0026-895X/92/050831-7$03.00/0

MOLECULAR PHARMACOLOGY, 42:831-837

Modulation of Cardiac Cyclic AMP Metabolism by Adenosine Receptor Agonists and Antagonists

HUI MA and RICHARD D. GREEN

Department of Pharmacology, University of Illinois at Chicago, Chicago, Illinois 60612

Received February 25, 1992; Accepted August 24, 1992

SUMMARY

The mechanism(s) underlying adenosine receptor-mediated modulation of cardiac cAMP levels has been investigated using detergent-permeabilized embryonic chick ventricular myocytes. The β-adrenergic receptor agonist isoproterenol (ISO) stimulated adenylyl cyclase activity in detergent-permeabilized cells by 5–10-fold, with an EC_{50} value of 0.3 μM. Three adenosine receptor agonists, (*R*)-N^6-phenylisopropyladenosine, N^6-(3-iodo-4-aminobenzyl)adenosine, and 5′-*N*-ethylcarboxamidoadenosine, inhibited ISO (10 μM)-stimulated adenylyl cyclase activity in a concentration-dependent manner. The maximum inhibition of the ISO-stimulated adenylyl cyclase activity by (*R*)-N^6-phenylisopropyladenosine (10 μM) was 30–40%. This inhibition was antagonized by the adenosine receptor antagonists xanthine amine congener and 8-cyclopentyl-1,3-dipropylxanthine and was abolished by pertussis toxin treatment, suggesting that the inhibition of adenylyl cyclase activity is mediated by A_1 adenosine receptors acting via a pertussis toxin-sensitive guanine nucleotide-binding protein (G protein). Because the adenosine receptor agonists had no detectable effect on phosphodiesterase activity, the adenosine receptor-mediated inhibition of adenylyl cyclase activity appears to account for the cAMP-lowering effect of adenosine receptor agonists seen in intact cardiac myocytes. Moreover, two A_1 adenosine receptor antagonists, 8-cyclopentyl-1,3-dipropylxanthine and 3-(4-amino)phenethyl-1-propyl-8-cyclopentylxanthine, stimulated basal adenylyl cyclase activity in the absence of an adenosine receptor agonist; this stimulation was abolished by pretreatment of the cells with pertussis toxin. We postulate that "precoupled" A_1 adenosine receptor-G protein complexes, present in the cardiac myocytes, exert a tonic inhibitory influence on adenylyl cyclase activity and that some adenosine receptor antagonists remove this tonic inhibition by destabilizing these precoupled receptor-G protein complexes.

It is well documented that β-adrenergic receptor agonists such as ISO and epinephrine increase the cAMP content of the myocardium and that this effect is well correlated with the positive inotropic effect of these agents. In contrast, adenosine and its analogs exert potent negative inotropic effects on the heart (1) and decrease ISO-elevated cAMP levels in guinea pig (2–5), rat (6, 7), and chick (8, 9) myocardium. Radioligand binding experiments have shown that A_1 AdoRs, known to couple to the inhibition of adenylyl cyclase in brain and adipocyte membranes (10, 11), are present in membranes prepared from cardiac tissue from multiple species (12–16) including chick (17–19). Although AdoR-mediated inhibition of adenylyl cyclase activity in cardiac membranes has been demonstrated (12, 17–21), the magnitude of this inhibition has generally been small when expressed as a percentage of the total adenylyl cyclase activity in the presence of ISO (17–19). Some investigators have been unable to demonstrate an effect of AdoR agonists on adenylyl cyclase activity in cardiac membranes (22–24).

Two explanations for the disparity between the effects of AdoR agonists on cAMP levels in intact cardiac tissue versus those on adenylyl cyclase activity in cardiac membranes are apparent. First, it is possible that the cAMP-lowering effect of AdoR agonists in intact cells results both from the inhibition of adenylyl cyclase and from the stimulation of a PDE. The latter mechanism is credible in view of reports that (*R*)-PIA stimulates a membrane-bound PDE in rat brain and fat cells (25, 26) and in 3T3-L1 fibroblasts (27). Alternatively, it is possible that the cardiac AdoR that couples to adenylyl cyclase is particularly vulnerable to uncoupling during the preparation of membranes and that the entire cAMP-lowering effect of AdoR agonists in whole cells is due to the inhibition of adenylyl cyclase.

We herein report studies using detergent-permeabilized embryonic chick myocytes to probe further the mechanisms underlying modulation of cardiac cAMP levels by agents that act

These studies were supported by a grant from the National Science Foundation (BNS-8719594).

ABBREVIATIONS: ISO, isoproterenol; BW-A844U, 3-(4-amino)phenethyl-1-propyl-8-cyclopentylxanthine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; IABA, N^6-(3-iodo-4-aminobenzyl)adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; PTX, pertussis toxin; (*R*)-PIA, (*R*)-N^6-phenylisopropyladenosine; XAC, xanthine amine congener; PDE, phosphodiesterase; AdoR, adenosine receptor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; G protein; guanine nucleotide-binding protein; dcAMP, deoxy-cAMP.

at AdoRs, i.e., we have assayed the effects of AdoR agonists and antagonists on adenylyl cyclase and PDE activities in permeabilized myocytes. This approach was taken based on the report that receptor-mediated effects on adenylyl cyclase that cannot be detected in membrane preparations can be demonstrated successfully in detergent-permeabilized cells (28). We have also used this preparation to test the hypothesis that alkylxanthine AdoR antagonists act at A_1 AdoRs to elevate basal adenylyl cyclase activity, as predicted from previous studies in our laboratory (29, 30).

Experimental Procedures

Materials. Fertilized White Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL). (*R*)-PIA was purchased from Boehringer-Mannheim (Indianapolis, IN). NECA, adenosine deaminase, ATP, dATP, dcAMP, cAMP, creatine phosphate, creatine phosphokinase, ISO, snake venom, saponin, and β-escin were obtained from Sigma (St. Louis, MO). XAC and CPX were from RBI (Natick, MA). BW-A844U and IABA were kindly provided by Dr. J. Linden (University of Virginia, Charlottesville, VA). Milrinone was a kind gift from Sterling-Winthrop Research Institute (Rensselaer, NY). Rolipram was provided by Berlex Laboratories, Inc. (Cedar Knolls, NJ). [α-^{32}P]ATP, [α-^{32}P]dATP, [^{3}H]dcAMP, [^{3}H]cAMP, [^{3}H]adenine, and [^{14}C]cAMP were purchased from ICN (Irvine, CA). PTX was from List Biologicals, Inc. (Campbell, CA). Culture medium, Earle's balanced salt solution, trypsin solution, and fetal bovine serum were purchased from Hazleton (Denver, PA) and Biologos Inc. (Naperville, IL).

