T_{84} cell receptor binding and guanyl cyclase activation by *Escherichia coli* heat-stable toxin

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GUARINO, ALFREDO, MITCHELL COHEN, MICHAEL THOMPSON, KIERTISIN DHARMSATHAPHORN, AND RALPH GIANNELLA. *T_{84} cell receptor binding and guanyl cyclase activation by Escherichia coli heat-stable toxin.* Am. J. Physiol. 253 (Gastrointest. Liver Physiol. 16): G775–G780, 1987.—*Escherichia coli* heat-stable enterotoxin (STa) induces intestinal secretion by binding to enterocyte receptors and activating the guanylate cyclase-guanosine 3′,5′-cyclic monophosphate (cGMP) system. The intermediate steps between binding of STa and secretion are poorly understood, due in part to the lack of a convenient system to study the effects of STa at the cellular level. To establish such a model, we investigated the binding of ^{125}I-STa, STa activation of guanylate cyclase, and STa-induced increase in cGMP production in a well-characterized human colonic cell line, T_{84}. Binding was specific, linear with cell number, and time, temperature and pH dependent, and reversible. ST may also be internalized by these cells. Addition of unlabeled STa competitively inhibited binding of ^{125}I-STa. These parameters closely resemble those described in intact rat enterocytes and cell-free membrane preparations. STa stimulated guanylate cyclase and cGMP production in a dose-related manner. The similar dose-response relationships for binding, guanylate cyclase stimulation by STa, and cGMP production suggest that the guanylate cyclase-cGMP system is coupled to ST occupancy of specific receptors. These data, together with the fact that STa induces chloride secretion from T_{84} cells suggest that T_{84} cells are a suitable and convenient system to study the cellular mechanism of action of STa.

intestinal secretion; diarrhea; high-performance liquid chromatography

ESCHERICHIA COLI can cause diarrhea in humans by producing one or more enterotoxins. These are a large molecular weight heat-labile enterotoxin (LT) and small molecular weight heat-stable enterotoxins (ST) (16). The latter have been divided into two groups, designated STa and STb, based on different biochemical characteristics and on differing host susceptibility (2). STa is elaborated in two forms, an 18- or 19-amino acid peptide, both of which have been sequenced (1, 3, 21). STa induces diarrhea through a mechanism that has been investigated in several animal models. The toxin binds to specific receptors located on the brush border of small intestinal enterocytes (9). This induces a prompt increase in guanosine 3′,5′-cyclic monophosphate (cGMP), through the activation of particulate guanylate cyclase activity (5), and impaired sodium chloride absorption and net chloride secretion (15). The binding of STa to rat small intestinal cells and to brush-border membranes has been previously described by us and others (7, 9, 11). However, the biological processes that follow the binding of STa are poorly understood, due in part to the lack of a convenient and suitable model to study the effects of STa at the cellular level.

We have used a human colonic cell line, the T_{84} cell line (4), to establish a model suitable to study the specific mechanisms of STa receptor-effector interactions. We report here the characterization of the binding of ^{125}I-STa to T_{84} cells, as well as STa-induced stimulation of guanylate cyclase and cGMP production. We chose the T_{84} cell line because these cells maintain the morphological characteristics of well-differentiated colonic epithelial cells. In addition, they retain vectorial electrolyte transport properties in response to various hormones (4). Furthermore, these cells have been recently shown to secrete chloride in response to STa (13). The T_{84} cells could therefore provide an experimental model to study STa-cell interactions from the initial binding to the final secretion.

METHODS

Cell culture. T_{84} cells were maintained and grown as previously described (4). Briefly, cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, 1.2 g of $NaHCO_3$, 40 mg penicillin, 8 mg ampicillin, and 90 mg streptomycin per liter plus 5% newborn calf serum. After 1 wk, confluent monolayers were split from one 100-mm tissue culture plate to two 24-well plates, using 0.1% trypsin and 0.9 mM EDTA in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS).

