Failure of Pertussis Toxin To Inhibit Activation of Guanylate Cyclase by the Heat-Stable Enterotoxin of Escherichia coli (STa) in the T84 Cell Line

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The heat-stable enterotoxin (STa) of Escherichia coli causes intestinal secretion by stimulating particulate guanylate cyclase, an enzyme believed to be distinct from the STa receptor. Pertussis toxin (PT) has been reported to block the ability of STa to stimulate guanylate cyclase in rat intestinal mucosa (S. A. Epstein, R. A. Giannella, and H. J. Brandwein, FEBS Lett. 203:44–48, 1986). This suggested that a guanine nucleotide regulatory protein (G protein) coupled the STa receptor to guanylate cyclase, a function not previously recognized for G proteins. We sought to explore this phenomenon and, if possible, to identify this G protein. Initial experiments with the human colon carcinoma cell line T84 revealed that higher-than-expected concentrations (1 μg/ml) of PT were needed to intoxicate cells, as assessed by ADP-ribosylation of endogenous PT substrate, but that 90 to 100% intoxication could be achieved. Homogenates from fully intoxicated cells did not differ from controls in basal or STa-stimulated guanylate cyclase activity, and cyclic GMP accumulation in intact T84 cells was not changed by PT treatment. We conclude that a PT-sensitive G protein is not involved in the stimulation of cyclic GMP production by the enterotoxin STa.

The heat-stable enterotoxin of Escherichia coli (STa) causes intestinal fluid secretion by stimulating particulate guanylate cyclase (15, 17). Although the STa receptor and guanylate cyclase are apparently distinct macromolecules (20, 28), the mechanism of coupling between the two is unknown. Pertussis toxin (PT) has been reported to reduce the ability of STa to stimulate guanylate cyclase in rat intestinal mucosa (9). This suggested that a PT-sensitive guanine nucleotide regulatory protein (G protein) is linked to a stimulation of guanylate cyclase, a function not previously reported in the repertoire of G proteins. We wished to further explore these observations and the identity of a possible new G protein.

The T84 human colon carcinoma line is a cell line which maintains adenylate cyclase sensitive to toxins and hormones and also maintains vectorial chloride transport (5). It also expresses receptors for STa and possesses STa-stimulated guanylate cyclase (14), and therefore was chosen for use in this study.

In the present study, no effect of conventional doses of PT (10 to 500 ng/ml) was found on STa-stimulated guanylate cyclase or on STa-stimulated cyclic GMP accumulation in intact cells. An ADP-ribosylation assay was used to determine the extent of intoxication with various doses of PT. The ability of STa to stimulate cyclic GMP production was preserved in fully PT-intoxicated cells and homogenates made from these cells.

(This study was presented in part at the national meeting of the American Federation of Clinical Research held in Washington, D.C., in May 1988.)

MATERIALS AND METHODS

Cell culture. T84 cells were obtained from the American Type Culture Collection, Rockville, Md., and were grown in medium consisting of a 1:1 mixture of Dulbecco modified Eagle medium and Ham F12. The medium was supplemented with 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (pH 7.4)–12.8 mM NaHCO3–105 U of penicillin per liter–100 mg of streptomycin per liter–5% newborn calf serum. Cells were grown in monolayers in polystyrene flasks and were split when confluent by using 0.25% trypsin–0.9 mM EDTA in phosphate-buffered saline (PBS). Cells were incubated at 37°C in a humidified atmosphere containing 5 to 7% CO2. For the preparation of cell fractions, cells were harvested by scraping in ice-cold PBS containing 1 mM EDTA. For experiments involving intact cells, 5 × 105 cells in 1 ml of medium were plated into each well (1.7 cm2) of multilwell plates and were allowed to grow for 6 to 8 days, at which time they were at or near confluency. The T84 cells were at passage number 49 upon receipt from the American Type Culture Collection and were maintained for an additional 20 passages.