Preparation of embryonic chick myocytes and cell culture. Fertilized chicken eggs were maintained in a humidified egg incubator at 38° until the embryos reached the desired state of development. The hearts were removed quickly from 11–12-day-old chick embryos and the ventricles were cut into small fragments (1–0.5 mm) and placed in a Ca^{2+}- and Mg^{2+}-free Earle's salt solution. The ventricular myocytes were gently dissociated by trypsinization (in 10 ml of 0.025%, w/v, trypsin in the Ca^{2+}/Mg^{2+}-free Earle's salt solution, for 8 min at 37°, for five or six cycles). The dissociated cells were then placed in 15 ml of ice-cold stop solution, i.e., serum-free culture medium containing 0.015% (w/v) trypsin inhibitor and 2.5% (w/v) bovine albumin. Cells to be studied immediately were pelleted, washed in serum-free medium (see below), and resuspended in the appropriate assay buffer. Cells to be put into culture were centrifuged at 350 × *g* for 4 min and sequentially washed with the stop solution and a medium 199-based culture medium consisting of 6% heat-inactivated fetal bovine serum (Biologos), 40% medium 199 (Hazleton), and 54% salt solution. Final salt concentrations in the culture medium were 117 mM NaCl, 4 mM KCl, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 0.5 mM NaH_2PO_4, 18 mM $NaHCO_3$, and 0.1 mM Na_2HPO_4 (31). The cells were resuspended in the culture medium, placed in 24-well culture dishes (0.8–1 × 10^6 cells/well), and incubated in a humidified 5% CO_2/95% air incubator at 37° for 2–4 days.

Measurement of cAMP accumulation in intact myocytes. Freshly isolated myocytes or cells maintained in culture for 2–4 days were studied. Relative changes in cAMP levels were determined by labeling the cells with [^{3}H]adenine and measuring [^{3}H]cAMP formation from [^{3}H]ATP (32, 33). Briefly, cells were incubated with [^{3}H]adenine (2 μCi/ml) at 37° for 1 hr in a buffer solution containing 140 mM NaCl, 5 mM KCl, 0.45 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM HEPES/Tris (pH 7.4), and 5 mM glucose. The cells were then washed once and placed in the same HEPES-buffered solution plus adenosine deaminase (2 Units/ml) (~0.8–1 × 10^6 cells/sample; 200–250 μg of protein/10^6 cells). After a 3-min incubation at 37°, reactions were initiated by the addition of various drug solutions and were incubated at 37° for 5 min (in a total volume of 900 μl for cells in suspension or 400 μl for cells in culture). Incubations were terminated by the addition of ice-cold TCA (5% final concentration). [^{14}C]cAMP was added and the samples were then kept at 4° for 3 hr, after which the supernatant was loaded onto Dowex 50W columns. The [^{3}H]cAMP formed was separated from [^{3}H]ATP by sequential chromatography on Dowex 50W and alumina (34). [^{14}C]cAMP was used to correct for the recovery (60–80%) of product, [^{3}H]cAMP. The results are expressed as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP. All assays were performed in triplicate.

Preparation of detergent-permeabilized myocytes. Chick myocytes were permeabilized with the nonionic detergent saponin or the saponin ester β-escin (35, 36). For permeabilization of cells in culture, the medium was replaced with 300 μl of a HEPES-buffered solution containing 120 mM NaCl, 1 mM KH_2PO_4, 1 mM Na_2EGTA, 25 mM HEPES/Tris (pH 7.4), and 1 mM $MgSO_4$. After a 5-min incubation at 30°, saponin (100 μg/ml) or β-escin (80 μM) was added and the incubation was continued at 30° for 4.5 min. The cells were washed twice with the ice-cold HEPES-buffered solution and placed in an appropriate assay buffer. For permeabilization of freshly isolated myocytes, cells were resuspended in the HEPES-buffered solution (~1.5 × 10^7 cells/ml) and maintained at 37° for 5 min. The detergent (β-escin, 40 μM, or saponin, 100 μg/ml) was added to the cell suspension and incubated at 37° for 1 (β-escin) or 4.5 (saponin) min. Ice-cold HEPES-buffered solution was then added and the cell suspension was centrifuged at 350 × *g* at 25° for 3 min. The cells were washed twice with ice-cold buffer (3-min centrifugation at 350 × *g* at 25°) and resuspended in the appropriate assay buffer. Both freshly isolated and cultured cells could be effectively permeabilized with either saponin or β-escin. The results on cells permeabilized with β-escin were more consistent, suggesting that the β-escin treatment was less disruptive to receptor-effector coupling.

Adenylyl cyclase assay in detergent-permeabilized myocytes. Adenylyl cyclase activity in detergent-permeabilized myocytes was assayed using a method modified from those of Salomon *et al.* (34) and Cooper and Londos (37). Generally, permeabilized cells were placed in a reaction mixture consisting of 120 mM NaCl, 1 mM KH_2PO_4, 1 mM Na_2EGTA, 1 mM $MgSO_4$, 100 μM GTP, 16 mM creatine phosphate, 12 units/ml creatine phosphokinase, and 0.2 mM ATP (or dATP), in a total volume of 300 μl/sample (0.8–1 × 10^6 cells or 200–250 μg). The reaction was initiated by the addition of [α-^{32}P]ATP (or [α-^{32}P]dATP) and carried out at 30° for 10 min. Ice-cold TCA was added to stop the reaction when [α-^{32}P]ATP was used (5% TCA in a total volume of 1 ml). This procedure did not work well when [α-^{32}P]dATP was used as the substrate. In this case the reaction was stopped by transferring the reaction medium overlying the cells in culture into a tube containing 100 μl of 1 mM ATP plus 1.3 mM cAMP and 2% sodium dodecyl sulfate (34) and then boiling the mixture for 3 min. Preliminary experiments with [α-^{32}P]ATP showed that all of the [α-^{32}P]cAMP formed was present in the overlay. In both cases [^{3}H]cAMP or [^{3}H]dcAMP was added to correct for the recovery and the samples were processed as previously discussed. Initial experiments in which permeabilized cells were incubated with [^{3}H]cAMP showed that rolipram (0.1 mM) plus cGMP (0.3 mM) completely inhibited PDE activity, and all adenylyl cyclase assays were performed in the presence of these agents. Additional preliminary experiments demonstrated that 1) the effects of AdoR agonists and antagonists on adenylyl cyclase activity were not significantly affected when various amounts of NaCl in the incubation mixture were replaced by KCl, i.e., when the concentrations of Na^+ and K^+ were adjusted to more closely approximate intracellular concentrations, and 2) dose-response curves for ISO and (*R*)-PIA were the same whether [α-^{32}P]dATP or [α-^{32}P]ATP was used as the substrate. Results are expressed as [α-^{32}P]cAMP or [α-^{32}P]dcAMP formation (pmol/well or 10^6 cells/10 min; 200–250 μg of protein/10^6 cells) or percentages of adenylyl cyclase activity in the presence of ISO. All adenylyl cyclase assays were performed in quadruplicate.

