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EFFECTS OF α_2-ADRENERGIC AGONIST PREINCUBATION ON SUBSEQUENT FORSKOLIN-STIMULATED ADENYLATE CYCLASE ACTIVITY AND [^{3}H]FORSKOLIN BINDING IN MEMBRANES FROM HT29 CELLS

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Abstract—α_2-Adrenergic agonist preincubation resulted in a leftward shift in the subsequent concentration–response curve to forskolin-stimulated adenylate cyclase activity in membranes from HT29 cells, a human colon adenocarcinoma cell line. This effect was much less pronounced than the effect seen in the intact cell cyclic AMP production assays. Removal of GTP from the assay caused a further slight leftward shift in the concentration–response curve. In [^{3}H]forskolin binding experiments, α_2-adrenergic agonist preincubation caused a doubling of the maximal number of binding sites (80 vs 31 fmol/mg protein) compared to control. The addition of $MgCl_2$ and NaF to the assay buffer increased control binding 5-fold. With agonist preincubation, there was a further increase in binding in the presence of $MgCl_2$ and NaF which was not significantly different from the appropriate control. Pertussis toxin pretreatment blocked both the leftward shift in the forskolin concentration–response curve and the increase in maximal number of binding sites, indicating that a pertussis toxin sensitive protein is involved in these changes. Activation of cyclic AMP production in the intact cell by cholera toxin followed by norepinephrine preincubation and then stimulation by forskolin resulted in a degree of sensitization similar to that seen in the membrane adenylate cyclase and binding assays. Pertussis toxin also blocked this sensitization. It appears that if the cyclase system is highly activated, then the degree of sensitization is similar in the membrane and intact cell assay.

Preincubation with an α_2-adrenergic receptor agonist sensitizes subsequent forskolin- or hormone-stimulated cyclic AMP production in intact HT29 cells, a human colonic adenocarcinoma cell line [1, 2]. Sensitization causes up to a 20-fold greater stimulation with forskolin compared to control, but only 2-fold with vasoactive intestinal peptide (VIP) stimulation. Similar sensitization phenomena have been observed in other receptor systems which are negatively coupled to adenylate cyclase [3]. A sensitization phenomenon mediated by inhibitory adenosine or somatostatin receptors has been examined in intact cell assays and membrane preparations in rat adipocytes and the mouse anterior pituitary AtT-20 cell line respectively. In adipocytes, the degree of phenylisopropyl-adenosine-induced sensitization observed with forskolin stimulation is much less in membrane preparations (2-fold) than in the intact cell assay (10-fold) [4, 5]. Similarly, in AtT-20 cells, somatostatin preincubation causes a 1.5-fold increase in forskolin-stimulated adenylate cyclase activity in broken cells compared to a 3.8-fold increase in cyclic AMP content [6]. To determine whether or not α_2-adrenergic receptor-mediated sensitization could be retained in a broken cell preparation, we studied the effect of α_2-adrenergic receptor agonist preincubation of cells on subsequent forskolin-stimulated adenylate cyclase activity under a variety of conditions. Consistent with the above studies, we report here that sensitization was much less pronounced in membrane assays than in intact cell assays.

Studies concerning the sensitization phenomenon have concentrated exclusively on membrane adenylate cyclase activity or cellular cyclic AMP formation. The molecular basis for the increased sensitivity to forskolin remains to be determined. Studies of sensitization in HT29 cells have indicated that the greater degree of forskolin stimulation may be due to an increased potency of forskolin in agonist-preincubated cells [1]. The increased potency could be due to either an increased affinity of forskolin for its binding site or to an increased availability of forskolin binding sites. One approach to distinguish between these alternatives is to label the forskolin binding site using [^{3}H]forskolin in membrane preparations. Radioligand binding studies utilizing [^{3}H]forskolin have been reported in rat brain, rat myocardium and human platelets [7–10]. In the present study, we characterized the binding of [^{3}H]forskolin to HT29 cell membranes from control and agonist-preincubated cells. We report that agonist preincubation increased the number of [^{3}H]forskolin binding sites but did not change the apparent affinity of forskolin for these sites.

