0026-895X/90/050667-07$03.00/0

MOLECULAR PHARMACOLOGY, 38:660-666

Characterization of Adenosine A_1 Receptors in Intact DDT_1 MF-2 Smooth Muscle Cells

P. GERWINS, C. NORDSTEDT,[1] and B. B. FREDHOLM

Department of Pharmacology, Karolinska Institutet, S-104 01 Stockholm, Sweden

Received May 21, 1990; Accepted August 6, 1990

SUMMARY

Adenosine receptors in the smooth muscle cell line DDT_1 MF-2 were studied by radioligand binding using the A_1 receptor-selective antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) as the ligand. Binding characteristics were similar in intact cells and in membranes (K_D value of approximately 1 nM). The maximum binding amounted to 183 fmol/10^6 intact cells or 344 fmol/mg of membranes. To characterize the receptor, competition experiments were performed by inhibiting [^{3}H]DPCPX binding with several adenosine agonists and antagonists. Adenosine receptor antagonists appeared to bind to a single class of binding site, both in membranes and intact cells. The order of potency was DPCPX = CGS 15943A > 8-cyclopentyl-1,3-dimethylxanthine > 8-(*p*-sulfophenyl)-theophylline > 3-isobutyl-1-methylxanthine > theophylline. Competition curves with adenosine agonists in membranes were best described by a two-site rather than a one-site model. At equilibrium in intact cells, only a single site was detected at both 4° and 25°. However, short term incubations (1–4 min) at 25° showed biphasic binding curves in intact cells. The equilibrium K_D values for intact cells were similar to the low affinity K_D values in membranes (K_L). The order of potency was N^6-cyclopentyladenosine ≥ (−)-(*R*)-N^6-phenylisopropyladenosine[(*R*)-PIA] ≥ N^6-cyclohexyl adenosine > 5′-*N*-ethylcarboxamidoadenosine (NECA) > 2-chloroadenosine > adenosine (intact cells only) > 2-phenylaminoadenosine (CV 1808). Treatment of cells with pertussis toxin ADP-ribosylated GTP-binding proteins and eliminated the high affinity agonist binding in membranes but did not affect binding to intact cells. The addition of GTP (100 μM) also shifted the competition curves from bi- to monophasic curves in membranes. Adenosine receptor agonists inhibited the formation of cAMP induced by isoprenaline (IC_{50} for (*R*)-PIA, 0.4 nM). This inhibition could be prevented with adenosine receptor antagonists. Pretreatment with pertussis toxin also reversed these effects and actually revealed functional A_2 receptors, as shown by the formation of cAMP induced by NECA. In conclusion, the equilibrium binding of A_1 receptor agonists to intact smooth muscle cells is similar to the low affinity binding observed in membranes. In addition, it is suggested that agonists may transiently convert the A_1 receptor from a "resting" low affinity state to a high affinity state coupled to a GTP-binding protein. DDT_1 MF-2 cells should prove useful for studying regulation of A_1 receptor signalling in intact cells.

Adenosine acts on two subtypes of membrane-bound receptors (A_1 and A_2) that are subdivided on the basis of the relative order of potencies of agonists and antagonists (1). Adenosine acting on A_1 receptors can induce a wide range of physiological effects, including bradycardia, inhibition of transmitter release, sedation, and inhibition of lipolysis (2). At least some of these effects are believed to be mediated by the inhibition of adenylate cyclase but there are also other effector systems such as K^+ channels (3).

Adenosine A_1 receptors have recently been purified (4–6) and have an apparent molecular weight of approximately 32,000–38,000, depending on glycosylation state. The adenosine A_1 receptor belongs to the class of G protein-coupled receptors. When it is purified by using agonist affinity chromatography it co-elutes with a G protein (6), but when it is purified by using antagonist affinity chromatography no G protein is co-purified (4, 5). With photoaffinity probes, distinct agonist and antagonist conformations have also been identified (7). Further biochemical evidence indicating that the A_1 receptor is firmly associated with G proteins includes the demonstration that GTP and stable GTP analogues decrease the apparent affinity of binding of agonists to membranes (8–11), solubilized receptors (12–15), and sections of brain tissue (16, 17). In addition,

These experiments were supported by grants from the Swedish Medical Research Council (Project 2553), Hedlunds Foundation, Karolinska Institutet, and AB Astra.

[1] Present address: Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Ave., New York, NY 10021-6399.

ABBREVIATIONS: G protein, GTP-binding protein; ADA, adenosine deaminase; BSA, bovine serum albumin; 2-CADO, 2-chloroadenosine; CHA, N^6-cyclohexyladenosine; CPA, N^6-cyclopentyladenosine; 8-CPT, 8-cyclopentyl-1,3-dimethylxanthine; CV 1808, 2-phenylaminoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; G_s, stimulatory GTP-binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate; IBMX, 3-isobutyl-1-metylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; 8-PST, 8-(*p*-sulfophenyl)-theophylline; PTX, pertussis toxin; (*R*)-PIA, (−)-(*R*)-N^6-phenylisopropyladenosine; CGS 15943A, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5-c]quinazolin-5-imine monomethanesulfonate; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

treatment with *N*-ethylmaleimde at concentrations that block several G proteins inhibits the action of adenosine receptor agonists without affecting the binding of antagonists (18). Furthermore, PTX, which ADP-ribosylates and inactivates several G proteins (19), blocks the ability of the A_1 receptor to inhibit adenylate cyclase (20).

In membrane preparations the A_1 receptor, like several other G protein-coupled receptors, has one high and one low affinity state (20, 21). Addition of GTP or pretreatment with PTX shifts all the receptors to a low affinity state (20, 21). However, in intact cells there may be sufficient amounts of guanine nucleotides to rapidly shift the binding to the low affinity state, so that the high affinity form can no longer be detected under equilibrium conditions. It is, therefore, of interest to examine the characteristics of adenosine receptors in membranes and in intact cells. Another reason to examine binding to intact cells is that this offers an opportunity to examine the potency of adenosine, itself a ligand at these receptors. Such studies are very difficult with membrane preparations (22, 23).

