 μl of 0.5 mM Na_2HPO_4. Total accumulated cAMP in the samples was determined by radioimmunoassay as described by Frandsen and Krishna (17).

Preparation of membranes. Rabbit brain and renal medullary membranes were prepared as described earlier (23). Membranes from the 28A cells were prepared as described for rat glioma C6 cells (35). Confluent 100-mm culture dishes of 28A cells of passages 7-10 were washed twice with cold phosphate-buffered saline (in mM: 138 NaCl, 1.5 KH_2PO_4, 3 KCl, and 8.1 Na_2HPO_4, pH 7.4). The cells were washed once more with cold lysing buffer (5 mM HEPES and 1 mM $MgSO_4$, pH 8.0) and kept in lysing buffer for 10 min at 4°C. The cells were scraped from the plates and vortexed before being centrifuged at 1,500 *g* for 5 min. The cells were washed once more with lysing buffer, and the pellet was resuspended in 2 mM tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.4), 1 mM EDTA, and 0.2 mM dithiothreitol before being disrupted with a Brinkmann Polytron (setting 6, 5-10 s). The cells were centrifuged at 600 *g* for 12 min, and the supernatant was collected and centrifuged at 36,000 *g* for 30 min. The membranes were resuspended in Tris·HCl (pH 7.4) and stored at −20°C until use. Membrane protein was determined as described previously (27).

Radioligand binding. The binding of [^{3}H]DPCPX to membranes prepared from the 28A cells was carried out in an assay volume of 250 μl Tris·HCl, pH 7.4, containing 0.01-0.4 nM [^{3}H]DPCPX in saturation experiments and 0.4 nM in the competition experiments. Binding of [^{3}H]DPCPX was measured under the same conditions as described earlier (20). The protein content was ~100-150 μg. The incubation lasted for 2 h at 12°C and was terminated by filtration through Whatman GF/B glass-fiber filters (25 mm), and the filter radioactivity was determined by liquid scintillation counting for 10 min. The nonspecific binding was defined by the presence of 100 μM *R*-N^6-phenylisopropyladenosine (*R*-PIA), and typically total binding was ~200 counts/min compared with 40 counts/min nonspecific binding.

Data analysis. Data were analyzed by nonlinear curve fitting using the program SCTFIT by DeLean et al. (12). Two affinity states were assumed when the corresponding fit was significantly better at the $P < 0.001$ level.

Materials. [^{3}H]DPCPX was purchased from Amersham Buchler, Braunschweig, FRG. *R*-PIA and *S*-N^6-phenylisopropyladenosine (*S*-PIA) were donated by Dr. K. H. Stegmeier, Boehringer Mannheim, Mannheim, FRG, and NECA was provided by Prof. G. Klemm, Byk Gulden Lomberg Chemishe Fabrik, Konstanz, FRG. 2-chloro-N^6[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) was synthesized and provided by Dr. Gloria Cristalli as described by Klotz et al. (unpublished observations). Guanosine 5'-triphosphate (GTP) and ADA (200 U/mg) were obtained from Boehringer Mannheim. Trypsin (1:250), DMEM, and fetal bovine serum were purchased from GIBCO Laboratories, Grand Island, NY. Fura-2/AM was purchased from Molecular Probes, Junction City, OR. Isoproterenol was purchased from Sigma. DPCPX was purchased from Research Biochemicals, Wayland, MA. Pertussis toxin was purchased from List Biological Industries, Campbell, CA. The phosphodiesterase inhibitor, Ro 20-1724, was purchased from BIOMOL Research Laboratories, Plymouth Meeting, PA. ^{125}I-labeled adenosine 3',5'-cyclic monophosphoric acid was from ICN Biomedicals, Irvine, CA. Goat anti-cAMP antiserum was from Research Products, Mt. Prospect, IL. Other chemicals of reagent grade or better were obtained from standard sources.

RESULTS

DPCPX functional studies. As demonstrated previously in RCCT cells and the 28A cell line (3, 4), adenosine analogues inhibit hormone-stimulated cAMP accumulation. Figure 1*A* shows inhibition of isoproterenol-stimulated cAMP by N^6-cyclohexyladenosine (CHA) and the effect of the A_1 antagonist, DPCPX, on this inhibition. DPCPX (100 nM) completely blocks the inhibitory effect of CHA except at 1 μM CHA. The effect of DPCPX on the adenosine analogue-induced increase in $[Ca^{2+}]_i$ is shown in Fig. 1*B*. CHA increases $[Ca^{2+}]_i$ with an ~50% effective concentration of 1 μM, as previously described for RCCT cells (2, 3), and 1 μM DPCPX completely inhibits this effect.