PDE assay in detergent-permeabilized myocytes. Potential effects of (*R*)-PIA on PDE activity were probed by 1) treatment of cells with (*R*)-PIA before permeabilization and assay of PDE and 2) addition of (*R*)-PIA to assays of PDE activity in permeabilized cells. In both cases, freshly isolated myocytes were permeabilized and resus-

pended in the HEPES-buffered solution used for the adenylyl cyclase assay. The cell density was adjusted so that product formation was <15% of the initial substrate concentration; PDE activity was determined by a method adapted from the literature (38, 39). The reaction mixture was composed of 2 units/ml adenosine deaminase, cell suspension, 0.25 μM cAMP, and [^{3}H]cAMP, with or without rolipram or milrinone, in a volume of 200 μl. The reactions were carried out at 30° for 10 min and were terminated by boiling for 3 min. The samples were cooled to room temperature and incubated with snake venom (0.2 mg/ml) at 30° for 10 min, after which the ^{3}H-labeled products (adenosine and inosine) were isolated using Dowex 1-X8 (200–400-mesh) columns. Preliminary experiments showed that the recovery of the ^{3}H-labeled product was consistently 90–95%. Results are expressed as percentages of total PDE activity (i.e., PDE activity in the absence of PDE inhibitors). All PDE assays were performed in triplicate.

The method of Lowry *et al.* (40) was used to determine the protein concentration; bovine serum albumin was utilized as the standard. Inhibition curves were analyzed by a computer program that provides the n_H value and IC_{50} determined from a Hill plot. Student's *t* test (paired or nonpaired) was used for statistical evaluations. The *p* values of <0.05 were accepted as significant.

Results

Effects of adenosine analogs on cAMP accumulation in intact cardiac myocytes. The effects of the β-adrenergic receptor agonist ISO and the AdoR agonist (*R*)-PIA on cAMP levels in intact myocytes were initially evaluated in order to ensure that the receptors in our intact isolated myocytes retain their abilities to modulate cAMP levels. Fig. 1, *inset*, shows the dose-response curve for ISO effects on cAMP levels in intact myocytes in the presence of the PDE inhibitor rolipram (0.1 mM). ISO increased the cAMP level in a dose-dependent manner, with an EC_{50} of 0.3 μM. The main panel of Fig. 1 shows that (*R*)-PIA (0.01–10 μM) produced a concentration-dependent reduction of the ISO-elevated cAMP level; the IC_{50} for (*R*)-PIA was 0.1–0.3 μM. The AdoR antagonist XAC (0.1 μM) attenuated the (*R*)-PIA responses (data not shown), suggesting that this effect was mediated via A_1 AdoRs. Similar results were obtained when cells in culture were used (data not shown). It should be noted that these measurements of cAMP levels in the presence of rolipram are not quantitative reflections of adenylyl cyclase activity, because the addition of propranolol to cells exposed to ISO plus rolipram resulted in a rapid fall in cAMP levels, suggesting the presence of significant residual PDE activity in the cells (data not shown).

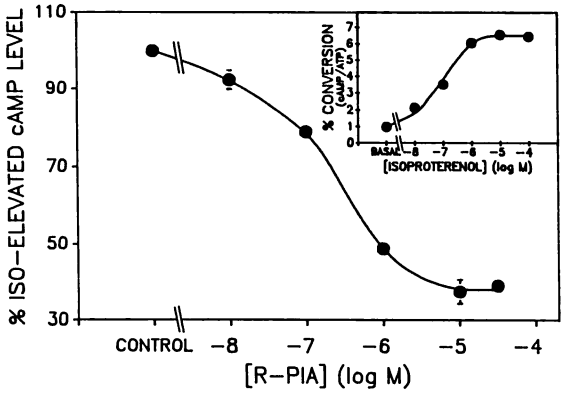

Fig. 1. *Inset*, dose-response curve for ISO for elevation of cAMP levels in the presence of rolipram (0.1 mM). This is one of four identical experiments. *Main panel*, dose-response curve for (*R*)-PIA for attenuation of ISO-elevated cAMP levels. The results are expressed as percentages of the ISO-elevated (ISO, 0.3 μM; rolipram, 0.1 mM) cAMP level. The values shown are means ± standard errors of eight experiments. *Standard error bars* are shown unless they are smaller than the *symbols*.

Effects of AdoR agonists on adenylyl cyclase activity in detergent-permeabilized myocytes. The accurate assessment of the effects of AdoR agonists on adenylyl cyclase in permeabilized myocytes requires conditions in which all PDE activity is inhibited and no "contaminating" adenosine is present. Residual PDE activity was not detectable in the presence of 0.1 mM rolipram and 0.3 mM cGMP (see Experimental Procedures), and these agents were present in all assays. The addition of adenosine deaminase to assays to metabolize endogenous or contaminating adenosine to inosine, which is inactive at AdoRs, is a common method used in studies on AdoRs. Fig. 2 contrasts the effects of adenosine deaminase on adenylyl cyclase activity when different adenylyl cyclase substrates were used. Adenosine deaminase (0.25–5 units/ml) did not affect adenylyl cyclase activity in the absence (Fig. 2A) or presence (Fig. 2B) of ISO when dATP was used. When ATP was utilized as the substrate (Fig. 2C), adenosine deaminase increased adenylyl cyclase activity in a dose-dependent manner; a concentration of adenosine deaminase of 0.5 units/ml maximally increased adenylyl cyclase activity. The following experiments were performed using a high concentration of adenosine deaminase (2 units/ml) and dATP as the substrate. Fig. 3, *inset*, shows that ISO stimulated adenylyl cyclase activity in a concentration-dependent manner; the EC_{50} for ISO was 0.3 μM. The main panel of Fig. 3 shows the effects of three AdoR agonists [(*R*)-PIA, IABA, and NECA] on adenylyl cyclase activity. All three AdoR agonists inhibited adenylyl cyclase activity in the presence of ISO (10 μM); the maximal inhibition by (*R*)-PIA (10 μM) was 30–40%. It was not possible to obtain complete dose-response curves for NECA, due to its low potency and limited solubility. The A_1 AdoR antagonists XAC and CPX (10 nM) shifted the inhibition curve of (*R*)-PIA to the right (Fig. 4), further suggesting that the inhibition of adenylyl cyclase by (*R*)-PIA was mediated via A_1 AdoRs. Treatment of cells with PTX (10 ng/ml, 20 hr) abolished the (*R*)-PIA- and NECA-induced inhibition of adenylyl cyclase (Fig. 5), indicating that this inhibition was mediated via a PTX-sensitive G protein.

