Activation of Particulate Guanylate Cyclase by *Escherichia coli* Heat-Stable Enterotoxin Is Regulated by Adenine Nucleotides

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Guanylate cyclase is regulated by adenine nucleotides in membranes of intestinal mucosal cells. Basal guanylate cyclase was activated about twofold by adenine nucleotides. Activation was specific for adenine, as compared with the pyrimidine nucleotides UTP and CTP. In addition, enzyme activation was obtained in the presence of saturating concentrations of GTP, the substrate for guanylate cyclase. The most potent adenine nucleotide was the nonhydrolyzable analog of ATP, adenosine 5'-O-(3-thiotriphosphate). Adenine nucleotide activation was specific for the particulate form of guanylate cyclase, as compared with the soluble form. Also, adenine nucleotides potentiated the activation of guanylate cyclase by the heat-stable enterotoxin produced by *Escherichia coli*. Indeed, enzyme activation by adenine nucleotides and toxin was greater than the sum of individual activations by these agents. Adenine nucleotides regulate guanylate cyclase by increasing the maximum velocity of the enzyme without altering its affinity for substrate or its cooperativity. In addition to stimulating guanylate cyclase, adenine nucleotides decreased the specific binding of the heat-stable enterotoxin to receptors in intestinal membranes. The coordinated regulation of the toxin-receptor interaction and guanylate cyclase activity by a process utilizing nonhydrolyzable analogs of a purine nucleotide is similar to the mechanisms involved in the hormone regulation of adenylate cyclase by guanine nucleotide-binding proteins. These data suggest that an adenine nucleotide-dependent protein may couple the toxin-receptor interaction to the regulation of particulate guanylate cyclase in intestinal membranes.

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**MATERIALS AND METHODS**

Membranes were prepared from homogenates of rat intestinal mucosal cells as described previously (31). In brief, mucosa from the intestines of one to three rats were pooled and homogenized in buffer containing 50 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 1 mM EDTA, 0.25 M sucrose, and 1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 100,000 × g for 60 min, and the resulting pellets were consecutively washed in (i) 50 mM Tris-HCl (pH 7.6)–1 mM EDTA–1 mM dithiothreitol (TED) supplemented with 500 mM KCl and (ii) TED. Washed pellets were suspended in TED and stored at −80°C until used.

Guanylate cyclase activity was assayed as described previously (12, 18, 31). In brief, incubations were conducted at 37°C in the presence of 50 mM Tris-HCl (pH 7.6)–0.5 mM isobutylmethylxanthine, a regenerating system including 7.5 mM creatine phosphate and 20 μg of creatine phosphokinase (160 U/mg of protein), 10 to 20 μg of membrane or detergent extract, manganese- or magnesium-GTP (3 mM excess of free metal), and ST when needed. Some incubations contained different concentrations of various nucleotides or their analogs. Reactions were initiated by the addition of metal-GTP, incubated for 5 min, and terminated by the addition of 0.4 ml of ice-cold 50 mM sodium acetate (pH 4.0). Samples were placed in a boiling water bath for 3 min. Generated cyclic GMP was quantified by radioimmunoassay (25) as described previously (12, 15, 16, 18, 25, 31). All reactions were performed at least in duplicate, and the data are representative of at least duplicate experiments. Enzyme activity was linear with respect to protein concentration and time throughout these experiments.

ST receptor binding was determined by a modification of the procedure described previously (20). In brief, intestinal membranes (20 to 40 μg of protein) were incubated in the reaction mixture used for guanylate cyclase assays, as outlined above, with 125I-ST (0.1 pmol) in a final volume of 60 μl. Nonspecific binding was measured in the presence of a 100-fold molar excess of unlabeled toxin and was less than

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**TABLE 1. Effect of nucleotides on particulate guanylate cyclase in rat intestinal membranes**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fold activation with: No ST</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0 ± 0.0</td>
<td>5.1 ± 1.0 (6)</td>
</tr>
<tr>
<td>ATP</td>
<td>2.7 ± 0.2 (7)</td>
<td>13.4 ± 4.0 (6)</td>
</tr>
<tr>
<td>ADP</td>
<td>1.8 ± 0.2 (5)</td>
<td>11.0 ± 3.2 (5)</td>
</tr>
<tr>
<td>Adenosine-5'-O-(2-thiodiphosphate)</td>
<td>1.5 ± 0.1 (5)</td>
<td>9.6 ± 3.2 (5)</td>
</tr>
<tr>
<td>dATP</td>
<td>2.5 ± 0.3</td>
<td>11.0 ± 3.2 (5)</td>
</tr>
<tr>
<td>CTP</td>
<td>1.1 ± 0.4</td>
<td>5.1 ± 1.0 (6)</td>
</tr>
</tbody>
</table>