Purification and labeling of STa. STa enterotoxin was purified from *E. coli* strain 18D as previously described (20). Pure STa, quantitated by amino acid analysis and by a specific enzyme-linked immunosorbent assay (19), was radioiodinated by a lactoperoxidase method and

purified using a C-18 Sep-Pak cartridge (Millipore) and high-performance liquid chromatography as previously described (20). ^{125}I-STa monoiodinated in the 4-tyrosine position was used for binding studies, since it has been shown to be stable, homogeneous, and retain its biological activity (20)

Binding of ^{125}I-STa to T_{84} cells. Binding assays were performed in tissue culture plates containing 24 × 16-mm wells. Each well contained approximately 6×10^5 cells or 200 μg protein. The variation in number of cells from well to well did not exceed 15%. Prior to the start of each experiment, cells were washed three times with PBS and placed for 30 min at 37°C in a humidified CO_2 incubator. Each well received 1 ml of F-12 modified Eagle's medium buffered with 15 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and containing 100,000–150,000 cpm (48–73 pM) ^{125}I-STa. Incubation was performed at 37°C for 1 h or other times as stated in the RESULTS. Particular experiments were also performed at 4°C as well. The pH of the medium used for the binding studies was 5.8 and did not change during the course of the experiment. After incubation, cells were rinsed three times with PBS and solubilized by incubation with 0.5 M NaOH for 1 h at room temperature. The remaining cell-associated radioactivity was then quantitated by counting the cell extracts in a Packard scintillation spectrometer. Specific binding was determined by measuring binding of ^{125}I-STa to T_{84} cells in the presence of excess unlabeled STa (1 μg/ml) and subtracting this nonspecific binding from total binding (binding in the presence of ^{125}I-STa alone). Protein was measured by the method of Lowry et al. (14). Cells were counted using a hemocytometer.

Binding of ^{125}I-STa to T_{84} membranes. T_{84} cell membranes were prepared as follows. T_{84} cells were twice washed with (in mM) 5 EDTA, 1 HEPES, 0.1 phenylmethylsulfonyl fluoride, and 8.2 tris(hydroxymethyl)aminomethane (Tris), pH 7.5, and harvested by scraping with a glass pestle. The scrapings were homogenized in EDTA buffer for 1.5 min in an Omni Sorvall mixer at maximum speed and centrifuged at 11,500 *g* for 30 min. The pellet was resuspended in F-12 modified Eagle's medium and used immediately. Binding assays were performed in 12 × 75 glass test tubes containing a total volume of 1 ml of modified F-12 medium, pH 5.8, containing 15 μg/ml bovine serum albumin (BSA), ~190,000 cpm (92 pM) ^{125}I-STa, and membranes. Incubations were performed at 37°C. The reaction was terminated by suction filtration through Whatman GF/B filters, each filter was washed three times with ice-cold F-12 medium, and the radioactivity was counted. Specific and nonspecific binding were calculated as previously described (9).

Guanylate cyclase assay. Confluent cells from two 100-mm flasks were washed twice with PBS, removed by scraping, and hand homogenized with a Wheaton A pestle for 60 s (18 strokes) in 10 ml of sucrose-EDTA-dithiothreitol (DTT) buffer (0.25 M sucrose, 50 mM Tris·HCl, 1 mM EDTA, 1 mM DTT, pH 7.9). The homogenate was diluted twofold in the same buffer and centrifuged at 32,000 *g* for 20 min. The pellet was washed in 5 mM Tris, pH 7.6, and resuspended to yield a final protein concentration of 15–30 μg/10 μl. Guanylate cyclase was determined as reported by Waldman et al. (22). The assay was performed by incubating the samples for 10 min at 37°C in the presence of GTP and a GTP-regenerating mixture (22). (Preliminary experiments demonstrated that 10 min, 37°C, pH 7.6, and 10–40 μg of protein are optimal conditions for the assay.) cGMP formed was determined using an radioimmunoassay (RIA) assay as previously described (8).