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MATERIALS AND METHODS

Cell culture and preparation of membranes. HT29

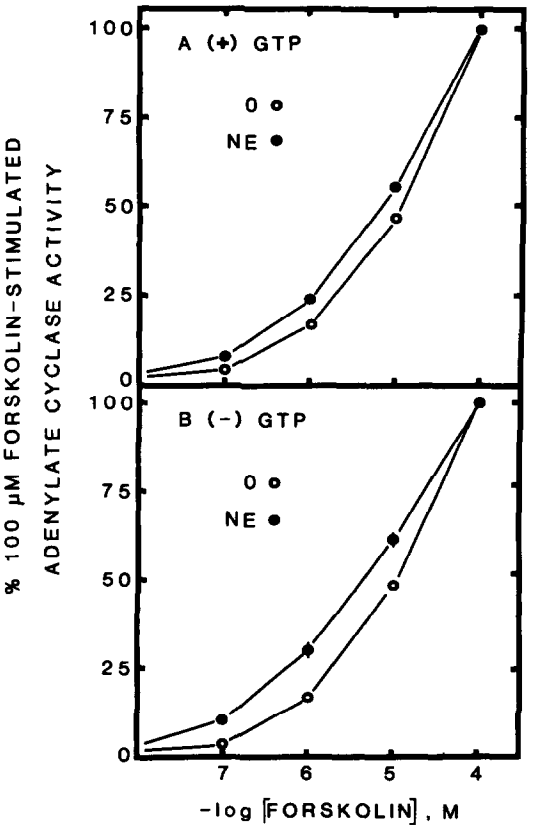

Fig. 1. Concentration–response curves for forskolin-stimulated adenylate cyclase activity without or with norepinephrine preincubation with (A) or without (B) GTP. (A) Forskolin-stimulated adenylate cyclase activity was determined in membranes from cells preincubated for 60 min without (○) and with (●) 10 μM norepinephrine. The data are expressed as percent of 100 μM forskolin-stimulated adenylate cyclase activity for each treatment. The average 100 percent values were 6130 ± 1230 and 6260 ± 1370 pmol cyclic AMP/mg protein/10 min for control and agonist-preincubated cells respectively. Each point is the mean ± SE for three separate experiments done in triplicate. (B) The average 100 percent values were 6830 ± 1380 and 6640 ± 1420 pmol cyclic AMP/mg protein/10 min for control and agonist-preincubated cells respectively.

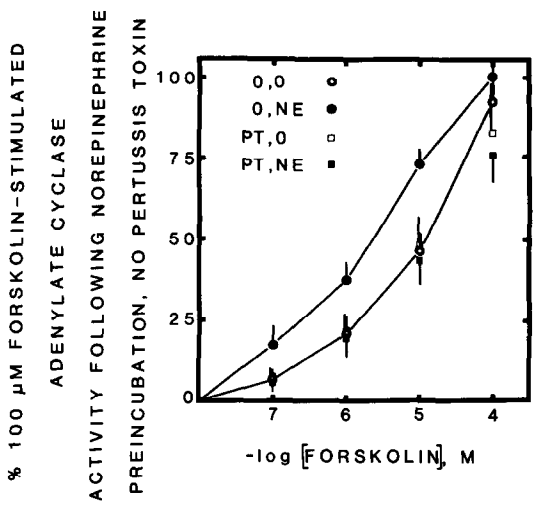

Fig. 2. Concentration–response curves for forskolin-stimulated adenylate cyclase activity without and with pertussis toxin pretreatment, and without and with norepinephrine preincubation. Forskolin-stimulated adenylate cyclase activity was determined in membranes from cells pretreated for 18 hr without (○, ●) or with (□, ■) 100 ng/mL pertussis toxin, and preincubated without (○, □) or with (●, ■) 10 μM norepinephrine for 60 min. Data are presented as percent of 100 μM forskolin-stimulated adenylate cyclase activity following norepinephrine preincubation in the absence of pertussis toxin (8904 ± 1935 pmol cyclic AMP/mg protein/10 min). Each point is the mean ± SE for three separate experiments done in triplicate.

cells, a human colonic adenocarcinoma cell line, were grown routinely in Dulbecco's modified Eagle's medium with high glucose (DMEM-H) supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) newborn bovine serum in 75-cm^2 disposable tissue culture flasks in a humidified atmosphere of 5% CO_2/95% air [11]. Cells were subcultured with 0.05% (w/v) trypsin/0.01% (w/v) EDTA and were seeded at a moderate density (approximately 160,000 cells/cm^2) in 150-mm tissue culture dishes with confluence being reached in approximately 8 days.