Pertussis toxin functional studies. In an attempt to characterize the G protein(s) involved in the inhibition of cAMP and the increase in $[Ca^{2+}]_i$, detailed concentration-effect experiments were performed with varying concentrations of pertussis toxin. It could be expected that if different G proteins were associated with a single A_1 receptor type, the concentration of pertussis toxin required to produce 50% inhibition (IC_{50}) would be different for each response. 28A cells were incubated for 18 h with concentrations of pertussis toxin ranging from 0.1 to 10 ng/ml (Table 1). After this treatment with pertussis toxin, the cells were used either for studying the ability of CHA to inhibit 1 μM isoproterenol-stimulated cAMP or to stimulate an increase in cytosolic Ca^{2+} (Table 1).

In the absence of pertussis toxin, 1.0 μM CHA reduced the isoproterenol-stimulated increase in cAMP to 19% of that demonstrated with isoproterenol alone (100% stimulation). Treatment with 0.1 ng pertussis toxin/ml media

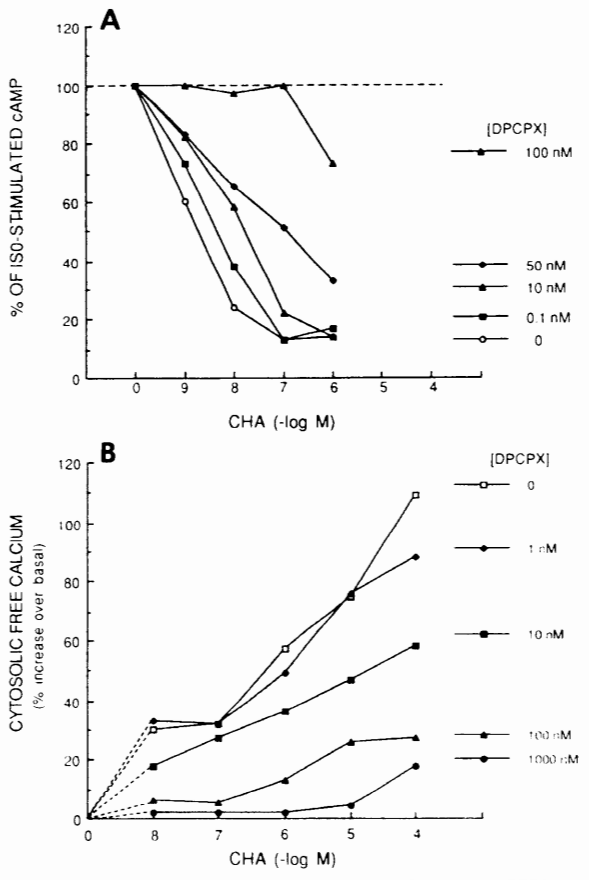

Fig. 1. *A*: effect of DPCPX on N^6-cyclohexyladenosine (CHA) inhibition of hormone-stimulated cAMP accumulation. Basal cAMP accumulation was 33.9 ± 4.7 pmol cAMP/mg protein, and isoproterenol (Iso)-stimulated cAMP accumulation was 136 ± 28 pmol cAMP/mg protein. Values are means of duplicate samples from 4 experiments. *B*: effect of DPCPX on CHA-induced increase in cytosolic free calcium. Control calcium concentration was 117 ± 7 nM. Values are means of 3 experiments.

Table 1. *Effect of pertussis toxin on inhibition of isoproterenol-stimulated cAMP or increase in cytosolic free calcium*

[CHA], −log M	[PTX], ng/ml			
	0	0.1	1.0	10
	Inhibition of cAMP, % of Iso-stimulated level			
9	91	88	95	101
8	47	43	78	101
7	21	23	61	73
6	19	21	62	75
	Cytosolic calcium, %increase over basal			
8	18	20	10	0
7	51	42	26	1
6	63	58	40	5
5	66	61	45	6

[CHA], N^6-cyclohexyladenosine concentration; [PTX], pertussis toxin concentration; Iso, isoproterenol. See also Fig. 2.

did not affect the inhibition of cAMP, whereas increasing the pertussis toxin concentration to 1 ng/ml resulted in a marked reduction of the 0.1 and 1.0 μM CHA inhibition, to 60% of isoproterenol stimulation without CHA. Treatment with 10 ng pertussis toxin/ml resulted in further interference with the ability of 0.1 and 1.0 μM CHA to inhibit cAMP, resulting in only 30% inhibition, and completely blocked the inhibition of cAMP produced by 0.01 μM and 1.0 nM CHA. Pertussis toxin treatment did not significantly alter the ability of isoproterenol alone to increase cAMP (270 ± 34 to 1,680 ± 241 vs. 295 ± 49 to 2,156 ± 406 pmol cAMP/mg protein for no pertussis toxin and 10 ng pertussis toxin, respectively).

