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α_1-Adrenergic Receptor-Linked Guanine Nucleotide-Binding Protein in Muscle and Kidney Epithelial Cells

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SUMMARY

We have studied the interaction of guanine nucleotides with α_1-adrenergic receptors of two cloned cell lines, the Madin Darby canine kidney (MDCK-D1) cells and BC3H-1 muscle cells. Although guanylylimidodiphosphate, Gpp(NH)p, had no effect on the affinity or the total number of [^{3}H]prazosin-binding sites in membranes prepared from these cells, the nucleotide decreased the apparent affinity of the agonists (−)-epinephrine and (−)-norepinephrine in competing for [^{3}H]prazosin-binding sites in both cell types. A maximal effect of Gpp(NH)p occurred at 10 μM. Guanine nucleotides were significantly more effective in shifting agonist affinity for the α_1 receptor than adenine nucleotides, and Mg^{2+} was required to observe a maximal effect. Binding of agonist to α_1-adrenergic receptors activated phosphatidylinositol (PI) hydrolysis in both cell types but had no effect on membrane adenylate cyclase activity. Incubation of MDCK cells for 19 hr with 100 ng/ml pertussis toxin, which eliminated the ability of pertussis toxin added to membranes to ADP-ribosylate 39–41-KDa substrate(s), failed to alter binding of agonists to α_1-adrenergic receptors, the ability of Gpp(NH)p to regulate agonist binding to these receptors, or epinephrine-stimulated PI hydrolysis and prostaglandin E_2 production. Incubation of BC3H1 cells with pertussis toxin had no effect on the ability of epinephrine to stimulate PI turnover. These results show that binding of agonists to α_1-adrenergic receptors in mammalian kidney and muscle cells is regulated by guanine nucleotides, presumably by interaction with a guanine nucleotide-binding (G) protein. The failure of the G-protein to regulate adenylate cyclase activity and the lack of effect of pertussis toxin to alter receptor-mediated binding or functional activity suggests that a G-protein other than G_s, G_i, or G_o interacts with α_1-adrenergic receptors in kidney and smooth muscle.

A requirement for guanine nucleotides in activation of cells by catecholamines has been associated with adrenergic receptors that regulate adenylate cyclase activity (recently reviewed in Ref. 1). GTP-binding proteins, G_s and G_i, couple β-adrenergic receptors to activation and α_2-adrenergic receptors to attenuation of cAMP formation, respectively (1). In addition, guanine nucleotides decrease the affinity of adrenergic agonists for these two types of receptors (1).

α_1-Adrenergic receptors typically stimulate target cells by mechanisms other than activation or attenuation of adenylate cyclase activity (2). The initial event in agonist occupancy of these receptors appears to be a phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate which leads to formation of diaglycerol and inositol 1,4,5-trisphosphate, both of which may act as intracellular second messengers (2). Molecular details whereby receptor occupancy produces these effects are poorly understood. The possibility that guanine nucleotides regulate binding of agonists to α_1-adrenergic receptors (and, by inference, that receptors would be linked to a G-protein) has been suggested from two types of experiments. First, guanine nucleotides can decrease agonist binding affinity to α_1 receptors in membranes prepared from rat kidney (3, 4), liver (5, 6), and heart (7, 8), although some investigators have been unable to show such regulation (e.g., Refs. 9 and 10). In addition, other evidence implicating a G-protein in the action of hormones that activate phospholipase C is from experiments demonstrating guanine nucleotide-stimulated PI hydrolysis in permeabilized cells and plasma membrane preparations (11, 12).

Studies directed toward identifying the putative G-protein have yielded controversial results. In neutrophils and mast cells, agonist-stimulated changes in phospholipid metabolism (including PI hydrolysis and arachidonic acid release) are virtually eliminated if cells are treated with pertussis toxin [also known as IAP (13, 14)]. These and other results have suggested

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ABBREVIATIONS: G-protein, guanine nucleotide-binding protein [also called N protein by other workers (see Ref. 40)]; G_s, stimulatory guanine nucleotide-regulatory protein; G_i, inhibitory guanine nucleotide-regulatory protein; G_o, 39,000-Da pertussis toxin substrate (described in Refs. 39 and 40); PI, phosphoinositide; IAP, islet-activating protein; DME, Dulbecco's modified Eagle's medium; PGE_2, prostaglandin E_2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Gpp(NH)p, guanylylimidodiphosphate.

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that a G-protein of 41 kDa (perhaps G_i) is a substrate for ADP-ribosylation by pertussis toxin in these cells. Somewhat different results have been obtained in other cell types. For example, in 3T3 fibroblasts, IAP inhibits thrombin-induced decreases in cAMP, increases in release of arachidonic acid, and stimulation of $^{45}Ca^{2+}$ uptake, but not PI hydrolysis or $^{86}Rb^{+}$ uptake (15). Also, IAP treatment does not alter binding of agonists to α_1-adrenergic receptors in acutely isolated cardiac myocytes (8), has no effect on muscarinic cholinergic-stimulated PI hydrolysis in chick heart cells or 1321N1 human astrocytoma cells (16), and has no effect on thyrotropin-releasing hormone-stimulated PI hydrolysis (17).

Our laboratory has utilized cloned cell lines for studying the expression of adrenergic receptors. Two of these cell lines, the Madin Darby canine kidney MDCK-D1 epithelial cell line (18) and the smooth muscle cell line BC3H-1 (19), co-express both α_1- and β_2-adrenergic receptors. Previous studies have documented that α_1 receptors in MDCK cells promote PGE_2 formation, arachidonic acid formation, and enhanced labeling of phosphatidylinositol (20, 21). α_1 Receptors in BC3H-1 cells promote PI hydrolysis and calcium mobilization, and there is a close coupling between receptor occupancy and these responses (22).

In this study, we report that guanine nucleotides regulate agonist binding to α_1-adrenergic receptors prepared from both MDCK-D1 and BC3H-1 cells, and that treatment with pertussis toxin fails to alter binding and cellular effects of α_1-adrenergic agonists. The possibility that a protein, distinct from G_s, G_i, and G_o, mediates α_1-adrenergic receptor responses in these and other cell types is discussed.

Experimental Procedures

Materials. The following compounds were received as gifts from the sources indicated: (−)-epinephrine (Sterling Winthrop Research Institute, Rensselaer, NY); phentolamine mesylate (Geigy Pharmaceutical, Summit, NJ); (−)-propranolol hydrochloride (Ayerst Laboratories, New York, NY); cyanopindolol hydrochloride (Sandoz, Inc., Basel, Switzerland); and prazosin (Pfizer Inc., Groton, CT). *myo*-[^{3}H]Inositol and [^{3}H]prazosin were purchased from Amersham Corp. (Arlington Heights, IL). Formula 946 liquid scintillation cocktail was from New England Nuclear. Cell culture media and sera were from Grand Island Biological Co. (Grand Island, NY). All other reagents were from standard sources.

Cell culture of MDCK cells. Late passage MDCK-D1 cells (18) were grown in DME with 7.5% heat-inactivated horse serum, 2.5% heat-inactivated fetal calf serum, and 15 mM HEPES. Subconfluent cells were subcultured every 3–4 days with a trypsin-EDTA solution by diluting 1:3 into 250-ml culture flasks. For experiments, 250-ml culture flasks were treated with trypsin-EDTA and cells were diluted 1:5 into 50-mm culture dishes. Cells were used after 3–4 days when 70–90% confluence was reached. These culture conditions lead to maximal binding to α_1-adrenergic receptor (data not shown).