Following various pretreatments at 37° (18 hr ± 100 ng/mL pertussis toxin and/or 60 min ± 10 μM norepinephrine), confluent dishes were placed on ice and washed four times with ice-cold phosphate-buffered saline, pH 7.4, and harvested with a rubber policeman. Cells were pelleted by centrifugation (1000 *g*, 2 min), resuspended in 20–30 mL of ice-cold 50 mM Tris, pH 8.0, and homogenized with a Tissumizer (Tekmar Co., Cincinnati, OH) for 30 sec at setting 90. The pellet resulting from centrifugation for 10 min at 49,000 *g* was washed once by resuspension in ice-cold Tris followed by a second centrifugation. The pellet, a crude particulate fraction, was resuspended in either 40 mM Tris, pH 7.4, for radioligand binding, or in 25 mM Tris, pH 7.6, for adenylate cyclase assays. Protein was measured by the method of Lowry *et al.* [12].

Adenylate cyclase assay. Adenylate cyclase activity in cell lysates was measured by the method described by Nickols *et al.* [13]. Briefly, 50–100 μg of protein was 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, 30 mM NaCl, 0.7 mM isobutylmethylxanthine, and 2 mg/mL of bovine serum albumin. Drugs were added in 5 μL of 5 mM HCl or, for forskolin, in 50% dimethyl sulfoxide. The incubation was for 10 min at 30° in the presence or absence of 10 μM GTP. Reactions were stopped by addition of 1 mL of 0.167 N perchloric acid. Dowex-50 alumina chromatography [14] was used to separate [^{32}P]cyclic AMP with [^{3}H]cyclic AMP as an internal standard for measuring recovery.

The [^{3}H]adenine prelabeling assay, utilized for measuring cyclic AMP production in intact cells, was as previously described [2].

Radioligand binding studies. Radioligand binding

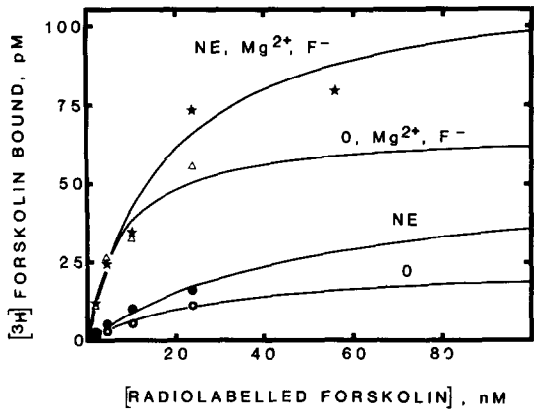

Fig. 3. [^{3}H]Forskolin binding to membranes from HT29 cells without or with norepinephrine preincubation. Confluent monolayers of cells were preincubated without (○, △) or with (●, ★) 10 μM norepinephrine for 60 min. The cells were harvested and prepared for binding without (○, ●) and with (△, ★) 5 mM $MgCl_2$ and 10 mM NaF. These data are representative of four such experiments.

