

Mechanism of action of Escherichia coli heat stable enterotoxin in a human colonic cell line.

P A Huott, ... , R A Giannella, K Dharmasathaphorn

J Clin Invest. 1988;82(2):514-523. <https://doi.org/10.1172/JCI113626>.

Research Article

Escherichia coli heat stable enterotoxin (STa) caused Cl⁻ secretion across T84 cell monolayers in a dose-dependent manner only when applied to the apical membrane surface and not when applied to the basolateral surface. Measurement of cAMP, cGMP, and free cytosolic Ca²⁺ in response to STa suggested that cGMP alone mediated the Cl⁻ secretory response. Studies utilizing blockers of the Na⁺,K⁺-ATPase pump, a Na⁺,K⁺,Cl⁻ cotransport system, a K⁺ channel, and a Cl⁻ channel suggest that all of them participate in the Cl⁻ secretory process induced by STa. The results suggest that the Cl⁻ secretory response induced by STa is mediated by cGMP after the enterotoxin binds to its receptor on the apical membrane. The enterotoxin, by increasing cGMP, opens a K⁺ channel on the basolateral membrane as well as a Cl⁻ channel on the apical membrane. The activation of these ion exit mechanisms, together with activations of the Na⁺,K⁺,Cl⁻ cotransporter and the Na⁺,K⁺-ATPase pump drives Cl⁻ exit through the Cl⁻ channel on the apical membrane.

Find the latest version:

<https://jci.me/113626/pdf>



Mechanism of Action of *Escherichia coli* Heat Stable Enterotoxin in a Human Colonic Cell Line

Patrick A. Huott, Wilson Liu, James A. McRoberts, Ralph A. Giannella, and Kiertisin Dharmasathaphorn

Division of Gastroenterology, Department of Medicine, University of California at San Diego Medical Center, University of California, San Diego, California 92103; Division of Digestive Diseases, Department of Internal Medicine, University of Cincinnati Medical Center, Cincinnati, Ohio 45267; and Veterans Administration Hospital, Cincinnati, Ohio 45220

Abstract

Escherichia coli heat stable enterotoxin (ST_a) caused Cl^- secretion across T_{84} cell monolayers in a dose-dependent manner only when applied to the apical membrane surface and not when applied to the basolateral surface. Measurement of cAMP, cGMP, and free cytosolic Ca^{2+} in response to ST_a suggested that cGMP alone mediated the Cl^- secretory response. Studies utilizing blockers of the Na^+, K^+ -ATPase pump, a Na^+, K^+, Cl^- cotransport system, a K^+ channel, and a Cl^- channel suggest that all of them participate in the Cl^- secretory process induced by ST_a . The results suggest that the Cl^- secretory response induced by ST_a is mediated by cGMP after the enterotoxin binds to its receptor on the apical membrane. The enterotoxin, by increasing cGMP, opens a K^+ channel on the basolateral membrane as well as a Cl^- channel on the apical membrane. The activation of these ion exit mechanisms, together with activations of the Na^+, K^+, Cl^- cotransporter and the Na^+, K^+ -ATPase pump drives Cl^- exit through the Cl^- channel on the apical membrane.

Introduction

Escherichia coli heat stable enterotoxin (ST_a)¹ causes intestinal secretion of fluid and electrolytes and an increase in cellular cGMP levels (1, 2). Studies by other investigators suggest that cGMP is the secondary messenger mediating the secretory response induced by the enterotoxin (3–6). The mechanism of action beyond the step of cGMP formation, including the transport pathways involved in the secretory process, has not been fully elucidated. Evidence in the literature suggests that cGMP inhibits a “neutral” Na^+, Cl^- absorptive process, and stimulates an “electrogenic” Cl^- secretory mechanism (4–6). Our laboratory has utilized a cultured human colonic epithe-