Culture of BC3H-1 muscle cells. The BC3H-1 muscle cells used in this study were a gift from Drs. J. P. Mauger and J. Bockaert, College de France (Paris, France). The cell line was originally isolated at the Salk Institute (La Jolla, CA) from a neoplasm extracted from offspring of pregnant mice that had received injections of nitrosoethylurea (23). The cells were routinely grown in Falcon T-flasks in DME supplemented with 10% (v/v) heat-inactivated fetal calf serum and maintained at 37° in a humidified atmosphere of 10% CO_2/90% air. The cells were subcultured at 3–4-day intervals, using cold custom ATV solution (Irvine Scientific) to dissociate them from the plastic substrate. For experimental purposes, approximately 2×10^6 cells were seeded into 150-mm-diameter culture dishes and grown for 4–6 days, at which time they reached confluence.

Membrane preparation. Cell membranes were prepared by hypotonic lysis of BC3H-1 and MDCK-D1 cells grown in culture dishes followed by homogenization. Each dish was initially washed twice with 5 ml of ice-cold isotonic buffer (0.32 M sucrose, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% phenylmethylsulfonyl fluoride). The cells were removed by scraping with a Costar cell scraper, the dishes were washed with 5 ml of the same buffer, and the pooled cells were pelleted at 1,000 × *g* for 10 min. The cells were resuspended in hypotonic buffer (20 mM Tris-HCl, 2 mM EDTA, 0.1% phenylmethylsulfonyl fluoride), 5 ml/dish, and allowed to sit for 10 min on ice. The cells were then homogenized with five strokes of a Potter-Elvehjem Teflon-glass tissue homogenizer; the homogenate was centrifuged at 1,000 × *g* for 10 min. The resulting supernatant was centrifuged at 40,000 × *g* for 15 min, washed three times in hypotonic buffer, and finally resuspended at 2 mg of protein/ml. Membranes were used immediately after preparation.

[^{3}H]Prazosin binding. Binding assays were performed by incubating 0.05–0.1 ml of freshly prepared membranes with 0.05 ml of [^{3}H]prazosin and 0.05 ml of various drugs in a final volume of 0.5 ml in polypropylene test tubes (16 × 105 mm; Walter Sarstadt, Inc., Princeton, NJ). Assays were initiated by the addition of membranes and were usually carried out in incubation buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) for 60 min at 25° in a shaking water bath. In some experiments $MgCl_2$ was omitted as indicated in the text. The incubation was terminated by separating bound from free radioligand by rapid filtration over glass-fiber filters (Whatman GF/C) on a Brandel M-48 cell harvester. The filters were then washed with 20 ml of 10 mM Tris-HCl, pH 7.4. Radioactivity retained on the filters was counted at 40% efficiency in plastic minivials containing 4.5 ml of New England Nuclear Formula 947 liquid scintillation cocktail. Specific binding was defined as the difference in the amount of radioligand bound in the presence and absence of 10 μM phentolamine. Typically, 750–1000 cpm of [^{3}H]prazosin bound to the filters; 10 μM phentolamine reduced that value to 150–200 cpm. [^{3}H]Prazosin binding assays have previously been validated as assays of α_1-adrenergic receptors in membranes prepared from both cell types (18, 19). When catecholamines were added, superoxide dismutase and catalase were added to prevent oxidation (24). The results shown are the mean of triplicate samples. Replicate samples varied less than 10%. Membrane protein content was measured by the method of Lowry *et al.* (25).

[^{3}H]Arachidonic acid release from MDCK cells. For deacylation studies, 40–60% confluent MDCK cells grown in 35-mm dishes were labeled 18 hr overnight with 0.3 μCi/ml [^{3}H]arachidonic acid in 0.5% serum in DME (20 mM HEPES, pH 7.4, 0.5% fetal calf serum). The purpose of the low serum was to increase the specific activity of the arachidonic acid. At the end of the labeling period, cells were washed three times with 0.5% DME; then, 1 ml of 0.5% DME (without bicarbonate), with or without 10 μM epinephrine, was added and cells were incubated at 37° for 20 min. At this time, the medium was aspirated and counted in a liquid scintillation counter.

Simultaneous measurement of PI hydrolysis and PGE_2 production of MDCK cells. The same set of 35-mm dishes was used for simultaneous measurement of PI hydrolysis and PGE_2 formation. The cells were labeled for 48 hr with *myo*-[^{3}H]inositol, 3.0 μCi/ml, in MDCK medium (DME-HCO_3, 10% serum, 20 mM HEPES, pH 7.4). Experiments were performed after cells were washed three times with DME containing 20 mM HEPES, pH 7.4, and 50 mM LiCl. For the incubation, 1.0 ml of medium with various concentrations of hormone was added and cells were incubated at 37° for 20 min. The medium was then aspirated and frozen at −70° for subsequent assay of PGE_2 by radioimmunoassay (New England Nuclear); ^{3}H in the medium did not interfere with the ^{125}I radioimmunoassay. The reaction in cells was terminated with 0.5 ml of cold methanol; the cells were then scraped and transferred to a centrifuge tube. Chloroform and water were then added to a final ratio of $CHCl_3$/MeOH/H_2O (1:1:0.8). The samples were then

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centrifuged and the aqueous phase was applied to AGx8 Dowex columns.

Columns for separation of inositol phosphates were prepared in glass wool plugged Pasteur pipettes with 200 mg of thoroughly washed Dowex (AG1x8-formate form). The aqueous samples were applied and free inositol was eluted with 20 ml of H_2O. Inositol monophosphate was eluted with 10 ml of 150 mM ammonium formate/100 mM formic acid, inositol bisphosphate was eluted with 10 ml of 400 mM ammonium formate/100 mM formic acid, and inositol trisphosphate was eluted with 5 ml of 2 M ammonium formate/100 mM formic acid. These fractions were counted in at least equal volumes of Aquasol.

Measurement of phosphatidylinositol turnover. Phosphatidylinositol turnover was estimated by a method similar to that described earlier for BC3H-1 cells (26). Cells were seeded into 35-mm dishes and grown until confluent. The growth medium was aspirated and replaced with 0.75 ml of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM D-glucose, 25 mM HEPES, pH 7.4, 0.06% (w/v) bovine serum albumin, 4 μCi/ml of *myo*-[^{3}H]inositol, 10 μg/ml of catalase, 10 μg/ml (−) of superoxide dismutase, 1 μM (±)-propranolol with or without 10 μM (−)-epinephrine. Following a 30-min incubation at 37°, the medium was aspirated and the cells were washed with two 1-ml aliquots of buffer that contained no radioligand, drugs, or enzymes. The cells were then covered with 1 ml of ice-cold methanol and removed from the dishes with the aid of a cell scraper. The samples were mixed with 1 ml of chloroform and 0.9 ml of 2 M KCl and centrifuged for 15 min at 2000 × *g* to separate the two phases. One ml of the upper phase was reserved for determination of its content of tritium and the remainder of the upper phase was aspirated. The chloroform was washed twice with 1 ml of chloroform/methanol/2 M KCl (3:48:47) before being transferred to scintillation vials and evaporated to dryness. The amount of radiolabel in the chloroform phase was corrected using the amount of radiolabel in the sample of upper phase, assuming proportional recovery of radiolabel in both phases.