Coupling between ^{125}I-STa binding to T_{84} cells and STa-induced increase in cGMP production. To determine whether binding of STa to T_{84} cells was coupled to cGMP production, cells were incubated at 37°C in 24-well plates with ^{125}I-STa in the presence of increasing concentrations of unlabeled STa in modified F-12 medium, pH 5.8. After 15 min the reaction was terminated, and binding was measured as described above. In parallel wells, the same increasing concentrations of STa in modified F-12 medium, pH 5.8, were added and production of cGMP was determined as follows. After 15 min at 37°C, each well received 1 ml ice-cold 10% trichloroacetic acid (TCA), and after 30 min, the supernatant from each well was collected and centrifuged. cGMP in the supernatant was purified by column chromatography on Dowex AG50W-X8 resin and measured by RIA as previously described (8). [^{3}H]cGMP added to TCA was used as a recovery marker.

Data presentation and analysis. All experimental points of binding studies were performed in triplicate and each experiment was repeated at least three times. Results are expressed as means ± SE of the counts per minute of ^{125}I-STa specifically bound per 100 μg of cell protein.

ST-stimulated guanylate cyclase activity, measured as picomoles of cGMP formed per minute per milligram of protein, is expressed as fold increase over basal level. Incubations with STa were performed in triplicate and each cGMP determination was assayed in duplicate. Production of cGMP after addition of STa was assayed in triplicate and results are expressed as picomoles of cGMP formed per milligram of protein. Results of the dose-response curves were calculated using the computer program "Allfit" (17). Each experiment was performed at least three times, and the data are presented as means ± SE.

RESULTS

Effect of time and protein concentration. The time courses of total, specific, and nonspecific binding of ^{125}I-STa to T_{84} cells are shown in Fig. 1. The rate of binding was highest in the initial 15 min, and binding reached a plateau at ~3 h. After 3 h of incubation no further binding was observed. In other experiments, incubation was continued for up to 5 h, and the radioactivity that was bound remained constant (data not shown). Nonspecific binding did not exceed 15% of total binding at any point in time. Binding was linear with protein concentration, in the range of 150–350 μg/ml (data not shown).

Effect of temperature. The effect of temperature on

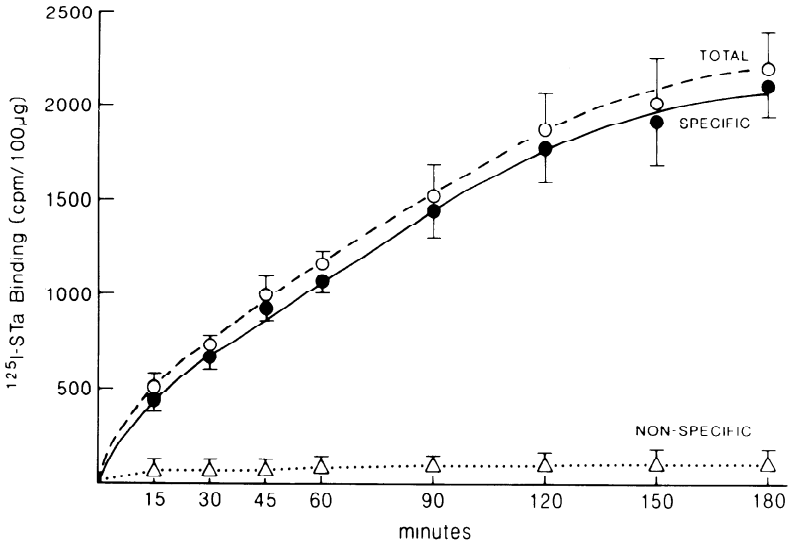

FIG. 1. Time course of binding of ^{125}I-heat-stable enterotoxin (STa) to T_{84} cells. Cells were incubated in 1 ml of F-12 Dulbecco's modified Eagle's medium buffered with 2-(*N*-morpholino)ethanesulfonic acid, containing ^{125}I-STa with and without 1 μg (500 nM) of unlabeled STa at 37°C. Total, nonspecific, and specific binding were determined as described in METHODS. Results are expressed as counts per minute bound per 100 μg protein and represent means ± SE of 3 separate experiments, each carried out in triplicate.