Preparation of cellular fractions. The T84 cell suspension obtained by scraping was pelleted by centrifugation at 2,000 × g and was suspended at 4°C in a hypotonic medium of 50 mM Tris (pH 7.4 at 25°C)–2 mM EDTA–0.9 mM benzamidine–5 μM aprotinin–60 μg of leupeptin per ml–0.1 mM phenylmethylsulfonylfluoride. The suspension was homogenized at 4°C in a glass Dounce homogenizer with a tight-fitting pestle for 35 strokes and was allowed to sit on ice for 10 min. This material is referred to as the crude homogenate. The supernatant obtained from centrifugation at 600 × g for 10 min is designated the low-speed supernatant, and the pellet obtained from centrifuging the latter at 82,000 × g for 1 h is the particulate fraction.

Rabbit ileum preparation. A 1.7-kg male New Zealand White rabbit was anesthetized, and a 10-cm portion of ileum

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was isolated with double ties. The segment was excised and washed with cold PBS. The segment was cut lengthwise along the antimesenteric border and laid flat. Mucosa was scraped off with a glass slide and placed in ice-cold PBS. This material was pelleted at 600 × g and was suspended into hypotonic lysing buffer and homogenized just as described above for T84 cells.

PT intoxication of cells. PT was prepared by the method of Cronin et al. (4) with modifications making it comparable to that prepared by List Laboratories and used by Epstein et al. (9), and its activity was tested by hemagglutination and by clumping of Chinese hamster ovary (CHO) cells (16).

Monolayers of T84 cells, near or at confluency at 6 or 7 days of culture, were exposed to PT by removing the old culture medium, rinsing the flask with 5 ml of PBS, and then adding 5 ml of PBS to the flask again. A stock of PT was added to yield a concentration of toxin in PBS 10 times that of the final desired concentration. The PT-PBS mixture was incubated over the cells for 15 min at room temperature. Control flasks were treated identically except that vehicle (100 mM potassium phosphate [pH 7.0]–500 mM NaCl) was used instead of toxin. Then 45 ml of fresh medium was added to the flasks to yield the final toxin concentration, usually 1 µg/ml, and the cells were incubated for 24 to 48 h.

For the experiments performed with intact T84 cells, PT was added directly to the tissue culture wells to yield the appropriate final concentration, usually 1 µg/ml, and the cells were incubated for 24 h.

Treatment of homogenates in vitro with PT. Homogenates from control T84 cells were prepared as described above. PT was activated before use by incubation with 10 mM dithiothreitol (DTT) (termed the low-DTT condition) or 50 mM DTT (high-DTT condition) and 2 mM ATP at 30°C for 30 min. Activation is necessary to dissociate the A subunit from the B oligomer and thereby reveal the A (catalytic)-subunit activity. Carry-over of DTT into the guanylate cyclase assay strongly inhibited the enzyme and was controlled for by also treating homogenates with a sham activation mixture containing the DTT and ATP but lacking PT. The in vitro ADP-ribosylation was performed by mixing the homogenate, 20 µg of activated PT or sham mix per ml, 1 mM NAD, 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 2.5 mM MgCl₂, and 1 mM EDTA in 100 mM Tris (pH 8.0) at 30°C for 30 min (18, 26, 30, 31). Then the sham- or PT-treated homogenates were diluted into tubes for the guanylate cyclase assay. Control homogenates were not incubated with activation mix or NAD. The final concentrations of DTT which carried over into the guanylate cyclase assay were 0.4 and 2 mM in the low- and high-DTT conditions, respectively; the final concentration of ATP in the guanylate cyclase reaction was 80 µM.

Guanylate cyclase assay. Homogenates or other cell fractions were assayed for guanylate cyclase in a reaction mixture of 50 mM Tris (pH 7.4)–0.5 mg of bovine serum albumin per ml–20 µg of creatine phosphokinase per ml–15 mM phosphocreatine–0.4 mM 3-isobutyl-1-methylxanthine (IBMX)–1 mM GTP–4 mM MgCl₂. The reaction volume was 100 µl, and the reaction was begun by adding the GTP-MgCl₂ and was carried out at 37°C for 10 min. The reaction was terminated by adding 0.9 ml of 0.1 M HCl. Pilot experiments showed that the assay was linear with time for 14 min and linear with protein concentration from 10 to 55 µg of particulate fraction and from 2.5 to at least 25 µg of crude homogenate. Samples obtained were frozen and assayed for cyclic GMP by radioimmunoassay.