Pertussis toxin-stimulated [^{32}P]ADP-ribosylation of membrane proteins. Pertussis toxin (List Biochemical or kindly provided by Dr. Erik Hewlett, University of Virginia) was activated by incubation for 30 min at 30° with 20 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 50 mM glycine, pH 7.5. Covalent modification of cell membranes was carried out by incubating 50 μg of membrane protein in a total volume of 100 μl containing activated toxin (2 μg), 40 mM potassium phosphate, pH 7.5, 15 μM NAD (2 μCi of [^{32}P]NAD), and 1 mM ATP for 30 min at 30°. The assay was terminated by adding 1.0 ml of ice-cold 10% trichloroacetic acid and after 10 min the samples were centrifuged in a Beckman Microfuge. The supernatants were discarded and the membrane pellets were solubilized by boiling in 50 μl of 50 mM Tris, pH 6.9, 2% SDS, 10% glycerol, and 0.02% bromophenol blue. The samples were applied to 10% SDS-polyacrylamide gels prepared and run as described by Laemmli (27). Molecular weight markers (Bio Rod Catalogue No. 161–0304) were applied to the gel as well and electrophoresis was terminated when the dye front left the bottom of the gel. The gels were stained with 50% methyl alcohol, 3.75% acetic acid, 0.25% Coomassie blue, and then destained in this solvent. The gels were dried and subjected to autoradiography using Kodak XAR5 film at −70°.

Adenylate cyclase activity. Assays (total volume 100 μl) were conducted with freshly prepared membranes plus 40 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 100 μM cAMP, 1.0 mM creatine phosphate, 2.5 units of creatine phosphokinase, 200 μM 3-isobutylmethylxanthine, 100 μM GTP, 200 μM ATP (0.3 μCi of [α-^{32}P]ATP), and other components as indicated. Assays were initiated by addition of 25-μl membranes and proceeded for 20 min at 30°. The reaction was stopped by addition of a solution containing 40 mM ATP, 1.4 mM cAMP ([^{3}H]cAMP, ~10,000 cpm), 2% SDS, and 50 mM Tris-HCl, pH 7.5, and cAMP was isolated as described by Salomon *et al.* (28). Adenylate cyclase activity is expressed as the mean of values from triplicate assays.

Data analysis. The dissociation constant (K_D) and maximum number of binding sites (B_{max}) were determined from Scatchard analysis (29) of saturation binding isotherms. The line of the Scatchard plot was fitted by linear regression analysis.

Competition binding curves were analyzed by a computer program (LIGAND) that performs iterative nonlinear regression (30).

Unless stated otherwise, all results shown here are typical of those obtained in at least three independent experiments.

Results

Radioligand binding to α_1 receptors in BC3H-1 and MDCK-D1 cells. Previous studies from this laboratory have shown that membranes prepared from BC3H-1 and MDCK-D1 cells contain a single class of [^{3}H]prazosin-binding sites

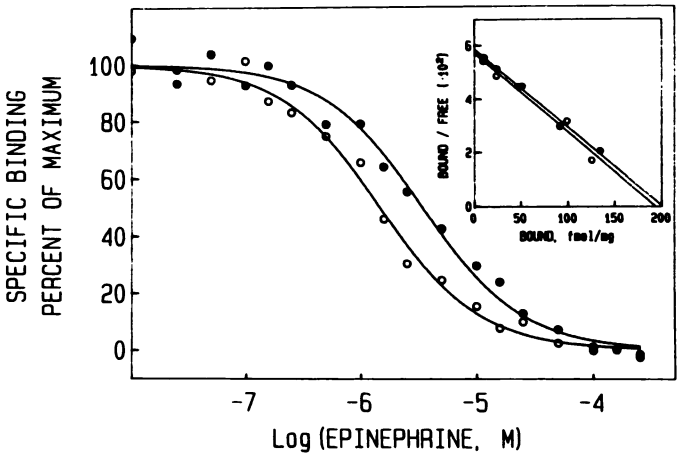

Fig. 1. Effect of Gpp(NH)p on [^{3}H]prazosin binding and (−)-epinephrine competition of [^{3}H]prazosine-binding sites in BC3H-1 cell membranes. Cell membranes were incubated with 0.5 nM [^{3}H]prazosin in the presence of increasing concentrations of (−)-epinephrine in the absence (○) and presence (●) of 0.1 mM Gpp(NH)p. The data were fit by LIGAND to a single class of binding sites, as described in the text. In the *inset*, membranes were incubated with 25–1000 pM [^{3}H]prazosin in the absence (○) and presence (●) of 0.5 mM Gpp(NH)p. Nonspecific binding was determined with 10 μM phentolamine.

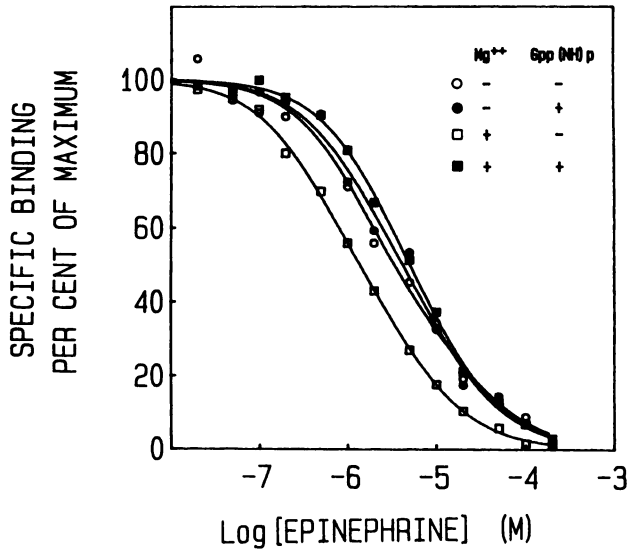

Fig. 2. Magnesium requirement for the effect of Gpp(NH)p on epinephrine competition of [^{3}H]prazosin binding in MDCK-D1 cell membranes. Membranes were incubated with varying concentrations of (−)-epinephrine and 0.5 nM [^{3}H]prazosin in the absence (○, ●) or presence (□, ■) of 10 mM $MgCl_2$ and in the absence (○, □) or presence (●, ■) of 0.5 mM Gpp(NH)p.

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TABLE 1

The effect of Gpp(NH)p on agonist and antagonist binding to BC3H-1 and MDCK-D1 cell membranes

The affinity of α_1 receptors for (−)-epinephrine, (−)-norepinephrine, and phentolamine was determined by computer analysis (30) of ligand competition for [^{3}H]prazosin binding (0.5 nM) to membranes prepared from both cell types. The affinity of the receptors for prazosin was determined by Scatchard analysis (29) of [^{3}H]prazosin binding as described in Experimental Procedures.

