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α_2-Adrenergic Receptor Stimulation of Phospholipase A_2 and of Adenylate Cyclase in Transfected Chinese Hamster Ovary Cells Is Mediated by Different Mechanisms

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SUMMARY

The effect of α_2-adrenergic receptor activation on adenylate cyclase activity in Chinese hamster ovary cells stably transfected with the α_{2A}-adrenergic receptor gene is biphasic. At lower concentrations of epinephrine forskolin-stimulated cyclic AMP production is inhibited, but at higher concentrations the inhibition is reversed. Both of these effects are blocked by the α_2 antagonist yohimbine but not by the α_1 antagonist prazosin. Pretreatment with pertussis toxin attenuates inhibition at lower concentrations of epinephrine and greatly potentiates forskolin-stimulated cyclic AMP production at higher concentrations of epinephrine. α_2-Adrenergic receptor stimulation also causes arachidonic acid mobilization, presumably via phospholipase A_2. This effect is blocked by yohimbine, quinacrine, removal of extracellular Ca^{2+}, and pretreatment with pertussis toxin. Quinacrine and removal of extracellular Ca^{2+}, in contrast, have no effect on the enhanced forskolin-stimulated cyclic AMP production. Thus, it appears that the α_2-adrenergic receptor in these cells can simultaneously activate distinct signal transduction systems; inhibition of adenylate cyclase and stimulation of phospholipase A_2, both via G_i, and potentiation of cyclic AMP production by a different (pertussis toxin-insensitive) mechanism.

In most systems, activation of the α_2-adrenergic receptor results in the inhibition of adenylate cyclase activity. This phenomenon was first reported in membranes from the human platelet (1) and has since been reported in many cells and tissues, including NG108 neuroblastoma × glioma cells, HT29 human colon carcinoma cells, OK opossum kidney cells, pancreatic islets, and human adipocytes (2–6). Inhibition of adenylate cyclase by the α_2-adrenergic receptor is mediated by the G protein G_i (5, 7). Pretreatment of cells with pertussis toxin, which ADP-ribosylates the α subunit of G_i, causes attenuation of the inhibition by an α_2 agonist (8). It has become clear, however, that in many systems a decrease in cyclic AMP levels does not account for the physiological response brought about by α_2-adrenergic receptor activation (9). In the human platelet, epinephrine increases the activity of the Na^+/H^+ antiporter and increases the release of AA, in addition to inhibiting adenylate cyclase (10). Further studies indicate that epinephrine causes mobilization of AA through α_2 receptor activation of phospholipase A_2 subsequent to an intracellular alkalinization brought about by accelerated Na^+/H^+ exchange (11, 12).

α_2-Adrenergic agonists, in addition to inhibiting adenylate cyclase, have also been reported to enhance isoproterenol- or forskolin-stimulated cyclic AMP production. In rat brain slices, this enhanced effect of agonist co-incubation was blocked by the nonselective phospholipase A_2 inhibitor quinacrine (13). Quinacrine, however, had no effect on the enhanced forskolin-stimulated cyclic AMP production in HT29 cells resulting from agonist preincubation. This α_2-adrenergic receptor sensitization of forskolin-stimulated cyclic AMP production in HT29 cells is mediated by a regulatory G protein, because this effect is blocked by pretreatment with pertussis toxin (14).

CHO cells have been transfected with the human α_{2A}-adrenergic receptor gene (15). The receptor is stably expressed and mediates inhibition of adenylate cyclase as well as a potentiation of forskolin-stimulated cyclic AMP production. The inhibition of adenylate cyclase was blocked by pertussis toxin and, in this initial study, the potentiation was blocked by quinacrine (15). We have characterized further the α_2-adrenergic receptor in these cells and found it to be coupled to three pathways; inhibition of adenylate cyclase activity, which is attenuated by pertussis toxin pretreatment but not by quinacrine; activation of AA mobilization, which is attenuated by pertussis toxin

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; CHO, Chinese hamster ovary; AA, arachidonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

pretreatment and by quinacrine; and potentiation of forskolin-stimulated cyclic AMP production, which is enhanced by pretreatment with pertussis toxin but is not attenuated by quinacrine.

Materials and Methods

Cell culture. CHO cells (CHO-K1) transfected with pSVL containing the α_2-adrenergic receptor (CHO-A2AR) were kindly provided by Drs. Fraser and Venter (National Institutes of Health, Bethesda, MD) and were grown routinely in Ham's F-12 medium, supplemented with 10% fetal bovine serum, 50 μg/ml penicillin/streptomycin, and 50 μg/ml geneticin, in 75-cm^2 disposable tissue culture dishes in a humidified atmosphere of 5% CO_2/95% air (15). The clone used in these experiments is identical to the one labeled "1200 fmol of receptor/mg of protein" described by Fraser *et al.* (15). Cells were subcultured with 0.05% trypsin and were seeded at a moderate density in 35-mm or 150-mm tissue culture dishes, with confluence being reached in approximately 4 days.