Cyclic GMP accumulation in intact cells. T84 cells grown in multwell plates were used in experiments by removing the standard medium and replacing it with 0.5 ml of medium containing 1 mM IBMX and lacking calf serum. For STa-stimulated conditions, the STa toxin was added to a final concentration of 1 µg/ml (0.5 µM) to initiate the experiment, and the plates were incubated in a 37°C water bath for 30 min. The dose of 1 µg/ml was determined to be a maximal dose in experiments not shown. The cyclic GMP accumulation reaction was stopped by adding 0.5 ml of 0.2 M HCl for 30 min; this method measures total cyclic GMP, intracellular plus that in the medium. The clear cell extracts obtained were assayed for cyclic GMP directly, and the results obtained were comparable to those seen when the incubation was stopped with 5% trichloroacetic acid and the samples were then repeatedly extracted with diethyl ether to remove the trichloroacetic acid. Several wells from each plate were used to measure cellular protein instead of cyclic GMP. Cellular protein measurements had a coefficient of variation of 10%, and cyclic GMP determinations were made in quintuplicate.

Radioimmunoassay of cyclic GMP. Cyclic GMP was measured by acetylation each sample and standard with 45 µl of a 3.5:1 mixture of triethylenamine and acetic anhydride per ml of sample, followed by automated radioimmunoassay as described elsewhere (3). In guanylate cyclase assays, the 1 mM GTP cross-reacted with antibody sufficiently to yield a GTP blank reading of 3.3 ± 0.2 pmol/ml (n = 7 experiments). This GTP blank was determined for each experiment and was subtracted from the raw cyclic GMP value of all tubes.

ADP-ribosylation assay. Particulate fractions or homogenates from control or PT-treated T84 cells were assayed for their degree of intoxication with an ADP-ribosylation procedure in vitro, using [³²P]NAD by the method of Pobiner et al. (23). Briefly, 3 to 4 µg of a cell fraction was pelleted at 12,000 × g for 15 min, and then detergent was extracted by suspending the pellet in a solution of 1% sodium cholate–50 mM Tris (pH 8.0)–1 mM EDTA–1 mM DTT–100 mM NaCl and shaking on ice for 1 h. PT was activated by incubating 120 µg of PT per ml in 50 mM DTT–0.1 mg of bovine serum albumin per ml–250 mM glycine buffer (pH 8.0) for 30 min at 30°C.

The ADP-ribosylation reaction was carried out by adding 4 to 30 µg of protein in cholate detergent to a tube containing (in a final volume of 100 µl) 2 µM unlabeled NAD, 2 µCi of [³²P]NAD, 100 mM Tris (pH 8.0), 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 2.5 mM MgCl₂, 1 mM EDTA, 3 mM sonicated dimyristoyl phosphatidylcholine, and 22.5 µg of preactivated PT per ml. The reaction was carried out at 30°C for 30 min and was terminated by adding 50 µl of 3 × Laemmli sample buffer (21), followed by boiling for 5 min. The samples were then applied to 12% polyacrylamide–sodium dodecyl sulfate (SDS) vertical slab gels and were electrophoresed at a constant current (21). The gels were stained with Coomassie blue, and radioactive bands were visualized by autoradiography.

Purified transducin was used as a 39-kilodalton (kDa) molecular size marker on autoradiograms and was a generous gift from James Miller of the Department of Biochemistry, University of Virginia, who prepared it from bovine retina by the method of Kuhn (19) with additional purification on DEAE-Sepharose by the method of Baehr et al. (1). Bovine brain membranes were used as a source of 39-, 40-, and 41-kDa PT substrates (24) and were a gift from the laboratory of Joel Linden, Department of Physiology, University of Virginia, where they were prepared by the method of Linden et al. (22).
Protein was assayed by the method of Lowry et al. (23), using a bovine serum albumin standard, except for the detergent extracts which were assayed by the method of Bradford (2), using a kit from Bio-Rad Laboratories, Richmond, Calif.