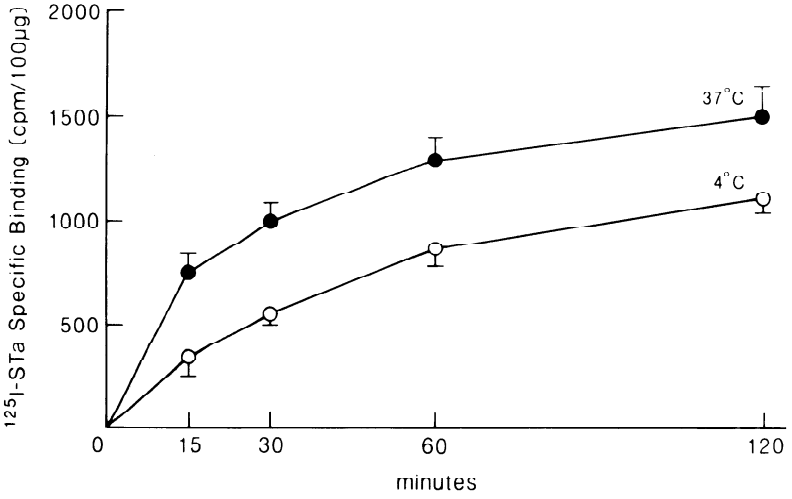

FIG. 2. Effect of temperature on specific binding of ^{125}I-heat-stable enterotoxin (STa) to T_{84} cells. Results are means ± SE of counts per minute specifically bound per 100 μg protein of 3 separate experiments.

binding of ^{125}I-STa to T_{84} cells is shown in Fig. 2. Binding was temperature dependent. At 37°C, the initial rate of binding was greater than at 4°C, such that after 15 min binding was twofold greater at 37 than at 4°C. If the incubation was continued, the rate of binding became parallel at both temperatures, and after 2 h, the binding at 4°C was ~75% of that at 37°C.

Effect of pH. The effect of pH is shown in Fig. 3. The pH of the medium was adjusted immediately prior to the experiment by titration with HCl or NaOH as appropriate. Binding was dependent on the pH of the medium. At pH 4.5 (the lowest pH tested) binding was maximal and then fell rapidly as the pH was increased. The pH of the media did not change during the course of the experiment.

Dissociation of ^{125}I-STa bound to T_{84} membranes. As shown in Fig. 4, the addition of excess unlabeled STa (1 μg/ml) resulted in the prompt dissociation of ^{125}I-STa bound to T_{84} membranes. When native toxin was added 15 min after the initiation of binding, ~92.6% of ^{125}I-STa dissociated by 6 h; when native toxin was added 30 min after binding, ~83.7% of ^{125}I-STa dissociated by 6 h; and when native STa was added 60 min after binding, ~64.4% of ^{125}I-STa dissociated by 6 h. However, when the three dissociation curves were linearized by logarithmic transformation (plotted as logarithm of counts per minute vs. time), all three curves intersected the *X*-axis, indicating complete dissociation (data not shown).

Competitive inhibition of binding. Native STa competed with ^{125}I-STa for binding to T_{84} cells. As shown in Fig. 5, when T_{84} cells were incubated with ^{125}I-STa at either 37 or 4°C, together with increasing concentrations of unlabeled ST, a dose-dependent inhibition of ^{125}I-STa binding was observed. Although the 37 and 4°C binding curves are parallel (suggesting similar K_as), maximal binding at 4°C was only approximately 63.8 ± 13.5% of that seen at 37°C ($P < 0.05$).

Effect of ST on guanylate cyclase activity. Addition of STa to homogenates of T_{84} cells resulted in a dose-related increase in guanylate cyclase activity (Fig. 6), reaching a plateau at an STa concentration of ~10^{-7} M. The maximal stimulation of guanylate cyclase activity was ninefold over basal level. The effect of STa on guanylate cyclase was extremely rapid. A significant stimulatory

