Characterization and Possible Mechanisms of $\alpha_2$-Adrenergic Receptor-mediated Sensitization of Forskolin-stimulated Cyclic AMP Production in HT29 Cells

Susan B. Jones and David B. Bylund

From the Department of Pharmacology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

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Preincubation with an $\alpha_2$-adrenergic agonist sensitized subsequent forskolin- and vasoactive intestinal peptide-stimulated cyclic AMP production in HT29 cells, a human colonic adenocarcinoma cell line. Preincubation with somatostatin, another agonist negatively coupled to adenylate cyclase, sensitized forskolin-stimulated cyclic AMP production to a lesser extent. $\alpha_2$-Adrenergic agonist preincubation also resulted in desensitization as indicated by a shift to the right in the dose-response curve of a subsequent challenge by an $\alpha_2$-adrenergic agonist.

In an effort to elucidate the mechanism for sensitization, we examined protein kinase C and the Na$^+$/H$^+$ antiporter. Whereas these components had marked effects on forskolin stimulation, there was no effect on sensitization. Changes in the concentration of extracellular Ca$^{2+}$ or Mg$^{2+}$ had no effect on either forskolin stimulation or sensitization. Pertussis toxin pretreatment caused a time-dependent decrease in sensitization, an attenuation of inhibition of cyclic AMP production, and a decrease in subsequent $[35]^{P}$ADP-ribosylation by pertussis toxin. The time course for these three events was similar, implicating the inhibitory guanine nucleotide regulatory protein in the mechanism for $\alpha_2$-adrenergic receptor-mediated sensitization of forskolin-stimulated cyclic AMP production. In addition, pertussis toxin dramatically decreased forskolin-stimulated cyclic AMP production, although with a different time course. These results suggest that the mechanism of sensitization is via an as yet undefined sequence of biochemical events that includes the inhibitory guanine nucleotide regulatory protein, but does not include inhibition of adenylate cyclase nor activation of the Na$^+$/H$^+$ antiporter.

We have recently reported that preincubation with an $\alpha_2$-adrenergic agonist sensitizes subsequent forskolin-stimulated cyclic AMP production in HT29 cells, a human colonic adenocarcinoma cell line (1). An apparently similar phenomenon has been observed in several receptor systems which are coupled in an inhibitory manner to adenylate cyclase (2). However, in these other systems, as compared to the HT29 cells, the time of preincubation needed to cause sensitization is longer and the degree of sensitization is much lower. In other systems, sensitization is mediated by receptors which, like the $\alpha_2$-adrenergic receptor, are negatively coupled to adenylate cyclase. Preincubation with muscarinic cholinergic agonists or somatostatin in AtT20 mouse pituitary tumor cells causes sensitization of both forskolin- and hormone-stimulated cyclic AMP production (3-5). Since hormonal stimulation of cyclic AMP production is also increased by agonist preincubation, sensitization is not exclusively an effect on forskolin stimulation in this cell line.

Activation of the $\alpha_2$-adrenergic receptor results in several biochemical effects. Inhibition of adenylate cyclase in the human platelet, NG108-15 and HT29 cell lines, as well as in brain and other tissues, is the most well-characterized effect (6-8). The inhibition of adenylate cyclase is mediated via the guanine nucleotide regulatory protein $G_i$, which is composed of three subunits called $\alpha_i$, $\beta$ and $\gamma$. The $\alpha_i$ subunit is unique to this protein, whereas the $\beta$ and $\gamma$ subunits are similar if not identical to those associated with $G_o$ and $G_s$ (9). Pertussis toxin (islet-activating protein) causes ADP-ribosylation of $\alpha_i$ with a resulting loss or attenuation of the inhibition of adenylate cyclase in those receptor systems which inhibit adenylate cyclase (10). It has become, therefore, a useful tool for examining systems coupled in an inhibitory manner to adenylate cyclase.

In the human platelet and S49 cyc$c^+$ cells, phorbol ester pretreatment also attenuates hormone-sensitive inhibition of adenylate cyclase, implicating protein kinase C in this event (11, 12). The attenuation of inhibition in the platelet by phorbol ester treatment is correlated with the phosphorylation of $G_o$, further implicating protein kinase C intervention (13). In addition, phorbol ester pretreatment enhances hormone-stimulated cyclic AMP levels in anterior pituitary as well as hormone and forskolin-stimulated adenylate cyclase activity in S49 lymphoma cells (14, 15).

A second effect of $\alpha_2$-adrenergic receptor activation in the human platelet is stimulation of the Na$^+$/H$^+$ antiporter and...
the resulting activation of phospholipase A2 (16-18). α2-Adrenergic stimulation of the Na+/H+ antiporter has also been characterized in the neuroblastoma glioma cell line NG108-15 (19). In this system, the α2-adrenergic receptor-mediated stimulation of Na+/H+ exchange is not blocked by pertussis toxin pretreatment (20). In addition, these events are not dependent on a decrease in cyclic AMP levels since increasing cyclic AMP levels in these cells do not alter the α2-agonist-induced antiporter effects.

We have examined the specificity of sensitization in HT29 cells and report that somatostatin preincubation also sensitizes forskolin-stimulated cyclic AMP production, although the degree of sensitization is much lower. α2-Adrenergic agonist preincubation results in sensitization of vasoactive intestinal peptide (VIP)-stimulated cyclic AMP production which is lower in magnitude and perhaps also different in mechanism than forskolin sensitization. In an effort to elucidate the mechanism for sensitization, we have examined protein kinase C, the Na+/H+ antiporter, changes in extracellular calcium concentration, and the guanine nucleotide regulatory protein, Gi. We report that neither the Na+/H+ antiporter nor protein kinase C appears to be involved in sensitization, but that pertussis toxin pretreatment causes a time-dependent attenuation of sensitization, implicating Gi in the mechanism of sensitization. We also found a unique effect of pertussis toxin on forskolin stimulation which may lead to a better understanding of forskolin's mechanism of action.