In most studies so far the A_1 receptors have been characterized in intact tissues from animals and humans. In order to prepare intact cells from such tissues, proteolytic enzymes are often used (21, 24), which may affect the receptors. Thus, in the two studies so far performed comparing A_1 receptors in intact cells and membranes from fat cells (24) and rat myocytes (21), limited collagenase digestion was used. We wanted to compare these findings using a cell line in culture, which also offers the advantages of greater cellular homogeneity and reproducibility. It is known that the vas deferens from several types of rodents expresses adenosine A_1 receptors (25, 26). Therefore, we chose a steroid-induced leiomyosarcoma from hamster vas deferens (27) in order to characterize a putative adenosine A_1 receptor. In a very recent publication it was shown that this cell line expresses both A_1 and A_2 receptors (28).

The aim of the present study was to characterize A_1 receptors in this cell line, with both receptor binding studies and functional studies on adenylate cyclase. We have investigated the affinity states of the receptors in membranes, as compared with intact cells, and also examined the effects of GTP and PTX.

Experimental Procedures

Materials. Cell culture media, fetal calf serum, and cell culture flasks were from NordVacc (Sweden). [^{3}H]DPCPX (80–120 Ci/mmol) and [^{32}P]NAD (30 Ci/mmol) were from New England Nuclear). DPCPX, 8-PST, 8-CPT, 2-CADO, and CPA were purchased from Research Biochemicals Incorporated. CGS 15943 A was a gift from Ciba Geigy. Theophylline, NECA, GTP, ATP, and BSA were all from Sigma. IBMX was from EGA-Chemie (FRG), adenosine from Aldrich-Europe (Belgium), and CV-1808 from Takeda Chemical Industries, Ltd. (Japan). CHA, (*R*)-PIA, ADA, and dithiothreitol were purchased from Boehringer Mannheim (FRG). PTX was from List Campbell. All other reagents and chemical were of analytical grade.

Cell culture. DDT_1 MF-2 smooth muscle cells, originally isolated from a steroid-induced leiomyosarcoma of Syrian hamster vas deferens (27), were obtained from the American Type Culture Collection. Cells were grown in suspension, maintained in DMEM with 4.5 g of glucose/liter that contained 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, at 37° in 5% CO_2/95% air. Cells were subcultured three times weekly at a ratio of 1:10 and experiments were initiated at a cell density of 1–2 × 10^5 cells/ml. Viability was more than 90%, as assessed by the exclusion of trypan blue.

Binding of [^{3}H]DPCPX to intact cells. Cells were harvested by centrifugation for 5 min at 800 × *g* and washed twice in PBS. The pellet was then resuspended in serum-free DMEM that was buffered with 20 mM HEPES, pH 7.4, and supplemented with 0.1% BSA, at a density of 1.5 × 10^6 cells/ml, and placed on ice. Aliquots (0.1 ml) containing indicated drugs, in a final volume of 0.3 ml, were added to wells in 96-well microtiter plates. All drugs were dissolved in the above-described medium. Nonspecific binding was defined as that occurring in the presence of 40 μM CHA. Except for short term incubations and association/dissociation experiments, the incubations lasted for 4 hr at 4°. Assays were terminated by rapid filtration over glass fiber filters for receptor binding (Skatron AS, Norway). Short term incubations were performed as described above, with the exception that they were carried out at 25°. Filtration was performed with a Skatron 1719 cell harvester (Skatron AS) and filters were further washed with 5 ml of ice-cold PBS. Filters were then transferred to scintillation vials (Skatron AS) and, after the addition of 3 ml of scintillation fluid (OptiPhase HiSafe II; LKB, Sweden), counted in a β-counter (1209 Rackbeta; LKB).

Binding of [^{3}H]DPCPX to membranes. After cells were washed, as described for intact cells, the pellet was resuspended in 1 ml of ice-cold homogenization buffer (50 mM Tris, pH 7.4, 7.5 mM $MgCl_2$, 5 mM EDTA). Cells were disrupted by sonication (MSE 100-W ultrasonic disintegrator, MSE London) at maximum setting, 4 times for 10 sec at 1-min intervals, at 4°. Unbroken cells and nuclei were sedimented at 1000 × *g* for 10 min and discarded. Plasma membranes and the cytosolic fraction were then separated by centrifugation at 30,000 × *g* for 60 min. The membrane pellet was resuspended in assay buffer (50 mM Tris, pH 7.4, 2 mM $MgCl_2$) at approximately 1 mg of protein/ml and preincubated with 5 IU/ml ADA for 60 min at 25° to remove endogenous adenosine. Aliquots (100 μl = 40–50 μg of protein) containing 3 IU/ml ADA, with indicated drugs in a final volume of 0.3 ml, were then added to wells in a 96-well microtiter plate. Except for association and dissociation experiments, membranes were incubated for 120 min at 25°. Nonspecific binding was defined as that occurring in the presence of 40 μM CHA. The binding was terminated by filtration over glass fiber filters as described for intact cells, with the exception that filters were further washed with assay buffer instead of PBS. Protein determinations were performed with the Bio-Rad protein assay, using BSA as a standard.

cAMP assay. After two washes with PBS, cells were resuspended in DMEM supplemented with 20 mM HEPES, pH 7.4, and 0.1% BSA. Aliquots (0.45 ml = 0.1–0.2 × 10^6 cells) were transferred to test tubes and indicated drugs were added. Reactions were terminated after a 10-min incubation at 37° by the addition of perchloric acid to a final concentration of 0.1 M. Samples were neutralized with KOH and the cAMP content in the supernatants was determined with a protein-binding assay as described (29), with the exception that bound cAMP was separated from free cAMP by rapid filtration over glass fiber filters (Skatron AS) rather than by charcoal precipitation.

ADP-ribosylation with PTX. Preparation of membranes from control cells and cells treated with PTX (200 ng/ml, 4 hr) was performed as described above for binding of [^{3}H]DPCPX to membranes. The final membrane pellet was resuspended in 50 mM Tris·HCl, pH 7.4, to a protein concentration of 12 mg/ml. PTX was preactivated for 60 min at room temperature with 50 mM dithiothreitol. The incubation mixture (100 μl) contained 20 μg/ml PTX, 300 μg/ml membrane protein, 50 mM Tris·HCl, pH 7.4, 1 mM ATP, 10 mM thymidine, 0.5 mM GTP, and 1.1 μM [^{32}P]NAD. The reaction (45 min at 30°) was stopped by the addition of 1 ml of ice-cold 50 mM Tris·HCl, pH 7.4, and samples were pelleted by centrifugation for 10 min at 10,000 × *g*. Pellets were resuspended in 30 μl of Laemmli buffer and the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The dried gel was exposed for 2 days to Fuji RX film.

