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# Solubilization and Partial Characterization of the Intestinal Receptor for *Escherichia coli* Heat-Stable Enterotoxin

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**Binding of *Escherichia coli* strain 431 heat-stable enterotoxin ($ST_a$) and activation of intestinal particulate guanylate cyclase by *E. coli* $ST_a$ were studied with rat intestinal epithelial cells and brush border membranes (BBMs). The rates of guanylate cyclase stimulation by 431 $ST_a$ in cells and BBMs were rapid, with maximal levels of cyclic GMP observed within 5 min. Specific binding of $^{125}$I-labeled $ST_a$ from *E. coli* 431 (431 $^{125}$I-$ST_a$) and activation of guanylate cyclase by unlabeled 431 $ST_a$ were observed with intestinal BBMs; however, neither was detected with membranes from nonintestinal tissues. The $ST_a$ receptor was solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a nondenaturing dipolar ionic detergent, in yields of approximately 50%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the detergent-solubilized receptor–431 $^{125}$I-$ST_a$ complex, followed by autoradiography, showed that 431 $^{125}$I-$ST_a$ bound to a single BBM component with a molecular weight of about 100,000. Binding of 431 $ST_a$ to its solubilized receptor was saturable, specific, and essentially irreversible. Pretreatment of the soluble receptor with trypsin and pronase but not chymotrypsin decreased binding of 431 $^{125}$I-$ST_a$. The 431 $ST_a$-receptor complex was dissociated by boiling in the presence of 1% sodium dodecyl sulfate, incubation with 0.5 M acetic acid, or reduction with dithiothreitol. In contrast to the residual particulate guanylate cyclase activity of detergent-treated membranes, solubilized guanylate cyclase was not stimulated by $ST_a$. Membrane structure appears to play an important role in the coordination of $ST_a$ binding and stimulation of guanylate cyclase activity.**

Diarrheal disease caused by enterotoxigenic strains of *Escherichia coli* results from the ingestion of contaminated water or food, colonization of the small intestine, and elaboration of one or more protein enterotoxins (41, 42). The heat-labile enterotoxin of *E. coli* is a multimeric-protein enterotoxin similar to cholera toxin with respect to subunit structure, ability to bind $GM_1$ ganglioside, antigenic properties, and mechanism of intestinal secretory action (3, 17–19, 26, 35). In contrast, the heat-stable enterotoxin ($ST_a$) produced by enterotoxigenic *E. coli* with biological activity in suckling mice and piglets is a low-molecular-weight, heat-stable protein of 18 to 19 amino acids (8, 46). Unlike heat-labile enterotoxin and cholera toxin, which induce secretion by activation of intestinal adenylate cyclase, $ST_a$ causes secretion by increasing mucosal levels of intracellular cyclic GMP (cGMP) caused by activation of particulate intestinal guanylate cyclase (11, 21, 27). Stimulation of guanylate cyclase by $ST_a$ is tissue specific; that is, only the intestinal form of the particulate enzyme responds to $ST_a$ (11, 21, 40). These data suggest that the tissue specificity exhibited by $ST_a$ is due to a specific $ST_a$ receptor present on the surface of intestinal epithelial cells or the fact that the intestinal form of guanylate cyclase is unique compared with other particulate guanylate cyclases (15, 22, 31, 49).

A high-affinity receptor for $ST_a$ associated with rat brush border membranes (BBMs), intestinal epithelial cells (12), and 7-day-old and 7-week-old piglets (L. Jaso-Friedmann, L. A. Dreyfus, S. C. Whipp, and D. C. Robertson, manuscript in preparation) has been detected. Even though the BBM microvillus is the predominant location of intestinal particulate guanylate cyclase (6, 7, 50), coupling of intestinal guanylate cyclase and the $ST_a$ receptor have not been established.

In this study, we examined the biochemical properties of the $ST_a$ receptor and its relation to particulate guanylate cyclase in isolated intestinal epithelial cells and BBMs. A specific correlation between binding of $ST_a$ and stimulation of particulate guanylate cyclase was observed. The $ST_a$ receptor was solubilized with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) detergent (45) and found to exhibit binding properties like those observed with the particulate form associated with BBMs and intestinal epithelial cells. The disulfide bonds of $ST_a$ were necessary for binding to the soluble receptor, which provided additional evidence that one of the early events in stimulation of particulate guanylate cyclase by the toxin involves a thiol-disulfide exchange reaction.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Enterotoxigenic *E. coli* strain 431, a class 2 porcine enteropathogen, was supplied by H. Moon, National Animal Disease Center, Ames, Iowa. The preparation of media, growth conditions, and maintenance of stock cultures were as described previously (1, 8, 12).