Effects of AdoR agonists on PDE activity in chick cardiac myocytes. PDE activities in control and (*R*)-PIA-pretreated myocytes were measured in permeabilized cells in the absence or presence of rolipram or milrinone so that we could partition total PDE activity into rolipram-sensitive and milrinone-sensitive PDE activities. Rolipram (0.1 mM) inhibited the total PDE activity by 43 ± 1.9% (mean ± standard error, 12 experiments). Milrinone (10 μM), a selective inhibitor of the cGMP-inhibited PDE isozyme (41), attenuated the total PDE activity by 61 ± 1.3% (mean ± standard error, 12 experiments). Milrinone (10 μM) plus rolipram (0.1 mM) reduced PDE activity by 83 ± 3.5% (four experiments). (*R*)-PIA (10 μM) had no significant effect on PDE activity measured under any of these conditions. We were concerned that an effect of (*R*)-PIA on PDE could be rapidly reversible and thus lost during the preparation of the permeabilized cells and that GTP and/or ATP might be required for a G protein-mediated event (25, 26) or a protein phosphorylation reaction (42). Therefore, we also determined the direct effect of (*R*)-PIA on PDE activity in detergent-permeabilized cells assayed with or without GTP

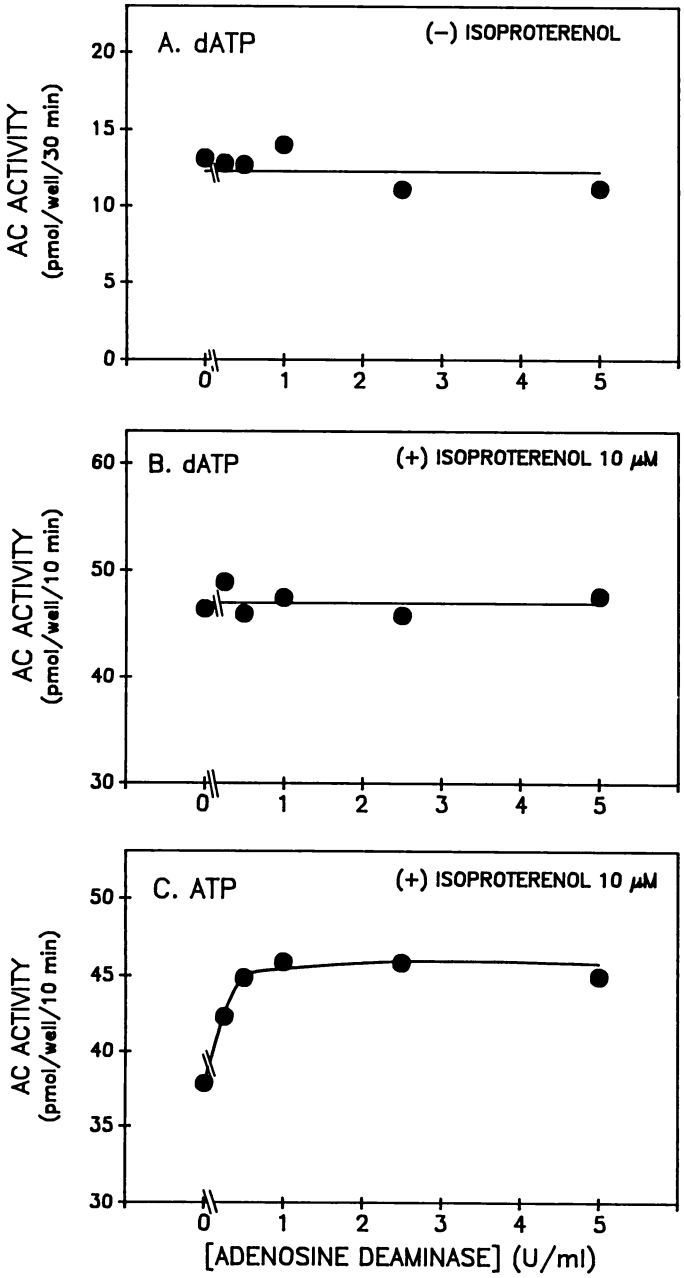

Fig. 2. Effects of adenosine deaminase on adenylyl cyclase activity in detergent-permeabilized myocytes. The assay was carried out using either [α-^{32}P]ATP (C) or [α-^{32}P]dATP as the substrate in the absence (A) or presence of ISO (10 μM) (B). The results are expressed as cAMP formed (pmol/well/10 min). The values shown are means of two or three identical experiments. *AC*, adenylyl cyclase.

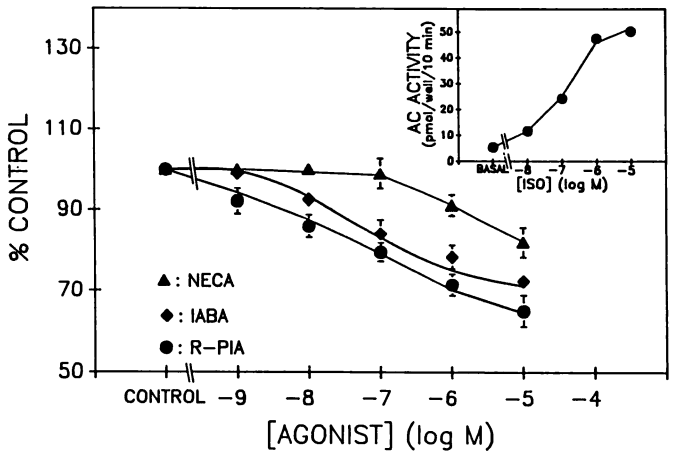

Fig. 3. *Main panel*, dose-response curves for three AdoR agonists, (*R*)-PIA, IABA, and NECA, for inhibition of adenylyl cyclase activity in detergent-permeabilized myocytes. The experiments were carried out in the presence of ISO (10 μM), cGMP (0.3 mM), rolipram (0.1 mM), and adenosine deaminase (2 units/ml). The results are expressed as percentages of total adenylyl cyclase activity in the presence of ISO (10 μM). The *points* shown are means ± standard errors of three to 12 experiments [(*R*)-PIA, 12 experiments; IABA, three experiments; NECA, seven experiments]. *Standard error bars* are shown unless they are smaller than the *symbols*. *Inset*, dose-response curve for ISO for stimulation of adenylyl cyclase activity under the same assay conditions. Adenylyl cyclase activity is expressed as cAMP formed (pmol/well/10 min; 200–250 μg of protein/well). The experiment shown is one of four identical experiments.