* Guanylate cyclase was assayed as outlined in Materials and Methods in the presence of a 1 mM concentration of the indicated nucleotide. The regenerating system was omitted from assays to minimize nucleotide phosphorylation. ST (30 μM) was added to incubations as indicated. Basal activity in these experiments was 3.7 ± 0.5 (seven experiments) pmol of cyclic GMP produced per min per mg of protein.

* Calculated as the ratio of enzyme activity in the presence and in the absence of the indicated nucleotide and reported as the mean ± standard error (number of experiments).

P < 0.0005.

P < 0.02.

P < 0.05.

20% of total binding. Reactions were incubated for 15 min at 37°C, and bound 125I-ST was separated from the free ligand by vacuum filtration through polyethyleneimine-treated Whatman GF/B glass fiber filters. Reaction tubes and filters were washed three times with ice-cold phosphate-buffered saline (pH 7.2). The radioactivity on the filter was determined by counting in a Beckman 5500 gamma counter. All binding assays were performed at least in duplicate.

Toxins used in these experiments included native ST (Sigma Chemical Co.) and a synthetic 18-amino-acid analog of native ST. These peptides were equally efficacious in receptor binding and guanylate cyclase activation assays. Native ST was radioiodinated by the lactoperoxidase method (Enzymobeads; Bio-Rad Laboratories) to a specific activity of about 100 Ci/mmole as described previously (20). Statistical significance was calculated with Student’s t test for paired samples (2).

**RESULTS**

The effects of various nucleotides on basal and ST-stimulated guanylate cyclase activity in membranes of intestinal mucosal cells were examined (Table 1). Significant activation of basal enzyme activity was observed with the following nucleotides in descending order of potency: adenosine 5'-O-(3-thiodiphosphate) (ATPdG) > ATP > adenylimidodiphosphate, adenosine 5'-O-(2-thiodiphosphate) > dATP, ADP, AMP, adenosine. Maximum activation of basal guanylate cyclase was two- to threefold with ATPdG. Adenine nucleotides which activated basal guanylate cyclase were tested for their ability to potentiate enzyme activation by ST (Table 2). These nucleotides potentiated the activation of guanylate cyclase by ST with an order of potency similar to that for the activation of the enzyme. Maximum activation of guanylate cyclase with the combination of ST and ATPdG was 10- to 20-fold, 3- to 4-fold greater than with ST alone. The activation of basal and ST-stimulated guanylate cyclase was specific for adenine nucleotides: UTP and CTP had no effect. Studies of the effects of GTP are technically difficult, since this nucleotide is the substrate for guanylate cyclase, and are addressed below.

The effects of adenine nucleotides and ST on particulate and soluble guanylate cyclase were compared with ATPdG, since it produced the greatest enzyme stimulation (Table 3). This nucleotide inhibited the soluble enzyme about 40%, whereas it stimulated the particulate enzyme 2.5-fold. ST did not alter the activity of soluble guanylate cyclase but activated the particulate enzyme about fivefold. Together, ST and ATPdG increased particulate enzyme activity 14-fold but inhibited soluble enzyme activity about 40%. These data are consistent with previous reports describing the inhibition of soluble guanylate cyclase by ATP (14, 22). They suggest that the regulation of guanylate cyclase by ST and adenine nucleotides is specific for the particulate isoenzyme as opposed to the soluble isoenzyme.

