

Characterization of the Mechanism of Action of *Escherichia coli* Heat-Stable Enterotoxin

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The mechanism of activation of intestinal guanylate cyclase by *Escherichia coli* heat-stable enterotoxin (ST_a) has been studied by using isolated rat intestinal epithelial cells and purified brush border membrane (BBM) preparations. Inhibitors of prostaglandin biosynthesis, quinacrine and 5,8,11,14-eicosatetraynoic acid (ETYA), significantly reduced intracellular levels of cyclic guanosine 3', 5'-monophosphate in isolated cells treated with ST_a. Although these data suggested that activation of phospholipase A₂ and metabolism of arachidonic acid are involved in the mechanism of action of ST_a, other data ruled out such a mechanism. (i) The rate of release of [³H]arachidonic acid by prelabeled intestinal cells incubated with ST_a was the same as control cells not treated with ST_a. (ii) Thin-layer chromatography of lipid extracts of intestinal cells treated with ST_a and untreated cells did not reveal any quantitative or qualitative differences in free fatty acids, neutral lipids, and phospholipids. (iii) Amounts of prostaglandin PGE₂, prostaglandin PGF_{2α}, and thromboxane B₂ in intestinal cells and BBM incubated with ST_a did not increase compared with controls not incubated with ST_a. When purified BBM preparations were incubated with phospholipase A₂ inhibitors (*p*-bromophenacyl bromide and quinacrine) or cyclooxygenase inhibitors (ETYA and indomethacin), basal and ST_a-induced guanylate cyclase activities were significantly reduced. Inhibitors of calcium-calmodulin-mediated reactions (EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid], trifluoperazine, and chlorpromazine) and calcium channel blockers (verapamil and nifedipine) also nonspecifically inhibited both basal and ST_a-stimulated guanylate cyclase in BBM preparations. Lanthanum, a competitive inhibitor of membrane-bound calcium, did not affect either basal or ST_a-stimulated guanylate cyclase of BBM preparations. Oxygen was not required for stimulation of particulate BBM guanylate cyclase by ST_a. Binding of ST_a to a specific receptor and subsequent activation of guanylate cyclase were both inhibited by thiol reagents [5,5'-dithiobis-(2-nitrobenzoic acid), *N*-ethylmaleimide, and cystamine]. The inhibition of ST_a binding and guanylate cyclase by cystamine were reversed by incubation of cystamine-treated BBM with dithiothreitol. A thiol-disulfide exchange may be one of the early reactions in the ST_a-induced activation of guanylate cyclase.

The heat-stable enterotoxin (ST) is one of two major types of enterotoxins which cause watery diarrhea in neonatal animals and humans after colonization of the small intestine by enterotoxigenic *Escherichia coli*. One enterotoxin type is a heat-labile oligomeric protein (LT), composed of two kinds of subunits, which stimulates adenylate cyclase in cells with G_{M1} ganglioside on their surfaces (17, 31). The other type of enterotoxin is a low-molecular-weight nonantigenic heat-stable molecule with biological activity in suckling mice, rats, rabbits, and piglets, referred to as ST_a (37, 39). In contrast to LT, which activates adenylate cyclase of intestinal and nonintestinal cells, ST_a stimulates only the particulate form of intestinal guanylate cyclase (22, 36). A second type of ST, referred to as ST_b, with biological activity only in piglet loops, has been detected (7, 34); however, its role in pathogenesis remains to be established. The mechanism of action of ST_b is unknown, but it does not appear to involve increased intracellular levels of either cyclic adenosine 3',5'-monophosphate or cyclic guanosine 3',5'-monophosphate (cGMP) (R. N. Greenberg, D. J. Kennedy, A. H. Stephenson, A. J. Lönigro, F. Murad, and R. L. Guerrant, *Clin. Res.* 30:367A, 1982; L. A. Dreyfus, L. Jaso-Friedmann, and D. C. Robertson, unpublished observations).

Inhibitors of prostaglandin biosynthesis and calmodulin-mediated reactions have been used to block fluid secretion in

suckling mice induced by ST_a. Indomethacin, chlorpromazine, quinacrine, and zomepirac sodium reduced fluid secretion in mice treated with submaximal doses, but not maximal doses of ST_a (1, 19, 20, 26, 30, 47). However, others have noted that the inhibitory effects of chlorpromazine on the ST_a-induced secretory response were not specific and independent of guanylate cyclase (38, 46). Inhibitors of calcium uptake (cromolyn sodium, diltiazem, and nifedipine) also decreased the ST_a-induced fluid response in suckling mice (26, 47). Trifluoperazine, a neuroleptic agent which inhibits calmodulin-mediated reactions (51), reduced short-circuit current and net Cl fluxes, but not cGMP concentrations, in rabbit intestinal mucosa treated with ST_a (45) and decreased ST_a-induced secretion in suckling mice (26). Based on these results and since quinacrine and indomethacin are specific inhibitors of phospholipase A₂ and cyclooxygenase, respectively, it was proposed that activation of phospholipase A₂, release of arachidonic acid, and synthesis of prostaglandins may be involved in the mechanism of action of ST_a (19, 20, 26, 27, 47, 48).

Since it is difficult to sort out cause and effect relationships in whole animal models, we used isolated rat intestinal epithelial cells and purified brush border membranes (BBM) to study the mechanism of *E. coli* ST_a-induced activation of particulate guanylate cyclase. Levels of intracellular cGMP in isolated rat intestinal epithelial cells induced by ST_a decreased when incubated with inhibitors which blocked the secretory response in suckling mice. However, other experiments with isolated cells did not indicate that phospholipase

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A₂ was activated or synthesis of prostaglandins occurred in response to treatment by ST_a. Based on results obtained with BBM, inhibitors which block ST_a-mediated secretion appeared to nonspecifically inhibit guanylate cyclase by reacting with an essential amino acid involved in catalysis or by perturbing the hydrophobic domain within the membrane. Inhibitors of calcium and calmodulin-mediated reactions exhibited nonspecific inhibition of ST_a-induced activation of guanylate cyclase activity. Interactions between disulfide bonds of ST_a and sulfhydryl groups of its high-affinity receptor and particulate guanylate cyclase appear to play an important role in the mechanism of action.

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. Preparation of media and maintenance of stock cultures have been described previously (4).

Purification of ST_a and bioassays. The ST_a produced by enterotoxigenic *E. coli* strain 431 was purified as described by Dreyfus et al. (10). Purified ST_a preparations were homogeneous by several parameters: (i) a single homogeneous peak on gel filtration, (ii) a single fluorescent band after reaction with dansyl chloride and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis, (iii) single amino-terminal and carboxyl-terminal residues, and (iv) amino acid analysis data which indicated a stoichiometric relationship between the amino acids. Purified preparations of 431 ST_a exhibited an effective dose of 0.4 ng by the suckling mouse assay performed as described previously (4, 10).