Preparation of membranes and radioligand assays. Confluent 150-mm dishes were washed several times with phosphate-buffered saline, pH 7.4, and then harvested with a rubber policeman. Cells were pelleted by centrifugation (1000 × *g*, 5 min), resuspended in 25 ml of 50 mM ice-cold Tris·HCl buffer, pH 8.0, and homogenized with a Tissumizer (Tekmar Co., Cincinnati, OH) for 20 sec at setting 90. The pellet obtained by centrifugation for 10 min at 49,000 × *g* was washed once by resuspension in the Tris·HCl buffer and was centrifuged as before. The pellet, a crude particulate fraction, was resuspended in either 25 mM glycylglycine buffer, pH 7.6, or 25 mM Tris·HCl buffer, pH 7.6, for radioligand and adenylate cyclase assays, respectively. Protein was measured by the method of Lowry *et al.* (16).

Binding assays were similar to those reported previously (3). For saturation experiments, total binding was determined with one set of incubation tubes containing 475 μl of membrane suspension (approximately 60 μg of membrane protein) and 20 μl of an appropriate concentration of [^{3}H]yohimbine in 5 mM HCl. To a parallel set of tubes, 5 μl of (−)-norepinephrine (final concentration, 10 μM) were added for determination of nonspecific binding. Specific binding was calculated as the difference between total and nonspecific binding. After a 30-min incubation at 23°, the suspensions were filtered through GF/B glass fiber filter strips, using a 24-sample manifold (Brandel cell harvester; Biomedical Research and Development, Gaithersburg, MD). The tubes and filters were washed twice with 5 ml of ice-cold Tris·HCl, and the radioactivity retained on the filters was determined by scintillation counting. The K_D and B_{max} values were calculated from a computer-assisted nonlinear regression of bound versus free ligand concentrations.

For inhibition experiments, a fixed concentration of radioligand (20 μl) and various concentrations of unlabeled drug (in 10 μl) were added to 470 μl of the membrane suspension. Inhibition data were analyzed using a nonlinear, least-squares, parametric curve-fitting program (CDATA; EMF Software, Inc., Knoxville, TN) to obtain IC_{50} values. K_i values were calculated from these IC_{50} values using the Cheng-Prusoff correction (17). The inhibition binding data were fitted to one- and two-site binding models, as previously described (18).

Cyclic AMP production assay. Confluent cultures of CHO-A2AR cells in 35-mm dishes were utilized for cyclic AMP assays, using a modification of the method of Shimizu *et al.* (19). Cell incubations and washes of cells were in serum-free Dulbecco's modified Eagle's medium, pH 7.4, with 20 mM HEPES. Cells were washed twice with 2 ml of medium and then prelabeled with [^{3}H]adenine (5 μCi/dish) in 1 ml for 60 min at 37°. Quinacrine was added for the last 20 min of the prelabeling period, in those experiments in which it was included. The medium was then removed and the cells were washed twice with 2 ml of medium. Cells were incubated for 20 min with the indicated drugs in 1 ml of medium. A phosphodiesterase inhibitor (0.5 mM Ro20-1724 or 0.5 mM isobutylmethylxanthine) was included in this part of the assay. At the end of the incubation period, the medium was aspirated and 1 ml of 5% trichloroacetic acid was added. The samples were then passed sequentially over Dowex 50 columns, to isolate [^{3}H]ATP, and alumina columns, to isolate [^{3}H]cAMP. Each fraction was collected in a scintillation vial, to which 10 ml of BudgetSolve (Research Products International, Mount Prospect, IL) were added. The radioactivity of the samples was determined by standard scintillation counting, with an efficiency of 35%. Values are expressed as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP [[^{3}H]cAMP/([^{3}H]cAMP + [^{3}H]ATP)].

For one experiment, a radioimmunoassay kit from New England Nuclear (Boston, MA) was used to measure levels of cyclic AMP. Preparation of samples was similar to the stimulation phase of the cyclic AMP production assay, with the incubation being stopped with 0.3 N perchloric acid and then neutralized with 2 N KOH.

Adenylate cyclase assay. Adenylate cyclase activity in cell membranes was measured by the method described by Nichols *et al.* (20). Briefly, 80 μg of protein were incubated with 1.2 mM [α-^{32}P]ATP in 75 μl of a medium containing 50 mM Tris·HCl (pH 7.6), 6.7 mM $MgCl_2$, 25 mM creatine phosphate, 5 units of creatine phosphokinase, 1 mM cyclic AMP, 10 μM GTP, 30 mM NaCl, 0.7 mM isobutylmethylxanthine, and 2 mg/ml bovine serum albumin. Drugs were added in 5 μl of 5 mM HCl or, for forskolin, in 50% dimethylsulfoxide. The incubation was for 15 min at 30°. Dowex-50 alumina chromatography (21) was used to separate [^{32}P]cAMP, with [^{3}H]cAMP as an internal standard for measuring recovery.

AA mobilization experiments. CHO cells were grown on 150-mm tissue culture dishes and then detached by incubation for 5 min at 37°, in phosphate-buffered saline containing 2 mM EDTA, followed by gentle pipetting with a plastic transfer pipet. Suspended cells were harvested by centrifugation at 160 × *g* for 10 min and were resuspended in approximately 5 ml of incubation medium containing (in mM): 134 NaCl, 2.9 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 4 $NaHCO_3$, 0.36 NaH_2PO_4, 5 dextrose, and 10 HEPES (pH 7.4 at 37°), with 0.2 mg/ml fatty acid-depleted bovine serum albumin. [^{3}H]AA (0.2 μCi/ml) was added, and the cells were incubated for 2 hr at 37° to label cellular phospholipids. After being washed twice, labeled cells were resuspended in incubation medium at a final density of 1–2 × 10^6 cells/ml.