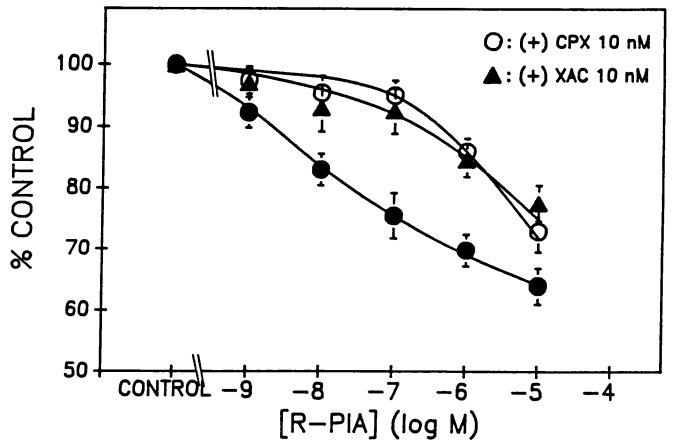

Fig. 4. Effects of the two AdoR antagonists, XAC and CPX, on (*R*)-PIA-induced inhibition of adenylyl cyclase activity in detergent-permeabilized myocytes. The experiments were carried out in the presence of ISO (10 μM) with or without XAC (10 nM) or CPX (10 nM). The results are expressed as percentages of total adenylyl cyclase activity in the presence of ISO (10 μM). The control dose-response curve for (*R*)-PIA (eight experiments) summarizes (means ± standard errors) four control experiments in conjunction with CPX and four control experiments in conjunction with XAC. *Standard error bars* are shown unless they are smaller than the *symbols*.

and with or without GTP plus ATP. We were unable to detect an effect of (*R*)-PIA on PDE activity under any condition tested (data not shown).

Effects of AdoR antagonists on basal adenylyl cyclase activity. Previous experiments on bovine brain A_1 AdoRs (29, 30) led us to determine whether AdoR antagonists affect basal adenylyl cyclase activity through a G protein-dependent mechanism. Considerable experimentation, as described in Experimental Procedures, established conditions in which the assay was not influenced by "endogenous" adenosine. In these experiments, we determined the effects of AdoR antagonists on basal adenylyl cyclase activity in the presence of a high concentration of adenosine deaminase (2 units/ml), using [α-^{32}P]dATP as the substrate. Fig. 6 shows that two AdoR antagonists, CPX and BW-A844U, stimulated basal adenylyl cyclase activity in the absence of agonists. Both antagonists maximally stimulated basal adenylyl cyclase by about 50%. For unknown reasons, CPX responses were decreased at higher concentrations (>0.1 μM). In addition, the stimulatory effect of the antagonists was blocked or reversed in the presence of 10 μM (*R*)-PIA (data not shown). Pretreatment with PTX increased basal adenylyl cy-

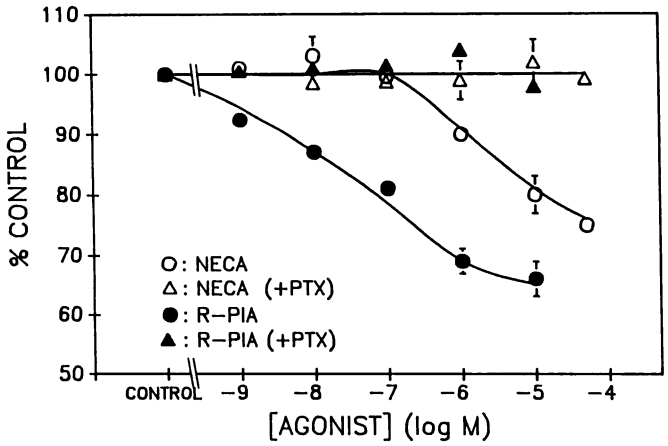

Fig. 5. Effect of PTX on (*R*)-PIA- or NECA-induced inhibition of adenylyl cyclase activity in detergent-permeabilized myocytes. Cells in culture were incubated with or without PTX (10 ng/ml) for 20 hr and the adenylyl cyclase assay was performed in the presence of ISO (10 μM) with or without (*R*)-PIA or NECA. The results are expressed as percentages of total adenylyl cyclase activity in the presence of ISO (10 μM). The *points* shown are means ± standard errors of three experiments. *Standard error bars* are shown unless they are smaller than the *symbols*.

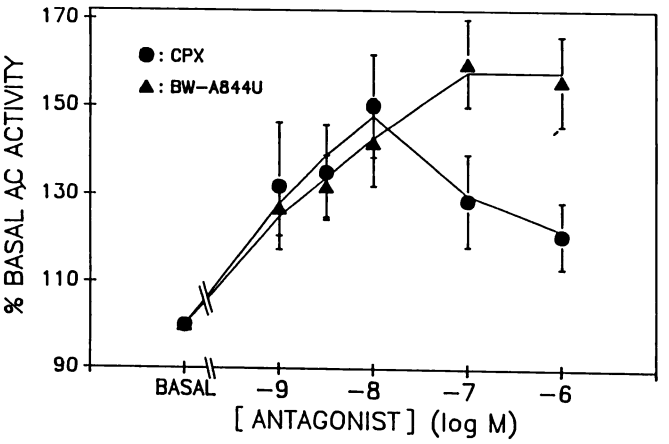

Fig. 6. Effects of two AdoR antagonists, CPX and BW-A844U, on basal adenylyl cyclase activity in detergent-permeabilized myocytes. The results are expressed as percentages of basal adenylyl cyclase activity. The *points* shown are means ± standard errors of seven experiments.