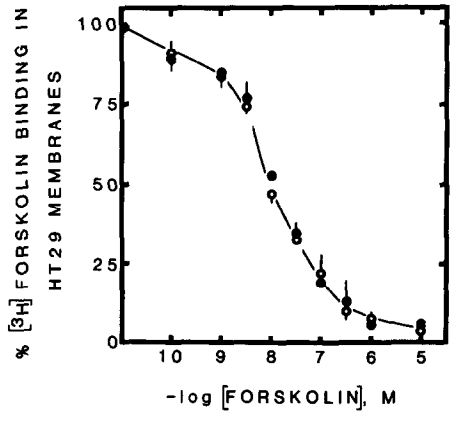

Fig. 4. Forskolin inhibition of [^{3}H]forskolin binding in membranes from HT29 cells without or with previous norepinephrine preincubation. Cells were preincubated without (○) or with (●) 10 μM norepinephrine for 60 min. The cells were harvested and membranes were prepared for binding. Data are presented as percent of [^{3}H]forskolin binding in the absence of competing drug. Each point is the mean of two separate experiments done in duplicate. The concentration of [^{3}H]forskolin used was 8 nM.

assays were performed as follows. The crude particulate fraction (approximately 600 μg of membrane protein) was incubated at 22° with 20 μL of the appropriate concentration of [^{3}H]forskolin for 60 min in a final volume of 500 μL. A parallel set of tubes containing 100 μM unlabeled forskolin was used to determine nonspecific binding. Specific binding was calculated as the difference between total and nonspecific binding. Following incubation, 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 ice-cold Tris buffer, pH 8.0, and the radioactivity retained on the filter was determined by liquid scintillation spectroscopy. K_D (dissociation constant) and B_{max} (maximal number of binding sites) values were calculated from a computer-assisted nonlinear regression analysis of bound versus free radioligand.

For inhibition experiments, a single concentration of [^{3}H]forskolin (approximately 8 nM) and various concentrations of unlabeled forskolin were incubated in a similar manner. The data were analyzed using a nonlinear least-squares parametric curve-fitting program (CDATA, EMF Software, Inc., Knoxville, TN) to obtain IC_{50} (50% inhibition concentration) values.

Materials. (−)-Norepinephrine bitartrate, creatine phosphate, creatine phosphokinase, isobutylmethylxanthine, cyclic AMP, GDPβS and GTP were purchased from Sigma (St. Louis, MO). Forskolin was obtained from Calbiochem-Behring and pertussis toxin from List Biological Laboratories, Inc. (Campbell, CA). [α-^{32}P]ATP (24 Ci/mmol) and [^{3}H]adenine (20 Ci/mmol) were purchased from International Chemical and Nuclear Radiochemicals (Irvine, CA), and [^{3}H]forskolin (49.2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). (−)-Propranolol hydrochloride was donated by Ayerst Laboratories (New York, NY).

RESULTS

Forskolin caused a concentration-dependent increase in adenylate cyclase activity in membranes from HT29 cells (Fig. 1). Because of the limited solubility of forskolin, the EC_{50} could not be determined accurately. The approximate half-maximal stimulation was with 10 μM forskolin. Norepinephrine preincubation of intact cells with subsequent forskolin stimulation of adenylate cyclase activity in membranes from those cells caused a slight, but significant, leftward shift in the concentration–response curve (Fig. 1A; $P < 0.0001$ utilizing a covariance model for a randomized design with fixed treatment effects). This effect was much less dramatic than the results of agonist preincubation on subsequent forskolin-stimulated cyclic AMP production seen in intact cells where there is a 40-fold leftward shift in the concentration–response curve compared to control cells [1].

In an effort to understand the difference in results between the intact cell and membrane assays, we repeated the experiment shown in Fig. 1A in the absence of GTP. We reasoned that the high concentrations of GTP used for optimal conditions in the adenylate cyclase assay might interfere with our ability to see differences caused by agonist preincubation, whereas such differences might become apparent under "suboptimal" conditions. Removing GTP from the adenylate cyclase assay did not change control adenylate cyclase activity (Fig. 1, A and B; $P > 0.05$). However, in membranes from agonist-preincubated cells, there was a further leftward ($P < 0.0002$) shift in the concentration–response curve in the absence of GTP, indicating a possible effect of GTP on sensitization. α_2-Adrenergic receptor-mediated sensitization was most pronounced at 0.1 μM forskolin (3-fold as compared to maximal

Table 1. [^{3}H]Forskolin binding in membranes from HT29 cells without and with agonist preincubation

	K_D (nM)	B_{max} (fmol/mg protein)	N
O	14 ± 3	32 ± 9	6
NE	16 ± 4	78 ± 9*	5
O, $MgCl_2$, NaF	16 ± 4	157 ± 24†	6
NE, $MgCl_2$, NaF	14 ± 4	221 ± 34	5

Cells in 150-mm dishes were preincubated without or with 10 μM norepinephrine (NE) for 60 min. Cells were then harvested and processed as described in Materials and Methods. $MgCl_2$ and NaF (5 and 10 mM, respectively) were added to half of the crude particulate membrane preparation, and binding of [^{3}H]forskolin was determined. Values are means ± SE.