Table 1 demonstrates the effect of varying concentrations of pertussis toxin on the CHA-induced increase in $[Ca^{2+}]_i$. As with the inhibition of cAMP, treatment with pertussis toxin at 0.1 ng/ml had no effect on the ability of CHA to increase Ca^{2+}. Pertussis toxin treatment at 1.0 ng/ml significantly reduced the increase in Ca^{2+} produced by 0.1–10 μM CHA, while 10 ng pertussis toxin/ml completely inhibited the increase in Ca^{2+} in response to 0.01 and 0.1 μM CHA and significantly reduced the increase by 1.0 and 10 μM CHA. Figure 2 directly compares the effects of pertussis toxin on the inhibition of cAMP and the increase in $[Ca^{2+}]_i$. The various concentrations of pertussis toxin are plotted against the response to maximally effective concentrations of CHA. Maximum inhibition of isoproterenol-stimulated cAMP occurred with 1 μM CHA, and the maximum increase in $[Ca^{2+}]_i$ occurred with 10 μM CHA. The effects of these concentrations on either inhibition of cAMP or increase in Ca^{2+} are presented as 100% of maximum. There is no apparent difference in the concentration of pertussis toxin required to inhibit these two effects of CHA.

28A cell membranes. Saturation binding of [^{3}H]-DPCPX in 28A membranes (Fig. 3), analyzed by nonlinear curve fitting, gave a one-site model with an apparent K_d value of 1.4 nM and a maximum number of binding sites (B_{max}) of 64 fmol/mg protein, indicating the presence of only one homogeneous population of binding

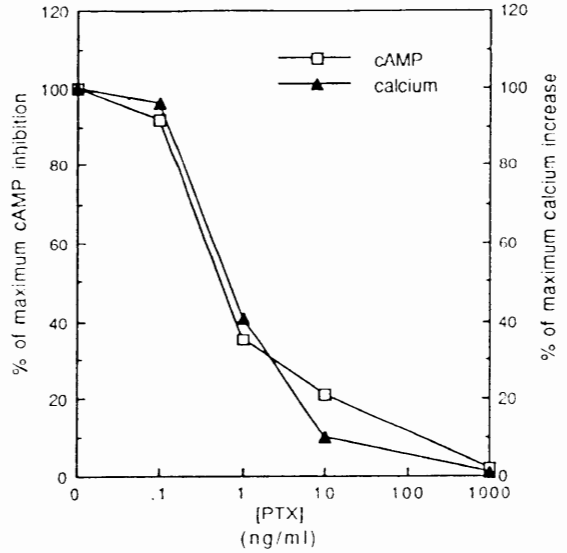

Fig. 2. Effect of pertussis toxin (PTX) on CHA-induced inhibition of cAMP or increase in cytosolic free calcium. Inhibition of cAMP by CHA is presented as percent of maximum inhibition of 1 μM isoproterenol-stimulated cAMP levels, with 100% representing inhibition produced by 1 μM CHA (0% represents full isoproterenol stimulation, i.e., full block of inhibition). Inhibition of increase in calcium is presented as percent of maximum calcium increase, with 100% representing increase in calcium in response to 10 μM CHA.

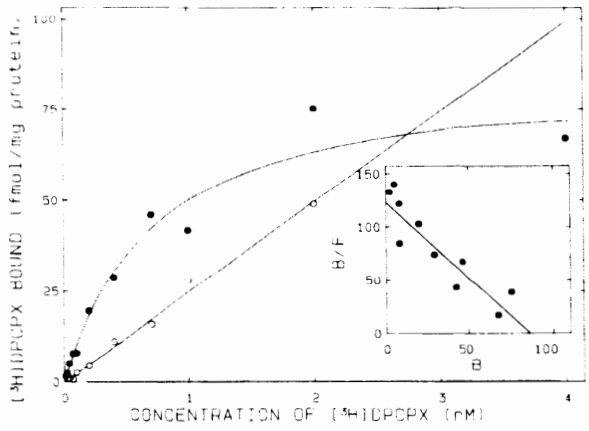

Fig. 3. Saturation of [^{3}H]DPCPX binding to RCCT-28A cell membranes. ●, specific binding; ○, nonspecific binding. Nonlinear curve fitting gave a K_d value of 1.4 nM and a maximal binding capacity value of 64 fmol/mg protein. *Inset*: Scatchard plot from data. Values are means of triplicate determinations, and figure is representative of 5 experiments utilizing different lots of cells. B, bound; F, free.

sites. The nonspecific binding was 20–30% of the total at the K_d, and saturation of specific binding was reached with 2 nM [^{3}H]DPCPX.

Competition of several agonists for the [^{3}H]DPCPX binding was measured to confirm that [^{3}H]DPCPX binds to the A_1 receptor. Competition of adenosine agonists for [^{3}H]DPCPX binding resulted in biphasic displacement curves (Fig. 4), indicating the presence of two affinity states for the agonists, with approximately half of the binding sites being in the high-affinity state and the other half in the low-affinity state. The K_i values for the various adenosine receptor agonists exhibit the typical pharmacological profile for A_1 receptors and the marked stereoselectivity for the PIA enantiomers.