Materials. Purified STa was obtained from Donald C. Robertson, University of Kansas, Lawrence, and was prepared as described by Dreyfus et al. (6). [32P]NAD was from NEN-Du Pont, Boston, Mass. Fraction V bovine serum albumin, leupeptin, and aprotinin were from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The following were obtained from Sigma Chemical Co., St. Louis, Mo.: GTP, lithium salt; ATP, disodium salt; creatine phosphokinase, rabbit muscle; phosphocreatine; dimyristoyl phosphatidylcholine, synthetic; β-NAD, sodium salt, from yeast; and IBMX. Electrophoresis reagents were from Hoefer Scientific and X-ray film was XK-1 from Eastman Kodak Co., Rochester, N.Y.

Data analysis. The data presented are expressed as means ± standard error of the mean. The Student t test was used to test statistical significance.

RESULTS

PT did not alter STa-stimulated cyclic GMP accumulation or STa-stimulated guanylate cyclase activity. PT was tested at doses of 10, 20, 100, and 500 ng/ml for 16 h in these early experiments, on the basis of concentrations reported to be effective in cultured pituitary cells (4), rat C6 glioma cells (27), and in the T84 cell line (J. Kiely and H. Brandwein, Fed. Proc. 46:2249, 1987). Because of the consistently negative results, we quantitated the activity of the PT by using an ADP-ribosylation assay to determine the degree of intoxication achieved by incubation of intact cells with PT. In this assay, cells intoxicated with PT should be resistant to incorporation of [32P]ADP-ribose in vitro because the G-protein substrates for PT have already been ADP-ribosylated by endogenous (unlabeled) NAD. The ADP-ribosylation method of Pobiner et al. (26) was used because it is adapted to small sample sizes and because the cholate extraction step removes endogenous NAD hydrolases found in crude cellular fractions. The cholate extraction step was found necessary for good labeling of G proteins in homogenates of T84 cells (experiments not shown; see Fig. 2 below).

Figure 1A demonstrates an autoradiogram obtained after electrophoresis on an SDS-polyacrylamide gel of crude homogenates of T84 cells (experiments not shown; see Fig. 2 below). Figure 1A demonstrates an autoradiogram obtained after electrophoresis on an SDS-polyacrylamide gel of crude homogenates of T84 cells that had been preincubated with 1 μg of PT per ml or with vehicle for 24 h. Lane 1 demonstrates heavy labeling of a protein with a molecular size of 39 to 41 kDa by PT in vitro in control T84 homogenates. This labeling was dependent on activated PT in vitro (lane 2). No incorporation of labeled ADP-ribose was seen in homogenates made from PT-treated T84 cells, indicating that the complete intoxication had occurred (lane 3). Purified transducin (39 kDa) was labeled in vitro as well (lane 4). For full intoxication of T84 cells, 1 μg of PT per ml was needed, since lower doses (10, 20, and 100 ng/ml) of PT did not detectably affect the subsequent incorporation of the radiolabel. In three separate experiments performed with 1 μg of PT per ml, the degree of ADP-ribosylation achieved in intact, PT-treated cells was determined by comparing densitometer readings of intoxicated preparations with controls. In these experiments, the degrees of ADP-ribosylation were 98.9, 98.3, and 100% after exposures to PT of 48, 24, and 24 h, respectively.

Figure 1B shows the relevant portion of an autoradiogram from an SDS-polyacrylamide gel electrophoresis performed to improve resolution and better distinguish the size of the target protein(s) labeled in vitro by PT. The labeled substrates in T84 cells were compared with G proteins in other tissues. In homogenates of mucosal scrapings from rabbit ileum, label was incorporated into a closely spaced doublet (lanes 1 and 2), both components of which are larger than transducin (lane 5). The bands in rabbit ileum may correspond to the alpha41 and alpha40 species separated chromatographically and immunologically by Mumby et al. (24) and collectively referred to as Gc. Lane 3 shows the pattern of labeling seen with T84 cells. Lane 4 represents bovine brain membranes, a rich source of Gc (39 kDa) as well as 40- and 41-kDa PT substrates. No 39-kDa PT substrate was identified in the T84 cells or rabbit ileal mucosa.