Isolation of intestinal epithelial cells and BBM. Intestinal epithelial cells were isolated from the resected small intestine of 120- to 180-g female Sprague-Dawley rats by the procedure of Weiser (50). BBM were prepared by a modification of the procedure of Hauser et al. (24) as described previously (15).

Determination of intracellular cGMP in rat intestinal epithelial cells. Accumulation of cGMP in isolated intestinal epithelial cells was measured in 5-ml incubation mixtures which contained cells (5.0×10^6 /ml), Hanks balanced salts solution (HBSS), 10% fetal calf serum, 431 ST_a (0 to 250 ng/ml), and theophylline (10 mM). 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 aliquots 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, resuspended 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 present in rat intestinal BBM was assayed as described by Kimura and Murad (25). Briefly, the reaction mixtures contained BBM, 50 to 100 µg; Tris-hydrochloride (pH 7.4), 5 µmol; theophylline, 1 µmol; phosphocreatine, 0.75 µmol; creatine phosphokinase, 3.5 U; guanosine triphosphate, 0.1 µmol; MgCl₂, 0.5 µmol; and ST_a, 4 to 400 ng/ml, in a total volume of 0.1 ml. The reaction was started by the addition of guanosine triphosphate-MgCl₂ 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 using a radioimmunoassay (RIA) procedure.

RIA for cGMP. The RIA for cGMP was performed essentially as described by Brooker et al. (6) with acetylation of samples (23). After overnight incubation at 4°C, normal rabbit immunoglobulin G (0.5 mg) was added as a carrier and

bound ¹²⁵I-labeled succinyl GMP tyrosine methyl ester was separated from unbound ¹²⁵I-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.

Labeling of rat intestinal epithelial cells with [³H]arachidonic acid. Rat intestinal epithelial cells were labeled with [³H]arachidonic acid (New England Nuclear Corp., Boston, Mass.; specific activity, 78.2 Ci/mmol) by incubating a cell suspension (2×10^6 cells per ml) in HBSS containing 10% fetal calf serum and [³H]arachidonic acid (1 to 5 µCi) in a total volume of 50 ml. After 45 min of incubation at 37°C in an air atmosphere with gentle shaking, the cells were centrifuged at $200 \times g$ and washed three times with 50 ml of cold HBSS containing 10% fetal calf serum. Cell viability determined by trypan blue exclusion was greater than 80%, with about 60% of the total [³H]arachidonic acid associated with intestinal cells.

Analysis of intestinal epithelial cell lipids. Release and metabolism of [³H]arachidonic acid by prelabeled intestinal epithelial cells were assayed in 5-ml reaction mixtures which contained: cells (5.0×10^6 /ml), HBSS, 10% fetal calf serum, 431 ST_a (400 ng/ml), and theophylline (10 mM). At timed intervals, 1 volume of reaction mixture was extracted with 3.75 volumes of chloroform-methanol (1:2[vol/vol]). The phases were broken by centrifugation, and the upper layer was discarded. The lower chloroform phase of each tube was dried under a stream of nitrogen. Lipids were dissolved in 0.05 ml of chloroform, spotted on plastic-backed Silica Gel G thin-layer chromatography plates, and developed with the upper phase of ethylacetate-2,2,4-trimethyl pentane-acetic acid-water (80:50:20:100[vol/vol/vol/vol]) (28). Air-dried thin-layer chromatography plates were sprayed with a fluorography enhancer reagent (EN³HANCE spray; New England Nuclear), and radioactive areas were detected by fluorography using Kodak X-OMAT AR X-ray film.

Determination of prostaglandins. Prostaglandin E₂ (PGE₂), prostaglandin F₂ (PGF_{2α}), and thromboxane B₂ (TXB₂) produced by isolated intestinal epithelial cells and BBM preparations were determined at the Ligand Laboratory, University of Michigan, Ann Arbor, by a RIA using antibodies and methods developed by Fitzpatrick et al. (12-14). Reaction mixtures in a total volume of 10 ml contained epithelial cells (5×10^6 /ml), HBSS-1% fetal calf serum, and ST_a (400 ng/ml). At timed intervals, 1-ml aliquots were removed and frozen in acetone-dry ice. Before assay, supernatants and cell debris were extracted for prostaglandins and to remove protein and free fatty acids. Bound ligand was separated from free ligand using dextran-coated charcoal. The limits of sensitivity for PGE₂, PGF_{2α}, and TXB₂ were 8, 4, and 2 pg, respectively. All samples were assayed in triplicate.

Binding assays. Binding of ¹²⁵I-labeled 431 ST_a to rat intestinal BBM was measured as described previously (15). Briefly, reaction mixtures containing BBM (30 to 50 µg), ¹²⁵I-labeled 431 ST_a (2.0 nM), and 12.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-1.0 mM EDTA, pH 7.4, in a final volume of 200 µl were incubated at 37°C for 10 min. Free ¹²⁵I-labeled 431 ST_a was separated from bound toxin by filtration on EGWP filters (Millipore Corp., Bedford, Mass.).

Radiolabeling of 431 ST_a. Purified 431 ST_a was radioiodinated enzymatically as described previously (15).

Protein assay. BBM protein was determined by the method of Lowry et al. (29). Membranes were solubilized by boiling in 1 N NaOH and neutralization with 1 N HCl before assay.

RESULTS

Inhibition of ST_a-induced activation of particulate guanylate cyclase in isolated rat intestinal epithelial cells. A rapid dose-dependent stimulation of guanylate cyclase was observed in rat intestinal epithelial cells incubated with ST_a (Fig. 1). The concentration of intracellular cGMP in reaction mixtures containing ST_a increased four to fivefold when treated with 100 ng of ST_a per ml and reached a maximum at about 15 min. The gradual decline over the next 20 to 30 min was presumably due to phosphodiesterase activity. The results show that quinacrine, a specific inhibitor of phospholipase A₂, inhibited ST_a-induced activation of intestinal cell guanylate cyclase and significantly reduced intracellular levels of cGMP. An inhibitor of cyclooxygenase, 5,8,11,14-eicosatetraenoic acid (ETYA) (49) also reduced intracellular cGMP levels in ST_a-treated cells to about 10% of amounts observed in the absence of inhibitor. The kinetics of intracellular cGMP accumulation induced by ST_a correlated with binding rates of radiolabeled ST_a to a specific high-affinity receptor (15; L. A. Dreyfus and D. C. Robertson, manuscript in preparation).