**Purification of $ST_a$, chemical modification, and bioassays.** The $ST_a$ produced by enterotoxigenic *E. coli* 431 was purified as described by Dreyfus et al. (8). Purified preparations of 431 $ST_a$ exhibited an effective dose of 0.4 ng by the suckling mouse assay performed as described previously (13). *S*-Carboxymethyl–$ST_a$ was prepared by reduction and reaction with iodoacetate by the method of Crestfield et al. (5) in a reaction mixture which contained the following: 50 to 100 μg of purified, salt-free 431 $ST_a$, 200 μl of 0.5 M Tris-hydrochloride–10.0 M urea (Schwartz/Mann, Orangeburg, N.Y.)–0.2% EDTA (pH 8.5), 30 μl of 0.5 M dithi-

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othreitol–0.5 M Tris-hydrochloride–0.2% EDTA (pH 8.5), and 20 μl of 0.5 M Tris-hydrochloride–0.2% EDTA (pH 8.5). The reaction was thoroughly flushed with $N_2$ and incubated for 4 h with continuous stirring in the dark. A 33-μl amount of freshly prepared, neutralized 1 M iodoacetic acid was added, followed by 30 min of incubation at 37°C with stirring. The mixture was applied to a Bio-Rad P-2 column (0.8 by 12 cm) equilibrated with distilled water and wrapped with aluminum foil. Fractions containing S-carboxymethyl–$ST_a$ were pooled, lyophilized, and stored at −20°C until use.

**Radiolabeling of 431 $ST_a$.** Purified 431 $ST_a$ was radioiodinated enzymatically as described previously (13). Briefly, a reaction mixture was used which contained the following: 1.0 μg of $ST_a$, 45 μl of 0.2 M sodium phosphate (pH 7.2), 50 μl of Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.), 1.0 mCi of $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.), and 25 μl of 1% D-glucose. Free $^{125}I$ label was removed from $^{125}I$-labeled $ST_a$ of *E. coli* 431 (431 I-$ST_a$) by XAD-2 resin adsorption chromatography.

**Isolation of intestinal cells and BBMs.** Intestinal epithelial cells were isolated from the resected small intestine of 120- to 180-g female Sprague-Dawley rats by the method of Weiser (52). The proximal segment of each intestinal segment was connected to peristaltic pump tubing and washed with 0.9% NaCl–0.1 M dithiothreitol until all fecal matter was removed. The segments were filled with solution A and incubated at 37°C for 15 min and then flushed, filled with solution B, and incubated at 37°C. Fractions were collected at 10, 30, and 50 min, which corresponded to a gradient of villus tip cells, a mixture of villus and crypt cells, and crypt cells, respectively. Membranes were prepared from each fraction as described previously (12) and analyzed for the relative contents of guanylate cyclase and sucrase. BBMs were prepared by a modification of the procedure of Hauser et al. (25) as described previously (12).

**Determination of intracellular cGMP in rat intestinal epithelial cells.** Accumulation of cGMP in isolated intestinal epithelial cells was measured as described previously (9) with 5-ml incubation mixtures containing $5.0 \times 10^6$ cells per ml, Hanks balanced salt solution, 10% fetal calf serum, 0 to 250 ng of 431 $ST_a$ per ml, and 10 mM theophylline. Mixtures in 25-ml Erlenmeyer flasks were incubated at 100 rpm on a shaking water bath at 37°C. At timed intervals, 0.5-ml samples were pipetted into tubes containing 1.0 ml of absolute ethanol at 0°C. Cells and debris were removed by centrifugation. Supernatants were dried under a stream of air, suspended in 0.5 ml of 50 mM acetate buffer (pH 6.2), and assayed for cGMP as described below.

**Guanylate cyclase assay.** Guanylate cyclase activity associated with rat intestinal BBMs was assayed as described by Kimura and Murad (29). Reaction mixtures contained 50 to 100 μg of BBMs, 5 μmol of Tris-hydrochloride (pH 7.4), 1 μmol of theophylline, 0.75 μmol of phosphocreatine, 3.5 U of creatine phosphokinase, 0.1 μmol of GTP, 0.5 μmol of $MgCl_2$, 4 to 400 ng of $ST_a$ per ml in a total volume of 0.1 ml. Reactions were started by the addition of GTP-$MgCl_2$ and stopped after 10 min of incubation at 37°C by the addition of 0.1 ml of 0.5 M sodium acetate (pH 4.0). The cGMP formed was measured by a radioimmunoassay procedure.

**Radioimmunoassay for cGMP.** The radioimmunoassay for cGMP was performed essentially as described by Brooker et al. (2) and Steiner et al. (47) with acetylation of samples (24). After overnight incubation at 4°C, 0.5 mg of normal rabbit immunoglobulin G was added as a carrier, and bound $^{125}I$-labeled succinyl cGMP tyrosine methyl ester was separated from unbound $^{125}I$-labeled cGMP tyrosine methyl ester by ammonium sulfate precipitation. Normal rabbit immunoglobulin G was purified by sodium sulfate precipitation followed by ion-exchange chromatography on DEAE–Sephadex A-50.