	K_D	
	−Gpp(NH)p	+Gpp(NH)p
	nM	
MDCK-D1 cells		
(−)-Epinephrine	110	565
(−)-Norepinephrine	360	1250
Prazosin	0.076	0.078
Phentolamine	48	58
BC3H-1 cells		
(−)-Epinephrine	120	610
(−)-Norepinephrine	890	2400
Prazosin	0.22	0.22
Phentolamine	52	60

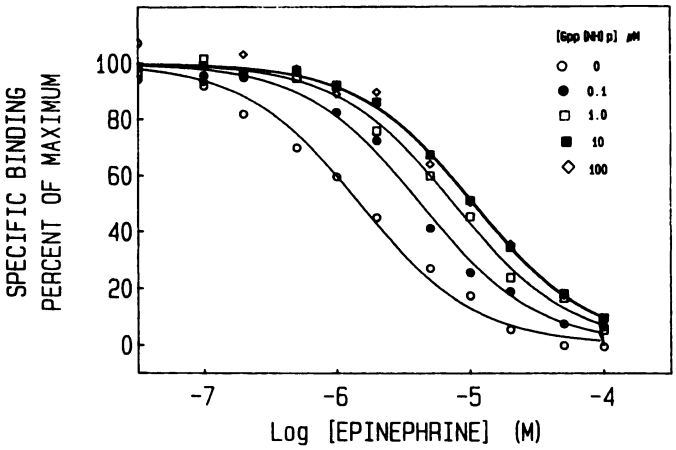

Fig. 3. Effect of Gpp(NH)p on (−)-epinephrine competition of [^{3}H]prazosin-binding sites in MDCK-D1 cell membranes. This experiment was done as described in Fig. 1, but with MCCK-D1 cell membranes and in the presence of varying concentrations of Gpp(NH)p as indicated. The concentration of [^{3}H]prazosin was 0.5 nM.

TABLE 2

The effect of α_1 agonists on adenylate cyclase activity in BC3H-1 cell membranes

Adenylate cyclase activity in BC3H-1 cell membranes was carried out as described in Experimental Procedures. The concentrations of ligands used were: (−)-epinephrine, 50 μM; (−)-isoproterenol, 50 μM; phentolamine, 10 μM; (−)-propranolol, 5 μM.

Addition	Receptor occupancy	Adenylate cyclase activity
		pmol cAMP/min/mg protein
None		42 ± 2.3
Epinephrine	α_1, β	118 ± 7.2
Epinephrine, phentolamine	β	117 ± 8
Epinephrine, propranolol	α_1	43 ± 1.7
Isoproterenol	β	120 ± 10.4
Isoproterenol, epinephrine	α_1, β	108 ± 2.3

that have the pharmacological properties expected of α_1-adrenergic receptors (18, 19). The *inset* to Fig. 1 shows that addition of 0.5 mM Gpp(NH)p had no effect on the affinity or the total number of [^{3}H]prazosin-binding sites to BC3H-1 cell membrane receptors. By contrast, 0.1 mM Gpp(NH)p decreased affinity of the agonist (−)-epinephrine in competing for [^{3}H]

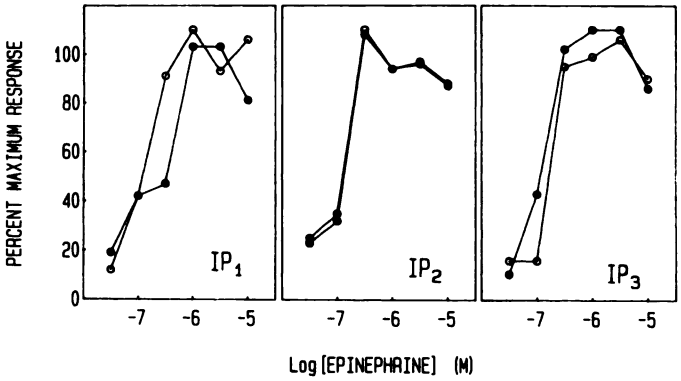

Fig. 4. Epinephrine-stimulated inositol phosphate (*IP*) formation in control and pertussis toxin-treated MDCK-D1 cells. Cells were incubated with *myo*-[^{3}H]inositol (3.0) μCi/ml) for 48 hr. Pertussis toxin (100 ng/ml) was added to one half of the cells for the final 19 hr and cells were incubated with an additional 100 ng/ml for the final 3 hr. Cells were washed three times with DME/HEPES (15 mM, pH 7.4) containing 50 mM LiCl. Varying concentrations of (−)-epinephrine were then incubated with cells for 20 min and inositol phosphate formation was measured in both control (○) and pertussis toxin-treated (●) cells. The 0% and 100% values for IP_1 correspond to 1580 cpm and 4360 cpm, respectively. For IP_2 they were 220 cpm and 730 cpm. For IP_3 they were 405 cpm and 1570 cpm. Each data point represents the mean of triplicate samples. The values within each set of triplicate samples did not vary by more than 10%.

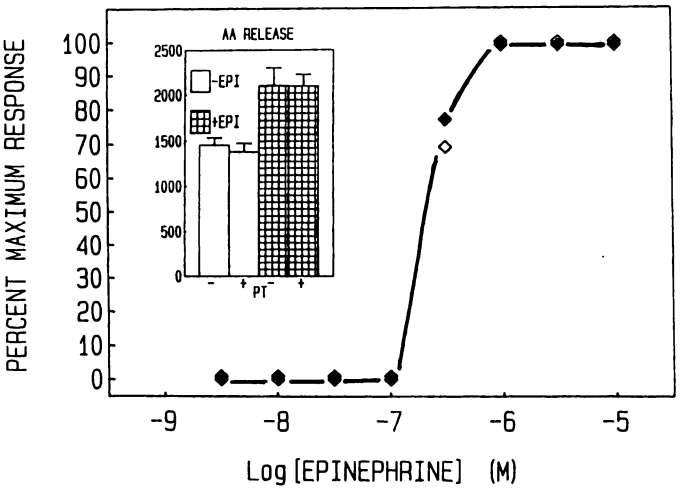

Fig. 5. Effect of pertussis toxin on epinephrine-stimulated arachidonic acid release and PGE_2 formation in MDCK-D1 cells. Cells were incubated with pertussis toxin as described in the legend to Fig. 4. Epinephrine-stimulated PGE_2 formation was measured in both control (◇) and pertussis toxin-treated (◆) cells. The *inset* shows the effect of pertussis toxin (*PT*) treatment of cells on subsequent epinephrine (*EPI*)-stimulated arachidonic acid (*AA*) release. For PGE_2 formation, the 100% value represents 6.4 pg of PGE_2 per dish. Epinephrine-stimulated PGE_2 formation and arachidonic acid release were determined in separate experiments done on separate days.

prazosin sites in membranes prepared from this cell type (Fig. 1). Thus, Gpp(NH)p increased the IC_{50} for competition by epinephrine from 1450 nM to 3400 nM. Computer analysis using LIGAND showed that the experimental data for competition by epinephrine in both the absence and presence of Gpp(NH)p were best fit by a model described by one class of binding sites with K_D values of 610 nM and 565 nM, respectively. In neither case did models with two classes of sites more accurately fit the experimental data. Fig. 2 shows that Gpp(NH)p also regulated binding of epinephrine to α_1-adrenergic receptors in MDCK-D1 cell membranes. In the absence of guanine nucleotides,

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TABLE 3

Phosphatidylinositol turnover in control and pertussis toxin-treated BC3H-1 cells

Cells were incubated with and without 200 ng/ml pertussis toxin for 16 hr at which time another 200 ng/ml toxin was added after 3 hr. The growth medium was aspirated and phosphatidylinositol turnover was measured in both control and pertussis-treated cells as described in Experimental Procedures. Triplicate samples were used for each piece of data and the results of two experiments done on separate days are given. Column A gives the average cpm in the chloroform phase for the triplicate samples. Column B gives the average cpm incorporated into the cells. For each sample, the amount of label in the chloroform phase was divided by the total amount in the cell. This ratio was averaged for the triplicate samples and the averaged ratio is given in column C. The value in column C for epinephrine-treated cells divided by that for cells not incubated with agonist is given in column D.