Data analysis. Data from receptor binding studies were analyzed with the commercial version of the LIGAND program (30) adapted for the IBM PC. Dose-response curves were generated by using the

GraphPad (ISI Software) program. Data are given as mean ± standard deviation and each experiment was performed in triplicate.

Results

Binding of [^{3}H]DPCPX to intact cells. Saturation analysis of [^{3}H]DPCPX binding to intact DDT$_1$ MF-2 cells showed a saturable specific binding, whereas nonspecific binding increased linearly with increasing concentrations of [^{3}H]DPCPX (Fig. 1A). Nonspecific binding, determined in the presence of 40 μM CHA, was less than 10% at the K_D concentration (1 nM). The K_D was calculated to be 0.93 ± 0.30 nM (four experiments) with a maximal binding (B_{max}) of 183 ± 53 fmol/10^6 cells, corresponding to 110,000 sites/cell. The binding data could be adequately fitted to a one-site model. The corresponding Scatchard plot was linear (Fig. 1B), indicating that [^{3}H]DPCPX bound to a single class of sites. Specific binding at 4° of 1 nM [^{3}H] DPCPX increased with time and approached equilibrium at about 4 hr, but there was a progressive increase in binding even after that time point (Fig. 2). Because this second slow increase in binding could be due to slow dissociation of endogenous adenosine, we also carried out experiments in the presence of a high concentration of ADA (2 units/ml). However, this did not alter the time course of binding and did not affect the K_D or the B_{max} (data not shown). Nonspecific binding was maximal after 10 min.

It was not possible to displace more than 5% of bound [^{3}H] DPCPX with an excess of unlabeled CPA (100 μM) or DPCPX (1 μM) when the experiment was carried out at 4° for 3 hr (data not shown). If the incubation with CPA or DPCPX was prolonged to 6 hr, the amount displaced was increased to 15%. The slow rate of association and dissociation means that there are large errors in the calculation of a kinetic K_D but it is between 0.2 and 0.6 nM. However, at 25° all bound [^{3}H]DPCPX could be displaced, with a half-time of 26 min (Fig. 3A). It was also possible to displace all [^{3}H]DPCPX bound at 4° with CPA or DPCPX after warming samples to 25° (data not shown). This proves that [^{3}H]DPCPX bound to cell surface receptors and that specific binding was not due to internalization of the radioligand. At 25° the specific binding reached equilibrium at 60 min (Fig. 3A) and the nonspecific binding was maximal after 10 min. Half-maximal binding occurred after 7 min. Calculations of the kinetic data at 25° gave an observed association rate constant (k_{obs}) of 0.099 ± 0.005 min^{-1} (three experiments), with a corresponding association rate constant (k_{+1}) of 7.6 × 10^7 M^{-1} min^{-1}. Dissociation occurred with a half-time of 25 min, giving a dissociation rate constant (k_{-1}) of 0.023 ± 0.001 min^{-1} (three experiments). Using the ratio k_{-1}/k_{+1}, the kinetic K_D was calculated to be 0.30 nM.

Binding of [^{3}H]DPCPX to membranes of DDT$_1$ MF-2 cells. We next compared the binding of [^{3}H]DPCPX to membranes with binding in intact cells. The specific binding was saturable (Fig. 1C), with a K_D of 0.56 ± 0.22 nM (three experiments) and a B_{max} of 344 ± 144 fmol/mg of protein. This value is lower than that reported by Ramkumar and co-workers (28), possibly indicating a less pure plasma membrane preparation than they used. The Scatchard plot was linear (Fig. 1D). Nonspecific binding was <10%. Kinetic studies showed that the binding of [^{3}H]DPCPX to membranes at 25° reached equilibrium after about 60 min, with half-maximal binding at 4 min (Fig. 3B). The k_{obs} was 0.15 ± 0.005 min^{-1} (three experiments), which gives an association rate constant (k_{+1}) of 1.13 × 10^8 M^{-1} min^{-1}. Dissociation of bound [^{3}H]DPCPX (Fig. 3B) was