**Detergent fractionation of BBMs.** Intestinal-cell BBMs (2.5 to 3.5 mg/ml) were suspended in an equal volume of detergent solution to give final concentrations of 5 mM CHAPS, 154 mM NaCl, 1.0 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4), and 1 mM phenylmethylsulfonyl fluoride and homogenized with 10 strokesof a Potter-Elvehjem homogenizer. After centrifugation at 150,000 × *g* for 1 h, the supernatant was removed with a Pasteur pipette and the pellet was extracted again with the CHAPS solution described above. Insoluble material was removed by centrifugation. Supernatant fractions from both extractions were combined and concentrated about fourfold by ultrafiltration at 4°C with an Amicon PM-10 membrane (Amicon Corp., Danvers, Mass.).

**Binding assays.** Binding of 431 $^{125}I$-$ST_a$ to rat intestinal BBMs was measured as described previously (12). Briefly, reaction mixtures containing 30 to 100 μg of BBMs, 2.0 nM 431 $^{125}I$-$ST_a$, and 12.5 mM HEPES–1.0 mM EDTA, pH 7.4, in a final volume of 200 μl were incubated at 37°C for 10 min. Free 431 $^{125}I$-$ST_a$ was separated from bound 431 $^{125}I$-$ST_a$ by filtration on EGWP filters (Millipore Corp., Bedford, Mass.).

Soluble $ST_a$ receptor activity extracted from BBMs was assayed by a gel filtration binding assay. The receptor was radiolabeled by incubating CHAPS-soluble fraction of BBMs containing 50 to 100 μg of protein with 431 $^{125}I$-$ST_a$ (100,000 cpm, 0.3 ng) in a total volume of 0.1 ml with shaking for 15 min at 37°C. Bound 431 $^{125}I$-$ST_a$ was separated from free 431 $^{125}I$-$ST_a$ by gel filtration with a Bio-Gel P-6DG column (0.80 by 12 cm) equilibrated with 2.5 mM CHAPS, 10 mM Tris-hydrochloride (pH 7.4), and 154 mM NaCl. Fractions (10 drops) were collected and scanned for radioactivity with a Packard 5110 gamma scintillation spectrometer. Receptor activity was expressed as the percentage of 431 $^{125}I$-$ST_a$ in the void volume of the column relative to the total radioactivity (counts per minute) added to the reaction mixture. All binding assay data were expressed as the average of triplicate determinations with variations of ±5% from the mean.

In experiments to determine the stability of the receptor-$ST_a$ complex, reaction mixtures were incubated under various conditions for 30 min at 37°C. Dissociated 431 $^{125}I$-$ST_a$ was separated from bound radioactivity by gel filtration on Bio-Gel P-6PG columns as described above; however, the columns were equilibrated with the test solution rather than the CHAPS-HEPES-NaCl solution.

Protease treatment of the soluble $ST_a$ receptor was performed in reaction tubes (12 by 75 mm) with 25 μg of BBMs and 2.5 μg of protease in a total volume of 0.2 ml. After incubation with mixing at 37°C for 1 h, 431 $^{125}I$-$ST_a$ was added. Bound and free 431 $^{125}I$-$ST_a$ were separated after 15 min of incubation by gel filtration.

**SDS-PAGE.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (32) with 5 to 15% gradient slab gels. Samples were prepared by mixing 1 volume of the 431 $^{125}I$-$ST_a$–soluble-receptor complex with 1 volume of a twofold concentration of sample buffer containing 2% SDS, 20% glycerol, 0.01% bromphenol blue, and 0.125 M Tris-hydrochloride (pH 6.8). Samples were not boiled before electrophoresis. After electrophoresis at 20 mA of constant current, autoradiography of the slab gel was carried out by standard methods with Kodak X-Omat AR X-ray film.

**Protein determinations.** Protein determination of $ST_a$ preparations was performed by the method of Ehreshmann et al. (10) or of Warburg and Christian (51). BBM protein was determined by the method of Lowry et al. (34) after boiling for 10 min in 1 N NaOH and neutralization with 1 N HCl before assay.