Release and metabolism of arachidonic acid by rat intestinal cells. Since quinacrine inhibited ST_a-induced formation of intracellular cGMP in rat intestinal epithelial cells, and

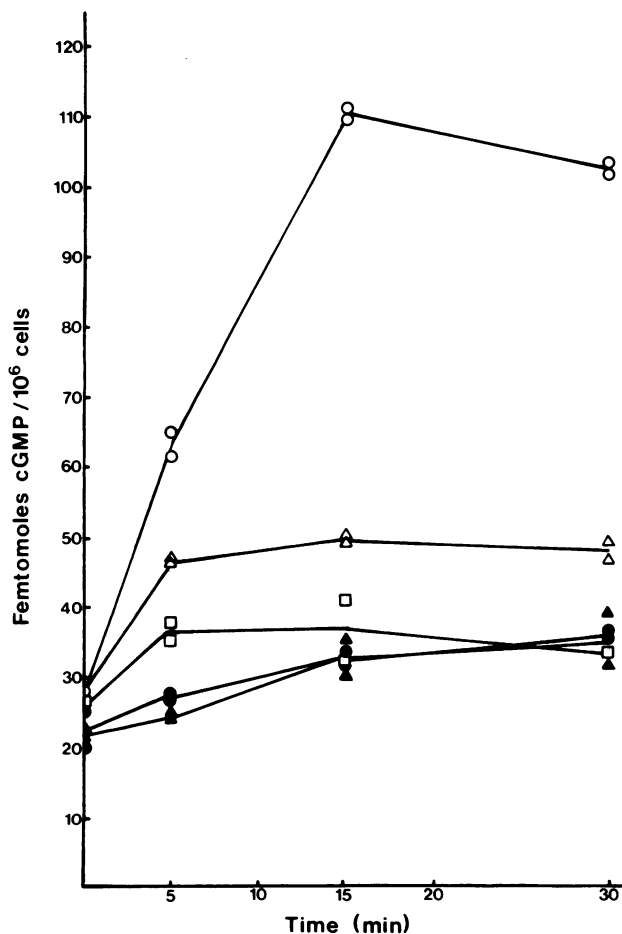


FIG. 1. Effects of quinacrine and ETYA on intracellular cGMP levels in isolated rat intestinal epithelial cells. Symbols: ○, ST_a (100 ng/ml); △, ST_a (100 ng/ml) and quinacrine (1.0 mM); □, ST_a (100 ng/ml) and ETYA (0.25 mM); ●, basal (no ST_a); ▲, basal (no ST_a) and quinacrine (1.0 mM) or ETYA (0.25 mM).

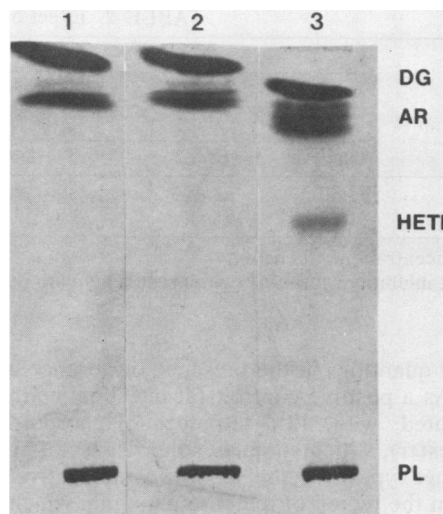


FIG. 2. Thin-layer chromatography of lipid extracts of rat intestinal epithelial cells. Cells were incubated with [³H]arachidonic acid, washed, and treated with *E. coli* ST_a or phorbol myristic acid. Lane 1, control cells not incubated with ST_a; lane 2, cells incubated with ST_a; lane 3, cells incubated with phorbol myristic acid. DG, Diglyceride; AR, arachidonic acid; HETE, 5-hydroxyeicosatetraenoic acid; PL, phospholipid.

because the drug has been shown to decrease secretion in suckling mice treated with 431 ST_a (20), it was of interest to determine whether activation of phospholipase A₂ occurred as a result of ST_a treatment and whether arachidonic acid metabolites could be detected. Freshly isolated rat intestinal cells were radiolabeled with [³H]arachidonic acid, followed by incubation with ST_a and extraction of free fatty acids and total lipids. Rates of [³H]arachidonic acid release were measured by scraping areas of thin-layer plates which contained radioactivity and comigrated at a rate corresponding to authentic arachidonic acid. The amount of arachidonic acid released due to hydrolysis by phospholipase A₂ in intestinal cells treated with 431 ST_a was identical to control cells not treated with 431 ST_a (data not shown). Furthermore, the amounts of radioactivity in diglyceride and phospholipid fractions were the same in lipid extracts of intestinal cells treated with 431 ST_a compared with controls not treated with toxin.

Even though it appeared that phospholipase A₂ was not stimulated by 431 ST_a, lipid extracts were subjected to thin-layer chromatography using a solvent system developed to resolve metabolites of arachidonic acid (28). Viable intestinal cells did not produce arachidonic acid metabolites in

TABLE 1. Prostaglandin levels in rat intestinal epithelial cells treated with ST_a^a

Time (min)	Prostaglandin level ^b (mg/ml)					
	PGE ₂		PGF _{2α}		TXB ₂	
	-ST _a	+ST _a	-ST _a	+ST _a	-ST _a	+ST _a
0	1.00	0.90	0.09	0.10	0.01	0.01
0.25	1.23	1.14	0.08	0.11	0.01	0.01
0.5	1.18	0.93	0.10	0.10	0.01	0.01
2.5	0.84	0.98	0.09	0.09	0.01	0.01

^a ST_a concentration, 400 ng/ml.

^b Mean of triplicate data points.

TABLE 2. Effect of oxygen on ST_a-stimulated guanylate cyclase

Atmosphere	Guanylate cyclase activity (pmol of cGMP/min/mg of protein)								
	Basal (-ST _a)						+ST _a ^a		
	Mg ²⁺			Mn ²⁺			Expt 1	Expt 2	\bar{x}^b
	Expt 1	Expt 2	\bar{x}^b	Expt 1	Expt 2	\bar{x}^b			
Air	1.38	1.68	—	6.98	6.18	—	5.98	6.38	—
Argon	1.22	1.68	5.8	6.98	6.38	-1.6	6.38	7.48	-12.0

^a ST_a concentration, 400 ng/ml.^b Percent inhibition; mean of two experiments with duplicate data points in each experiment.

detectable quantities in the presence or absence of 431 ST_a (Fig. 2). As a positive control, rat intestinal epithelial cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester), which stimulates the release of arachidonic acid in other types of cells (32, 53). A radioactive band was detected in the region of the chromatogram which migrated with a mobility corresponding to a 5-hydroxyeicosatetraenoic acid (HETE). Since it was possible that our assay system was not sensitive enough to detect low levels of arachidonic acid metabolites which might be responsible for activation of guanylate cyclase by ST_a, intestinal cells and membranes were incubated with ST_a and assayed for prostaglandins using a sensitive RIA. The levels of PGE₂, PGF_{2α}, and TXB₂ released by intestinal cells treated with ST_a were similar to controls (Table 1). Similar results were obtained with BBM preparations (data not shown). Cells in these experiments were incubated with high doses of ST_a (400 ng/ml) which maximally stimulated guanylate cyclase, even though there was no detectable increase in prostaglandin levels.