,† Significantly different ($P < 0.05$; †$P < 0.025$) from control (*t*-test for two means).

Table 2. Effects of pertussis toxin pretreatment on [^{3}H]forskolin binding in membranes from HT29 cells without or with agonist preincubation

Pretreatment, Preincubation	K_D (nM)	B_{max} (fmol/mg protein)	N
O			
O, O	17 ± 2	40 ± 5	5
O, NE	23 ± 5	78 ± 17*	5
PT, O	13 ± 5	28 ± 15	4
PT, NE	20 ± 3	18 ± 4*	4
$MgCl_2$, NaF			
O, O	12 ± 3	182 ± 35	4
O, NE	14 ± 3	207 ± 25	5
PT, O	12 ± 3	176 ± 31	4
PT, NE	12 ± 3	152 ± 31	5

Cells in 150-mm dishes were pretreated without or with 100 ng/mL pertussis toxin (PT) for 18 hr followed by preincubation without or with 10 μM norepinephrine (NE) for 60 min. Cells were harvested and processed, and binding assays were performed as described in Materials and Methods. Values are means ± SE and were not significantly different within pretreatment groups except as indicated.

* Significantly different ($P < 0.05$) from control (*t*-test for two means).

sensitization (20-fold) at 10 μM forskolin in the intact cell assay [1]. These experiments were repeated in the presence of GDPβS, a GTP antagonist, to be certain that the effects of residual GTP in the membranes were eliminated. These results were similar to those seen with assays performed in the absence of GTP.

Pretreatment of HT29 cells with pertussis toxin prevents α_2-adrenergic receptor mediated sensitization of forskolin-stimulated cyclic AMP production in intact cells [2]. In addition, pertussis toxin caused an unexpected decrease in forskolin-stimulated cyclic AMP production. We examined the effects of pertussis toxin pretreatment of cells on subsequent forskolin-stimulated adenylate cyclase activity without or with agonist preincubation. The results of these experiments are shown in Fig. 2. Pertussis toxin pretreatment prevented α_2-agonist-induced sensitization of forskolin-stimulated adenylate cyclase activity but had no effect on forskolin stimulation *per se*. These results are consistent with the involvement of a pertussis toxin sensitive guanine nucleotide regulatory protein, perhaps G_i, in the sensitization phenomenon.

The results from both membrane adenylate cyclase assays and the intact cell cyclic AMP production assays indicate that the effect of agonist preincubation is to shift the forskolin concentration–response curve to the left, resulting in an increased potency [1]. We examined [^{3}H]forskolin binding in membranes from HT29 cells without or with prior agonist preincubation to see if this change in activity was reflected in the binding parameters. Agonist preincubation resulted in a doubling of the number of binding sites ($P < 0.05$) with no change in affinity (Table 1; Fig. 3). The inclusion of 5 mM $MgCl_2$ and 10 mM NaF in the binding assays of control membranes increased the B_{max} 5-fold ($P < 0.025$) with no change in K_D (Table 1). In the presence of $MgCl_2$ and NaF, agonist preincubation had no significant effect on either K_D or B_{max}. However, the trend was an increased number of binding sites following agonist preincubation.