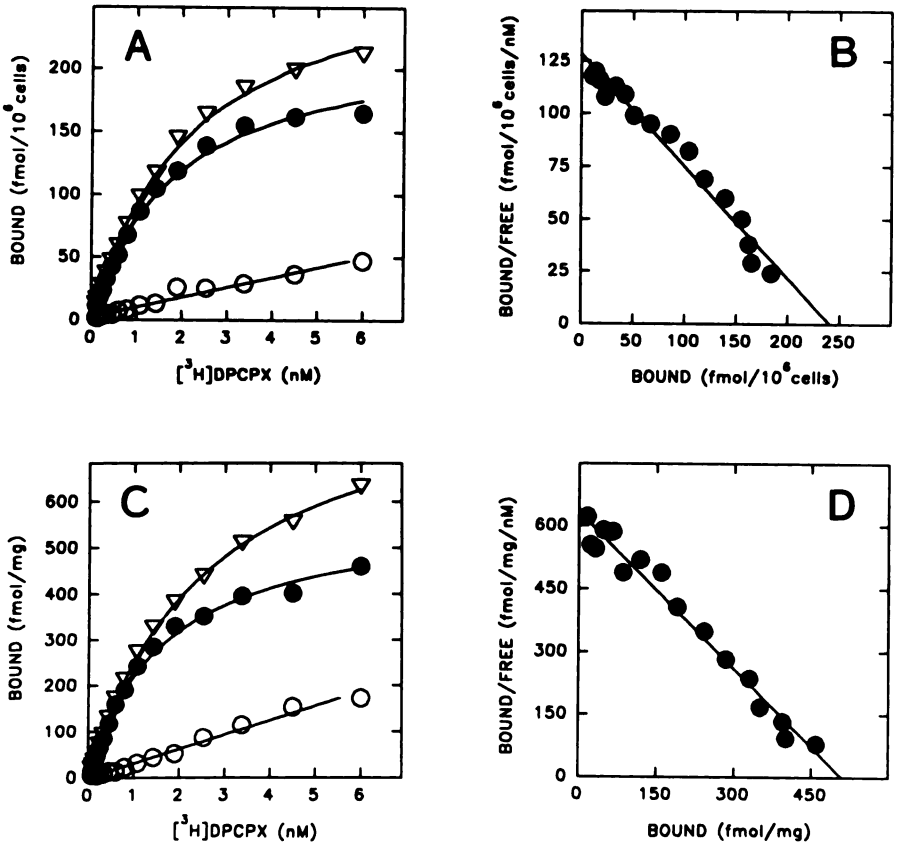

Fig. 1. Saturation (A and C) and Scatchard (B and D) analysis of [^{3}H]DPCPX binding to intact DDT$_1$ MF-2 cells (A and B) and membranes prepared from the same cell line (C and D). The experiments with intact cells were carried out at 4° for 4 hr and the experiments with membranes were carried out at 25° for 2 hr, as described in Experimental Procedures. The presence and absence of 40 μM CHA was used to determine nonspecific (○) and total (▽) binding, respectively; specific binding (●) was the difference between total and nonspecific binding. The average K_D was 0.93 ± 0.30 nM (four experiments) for intact cells and 0.56 ± 0.22 nM (three experiments) for membranes. Corresponding B_{max} values were 183 ± 53 fmol/10^6 cells and 344 ± 144 fmol/mg of protein, respectively. Results shown are from one experiment performed in triplicate.

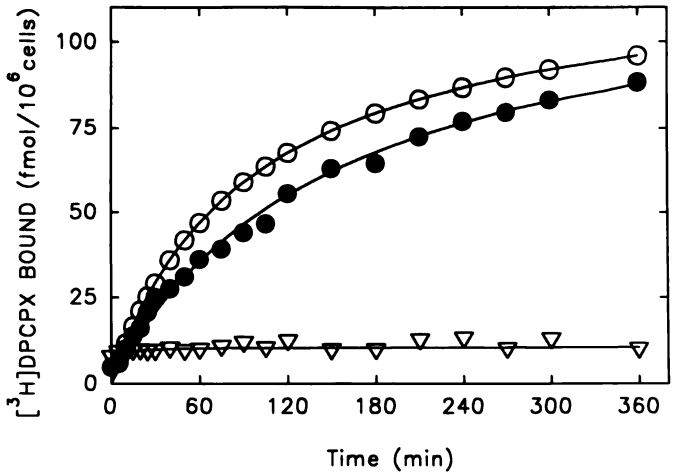

Fig. 2. Association of [^{3}H]DPCPX (1 nM) to intact DDT$_1$ MF-2 cells at 4°. For further details, see Experimental Procedures. ●, Specific; ○, total; and ▽, nonspecific (40 μM CHA) binding. Results are from one typical experiment of three, each carried out in triplicate.

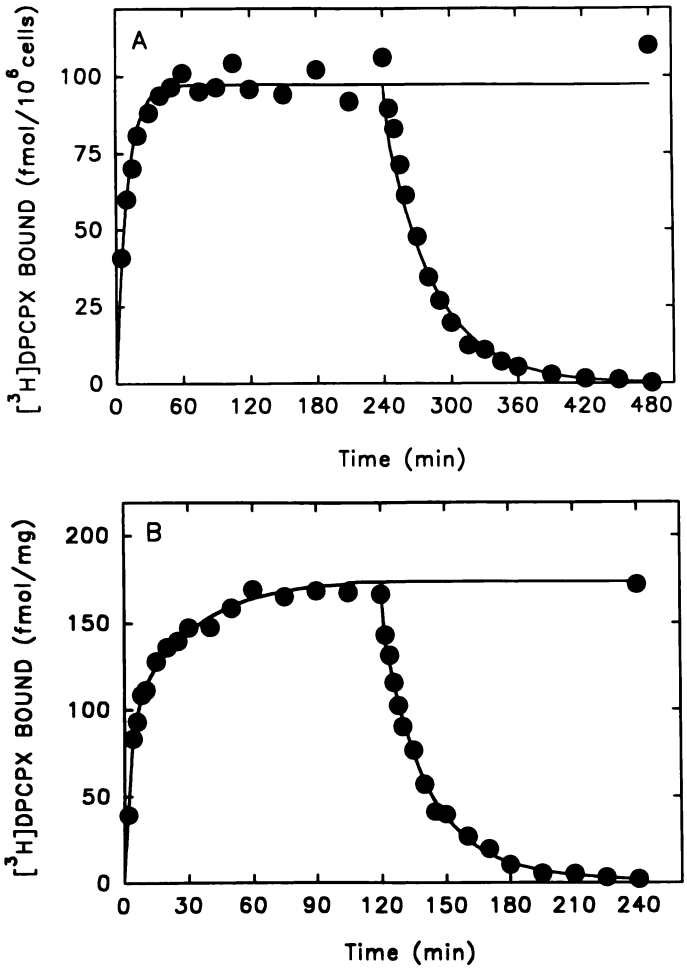

Fig. 3. Association and dissociation of [^{3}H]DPCPX (1 nM) in intact DDT$_1$ MF-2 cells (A) and membrane preparations (B) from the same cell line. The experiments were carried out at 25°. For details, see Experimental Procedures. Dissociation was initiated by addition of 100 μM CPA to cells that previously had been incubated with 1 nM [^{3}H]DPCPX for 4 hr and to membranes that had been incubated for 2 hr. Data are means of triplicate determinations from one of three experiments.

achieved by the addition of 10 μM CPA to membranes that have previously been incubated with 1 nM [^{3}H]DPCPX for 2 hr at 25°. Dissociation occurred with a half-time of 11 min, which corresponds to a dissociation rate constant (k_{-1}) of 0.038 ± 0.01 min^{-1} (three experiments). From the rates of association and dissociation, the equilibrium constant $K_D = k_{-1}/k_{+1}$ was calculated to be 0.34 nM.

Competition studies with adenosine agonists and antagonists. To confirm that the adenosine receptors present on the DDT$_1$ MF-2 cells were of the A_1 subtype, we evaluated the rank order of potency for a series of adenosine receptor agonists and antagonists. In both intact cells and membranes, the order of potency for agonists was CPA ≥ (*R*)-PIA ≥ CHA > NECA > 2-CADO > adenosine (intact cells only) > CV 1808 (Table 1A), and for antagonists DPCPX = CGS 15943A > 8-CPT > 8-PST > IBMX > theophylline (Table 1B). Adenosine receptor antagonists appeared to bind to a single class of sites both in membranes and in intact cells. At equilibrium in intact cells adenosine agonists also bound to a single class of sites, but in membrane preparations the data fitted significantly better to a two-site model than to a one-site model (LIGAND, $p < 0.01$) (Fig. 4). The low affinity site in membranes had a K_D very similar to that found in intact cells (Table 1A).