EXPERIMENTAL PROCEDURES

Cell Culture—HT29 cells, a human colonic adenocarcinoma cell line, were obtained from J. Fogh (Sloan-Kettering Institute, Rye, NY). Cells were grown routinely in Dulbecco's modified Eagle's medium with high glucose supplemented with 5% (v/v) fetal bovine serum and 1% (v/v) non-essential amino acids in a 75-cm² tissue culture flask in a humidified atmosphere of 5% CO₂/95% air. Cells were subcultured with 0.05% (v/v) trypsin/0.01% (v/v) EDTA and were seeded at a moderate density in 35-mm tissue culture dishes with confluence being reached in approximately 5 days (8).

Cyclic AMP Production Assay—Confluent cultures of HT29 cells in 35-mm dishes were utilized for cyclic AMP assays by a modification of the method of Shimizu et al. (21). All incubations and washes of drugs in 1 ml of medium. 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 and alumina columns to isolate cyclic AMP and [3H]ATP. Each fraction was collected in a scintillation vial to which 10 ml of Budget Solve (Research Products International, Mount Prospect, IL) was added. The radioactivity of the samples was determined by standard scintillation spectroscopy with an efficiency of 35%. Values are expressed as percentage conversion of [3H]ATP to cyclic [3H]AMP ( disarm [3H]AMP/ [3H]AMP + [3H]ATP).

Adenylate Cyclase Assay—Adenylate cyclase activity in cell lysates was measured by the method described by Nickols et al. (22). Briefly, 50–100 µg of protein were incubated with 1.2 µM [α-32P]ATP in 75 µl of a medium containing 50 mM Tris-HCl (pH 7.6), 6.7 mM MgCl₂, 25 mM creatine phosphate, 5 units of creatine phosphokinase, 1 mM cysteine, 10 mM ATP, 30 mM NaCl, 0.5 mM 3-isobutyl-1-methylxanthine, and 2 mg/ml 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°C. Dowex-50 alumina chromatography (23) was used to separate [32P]cyclic AMP with [3H]cyclic AMP as internal standards for measuring potency.

ADP Ribosylation of HT29 Cell Membranes—The ADP ribosylation of HT29 cell membranes was done essentially as described by Yajima et al. (24). The cells were harvested in phosphate-buffered saline (pH 7.4) and pelleted by centrifugation. The pellet was then suspended for 10 min in ice-cold suspension buffer consisting of 5 mM Tris (pH 7.5) containing 1 mM each dithiobiotin and EDTA. The suspension was homogenized with a Teflon Tissumizer for 10 s at setting 9. The homogenate was centrifuged at 100 x g for 2 min to remove undisrupted cells and nuclei. The supernatant was then centrifuged at 35,000 x g for 15 min at 4°C followed by resuspension of the pellet in freezing buffer consisting of 5 mM Tris (pH 7.5), 1 mM each dithiobiotin and EGTA, and 10% glycerol. The samples were frozen at −80°C until used. On the day of the assay, the membrane preparation was thawed and washed once with suspension buffer. Approximating 20 µg of protein were incubated at 30°C for 50 min with 6.5 µM [32P]NAD in the presence of 2.5 µg of pertussis toxin in a reaction mixture consisting of 100 mM potassium phosphate buffer (pH 7.5), 10 mM thymidine, 1 mM ATP, 0.5 mM EDTA, and 20 mM dithiobiotin in a final volume of 100 µl. The reaction was terminated by diluting with 1.0 ml of ice-cold 5 mM Tris-HCl (pH 7.5), and centrifuging at 35,000 x g for 15 min. The pellet was washed once with the same buffer and dissolved in Laemmli's sample buffer by heating at 100°C for 3 min. The samples were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels (25), stained with Coomassie Brilliant Blue R-250, destained, and exposed to Kodak XR-2 film for 24–48 h at −80°C. Appropriate bands were excised and radioactivity determined by liquid scintillation spectroscopy.

Materials—(-)-Norepinephrine bitartrate, creatine phosphate, creatine phosphokinase, isobutylmethylxanthine, cyclic AMP, GTP, phorbol 12-myristate 13-acetate, A23187, quinacrine, and indomethacin were purchased from Sigma. Forskolin was obtained from Behring Diagnostics, pertussis toxin from List Biological Laboratories, Inc. (Campbell, CA), and vasoactive intestinal peptide from Peninsula Laboratories (Belmont, CA). [α-32P]ATP (25 Ci/mmol) and adenosine (46 Ci/mmol) were purchased from International Chemical and Nuclear Radiochemicals (Irvine, CA) and [3H]NAD (30 Ci/mmol) was purchased from Du Pont/New England Nuclear (Boston, MA). The following drugs were graciously donated by the respective companies: UK14,304 (Pfizer, Groton, CT), and propanolol hydrochloride (Ayerst Laboratories, NY).