All experiments were carried out at 37°. CHO-A2AR cell suspensions (0.5 ml) were incubated in siliconized 13- × 100-mm test tubes with various treatments, as indicated in the figure legends, and the incubations were terminated at the appropriate times by addition of 1.9 ml of ice-cold chloroform/methanol (1:2, v/v). Lipids were extracted according to the method of Bligh and Dyer (22) and separated by thin layer chromatography on Whatman LK-6D plates (20 cm), in a solvent system consisting of 100 ml of the upper phase of ethyl acetate/isooctane/acetic acid/water (90:50:20:100, v/v) plus 1 ml of acetic acid (23). The free AA band was identified by comigration with an authentic standard (after spraying of the plate with 1 mM toluidinylnaphthalene sulfonate and visualization under UV light), scraped from the plate, and quantitated by scintillation counting.

Materials. Epinephrine bitartrate, (−)-norepinephrine bitartrate, creatine phosphate, creatine phosphokinase, isobutylmethylxanthine, cyclic AMP, GTP, quinacrine, and yohimbine were purchased from Sigma Chemical Co. (St. Louis, MO). [α-^{32}P]ATP (24 Ci/mmol) and [^{3}H]adenine (40 Ci/mmol) were purchased from International Chemical and Nuclear Radiochemicals (Irvine, CA). Forskolin was purchased from Calbiochem Behring (San Diego, CA), and pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). Geneticin was purchased from GIBCO (Grand Island, NY). [^{3}H]Yohimbine (80 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H]AA (76 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA). A23187, fatty acid-depleted bovine serum albumin, and HEPES were obtained from United States Biochemicals (Cleveland, OH), and Whatman LK-6D plates were from Baxter (McGaw Park, IL). The following drugs were graciously donated by the respective companies: prazosin hydrochloride (Pfizer, Groton, CT) and Ro20-1724 [4-(3-butoxy-4-methox-

ybenzyl)-2-imidazolidinedione] (Hoffman-LaRoche Laboratories, Nutley, NJ).

Statistics. The paired t test in GraphPad InStat was used for statistical analysis. A $p < 0.05$ was required for significance.

Results

Saturation binding experiments with membranes from CHO-A2AR cells and the α_2-adrenergic antagonist [^{3}H]yohimbine indicated a B_{max} (receptor density) of 1540 ± 240 fmol/mg of protein, with a K_D (affinity) of 0.30 ± 0.02 nM (mean ± standard error from seven experiments). Fig. 1 is a representative Rosenthal plot of one such experiment. The radioligand bound to a single class of sites, with an affinity typical of an α_{2A}-adrenergic receptor. The rank order of potency of antagonists in inhibiting [^{3}H]yohimbine binding was consistent with that of an α_2-adrenergic receptor (Table 1). The prazosin/yohimbine ratio indicates an α_{2A} subtype, as expected, because the cells were transfected with the gene for the α_{2A} receptor. We also examined the inhibition of [^{3}H]yohimbine binding by three agonists, UK14,304, epinephrine, and norepinephrine. The data for the agonists were fit significantly better by a two-site than a one-site model in all cases. The results of these experiments are shown in Table 1.

In control cells, epinephrine inhibited forskolin-stimulated

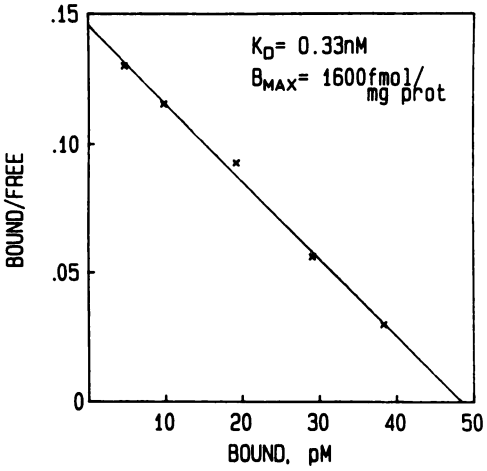

Fig. 1. Rosenthal plot of [^{3}H]yohimbine binding in CHO cells transfected with the α_{2A}-adrenergic receptor. Cells were grown to confluence in 150-mm dishes. After harvesting, membranes were prepared as described in Materials and Methods, and [^{3}H]yohimbine binding was assessed. This is a representative experiment of seven such experiments.

TABLE 1

Inhibition of [^{3}H]yohimbine binding

Values are means ± standard errors for the indicated number of experiments (n).