lial cell line, T_{84} , as a model system to study colonic secretion (7–18). The cell line, derived from a lung metastasis of a human colonic carcinoma, resembles crypt cells morphologically (7, 8) and secretes Cl^- in response to a variety of secretagogues whose actions are mediated via cAMP- or Ca^{2+} -related mechanisms (7, 9–14, 17, 18). The T_{84} cell line is a secretory cell line without absorptive function and thus may allow better elucidation of the secretory mechanism. Previous studies indicate that Cl^- secretion across T_{84} cell monolayers results from a coordinated interaction of four transport pathways: (a) The Na^+, K^+ -ATPase pump which provides the driving force, (b) a Na^+, K^+, Cl^- cotransport system that serves as the Cl^- uptake pathway (9, 11, 13), (c) A K^+ channel that serves to recycle the K^+ (10, 11, 13, 15), and (d) A Cl^- channel that serves as the Cl^- exit step (11, 16). The first three pathways are located basolaterally while the Cl^- exit channel is located apically. Regulation by secretagogues is at the K^+ and/or Cl^- channels (10–13, 15, 16). These channels are regulated directly and independently of the Na^+, K^+, Cl^- cotransporter or the Na^+, K^+ -ATPase pump. cAMP (vasoactive intestinal polypeptide, VIP, or PGE_1) opens both the apically localized Cl^- channel as well as a basolaterally localized K^+ channel. On the other hand, Ca^{2+} (carbachol) opens another type of K^+ channel on the basolateral membrane and we could not detect opening the apical Cl^- channel with the technique we used. The interaction between these two different mediators (cAMP and Ca^{2+}) results in a synergistic action that promotes increased Cl^- secretion (12, 13). Because ST_a , which increases cGMP, may activate Cl^- secretion by a mechanism different from that activated by cAMP or Ca^{2+} , we decided to investigate the mechanism of action of ST_a and compare the results with cAMP- or Ca^{2+} -mediated secretory responses.

Methods

Growth and maintenance of T_{84} cells, preparation of the collagen-coated (Nuclepore, Pleasanton, CA) filters, transepithelial electrolyte transport studies, $^{86}Rb^+$ efflux from monolayers mounted in Ussing chambers and extraction and measurement of cAMP followed the procedures previously described (9–11, 15, 16). Extraction and measurement of cGMP used similar procedures as those described for cAMP except that cGMP standard and cGMP antisera from New England Nuclear (Boston, MA) were used instead of the cAMP standard and cAMP antisera. The antisera were specific for cAMP or cGMP and there was no cross-reactivity (see Results).

Short-circuit current and transepithelial chloride transport studies. Although the detailed methodology has been described repeatedly elsewhere, it appears appropriate to define the short-circuit current (I_{sc}) for readers. Our experiments were carried out across T_{84} monolayers mounted between two fluid-filled reservoirs (Ussing chambers) that allowed active transport mechanisms to occur unimpeded while eliminating passive forces that might confound the observation. I_{sc} is the electrical current needed to nullify the electrical potential differences

Address reprint requests to Dr. Dharmasathaphorn, Division of Gastroenterology, UCSD Medical Center (H-811-D), 225 West Dickinson Street, San Diego, CA 92103.

Presented in abstract form at the 86th Annual Meeting of the American Gastroenterological Association in San Francisco, May 1986.

Received for publication 10 July 1986 and in revised form 3 March 1988.

1. Abbreviations used in this paper: F_0 , fluorescence in absence of calcium; F_s , fluorescence in saturating calcium; I_{sc} , short circuit current; ST_a , *E. coli* heat stable enterotoxin; VIP, vasoactive intestinal polypeptide.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/88/08/0514/10 \$2.00

Volume 82, August 1988, 514–523

(P.D.) resulted from active transport of ions; the I_{sc} maintained the P.D. at zero. In our experimental system, P.D. across T_{84} cell monolayers have been shown to result from the transepithelial secretion of chloride ions since they are the only ions transported transcellularly by T_{84} cells. Cl^- ions carry negative charges with them. Hence, the amount of Cl^- secreted can be easily quantitated by recording the electrical current (I_{sc}) needed to nullify the electrical charges carried by Cl^- ions from one reservoir to another. For practical purposes readers can equate I_{sc} with Cl^- secretion in this series of studies. Results in Table I support our conclusion in this regard.