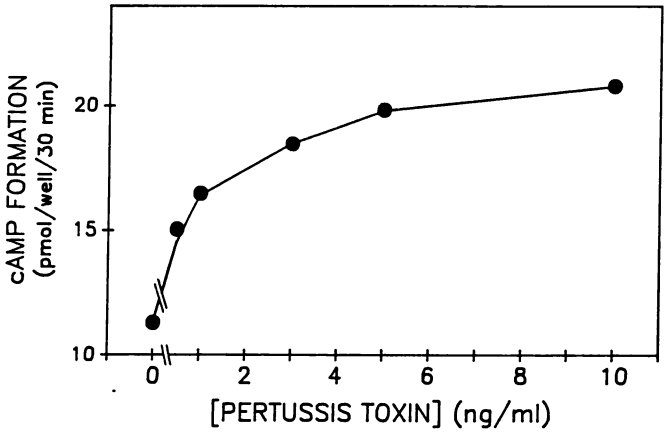

Fig. 7. Effects of PTX on basal adenylyl cyclase activity in detergent-permeabilized myocytes. Cells in culture were incubated with or without PTX (0–10 ng/ml) for 20 hr, permeabilized, and assayed for adenylyl cyclase activity. This is one of three identical experiments.

clase activity (Fig. 7) and blocked the abilities of the AdoR antagonists to stimulate the basal adenylyl cyclase (Fig. 8). Interestingly, the depressant effect of high concentrations of CPX on adenylyl cyclase activity did not appear to be blocked by the PTX pretreatment.

Discussion

We report herein that ISO (10 μM) stimulates adenylyl cyclase activity in detergent-permeabilized embryonic chick heart myocytes by 5–10-fold (Fig. 1). ISO has been reported to stimulate adenylyl cyclase activity in embryonic chick heart membranes by 1.3–2-fold (17–19). AdoR agonists such as (*R*)-PIA, IABA, and NECA inhibited adenylyl cyclase activity in the presence of ISO (10 μM). Maximal inhibition by (*R*)-PIA was 30–40% of total (plus ISO) adenylyl cyclase activity (Fig. 1). (*R*)-PIA inhibits total adenylyl cyclase (plus ISO) in embryonic chick heart membranes by 0–15% (17, 18).[1] It is thus clear that receptor-adenylyl cyclase coupling is much less perturbed in permeabilized myocytes, compared with membrane preparations. This is true in spite of extensive unpublished experimentation to try to make "better" cardiac membrane preparations.

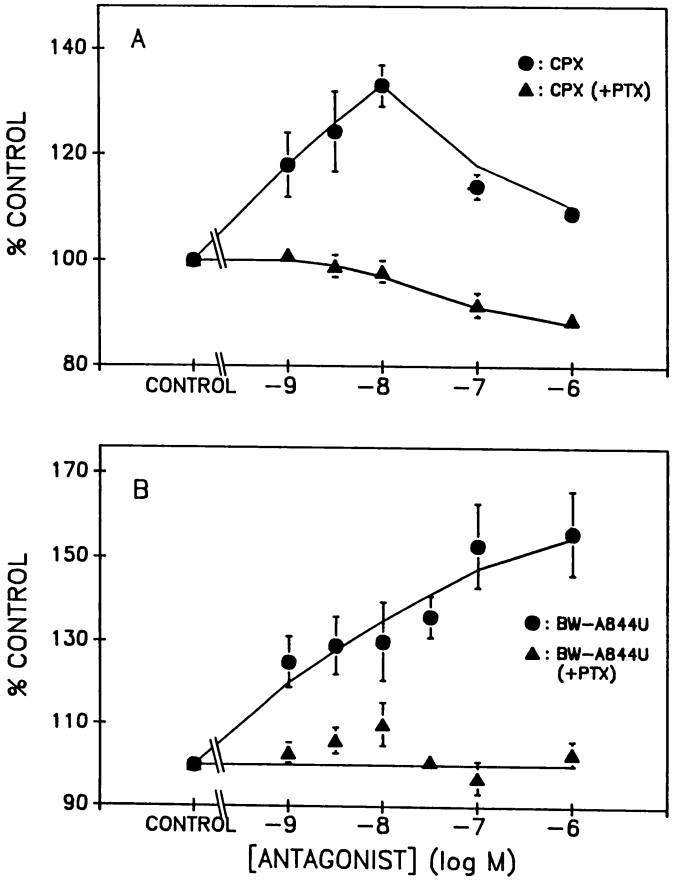

Fig. 8. Effects of PTX on AdoR antagonist-induced stimulation of basal adenylyl cyclase activity in detergent-permeabilized myocytes. Cells in culture were incubated with or without PTX (10 ng/ml) for 20 hr, permeabilized, and assayed for adenylyl cyclase activity in the presence of CPX (A) or BW-A884U (B). The results are expressed as percentages of the basal adenylyl cyclase activity. The *points* shown are means ± standard errors of three experiments.

[1] R. D. Green, unpublished observations.

Experiments showing that 1) CPX, a selective A_1 AdoR antagonist, attenuates the (*R*)-PIA responses, 2) (*R*)-PIA is more potent than NECA, and 3) the responses to (*R*)-PIA are blocked by PTX establish that the inhibition of adenylyl cyclase by AdoR agonists is mediated by cardiac A_1 AdoRs acting via a PTX-sensitive G protein. It could be argued that the relative impotency of NECA, compared with (*R*)-PIA, in the adenylyl cyclase assays could be due to the presence of A_2 AdoRs in the myocyte preparations. Indeed, Xu *et al.* (43) recently reported that NECA stimulates adenylyl cyclase activity in membranes prepared from embryonic chick ventricular myocytes when 1) CPX is present to block A_1 AdoR or 2) membranes are prepared from myocytes treated for 12 hr with PTX to inactivate G_i and thus block A_1 AdoRs effects on adenylyl cyclase. We have performed similar experiments and found 1) for two experiments with control cells the adenylyl cyclase activity in the presence of 0.3 μM CPX plus 10 μM NECA was 90.4 and 106.6% of that in the presence of CPX alone and 2) for four experiments with PTX-treated cells (10 ng/ml, 18 hr) adenylyl cyclase activities in the presence of 10 μM NECA were 108 ± 4 and 101 ± 14% of control in the absence and presence of 0.3 μM CPX, respectively. Thus, unlike Xu *et al.* (43), we were unable to demonstrate a convincing stimulatory effect of NECA under these experimental conditions. Although the reason for this discrepancy is not apparent, we can confidently conclude that a stimulatory effect of NECA on adenylyl cyclase was not the basis for the relatively low potency of NECA, compared with (*R*)-PIA, in our experiments.

Attempts to detect effects of (*R*)-PIA on PDE activity in permeabilized myocytes all gave negative results. Thus, even though we cannot rule out the possibility that AdoR agonists may modulate cAMP levels in whole myocytes by affecting the activity of a PDE isozyme, our results from detergent-permeabilized cells and from membrane preparations (data not shown) do not support the existence of such a mechanism. Therefore, the apparent discrepancy between the effects of AdoR agonists on cAMP levels in intact cells and the effects of the same agents on adenylyl cyclase activity in cardiac membrane preparations appears to be due to receptor-effector uncoupling in the membrane preparations.