Antagonists	K_i	n
	nM	
Yohimbine	0.23 ± 0.04	4
Phentolamine	1.86 ± 0.36	3
Prazosin	212 ± 44	3

Agonists	High affinity site	K_i		n
		High affinity site	Low affinity site	
	%	nM		
UK14,304	43	1.0 ± 0.67	45 ± 21	3
Epinephrine	36	1.3 ± 0.87	156 ± 90	3
Norepinephrine	29	1.8 ± 0.87	339 ± 100	3

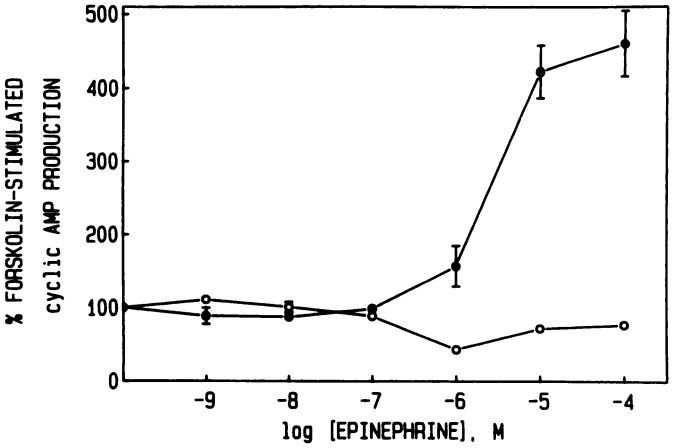

Fig. 2. Epinephrine inhibition of forskolin-stimulated cyclic AMP production without and with prior pertussis toxin pretreatment. Cells grown in 35 mm dishes were pretreated without (○) or with (●) pertussis toxin (200 ng/ml) for 18–20 hr. Cells were washed, and 5 μCi of [^{3}H]adenine in 1 ml of Dulbecco's modified Eagle's medium/HEPES were added for 60 min. After another wash, 1 ml of Dulbecco's modified Eagle's medium/HEPES containing 10 μM forskolin, without or with various concentrations of epinephrine, was added for 20 min. Separation and quantitation of [^{3}H]ATP and [^{3}H]cAMP were as described in Materials and Methods. Forskolin-stimulated values were 2.09 ± 0.19 and 1.19 ± 0.08% of conversion for control and pertussis toxin-pretreated cells, respectively. Values are presented as percentage of forskolin-stimulated cyclic AMP production and are the mean ± standard error for six to eight experiments. The absence of an *error bar* means that the standard error was less than the *symbol size*.

cyclic AMP production in a dose-dependent manner, up to 1 μM (Fig. 2). At higher concentrations of epinephrine, there was an attenuation of the inhibition. In cells that had been pretreated for 18 hr with 200 ng/ml pertussis toxin, the epinephrine-induced inhibition was completely attenuated, presumably due to inactivation by ADP-ribosylation of the regulatory G protein G_i. At higher concentrations of epinephrine, however, forskolin-stimulated cyclic AMP production following pertussis toxin pretreatment was 500% of that from forskolin stimulation alone (Fig. 2).

To determine whether the inhibition and/or potentiation of forskolin-stimulated cyclic AMP production were α_2-adrenergic receptor mediated, the α_1-adrenergic receptor antagonist prazosin or the α_2-adrenergic receptor antagonist yohimbine was included in the stimulation phase of the assay. Prazosin had little, if any, effect on either the epinephrine-induced inhibition or potentiation of cyclic AMP production (Fig. 3). Yohimbine, on the other hand, shifted the control dose-response curve to the right, as would be expected for an α_2-adrenergic receptor-mediated response, as well as blocking the attenuation of inhibition observed at higher concentrations of epinephrine. In those cells that were pretreated with pertussis toxin, the enhanced forskolin-stimulated response was blocked at lower concentrations of epinephrine and partially blocked at higher concentrations, indicating a shift to the right in the epinephrine dose-response curve in the presence of yohimbine. These data support the conclusion that the α_2-adrenergic receptor mediates both inhibition and potentiation of forskolin-stimulated cyclic AMP production.

Because receptor mechanisms are often better studied in a broken cell preparation, we wondered whether the enhancing effect we observed in intact CHO-A2AR cells was retained in a membrane preparation. In membranes from control cells, epi-

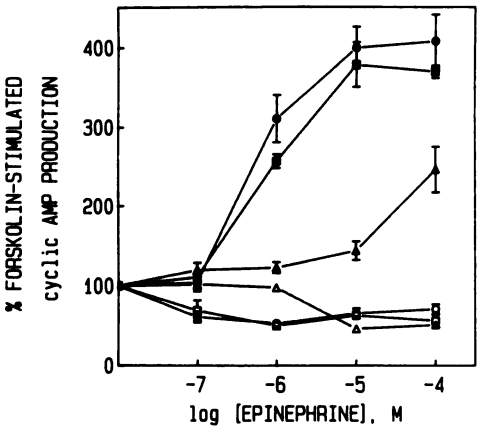

Fig. 3. Effect of antagonists on epinephrine inhibition of forskolin-stimulated cyclic AMP production without and with prior pertussis toxin pretreatment. Cells grown in 35-mm dishes were pretreated without (*open symbols*) or with (*closed symbols*) pertussis toxin and were prelabeled with [^{3}H]adenine as described for Fig. 2. After washing, 1 ml of 10 μM forskolin without or with epinephrine, in the absence (*circles*) or presence of yohimbine (*triangles*) or prazosin (*squares*), was added for 20 min. After removal of drugs, the cyclic AMP production was determined as described for Fig. 2. Values are presented as percentage of forskolin-stimulated cyclic AMP production and are the mean ± standard error for three or four experiments.