Radionuclide efflux and uptake studies. Bumetanide-sensitive and insensitive $^{86}Rb^+$ efflux, bumetanide-sensitive $^{86}Rb^+$ uptake and $^{36}Cl^-$ uptake were studied to determine the effect of ST_a on specific ion transport pathways. Radionuclide uptake or efflux was assayed using replicate confluent monolayer cultures of T_{84} cells attached to 35 mm culture dishes.

Bumetanide-sensitive and insensitive $^{86}Rb^+$ efflux was assayed in the presence of 0.5 mM ouabain as described previously (15) to determine whether the K^+ channels and/or the Na^+, K^+, Cl^- cotransporter were activated. Confluent monolayer cultures of T_{84} cells were loaded to steady state with $^{86}Rb^+$ by incubation for 4 h in buffer containing 138 mM NaCl, 10 mM Hepes-Tris, pH 7.4, 10 mM glucose, 2 mM RbCl, 1 mM $MgCl_2$, and 0.25 $\mu Ci/ml$ $^{86}Rb^+$. The monolayers were washed and incubated with buffer containing 140 mM choline chloride, 10 mM Hepes-Tris, pH 7.4, 1 mM $MgCl_2$, and 0.5 mM ouabain for 1 h. Effluxes were initiated by aspirating this buffer and adding buffer containing 140 mM NaCl, 10 mM Hepes-Tris (pH 7.4), 1 mM $MgCl_2$, and 0.5 mM ouabain with or without 0.1 mM bumetanide and with or without ST_a or VIP. Effluxes were stopped after 15 and 30 min by washing rapidly four times with ice-cold 100 mM $MgCl_2$ 10 mM Hepes-Tris, pH 7.4.

Bumetanide-sensitive $^{86}Rb^+$ uptakes were determined in the presence of ouabain as described previously (9) to assess whether the Na^+, K^+, Cl^- cotransport pathway is directly activated by ST_a . The cells were first incubated in KCl buffer for 1 h followed by incubation in isotonic sucrose buffer (241 mM sucrose, 10 mM Hepes-Tris, 1 mM $MgCl_2$) containing 0.5 mM ouabain for 1.0 h. This double preincubation procedure eliminated Na^+, K^+ ATPase activity as well as removing all extracellular ions while maintaining relatively high cellular K^+ concentrations (9, 16). $^{86}Rb^+$ uptakes were determined after prestimulating the cells with ST_a in sucrose buffer by aspirating the preincubation buffer and replacing it with uptake buffer (135 mM NaCl, 10 mM Hepes-Tris, pH 7.4, 5 mM KCl, 1 mM $MgCl_2$) containing 2 $\mu Ci/ml$ $^{86}Rb^+$ with or without 0.1 mM bumetanide. The initial rate of $^{86}Rb^+$ uptake under these conditions is largely (> 95%) bumetanide sensitive and Na^+ and Cl^- dependent (9). Both $^{86}Rb^+$ and $^{36}Cl^-$ uptakes were terminated using the $MgSO_4$ /sucrose wash procedure described previously (9, 11, 15, 16).

The initial rate of $^{36}Cl^-$ uptake mediated by the Cl^- channel was estimated using a modification of the method originally reported to test whether the Cl^- channel is opened directly by ST_a (16). An example of the method is shown in Fig. 1. Replicate, confluent monolayers of T_{84} cells were preincubated in KCl buffer (140 mM KCl, 10 mM Hepes-Tris, pH 7.4, 1 mM $MgCl_2$). After 1 h intracellular K^+ and Cl^- concentrations approached 140 and 100 mM, respectively (16). The cells were stimulated with either ST_a or VIP by preincubating for the stated times in KCl buffers containing given concentrations of these effectors. $^{36}Cl^-$ uptake was assayed by aspirating this preincubation buffer and replacing it with K gluconate buffer (140 mM K gluconate, 10 mM Hepes-Tris, pH 7.5, 1 mM Mg gluconate) containing 1 $\mu Ci/ml$ $^{36}Cl^-$ (1.2 mM final $^{36}Cl^-$). Under these conditions a transient Cl^- diffusion potential is created that favors uptake of $^{36}Cl^-$ through an electrogenic (i.e., channel) mediated mechanism (19). When the cells have been stimulated with effectors that open the apically localized Cl^- channels, the time course of $^{36}Cl^-$ uptake is biphasic reaching a peak after 90 s and declining to control values within 6 min. Uptakes are linear during the first 30 s. Triplicate values determined after 5, 15, and 30 s. in the absence of effectors were used to extrapolate zero time