Agonist binding was further characterized by measuring the competition of *R*-PIA for [^{3}H]DPCPX binding in the presence and absence of GTP (100 μM). In the absence of GTP, the competition of [^{3}H]DPCPX by *R*-PIA resulted in a biphasic displacement curve with an apparent K_d value of 0.5 nM and B_{max} value of 16.1 pmol/mg protein for the high-affinity state and a low-affinity K_d value of 10.5 nM and B_{max} value of 20.2 fmol/mg protein (Fig. 5).

When the competition experiment was carried out in the presence of 100 μM GTP (Fig. 5), a monophasic curve was obtained, indicating a single affinity state with a K_d value of 17.7 nM and a B_{max} value of 54.1 fmol/mg protein. Control binding (100%) increased from 36.3 to 54.1 fmol/mg protein with the addition of 100 μM GTP.

Rabbit renal medulla membranes. Saturation and competition binding curves were performed in membranes from rabbit renal medulla to determine whether the binding characteristics of the intact tissue and the cultured cells were similar. Saturation binding of [^{3}H]DPCPX in medullary membranes, analyzed by nonlinear curve fitting, gave a one-site model with an apparent K_d value of 2.4 nM (1.8–3.09, 95% confidence limits) and a B_{max} value of 17 ± 1.4 (SE) fmol/mg protein (data not shown), indicating the presence of only one homogeneous population of binding sites.

Competition for [^{3}H]DPCPX binding by adenosine agonists yielded biphasic displacement curves that were similar in binding parameters to those described for the 28A cell membranes (Fig. 6).

Rabbit brain membranes. To compare the adenosine receptor binding in renal medullary and 28A cell membranes with tissue in which adenosine binding is well characterized, we measured binding of [^{3}H]DPCPX and its competition for binding with various adenosine

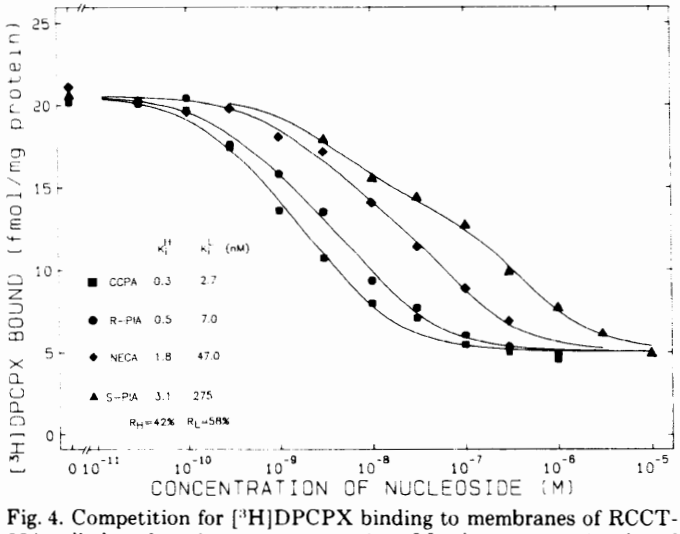

Fig. 4. Competition for [^{3}H]DPCPX binding to membranes of RCCT-28A cells by adenosine receptor agonists. Membranes were incubated with 0.4 nM [^{3}H]DPCPX in presence of increasing concentrations of CCPA, *R*-PIA, NECA, and *S*-PIA. Competition curves were simultaneously fitted with SCTFIT program. Data were best fitted assuming a 2-site model, and proportions of receptors in high- (R_H) and low- (R_L) affinity states were 42 and 58%, respectively. K_i^H and K_i^L affinity constants for high- and low-affinity states, respectively. Values are means of duplicate determinations, and figure is representative of 4 experiments utilizing different lots of cells.