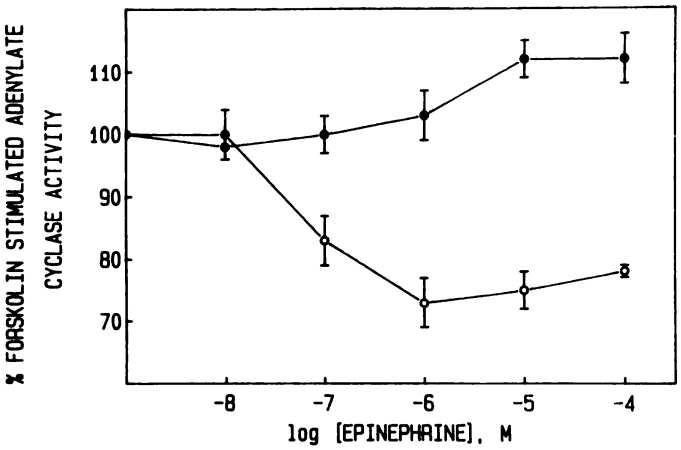

Fig. 4. Epinephrine inhibition of forskolin-stimulated adenylate cyclase activity without and with prior pertussis toxin pretreatment. Confluent cells in 150-mm dishes were treated without (○) or with (●) 200 ng/ml pertussis toxin for 18–20 hr. Cells were washed four times with phosphate-buffered saline and membranes were prepared for measurement of adenylate cyclase activity. Values are presented as percentage of forskolin-stimulated adenylate cyclase activity and are the mean ± standard error for three experiments. Forskolin-stimulated values were 694 ± 126 and 711 ± 98 pmol/mg of protein/15 min for control and pertussis toxin-pretreated cells, respectively.

nephrine inhibited forskolin-stimulated adenylate cyclase in a dose-dependent manner, with maximal inhibition at 1 μM epinephrine and little, if any, attenuation of inhibition at higher concentrations (Fig. 4). In membranes from cells that had been pretreated with pertussis toxin, there was only a slight enhancement (10%) of forskolin-stimulated adenylate cyclase activity at high concentrations of epinephrine, indicating that the potentiating effects were not retained in the broken cell preparation. It is, nevertheless, possible that the effect might be seen under some other set of assay conditions, such as low Mg^{2+}.

Previous work with these cells has indicated that the enhanced α_2-mediated cyclic AMP production is sensitive to quinacrine, a nonspecific inhibitor of phospholipase A_2 (15). To test the hypothesis that the potentiation of cyclic AMP production is mediated via phospholipase A_2 activation, we measured epinephrine-induced AA mobilization in the transfected CHO cells. The time course for epinephrine-induced AA mobilization was very rapid (half-maximal at 30 sec), indicating a receptor-mediated event (Fig. 5). AA mobilization was maximal at 5 min and was sustained for at least 20 min. To determine whether epinephrine-induced AA mobilization was α_2-adrenergic receptor mediated, we tested the effect of yohimbine, an α_2-adrenergic receptor antagonist. Yohimbine blocked AA mobilization in a dose-dependent manner (Table 2). To confirm that epinephrine was activating phospholipase A_2, similar experiments were done in the presence of quinacrine (a nonspecific phospholipase A_2 inhibitor) or in the absence of extracellular Ca^{2+} (EGTA). Both of these treatments blocked

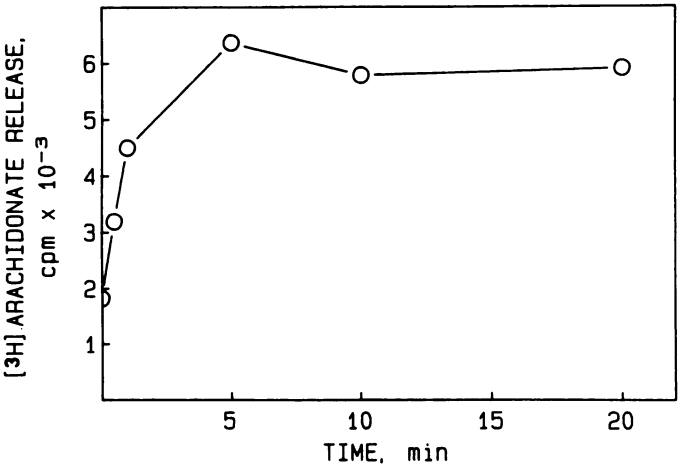

Fig. 5. Time course of AA mobilization in transfected CHO cells. Cells were grown in 150-mm dishes. After detachment with 2 mM EDTA, cells were pelleted and resuspended in incubation medium containing 0.2 μCi/ml [^{3}H]AA. After a 2-hr incubation, cells were washed twice and resuspended in incubation medium. Aliquots of cells (1–2 × 10^6 cells/ml) were incubated for the indicated times with 100 μM epinephrine. Lipids were extracted and separated by thin layer chromatography. Bands were identified and scraped, and the radioactivity was determined by scintillation counting. This is a representative experiment of three such experiments done in triplicate. Basal value at 10 min was 2900 cpm.