Role of oxygen in activation of guanylate cyclase by ST_a. Activation of intestinal particulate guanylate cyclase by ST_a could result from free radicals generated through metabolic reactions involving molecular oxygen, as reported for the soluble form of the enzyme (33). To ascertain whether ST_a-induced activation of guanylate cyclase is due to such a

mechanism, 25-ml Erlenmeyer flasks containing BBM and other reaction components were fitted with a rubber septum and thoroughly flushed with argon, followed by the addition of ST_a. There were no differences between the stimulation of intestinal guanylate cyclase by ST_a in an argon atmosphere compared with an air atmosphere (Table 2). Furthermore, basal levels of guanylate cyclase measured in the presence of either Mg²⁺ or Mn²⁺ were not altered by the absence of molecular oxygen.

Inhibition of ST_a-induced activation of guanylate cyclase in BBM. BBM preparations were used in an attempt to resolve data which indicated that various inhibitors of prostaglandin synthesis decreased intracellular cGMP levels in isolated intestinal cells treated with ST_a, yet there was no detectable stimulation of phospholipase A₂ or production of arachidonic acid metabolites. All drugs were incubated with BBM over 100-fold concentration ranges in the presence and absence of ST_a (Table 3). Without exception, basal and ST_a-induced levels of guanylate cyclase were reduced by low and intermediate amounts of each inhibitor. Significant inhibition of basal and ST_a-stimulated guanylate cyclase activities by *p*-bromophenacyl bromide was observed at low concentrations (0.01 mM). In contrast, indomethacin and ETYA had no effect on either activity at low concentrations. The lipooxygenase inhibitor nordihydroguaiaretic acid did not inhibit

TABLE 3. Effect of inhibitors of arachidonic acid metabolism on basal and ST_a-stimulated guanylate cyclase

Inhibitor and concn (mM)	Guanylate cyclase activity (pmol of cGMP/min/mg of protein)					
	Basal (-ST _a)			+ST _a ^a		
	Expt 1	Expt 2	\bar{x}^b	Expt 1	Expt 2	\bar{x}^b
<i>p</i>-Bromophenacyl bromide						
0	3.2	4.4	—	16.8	21.1	—
0.1	0.1	0.1	97.0	0.3	0.4	98.0
1.0	0.0	0.1	99.0	0.3	0.3	98.0
Indomethacin						
0	4.2	6.1	—	15.3	17.4	—
0.1	4.1	3.6	22.0	10.9	13.0	22.0
1.0	2.5	2.9	46.0	2.6	4.8	78.0
ETYA						
0	8.9	9.0	—	26.5	26.0	—
0.1	2.5	2.6	72.0	15.6	15.7	41.0
1.0	2.5	2.5	72.0	8.2	7.5	70.0
Nordihydroguaiaretic acid						
0	3.7	5.4	—	20.9	26.2	—
0.1	4.4	4.5	-1.1	25.7	19.4	1.5
1.0	3.2	5.1	9.6	20.2	25.8	2.4

^a Concentration of ST_a, 400 ng/ml.^b Percent inhibition; mean of two experiments with duplicate data points in each experiment.

TABLE 4. Effect of calcium and calmodulin inhibitors on basal and ST_a-stimulated guanylate cyclase

Inhibitor and concn (mM)	Guanylate cyclase activity (pmol of cGMP/min/mg of protein)						
	Basal (-ST _a)			+ ST _a ^a			
	Expt 1	Expt 2	\bar{x}^b	Expt 1	Expt 2	\bar{x}^b	
EGTA							
0	1.6	2.0		7.0	8.0		
1.0	1.6	2.0	0.0	7.2	7.4		2.3
2.0	1.2	2.2	7.5	1.0	2.7		76.0
EDTA							
0	3.2	3.1		17.8	18.7		
2.0	3.1	3.0	3.2	15.4	17.8		9.2
Trifluoperazine							
0	3.0	3.5		8.6	11.5		
0.1	1.6	1.0	46.2	6.4	9.2		23.8
1.0	0.2	0.4	91.0	1.4	1.9		83.5
Chlorpromazine							
0	6.0	7.0		15.6	16.8		
0.1	2.8	2.2	61.0	6.6	8.0		55.0
1.0	0.2	0.8	92.6	0.3	0.6		97.2
Verapamil							
0	3.0	3.5		8.6	11.5		
0.1	1.9	1.8	42.6	7.0	7.5		26.7
1.0	1.8	1.6	1.6	5.0	6.8		40.4
Nifedipine							
0	3.0	3.5		8.6	11.5		
0.1	1.0	0.8	71.9	4.6	3.6		57.6
1.0	0.1	0.2	95.5	0.9	1.0		90.4
Lanthanum							
0	2.0	2.0		8.0	8.3		
0.1	1.7	2.0	7.5	8.0	9.3		-6.0

^a Concentration of ST_a, 400 ng/ml.

^b Percent inhibition; mean of two experiments with duplicate data points in each experiment.

either basal or ST_a-induced guanylate cyclase to any significant extent. Basal enzyme activities and the fold stimulation of guanylate cyclase by ST_a depended on the age of membrane preparations; however, the amount of inhibition caused by drugs was reproducible ($\pm 5\%$).

The role of calcium and calmodulin in the activation of guanylate cyclase by ST_a was examined by using pharmacological agents which chelate calcium (EGTA), inhibit calmodulin-mediated reactions (trifluoperazine and chlorpromazine), act as calcium channel blockers (verapamil and nifedipine), or displace calcium from membrane-binding sites (La³⁺). BBM were incubated with each inhibitor or drug for 10 min at 4°C before the addition of Mg²⁺-guanosine triphosphate and incubation at 37°C for 10 min. EGTA had no effect on the stimulation of guanylate cyclase by ST_a at a concentration of 1 mM but exhibited significant inhibition at 2.0 mM (Table 4). The inhibitory effect of EGTA at the higher concentration could not be reversed by the addition of calcium or other divalent cations. Inhibitors of calmodulin-mediated reactions and calcium channel blockers appeared not to be specific based on inhibition of basal enzyme activity and ST_a-induced activation of guanylate cyclase. Lanthanum was not inhibitory in the presence or absence of ST_a at concentrations shown to be inhibitory in other calcium-dependent systems (52).