The ADP-ribosylation assays showed that higher-than-expected concentrations of PT were required to achieve full intoxication of T84 cells. Therefore, the effect of high-dose
FAILURE OF PT TO INHIBIT GUANYLATE CYCLASE ACTIVATION

TABLE 1. STa-stimulated guanylate cyclase in homogenates made from control and PT-intoxicated T84 cells

<table>
<thead>
<tr>
<th>Expt</th>
<th>Treatment*</th>
<th>Guanylate cyclase activitya (pmol/min per mg ± SE)</th>
<th>% Change in STa-stimulated activityb by PTc</th>
<th>% ADP-ribosylationd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>STa-stimulated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>4.3 ± 6.2</td>
<td>52.7 ± 6.2</td>
<td>−36</td>
</tr>
<tr>
<td></td>
<td>PT</td>
<td>8.7 ± 2.8</td>
<td>33.8 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>6.1 ± 0.01</td>
<td>113.0 ± 1.8</td>
<td>−4</td>
</tr>
<tr>
<td></td>
<td>PT</td>
<td>9.0 ± 1.6</td>
<td>109.0 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>14.6 ± 1.0</td>
<td>122.0 ± 5.1</td>
<td>+14</td>
</tr>
<tr>
<td></td>
<td>PT</td>
<td>23.0 ± 2.2</td>
<td>139.0 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

* PT, 1 μg/ml for 24 h.

b The values for the GTP blank (see Materials and Methods) were 2.62, 4.4, and 3.21 pmol/ml for experiments 1, 2, and 3, respectively, and were subtracted from the raw cyclic GMP data. STa, 1 μg/ml.

c Taking the three experiments together, using the paired t test, PT had no significant effect on STa-stimulated guanylate cyclase. Activity was inhibited in experiments 1 and 2 and was stimulated in experiment 3.

d ND, Not done.

PT on STa-stimulated guanylate cyclase and cyclic GMP accumulation was tested.

Table 1 shows basal and STa-stimulated guanylate cyclase activities in homogenates made from control and PT-treated cells. Crude homogenates were used in this experiment and most others because guanylate cyclase activities were higher than in the low-speed supernatant and particulate fraction, but similar patterns were seen in those fractions as well. Table 1 presents results from three experiments, each done in triplicate. In experiment 1, there was a small decrease (36%) in STa-stimulated guanylate cyclase activity with the PT treatment, in experiment 2 there was no effect, and in experiment 3 there was a small increase. The degrees of ADP-ribosylation achieved were 98.9 and 100% in experiments 1 and 2, respectively. In this series of experiments, no consistent effect of PT on STa-stimulated guanylate cyclase activity was noted. There also was not a statistically significant effect of PT treatment on basal guanylate cyclase activity.

High-dose PT treatment was also tested for its ability to alter STa-stimulated cyclic GMP accumulation in intact T84 cells. In these experiments, cells were treated with 1 μg of PT per ml for 24 h or with vehicle for controls, and cyclic GMP levels were measured in the basal state and 30 min after the addition of 1 μg of STa per ml, both in the presence of 1 mM IBMX. The basal cyclic GMP accumulation (in pmoles per milligram ± standard error of the mean) was 2.19 ± 0.04 and 2.41 ± 0.17 for control and PT-treated cells, respectively; the STa-stimulated cyclic GMP accumulation was 238 ± 21 and 278 ± 8.8 for control and PT-treated cells, respectively. Thus, basal and STa-stimulated cyclic GMP accumulation in the PT-treated cells was unchanged compared with that of control cells.

In some experimental systems, PT treatment results in enhanced accumulation of cyclic AMP. In the T84 cells, basal cyclic AMP levels were 3 to 5 pmol/mg of protein and were unchanged by PT treatment. Prostaglandin E2 (10 μM) stimulated cyclic AMP accumulation in T84 cells to 1,600 to 2,000 pmol/mg; this accumulation was slightly enhanced in PT-treated cells, but the difference did not achieve statistical significance.