TABLE 2

Effects of yohimbine, quinacrine, or EGTA on epinephrine-stimulated AA mobilization

Values are percentage of basal and are the mean ± standard error for the indicated number of experiments (*n*) or individual values for two experiments. Basal value was 1750 ± 490 cpm.

Treatment	AA mobilization			
	Basal	(*n*)	10 μM Epinephrine	(*n*)
	% of basal			
0	100	(5)	147 ± 8[a]	(5)
0.1 μM Yohimbine	94, 124	(2)	120, 131	(2)
10 μM Yohimbine	100 ± 9	(3)	108 ± 8[b]	(3)
2 mM EGTA	97 ± 12	(3)	97 ± 6[b]	(3)
0.1 mM Quinacrine	73, 77	(2)	57, 65	(2)
1 μM A23187	196, 154	(2)	ND[c]	
1 μM A23187 + 0.1 mM quinacrine	58, 54	(2)	ND	

[a] Significantly different from basal, $p = 0.009$.
[b] Significantly different from 10 μM epinephrine with 0 treatment, $p < 0.05$.
[c] ND, not done.

AA mobilization by epinephrine as well as by A23187 (a Ca^{2+} ionophore), supporting the hypothesis that phospholipase A_2 activation is the mechanism of epinephrine-induced AA mobilization (Table 2).

In order to test the hypothesis that the α_2-adrenergic potentiation of cyclic AMP production is mediated by phospholipase A_2, we determined the epinephrine dose dependency of AA mobilization in cells that were pretreated without or with pertussis toxin (Fig. 6). Pertussis toxin pretreatment completely blocked epinephrine-induced AA mobilization. In contrast, pertussis toxin had no effect on AA mobilization caused by the Ca^{2+} ionophore A23187, which activates phospholipase A_2 directly. These results indicate that the epinephrine-induced AA mobilization is mediated via a pertussis toxin-sensitive regulatory G protein. Because the α_2 receptor potentiation of cyclic AMP production is enhanced following pertussis toxin pretreatment, phospholipase A_2 does not appear to be involved in the mechanism that causes potentiation of cyclic AMP production.

To examine further whether activation of phospholipase A_2 is involved in the potentiation of cyclic AMP production, we tested the effects of addition of quinacrine and of removal of extracellular Ca^{2+} by EGTA on the α_2-adrenergic receptor enhancement of cyclic AMP production following pertussis toxin pretreatment (Fig. 7). Neither quinacrine nor extracellular Ca^{2+} removal had an inhibitory effect on the enhanced cyclic AMP production, confirming that this effect is not mediated by phospholipase A_2-mediated AA mobilization.

In contrast to the lack of an effect of quinacrine on the potentiation of cyclic AMP production reported here, Fraser *et al.* (15) previously reported a dose-dependent inhibition by quinacrine of the potentiation of cyclic AMP production. In order to attempt to resolve this difference, we tested several different batches of quinacrine, including quinacrine from the laboratory of Fraser and Venter, at various concentrations up to 1 mM in control and pertussis toxin-pretreated cells. No

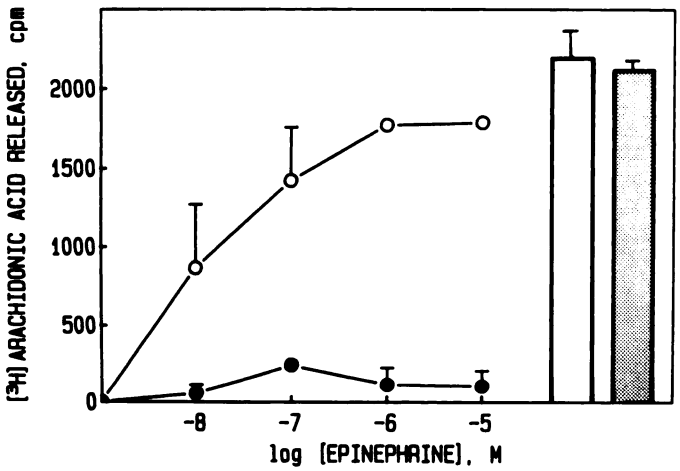

Fig. 6. Effect of pertussis toxin pretreatment on epinephrine-induced AA release in CHO cells. Cells were grown in 150-mm dishes. One group of cells was pretreated for 24 hr with 200 ng/ml pertussis toxin. After harvesting and labeling as described for Fig. 5, cells were incubated for 10 min with the indicated concentrations of epinephrine. ○, Control; ●, pertussis toxin pretreated. *Bars to the right*, A23187-induced AA release without (□) and with (■) pertussis toxin pretreatment. Values are presented as cpm of [^{3}H]AA released and are the mean ± standard error of two experiments done in triplicate. Basal values were 2800 ± 300 and 2400 ± 400 for control and pertussis toxin-pretreated cells, respectively.