	A cpm of Chloroform	B Total cpm of cells	C Ratio $\times 10^{-3}$	D-Fold stimulation
Experiment 1				
Control cells				
−Epinephrine	804	208,000	3.85 ± 0.9	
+Epinephrine	2,520	202,400	12.45 ± 1.85	3.23
Toxin-treated cells				
−Epinephrine	508	189,400	2.7 ± 0.65	
+Epinephrine	1,652	171,200	9.65 ± 0.9	3.57
Experiment 2				
Control cells				
−Epinephrine	241	95,600	2.5 ± 0.6	
+Epinephrine	1,240	93,500	13.35 ± 0.85	5.3
Toxin-treated cells				
−Epinephrine	191	82,200	2.3 ± 0.2	
+Epinephrine	901	85,000	10.6 ± 0.3	4.6

epinephrine competition for [^{3}H]prazosin-binding sites could be fit to a single class of binding sites with a K_D = 110 nM epinephrine. Addition of 0.5 mM Gpp(NH)p increased the K_D to 565 nM. Table 1 summarizes the effect of 0.1 mM Gpp(NH)p on agonist (epinephrine, norepinephrine) and antagonist (prazosin, phentolamine) binding in BC3H-1 and MDCK-D1 cell membranes. It is clear from the data that the effect of Gpp(NH)p is specific for agonists.

An effect of guanine nucleotides on agonist binding to α_1 receptors has been reported in other membrane systems (3–8), although some investigators have failed to observe such an effect (9, 10). We also failed initially to observe an effect of guanine nucleotides on α_1 agonist binding in membranes from MDCK-D1 (18) and BC3H-1 muscle cells (19). One change we made which was important in bringing out the effect of guanine nucleotide was the inclusion of protease inhibitors and EDTA during membrane preparation, as well as repeated washing of the membranes. Our results are consistent with the report of Lynch *et al.* (6), who observed no effect of Gpp(NH)p on agonist binding in liver membranes when the membranes were prepared in buffer that lacked protease inhibitors and contained calcium. Lynch *et al.* (6) concluded that, under these conditions, a calcium-dependent protease inhibits the effect of Gpp(NH)p.

The ability of guanine nucleotides to regulate agonist binding to β- and α_2-adrenergic receptors is Mg^{2+} dependent (1). In the absence of Mg^{2+}, we found (Fig. 2) that the IC_{50} for competition by epinephrine for [^{3}H]prazosin binding to MDCK cell membranes was 2960 nM and that addition of 0.5 mM Gpp(NH)p had little effect on affinity of epinephrine. In contrast to these results, the presence of 10 mM $MgCl_2$ in the absence of Gpp(NH)p increased the affinity of epinephrine (IC_{50} = 1320 nM) for the receptor. Addition of 0.5 mM Gpp(NH)p together with 10 mM $MgCl_2$ decreased the affinity of epinephrine and yielded a binding curve similar to that seen in the absence of Mg^{2+}. Thus, detection of an effect of guanine nucleotides on agonist binding to α_1 receptor binding to α_1 receptors appears to be dependent on the presence of Mg^{2+}.

The dose dependence for Gpp(NH)p in altering agonist binding to α_1 receptors in MDCK-D1 membranes (Fig. 3) indicated that as low as 0.1 μM significantly decreased agonist affinity to the receptor and that a maximal effect was seen at 10 μM. The effect of other guanine nucleotide as well as adenine nucleotides was tested for (data not shown) in the following manner. MDCK-D1 cell membranes were incubated with 0.5 nM [^{3}H] prazosin, 3 μM epinephrine, and a 100 μM concentration of the desired nucleotide. In the absence of nucleotide, 350 ± 16 cpm bound to the filters; the addition of 5′-adenylylimidodiphosphate increased that value of 535 ± 11 cpm, indicating a reduced affinity of the receptor for agonist. GTP and guanosine 5′-*O*-(3-thiotriphosphate) produced an effect almost identical to that of Gpp(NH)p. The addition of Gpp(NH)p and ATP yielded 420 ± 4 and 380 ± 14 cpm, respectively; ADP and GDP yielded values not significantly different from those of samples not incubated with nucleotide. We conclude that the observed effect is selective guanine nucleotides (data not shown).

The results presented thus far suggest that α_1 receptors in muscle and kidney cells interact with a protein having characteristics similar to those of G_s and G_i, proteins which mediate stimulation and attenuation of adenylate cyclase activity, respectively. In view of this, a series of experiments was done 1) to clarify the functional consequences of the α_1 receptor-associated protein, and 2) to test whether the GTP-binding protein is distinct from or identical to previously described G-proteins.

α_1-Adrenergic-mediated functional activity. Although guanine nucleotides are able to mediate functional activity of adrenergic agonists that activate or attenuate adenylate cyclase activity, we found that agonist occupancy of α_1 receptors in BC3H-1 cell membranes had no effect of basal or β-adrenergic-stimulated adenylate cyclase activity (Table 2). Thus, adenylate cyclase activity in the presence of epinephrine plus propranolol (occupancy of α_1 receptors) was the same as basal activity, and activity in the presence of epinephrine (occupancy of both α_1 and β receptors) was the same as that seen with epineprhine plus phentolamine or isoproterenol (occupancy of β-adrenergic receptors). In addition, occupancy of α_1 receptors by agonists had no effect on forskolin-stimulated adenylate cyclase activity in BC3H-1 cell membranes, or on basal activity or forskolin-stimulated activity in MDCK-D1 cell membranes (data not shown).

As shown in Figs. 4 and 5, epinephrine produced a concentration-dependent enhancement in PI hydrolysis and PGE_2 production by MDCK-D1 cells. Agonist binding to α_1-adrenergic receptor in BC3H-1 cells stimulates phosphatidylinositol turnover (Table 3) and has previously been reported to stimulate PI hydrolysis in those cells (26).

The effect of pertussis toxin on α_1 agonist-mediated functional activity. Pertussis toxin inhibits the action of hormones that attenuate adenylate cyclase activity by catalyzing the ADP-ribosylation of G_i, and IAP also decreases the binding affinity of agonists and agonist-stimulated PI hydrolysis and arachidonic acid release in some systems (13–15). We therefore tested the ability of pertussis toxin to alter agonist binding in MDCK cell membranes and agonist-mediated functional activity in intact cells. Fig. 6 shows that treatment of

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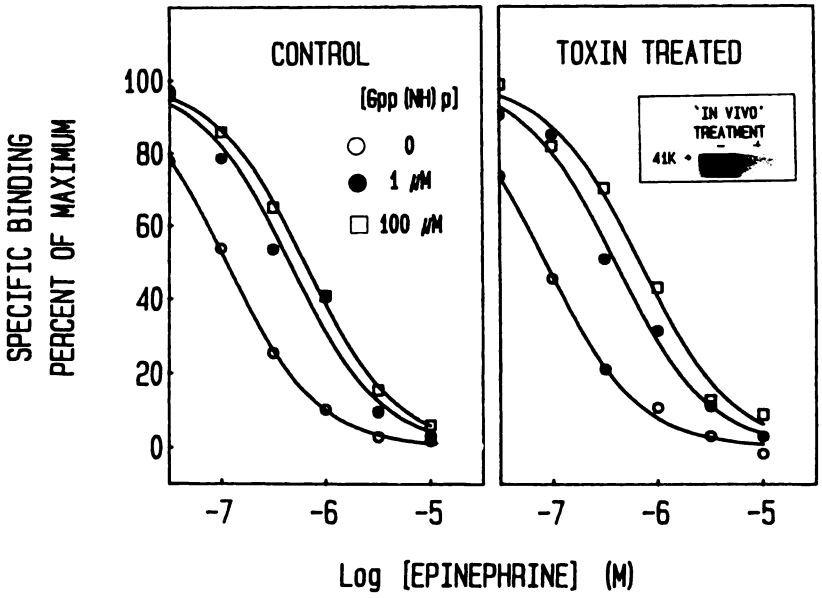