Role of sulfhydryl groups in activation of guanylate cyclase. The effects of several thiol-reactive reagents on the binding

of ST_a to BBM and activation of intestinal guanylate cyclase were examined since intact disulfide bonds are required for the biological activity of ST_a (10) and sulfhydryl groups are important in the regulation and function of guanylate cyclase (5, 33). The results show that reagents which react with free sulfhydryl groups, *N*-ethylmaleimide (NEM) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), inhibited specific binding about 70% (Fig. 3). More important, cystamine, a disulfide compound, also inhibited binding. The effects of cystamine or ST_a-induced activation of guanylate cyclase are shown in Fig. 4. Cystamine inhibited ST_a-induced guanylate cyclase activity by greater than 95% at a concentration of 1 mM. Inhibition of ST_a-induced particulate guanylate cyclase by low concentrations (0.001 mM) of NEM and DTNB was greater compared with cystamine; however, at higher concentrations (0.1 mM), amounts of inhibition by the thiol-reactive compounds were similar (data not shown). In reaction mixtures containing 0.01 mM cystamine, both binding and ST_a-induced stimulation of guanylate cyclase were inhibited; however, there was little effect on basal enzyme levels. In an attempt to determine whether the inhibition of binding and ST_a-induced stimulation of guanylate cyclase were reversible, membranes were incubated with cystamine, washed, and incubated with the reducing agent dithiothreitol. Inhibition of ST_a binding and inhibition of ST_a-induced activation of particulate guanylate cyclase by cystamine were readily reversible (Table 5).

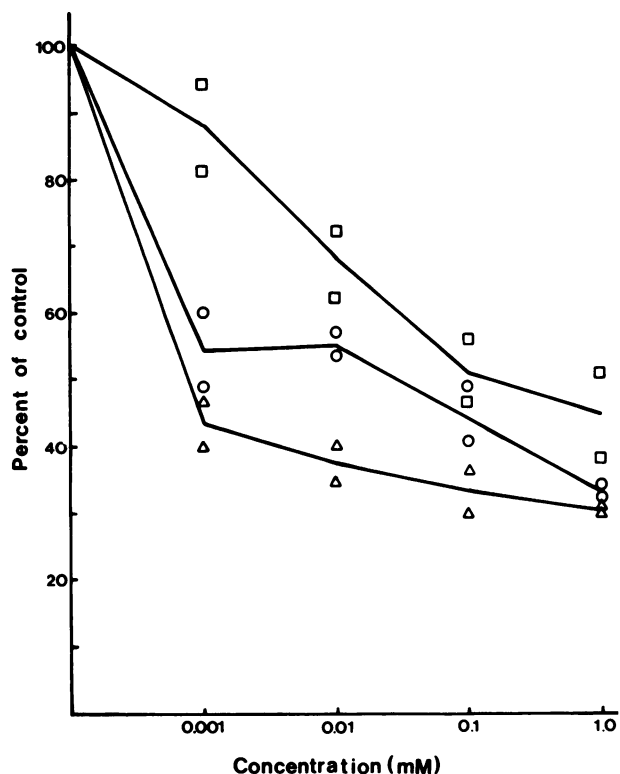


FIG. 3. Effect of thiol-reactive compounds on binding of *E. coli* ^{125}I -labeled ST_a to rat BBM. Symbols: \square , cystamine; \circ , NEM; \triangle , DTNB.

DISCUSSION

Previous studies have established the importance of cGMP and stimulation of particulate intestinal guanylate cyclase in the secretory response induced by ST_a (16, 22, 36, 41). Since many compounds which activate particulate guanylate cyclase are known (33), inhibitors of phospholipase A_2 , prostaglandin biosynthesis, and calcium transport have been used to block the secretory activity induced by ST_a in suckling mice and piglets. Quinacrine, indomethacin, and chlorpromazine, inhibitors of phospholipase A_2 , cyclooxygenase, and calmodulin-mediated reactions, respectively, reduced the secretory response of suckling mice to submaximal doses of ST_a , but had no effect when animals were challenged with maximal doses of ST_a (1, 19, 20, 26, 30, 47). In contrast, indomethacin did not inhibit ST_a -induced net water and electrolyte loss in perfused pig jejunum (2), but α -adrenergic agonists and opiate agonists reduced the secretory response and loss of chloride (3). Calcium channel blockers decreased the fluid response in suckling mice induced by ST_a (48). Also, trifluoperazine, an inhibitor of calmodulin-mediated reactions (51), reduced short-circuit current and net Cl fluxes, but not cGMP concentrations, in rabbit intestinal mucosa treated with ST_a (45). Despite the evidence pointing to stimulation of prostaglandin biosynthesis and calcium in the mechanism of action of *E. coli* ST_a , Field et al. (11) could not demonstrate that calcium was required to elicit an electrical signal in isolated rabbit ileal mucosa incubated with the toxin. Also, stimulation of phospholipase A_2 is usually involved in the mechanism of action of cytolytic toxins (42). Thus, based on these results, it is difficult to assign a site of action for each inhibitor.

To avoid complex cause and effect relationships observed with animal models, we used isolated rat intestinal epithelial cells and BBM to study the activation of intestinal guanylate cyclase by ST_a . Experiments with isolated rat intestinal cells described in this report supported data obtained with suckling mice; that is, quinacrine and ETYA inhibited ST_a -induced formation of intracellular cGMP in isolated rat intestinal epithelial cells. Although these data suggested that activation of phospholipase A_2 and synthesis of prostaglandin intermediates occurred in intestinal tissues treated with ST_a , several contradictory observations indicated that such a mechanism was not involved. (i) The rate of release of ^3H arachidonic acid by prelabeled intestinal epithelial cells incubated with ST_a was identical to that of cells in reaction mixtures which did not contain ST_a . (ii) There were no differences in the distribution of radioactivity in major classes of lipids (fatty acids, neutral lipids, and phospholipids) extracted from intestinal epithelial cells incubated with ST_a compared with controls (no ST_a). (iii) Levels of prostaglandins PGE_2 , $\text{PGF}_{2\alpha}$, and TXB_2 did not increase in supernatants containing either cells or BBM treated with ST_a . When intestinal epithelial cells were treated with phorbol ester (32, 53), a faint band was observed with a mobility corresponding to 5-hydroxyeicosatetraenoic acid. Thus, even though there was significant incorporation and release of ^3H arachidonic acid by intestinal epithelial cells (50% uptake in 45 min and greater than 20% release in 30 min), there was no detectable stimulation of phospholipase A_2 as measured by comparing rates of release of ^3H arachidonic acid by ST_a -treated and untreated cells, formation of arachidonic metabolites, or using a sensitive RIA for prostaglan-