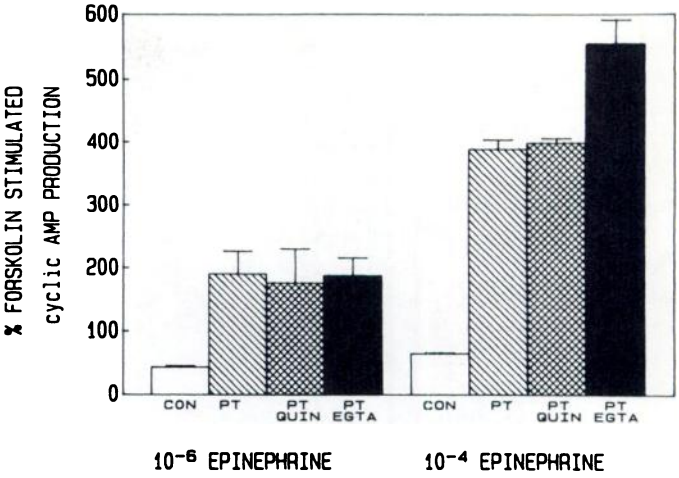

Fig. 7. Effect of quinacrine and EGTA on epinephrine inhibition of forskolin-stimulated cyclic AMP production without and with prior pertussis toxin pretreatment. Cells in 35-mm dishes were pretreated without or with 200 ng/ml pertussis toxin (*PT*) for 18–20 hr. Cells were washed and prelabeled as before. Quinacrine (*Quin*) (1 mM) was added for the last 20 min of the prelabeling period. EGTA (3 mM) was included throughout the assay (including washes) for those cells receiving it. Following prelabeling and washing, cells were stimulated with 10 μM forskolin without or with the indicated concentrations of epinephrine. Values are presented as percentage of forskolin-stimulated cyclic AMP production and are the mean ± standard error of three or four experiments. *Con*; control.

inhibition of the potentiation of cyclic AMP production was observed in any of these experiments. However, all batches of quinacrine tested did inhibit AA mobilization. To test the unlikely possibility that the opposite results were due to the different assays used for measurement of cyclic AMP production, we repeated our experiment utilizing radioimmunoassay, the procedure used by Fraser *et al.* (15). Again we found no inhibition by quinacrine of the increased cyclic AMP production. In subsequent experiments in Fraser's laboratory, the inhibitory effect of quinacrine on cyclic AMP potentiation was not consistently observed.[3]

Discussion

Our characterization of the α_2-adrenergic receptor in CHO-A2AR cell membranes using the radioligand binding technique is consistent with that previously reported by Fraser *et al.* (15). The receptor density in these cells is about 5 times higher than that found in tissues and in cultured cells used to study α_2 receptors. The K_D value of [^{3}H]yohimbine is lower (higher affinity) than that reported by Fraser *et al.* (15) but is similar to the K_D values we have previously reported for the α_{2A}-adrenergic receptor in human platelets, HT29 cells, and human cortex (3, 24, 25). The higher K_D value obtained by Fraser results from the use of Tris buffer and NaCl in the binding assay, both of which reduce the affinity of [^{3}H]yohimbine for the receptor. In contrast, we routinely use a glycylglycine buffer without NaCl. The rank order of potency of antagonists in inhibiting [^{3}H]yohimbine binding was similar to that reported previously in these cells and is consistent with that of an α_{2A}-adrenergic receptor subtype (26, 27). Agonist inhibition of [^{3}H] yohimbine binding in CHO-A2AR cells could be resolved into

[3] C. M. Fraser, personal communication.

high and low affinity components and was similar to that observed for α_{2A} receptors in HT29 cell membranes (3).

Lower concentrations of epinephrine inhibited forskolin-stimulated cyclic AMP production in a dose-dependent manner. Thus, the transfected α_2-adrenergic receptor maintained the inhibitory functional response. Inhibition was attenuated after pretreatment with pertussis toxin, as would be expected in a system coupled to inhibition of adenylate cyclase through G_i. Inhibition of cyclic AMP production was mediated by the α_2-adrenergic receptor, because yohimbine, an α_2 antagonist, shifted the epinephrine dose-response curve to the right, whereas prazosin, an α_1 antagonist, had no effect. The increase in cyclic AMP production at higher concentrations of epinephrine following pertussis toxin pretreatment was also an α_2-adrenergic receptor-mediated event, as indicated by the rightward shift in the dose-response curve produced by yohimbine but not by prazosin.

The epinephrine-induced increase in forskolin-stimulated cyclic AMP production observed in CHO-A2AR cells has some similarities to the effects of inhibitory agonist preincubation on subsequent forskolin- or hormone-stimulated cyclic AMP production. This phenomenon, which is called sensitization, has been observed with several receptor systems that are negatively coupled to adenylate cyclase (28). However, with at least two of these receptors, the α_2 receptor in HT29 cells and the muscarinic receptor in NG108 cells, the effect is blocked by prior treatment of the cells with pertussis toxin (14, 29). Thus, it seems unlikely that the mechanism of the increase observed in the present study is similar to the sensitization phenomenon described previously.