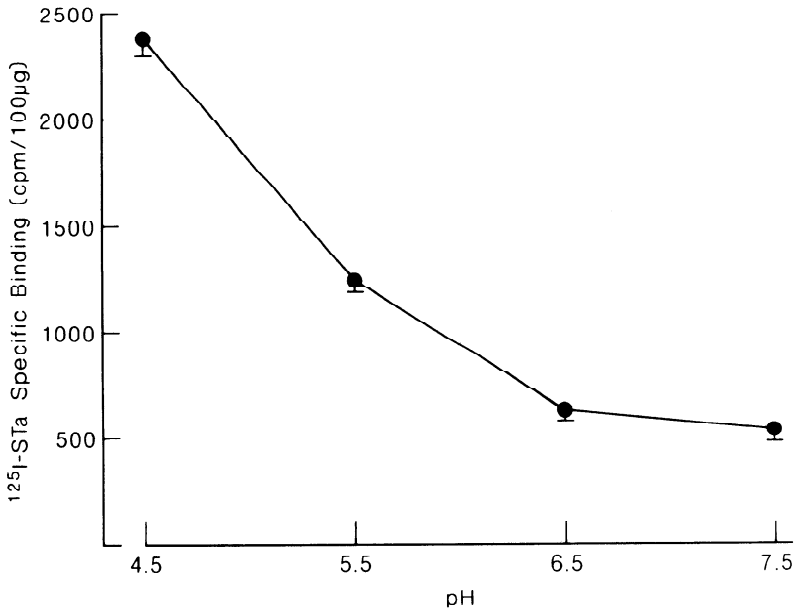

FIG. 3. Effect of pH on binding of ^{125}I-heat-stable enterotoxin (STa) to T_{84} cells. Incubation was performed at 37°C for 1 h in parallel wells containing 1 ml of medium, adjusted to appropriate pH. Results are means ± SE of 3 different experiments.

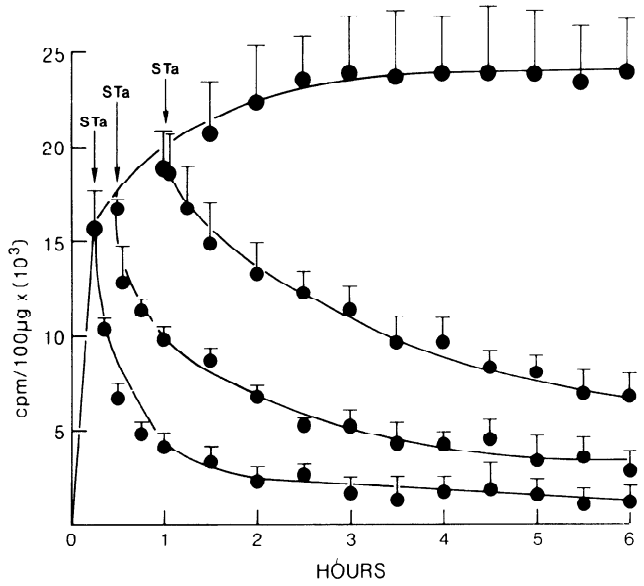

FIG. 4. Dissociation of ^{125}I-heat-stable enterotoxin (STa) bound to T_{84} membranes by addition of excess nonlabeled STa. T_{84} membranes were incubated with ^{125}I-STa at 37°C, and at the times indicated by *arrows* excess cold toxin (500 nM) was added and binding was measured for a total of 6 h. Results are means ± SE of 3 separate experiments.

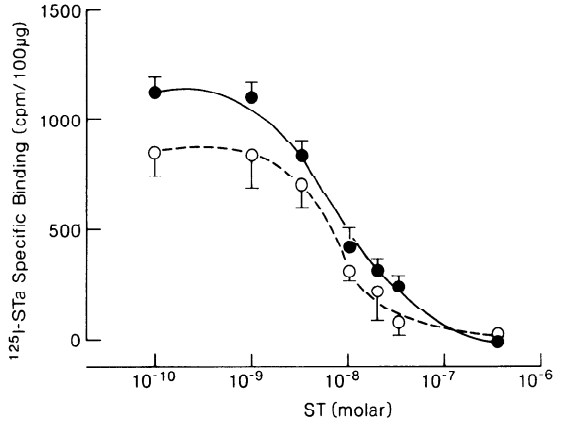

FIG. 5. Competitive inhibition of ^{125}I-heat-stable enterotoxin (STa) binding by unlabeled STa. Cells were incubated with ^{125}I-STa and with increasing concentrations of STa at 37°C (*closed circles*) or 4°C (*open circles*) for 1 h. Results are means ± SE of 3 separate experiments.

effect was seen as early as 2 min (the shortest time tested) after the addition of STa (data not shown).