## RESULTS

**Activation of rat intestinal epithelial cell and BBM guanylate cyclase by $ST_a$.** A rapid, dose-dependent accumulation of intracellular cGMP was observed in rat intestinal epithelial cells incubated with $ST_a$ (Fig. 1). The results show that a concentration of 10.0 ng of $ST_a$ per ml (5 pmol/ml) was sufficient to elicit a detectable response in isolated rat intestinal epithelial cells. Maximal levels of intracellular cGMP were observed at 2.5 min, which correlated with the rapid binding rate described previously (12). The decreased cGMP concentrations at longer incubation times were probably due to cellular phosphodiesterase activity. Stimulation of guanylate cyclase associated with BBMs by $ST_a$ was found to be dose dependent with respect to concentrations of $ST_a$ (Fig. 2) and membrane protein (data not shown). Formation of cGMP by BBMs treated with $ST_a$ was linear for approximately 10 min and reached a plateau by 15 min.

In our initial experiments on the stimulation of particulate guanylate cyclase by $ST_a$, we used membranes prepared from a mixture of crypt, mid-villus, and villus tip cells; thus, it was of interest to determine whether increased numbers of $ST_a$ receptors associated with rat villus intestinal cells (12) could be correlated with higher levels of guanylate cyclase activity. When BBM preparations obtained from cells corresponding to villus tip, mid-villus, and crypt cells were incubated with 431 $ST_a$, the highest levels of guanylate cyclase activity were obtained with BBMs derived from villus tip cells (Table 1). Consequently, in all subsequent experiments, BBMs derived from villus tip cells were used.

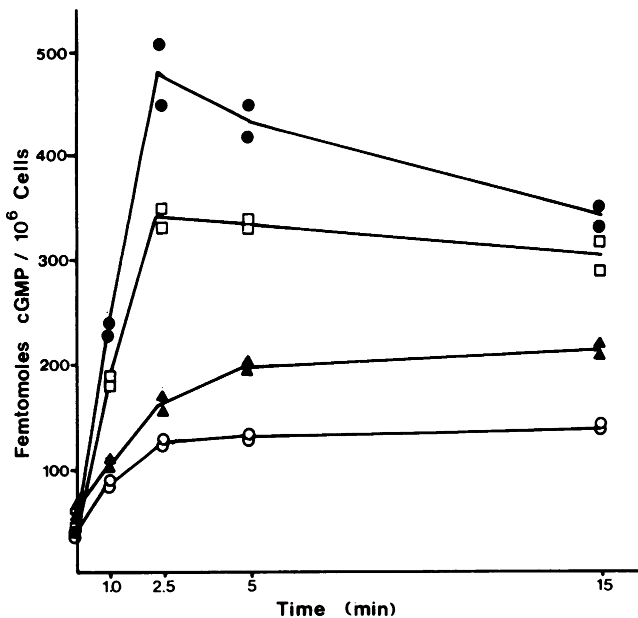


FIG. 1. Accumulation of intracellular cGMP in isolated rat intestinal epithelial cells treated with *E. coli* $ST_a$ at concentrations of 100 (●), 50 (□), 10 (▲), and 0 (basal) (○) ng/ml. At timed intervals, samples of reaction mixtures were pipetted into cold ethanol for determination of intracellular cGMP. Each datum point represents the mean of duplicate experiments.

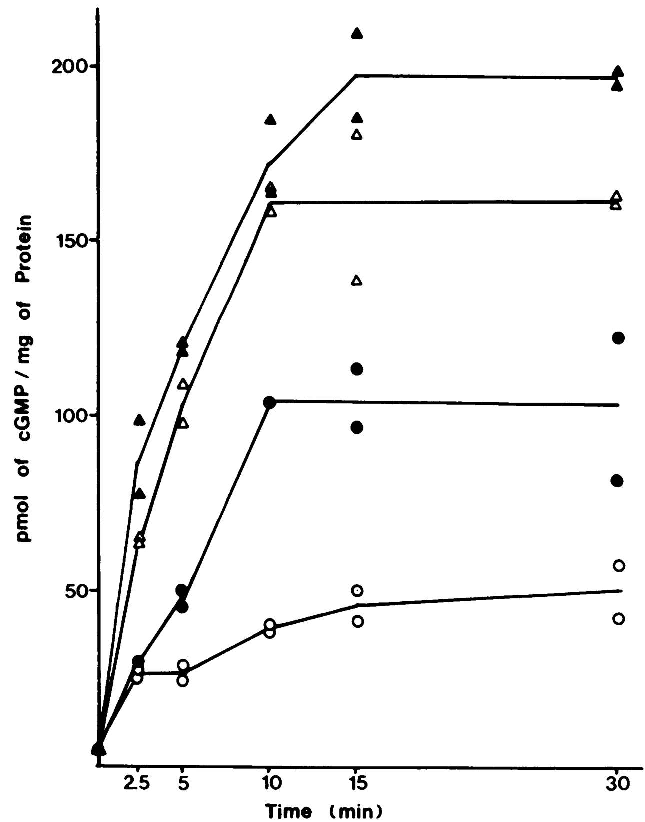


FIG. 2. Activation of guanylate cyclase associated with purified rat intestinal BBMs by *E. coli* $ST_a$ at concentrations of 400 (▲), 40 (△), 4.0 (●), and 0 (basal) (○) ng/ml. Each datum point represents the mean of duplicate experiments.