RESULTS

Preincubation of HT29 cells with norepinephrine causes a marked (10–20-fold) sensitization of subsequent forskolin-stimulated cyclic AMP production. This event is mediated via the α2-adrenergic receptor since yohimbine, an α2-adrenergic-selective antagonist blocks sensitization, whereas prazosin and sotalol (α1- and β-adrenergic antagonists, respectively) do not. Sensitization is rapid in onset and reversible with half-maximal sensitization occurring at 5 min and half-maximal reversal occurring at 10 min (1).

Characterization of Sensitization—To characterize further the sensitization phenomenon, we determined the dose-response relationship for norepinephrine, an α2-adrenergic agonist, to induce sensitization of forskolin-stimulated cyclic AMP production. The effects of norepinephrine were dose-dependent, with maximal sensitization occurring at 10 µM norepinephrine (Fig. 1). The EC₅₀ for sensitization was 600 nM, which is similar to the EC₅₀ for norepinephrine inhibition of forskolin-stimulated cyclic AMP production in these cells (500 nM) (1).

Whereas most studies of the sensitization phenomenon have utilized whole cell assays, Parsons and Stiles (26) have reported a 2-fold sensitization of hormone- and forskolin-stimulated adenylyl cyclase by phenylisopropyladenosine, an adenosine-1 receptor agonist, in membrane preparations from fat cells from rats treated with the agonist. To determine whether or not α2-adrenergic receptor-mediated sensitization was retained in a broken cell preparation, we assessed the effect of α2-adrenergic receptor agonist preincubation of cells on subsequent adenylyl cyclase activity. There was a slight increase in forskolin-stimulated adenylyl cyclase following agonist preincubation (2450 ± 550 versus 3130 ± 300 pmol of cyclic AMP/mg of protein/10 min; p < 0.05 by paired t test).
but rather from an increase in efficacy. This differs from adenine without (○) and with (●) 10 μM norepinephrine for 30 min. Cells were washed and the indicated concentrations of VIP were added for 2 min. Cyclic [3H]AMP content was then determined. Values are means ± S.E. for three separate experiments. Basal content was subtracted.

Thus, an intact cell system may be required to observe sensitization.

**Specificity of Sensitization**—In other systems, sensitization of cyclic AMP production is not limited to forskolin stimulation. Others have shown hormone-stimulated cyclic AMP production to be sensitized following agonist preincubation (27, 28). Since HT29 cells have vasoactive intestinal peptide (VIP) receptors which stimulate adenylate cyclase, we assessed the effect of agonist preincubation on the VIP dose-response curve. Norepinephrine preincubation caused a 2-fold sensitization of VIP-stimulated cyclic AMP production (Fig. 2). VIP sensitization did not result from a shift in VIP potency but rather from an increase in efficacy. This differs from forskolin sensitization in which potency appears to be affected (1).

To test the hypothesis that all receptors negatively coupled to adenylate cyclase cause sensitization, we looked for another inhibitory agonist in HT29 cells. Somatostatin, which inhibits adenylate cyclase in other systems, has been reported to cause sensitization of forskolin- and hormone-stimulated cyclic AMP production in AtT20 cells, (4, 5). Somatostatin also inhibited forskolin- and VIP-stimulated cyclic AMP production in HT29 cells, (4, 5). Somatostatin also inhibited forskolin- and VIP-stimulated cyclic AMP production in HT29 cells with EC50 values of 3 ± 7 and 14 ± 3 nM, respectively, but only 65–65% compared with 80–90% inhibition seen with α2-adrenergic agonists. Pretreatment of cells under our usual sensitization conditions with somatostatin at a concentration which maximally inhibits cyclic AMP production (1 μM) resulted in a 3.5-fold increase in forskolin-stimulated cyclic AMP production compared with the 15-fold increase observed with norepinephrine preincubation in the same experiment (Table I). These results indicate that, whereas somatostatin does cause sensitization, the degree of sensitization is markedly lower than that caused by α2-adrenergic agonists.

**Desensitization of the α2-Adrenergic Receptor-mediated Response**—Initial experiments with α2-adrenergic agonists at a single, high concentration indicated that there was no subsequent attenuation of the extent of α2 receptor-mediated inhibition of forskolin-stimulated cyclic AMP production following α2-adrenergic agonist preincubation (1). However, in experiments with full dose-response curves to UK14,304, an α2 agonist, we did observe an increase in EC50 from 2.0 to 30 nM following norepinephrine preincubation (Fig. 3). These data indicate that agonist preincubation induces desensitization of the α2-adrenergic receptor-mediated inhibition of cyclic AMP production. This explanation for the change in EC50 value is complicated by the fact that, under sensitization conditions, forskolin stimulation is 10-fold higher (see inset, Fig. 3). Thus, we repeated these experiments using VIP to stimulate cyclic AMP production. Norepinephrine preincubation caused only a doubling of the VIP stimulation, but there was a similar shift in the dose-response curve to UK14,304 (EC50 = 0.5 nM versus 5.6 nM, n = 2). These results suggest that there is a desensitization of the adenylate cyclase inhibitory response resulting from preincubation with an α2 agonist.

**Mechanism of Sensitization**—Sensitization of forskolin or hormone-stimulated cyclic AMP production by activation of receptors coupled in an inhibitory manner to adenylate cyclase is fairly ubiquitous (see Ref. 2 for review). However, the mechanism(s) for this event has remained elusive. To clarify the mechanism of sensitization in HT29 cells, we examined the possible involvement of protein kinase C, the Na+/H+ antipporter, changes in extracellular divalent cation concentration, and the guanine nucleotide binding protein Gs.