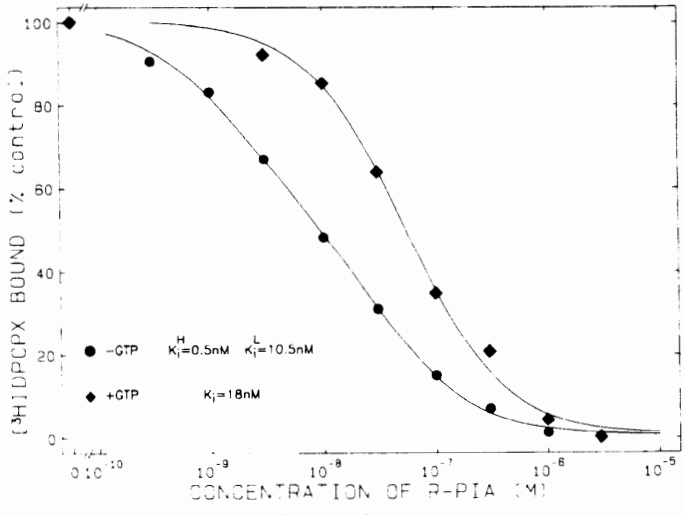

Fig. 5. Competition for [^{3}H]DPCPX binding to A_1 adenosine receptors of 28A cell membranes by *R*-PIA. Binding of [^{3}H]DPCPX was measured in absence and presence of 100 μM GTP. Data are given as percentage of total binding of [^{3}H]DPCPX in absence of *R*-PIA. Control binding (100%) amounted to 36.3 and 54.1 fmol/mg protein in absence and presence of GTP, respectively. In absence of GTP, curve was best fitted according to a 2-site model, and K_i values of 0.5 (high-affinity state) and 10.5 nM (low-affinity state) were calculated. In presence of GTP, only 1 affinity state with a K_i value of 17.7 nM was detected.

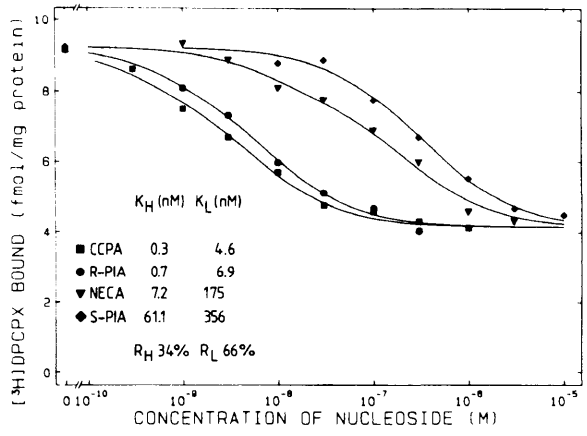

Fig. 6. Competition for [^{3}H]DPCPX binding to membranes of rabbit renal medullas by adenosine receptor agonists. Membranes were incubated with 0.4 nM [^{3}H]DPCPX in presence of increasing concentrations of CCPA, *R*-PIA, NECA, and *S*-PIA. Competition curves were simultaneously fitted with SCTFIT program. Data were best fitted assuming a 2-site model, and proportions of receptors in high- and low-affinity states were 34 and 66%, respectively. See legend to Fig. 4 for abbreviations. Values are means of duplicate determinations, and figure is representative of 5 experiments utilizing 4 different lots of membranes.

agonists in rabbit brain membranes (Fig. 7). Competition for [^{3}H]DPCPX binding by adenosine agonist ligands yielded biphasic displacement curves with similar K_d values to those observed in the binding studies of 28A cell and renal medullary membranes.

DISCUSSION

The demonstration of two signaling mechanisms (i.e., inhibition of adenylyl cyclase and stimulation of phospholipase C) associated with the activation of adenosine A_1 receptors in the cortical collecting tubule cell and the RCCT-28A cell line and the finding that both responses can be blocked by pertussis toxin treatment (2, 3, 4) and by the A_1 receptor antagonist DPCPX raise the question

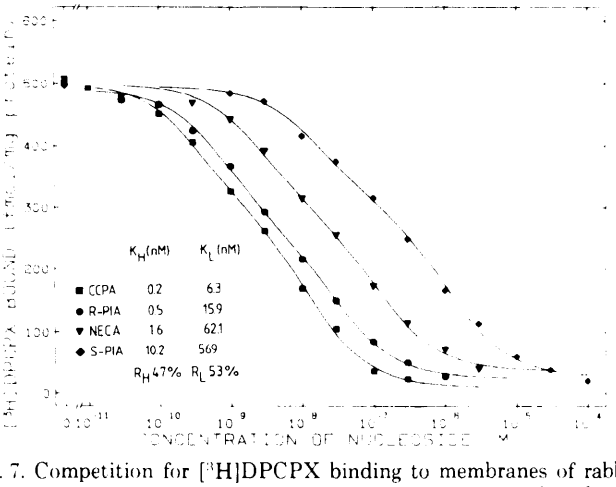

Fig. 7. Competition for [^{3}H]DPCPX binding to membranes of rabbit brain by adenosine receptor agonists. Membranes were incubated with 0.4 nM [^{3}H]DPCPX in presence of increasing concentrations of CCPA, *R*-PIA, NECA, and *S*-PIA. Competition curves were individually fitted with SCTFIT program. Data were best fitted assuming a 2-site model, and proportions of receptors in high- and low-affinity states were 47 and 53%, respectively. See legend to Fig. 4 for abbreviations. Values are means of duplicate determinations, and figure is representative of 3 experiments utilizing different lots of rabbit brain membranes.

of whether two subtypes of A_1 receptors exist. Several receptor types have been shown to couple to different signaling pathways, whereas other receptor populations contain many subtypes, each of which is monogamous in its coupling to signaling pathways.