Because of the discrepancy between our results and those of Epstein et al. (9), we attempted to follow their experimental conditions more closely in the method of PT intoxication. Epstein et al. treated rat mucosal homogenates in vitro with PT and assayed guanylate cyclase activity directly without removing the PT. In their work, the PT was activated before use but the exact method was not specified; DTT apparently was not used. Pilot ADP-ribosylation experiments in our laboratory consistently showed poor labeling of G proteins when we omitted DTT in the activation of PT; other investigators have used 20 to 50 mM DTT to activate PT (26, 30, 31). Therefore, we compared two methods of activating PT, a high-DTT method with 50 mM DTT and 2 mM ATP and a low-DTT method with 10 mM DTT and 2 mM ATP. The concentrations of DTT which carried over into the guanylate cyclase assay were 2 and 0.4 mM for the high- and low-DTT conditions, respectively. Because of the known effects of sulphydryl reducing agents on STa toxin and guanylate cyclase (8, 29), each PT treatment was accompanied by a sham treatment with the preactivation mix but no PT. The results of an experiment (typical of three) done in triplicate are summarized in Table 2. Basal and STa-stimulated guanylate cyclase activities were inhibited about 40% by addition of the low-DTT sham activation mixture and were profoundly inhibited by the high-DTT sham activation mixture. Apart from the effects of the activation mixture, PT had no additional inhibitory effect on basal or STa-stimulated guanylate cyclase even when applied to homogenates in vitro at a concentration of 20 μg/ml in the presence of NAD.

Interestingly, when we subjected the homogenates already treated in vitro with PT to cholate extraction and performed the ADP-ribosylation assay, additional [32P]ADP-ribose was incorporated into G proteins (Fig. 2). Lane 1 shows labeling of purified transducin. Lane 2 shows the labeling of a cholate extract of T84 cell homogenates previously treated with preactivated PT and unlabeled NAD in vitro, compared with the labeling seen in control T84 homogenates (lane 3). Lane 4 again shows labeling of bovine brain membranes. Densi-

TABLE 2. Effect on guanylate cyclase of treating homogenates in vitro with PT

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Low DTT</th>
<th>High DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>17.7 ± 0.1</td>
<td>6.8 ± 1.2a</td>
<td>6.0 ± 1.2</td>
</tr>
<tr>
<td>STa-stimulated</td>
<td>52.8 ± 1.9</td>
<td>31.8 ± 1.4c</td>
<td>28.8 ± 1.4</td>
</tr>
</tbody>
</table>

a This value is significantly different (P < 0.01) from that for the control basal condition but not from that for the low-DTT PT treatment basal condition.

b This value is not significantly different from the high-DTT sham treatment basal value.

c This value is significantly different (P < 0.001) from that for the control STa-stimulated condition but not from that for the low-DTT PT treatment STa-stimulated condition.

d This value is not significantly different from that for the high-DTT sham treatment STa-stimulated condition.
ADP-ribosylation of G proteins had no effect on STa-stimulated guanylate cyclase (Table 1). Cyclic GMP accumulation in PT-treated intact cells was, if anything, slightly enhanced compared with that of controls, although this was not statistically significant, as described in Results.

Our attempts to reproduce the methods used by Epstein et al. (9) were complicated by technical subtleties not addressed in that previous report. First, the method used to activate PT (which is necessary for an in vitro experiment) was critical (Table 2). Second, even though high concentrations of PT and NAD were used, we found that only 34% ADP-ribosylation of G proteins in the crude T84 cell homogenates was achieved in vitro. These findings suggest that the PT treatment used by Epstein et al. may also not have achieved full ADP-ribosylation of G proteins in rat intestine.

There are several possible explanations for the differences between our observations and those of Epstein et al. (9). First, there may be a species difference between rat and human intestinal tissues in regulation of guanylate cyclase. This would not, however, explain the preliminary report of Kiely and Brandwein (Fed. Proc., 1987) that PT also partly blocks STa stimulation of guanylate cyclase in the T84 cell. Second, our source of PT was different from that of Epstein et al., and this may affect the type of activation required for an in vitro effect. Third, there are many G proteins and several PT substrates already identified in mammalian cells; some of these G proteins may be better substrates for PT than others so that the effect one observes with gentle PT treatment could indeed be different from that observed with 100% intoxication. If this were the case, however, we should have observed a PT effect on guanylate cyclase activity or cyclic GMP accumulation in our experiments with 10, 20, 100, 200, and 500 ng of PT per ml, all of which showed no effect. Fourth, our T84 cells may represent a subclone different from those employed by Kiely and Brandwein (Fed. Proc., 1987). Even if this is true, however, the fact that guanylate cyclase remains highly sensitive to stimulation by STa in intoxicated cells implies that a PT substrate is not necessary for coupling of receptor to effector.