**Tissue specificity of $ST_a$ binding and stimulation of guanylate cyclase.** The mechanism of tissue specificity exhibited by $ST_a$ is unknown but may be due to a unique, high-affinity $ST_a$ receptor located on intestinal cells (12); thus, membrane preparations from various rat tissues were assayed for guanylate cyclase activity and 431 $^{125}I$-$ST_a$ binding capacity. Spleen preparations were low in particulate guanylate cyclase activity (Table 2), whereas lung membranes contained 5- to 15-fold more enzyme activity than did other membrane preparations. Guanylate cyclase activity associated with

TABLE 1. Stimulation of guanylate cyclase associated with intestinal cells by *E. coli* $ST_a$ [a]

| Cell type or membrane prepn | Guanylate cyclase activity (pmol of cGMP per min/mg of protein) | | |
|---|---|---|---|
| | Basal (without $ST_a$) | | With $ST_a$ [b] |
| | $Mg^{2+}$ | $Mn^{2+}$ [c] | |
| Villus tip | 9.8 | 55.4 | 40.8 (4.2) |
| Mid-villus | 5.4 | 33.6 | 22.7 (4.2) |
| Crypt | 4.6 | 27.1 | 16.4 (3.6) |

[a] Representative experiment expressed as the mean of duplicate determinations which varied by ±5%.
[b] $ST_a$ concentration, 2,000 ng/ml. Fold stimulation relative to basal level with $MgCl_2$ is shown in parentheses.
[c] $MnCl_2$ concentration, 0.4 mM.

TABLE 2. Binding specificity of *E. coli* $ST_a$ and stimulation of guanylate cyclase from various tissues

| Membrane type | 431 $^{125}I-ST_a$ binding (cpm) | | Guanylate cyclase activity (pmol of cGMP per min/mg of protein) | |
|---|---|---|---|---|
| | Without unlabeled $ST_a$ | With 100-fold excess of unlabeled $ST_a$ | Basal (without $ST_a$) | With $ST_a$[a] |
| Intestinal BBM | 22,300 ± 912 | 9,221 ± 303 | 1.9 | 9.6 (5.05) |
| Liver | —[b] | — | 2.4 | 3.2 (1.33) |
| Lung | 2,988 ± 617 | 2,409 ± 498 | 30.0 | 22.0 (0.73) |
| Spleen | 6,704 ± 722 | 7,704 ± 664 | 0.2 | 0.2 (1.0) |
| Kidney | 10,169 ± 2,603 | 10,743 ± 1,020 | 5.6 | 4.6 (0.82) |

[a] $ST_a$ concentration, 400 ng/ml. Fold stimulation due to $ST_a$ is shown in parentheses.
[b] —, No binding detected.

liver, lung, spleen, and kidney membranes was not stimulated by 431 $ST_a$; however, a fivefold stimulation of the intestinal enzyme was observed after the addition of toxin (Table 2). All membrane preparations except liver bound 431 $^{125}I-ST_a$, but specific binding was observed only with intestinal membranes; that is, binding of 431 $^{125}I-ST_a$ was inhibited by a 100-fold excess of unlabeled $ST_a$.

**Solubilization of the $ST_a$ receptor.** In an attempt to characterize the role of the $ST_a$ receptor in the mechanism of activation of particulate guanylate by $ST_a$, we extracted BBMs with a nondenaturizing dipolar ionic detergent (CHAPS) and assayed the soluble fraction for $ST_a$ binding activity. A significant amount (approximately 25%) of the total 431 $^{125}I-ST_a$ bound to a high-molecular-weight component which eluted in the void volume of the Bio-Gel P-6DG column (Fig. 3). Examination of the void volume fraction by SDS-PAGE and autoradiography showed that 431 $^{125}I-ST_a$ was associated with a single high-molecular-weight species which migrated with a mobility corresponding to a molecular weight of $10^5$ (Fig. 4, lane e). When the 431 $^{125}I-ST_a$–receptor complex was boiled (Fig. 4, lane f) or reduced with dithiothreitol (lane g) before electrophoresis, all radioactivity migrated with the same mobility as free $^{125}I-ST_a$.