Initially, studies of the α-adrenergic receptor demonstrated two receptor types: α_2-adrenergic receptors specifically coupled to the inhibition of adenylyl cyclase and α_1-adrenergic receptors coupled to the acceleration of phosphatidylinositol turnover; however, recently these receptors have been shown to consist of several subtypes, with each possibly interacting with more than one signaling pathway. It is becoming increasingly clear that a variety of cell surface receptors are coupled to more than one intracellular signaling mechanism. Angiotensin (7), prostaglandin E (29), and P_2 purinergic (31) receptors are examples of receptor types that appear to couple to both adenylyl cyclase and phosphoinositide metabolism without any conclusive evidence of different subclasses of receptors. The muscarinic cholinergic receptor also mediates several different biological responses (a decrease in cAMP by 2 independent mechanisms and increases in phosphoinositide hydrolysis, cyclic GMP accumulation, and K^+ permeability), but attempts to associate these with different receptor subtypes yielded equivocal results in several earlier studies (8, 15, 19, 33). There is also recent evidence that some muscarinic receptor subtypes couple to phosphoinositide turnover without affecting adenylyl cyclase (32). Thus a precedent exists for both models: separate but similar populations of receptors coupled to separate signaling mechanisms or one receptor population controlling divergent effector systems. The aim of the present study was to characterize the A_1 adenosine receptor population in RCCT-28A cells and in doing so attempt to determine the existence of two adenosine A_1 receptor subtypes.

DPCPX and pertussis toxin are both known to interfere with the ability of adenosine analogues to inhibit cAMP and to stimulate phosphoinositide turnover and subsequently increase Ca^{2+}. DPCPX is a highly specific A_1 adenosine receptor antagonist, while pertussis toxin ADP-ribosylates and inactivates the G_i protein associated with the A_1 receptor. DPCPX and pertussis toxin were used at several concentrations in the present study in an attempt to uncover a difference in IC_{50} of these agents to block the CHA-induced inhibition of cAMP vs. the stimulation of phosphoinositide turnover and increase in Ca^{2+}. A difference in the potency of DPCPX or pertussis toxin to inhibit these two phenomena would have provided evidence for different receptors or different G_i proteins, respectively. However, the potencies for DPCPX and pertussis toxin to inhibit the cAMP response compared with the respective IC_{50} values for the Ca^{2+} response were not appreciably different, therefore failing to provide support for the hypothesis that there are two different receptors or even two different G proteins associated with the same receptor. These results do not, however, preclude the possibility of receptors that are similar in their sensitivity to DPCPX or G proteins that are equally inactivated by pertussis toxin.

It is well established that adenosine A_1 receptors, like

other receptors that are coupled to G proteins, exist in two agonist affinity states. An aim of the present study was to determine whether structural differences in the A_1 receptor exist in addition to any conformational changes (high- and low-affinity states) induced by guanine nucleotides. To obtain an assessment of the A_1 receptors in collecting tubule cells, we utilized the antagonist radioligand [^{3}H]DPCPX, which binds to A_1 receptors with very high affinity and independently of the receptor interaction with guanine nucleotide regulatory proteins. The affinity of the radioligand in 28A cell membrane preparations and renal medullary membrane preparations was in good agreement with values obtained in rabbit brain membranes and with A_1 receptors described in other tissues (22). These binding data confirm the previously reported functional data that cells of the cortical collecting tubule have adenosine A_1 receptors. More importantly, saturation binding of [^{3}H]DPCPX to 28A cell membranes, analyzed by computer curve fitting, gave a one-site model.

Further characterization of the [^{3}H]DPCPX binding site on 28A cell membranes was accomplished by competition experiments with several agonists. Competition for [^{3}H]DPCPX binding with increasing concentrations of adenosine receptor agonists (Fig. 4) gave biphasic competition curves, with K_i values similar to those obtained in rabbit brain membranes in the present study (Fig. 7) and in previous studies (22). In the presence of GTP, the competition curve of [^{3}H]DPCPX binding with increasing concentrations of *R*-PIA was shifted from biphasic to monophasic (Fig. 3). This finding indicates that the A_1 receptor in the 28A cell membranes exists in both high- and low-affinity states for agonist binding in the absence of GTP and that addition of GTP converted all A_1 receptors into the low-affinity state. The rank order of potency for adenosine agonists and the effects of GTP on agonist binding observed in the present studies are entirely in keeping with what is known about the binding properties of the A_1 receptor in other tissues.