Previous experiments in our laboratory showed that AdoRs in bovine brain are tightly coupled to G proteins in the absence of an agonist (29, 30). We presented evidence that antagonists such as CPX and XAC preferentially bind to free receptors and cause dissociation of "precoupled" receptor-G protein complexes (29, 30). However, we did not attempt to probe the physiological consequences of these precoupled receptor-G protein complexes. We proposed that, if AdoRs are precoupled to G_i proteins in the absence of agonists, these receptor-G protein complexes may exert tonic inhibitory effects on adenylyl cyclase. If this is the case, antagonists should stimulate adenylyl cyclase activity by causing the dissociation of precoupled receptor-G protein complexes. We postulated that the abilities of different antagonists to cause the dissociation of precoupled receptors would be a function of their different relative affinities for coupled and uncoupled receptors and that different antagonists should exhibit different degrees of "negative efficacy." Antagonists with equal affinities for the coupled and uncoupled receptors would not cause the dissociation of precoupled receptors and would behave as classical competitive antagonists.

In the present study, we found that two antagonists, CPX and BW-A844U, increased adenylyl cyclase activity in the absence of agonists. This effect was reversed by (*R*)-PIA and prevented by PTX treatment. The experiments with PTX also revealed that PTX increased the basal adenylyl cyclase activity, an observation further supporting the concept of the tonic, receptor-driven inhibition of basal adenylyl cyclase activity. Stimulatory effects of alkylxanthine-type AdoR antagonists on adenylyl cyclase activity in the absence of agonists have been observed by others and attributed to a direct inhibition of G_i function (44, 45). However, neither our studies nor those of Stiles and co-workers (44, 45) differentiate between effects of the antagonists on G_i versus those on AdoR-G_i protein complexes, and we favor our interpretation of the data.

The postulate that antagonists can exert effects opposite to those of agonists in the absence of an agonist, i.e., exert negative efficacy, is not without precedent. Costa *et al.* (46, 47) reported that δ-opioid receptors spontaneously couple to PTX-sensitive G proteins and that some competitive antagonists exhibit negative efficacies, as measured by a reduction in basal high affinity GTPase activity. The maximum effects of different antagonists varied and one antagonist studied had no effect on GTPase activity, i.e., acted as a classical antagonist. This response was receptor mediated and could not be attributed to the antagonism of an endogenous agonist effect, because the inhibitory effects of the "active" antagonists were antagonized by the "inactive" antagonist. We have not yet attempted to test a larger number of alkylxanthines in the adenylyl cyclase assay to find an antagonist with zero efficacy. The only other antagonist that we have tested, XAC, increased basal adenylyl cyclase activity much the same as did the agents presented. It, therefore, remains to be determined whether different alkylxanthines that express different negative efficacies exist and, if so, whether these agents exhibit different characteristics in the whole animal. This could be the case, because antagonists with zero efficacy would act entirely by antagonizing endogenous adenosine, whereas antagonists that can exert negative efficacies would exert an additional effect, an effect not dependent on the presence of endogenous adenosine.

References

1. Belardinelli, L., J. Linden, and R. M. Berne. The cardiac effects of adenosine. *Prog. Cardiovasc. Dis.* **32**:73–97 (1989).
2. Schrader, J., G. Baumann, and E. Gerlach. Adenosine as inhibitor of myocardial effects of catecholamines. *Pflügers Arch.* **372**:29–35 (1977).
3. Neumann, J., W. Schmitz, H. Scholz, and B. Stein. Effects of adenosine analogues on contractile response and cAMP content in guinea-pig isolated ventricular myocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**:689–695 (1989).
4. Behnke, N., W. Müller, J. Neumann, W. Schmitz, H. Scholz, and B. Stein. Differential antagonism by 1,3-dipropylxanthine-8-cyclopentylxanthine and 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo (1,5-c)quinazolin-5-imine of the effects of adenosine derivatives in the presence of isoprenaline on contractile response and cyclic AMP content in cardiomyocytes: evidence for the coexistence of A_1 and A_2 adenosine receptors on cardiomyocytes. *J. Pharmacol. Exp. Ther.* **254**:1017–1023 (1990).
5. Wilken, A., H. Tawfik-Schlieper, K.-N. Klotz, and U. Schwabe. Pharmacological characterization of the adenylate cyclase-coupled adenosine receptor in isolated guinea pig atrial myocytes. *Mol. Pharmacol.* **37**:916–920 (1990).
6. Henrich, M., H. M. Piper, and J. Schrader. Evidence for adenylate cyclase-coupled A_1 adenosine receptors on ventricular cardiomyocytes from adult rat and dog heart. *Life Sci.* **41**:2381–2388 (1987).
7. Martens, D., M. J. Lohse, B. Rauch, and U. Schwabe. Pharmacological characterization of A_1 adenosine receptors in isolated rat ventricular myocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336**:342–348 (1987).
8. Belardinelli, L., S. Vogel, J. Linden, and R. M. Berne. Antiadrenergic action of adenosine on ventricular myocardium in embryonic chick hearts. *J. Mol. Cell. Cardiol.* **14**:291–294 (1982).
9. Thakkar, J. K., and N. Sperelakis. Changes in cyclic nucleotide levels during embryonic development of chick hearts. *J. Dev. Physiol.* **9**:497–505 (1987).