To assess further possible changes in forskolin potency following agonist preincubation, we examined forskolin inhibition of [^{3}H]forskolin binding in membranes from cells without or with prior agonist preincubation. The IC_{50} values for forskolin without

Table 3. Effect of pertussis toxin pretreatment with or without subsequent cholera toxin pretreatment on sensitization and on forskolin-stimulated cyclic AMP production

Pretreatment		% Conversion ([^{3}H]cAMP/[^{3}H]cAMP + [^{3}H]ATP)			
		0 Norepinephrine		10 μM Norepinephrine	
PT	CT	0 Forskolin	30 μM Forskolin	0 Forskolin	30 μM Forskolin
−	−	0.015 ± 0.003	0.24 ± 0.03	0.028 ± 0.003	2.84 ± 0.16
+	−	0.015 ± 0.003	0.07 ± 0.01	0.018 ± 0.003	0.09 ± 0.01
−	+	0.37 ± 0.05	4.75 ± 0.24	1.08 ± 0.09	6.54 ± 0.14*
+	+	0.20 ± 0.04	4.53 ± 0.20	0.27 ± 0.06	4.54 ± 0.16†

Confluent cells in 35-mm dishes were pretreated without or with 100 ng/mL pertussis toxin (PT) for 18 hr. Cholera toxin pretreatment (CT; 100 ng/mL) was for 30 min followed by 45 min in DMEH-H to allow penetration into the cell membrane. [^{3}H]Adenine without or with norepinephrine was then added for 60 min. Cells were washed, and forskolin or medium was added for 2 min. Values are means ± SE for three experiments.

* Significantly different ($P < 0.05$) from the same treatment in the absence of norepinephrine preincubation (t-test for two means).

† Significantly different ($P < 0.01$) from the same treatment in the absence of pertussis toxin pretreatment (t-test for two means).

or with agonist preincubation were 14 and 16 nM, respectively, again indicating no effect of agonist preincubation on binding affinity (Fig. 4).

We also examined the effects of pertussis toxin pretreatment on subsequent forskolin binding to membranes without or with agonist preincubation. In the assays in which $MgCl_2$ and NaF were present, there was no difference in K_D or B_{max} among any of the treatments, although the trend was an increased number of binding sites following agonist preincubation without previous pertussis toxin pretreatment (Table 2). However, without $MgCl_2$ and NaF, pertussis toxin significantly decreased B_{max} in membranes from norepinephrine-preincubated cells.

The effects of agonist preincubation in the membrane assays were decidedly less than those observed in the intact cell assay. In the membrane assay, 100 μM forskolin-stimulated adenylate cyclase activity was similar in membranes from control and agonist-preincubated cells (Fig. 1). In the intact cell, there is a 7-fold difference in forskolin-stimulated cyclic AMP production at this concentration [1]. One of the differences between the two assay systems is that in the membrane assay conditions have been optimized in terms of substrate concentration, ion concentrations and inhibition of phosphodiesterase, whereas in the intact cell assay, the intracellular conditions are not necessarily optimal for maximum adenylate cyclase activity. Thus, we reasoned that if cyclic AMP production were stimulated to a high level in the intact cell, then the effects of agonist preincubation might be similar to the magnitude of change observed in the membrane assay. In many systems when cells are stimulated with forskolin and an agent which activates G_s, a synergism occurs such that cyclic AMP production is greater than that with either agent alone [15]. We treated cells for 30 min with 100 ng/mL cholera toxin to activate G_s followed by a 45-min incubation in DMEH-H to allow penetration of the toxin, and then conducted the assay in the usual manner and stimulated without and with 30 μM forskolin. We chose a submaximal concentration of forskolin to be able to discern differences between control and agonist-preincubated conditions similar to the membrane assay. Cholera toxin alone caused a 25-fold increase over basal in cyclic AMP production and potentiated forskolin-stimulated production of cyclic AMP 20-fold (Table 3). In the absence of cholera toxin pretreatment, norepinephrine preincubation resulted in a 12-fold sensitization of forskolin-stimulated cyclic AMP production (0.24 to 2.84% conversion), whereas after pretreatment with cholera toxin the sensitization by norepinephrine of forskolin-stimulated cyclic AMP production was only 36% (4.75 to 6.54% conversion). Thus, the degree of sensitization (at least at 30 μM forskolin) observed in intact cells pretreated with cholera toxin is comparable to the sensitization observed in the membrane assay using cells pretreated without cholera toxin (Fig. 1). As expected, the sensitization observed both without and with cholera toxin pretreatment was blocked by prior pretreatment of cells with pertussis toxin (Table 3).