**Table 1**

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Conversion</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>1 μM somatostatin</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>10 μM norepinephrine</td>
<td>2.35 ± 0.16</td>
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</table>
**Effects of Protein Kinase C Activation and Down-regulation on Sensitization** —Since phosphorylation, which stimulates protein kinase C, have been shown to affect hormone and forskolin stimulation of cyclic AMP formation, we examined the effects of 1 and 20 h of phorbol 12-myristate 13-acetate (PMA) pretreatment on forskolin stimulation under control and sensitization conditions. One hour of PMA pretreatment had no effect on forskolin-stimulated cyclic AMP production in cells preincubated with norepinephrine, but did double in cells preincubated without norepinephrine (Table II). Pretreatment for 1 hr with 1 μM mezerein, an activator of protein kinase C, gave similar results (data not shown). If protein kinase C were important in sensitization, pretreatment with an inhibitor of the enzyme should block sensitization. We pretreated cells with H-7, an inhibitor of protein kinase C activity, and saw no effect on sensitization (data not shown).

In cells pretreated with PMA for 20 h, forskolin stimulation was decreased in the presence or absence of agonist pretreatment, indicating an effect of PMA on forskolin stimulation per se with no alteration of sensitization (Table II). The lack of an effect on 1 or 20 h of PMA treatment on sensitization was confirmed by dose-response experiments using forskolin at concentrations range of 1-250 μM (29).

We also examined the effects of 1 and 20 h of PMA pretreatment on UK14,304 inhibition of forskolin-stimulated cyclic AMP production without and with norepinephrine preincubation. We found no evidence for attenuation of inhibition following either 1 or 20 h of PMA pretreatment without or with subsequent norepinephrine preincubation (29), indicating that, in HT29 cells under these conditions, PMA, presumably through activation of protein kinase C, does not attenuate α2-adrenergic inhibition of cyclic AMP production.

**Effects of Inhibition of the Na+/H+ Antiporter on Sensitization** —The Na+/H+ antiporter is activated via the α2-adrenergic receptor in human platelets and NG108 neuroblastoma cells. In human platelets, this results in an intracellular alkalinization with subsequent phospholipase A2 activation. Activation of this enzyme results in release of arachidonic acid which can be converted to various metabolites by the enzymes cyclooxygenase and lipoygenase (16–19). To determine whether or not the Na+/H+ antiporter is important in sensitization, extracellular Na+ was replaced with choline in a Hanks’ balanced salt solution to block the antiporter activity. Forskolin was less effective in the balanced salt solution compared with DMEM-H under both sensitizing and nonsensitizing conditions (Table III). However, when we compared forskolin stimulation in the sodium balanced salt solution with the choline balanced salt solution curves, there appeared to be a slightly greater degree of sensitization in the presence of sodium as compared with the absence, with no discernible difference between the forskolin stimulation in the absence of agonist preincubation. These data indicated either a possible involvement of the Na+/H+ antiporter or an effect of Na+ on cyclic AMP production per se.

**TABLE II**

<table>
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<tr>
<th>Pretreatment</th>
<th>Norepinephrine</th>
<th>n</th>
<th>10 μM Norepinephrine</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.13 ± 0.05</td>
<td>3</td>
<td>2.18 ± 0.35</td>
<td>3</td>
</tr>
<tr>
<td>1 h of PMA</td>
<td>0.26 ± 0.12</td>
<td>3</td>
<td>2.28 ± 0.37</td>
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<tr>
<td>0</td>
<td>0.19 ± 0.10</td>
<td>3</td>
<td>2.60 ± 0.35</td>
<td>3</td>
</tr>
<tr>
<td>20 h of PMA</td>
<td>0.09 ± 0.04</td>
<td>3</td>
<td>1.21 ± 0.09</td>
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<tr>
<td>0</td>
<td>0.13 ± 0.04</td>
<td>2</td>
<td>2.38 ± 0.01</td>
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<tr>
<td>1 μM amiloride</td>
<td>0.16 ± 0.03</td>
<td>2</td>
<td>1.68 ± 0.06</td>
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<tr>
<td>0</td>
<td>0.13 ± 0.02</td>
<td>4</td>
<td>3.27 ± 0.71</td>
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<tr>
<td>10 μg/ml melittin</td>
<td>0.35 ± 0.03</td>
<td>4</td>
<td>3.93 ± 0.56</td>
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<tr>
<td>100 μM quinacrine</td>
<td>0.29 ± 0.11</td>
<td>3</td>
<td>3.74 ± 1.16</td>
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<tr>
<td>Melittin + quinacrine</td>
<td>0.42 ± 0.09</td>
<td>3</td>
<td>4.33 ± 1.17</td>
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</tr>
<tr>
<td>0</td>
<td>0.10 ± 0.02</td>
<td>2</td>
<td>1.64 ± 0.02</td>
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<tr>
<td>1 μM indomethacin</td>
<td>0.10 ± 0.02</td>
<td>2</td>
<td>1.72 ± 0.04</td>
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<tr>
<td>10 μM indomethacin</td>
<td>0.10 ± 0.02</td>
<td>2</td>
<td>1.61 ± 0.14</td>
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<tr>
<td>100 μM indomethacin</td>
<td>0.14 ± 0.04</td>
<td>2</td>
<td>1.45 ± 0.18</td>
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<tr>
<td>0.3 mM EGTA</td>
<td>0.09 ± 0.01</td>
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<td>2.16 ± 0.13</td>
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<tr>
<td>EGTA + A23187</td>
<td>0.18 ± 0.02</td>
<td>2</td>
<td>2.97 ± 0.16</td>
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**TABLE III**