The concentration dependence of guanylate cyclase activation by ATPdG was examined in the presence or absence of ST (Fig. 1). ATPdG activated the enzyme in a dose-dependent manner. Maximum activation was observed with 1 mM ATPdG, and the concentration yielding half-maximum activation was about 10⁻⁴ M in the presence or absence of maximally stimulating concentrations of ST. Consequently, adenine nucleotide concentrations used in subsequent experi-

**TABLE 2. Effect of ATPdG on particulate and soluble guanylate cyclase in rat intestines**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Particulate enzyme</th>
<th>Soluble enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sp actb</td>
<td>Fold activation</td>
</tr>
<tr>
<td>None</td>
<td>3.1 ± 1.0</td>
<td>10.00 ± 1.7</td>
</tr>
<tr>
<td>ST</td>
<td>16.0 ± 4.0</td>
<td>5.5 ± 2.2</td>
</tr>
<tr>
<td>ATPdG</td>
<td>7.7 ± 3.0</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>ST + ATPdG</td>
<td>41.7 ± 5.7</td>
<td>14.0 ± 4.9</td>
</tr>
</tbody>
</table>

* Guanylate cyclase activity in soluble and particulate fractions from intestines (15 μg of protein per assay) was measured in the presence or absence of 30 μM ST, 1 mM ATPdG, or 30 μM ST plus 1 mM ATPdG as outlined in Materials and Methods. Values are reported as means ± standard errors. The data are the means of three experiments.

* Picomoles of cyclic GMP produced per minute per milligram of protein.

* Specific activity in the presence of the indicated agent/basal specific activity.

**TABLE 3. Effect of ATPdG on the kinetics of rat intestine particulate guanylate cyclase**

<table>
<thead>
<tr>
<th>Addition</th>
<th>S0.5 (mM)</th>
<th>Vmax (pmol/min/mg of protein)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.2</td>
<td>32 ± 2.5</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>ST</td>
<td>4.7 ± 0.2</td>
<td>176 ± 46.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>ATPdG</td>
<td>4.3 ± 0.6</td>
<td>48 ± 9.4*</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>ST + ATPdG</td>
<td>3.8 ± 0.6</td>
<td>278 ± 50.7*</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>

* Guanylate cyclase activity was determined in the presence or absence of 1 mM ATPdG with MgCl₂-GTP as the substrate as outlined in Materials and Methods. Values are reported as means ± standard errors. The data are the means of three experiments.

* Concentration of substrate yielding half-maximum guanylate cyclase activity (3, 8a, 32).

* Maximum velocity of enzyme activity (picomoles of cyclic GMP produced per minute per milligram of protein) (3, 8a, 30).

* Calculated as described previously (3, 8a, 30).

* P < 0.10.

* P < 0.05.
Guanylate cyclase. Guanylate cyclase in the absence of ATPgS, and guanylate cyclase activity was determined as described in Materials and Methods. Assays were conducted in the absence (□) or the presence (△) of 1 µM ST.

The effect of ATPgS on the concentration dependence of guanylate cyclase activation by ST was examined (Fig. 2). ATPgS increased the maximum response of guanylate cyclase to ST, without a significant change in the concentration of this yielding half-maximum peptide enzyme activation, which was about 10⁻⁷ M.

The effects of ATPgS on basal and ST-activated guanylate cyclase were explored with magnesium or manganese as a cation cofactor (Fig. 3). ATPgS had no effect on basal or ST-stimulated guanylate cyclase in the absence of free magnesium. Maximum basal and ST- and ATPgS-stimulated activities required the presence of 5 mM free MgCl₂. In contrast, MnCl₂ inhibited the enzyme in the presence of ATPgS at each concentration of free manganese tested.

The effects of ATPgS on the kinetics of basal and ST-stimulated guanylate cyclase were examined (Fig. 4 and Table 3). Enzyme activity was concentration dependent and saturable in the absence or presence of maximally stimulating concentrations of ST. Activation by ATPgS in the presence of 1 mM GTP-MgCl₂ was reduced when saturating concentrations of the substrate were used. However, ATPgS significantly potentiated ST activation of guanylate cyclase in the presence of saturating concentrations of GTP. Indeed, ATPgS increased Vₘₕ without altering the affinity of the enzyme for the substrate or enzyme cooperativity (Table 3).

The ability of nucleotides to alter the specific binding of ¹²⁵I-ST to receptors in membranes of intestinal cells was measured. Adenine nucleotides inhibited the specific binding of ST to receptors with an order of potency that was similar to that observed for adenine nucleotide activation of guanylate cyclase (Table 1): ATPgS > ATP > other adenine nucleotides. Maximum inhibition of ST binding of 30 to 40% was observed with ATPgS. Inhibition of binding was specific for adenine nucleotides, since CTP and UTP had no effect, and all binding was performed in the presence of 1 mM GTP.
These data concern peptide-activated guanylate cyclase.