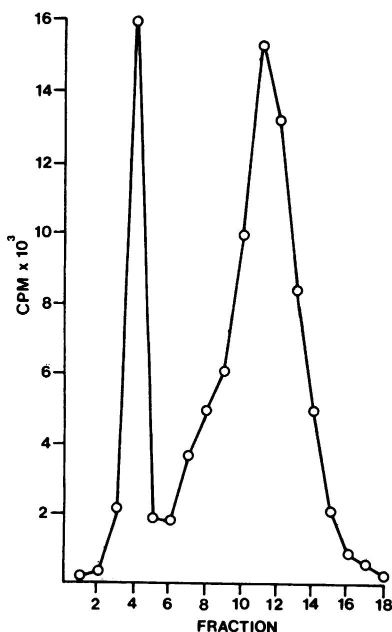


FIG. 3. Bio-Gel P-6 elution profile of CHAPS-soluble fraction of rat intestinal BBMs after incubation with 431 $^{125}I-ST_a$.

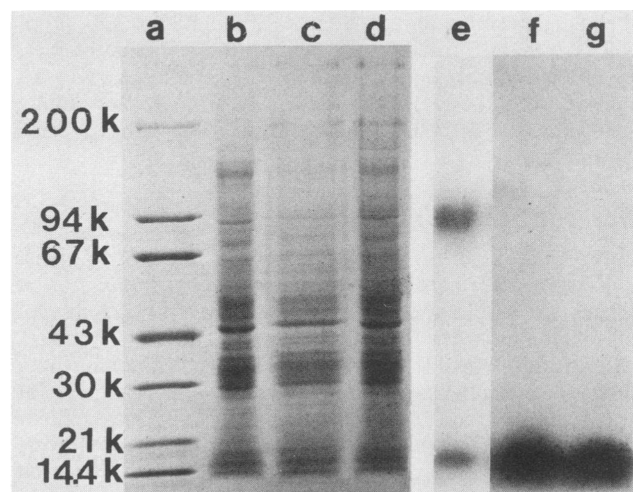


FIG. 4. SDS-PAGE analysis and autoradiograph of the CHAPS-soluble fraction of rat intestinal BBMs after incubation with 431 $^{125}I-ST_a$ and Bio-Gel P-6DG chromatography. Lane a, molecular weight standards; lane b, soluble membrane fraction (50 μg); lane c, soluble membrane fraction boiled for 5 min; lane d, soluble membrane fraction reduced with 50 mM dithiothreitol; lanes e through g, autoradiograph of lanes b through d. Positions corresponding to molecular standards are as indicated (k, molecular weight × $10^3$): 200k, myosin; 94k, phosphorylase B; 67k, bovine serum albumin; 43k, ovalbumin; 30k, carbonic anhydrase; 21k, soybean trypsin inhibitor; 14.4k, α-lactalbumin.

**Binding properties of the solubilized $ST_a$ receptor.** The effect of 431 $^{125}I-ST_a$ concentrations on binding to its soluble receptor was examined (Fig. 5). Binding in the presence of 25 μg of protein was linear up to a $ST_a$ concentration of 4.0 ng (2.0 pmol) per ml, and saturation binding was reached between 10.0 and 20.0 ng of $ST_a$ per ml. Binding specificity

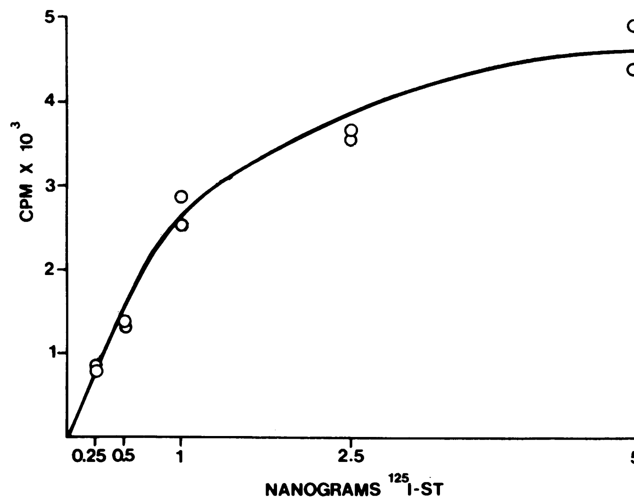


FIG. 5. Effect of concentration on binding of 431 $^{125}I-ST_a$ to CHAPS-solubilized receptor from rat BBMs. After incubation, free $^{125}I-ST_a$ was separated from bound $^{125}I-ST_a$ by a gel filtration assay described in the text.

was tested by incubating increasing amounts of unlabeled $ST_a$ and S-carboxymethyl–$ST_a$ in the presence of 431 $^{125}$I-$ST_a$ and its soluble receptor (Fig. 6). Addition of a 100-fold excess of unlabeled 431 $ST_a$ reduced binding by greater than 80%, which is in the range of specific binding observed with intact BBMs (12). Binding was inhibited about 50% by a fivefold excess of unlabeled 431 $ST_a$. In contrast, *S*-carboxymethyl–431 $ST_a$ which had been reduced and alkylated with iodoacetic acid did not inhibit binding of 431 $^{125}$I-$ST_a$ to its soluble receptor.