Fig. 6. The effect of pertussis toxin treatment of intact MDCK-D1 cells on Gpp(NH)p regulation of (−)-epinephrine competition for [^{3}H]prazosin-binding sites in cell membranes. Pertussis toxin (100 ng/ml) was added to 40–60% confluent MDCK-D1 cells. After 16 hr another 100 ng/ml of the toxin were added, and the cells were incubated for a further 3 hr. Membranes were prepared from both control and toxin-treated cells. The effect of Gpp(NH)p on (−)-epinephrine competition of [^{3}H]prazosin-binding sites was measured as described in Fig. 1 with no (○), 1 μM (●), or 100 μM (□) Gpp(NH)p. The *inset* shows the result of ADP-ribosylation of MDCK-D1 cell membranes with [^{32}P]NAD. The membranes used were from the same preparation used for measuring [^{3}H]prazosin binding. The area of the autoradiogram surrounding the 41-kDa proteins in this experiment is shown; autoradiography was done for 3 days.

intact MDCK-D1 cells with toxin IAP had no effect on the ability of Gpp(NH)p to regulate agonist binding in membranes prepared from these cells. In this experiment, membranes were prepared from cells that had been incubated with 100 ng/ml pertussis toxin for 16 hr and with an additional 100 ng/ml toxin for an additional 3 hr. We also tested the effect of pertussis toxin treatment on functional activity in MDCK-D1 cells. As shown in Figs. 4 and 5, pertussis toxin treatment had no effect on epinephrine-stimulated PI hydrolysis, arachidonic acid release, or PGE_2 production.

We also tested the effect of pretreating BC3H-1 cells with pertussis toxin on α_1-receptor agonist-mediated functional activity (Table 3). In control cells, 10 μM epinephrine stimulated the incorporation of [^{3}H]inositol into membranes (phosphatidylinositol turnover) some 3–5-fold. Although the total amount of [^{3}H]inositol incorporated into (the chlorformphase) lipids in the presence of agonist was decreased in toxin-treated cells, (Table 3, column A, +epinephrine), both the amount of label incorporated into the cells and the basal (absence of agonist) level of turnover were decreased (Table 3, columns B and A, −epinephrine). When the total amount of label within the cells was taken into account, little difference could be observed in the ability of epinephrine to stimulate phosphatidylinositol turnover in toxin treated and control cells (Table 3, columns C and D).

In order to be sure that the conditions used for incubation of intact cells with toxin allowed maximal covalent modification of potential substrates, we incubated membranes from both toxin-treated and control cells with [^{33}P]NAD and activated toxin and used SDS-PAGE autoradiography to test for loss of ability of pertussis toxin to ADP-ribosylate membranes from pertussis toxin-treated cells. Fig. 7 shows ADP-ribosylation of a 41-kDa protein in membranes prepared from control cells, but no apparent labeling was observed in toxin-treated MDCK-D1 or BC3H-1 cells. When the 35–45-kDa region of the dried gels was counted, no cpm were detected in the membranes prepared from toxin-pretreated cells, whereas 360 cpm were detected in membranes from control MDCK-D1 cells and 380 cpm were detected from control BC3H-1 cells. Assuming that we could have detected 15 cpm incorporated, we estimate that greater than 95% [(360 − 15) − 360 = 95.8%] of pertussis toxin substrates are modified by incubation of cells with toxin. This result, together with a lack of an effect of pertussis toxin on α_1 receptor-mediated functional activity, suggests that the α_1 receptor-associated G-protein is not a substrate for pertussis toxin.

Discussion

The results presented in this paper show that α_1-adrenergic receptors in membranes from MDCK-D1 and BC3H-1 cells exist in at least two forms which differ in their affinities for agonists. The high affinity form of the receptor is observed when radioligand binding is done in the absence of guanine nucleotide; addition of Gpp(NH)p or GTP causes a severalfold increase in the K_D for epinephrine binding. The ability of guanine nucleotides to alter agonist binding to α_1 receptors is similar in some respects to effects of guanine nucleotides on β- and α_2-adrenergic receptors. Thus, α_1-adrenergic receptors as well as other classes of adrenergic receptors show a similar requirement for Mg^{2+} in order to observe an effect of guanine nucleotides on agonist binding (1). In the absence of Mg^{2+} the

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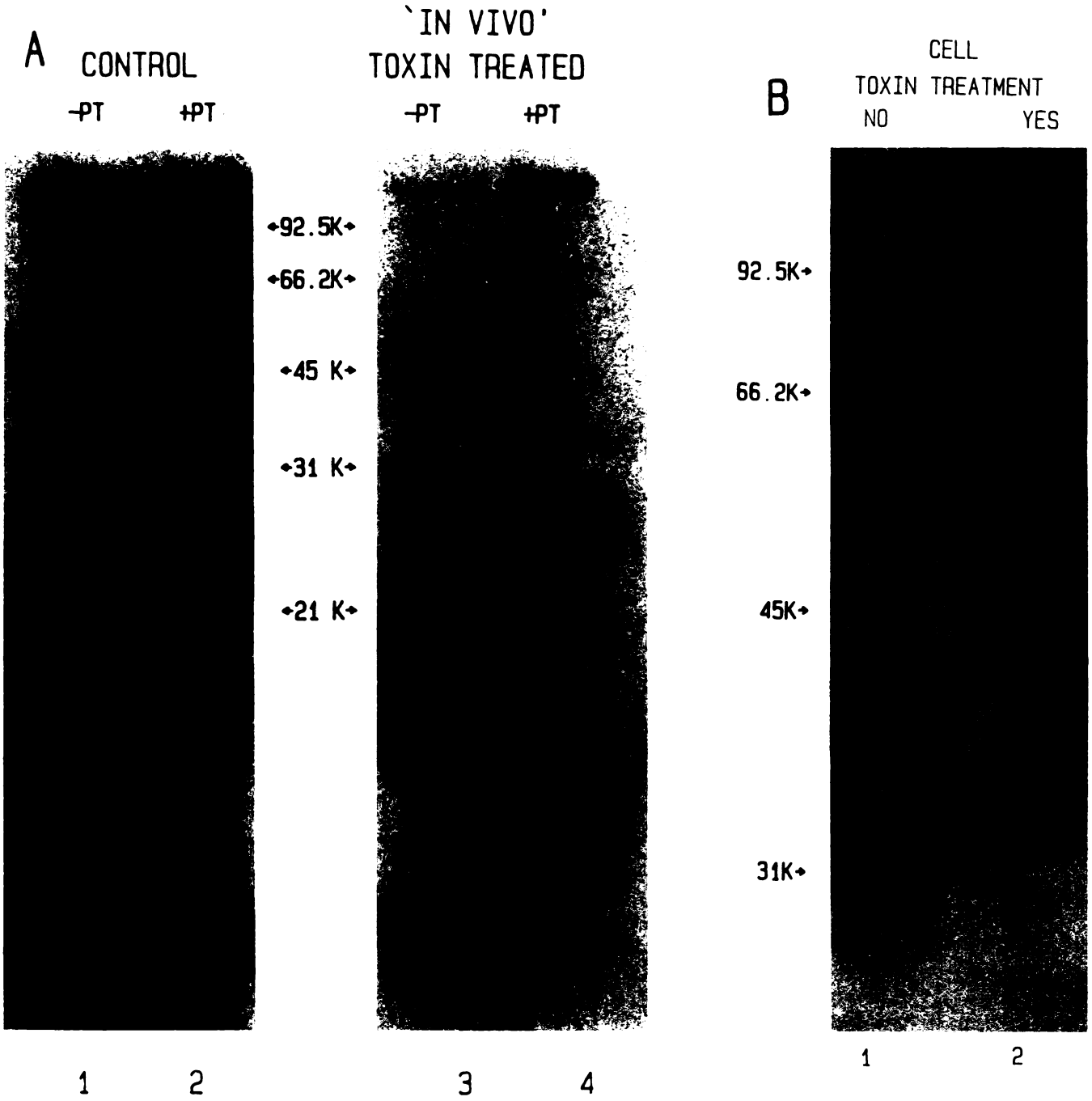