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. Londos, C., D. M. F. Cooper, and J. Wolff. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* **77:**2551–2554 (1980).
12. Romano, F. D., and J. G. Dobson, Jr. Adenosine modulates β-adrenergic signal transduction in guinea-pig heart ventricular membranes. *J. Mol. Cell. Cardiol.* **22:**1359–1370 (1990).
13. Böhm, M., B. Pieske, M. Ungerer, and E. Erdmann. Characterization of A_1 adenosine receptors in atrial and ventricular myocardium from diseased human hearts. *Circ. Res.* **65:**1201–1211 (1989).
14. Klotz, K.-N., M. J. Lohse, U. Schwabe, G. Cristalli, S. Vittori, and M. Grifantini. 2-Chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA), a high affinity agonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340:**679–683 (1989).
15. Lohse, M. J., 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).
16. Tawfik-Schlieper, H., K.-N. Klotz, V. A. W. Kreye, and U. Schwabe. Characterization of the K^+-channel-coupled adenosine receptor in guinea pig atria. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340:**684–688 (1989).
17. Hosey, M. M., K. K. McMahon, and R. D. Green. Inhibitory adenosine receptors in the heart: characterization by ligand binding studies and effects on β-adrenergic receptor stimulated adenylate cyclase and membrane phosphorylation. *J. Mol. Cell. Cardiol.* **16:**931–942 (1984).
18. 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).
19. Blair, T. A., M. Parenti, and T. F. Murray. Development of pharmacological sensitivity to adenosine analogs in embryonic chick heart: role of A_1 adenosine receptors and adenylyl cyclase inhibition. *Mol. Pharmacol.* **35:**661–670 (1989).
20. Romano, F. D., S. G. MacDonald, and J. G. Dobson, Jr. Adenosine receptor coupling to adenylate cyclase of rat ventricular myocyte membranes. *Am. J. Physiol.* **257:**H1088–H1095 (1989).
21. Hershberger, R. E., A. M. Feldman, and M. R. Bristow. A_1-adenosine receptor inhibition of adenylate cyclase in failing and nonfailing human ventricular myocardium. *Circulation* **83:**1343–1351 (1991).
22. Schütz, W., and E. Tuisl. Evidence against adenylate cyclase-coupled adenosine receptors in the guinea pig heart. *Eur. J. Pharmacol.* **76:**285–288 (1981).
23. Böhm, M., R. Brückner, I. Hackbarth, B. Haubitz, R. Linhart, W. Meyer, B. Schmidt, W. Schmitz, and H. Scholz. Adenosine inhibition of catecholamine-induced increase in force of contraction in guinea pig atrial and ventricular heart preparations: evidence against a cyclic AMP- and cyclic GMP-dependent effect. *J. Pharmacol. Exp. Ther.* **230:**483–492 (1984).
24. Brückner, R., A. Fenner, W. Meyer, T.-M. Nobis, W. Schmitz, and H. Scholz. Cardiac effects of adenosine and adenosine analogs in guinea-pig atrial and ventricular preparations: evidence against a role of cyclic AMP and cyclic GMP. *J. Pharmacol. Exp. Ther.* **234:**766–774 (1985).
25. deMazancourt, P., and Y. Giudicelli. Guanine nucleotide- and GTP-dependent N^6-phenylisopropyladenosine stimulation of the membrane-bound cyclic AMP high affinity phosphodiesterase in rat brain. *FEBS Lett.* **167:**142–146 (1984).
26. deMazancount, P., and Y. Giudicelli. Guanine nucleotides and adenosine "R_i"-site analogues stimulate the membrane bound low-K_m cyclic AMP phosphodiesterase of rat adipocytes. *FEBS Lett.* **173:**385–388 (1984).
27. Elks, M. L., M. Jackson, V. C. Manganiello, and M. Vaughn. Effects of N^6-(L-2-phenylisopropyl) adenosine and insulin on cAMP metabolism in 3T3-L1 adipocytes. *Am. J. Physiol.* **252:**C342–C348 (1987).
28. Murayama, T., and M. Ui. Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts. *J. Biol. Chem.* **260:**7226–7233 (1985).
29. Leung, E., and R. D. Green. Density gradient profiles of A_1 adenosine receptors labeled by agonist and antagonist radioligands before and after detergent solubilization. *Mol. Pharmacol.* **36:**412–419 (1989).
30. Leung, E., K. A. Jacobson, and R. D. Green. Analysis of agonist-antagonist interactions at A_1 adenosine receptors. *Mol. Pharmacol.* **38:**72–83 (1990).
31. Marsh, J. D., and D. J. Roberts. Adenylate cyclase regulation in intact cultured myocardial cells. *Am. J. Physiol.* **252:**C47–C54 (1987).
32. Shimizu, H., J. W. Daly, and C. R. Creveling. A radioisotopic method for measuring the formation of adenosine 3′,5′-cyclic monophosphate in incubated slices of brain. *J. Neurochem.* **16:**1609–1619 (1969).
33. Barber, R., K. P. Ray, and R. W. Butcher. Turnover of adenosine 3′,5′-monophosphate in WI-38 cultured fibroblasts. *Biochemistry* **19:**2560–2567 (1980).
34. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58:**541–548 (1974).
35. Kobayashi, S., T. Kitazawa, A. V. Somlyo, and A. P. Somlyo. Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. *J. Biol. Chem.* **264:**17997–18004 (1989).
36. Rasenick, M. M., and R. S. Kaplan. Guanine nucleotide activation of adenylate cyclase in saponin-permeabilized glioma cells. *FEBS Lett.* **207:**296–301 (1986).
37. Cooper, D. M. F., and C. Londos. Evaluation of the effects of adenosine on hepatic and adipocyte adenylate cyclase under conditions where adenosine is not generated endogenously. *J. Cyclic Nucleotide Res.* **5:**289–302 (1979).
38. Thompson, W. J., and M. M. Appleman. Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10:**311–316 (1971).
39. Boudreau, R. J., and G. I. Drummond. A modified assay of 3′,5′ cyclic-AMP phosphodiesterase. *Anal. Biochem.* **63:**388–399 (1975).
40. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
41. Nicholson, C. D., R. A. J. Challiss, and M. Shahid. Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.* **12:**19–27 (1991).
42. Macphee, C. H., D. H. Reifsnyder, T. A. Moore, K. M. Lerea, and J. A. Beavo. Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. *J. Biol. Chem.* **263:**10353–10358 (1988).
43. Xu, D., H. Kong, and B. T. Liang. Expression and pharmacological characterization of a stimulatory subtype of adenosine receptor in fetal chick ventricular myocytes. *Circ. Res.* **70:**56–65 (1992).
44. Parsons, W. J., V. Ramkumar, and G. L. Stiles. Isobutylmethylxanthine stimulates adenylate cyclase by blocking the inhibitory regulatory protein, G_i. *Mol. Pharmacol.* **34:**37–41 (1988).
45. Ramkumar, V., and G. L. Stiles. A novel site of action of a high affinity A_1 adenosine receptor antagonist. *Biochem. Biophys. Res. Commun.* **153:**939–944 (1988).
46. Costa, T., and A. Herz. Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. USA* **86:**7321–7325 (1989).
47. Costa, T., J. Lang, C. Gless, and A. Herz. Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: specific regulation by antagonists and sodium ions. *Mol. Pharmacol.* **37:**383–394 (1990).

Send reprint requests to: Richard D. Green, Department of Pharmacology, M/C 868, University of Illinois at Chicago, 835 S. Wolcott Ave., Chicago, IL 60612.