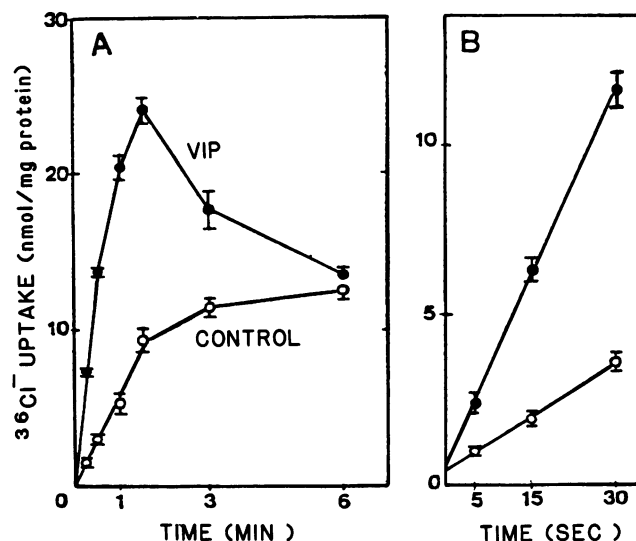


Figure 1. Stimulation by VIP of dilution potential-induced $^{36}Cl^-$ uptake. Replicate confluent monolayer cultures of T_{84} cells on 35 mm culture plates were incubated for 1.5 to 2 h in KCl buffer (140 mM KCl, 10 mM Hepes-Tris, pH 7.5, 1 mM $MgCl_2$). The monolayers were then incubated an additional 15 min in this KCl buffer in the presence (●) or absence (○) of 10^{-7} M VIP. $^{36}Cl^-$ uptakes were initiated by aspirating the preincubation buffer and replacing it with K gluconate buffer (140 mM K gluconate, 10 mM Hepes-Tris, pH 7.5, 1 mM Mg gluconate) containing 1 $\mu Ci/ml$ $^{36}Cl^-$ (1.64 mM final $^{36}Cl^-$ in this experiment). Uptakes were terminated at the given times by washing rapidly (within 5 s) four times with 2 ml ice-cold $MgSO_4$ -sucrose buffer (137 mM sucrose, 100 mM $MgSO_4$, 10 mM Hepes-Tris, pH 7.5). (A) Points are the average of duplicate determinations and bars the range. (B) Points are the mean of quadruplicate determinations and bars are the standard deviation from the mean.

background levels by linear regression (< 20% of the control 30-s time values). Uptakes determined after prestimulation by effectors were terminated after 15 and 30 s in duplicate. After subtraction of zero time values, and correction for the time increment, these values were averaged. This method insured that initial rates of uptake were measured. Linear regression of the data always gave values of $r > 0.92$.

Measurement of free cytosolic Ca^{2+} . The method for free cytosolic calcium ($[Ca^{2+}]_i$) measurement follows that described by Tsein et al. (20, 21) with some modifications. T_{84} monolayers were plated and grown as described previously. Monolayers were rinsed free of culture media and then incubated for 15 min at room temperature in calcium-free Ringer's solution, buffered with 30 mM Hepes containing 1.0 μM Fura-2/AM. 5 vol of identical Ringer's buffer containing calcium and Fura-2/AM (1.2 mM and 1.0 μM , respectively) were then added, and the incubation continued for an additional 75 min at 37°C. After the loading incubation, the entire ring assembly was incubated for 15 min at 37°C in the buffer used for fluorescence measurements. This buffer contained: NaCl 137.0 mM, KCl 5.4 mM, $CaCl_2$ 1.0 mM, KH_2PO_4 0.4 mM, $MgCl_2$ 0.5 mM, $MgSO_4$ 0.4 mM, $NaHCO_3$ 4.2 mM, Na_2HPO_4 0.3 mM, Hepes 10.0 mM, and glucose 0.6 mM, with the pH adjusted to 7.4 with NaOH. The final incubation step served to remove any dye remaining outside the cell or in contact with the cell membrane.