Nonequilibrium agonist binding to intact cells. In an attempt to resolve the apparent discrepancy between the high potency of adenosine in producing a biological effect and the low affinity of the binding to intact cells, further experiments were performed. Competition binding studies of (*R*)-PIA and [^{3}H]DPCPX with intact cells, performed at equilibrium at 4°

TABLE 1

Competition of adenosine agonists and antagonists with [^{3}H]DPCPX binding

Competition of adenosine agonists and antagonists with [^{3}H]DPCPX (1 nM) for binding to intact DDT$_1$ MF-2 cells (4°) and membranes (25°) prepared from this cell line is shown. Data were analyzed with a computer program (LIGAND). Results are mean ± standard deviation of two or three separate experiments, each performed in triplicate. A, Competition data for agonists. In membrane preparations there was a significantly better fit using a two-site fit, compared with a one-site fit ($p < 0.01$). In intact cells only a low affinity site could be detected. K_D, dissociation constant; K_H, K_D for high affinity state in membranes; K_L, K_D for low affinity state in membranes. B, Competition data for antagonists. In intact cells and in membrane preparations a single-site model best fit the data.

A

Agonist	Membranes		Intact cells, K_D
	K_H	K_L	
		nM	
CPA	0.45 ± 0.05	41 ± 5	58 ± 6
(*R*)-PIA	1.74 ± 0.08	93 ± 23	77 ± 26
CHA	0.76 ± 0.33	147 ± 32	144 ± 67
NECA	1.95 ± 0.78	362 ± 167	445 ± 199
2-CADO	2.3 ± 1.0	644 ± 157	801 ± 8
Adenosine			1,630 ± 150
CV 1808	76 ± 8	16,200 ± 6,720	11,200 ± 7,300
(*R*)-PIA + 100 μM GTP		127 ± 5	107 ± 13
(*R*)-PIA + PTX (200 ng/ml, 4 hr)		195 ± 19	129 ± 39

B

Antagonist	Membranes, K_D	Intact cells, K_D
	nM	
DPCPX	0.46 ± 0.16	0.95 ± 0.11
CGS 15943A	1.12 ± 0.39	0.72 ± 0.06
8-CPT	10 ± 0.66	4.9 ± 1.1
8-PST	690 ± 17	144 ± 30
IBMX	2,470 ± 1,390	1,390 ± 160
Theophylline	7,280 ± 160	3,730 ± 280

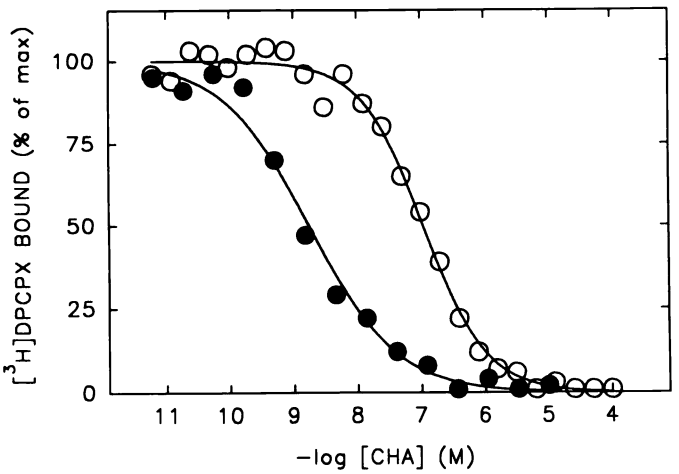

Fig. 4. Competition by increasing concentrations of the agonist CHA for [^{3}H]DPCPX (1 nM) binding to intact cells at 4° (○) or to membrane preparations of DDT$_1$ MF-2 cells at 25° (●). Data presented are from one of two experiments, each performed in triplicate.

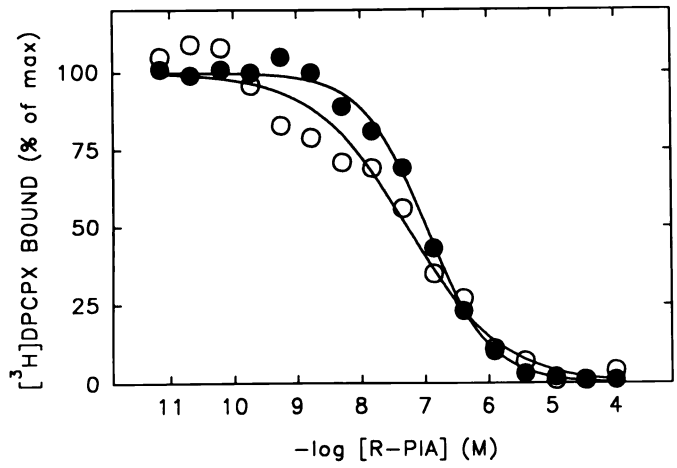

Fig. 5. Competition by (*R*)-PIA of [^{3}H]DPCPX (1 nM) binding to intact cells at 25° during nonequilibrium conditions. Increasing concentrations of (*R*)-PIA were added together with 1 nM [^{3}H]DPCPX to intact cells for 2 min (○) and 8 min (●). Data are from one experiment of four, each performed in triplicate.

(Table 1A) or 25° (data not shown), showed that all the receptors were in the low affinity state. Addition of ADA (2 units/ml) to remove possible endogenous agonist did not change these results. In order to examine whether a high affinity state could be detected under nonequilibrium binding conditions, short term incubations (2–4 min) at 25° were studied. As seen in Fig. 5, these experiments gave biphasic competition curves with both a high and a low affinity form of the receptor (LIGAND, $p < 0.05$). Calculations of four independent experiments gave a high affinity K_D of 13 ± 9 nM and a low affinity K_D of 305 ± 180 nM.