The potentiation of forskolin-stimulated cyclic AMP production at higher concentrations of epinephrine, particularly the marked increase observed after pertussis toxin pretreatment, is intriguing. We have considered several potential mechanisms that could account for these results. First, potentiation cannot be explained only by a tonic inhibition that exists in the CHO-A2AR cells such that, when cells are pretreated with pertussis toxin, the inhibition is removed and there is a greater production of cyclic AMP production. Forskolin stimulation (in the absence of epinephrine) should be elevated if a tonic inhibition is removed, but forskolin stimulation was actually decreased following pertussis toxin pretreatment. In addition, we observed attenuation of inhibition at higher concentration of epinephrine in control (i.e., not pertussis toxin-treated) cells.

A second potential mechanism would be activation of β-adrenergic receptors by epinephrine. However, previous work in our laboratory has indicated the HT29 cells do not express β-adrenergic receptors. In addition, the potentiation is not blocked by propranolol but does occur with UK14,304 and *p*-aminoclonidine (15).

A third potential mechanism for the potentiation of forskolin-stimulated cyclic AMP production is activation of phospholipase C. In transfected A9L cells, the M_1 muscarinic acetylcholine receptor may increase cyclic AMP accumulation by such a mechanism (30). However, there is no evidence for phospholipase C activity in CHO or CHO-A2AR cells (15). In addition, a high concentration of epinephrine caused only a slight increase (30–40% increase, as compared with a >10-fold increase caused by ATP or A23187) in intracellular Ca^{2+}, as measured by fura-2, in CHO-A2AR.[4] Thus, it is unlikely that phospholipase C is involved in the α_2-adrenergic receptor-mediated increase in cyclic AMP production in CHO-A2AR cells.

A fourth potential mechanism is the activation of phospholipase A_2. Fraser *et al.* (15) observed inhibition of this effect with quinacrine, indicating possible phospholipase A_2 involvement. Because we were unable to observe a similar effect of quinacrine, we chose to assess phospholipase A_2 involvement directly, by measuring AA mobilization in CHO-A2AR cells. Epinephrine caused a time- and dose-dependent increase in AA mobilization, which was blocked by yohimbine, quinacrine, EGTA, and pertussis toxin. From these data it appears reasonable to postulate that α_2-adrenergic receptor activation mobilizes AA by increasing the activity of phospholipase A_2, via a pertussis toxin-sensitive G protein. This is consistent with previous reports of G protein regulation of phospholipase A_2 activity in various cells (31–36). In some tissues the G protein is pertussis toxin sensitive (31, 36), but in others it is not (32). In human platelets, α_{2A}-adrenergic receptor-mediated stimulation of phospholipase A_2 activity may be secondary to the activation of the Na^+/H^+ antiporter (11, 12). However, in other cells, such as the HT29 cell line and the OK cell line, no activation of the Na^+/H^+ antiporter by the α_2 receptor has been detected (37, 38). Pertussis toxin pretreatment, which enhances the α_2 receptor-mediated potentiation of forskolin-stimulated cyclic AMP production, blocked α_2 receptor-mediated AA mobilization. These results imply that a G protein, possibly G_i, is involved in α_2 receptor-mediated AA mobilization. Because the A23187-induced AA mobilization was unaffected by pertussis toxin pretreatment, the activity of phospholipase A_2 itself is not altered by pertussis toxin. Because AA mobilization was inhibited by quinacrine and pertussis toxin and the epinephrine-induced increase in cyclic AMP production was not, we conclude that phospholipase A_2 activation is not involved in the mechanism of the potentiation of cyclic AMP production.

A fifth potential mechanism is that the greater than normal density of α_2-adrenergic receptors allows a direct interaction of the receptor with G_s, the regulatory G protein that stimulates adenylate cyclase. A high receptor density appears to be a factor, because in CHO-A2AR cells expressing only 200 fmol of receptor/mg of protein there was only a 25% increase in cyclic AMP production at high epinephrine concentrations after pertussis toxin pretreatment (15). This hypothesis is also supported by the observation that CHO cells have a high density of G_s.[5] The interaction between α_2-adrenergic receptors and G_s has been studied in artificial lipid vesicles reconstituted with purified proteins. Partially purified receptor from human platelets stimulated the GTPase activity of human erythrocyte G_s to about 20% of the level observed for human erythrocyte G_i (39). Similarly, an interaction between the β-adrenergic receptor and G_i has been observed both in artificial membranes and in S49 cyc^- cells (40, 41). Thus, whereas the potential for a direct interaction between the α_2-adrenergic receptor and G_s to stimulate adenylate cyclase appears to exist, it is not clear whether that is sufficient to explain the results reported here. If this hypothesis is correct, then the potentiation would be expected to be retained in broken cell preparations. Our obser-

[4] J. T. Turner, personal communication.
[5] M. Rodbell, personal communication.

vation that the effect is not retained in a membrane adenylate cyclase assay argues weakly against this hypothesis and suggests that an intact cell system may be necessary in order for the effect to be observed.

In summary, in CHO-A2AR cells the α_2-adrenergic receptor is coupled to three distinct signal transduction pathways; inhibition of forskolin-stimulated cyclic AMP production and activation of AA mobilization, both of which are attenuated by pertussis toxin pretreatment, and potentiation of forskolin-stimulated cyclic AMP production, which is enhanced by pertussis toxin pretreatment.

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