Fluorescence measurements were carried out in a fluorescence spectrophotometer (model 650-10S; Perkin-Elmer, Norwalk, CT). Excitation monochromator settings were 340 and 380 nm with a slit width of 5 nm. Emitted light was collected through a 495-515-nm interference filter with an emission slit of 10 nm. Monolayers were mounted for measurement as follows: the Nuclepore filter holding the monolayer was peeled off the Lexan ring and attached to a polystyrene

support, which was then placed in a standard 10-mm cuvette. The polystyrene support, with the attached monolayer, was exposed to the same buffer in the cuvette on both the apical and basolateral sides. Orientation of the monolayer within the cuvette itself was 45° to the excitation beam and angled 10–20° from the vertical plane to minimize the interference from reflected excitation light. Excitation monochromator settings were changed manually every 10 s with the wavelength drive control.

Free cytosolic calcium levels were calculated from the equation $[Ca^{2+}]_i = K_d(F_0/F_s)[(R - R_0)/(R_s - R)]$, where K_d is 224 nm, F_0 is the fluorescence at 380 nm in the absence of calcium, F_s is the fluorescence at 380 nm in saturating calcium, and the experimental ratio, R , is obtained by dividing the fluorescence at 340 nm by that at 380 nm after subtracting the respective autofluorescence levels. R_0 is the ratio of the signal at 340 nm to the signal at 380 nm in the absence of calcium and R_s is the ratio at saturating calcium. Autofluorescence for individual monolayers was measured at the end of each experiment after the addition of 1 mM MnCl₂ and ionomycin (10 μg/ml) to the buffer to allow the Mn²⁺ to enter the cells and quench the dye signal.

Materials. All radionuclides, cyclic AMP, and cyclic GMP antisera were obtained from New England Nuclear, Boston, MA. ST_a was prepared and purified by one of the authors, Dr. Ralph Giannella, according to the procedure previously described (22–24). Bumetanide was a gift from Dr. P. W. Feit of Leo Pharmaceutical Products, Ballerup, Denmark. Barium chloride dehydrate was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Ouabain was from Fluka Chemical Corp., Hauppauge, NY. 5-Nitro-2-(2-phenylpropylamino)-benzoic acid was a gift from Dr. R. F. Greger of Physiologisches Institut, Universität Freiburg, Freiburg, FRG. Carbachol was from ICN Biochemicals, Cleveland, OH. VIP was a gift from Dr. Jean Rivier of the Salk Institute, La Jolla, CA. Fura-2/AM and Fura-2 pentapotassium salt were purchased from Molecular Probes, Inc., Eugene, OR. Ionomycin was purchased from Calbiochem Biochemicals, La Jolla, CA.

Statistical analysis. Student's *t* tests were used as indicated (25).

Results

Stimulation of net Cl⁻ secretion across T₈₄ monolayers by ST_a. The addition of 2.5 × 10⁻⁷ M ST_a to the mucosal bathing solution caused an immediate increase in the *I*_{sc}, which reached a peak 30 to 35 min after addition and remained near maximal throughout the study period. Serosal addition of ST_a had no effect (Fig. 2). The *I*_{sc} response to ST_a was dose dependent with the threshold stimulation occurring at 10⁻⁸ M, and the maximal response at 5 × 10⁻⁷ M (Fig. 3). Half maximal stimulation occurred at ~ 4 × 10⁻⁸ M. Since our supply of ST_a was limited, a concentration of 2.5 × 10⁻⁷ M, which gave a near maximal response, was selected for subsequent studies unless otherwise indicated.

Unidirectional Na⁺ and Cl⁻ flux results are summarized in Table I. The time course of these data correspond to the time course of *I*_{sc} illustrated in Fig. 2. In the basal state, net flux of Na⁺ and Cl⁻ and *I*_{sc} remains at or near zero for more than 100 min (9–14, and Table I, group I). Following the addition of 2.5 × 10⁻⁷ M ST_a, an increase in net chloride secretion was observed and totally accounted for changes in *I*_{sc} (Table I, group II). No change in the unidirectional fluxes of Na⁺ was observed after the addition of ST_a. In contrast, both the serosal to mucosal and mucosal to serosal Cl⁻ fluxes increased, with the former being consistently greater, thus resulting in net Cl⁻ secretion (Table I, group II).