**Chemical properties of the solubilized $^{125}$I-$ST_a$ receptor complex.** Radiolabeled 431 $ST_a$ did not readily dissociate once bound to its receptor during incubation at pH 7.4 in the presence of CHAPS-HEPES-NaCl buffer. Dissociation was not increased by incubation with 0.1 M glycine–2 M NaCl (pH 3.0) or 4 M KSCN (Table 3). Addition of guanidine hydrochloride partially dissociated 431 $^{125}$I-$ST_a$ from its receptor. Treatment with 0.05 to 0.5 M dithiothreitol and boiling in the presence of 1% SDS caused complete dissociation.

Pretreatment of the solubilized membrane fraction with proteases reduced the amount of 431 $^{125}$I-$ST_a$ binding. Trypsin and pronase reduced binding by 51 and 48% of the total 431 $^{125}$I-$ST_a$ bound by untreated membranes, respectively. Chymotrypsin had no effect on binding of 431 $^{125}$I-$ST_a$ to the solubilized receptor.

Extracts of BBMs containing the $ST_a$ receptor and solubilized guanylate cyclase were not stimulated by $ST_a$, in contrast to residual enzyme activity associated with detergent-extracted membranes (data not shown).

## DISCUSSION

Most of the studies on the mechanism of action of *E. coli* $ST_a$ have employed animal models (e.g., suckling mice, rabbits, and piglets) (23, 30, 37, 44). In an attempt to develop a defined model system to study the mechanism of $ST_a$-mediated stimulation of particulate intestinal guanylate cyclase, we used isolated intestinal epithelial cells and partially

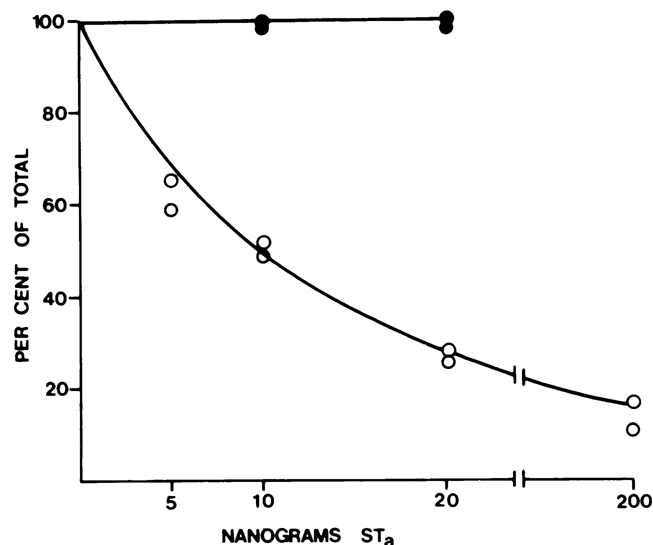


FIG. 6. Effect of unlabeled $ST_a$ (○) and carboxylmethyl-$ST_a$ (●) concentration on binding of 431 $^{125}$I-$ST_a$ to CHAPS-solubilized receptor. After incubation with $ST_a$ or carboxymethyl-$ST_a$, free 431 $^{125}$I-$ST_a$ was separated from bound 431 $^{125}$I-$ST_a$ by a gel filtration assay described in the text.

TABLE 3. Dissociation of 431 $^{125}$I-$ST_a$ from detergent-solubilized BBM $ST_a$ receptor

| Treatment[a] | % of bound $^{125}$I-$ST_a$[b] |
|---|---|
| None | 100.0 |
| 0.1 M glycine (pH 3.0, 2 M NaCl) | 98.7 |
| 4 M KSCN | 100.0 |
| 4 M guanidine-hydrochloride | 57.3 |
| 0.01 M dithiothreitol | 100.0 |
| 0.5 M dithiothreitol | 14.7 |
| 0.5 M acetic acid | 12.3 |
| 0.1% SDS (100°C) | 0.0 |

[a] After incubation of $^{125}$-I-$ST_a$ with detergent-solubilized membrane fraction, reaction mixtures were adjusted to the conditions listed, incubated for 15 min at 37°C, and assayed for amount of bound $ST_a$ by the gel filtration binding assay described in the text.

[b] Means of three separate experiments.

purified BBM preparations (9, 12). In this report, we describe results which further characterize the $ST_a$-mediated stimulation of guanylate cyclase of intestinal epithelial cells and BBMs and define the role of the high-affinity receptor in the mechanism of action of $ST_a$. The term receptor is used to define a binding component on the intestinal cell surface which facilitates the initial reaction and subsequent steps involved in the stimulation of guanylate cyclase by $ST_a$.