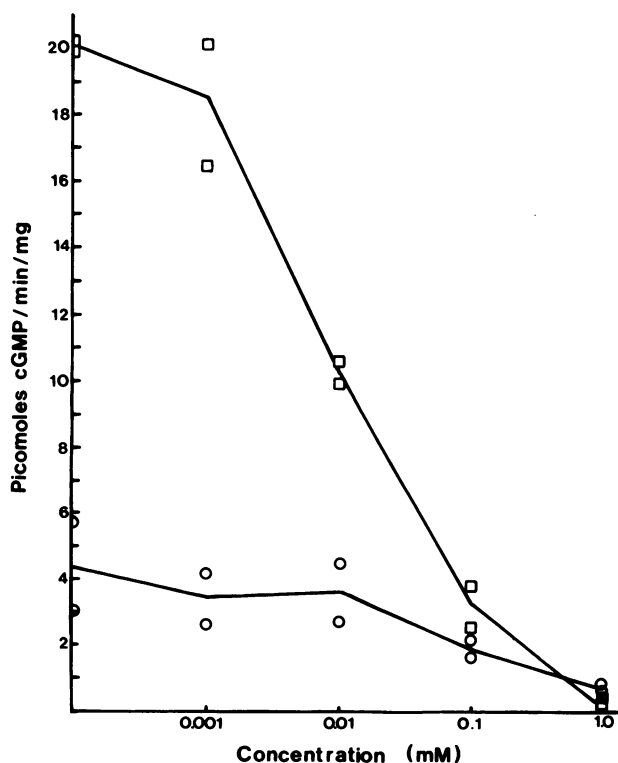


FIG. 4. Effect of cystamine on ST_a -stimulated guanylate cyclase of rat intestinal BBM. Symbols: \square , ST_a ; \circ , basal enzyme levels (no ST_a).

TABLE 5. Effect of dithiothreitol on ST_a binding and guanylate cyclase activity after inhibition by cystamine

Treatment	ST _a binding (% inhibition)			Guanylate cyclase activity (pmol of cGMP/min/mg of protein)					
	Expt 1	Expt 2	\bar{x}^b	Basal (-ST _a)			+ST _a ^a		
				Expt 1	Expt 2	\bar{x}^b	Expt 1	Expt 2	\bar{x}^b
Control (Untreated)				2.46	3.53		10.36	11.53	
+cystamine (10 μM)	52.0	39.8	45.9	1.26	1.86	48.9	5.76	6.17	45.4
+cystamine (10 μM)	4.0	-4.3	0.2	1.96	3.03	17.2	10.36	11.03	2.1
+dithiothreitol (10 μM)									

^a ST_a concentration, 400 ng/ml.

^b Percent inhibition; mean of two experiments with duplicate data points in each experiment.

dins under conditions in which guanylate cyclase was maximally activated.

To more directly study the ST_a-induced activation of particulate intestinal guanylate cyclase and to avoid secondary reactions which might yield equivocal results, purified BBM were incubated with ST_a and various pharmacological agents which inhibit prostaglandin biosynthesis. Without exception, compounds which reduced the ST_a-induced activation of intestinal guanylate cyclase also inhibited basal enzyme activity measured with either Mg²⁺ or Mn²⁺. Dose-response curves over a 100-fold range of concentration of each drug were tested. *p*-Bromophenacyl bromide, a specific inhibitor of phospholipase A₂, significantly reduced both basal and ST_a-activated levels of guanylate cyclase by ST_a at a final concentration of 0.1 mM. Likewise, inhibitors of cyclooxygenase (indomethacin and ETYA) reduced both basal and ST_a-activated levels of guanylate cyclase. The nonspecific action by nonsteroidal antiinflammatory agents which cannot be explained by inhibition of prostaglandin synthesis has been observed by other investigators (35, 43). Also, the antisecretory action of indomethacin in rabbit ileal mucosa treated with a number of secretory agents (cholera toxin, LT, and ST_a) could not be explained by inhibition of prostaglandin synthesis (44).

Calcium ions have been proposed as a second messenger in stimulus-response coupling, and the effects are often mediated by the calcium-binding protein calmodulin (8). Since calcium appears to play a key role in regulation of intestinal electrolyte transport (9), several pharmacological agents which inhibit either calcium or calmodulin-mediated reactions were incubated with purified BBM, Mg²⁺-guanosine triphosphate, and ST_a. At concentrations up to 1 mM, EGTA had no effect on ST_a-induced activation of particulate guanylate cyclase; however, at higher concentrations, significant inhibition was observed which could not be reversed by the addition of calcium or other divalent cations. As observed with antiinflammatory agents, inhibitors of calcium and calmodulin-mediated reactions exhibited a nonspecific mechanism of action and inhibited both basal and ST_a-induced guanylate cyclase. Trifluoperazine and chlorpromazine may perturb the hydrophobic domain of the enzyme. In contrast to other pharmacological agents, lanthanum, which displaces calcium from membrane-binding sites (52), did not inhibit either basal or ST_a-induced activation of guanylate cyclase. These data are similar to the results of Greenberg et al. (21). If calcium is involved in the regulation of particulate intestinal guanylate cyclase, calcium influx likely occurs after binding of ST_a to a specific receptor. However, at low concentrations, calcium had no effect on guanylate cyclase activity assayed in the presence of Mg²⁺ or Mn²⁺ and was inhibitory at higher concentrations (L. Jaso-Friedmann and D. C. Robertson, unpublished observations).

Since previous studies suggested that free radicals activate soluble guanylate cyclase (33), and because guanylate cyclase may be regulated by the oxidation-reduction potential of cells (5), the requirement for molecular oxygen in the mechanism of activation of particulate guanylate cyclase was examined. When argon was substituted for air, the guanylate cyclase activity in BBM induced by ST_a was identical to that in an air atmosphere. These data appear to rule out an involvement of cyclooxygenase and lipoxygenase in the mechanism of action of ST_a. It should be emphasized that these reaction mixtures contained only purified BBM, Mg²⁺ -GTP, and ST_a, and in contrast to adenylate cyclase (40), soluble cellular components do not appear to be required for ST_a-induced activation of particulate guanylate cyclase.

Several lines of evidence suggest that disulfide and thiol groups play an important role in the mechanism of action of ST_a; for example, the biological activity of ST_a is dependent on the presence of three intact disulfide bonds (10). Furthermore, several disulfide and thiol compounds inhibited ST_a-mediated secretion in suckling mice (18). When purified rat BBM were treated with thiol-reactive reagents (DTNB or NEM), binding of radiolabeled ST_a was inhibited about 70% and particulate guanylate cyclase activity was almost completely inhibited. At a concentration of 0.01 mM, which had no effect on basal enzyme activity, cystamine inhibited binding of ¹²⁵I-labeled ST_a by about 35%, and guanylate cyclase activity was reduced by 50%. Since both oxidized and reduced forms of ST_a do not bind to BBM or stimulate guanylate cyclase (L. A. Dreyfus and D. C. Robertson, manuscript in preparation), these data suggest that binding of ST_a must occur before activation of guanylate cyclase and that the primary effect of cystamine is on the ST_a receptor. A reaction between cystamine and particulate guanylate cyclase cannot be ruled out; however, inhibition of binding and guanylate cyclase activity were both reversed by incubating cystamine-treated membranes with dithiothreitol.