Fig. 7. The effect of pertussis toxin (*PT*) treatment of intact MDCK-D1 and BC3H-1 cells on subsequent [^{32}P]ADP-ribosylation. MDCK-D1 (50% confluent) and BC3H-1 cells (90% confluent) were incubated for 16 hr with pertussis toxin (100 ng/ml and 200 ng/ml, respectively) at which time another aliquot of toxin was added and the cells were incubated for another 3 hr. Membranes were prepared from both control and toxin-treated cells and [^{32}P]ADP-ribosylation of both sets of membranes was carried out as described in Experimental Procedures. A and B show the results for MDCK-D1 and BC3H-1 cells, respectively. Activated toxin was added in samples shown in *lanes 2* and *4* of A and samples from both lanes in B. A 10% acrylamide gel was used for MDCK-D1 cells, whereas a 7% gel was used for BC3H-1 cells. For A (MDCK-D1 membranes), autoradiography was done for 2 days; for B it was 4 days.

α_1 receptor in MDCK-D1 membranes binds agonist in a low affinity manner, and addition of 10 mM $MgCl_2$ shifts the affinity state of the receptor to a high affinity form. In the presence of both Mg^{2+} and guanine nucleotide, only the low affinity state is observed. Birnbaumer *et al.* (1), in describing this effect for β-adrenergic receptors, have suggested that guanine nucleotides either block an Mg^{2+}-dependent alteration in the affinity state of the receptor or reverse this process. A second similarity is that guanine nucleotides are much more effective than adenine nucleotides in altering agonist binding to α_1 receptors and other classes of adrenergic receptors. In addition, the concentration of guanine nucleotide required to observe maximal effects in this study (10 μM) is similar to that which has been reported to regulate agonist binding to several other types of receptors that bind agonist in a guanine nucleotide-dependent manner. Thus, 1–10 μM Gpp(NH)p caused maximal shifts in binding of agonists to β-adrenergic receptors (1), α_2-adrenergic receptors

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(31), muscarinic cholinergic receptors (32), and opiate receptors (33).

Computer analysis of agonist competition for [^{3}H]prazosin binding demonstrated a single class of binding sites in both the presence (low affinity state) and absence of guanine nucleotide (high affinity state). The results differ from those previously reported on β- and α_2-adrenergic (1, 34, 35) receptors where two affinity states of the receptor best explain the data seen in the absence of guanine nucleotide. The addition of guanine nucleotide in these studies destabilizes the high affinity form of the receptor. Computer modeling of α_1 agonist binding in the absence of guanine nucleotide in some systems (5, 6, 8) has revealed two affinity states. It is not clear why our results on α_1 agonist binding to MDCK-D1 and BC3H-1 cell membranes yield only a single affinity state.

The effect of guanine nucleotides on hormonal regulation of adenylate cyclase activity is mediated by the GTP-binding proteins G_s and G_i. The similarities between the effect of guanine nucleotides on α_1 agonist binding and the effect reported for other receptors would suggest that a G-protein is associated with α_1 receptors. Rather than altering adenylate cyclase activity, however, α_1-adrenergic receptors in MDCK and BC3H-1 cells appear to activate phospholipase C, and perhaps phospholipase A_2 as well.[1] Thus, our results imply that the coupling between α_1 receptors and phospholipase C (and perhaps phospholipase A_2)[1] involves a G-protein. Exton and colleagues previously proposed that effects of guanine nucleotides on agonist binding to α_1 receptors in liver may represent a linkage of these receptors with adenylate cyclase under certain conditions (2, 36), although alternative suggestions have been provided more recently (37).

Because of evidence that pertussis toxin treatment can inhibit compound 48/80-stimulated PI hydrolysis and arachidonic acid release in mast cells (13) and formyl-Met-Leu-Phe-dependent arachidonic acid release in neutrophils (14), and because of the close correlation between inhibition of these responses and ADP-ribosylation of a 41-kDa protein, other investigators have suggested that G_i or another IAP substrate of M_r = 41,000 is responsible for these effects. However, in other systems, e.g., chick heart cells and 1321N1 human astrocytoma cells (16, 17), cardiomyocytes (8), and liver membranes (37), pertussis toxin has no effect on hormone-stimulated PI hydrolysis.

In view of these contradictory data regarding pertussis toxin, G-proteins, and phospholipid metabolism, we reasoned that MDCK-D1 and BC3H-1 cells would provide a particularly useful system for studying effects of pertussis toxin on cellular function because these continuously growing cells could be incubated with toxin for extended periods so as to maximally modify substrates. Maximal modification of IAP substrates is crucial for accessing the effect of toxin on functional responses, as emphasized by a recent publication by Pobiner *et al.* (38). These authors showed that >80% ADP-ribosylation of G_i was required before a reduction in angiotensin-mediated inhibition of adenylate cyclase activity could be observed by IAP in hepatocytes. In our studies we incubated MDCK-D1 and BC3H-1 cells 16 hr with 100 ng/ml or 200 ng/ml pertussis toxin followed by an additional 3-hr incubation with fresh toxin; these protocols produced maximal covalent modification of the 41-kDa substrate (Fig. 6, *inset*, and Fig. 7). Even under these conditions we found no alteration in the guanine nucleotide-mediated shift in agonist binding to α_1-adrenergic receptors or in epinephrine-stimulated PI hydrolysis, PGE_2 formation, or arachidonic acid release. These results support the notion that guanine nucleotides exert their effect on α_1 receptor functional response by a mechanism independent of G_i [and perhaps G_o, which is also a substrate for pertussis toxin (39, 40)]. We conclude that a G-protein, distinct from G_s and G_i, is likely to be a key membrane component that links agonist occupancy of receptors to α_1-adrenergic-mediated alterations in phospholipid metabolism and presumably other cellular responses in kidney, muscle, and perhaps other cell types as well.