<table>
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<tr>
<th>Treatment</th>
<th>Norepinephrine</th>
<th>n</th>
<th>10 μM Norepinephrine</th>
<th>n</th>
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<tr>
<td>DMEM-H</td>
<td>0.33 ± 0.11</td>
<td>3</td>
<td>2.84 ± 0.94</td>
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<tr>
<td>Sodium BSS</td>
<td>0.11 ± 0.02</td>
<td>3</td>
<td>0.99 ± 0.05</td>
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<tr>
<td>Choline BSS</td>
<td>0.07 ± 0.003</td>
<td>3</td>
<td>0.29 ± 0.10</td>
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<tr>
<td>pH 7.4</td>
<td>0.16 ± 0.08</td>
<td>4</td>
<td>2.11 ± 0.36</td>
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<tr>
<td>pH 6.8</td>
<td>0.06 ± 0.007</td>
<td>4</td>
<td>0.53 ± 0.16</td>
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<tr>
<td>pH 8.0</td>
<td>0.42 ± 0.21</td>
<td>4</td>
<td>2.95 ± 0.33</td>
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</table>
To further investigate involvement of the Na\(^+\)/H\(^+\) antiporter in sensitization, we examined forskolin stimulation in DMEM-H at the normal pH of 7.4, at a pH of 6.8, which should block antiporter activity, and at pH 8.0. Forskolin stimulation without agonist preincubation was decreased at pH 6.8 compared with pH 7.4 and pH 8.0 (Table 111). At pH 8.0, forskolin stimulation in the absence of norepinephrine preincubation was higher than that seen with pH 6.8 following agonist preincubation. However, the fold sensitization between control and agonist preincubated cells was similar for all three pH levels (6-10-fold).

To further characterize the effects of pH on forskolin stimulation, we examined the time course of stimulation at pH 6.8, 7.4, and 8.0 (Fig. 4). At pH 6.8, forskolin stimulation was low and similar throughout the time course. At pH 7.4, forskolin stimulation increased with time up to 5 min where it leveled off. However, at pH 8.0, forskolin stimulation rose rapidly and linearly up to 5 min and began to level off at 10 min. The ATP levels from these experiments were similar at each pH, indicating that availability of substrate was not responsible for the difference in response. These data and the previous data indicate an effect of extracellular pH on forskolin stimulation, but not an effect on sensitization. It appears that both Na\(^+\) and H\(^+\) may be involved in the regulation of forskolin stimulation. However, it seems unlikely that the Na\(^+\)/H\(^+\) antiporter is involved in sensitization. Further, amiloride, which blocks antiporter activity had minimal effects on sensitization (Table II).

Lack of antiporter effects, however, does not preclude involvement of another component in the proposed pathway in the human platelet, namely phospholipase A\(_2\) activity. An additional reason for considering this enzyme is that phospholipase A\(_2\) has been shown to be involved in \(\alpha_2\)-receptor modulation of cyclic AMP accumulation in rat brain slices (30). We pretreated cells with melittin and/or quinacrine, a nonspecific activator and inhibitor, respectively, of phospholipase A\(_2\). Melittin slightly increased cyclic AMP production in cells preincubated both without and with agonist, indicating a possible effect on forskolin stimulation, but not on sensitization. Quinacrine, which should inhibit melittin's effect and/or sensitization if phospholipase A\(_2\) is involved, had no effect on either (Table II). To examine a further step in this pathway, we pretreated cells with a cyclooxygenase inhibitor, indomethacin, to determine whether or not metabolites of arachidonic acid (a product of phospholipase A\(_2\) activity) are important in sensitization. Indomethacin had no effect on either forskolin stimulation or sensitization (Table II).

Divalent Cation Effects on Sensitization—The divalent cation Mg\(^{2+}\) has been shown to affect \(\alpha_2\) agonist binding in membranes from HT29 cells (8). Furthermore, both Mg\(^{2+}\) and Ca\(^{2+}\) increase follicle-stimulating hormone binding to ovarian granulosa cells but have opposite effects, increasing and decreasing, respectively, cyclic AMP levels in these cells (31, 32). We tested the effects of increasing Mg\(^{2+}\) or Ca\(^{2+}\) concentration in DMEM-H and the effect of eliminating them with EDTA or EGTA, respectively, on the dose-response curve to forskolin without and with agonist preincubation. There was no effect of increasing either cation concentration or eliminating them on the forskolin dose-response curve in either control or agonist preincubated cells (Fig. 5). To test the effects of increasing the intracellular Ca\(^{2+}\) concentration, we utilized the calcium ionophore A23187 under control and sensitizing conditions and found a slight decrease in forskolin stimulation in both control and agonist preincubated cells (Table II). The results of these experiments indicate that, under these conditions, the extracellular concentration of Mg\(^{2+}\) or Ca\(^{2+}\) and intracellular concentration of Ca\(^{2+}\) are not important in sensitization.

Effects of Cycloheximide Pretreatment on Sensitization—Long-term somatostatin preincubation causes sensitization of...
forskolin and hormone stimulation of cyclic AMP accumulation in AtT20 cells (5, 33). This effect is blocked by cycloheximide pretreatment, indicating a requirement for protein synthesis in this event. To test whether or not protein synthesis is required for sensitization in our system, we pretreated cells without and with cycloheximide for 18 h and examined the dose response to forskolin under sensitizing and nonsensitizing conditions. There was a cycloheximide-dependent decrease in forskolin stimulation under both conditions, indicating an effect of protein synthesis on forskolin stimulation but not on sensitization (Fig. 6).