Comparison of the A_1 ligand binding characteristics of the 28A cell line membranes with membranes from the rabbit renal medulla and rabbit brain, a tissue in which adenosine A_1 receptors are well characterized, indicates that although the cell line and renal medulla have much lower receptor density than brain, adenosine agonists have similar affinity for A_1 receptors of renal origin as for brain A_1 receptors. Furthermore, the similar binding characteristics between the 28A cell line and the renal medulla and our previous report on the mobilization of $[Ca^{2+}]_i$ (2) and stimulation of inositol phosphate metabolism (3) indicate that the 28A cell line is a useful model in which to investigate the adenosine A_1 receptor and its signaling mechanisms.

The binding data in this study fail to provide support for the hypothesis that the inhibition of adenylyl cyclase and the stimulation of phospholipase C are coupled to two subpopulations of the A_1 receptor, although it is recognized that this conclusion may be a function of the inability of currently available ligands to differentiate between the A_1 receptor subtypes. We also recognize the possibility that a subpopulation of the A_1 receptor, if very small compared with the maximum number of binding sites for [^{3}H]DPCPX (i.e., <10%), might escape detection by saturation analysis. However, given the relatively low abundance of A_1 receptors presently found in these tissues (30–70 fmol/mg protein), it seems unlikely that a subpopulation with a receptor density of <10 pmol/mg protein could be responsible for the functional changes in Ca^{2+} mobilization and inositol phosphate formation that we have previously observed (2, 3). Failure to observe more than a single class of A_1 receptors by antagonist radioligand binding leads us to suggest that in the collecting tubule cell, adenosine mediates both the inhibition of adenylyl cyclase and activation of phospholipase C via a single population of A_1 receptors. An unequivocal test of the hypothesis that one receptor population mediates both responses would be the insertion of the cloned A_1 adenosine receptor into cells lacking this receptor and the demonstration that adenosine elicits both biological responses in these cells.

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK-39654.

Address for reprint requests: W. S. Spielman, 502C Biochemistry Bldg., Michigan State Univ., East Lansing, MI 48824.

Received 31 October 1991; accepted in final form 7 April 1992.

REFERENCES

1. **Arch, J. R. S., and E. A. Newsholme.** The control of the metabolism and the hormonal role of adenosine. *Essays Biochem.* 14: 82–123, 1978.
2. **Arend, L. J., M. A. Burnatowska-Hledin, and W. S. Spielman.** Adenosine receptor-mediated calcium mobilization in cortical collecting tubule cells. *Am. J. Physiol.* 255 (*Cell Physiol.* 24): C581–C588, 1988.
3. **Arend, L. J., J. S. Handler, J. S. Rhim, F. Gusovsky, and W. S. Spielman.** Adenosine-sensitive phosphoinositide turnover in a newly established cell line. *Am. J. Physiol.* 256 (*Renal Fluid Electrolyte Physiol.* 25): F1067–F1074, 1989.
4. **Arend, L. J., W. K. Sonnenberg, W. L. Smith, and W. S. Spielman.** A_1 and A_2 adenosine receptors in rabbit cortical collecting tubule cells: modulation of hormone-stimulated cAMP. *J. Clin. Invest.* 79: 710–714, 1987.
5. **Ashkenazi, A., J. W. Winslow, E. G. Peralta, G. L. Peterson, M. I. Schimerlik, D. J. Capon, and J. Ramachandran.** An M_1 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science Wash. DC* 238: 672–675, 1987.
6. **Birnbaumer, L., T. Nakahare, and P. C. Yang.** Studies on receptor-mediated activation of adenylyl cyclases. *J. Biol. Chem.* 259: 755–7566, 1974.
7. **Bouscarel, B., P. F. Blackmore, and J. H. Exton.** Characterization of the angiotensin II receptor in primary cultures of rat hepatocytes: evidence that a single population is coupled to two different responses. *J. Biol. Chem.* 263: 14913–14919, 1988.
8. **Brown, J. H., and S. B. Masters.** Muscarinic regulation of phosphatidylinositol turnover and cyclic nucleotide metabolism in the heart. *Federation Proc.* 43: 2613–2617, 1984.
9. **Burnatowska-Hledin, M. A., and W. S. Spielman.** Effects of adenosine on cAMP production and cytosolic Ca^{++} in cultured rabbit medullary thick limb cells. *Am. J. Physiol.* 260 (*Cell Physiol.* 29): C143–C150, 1991.
10. **Coulson, R., and S. J. Scheinman.** Evidence for renal tubular adenosine receptors (Abstract). *Kidney Int.* 27: 255, 1985.
11. **Daly, J. W.** Adenosine receptors: targets for future drugs. *J. Med. Chem.* 25: 197–207, 1982.
12. **DeLean, A., A. A. Hancock, and R. J. Lefkowitz.** Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* 21: 5–16, 1982.