In summary, data presented in this report strongly suggest that activation of phospholipase A₂, metabolism of arachidonic acid, and formation of prostaglandins are not involved in stimulation of intestinal particulate guanylate cyclase by ST_a. Furthermore, a mechanism mediated by free radicals appears unlikely, since the fold activation of guanylate cyclase by ST_a was the same in the presence and absence of an air atmosphere. No evidence was found to suggest that either calcium or calmodulin was required for ST_a-induced activation of guanylate cyclase, but these results do not rule out a requirement for calcium in later reactions of the secretory response. Most important, results of inhibition experiments designed to block the action of ST_a with neuroleptic agents, antiinflammatory agents, and inhibitors of prostaglandin biosynthesis and calcium metabolism indicated

that these drugs exert a nonspecific inhibitory effect directly on guanylate cyclase and probably have nothing to do with reactions which occur after binding of ST_a to its specific high-affinity receptor (15). As suggested by a previous report (18), results in this paper indicate that thiol groups of the ST_a receptor and disulfide bonds of ST_a play a critical role in the mechanism of action, and a disulfide interchange between ST_a and its receptor might precede activation of particulate guanylate cyclase. It remains to be determined whether the ST_a receptor and guanylate cyclase are separate proteins or a transmembrane glycoprotein with the ST_a receptor on the cell surface and guanylate cyclase on the cytoplasmic side of the membrane. Studies are in progress to characterize the interactions between the ST_a receptor and particulate guanylate cyclase.

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LITERATURE CITED

- Abbey, D. M., and F. C. Knoop. 1979. Effect of chlorpromazine on the secretory activity of *Escherichia coli* heat-stable enterotoxin. *Infect. Immun.* **25**:1000-1003.
- Ahrens, F. A., and B. Zhu. 1982. Effects of indomethacin, acetazolamide, ethacrynate sodium, and atropine on intestinal secretion mediated by *Escherichia coli* heat-stable enterotoxin in pig jejunum. *Can J. Physiol. Pharmacol.* **60**:1281-1286.
- Ahrens, F. A., and B. Zhu. 1982. Effects of epinephrine, clonidine, L-phenylephrine, and morphine on intestinal secretion mediated by *Escherichia coli* heat-stable enterotoxin in pig jejunum. *Can J. Physiol. Pharmacol.* **60**:1680-1685.
- Alderete, J. F., and D. C. Robertson. 1978. Purification and chemical characterization of the heat-stable enterotoxin produced by porcine strains of enterotoxigenic *Escherichia coli*. *Infect. Immun.* **19**:1021-1030.
- Brandwein, H. J., J. A. Lewicki, and F. Murad. 1981. Reversible inactivation of guanylate cyclase by mixed disulfide formation. *J. Biol. Chem.* **256**:2958-2962.
- Brooker, G., J. F. Harper, W. L. Terasaki, and R. C. Moylan. 1979. Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv. Cyclic Nucleotide Res.* **10**:1-32.
- Burgess, M. N., R. J. Bywater, C. M. Cowley, N. A. Mullan, and P. M. Newsome. 1978. Biological evaluation of a methanol-soluble, heat-stable *Escherichia coli* enterotoxin in infant mice, pigs, rabbits, and calves. *Infect. Immun.* **21**:526-531.
- Cheung, W. Y. 1982. Calmodulin: an overview. *Fed. Proc.* **41**:2253-2257.
- Donowitz, M., and J. L. Madara. 1982. Effect of extracellular calcium depletion on epithelial structure and function in rabbit ileum: a model for selective crypt or villus epithelial cell damage and suggestion of secretion by villus epithelial cells. *Gastroenterology* **83**:1231-1243.
- Dreyfus, L. A., J. C. Frantz, and D. C. Robertson. 1983. Chemical properties of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* of different host origins. *Infect. Immun.* **42**:539-548.
- Field, M., L. H. Graf, Jr., W. J. Laird, and P. L. Smith. 1978. Heat-stable enterotoxin of *Escherichia coli*: *in vitro* effects on guanylate cyclase activity, cGMP concentration, and ion transport in small intestine. *Proc. Natl. Acad. Sci. U.S.A.* **75**:2800-2804.
- Fitzpatrick, F. A., and G. L. Bundy. 1978. Hapten mimic elicits antibodies recognizing prostaglandin E₂. *Proc. Natl. Acad. Sci. U.S.A.* **75**:2689-2693.
- Fitzpatrick, F. A., P. R. Gorman, J. C. McGuire, R. C. Kelly, M. A. Wynalds, and F. F. Sun. 1977. A radioimmunoassay for thromboxane B₂. *Anal. Biochem.* **82**:1-7.
- Fitzpatrick, F. A., and W. A. Wynalds. 1976. A rapid, solid phase radioimmunoassay for prostaglandin F₂ and its main circulating metabolites. *Anal. Biochem.* **73**:198-208.
- Frantz, J. C., L. Jaso-Friedmann, and D. C. Robertson. 1984. Binding of *Escherichia coli* heat-stable enterotoxin to rat intestinal cells and brush border membranes. *Infect. Immun.* **43**:622-630.
- Giannella, R. A., and K. W. Drake. 1979. Effect of purified *Escherichia coli* heat-stable enterotoxin in intestinal cyclic nucleotide metabolism and fluid secretion. *Infect. Immun.* **24**:19-23.
- Gill, D. M., and S. H. Richardson. 1980. Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of *Escherichia coli*: comparison with cholera toxin. *J. Infect. Dis.* **141**:64-70.
- Greenberg, R. N., J. A. Dunn, and R. L. Guerrant. 1983. Reduction of the secretory response to *Escherichia coli* enterotoxin by thiol and disulfide compounds. *Infect. Immun.* **41**:174-180.
- Greenberg, R. N., R. L. Guerrant, B. Chang, D. C. Robertson, and F. Murad. 1982. Inhibition of *Escherichia coli* heat-stable enterotoxin effects on intestinal guanylate cyclase and fluid secretion by quinacrine. *Biochem. Pharmacol.* **31**:2005-2009.
- Greenberg, R. N., F. Murad, B. Chang, D. C. Robertson, and R. Guerrant. 1980. Inhibition of *Escherichia coli* heat-stable enterotoxin by indomethacin and chlorpromazine. *Infect. Immun.* **29**:908-913.
- Greenberg, R. N., F. Murad, and R. L. Guerrant. 1982. Lanthanum chloride inhibition of the secretory response to *Escherichia coli* heat-stable enterotoxin. *Infect. Immun.* **35**:483-488.
- Guerrant, R. L., J. M. Hughes, B. Chang, D. C. Robertson, and F. Murad. 1980. Activation of intestinal guanylate cyclase by heat-stable enterotoxin of *Escherichia coli*: studies of tissue specificity, potential receptors, and intermediates. *J. Infect. Dis.* **142**:220-227.
- Harper, J. F., and G. J. Brooker. 1975. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2:0-acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**:207-218.
- Hauser, H., K. Howell, R. M. C. Dawson, and D. E. Bowyer. 1980. Rabbit small intestinal brush border membrane preparation and lipid composition. *Biochim. Biophys. Acta* **602**:567-577.
- Kimura, H., and F. Murad. 1974. Evidence for two different forms of guanylate cyclase in rat heart. *J. Biol. Chem.* **249**:6910-6919.
- Knoop, F. C., and D. M. Abbey. 1981. Effect of chemical and pharmacological agents on the secretory activity induced by *Escherichia coli* heat-stable enterotoxin. *Can. J. Microbiol.* **27**:754-758.
- Knoop, F. C., and D. D. Thomas. 1983. Stimulation of calcium uptake and cyclic GMP synthesis in rat basophilic leukemia cells by *Escherichia coli* heat-stable enterotoxin. *Infect. Immun.* **41**:971-977.
- Lapetina, E. G., C. J. Schmitges, K. Chandrabrose, and P. Cuatrecasas. 1977. Cyclic adenosine 3',5'-monophosphate and prostacyclin inhibit membrane phospholipase activity in platelets. *Biochem. Biophys. Res. Commun.* **76**:828-835.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Madsen, G. L., and F. C. Knoop. 1978. Inhibition of the secretory activity of *Escherichia coli* heat-stable enterotoxin by indomethacin. *Infect. Immun.* **22**:143-147.
- Moss, J., J. C. Osborne, Jr., P. H. Fishman, S. Nakaya, and D. C. Robertson. 1981. *Escherichia coli* heat-labile enterotoxin: ganglioside specificity and ADP-ribosyltransferase activity. *J. Biol. Chem.* **256**:12861-12865.
- Mufson, R. A., D. DeFeo, and I. B. Weinstein. 1979. Effects of phorbol ester tumor promoters on arachidonic acid metabolism in chick embryo fibroblasts. *Mol. Pharmacol.* **16**:569-578.