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References

1. Birnbaumer, L., J. Codina, R. Mattera, R. A. Cerione, J. D. Hildebrandt, T. Sunyer, F. J. Rojas, M. G. Caron, R. J. Lefkowitz, and R. Iyengar. Regulation of hormone receptors and adenylyl cyclases by guanine nucleotide binding. *Recent Prog. Horm. Res.* **41**:41–99 (1985).
2. Exton, J. Mechanisms involved in α-adrenergic phenomena. *Am. J. Physiol.* **248**:E633–E647 (1985).
3. Snavely, M. D., and P. A. Insel. Characterization of alpha-adrenergic receptor subtypes in the rat renal cortex. *Mol. Pharmacol.* **22**:532–546 (1982).
4. Boyer, J. L., A. Garcia, C. Posadas, and J. A. Garcia-Sainz. Differential effect of pertussis toxin on the affinity state for agonists of renal α_1- and α_2-adrenoceptors. *J. Biol. Chem.* **259**:8076–8079 (1984).
5. Goodhardt, M., N. Ferry, P. Geynet, and J. Hanoune. α_1-Adrenergic receptors show agonist-specific regulation by guanine nucleotides. *J. Biol. Chem.* **257**:11577–11583 (1982).
6. Lynch, C. J., R. Charest, P. F. Blackmore, and J. H. Exton. Studies on the hepatic α_1-adrenergic receptor. *J. Biol. Chem.* **260**:1593–1600 (1985).
7. Colucci, W. S., M. A. Gimbrone, Jr., and R. W. Alexander. Regulation of myocardial and vascular α-adrenergic receptor affinity. *Circ. Res.* **55**:78–88 (1984).
8. Buxton, I. L. O., and L. L. Brunton. Studies on the hepatic α_1-adrenergic receptor. *J. Biol. Chem.* **260**:6733–6737 (1985).
9. Hoffman, B. B., T. Michel, D. Mullikin-Kilpatrick, R. J. Lefkowitz, M. E. M. Tolbert, H. Gilman, and J. N. Fain. Agonist versus antagonist binding to α_1-adrenergic receptors. *Proc. Natl. Acad. Sci. USA* **77**:4569–4573 (1980).
10. Cornett, L. E., D. W. Ball, and J. S. Norris. α-Adrenergic receptors of a smooth muscle cell line: guanine nucleotides do not regulate agonist affinities. *J. Recept. Res.* **2**:601–615 (1982).
11. Cockcroft, S., and B. D. Gomperts. Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature (Lond).* **314**:534–536 (1985).
12. Smith, C. D., B. C. Lane, I. Kusaka, M. W. Verghese, and R. Snyderman. Chemoattractant receptor induced hydrolysis of phosphatidylinositol 4,5-bisphosphate in human polymorphonuclear leukocyte membranes. *J. Biol. Chem.* **260**:5875–5878 (1985).
13. Nakamura, T., and M. Ui. Simultaneous inhibitions of inositol phospholipid breakdown, arachidonic acid release, and histamine secretion in mast cells by islet-activating protein, pertussis toxin. *J. Biol. Chem.* **260**:3584–3593 (1985).
14. Bokoch, G. M., and A. G. Gilman. Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. *Cell* **39**:301–308 (1984).
15. 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).
16. Hoffman, B. B., D. F. Dukes, and R. J. Lefkowitz. Alpha-adrenergic receptors in liver membranes: delineation with subtype selective radioligands. *Life Sci.* **28**:265–272 (1981).
17. Martin, T. F. J., D. O. Lucas, S. M. Bajjalieh, and J. A. Kowalchyk. Thyrotropin-releasing hormone activates a Ca^{2+}-dependent polyphosphoinositide phosphodiesterase in permeable GH_3 cells. *J. Biol. Chem.* **261**:2918–2927 (1986).
18. Meier, K. E., M. D. Snavely, S. L. Brown, J. H. Brown, and P. A. Insel. α_1- and β_2-Adrenergic receptor expression in the Madin-Darby kidney, epithelial cells. *J. Cell Biol.* **97**:405–415 (1983).
19. Hughes, R. J., M. R. Boyle, R. D. Brown, P. Taylor, and P. A. Insel. Characterization of coexisting alpha$_1$- and beta$_2$-adrenergic receptors on a cloned muscle cell line, BC3H-1. *Mol. Pharmacol.* **22**:258–266 (1982).
20. Meier, K. E., D. M. Sperling, and P. A. Insel. Agonist-mediated regulation of α_1- and β_2-adrenergic receptors in cloned MDCK cells. *Am. J. Physiol.* **246**:C69–C77 (1985).
21. Slivka, S. R., K. E. Meier, and P. A. Insel. Alpha-adrenergic stimulation of

[1] S. R. Slivka and P. A. Insel, manuscript in preparation.

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MDCK cells elicits faster arachidonic acid release than phosphatidylinositol breakdown. *Fed. Proc.* **44**:508 (1985).
22. Brown, R. D., K. D. Berger, and P. Taylor. α_1-Adrenergic receptor activation mobilizes cellular Ca^{2+} in a muscle cell line. *J. Biol. Chem.* **259**:7554–7562 (1984).
23. Schubert, D., A. J. Harris, C. E. Devine, and S. Heinemann. Characterization of a unique muscle cell line. *J. Cell Biol.* **61**:398–413 (1974).
24. Mahan, L. C., and P. Insel. Use of superoxide dismutase and catalase to protect catecholamines from oxidation in tissue culture studies. *Anal. Biochem.* **136**:208–216 (1984).
25. 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).
26. Ambler, S. K., R. D. Brown, and P. Taylor. The relationship between phosphatidylinositol metabolism and mobilization of intracellular calcium elicited by alpha$_1$-adrenergic receptor stimulation in BC3H-1 muscle cells. *Mol. Pharmacol.* **26**:405–413 (1984).
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
28. Salomon, Y., C. Londos, and M. Rodbell. (1984) A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:54–48 (1948).
29. Scatchard, G. The attraction of protein for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
30. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
31. Tsai, B. S., and R. J. Lefkowitz. Agonist-specific effects of guanine nucleotides on alpha-adrenergic receptors in human platelets. *Mol. Pharmacol.* **16**:61–68 (1979).
32. Berrie, C. P., N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Guanine nucleotides modulate muscarinic receptor binding in the heart. *Biochem. Biophys. Res. Commun.* **87**:1000–1005 (1979).
33. Blume, A. J. Interaction of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides. *Proc. Natl. Acad. Sci. USA* **775**:1713–1717 (1978).
34. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist specific binding properties of the adenylate clyclase coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
35. Michel, T., B. B. Hoffman, and R. J. Lefkowitz. Differential regulation of the α_2-adrenergic receptor by Na^+ and guanine nucleotides. *Nature (Lond).* **288**:709–711 (1980).
36. Morgan, N. G., P. F. Blackmore, and J. H. Exton. Age-related changes in the control of hepatic cyclic AMP levels by α_1- and β_2-adrenergic receptors in male rats. *J. Biol. Chem.* **258**:5103–5109 (1983).
37. Lynch, C. J., V. Prpic, P. F. Blackmore, and J. H. Exton. Effect of islet-activating pertussis toxin on the binding characteristics of Ca^{2+}-mobilizing hormones and on agonist activation of phosphorylase in hepatocytes. *Mol. Pharmacol.* **29**:196–203 (1986).
38. Pobiner, B. F., E. C. Hewlett, and J. C. Garrison. Role of Ni in coupling angiotension receptors to inhibition of adenylate cyclase in hepatocytes. *J. Biol. Chem.* **260**:16200–16209 (1985).
39. Sternweis, P. C., and J. D. Robishaw. Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* **259**:13806–13813 (1984).
40. Neer, E. J., J. M. Lok, and L. G. Wolf. Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. *J. Biol. Chem.* **259**:14222–14229 (1984).

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