DISCUSSION

α_2-Agonist preincubation of HT29 cells caused sensitization of subsequent forskolin-stimulated adenylate cyclase activity. Sensitization in the membrane adenylate cyclase assay was less dramatic than in the intact cell cyclic AMP accumulation assay, as indicated by a 3- vs a 40-fold change in apparent forskolin EC_{50} respectively. A partial explanation for this difference may be that, in the membrane assay, conditions are more nearly optimal, and thus because the relative activity is initially higher, the fold change is less.

The leftward shift in the subsequent forskolin concentration–response curve in membranes from agonist-preincubated cells was greater in the absence that in the presence of added GTP. One interpretation of these data is that the amount of GTP

added to the membrane assay is sufficient to "dampen" the amount of forskolin sensitization seen in agonist-preincubated cells. GTP at high concentrations will inhibit adenylate cyclase, presumably by interacting with the guanine nucleotide regulatory protein G_i [16]. Thus, it is reasonable that large amounts of GTP might interfere with full expression of the sensitization phenomenon.

In intact HT29 cells, pertussis toxin pretreatment results in a marked decrease in stimulation of cyclic AMP production by forskolin [2]. We expected that some degree of this effect might be retained in the broken cell preparation, but it was not. This lack of effect of pertussis toxin on forskolin stimulation in the membrane assay is consistent with the results of the dual toxin intact cell experiments where cholera toxin + forskolin stimulation was not decreased with pertussis toxin pretreatment. More importantly, it is clear that pertussis toxin blocks sensitization in the membrane preparation, as well as that in intact cells.

[^{3}H]Forskolin binding to membranes from cells without or with agonist preincubation indicated an increased number of forskolin binding sites following agonist preincubation with no change in affinity. The increase in the number of forskolin binding sites in the presence of $MgCl_2$ and NaF was similar to observations for [^{3}H]forskolin binding in membranes from the human platelet and rat brain [9, 10]. In these tissues and HT29 membranes, the number of forskolin binding sites is increased under conditions that promote the interaction of the catalytic subunit and G_s. The further increase in binding sites following agonist preincubation in the presence of NaF and $MgCl_2$, while not statistically significant, was consistent with the degree of sensitization observed in the membrane adenylate cyclase assay and the intact cell experiments utilizing cholera toxin. This degree of sensitization was similar to that observed by us with vasoactive intestinal peptide stimulated cyclic AMP production (2-fold [2]) in these cells, and with hormones in other cells [6, 17]. Taken together, these data imply that if adenylate cyclase is activated through G_s (e.g. hormone) or if the components for activating G_s (e.g. cholera toxin or NaF) are present, then the degree of the subsequent α_2-adrenergic agonist-mediated sensitization of forskolin activation is similar for the intact cell cyclic AMP production assay, the membrane adenylate cyclase assay, and the membrane binding assay.

One of the problems in elucidating the mechanism for sensitization of forskolin-stimulated adenylate cyclase is that the mechanism of forskolin activation is not well understood. Whereas many studies indicate forskolin directly activates the catalytic subunit of adenylate cyclase, other evidence suggests that forskolin may have more than one site of action [18]. Our forskolin stimulation and sensitization results are consistent with a two-site hypothesis. Pretreatment of cells with cholera toxin followed by forskolin stimulation resulted in a much greater cyclic AMP production than seen with either agent alone. Agonist preincubation further increased production, indicating that synergism and sensitization may be mediated differently.

The results of this study indicate that sensitization in membranes from HT29 cells results in a small leftward shift in the concentration–response curve to forskolin-stimulated adenylate cyclase activity. This shift may result from an increased number of available forskolin binding sites. The effect of pertussis toxin pretreatment to decrease the number of [^{3}H]forskolin binding sites is consistent with pertussis toxin blocking the norepinephrine sensitization of forskolin-stimulated adenylate cyclase activity in the membrane assay. It appears that if G_s is activated with cholera toxin, the degree of sensitization of forskolin stimulation in the intact cell is similar to that seen in the membrane preparations. The actual mechanism for the sensitization phenomenon remains elusive.

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