Coupling between binding of STa and cGMP stimulation. Experiments of competitive binding and cGMP stimulation by STa were performed in parallel plates under the same pH and other conditions. Shown in Fig. 7 are the dose-response relationships of the ability of unlabeled STa to inhibit binding of ^{125}I-STa to the T_{84} cells and to stimulate cGMP. Results are expressed as percent of maximal activity. The ability of STa to inhibit the binding of ^{125}I-STa closely correspond to its ability to stimulate cGMP formation. The concentrations of STa at which a half-maximal effect was obtained in each assay were $1.5 \pm 0.4 \times 10^{-8}$ and $3.1 \pm 0.3 \times 10^{-8}$ M, respectively.

DISCUSSION

The initial step in the induction of secretion by STa appears to involve binding of ST to a specific cell surface receptor (9). This binding step has been studied in isolated rat enterocytes (9), rat brush-border membranes (7), rat basophilic leukemia cells (18), and solubilized partially purified rat enterocyte receptor preparations (12). Although a great deal is known about STa interaction with its receptor (7, 9, 12) and the alterations in ion transport mechanisms induced by ST (5, 10, 15), relatively little is known about the intermediate mechanism of action linking receptor occupancy to secretion. In the present study we wish to determine whether the human colonic T_{84} cell line might be a suitable model to further investigate the mechanisms of action of STa. We have, therefore, examined whether these cells possess ST-re-

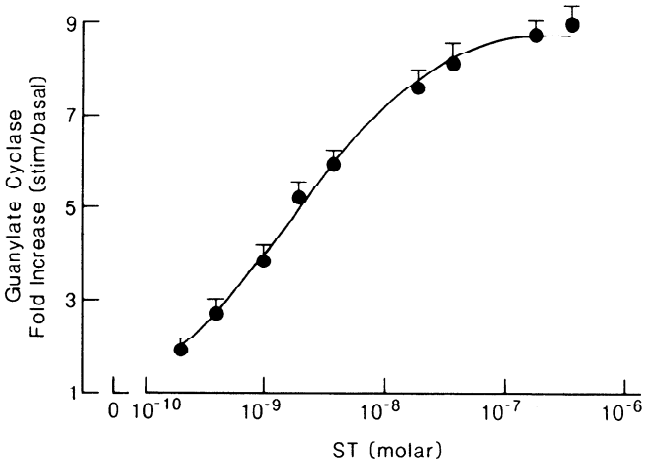

FIG. 6. Stimulation of guanylate cyclase activity induced by heat-stable enterotoxin (STa). A homogenate of T_{84} cells was incubated with increasing concentrations of STa at 37°C for 1 h. Guanylate cyclase activity is expressed as fold increase over basal level. Results are means ± SE of 3 separate experiments. Base-line level was 6.4 ± 1.7 pmol of cGMP formed per minute per milligram of protein.

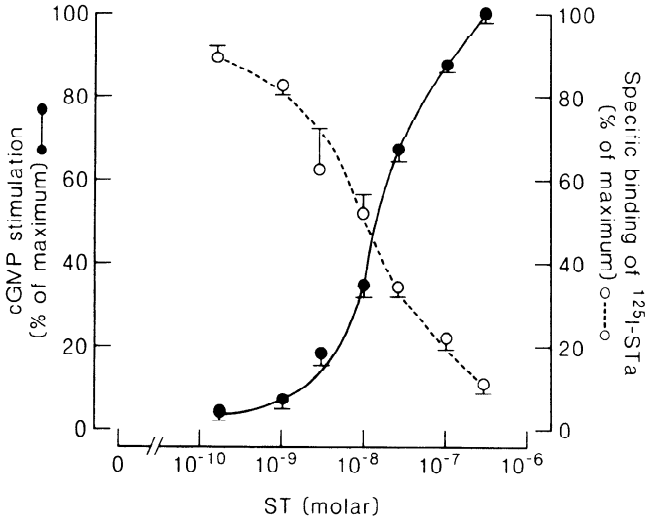

FIG. 7. Dose-response relationships between ability of nonlabeled-heat-stable enterotoxin (STa) to inhibit binding of labeled STa and to increase cGMP concentration. T_{84} cells were incubated with increasing concentrations of STa for 15 min at 37°C, and binding and cGMP concentration were measured as described in METHODS. Results, expressed as percent of maximal effect, are means ± SE of 3 separate experiments. Under these conditions respective ED_{50} were 1.5 and 3×10^{-8} M.