**DISCUSSION**

The primary event mediating ST-induced secretion is the binding of peptides to intestinal cell surface receptors. These receptors are coupled to the activation of guanylate cyclase and increases in intracellular cyclic GMP. ST receptors and guanylate cyclase are separate molecules, and little is known concerning their functional coupling (29). It is well established that guanine nucleotide-binding proteins couple hormone-receptor interactions and adenylate cyclase activity (11, 26, 27). Recently, ATP was reported to enhance basal guanylate cyclase activity in rat lungs (8a) as well as atrial peptide-activated guanylate cyclase in rat lungs and liver (4, 21). These data suggest that adenine nucleotides may regulate basal and receptor-stimulated guanylate cyclase in a manner which is similar to the regulation of adenylate cyclase by guanine nucleotides.

The data reported here demonstrate the activation of basal and ST-stimulated guanylate cyclase by adenine nucleotides. Activation of the enzyme was induced specifically by adenine, as compared with other nucleotides. Enzyme stimulation with ST and adenine nucleotides together was greater than the sum of individual activations by these agents. The most potent nucleotide regulating guanylate cyclase was the nonhydrolyzable analog of ATP, ATPγS. This effect is similar to the effects of nonhydrolyzable analogs of GTP on adenylate cyclase mediated by guanine nucleotide-binding proteins (11, 26, 27). These data suggest that an adenine nucleotide regulatory protein may couple ST-receptor interactions to guanylate cyclase activation. Adenine nucleotides which serve as phosphate donors through a kinase reaction (ATP) and those which do not (adenyl-imidophosphate) were effective in activating guanylate cyclase. Thus, it is unlikely that activation involves phosphorylation of the enzyme by a kinase with adenine nucleotides as phosphate donors.

Adenine nucleotides activate the particulate form of guanylate cyclase specifically, as compared with the soluble form. The stimulation of basal and ST-activated guanylate cyclase by adenine nucleotides was concentration depen-
Alternatively, adenine nucleotides may regulate particulate guanylate cyclase by a separate adenine nucleotide-binding protein. Previous studies demonstrated that adenine nucleotide regulation of ANP-stimulated particulate guanylate cyclase could be separated from that enzyme by sequential washing of crude membranes (4). Washing did not reduce the specific activity of the enzyme but did remove the ability of adenine nucleotides to potentiate the activation of guanylate cyclase by ANP. Similarly, recent studies demonstrated that adenine nucleotide regulation and guanylate cyclase activity could be separated by partial purification of this enzyme (8a). When detergent extracts of rat lung membranes were chromatographed on GTP-agarose, particulate guanylate cyclase was quantitatively purified. However, this partially purified enzyme was unresponsive to adenine nucleotides. These data support the suggestion that adenine nucleotide regulation of particulate guanylate cyclase is mediated by a protein which is separate from that enzyme (4, 8a).

In conclusion, the data presented here demonstrate that the activation of particulate guanylate cyclase by ST-receptor interactions is regulated by an adenine nucleotide-dependent event. Activation is potentiated by analogs of ATP which cannot serve as kinase substrates. Thus, it is unlikely that adenine nucleotide regulation of this system reflects alterations in the phosphorylation state of the constituents of the involved signal transduction cascade. Adenine nucleotides alter the maximum activity of basal and agonist-stimulated enzyme without altering the affinity of the enzyme for the substrate or the characteristic cooperativity of particulate guanylate cyclase. These nucleotides coordinately regulate both ST receptor and particulate guanylate cyclase activities. The nucleotide most potent in regulating these activities is the nonhydrolyzable analog of ATP, ATPγS. That both receptor and effector activities are regulated by a purine nucleotide-dependent mechanism which utilizes nonhydrolyzable analogs of ATP supports the suggestion that an adenine nucleotide-binding protein may regulate this system in a manner similar to the regulation of adenylate cyclase by guanine nucleotide-binding proteins. Identification and characterization of this coupling protein are currently being undertaken in this laboratory.

ACKNOWLEDGMENT

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REFERENCES