Effects of adenosine receptor agonists on cAMP accumulation. Addition of adenosine agonists inhibited the accumulation of cAMP induced by isoprenaline. (*R*)-PIA (100 nM) inhibited 76% of the cAMP accumulation induced by 10 μM isoprenaline, with an IC_{50} of 0.4 nM (Fig. 6). Addition of 10 nM DPCPX prevented (*R*)-PIA from inhibiting the isoprenaline-induced cAMP accumulation (data not shown). Pretreatment of cells with PTX (200 ng/ml, 4 hr) totally eliminated the ability of (*R*)-PIA to inhibit isoprenaline-induced cAMP

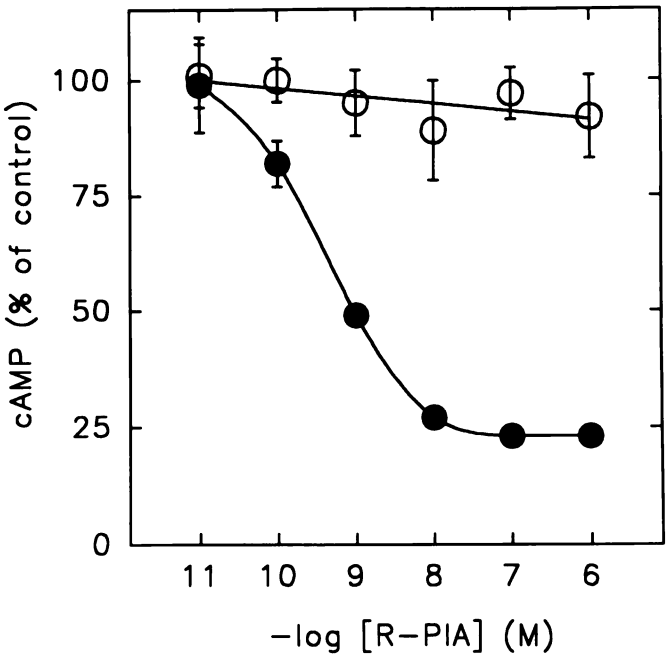

Fig. 6. Effects of (*R*)-PIA and PTX on isoprenaline-induced cAMP accumulation in intact DDT$_1$ MF-2 cells. Control cells (●) and cells treated with PTX (200 ng/ml for 4 hr) (○) were incubated with 10 μM isoprenaline for 10 min in the presence of different concentrations of (*R*)-PIA. (*R*)-PIA inhibited the accumulation of cAMP with an IC_{50} of 0.4 nM and a maximal inhibition of 76%. Data presented are mean ± standard error for one experiment of two.

accumulation (Fig. 6). The treatment with PTX also reveals a functional A_2 receptor, as measured by the ability of NECA to stimulate the accumulation of cAMP (increasing cAMP from 49.4 ± 8.1 to 72.7 ± 3.9 pmol/10^6 cells at 10 μM NECA and to 78.5 ± 3.3 pmol/10^6 cells at 100 μM NECA). Without PTX pretreatment, NECA was unable to stimulate cAMP accumulation (39.1 ± 2.4 at 10 μM NECA and 39.3 ± 1.7 at 100 μM, as compared with 36.5 ± 2.4 pmol/10^6 cells in control cells), probably due to the inhibitory effects of the A_1 receptor.

Effects of GTP and PTX on receptor affinity state. In order to determine whether a PTX-sensitive G protein was coupled to the A_1 receptor, we investigated the effects of addition of GTP or PTX. In intact cells, addition of GTP (100 μM) or pretreatment with PTX (200 ng/ml, 4 hr) did not change the shape of the competition curve with (*R*)-PIA for [^{3}H]DPCPX binding or the K_D (Table 1). In membranes, however, the above treatment shifted the competition curve with (*R*)-PIA from a biphasic to a monophasic curve (LIGAND, $p < 0.01$) (Table 1), with a K_D close to the low affinity K_D in membranes and the K_D in intact cells.

To show that treatment of cells with PTX caused an ADP-ribosylation of G proteins, we pretreated cells with PTX (200 ng/ml for 4 hr) and subsequently prepared membranes. The PTX-catalyzed [^{32}P]ADP-ribosylation of G proteins in these membranes was very small, which indicated that the treatment described above ADP-ribosylated G proteins in intact DDT$_1$ MF-2 cells (results not shown).

Discussion

In order to establish an experimental system for studies of the A_1 receptor signal transduction pathways in intact cells, we have characterized A_1 receptors in the smooth muscle cell line

DDT_1 MF-2. Because of its high selectivity for the A_1 receptor (approximately 700-fold), high specific activity, and low non-specific binding, we used [^{3}H]DPCPX as the ligand in these studies of intact cells and membranes (21, 31, 32). The binding sites on intact DDT_1 MF-2 cells showed the characteristics of adenosine A_1 receptors. Thus, the order of potency for both agonists and antagonists showed good agreement with previous reports of A_1 receptor binding to membranes (31). The binding was rapid and reversible, at least at higher temperatures. It was saturable, with a binding capacity of 110,000 sites/cell. This indicates that the number of A_1 receptors on DDT_1 MF-2 cells is similar to the number of β-receptors but larger than the number of A_1 receptors on cardiac myocytes (21, 33). The number is clearly below the number of histamine H_1 receptors on DDT_1 MF-2 cells, which amounts to 9.7×10^6 sites/cell (34). [^{3}H]DPCPX binding was studied both in intact cells and in membranes. The binding characteristics for antagonists were in excellent agreement with K_D values for DPCPX both in equilibrium binding and in kinetic experiments. With agonist binding, the situation was more complex. At equilibrium in intact cells, Scatchard plots were linear and association and dissociation curves could be best described by binding to a single class of receptors. In membranes, on the other hand, agonist binding was most adequately fitted to a two-site model, in agreement with several previous studies (20, 21, 32). The apparent affinities of agonists binding to the low affinity sites in membranes compared well with their affinities for the single class of sites on intact cells, in agreement with results obtained in ventricular myocytes (21). The finding that agonist binding to intact cells was similar to the low affinity binding seen in membrane preparation has also been reported for several other receptor systems, including muscarinic receptors (35) and β-adrenoceptors (36–38). A major reason for the discrepancy is that the concentration of GTP in intact cells is sufficiently high to shift the equilibrium binding to the low affinity state; in the present cells the intracellular GTP concentration was determined to be close to 1 mM.[2]

In the case of β-adrenoceptors, it has been proposed that the agonists may initially bind to the receptors in an apparent high affinity state but that the agonist-induced change in GTP binding then leads to dissociation of the drug-receptor complex (38). This model is supported by the finding (39) that there is a time-dependent shift in affinity state that becomes slower at lower temperatures. At 4° most of the β-adrenoceptor binding to intact cells was in the high affinity state, even after 3 hr of equilibration. This is clearly in contrast to the present data, where the binding even at 4° was in the low affinity state. At this low temperature the possibility of an agonist-induced transition to a low affinity state is unlikely. In DDT_1 MF-2 smooth muscle cells, all the receptors are in the low affinity form, which then should represent the unstimulated "resting" affinity state of the receptor. It should be mentioned also that in membranes the competition of adenosine receptor agonists shows low affinity at low temperatures (11). Short term incubations of intact DDT_1 MF-2 cells at 25° gave biphasic competition curves, which means that both the high and the low affinity states of the receptor are present. This indicates that agonists transiently convert the receptor from its resting low affinity state to its high affinity state, after which the receptor again resumes the low affinity form. This could also explain why the A_1 receptor co-elutes with G proteins when purified by using agonists but not when purified with antagonists. Thus, there may be a fundamental difference between the A_1 receptors on these cells and the β-adrenoceptors on most cells studied, with regard to the state that the receptors are in "at rest." Whether this reflects a difference between receptors associated with G_s and those associated with non-G_s proteins remains to be determined.