13. **Fain, J. N., and C. C. Malbon.** Regulation of adenylate cyclase by adenosine. *Mol. Cell. Biochem.* 25: 143-169, 1979.
14. **Feigl, E. D.** Coronary physiology. *Physiol. Rev.* 63: 1-205, 1983.
15. **Fleming, B. P., W. Giles, and J. Lederer.** Are acetylcholine-induced increases in ^{42}K efflux mediated by intracellular cyclic GMP in turtle cardiac pacemaker tissue? *J. Physiol. Lond.* 314: 47-64, 1981.
16. **Fox, I. H., and W. N. Kelley.** The role of adenosine and 2-deoxyadenosine in mammalian cells. *Annu. Rev. Biochem.* 47: 655-686, 1978.
17. **Frandsen, E. K., and G. Krishna.** A simple ultrasensitive method for the assay of cyclic AMP and cyclic GMP in tissues. *Life Sci.* 18: 529-541, 1976.
18. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440-3450, 1985.
19. **Harden, T. K., L. I. Tanner, M. W. Martin, N. Nakahata, A. R. Hughes, J. R. Hepler, T. Evans, S. B. Masters, and J. H. Brown.** Characteristics of two biochemical responses and stimulation of muscarinic cholinergic receptors. *Trends Pharmacol. Sci. Suppl.* II: 14-18, 1986.
20. **Klotz, K.-N., M. J. Lohse, U. Schwabe, G. Cristalli, S. Vittori, and M. Grifantini.** 2-Chloro-N^6-[^{3}H]cyclopentyladenosine—a high affinity agonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 340: 679-683, 1989.
21. **Libert, F., S. N. Schiffman, A. LeFort, M. Parmentier, C. Gerard, J. E. Dumont, J-J. Vanderhaeghen, and G. Vassart.** The orphan receptor cDNA RDC7 encodes an A_1 adenosine receptor. *EMBO J.* 10: 1677-1682, 1991.
22. **Lohse, M. J., K.-N. Klotz, J. Lindenborn-Fotinos, M. Reddington, U. Schwabe, and R. A. Olsson.** 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)-a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 336: 204-210, 1987.
23. **Lohse, M. J., V. Lenschow, and U. Schwabe.** Interaction of barbiturates with adenosine receptors in rat brain. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 326: 69-74, 1984.
24. **Lohse, M., D. Ukena, and U. Schwabe.** Demonstration of R_i-type adenosine receptors in bovine myocardium by radioligand binding. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 328: 310-316, 1985.
25. **Londos, C., D. M. F. Cooper, and J. Wolff.** Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* 77: 2551-2554, 1980.
26. **Maenhaut, C., J. VanSande, F. Libert, M. Abramowicz, M. Parmentier, J-J. Vanderhaegen, J. E. Dumont, G. Vassart, and S. Schiffman.** RDC8 codes for an adenosine A_2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* 173: 1169-1178, 1990.
27. **Markwell, M. A. K, S. M. Haas, L. L. Bieber, and N. E. Tolbert.** A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87: 206-210, 1978.
28. **McKenzie, S. G., and H. P. Bar.** On the mechanism of adenyl cyclase inhibition by adenosine. *Can. J. Physiol. Pharmacol.* 51: 190-196, 1973.
29. **Negishi, M., S. Ito, and O. Hataishi.** Prostaglandin E receptors in bovine adrenal medulla are coupled to adenylate cyclase via G_i and to phosphoinositide metabolism in a pertussis toxin-insensitive manner. *J. Biol. Chem.* 264: 3916-3923, 1989.
30. **Newman, M. E., and A. Levitzki.** Desensitization of normal rat kidney cells to adenosine. *Biochem. Pharmacol.* 32: 137-140, 1983.
31. **Okajima, F., Y. Tokumitsu, Y. Kondo, and M. Ui.** P_2-purinergic receptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of inositol trisphosphate in rat hepatocytes. *J. Biol. Chem.* 262: 13483 13490, 1987.
32. **Peralta, E. G., A. Ashkenazi, J. W. Winslow, J. Ramachandran, and D. J. Capon.** Differential regulation of PI hydrolysis and adenylate cyclase by muscarinic receptor subtypes. *Nature Lond.* 34: 434-437, 1988.
33. **Quist, E. E.** Evidence for a carbachol-stimulated phosphatidylinositol effect in heart. *Biochem. Pharmacol.* 31: 3130-3133, 1982.
34. **Woodcock, E. A., R. Loxley, E. Leung, and C. I. Johnston.** Demonstration of R_a-adenosine receptors in rat renal papillae. *Biochem. Biophys. Res. Commun.* 121: 434-440, 1984.
35. **Zaremba, T. G., and P. H. Fishman.** Desensitization of catecholamine-stimulated adenylate cyclase and down-regulation of beta-adrenergic receptors in rat glioma C6 cells. Role of cyclic AMP and protein synthesis. *Mol. Pharmacol.* 26: 206-213, 1984.