The degree of ADP-ribosylation of PT substrates required to abolish a G-protein-mediated effect has been studied in other systems and varies substantially. In neutrophils, activation of phagocytic functions by leukotriene $B_4$ is mediated by a putative G protein (12, 13). PT treatment sufficient to yield 60% ADP-ribosylation of 40-kDa substrate completely abolished the leukotriene $B_4$ effect. On the other hand, Pobiner et al., studying the angiotensin II-mediated inhibition of adenylate cyclase in rat liver, found that 90 to 95% ADP-ribosylation of $G_i$ was required to block the inhibition of adenylate cyclase by angiotensin (26). The lack of effect of PT on cyclic GMP production in the T84 cell, despite 98 to 100% ADP-ribosylation of substrate, is strong evidence against a role of a PT-sensitive G protein in the stimulation of guanylate cyclase in this system.

The mechanism of coupling of the STa receptor to guanylate cyclase remains unknown. Possibilities include their coupling directly, as might be the case if the STa receptor is a transmembrane-signaling protein whose function is perturbed by the toxin. Alternatively, the receptor and the guanylate cyclase may be coupled via a G protein which is

**DISCUSSION**

Although the receptor for the toxin STa and guanylate cyclase are believed to be separate molecules (20, 28), few studies have addressed the mechanism of coupling between them. G proteins comprise a large family of signal-transducing molecules whose functions include coupling receptors to a stimulation or inhibition of adenylate cyclase, visual and olfactory transduction, stimulation of phospholipase C, and coupling receptors to ion channels (11, 25). The findings of Epstein et al. (9) suggested that a PT-sensitive G protein might be involved in coupling the STa receptor to guanylate cyclase.

The levels of guanylate cyclase activity and the fold stimulation by STa reported here in the T84 cell homogenates compare favorably with those previously reported in the T84 cell line (14) and are several times higher than those observed in intestinal mucosal preparations from animals (7, 14, 10, 15).

Experiments to investigate the role of PT-sensitive G proteins in the action of STa showed no effect of conventional doses of PT treatment (10 to 500 ng/ml) on cyclic GMP production in T84 cells and homogenates. This begged the question of whether the PT treatment had been effective. An ADP-ribosylation assay revealed that cells treated with 1 μg of PT per ml for 24 h were 98.3 to 100% intoxicated, whereas cells treated with 100 ng/ml for 16 h were negligibly intoxicated (data not shown). Thus the half-maximal dose of PT lies between 100 ng/ml and 1 μg/ml. The reason that T84 cells require somewhat higher doses of PT than other tissues for full intoxication is unknown. A decrease in the number of toxin-binding sites (recognized by the B oligomer of PT) is a plausible explanation but could not be directly confirmed because of the difficulty to date of radiolabeling the toxin.

PT in amounts sufficient to achieve 98.9 to 100% ADP-ribosylation of G proteins had no effect on STa-stimulated guanylate cyclase. The levels of cyclic GMP showed that this is also the case in the T84 cell crude homogenates.

**FIG. 2. Autoradiogram showing PT intoxication of T84 cell homogenates in vitro.** In contrast to those for Fig. 1, T84 homogenates were initially treated in vitro with 20 μg of activated PT per ml and 1 mM unlabeled NAD as described in Materials and Methods. Next, the PT-treated (lane 2) and control (lane 3) homogenates were subjected to extraction with cholate detergent, and the supernatants were again exposed to PT in the presence of $^{32}$P$^\text{NAD}$ according to the usual ADP-ribosylation method. The samples loaded in each lane were as follows: lane 1, 0.14 μg of transducin; lane 2, 6.6 μg in cholate of T84 cell homogenate treated in vitro with PT; lane 3, 6.6 μg in cholate of control T84 cell homogenate; lane 4, 2.8 μg of cholate extract of bovine brain membranes. Molecular size marker (in kilodaltons) is shown on the left.
not a PT substrate. A last category of possibilities is that guanylate cyclase is activated in response to some other intracellular or membrane-bound messenger, such as a calcium ion or lipid, or through the action of a kinase. Further studies should reveal more of the mechanism of action of this interesting and hormonelike enterotoxin of E. coli.

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LITERATURE CITED