33. Murad, F., W. P. Arnold, C. K. Mittal, and J. M. Braugher. 1979. Properties and regulation of guanylate cyclase and some proposed functions for cyclic GMP. *Adv. Cyclic Nucleotide Res.* **11**:175-204.
34. Olson, E., and O. Soderland. 1980. Comparison of different assays for definition of heat-stable enterotoxigenicity of *Escherichia coli* porcine strains. *J. Clin. Microbiol.* **11**:6-15.
35. Pickett, W. C., K. F. Austen, and E. J. Goetzel. 1979. Inhibition by nonsteroidal anti-inflammatory agents on the ascorbate-induced elevations of platelet cGMP levels. *J. Cyclic Nucleotide Res.* **5**:197-209.
36. Rao, M. C., S. Guandalini, P. L. Smith, and M. Field. 1980. Mode of action of heat-stable *Escherichia coli* enterotoxin: tissue and subcellular specificity and role of cGMP. *Biochim. Biophys. Acta* **632**:35-46.
37. Robertson, D. C., and J. F. Alderete. 1980. Chemistry and biology of the heat-stable *Escherichia coli* enterotoxin, p. 115-126. In O. Ouchterlony and J. Holmgren (ed.), *Cholera and related diarrheas*. S. Karger, Basel.
38. Robins-Browne, R. M., and M. M. Levine. 1981. Effect of chlorpromazine on intestinal secretion mediated by *Escherichia coli* heat-stable enterotoxin and 8-Br-cyclic GMP in infant mice. *Gastroenterology* **80**:321-326.
39. Sack, R. B. 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. *Annu. Rev. Microbiol.* **29**:333-353.
40. Sahyoun, N., H. LeVine III, P. Stenbuck, and P. Cuatrecasas. 1983. Cytosolic activator of adenylate cyclase: reconstitution characterization, and mechanism of action. *Proc. Natl. Acad. Sci. U.S.A.* **80**:3646-3650.
41. Scoot, A., G. W. Forsyth, R. A. Kapitany, W. E. Roe, and D. L. Hamilton. 1980. Effects of isolated heat-stable enterotoxin produced by *Escherichia coli* on fluid secretion and cyclic nucleotide levels in the jejunum of the weanling pig. *Can. J. Physiol. Pharmacol.* **58**:772-777.
42. Shier, W. T. 1982. Cytolytic mechanisms: self-destruction of mammalian cells by activation of endogenous hydrolytic enzymes. *J. Toxicol. Toxin Rev.* **1**:1-32.
43. Siegel, M. I., R. T. McConnell, N. A. Porter, and P. Cuatrecasas. 1980. Arachidonate metabolism via lipoxygenase and 12 L-hydroperoxy -5, 8, 10, 14-eicosatetraenoic acid peroxidase sensitive to antiinflammatory drugs. *Proc. Natl. Acad. Sci. U.S.A.* **77**:308-312.
44. Smith, P. L., J. B. Blumberg, J. S. Stoff, and M. Field. 1981. Antisecretory effects of indomethacin on rabbit ileal mucosa *in vivo*. *Gastroenterology* **80**:356-365.
45. Smith, P. L., and M. Field. 1980. *In vitro* antisecretory effects of trifluoperazines and other neuroleptics in rabbit and human small intestine. *Gastroenterology* **78**:1545-1553.
46. Takeda, T., T. Honda, Y. Takeda, and T. Miwatani. 1981. Failure of chlorpromazine to inhibit fluid accumulation caused by *Escherichia coli* heat-stable enterotoxin in suckling mice. *Infect. Immun.* **32**:480-483.
47. Thomas, D. D., and F. C. Knoop. 1982. The effect of calcium and prostaglandin inhibitors on the intestinal fluid response to heat-stable enterotoxin of *Escherichia coli*. *J. Infect. Dis.* **145**:141-147.
48. Thomas, D. D., and F. C. Knoop. 1983. Effect of heat-stable enterotoxin of *Escherichia coli* on cultured mammalian cells. *J. Infect. Dis.* **147**:450-459.
49. Tobias, L. D., and J. G. Hamilton. 1978. The effects of 5,8,11,14-eicosatetraenoic acid on lipid metabolism. *Lipids* **14**:181-193.
50. Weiser, M. M. 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. *J. Biol. Chem.* **248**:2536-2541.
51. Weiss, B., and R. M. Levin. 1977. Mechanism of selectivity inhibiting the activation of cyclic nucleotide phosphodiesterase and adenylate cyclase by antipsychotic agents. *Adv. Cyclic Nucleotide Res.* **9**:285-303.
52. Weiss, G. B. 1974. Cellular pharmacology of lanthanum. *Annu. Rev. Pharmacol.* **14**:343-354.
53. Yamasaki, H. 1979. Phorbol ester-induced prostaglandin synthesis and [³H]-TPA metabolism by TPA-sensitive and TPA-resistant Friend erythroleukemic cells. *Biochem. Biophys. Res. Commun.* **89**:1018-1025.