Guanine Nucleotide Regulatory Protein Involvement in Sensitization—The $\alpha_2$-adrenergic receptor is coupled to adenylate cyclase via the guanine nucleotide regulatory protein $G_i$. In order to assess the involvement of $G_i$ in sensitization, we pretreated cells with 100 ng/ml pertussis toxin for various times and then examined forskolin stimulation, $\alpha_2$-adrenergic inhibition of forskolin stimulation, and $\alpha_2$-adrenergic agonist-induced sensitization. Pertussis toxin has been shown to ADP-ribosylate the $\alpha_i$ subunit of $G_i$, thus preventing the dissociation of $G_i$ into its subunits and blocking its action. Unexpectedly, forskolin-stimulated cyclic AMP production was decreased with increasing time of pertussis toxin pretreatment (Fig. 7A and B, give actual percent conversion data, whereas in Fig. 8 the data are presented as percent of control.)

Ten hours of pertussis toxin pretreatment maximally reduced forskolin stimulation to 30% of control levels (Fig. 8). The time needed to obtain 50% of this maximal effect was approximately 5 h. This result indicates a previously unrecognized role of a pertussis toxin-sensitive protein (perhaps, but not necessarily, $G_i$) in the mechanism of forskolin activation of adenylate cyclase. The effects of agonist preincubation (sensitization) were also decreased with pertussis toxin pretreatment (Figs. 7A and 8). After 18 h of pertussis toxin pretreatment, sensitization could not be demonstrated. The time needed to reach 50% inhibition of sensitization was about 8 h. The effects of pertussis toxin pretreatment on the inhibition of cyclic AMP production produced by UK14,304 were similar to the time course for decreases in sensitization (Figs. 7B and 8). Thus, 18 h of pertussis toxin preincubation completely prevented UK14,304 inhibition of cyclic AMP production and 8 h were needed to reach 50% of this maximal effect.

In view of these two different time courses of the effects of pertussis toxin pretreatment, one for forskolin stimulation and another for sensitization and inhibition of cyclic AMP production, it seemed important to determine the time course of pertussis toxin-induced ADP-ribosylation under these conditions. To do this, we measured $[^{32}P]$ADP-ribosylation of membranes prepared from cells pretreated with pertussis toxin for the indicated period of time.

For panel A, following pretreatment with pertussis toxin, cells were washed and preincubated with $[^{3}H]$adenine without (O) or with (●) 10 $\mu$M norepinephrine for 30 min. Following another wash, the cells were stimulated with 10 $\mu$M forskolin (C, ●). For panel B, following pretreatment with pertussis toxin, cells were washed and preincubated with $[^{3}H]$adenine for 30 min. Following another wash, the cells were stimulated with 10 $\mu$M forskolin without (O) or with 1 $\mu$M UK14,304 (●). Note that the forskolin-only data (C) are identical in the two panels. Results for both panels are presented as percent conversion $[^{3}H]$ATP to cyclic $[^{3}H]$AMP and are the means ± S.E. for three to five experiments. Basal content was subtracted.
**α2-Adrenergic Sensitization of Forskolin cAMP Production**

**Fig. 8.** Effect of pertussis toxin pretreatment on forskolin stimulation, sensitization, and α2-adrenergic inhibition of cyclic AMP production in intact cells and on [32P]ADP-ribosylation in membrane preparations. Cells were pretreated as described in Fig. 7. Results are presented as percent of control (absence of pertussis toxin pretreatment) and are the means ± S.E. for three to five experiments. Control values are as follows: forskolin stimulation percent conversion = 0.18 ± 0.05, forskolin stimulation following norepinephrine preincubation/forskolin stimulation (sensitization) = 17.4 ± 4; UK14,304 inhibition of forskolin-stimulated cyclic AMP production = 85 ± 4%. Statistical analysis utilizing a 2-factor analysis of variance along with examining interaction with treatments and time indicates that the forskolin and sensitization curves are statistically dissimilar (*P* < 0.01). Forskolin stimulation differs from inhibition from 3–8 h (*P* < 0.0002). At 18 h, sensitization is similar to inhibition but forskolin differs. The percent values for [32P]ADP-ribosylation were derived by cutting out the M2 = 41,000 bands, determining counts/minute by liquid scintillation spectrophotometry and dividing this value by the counts/minute of a 140-kDa band, where labeling was independent of the presence of pertussis toxin (see Fig. 9).

**Fig. 9.** ADP-ribosylation of membranes from pertussis toxin-pretreated cells. Cells in 35-mm dishes were pretreated with 100 ng/ml pertussis toxin for the indicated times. Cells were placed on ice and harvested as described under “Experimental Procedures.” On the day of the assay, a [32P]ADP-ribosylation experiment was conducted as described without (−) and with (+) 2.5 μg of pertussis toxin with subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis, drying, and exposure to XAR-2 film. The gel shown is representative of five such experiments. Molecular weight markers are on the left.

**DISCUSSION**

α2-Adrenergic agonist preincubation of the human colon carcinoma cell line HT29 causes a marked sensitization of subsequent forskolin-stimulated cyclic AMP production (1). We have shown previously that sensitization is rapid in onset (half-maximal in 5 min) and reversal (τ0 of 10 min) and does not appear to be caused by decreased cyclic AMP levels during the preincubation or to result from decreased degradation of cyclic AMP during the assay. In this paper we have explored the specificity of this phenomenon and possible mechanisms mediating sensitization in the HT29 cell.