The G protein that adenosine A_1 receptors couple to in intact DDT_1 MF-2 cells was PTX sensitive, in agreement with the situation in heart (40). In contrast, the adenosine A_1 receptors in rat hippocampus do not all show the same PTX sensitivity (41). It is possible that a single class of adenosine A_1 receptors may interact with several types of G proteins, some of which are PTX sensitive and some not. It is also possible that there are, in fact, several distinct types of A_1 receptors, even though we cannot discriminate between them on the basis of presently available pharmacological tools.

In the intact DDT_1 MF-2 cells, (*R*)-PIA was an extremely potent inhibitor of β-adrenoceptor-stimulated cAMP accumulation. Both the potency (IC_{50} of 0.4 versus 62 nM) and the magnitude of the inhibition (76 versus 31% inhibition at maximally effective doses) were much larger than in a membrane preparation (28). This could indicate that the coupling between the receptor and the effector system is compromised by homogenization.

Pretreatment with PTX not only prevented adenosine analogues from inhibiting isoprenaline-induced cAMP accumulation but also revealed the presence of functional adenosine A_2 receptors linked to the stimulation of cAMP formation. This supports the recent finding (28) that these cells possess both types of adenosine receptors, just as it was previously shown that primary cultures of human smooth muscle cells possess both A_1 and A_2 receptors mediating opposing actions not only on cAMP but also on cell growth (42). The results indicate that not only the cAMP rise induced by β-adrenoceptor stimulation but also that following A_2 stimulation could be blocked by A_1 receptor activation.

In conclusion, we have characterized adenosine A_1 receptors on the smooth muscle cell line DDT_1 MF-2, which are negatively coupled to adenylate cyclase via a PTX-sensitive G protein. The present paper also shows that, at equilibrium in membrane preparations (25°), there are two affinity states of the receptor, whereas in intact cells (4° or 25°) it is only possible to detect the low affinity form, probably due to the fact that intact cells have a sufficient amount of guanine nucleotides present to rapidly shift the receptors to their low affinity form. At 4° the receptors are believed to be in their resting state, which in these cells appears to be the low affinity state. We have proposed that short term incubations with agonists transiently convert the receptor to its high affinity form. Because DDT_1 MF-2 cells grow in suspension as well as adherently and because there are only a few cell lines available that express A_1 receptors, we believe that this cell line could be a useful tool when studying the role of A_1 receptors in signal transduction in intact cells.

Acknowledgments

We are grateful to Ms. Susanne Ahlberg and Dr. Ingeborg van der Ploeg for their assistance with the ADP-ribosylation and cAMP experiments.

[2] P. Gerwins and B. B. Fredholm, unpublished observations.