Only the particulate form of intestinal guanylate cyclase is stimulated by *E. coli* $ST_a$ (21, 40) and may be explained by a specific receptor that is required to mediate the biological activity of $ST_a$ or the molecular properties of intestinal particulate guanylate cyclase that are unique compared with other particulate guanylate cyclases (15, 22, 31, 39, 49). When membrane preparations from various tissues were examined in this study, only intestinal membranes exhibited specific binding of $ST_a$ and stimulation of particulate guanylate cyclase, although membrane preparations from other tissues contained measurable amounts of guanylate cyclase activity. These data suggest that nonintestinal guanylate cyclases do not respond to $ST_a$ because they lack the specific high-affinity receptor which mediates biological activity. More important, rates of guanylate cyclase activation described in this report correlated with binding kinetics (12); thus, activation of guanylate cyclase by $ST_a$ occurs without a significant lag period after binding of the toxin to its receptor.

To better define the molecular nature of the $ST_a$ receptor, we treated BBMs with CHAPS, a nondenaturing dipolar ionic detergent which has been used to solubilize active opiate receptors from rat brains (45). Treatment of BBMs with CHAPs yielded a clear supernatant fraction containing the $ST_a$ receptor and guanylate cyclase activity. SDS-PAGE of the $^{125}$I-$ST_a$–receptor complex revealed that $ST_a$ was firmly bound to a single high-molecular-weight component of the BBM, presumably its specific receptor. Once bound to its receptor, $ST_a$ was readily dissociated by dithiothreitol, which suggested that one of the disulfide bonds in native $ST_a$ (8, 46) reacts with a sulfhydryl group of the receptor. It is possible that reducing agents disrupt the tertiary structure of $ST_a$ and the disulfide bonds are not involved in binding to its receptor; however, thiol reagents [5,5 dithiobis-(2-nitrobenzoic acid), *N*-ethylmaleimide, and cystamine] inhibited binding of $^{125}$I-$ST_a$ to BBMs and $ST_a$-mediated stimulation of guanylate cyclase (9). The $ST_a$ receptor appears to be a protein or glycoprotein, on the basis of the following data: (i) as shown in this report, binding of $ST_a$ to its solubilized receptor was reduced by pretreatment with trypsin or pron-

ase; (ii) various carbohydrates, mixed gangliosides, and chloroform-methanol extracts of BBMs did not competitively inhibit binding of $ST_a$ to BBMs (12); and (iii) the $^{125}I-ST_a$–receptor complex comigrated during SDS-PAGE with a band which stained for protein and carbohydrate (12).

Although little is known about the molecular properties of particulate forms of guanylate cyclase, soluble liver guanylate cyclase is characterized by vicinal dithiol groups that are located at its active site and modulate its basal and activated levels (4, 28). Inhibitors and activators of liver guanylate cyclase covalently modify the active-site sulfhydryl groups and alter the oxidation state of the enzyme; for example, reduction and alkylation inactivated α-bungarotoxin and α-cobra toxin, which activate the soluble form of guanylate cyclase (33).

One or more of the disulfide bonds of $ST_a$ play a key role in the mechanism of *E. coli* $ST_a$-mediated stimulation of particulate guanylate cyclase (8, 46), and it appears that sulfhydryl groups mediate binding of $ST_a$ to its receptors and the enzymatic activity of particulate intestinal guanylate cyclase (9). It remains to be determined whether the $ST_a$ receptor and particulate guanylate cyclase are separate proteins or constitute a transmembrane glycoprotein, with the $ST_a$ receptor domain on the surface of the intestinal cell and guanylate cyclase on the cytoplasmic side of the membrane. The question can be resolved only by further purification and characterization of the $ST_a$ receptor and particulate intestinal guanylate cyclase.

Even though the mechanism of action of $ST_a$ cannot be precisely defined, several mechanisms appear not to be involved in the activation of particulate guanylate cyclase, i.e., activation of phospholipase $A_2$ and metabolism of arachidonic acid with formation of prostaglandins, stimulation of guanylate cyclase by free radicals derived from molecular oxygen, a calcium- or calmodulin-dependent reaction, phosphorylation coupled with dephosphorylation, and methylation of membrane phospholipids (9). The data presented in this paper strongly suggest that thiol groups of the $ST_a$ receptor react with at least one of the disulfide bonds present in $ST_a$. Metabolic regulation by thiol-disulfide reactions has been observed in other systems (14, 16, 36, 38, 43, 48), but such a reaction involved in regulation of guanylate cyclase may constitute a novel regulatory mechanism. Experiments are in progress to define and characterize the role of the thiol-disulfide exchange reaction in the $ST_a$-mediated stimulation of particulate guanylate cyclase.

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