**Characterization of Sensitization**—Sensitization is a dramatic increase in subsequent cyclic AMP production caused by agonist occupation of a receptor coupled in an inhibitory manner to adenylate cyclase. To assess the specificity of sensitization, we used both another activator of adenylate cyclase (VIP) and another receptor system negatively coupled to adenylate cyclase (somatostatin). α2-Adrenergic receptor sensitization of VIP-stimulated cyclic AMP production has different characteristics than sensitization of forskolin-stimulated cyclic AMP production. The stimulatory action of VIP is thought to be mediated directly by the α2 subunit of Gα, and the effect of VIP is sensitized only 2-fold. The stimulatory action of forskolin, by contrast, appears to involve both the catalytic unit directly, as well as another component (34, 35) and is sensitized 10–20-fold. In addition, the dose response to VIP clearly represents an increased efficacy following norepinephrine preincubation, whereas the left-shift in the dose-response curve to forskolin appears to be an increase in potency (1). These results are similar to the effects of somatostatin preincubation in AtT20 cells, where forskolin sensitization is greater in magnitude than VIP or isoproterenol sensitization (5). Furthermore, in AtT20 cells there is not a shift to the left in the dose-response curve (no change in EC50) for hormones activating cyclic AMP production, but there is a shift in the dose-response curve to forskolin. Similar effects have been reported in hormone stimulation of cyclic AMP production in fat cells from adenosine-1 agonist-treated rats (27). Taken together, these results may indicate that sensitization of α2-stimulated (hormone or cholera-toxin) cyclic AMP production may be mediated differently than sensitization of forskolin-stimulated cyclic AMP production.

Somatostatin receptors are negatively coupled to adenylate cyclase in HT29 as evidenced by somatostatin’s inhibition of both forskolin- and VIP-stimulated cyclic AMP production. However, somatostatin was less efficacious than norepinephrine both in its maximal inhibition of cyclic AMP production (60% versus 85%) and in causing sensitization (3.5-fold versus 15-fold). In AtT20 mouse pituitary cells somatostatin was both more potent and more efficacious in inhibiting cyclic AMP production as compared with HT29 cells (4, 5). These data could reflect either a lower density of somatostatin receptors or less efficient coupling of these receptors to the effector systems. Whereas the degree of somatostatin sensitization in HT29 cells and in AtT20 cells was similar, the time course was not, since at least 4 h are required to see any significant sensitization in AtT20 cells. The difference in the time of preincubation necessary to observe sensitization in the two systems could reflect either a difference in cell type or a difference in mechanism of sensitization.

Agonist preincubation of cells can result in a blunting of
the physiological response to a subsequent challenge by that agonist. In a system in which a receptor is negatively coupled to adenylate cyclase, one would expect to see an attenuation of inhibition following a subsequent challenge with agonist.

In the HT29 cells, the dose-response to an α2 agonist in inhibiting cyclic AMP production was shifted to the right (less potent) following norepinephrine preincubation. We have interpreted these data as indicating a desensitization of the α2-adrenergic receptor-adenylate cyclase system. Similar effects have been found with somatostatin and muscarinic cholinergic agonist pretreatment in AtT20 cells (5, 36). It is noteworthy under identical conditions that α1 agonist preincubation causes both desensitization of inhibition and sensitization of stimulation. Further experiments of both phenomena will be needed to understand the relationship between them.

Potential Mechanisms of Sensitization—Mechanistic studies are frequently more efficiently pursued in cell-free systems. Accordingly, we measured adenylate cyclase activity in broken cell preparations following preincubation of intact cells without and with agonist. Under these conditions, sensitization per se was not observable, as there was only a 1.3-fold increase in forskolin-stimulated cyclic AMP production compared to the 10-20-fold increase seen in the whole cell preparation. These results are similar to those involving the adenosine-1 receptor-mediated sensitization in fat cells, where there is a dramatic increase in hormone- and forskolin-stimulated (4- and 10-fold, respectively) cyclic AMP production in fat cells from animals pretreated for 6 days with the adenosine receptor agonist phenylisopropyladenosine (27). When adenylate cyclase was assayed in adipocyte membranes from animals treated with phenylisopropyladenosine, only a 2-fold sensitization of forskolin- or hormone-stimulated adenylate cyclase activity was observed (26). Our results in HT29 cells and those in the adipocyte indicate that the intact cell may be necessary for observing sensitization. An alternative explanation is that the membrane adenylate cyclase assay conditions may not be optimal for observing sensitization.

The mechanism for α2-adrenergic receptor-mediated sensitization of forskolin-stimulated cyclic AMP has not yet been elucidated. We have approached this issue by studying various components known to affect either forskolin stimulation of adenylate cyclase or the α2-adrenergic receptor system. Studies in brain, S49 lymphoma cells, bovine adrenal cells, and 3T3 fibroblasts have shown an involvement of protein kinase C in augmentation of hormone- or forskolin-stimulated cyclic AMP production (37-40). In addition, long-term treatment of cells with PMA has been shown to desensitize protein kinase C activity in BC3H-1 myocytes and 1321-N1 cells (41, 42). If activation of protein kinase C activity is important in sensitization, then long-term pretreatment with PMA should attenuate α2-adrenergic receptor sensitization of forskolin-stimulated cyclic AMP production. This did not occur, thus protein kinase C activation does not appear to be involved in the mechanism of sensitization. Our data, as well as those referenced above, do indicate that PMA pretreatment can either increase or decrease forskolin-stimulated cyclic AMP production, depending on the length of pretreatment.