References

1. Watson, S., and A. Abbot. Receptor nomenclature supplement. *Trends Pharmacol. Sci.* **11** (1990).
2. Fredholm, B. B., and B. Jonzon. Adenosine receptors: agonists and antagonists, in *Role of Adenosine in Cerebral Metabolism and Blood Flow* (V. Stefanovich and I. Okayayuz-Baklouti, eds.). VSP BV, Zeist, The Netherlands, 17–45 (1988).
3. Fredholm, B. B., and T. V. Dunwiddie. How does adenosine inhibit transmitter release? *Trends Pharmacol. Sci.* **9:** 130–134 (1988).
4. Nakata, H. Purification of A_1 adenosine receptor from rat brain membranes. *J. Biol. Chem.* **264:** 16545–16551 (1989).
5. Nakata, H. Affinity chromatography of A_1-adenosine receptors of rat brain membranes. *Mol. Pharmacol.* **35:** 780–786 (1989).
6. Munshi, R., and J. Linden. Co-purification of A_1 adenosine receptors and guanine nucleotide-binding proteins from bovine brain. *J. Biol. Chem.* **264:** 14853–14589 (1989).
7. Barrington, W. W., K. A. Jacobson, and G. L. Stiles. Demonstration of distinct agonist and antagonist conformations of the A_1 adenosine receptors. *J. Biol. Chem.* **264:** 13157–13164 (1989).
8. Bruns, R. F., J. W. Daly, and S. H. Snyder. Adenosine receptors in brain membranes: binding of N^6-cyclohexyl[^{3}H]adenosine and 1,3-diethyl-8-[^{3}H] phenylxanthine. *Proc. Natl. Acad. Sci. USA* **77:** 5547–5551 (1980).
9. Goodman, R. R., M. J. Cooper, M. Gavish, and S. H. Snyder. Guanine nucleotide and cation regulation of the binding of [^{3}H]cyclohexyladenosine and [^{3}H]diethylphenylxanthine to adenosine A_1 receptors in brain membranes. *Mol. Pharmacol.* **21:** 329–335 (1982).
10. Yeung, S.-M., and R. D. Green. Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5′-guanylylimidodiphosphate or *N*-ethylmaleimide. *J. Biol. Chem.* **258:** 2334–2339 (1983).
11. Lohse, M. J., V. Lenschow, and U. Schwabe. Two affinity states of Ri adenosine receptors in brain membranes: analysis of guanine nucleotide and temperature effects on radioligand binding. *Mol. Pharmacol.* **26:** 1–9 (1984).
12. Gavish, M., R. R. Goodman, and S. H. Snyder. Solubilized adenosine receptors in the brain: regulation by guanine nucleotides *Science* (*Washington DC*) **215:** 1633–1635 (1982).
13. Stiles, G. L. The A_1 adenosine receptor: solubilization and characterization of a guanine nucleotide-sensitive form of the receptor. *J. Biol. Chem.* **260:** 6728–6732 (1985).
14. Klotz, K. N., M. J. Lohse, and U. Schwabe. Characterization of the solubilized A_1 adenosine receptor from rat brain membranes. *J. Neurochem.* **46:** 1528–1534 (1986).
15. Ströher, M., C. Nanoff, and W. Schütz. Differences in the GTP-regulation of membrane-bound and solubilized A_1-adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340:** 87–92 (1989).
16. Goodman, R. R., and S. H. Snyder. Autoradiographic localization of adenosine receptors in rat brain using [^{3}H]cyclohexyladenosine. *J. Neurosci.* **2:** 1230–1241 (1982).
17. Fastbom, J., and B. B. Fredholm. Regional differences in the effect of guanine nucleotides on agonist and antagonist binding to adenosine A_1-receptors in rat brain, as revealed by autoradiography. *Neuroscience* **34:** 759–769 (1990).
18. Fredholm, B. B., E. Lindgren, and K. Lindström. Treatment with *N*-ethylmaleimide selectively reduces adenosine receptor-mediated decreases in cyclic AMP accumulation in rat hippocampal slices. *Br. J. Pharmacol.* **86:** 509–513 (1985).
19. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56:** 615–649 (1987).
20. Liang, B. T. Characterization of the adenosine receptor in cultured embryonic chick atrial myocytes: coupling to modulation of contractility and adenylate cyclase activity and identification by direct radioligand binding. *J. Pharmacol. Exp. Ther.* **249:** 775–784 (1989).
21. Martens, D., M. J. Lohse, and U. Schwabe. [^{3}H]-8-Cyclopentyl-1,3-dipropylxanthine binding to A_1 adenosine receptors of intact rat ventricular myocytes. *Circ. Res.* **63:** 613–620 (1988).
22. Schwabe, U., and T. Trost. Characterization of adenosine receptors in rat brain by [^{3}H]N^6-phenylisopropyladenosine. *Naunyn-Schmideberg's Arch. Pharmacol.* **313:** 179–187 (1980).
23. Linden, J. Adenosine deaminase for removing adenosine: how much is enough? *Trends Pharmacol. Sci.* **10:** 260–262 (1989).
24. Lohse, M. J., K.-N. Klotz, and U. Schwabe. Agonist photoaffinity labelling of A_1 adenosine receptors: persistent activation reveals spare receptors. *Mol. Pharmacol.* **30:** 403–409 (1986).
25. Hedqvist, P., and B. B. Fredholm. Effects of adenosine on adrenergic neurotransmission; prejunctional inhibition and postjunctional enhancement. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **293:** 217–223 (1976).
26. Paton, D. M. The pharmacological classification and structure of peripheral adenosine receptors, in *Adenosine and Adenine Nucleotides: Physiology and Pharmacology* (D. M. Paton, eds.). Taylor and Francis, London, 51–58 (1988).
27. Norris, J. S., J. Gorski, and P. O. Kohler. Androgen receptors in a Syrian hamster ductus deferens tumor cell line. *Nature* (*Lond.*) **248:** 422–424 (1974).
28. Ramkumar, V., W. W. Barrington, K. A. Jacobson, and G. L. Stiles. Demonstration of both A_1 and A_2 adenosine receptors in DDT$_1$ MF-2 smooth muscle cells. *Mol. Pharmacol.* **37:** 149–156 (1990).
29. Brown, B. C., J. D. M. Albano, R. P. Ekins, A. M. Sgherzi, and W. A. Tampion. A simple and sensitive saturation assay method for the measurement of adenosine 3,5-cyclic monophosphate. *Biochem. J.* **121:** 561–563 (1971).
30. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.* **107:** 220–239 (1980).
31. 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).
32. Bruns, R. F., J. H. Fergus, E. W. Badger, J. A. Bristol, L. A. Santay, J. D. Hartman, S. J. Hays, and C. C. Huang. Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335:** 59–63 (1987).
33. Limas, C. J., and C. Limas. Rapid recovery of cardiac β-adrenergic receptors after isoproterenol-induced "down"-regulation. *Circ. Res.* **55:** 524–531 (1984).
34. Mitsuhashi, M., and D. G. Payan. Characterization of functional histamine H_1 receptors on a cultured smooth muscle cell line. *J. Cell. Physiol.* **134:** 367–375 (1988).
35. Nathanson, N. M. Binding of agonists and antagonists to muscarinic acetylcholine receptors on intact cultured heart cells. *J. Neurochem.* **41:** 1545–1549 (1983).
36. Insel, P. A., L. C. Mahan, H. J. Motulsky, L. M. Stoolman, and A. M. Koachman. Time-dependent decreases in binding affinity of agonists for β-adrenergic receptors of intact S49 lymphoma cells. *J. Biol. Chem.* **258:** 13497–13605 (1983).
37. Toews, M. L., and J. P. Perkins. Agonist-induced changes in β-adrenergic receptors on intact cells. *J. Biol. Chem.* **259:** 2227–2235 (1984).
38. Marsh, J. D., and T. W. Smith. Receptors for β-adrenergic agonists in cultured chick ventricular cells: relationship between agonist binding and physiologic effect. *Mol. Pharmacol.* **27:** 10–18 (1984).
39. Schwarz, K. R., E. A. Carter, C. J. Homcy, and R. M. Graham. Agonist interactions at hepatic α_1- and β-adrenergic receptors: affinity-state regulation by guanine nucleotides and temperature. *Biochemistry* **25:** 7782–7788 (1986).
40. Böhm, M., R. Brückner, J. Neumann, W. Schmitz, H. Scholz, and J. Starbatty. Role of guanine nucleotide-binding protein in the regulation by adenosine of cardiac potassium conductance and force of contraction: evaluation with pertussis toxin. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **332:** 403–405 (1986).
41. Fredholm, B. B., W. Proctor, I. Van der Ploeg, and T. V. Dunwiddie. *In vivo* pertussis toxin treatment attenuates some, but not all, adenosine A_1 effects in slices of rat hippocampus. *Eur. J. Pharmacol.* 172: 249–262 (1989).
42. Jonzon, B., J. Nilsson, and B. B. Fredholm. Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J. Cell. Physiol.* **124:** 451–456 (1985).

Send reprint requests to: Pär Gerwins, Department of Pharmacology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.