ceptors and guanylate cyclase activity, whether the guanylate cyclase-cGMP system is responsive to ST, and whether receptor occupancy by STa is coupled to guanylate cyclase and cGMP stimulation.

T_{84} cells bound ^{125}I-STa and binding was highly specific, linear with cell number, and time, temperature and pH dependent. These characteristics are similar to those reported for ST binding to isolated rat small intestinal cells and brush-border membranes (9, 11). The effect of pH on binding of ^{125}I-STa to T_{84} cells closely resembles that which we have previously reported for rat intestinal brush-border membranes (11). Binding increased markedly as the pH of the medium was reduced. We have demonstrated that the increased binding seen at lower pH in the intestinal brush-border membrane system is explained by a greater number of receptors exposed at a lower pH value, while the apparent K_a of binding is unaltered (11).

The fate of receptor-bound ^{125}I-STa is uncertain. It is possible that a portion of ^{125}I-STa could be internalized by the T_{84} cells and/or irreversibly bound to the surface receptors. When the competitive inhibition experiments at 37°C were compared with those done at 4°C (Fig. 5), maximal binding at 4°C was only ~64% of that seen at 37°C. Thus it is possible that at 37°C a portion of the ^{125}I-STa bound to T_{84} cells is internalized by these cells.

To determine whether an element of irreversible receptor binding might also be occurring, experiments were performed to examine the dissociability of ^{125}I-STa from T_{84} membranes. Addition of excess native STa resulted in the dissociation of ^{125}I-STa bound to such membranes (Fig. 4). The rate of dissociation, however, was quite slow and receptor binding did not reach zero during the duration of the experiment. Visual examination of the curves, however, suggests that complete dissociation would ultimately occur. Log transformation of the three dissociation curves reveals all three to be straight lines and to intersect the X-axis, thereby confirming the visual impression. Thus dissociation from T_{84} membrane receptors, although slow, is ultimately complete.

STa increases cGMP levels through the activation of guanylate cyclase (5, 10). We have shown that T_{84} cells possess an STa-responsive guanylate cyclase activity and that activation of guanylate cyclase was dose and time dependent, closely resembling results obtained in animal models (5, 10, 22). The close correlation, as reflected by the similar ED_{50}s, between the dose-response relationship of STa in inhibiting binding of ^{125}I-STa to T_{84} cells and in stimulating cGMP formation suggest that these processes are coupled. We have previously described the same correlation in rat small intestinal cells (9), and the ED_{50} for both binding of STa and cGMP stimulation (1.25×10^{-8}) found in rat enterocytes are nearly identical to those found in T_{84} cells. Furthermore, we have also shown that the STa dose-response of activation of guanylate cyclase is also similar to the STa dose-response of inhibition of binding of ^{125}I-STa (Figs. 5 and 6). Although these experiments were done under different conditions than those employed in the cGMP-^{125}I-STa coupling experiments, these similarities in stoichiometry are consistent with the coupling of STa binding, activation of guanylate cyclase, and production of cGMP.

We have, therefore, characterized the binding of STa to T_{84} cells and established a reproducible model that involves the use of a monoiodinated, homogeneous radioligand (20). We have also shown that the T_{84} cell line responds to STa with an increase in guanylate cyclase activity and cGMP formation and that the stimulation of the guanylate cyclase-cGMP system is likely coupled to the occupancy of specific receptors by STa. Liu et al. (13) have recently shown that STa causes an increase in cGMP concentration coupled to chloride secretion in this the same cell line. The T_{84} cell line, therefore, seems a suitable system to study the mechanism of action of STa at the cellular level. The T_{84} cell line cannot only

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