Activation of the Na+/H+ antiporter is an α2-adrenergic receptor-mediated event in the human platelet and the neuroblastoma x glioma hybrid cell line NG108-15 (16, 19). However, an antiporter mechanism for sensitization can be discounted for the following reasons. First, there was no inhibition of sensitization at a pH of 6.8, which should block antiporter activity. Second, pretreatment with a nonspecific phospholipase A2 inhibitor, quinacrine or a cyclooxygenase inhibitor, indomethacin, had no effect on sensitization, indicating that the subsequent steps in this pathway do not mediate sensitization. Third, amiloride, a Na+/H+ antiporter inhibitor, did not block sensitization. Fourth, sensitization was eliminated by 18 h of pertussis toxin pretreatment, whereas α2-adrenergic receptor-mediated activation of the antiporter in the NG108-15 cells is pertussis toxin-insensitive (20).

In AtT20 mouse pituitary cells, a 4-h pretreatment with cycloheximide, an inhibitor of protein synthesis, appeared to block sensitization mediated by somatostatin receptors (5). However, a subsequent paper demonstrated a decrease in forskolin-stimulated cyclic AMP production with longer cycloheximide pretreatment such that at 16 h, the degree of sensitization induced by somatostatin is the same as in control cells (33). These data are consistent with our data, which indicate an effect of cycloheximide pretreatment on forskolin stimulation, but not on sensitization. These results are intriguing because Barovský et al. (34) have speculated that there are two forskolin binding sites. In their model, a high affinity binding site mediates synergism between forskolin and hormones or cholera toxin, which is similar in degree to sensitization. This site is cycloheximide-insensitive. The low affinity site is presumably on a protein with a shorter half-life as it is cycloheximide-sensitive. Our forskolin stimulation and sensitization results are consistent with the two-affinity site hypothesis. Whether or not the actual mechanisms for synergism and sensitization are similar, it would appear that the effects of cycloheximide on the two processes are similar.

Inhibitory Guanine Nucleotide Regulatory Protein—Pertussis toxin, presumably through ADP-ribosylation of Gi, blocked α2-adrenergic receptor-mediated sensitization of forskolin-stimulated cyclic AMP production as well as attenuated α2-adrenergic receptor-mediated inhibition of forskolin-stimulated cyclic AMP production. The inhibition of subsequent [32P]ADP-ribosylation of a protein, M, = 41,000, confirms that endogenous ADP-ribosylation occurred during the pretreatment. The inhibition of [32P]ADP-ribosylation followed the same time course as attenuation of sensitization and inhibition of cyclic AMP production in the HT29 cells. This effect of pertussis toxin on attenuation of inhibition is similar to that seen in NG108 and AtT20 cells (28, 36).

The effects of pertussis toxin pretreatment on sensitization were dramatic, resulting in a complete blockade. Similar results have been found for cholinergic receptor-induced sensitization of prostaglandin-E1-stimulated cyclic AMP in neuroblastoma x glioma cells (28). In addition, in vivo administration of pertussis toxin has been shown to abolish the naltrexone withdrawal effect normally seen in guinea pig ileum or myenteric plexus with chronic opioid administration (43, 44). The naltrexone withdrawal phenomenon is similar to sensitization and is also mediated through a receptor (the opioid receptor) that can couple in an inhibitory manner to adenylate cyclase.

The effects of pertussis toxin pretreatment on forskolin stimulation were unexpected. Two other studies have shown a slight decrease in forskolin stimulation following pertussis toxin pretreatment (40, 46), but not the 70% decrease we observed. A possible explanation for these results is that pertussis toxin is altering growth or protein synthesis in HT29 cells. This effect has been shown to occur in 3T3 fibroblast cells (46, 47). This does not seem likely because in both of these studies, the inhibition of growth effects of pertussis toxin required 2 days of pretreatment, whereas we saw effects on forskolin and VIP (data not shown) stimulation at 3 h. In addition, if alterations in cell growth were causing pertussis toxin effects, one might expect similar effects by cyclohexi-
mide pretreatment. There was, however, no effect of cycloheximide pretreatment on sensitization.

We considered the possibility that the effect of pertussis toxin on sensitization was merely a reflection of the reduced forskolin stimulation. However, forskolin stimulation is more sensitive to pertussis toxin pretreatment than sensitization based on the different time courses for blocking these events. The effects of pertussis toxin on sensitization follow the same time course as effects on α2-adrenergic receptor-mediated inhibition as well as inhibition of subsequent ADP-ribosylation of a protein, M* = 41,000.

A noteworthy generalization from these experiments is that forskolin stimulation in HT29 cells is altered by a variety of factors including pH, Na+ and other ions, cycloheximide, phorbol esters, and pertussis toxin, whereas only pertussis toxin alters sensitization. Thus, the HT29 cell may prove to be a useful system for defining the mechanism of forskolin action.

Preincubation of HT29 cells with agonists for receptors negatively coupled to adenylate cyclase causes sensitization of forskolin- and VIP-stimulated cyclic AMP production. It seems reasonable to suggest that cells exposed to an inhibitory agonist are adapting to this agonist challenge to maintain cellular homeostasis by overreacting to a subsequent stimulatory challenge. Neither protein kinase C nor the Na+/H+ antiporter appears to be involved in sensitization.

Although our data and those of others implicate a pertussis toxin-sensitive protein, probably, Gβ in the sensitization phenomenon, the actual mechanism remains elusive. Further studies of the multiple components involved in forskolin stimulation in those cells should lead to a better understanding of how these components could be modified to cause sensitization.

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