Because of the increase in monolayers' conductance after the addition of ST_a, questions arise as to whether ST_a increases paracellular permeability. Mannitol fluxes were, therefore,

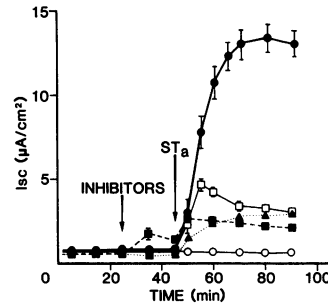


Figure 2. Time course of *I*_{sc} response to *E. coli* heat stable enterotoxin (ST_a) by T₈₄ monolayers mounted in the Ussing chamber. As shown in Table I, *I*_{sc} reflects net Cl⁻ secretion across T₈₄ monolayers. ST_a was added to the mucosal reservoir except for the serosal experiment (○) at 45 min after mounting. Bumetanide or barium chloride, if added, were to

the serosal reservoir, while 5-nitro-2-(2-phenylpropylamino)-benzoic acid was added to the mucosal reservoir. All inhibitor additions were made at 25 min after mounting. The results shown are from the same monolayers in Table I, except for 5-nitro-2-(2-phenylpropylamino)-benzoic acid experiment for which *n* = 4 and for the serosal addition of ST_a for which *n* = 3. All three monolayers in which ST_a was added serosally responded subsequently to mucosal addition with similar results as the group shown here for ST_a alone. The concentration of ST_a in all groups was 0.25 μM. Addition of 0.1 mM bumetanide (□) or 3 mM barium (▲) or 0.1 mM 5-nitro-2-(2-phenylpropylamino)-benzoic acid (■) significantly reduced ST_a-induced *I*_{sc} response as compared to that of ST_a alone (●). At 3 mM, precipitation of barium was visible. Precipitation of barium occurs at concentrations of 3 and 10 mM. Therefore, the concentrations were overestimated at these concentrations. 5-Nitro-2-(2-phenylpropylamino)-benzoic acid also inhibited the ST_a-induced increase in cGMP. Thus it is unclear whether its ability to inhibit the *I*_{sc} induced by ST_a was attributable to its effect on the Cl⁻ channel or its effect on cGMP.

carried out as reported previously (8) over the same time frame as for Na⁺ and Cl⁻ fluxes. There was no significant changes of mannitol flux after the addition of ST_a: $J_{mannitol}^{m \rightarrow s}$ and $J_{mannitol}^{s \rightarrow m}$ were 0.002 ± 0.001, 0.003 ± 0.001 μmol/h · cm², respectively, in period I–II (prior to the addition of 2.5 × 10⁻⁷ M ST_a) and 0.003 ± 0.002, 0.003 ± 0.003 μmol/h · cm² in period III (after the addition of ST_a, *n* = 3 pairs).

Another question that arose was whether the increase in monolayer's conductance was reversible. When ST_a antibodies were used to reserve ST_a's action in another study (26), monolayers conductance was also reversed to control level in 30 min (ST_a antibodies reversed 97 ± 3% of ST_a-induced increase in *I*_{sc} and 93 ± 7% of ST_a-induced increase on conductance in that study, *n* = 3). In another experiment the bathing media were replaced with ST_a-free media after ST_a had induced an increase in *I*_{sc} and conductance. 30 min after washing 88 ± 7% of ST_a-induced increase in *I*_{sc} and 62 ± 19% of ST_a-induced increase in

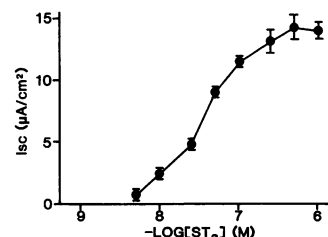


Figure 3. Graded dose effect of ST_a on the changes in *I*_{sc}. As shown in Table I, *I*_{sc} reflects net Cl⁻ secretion across T₈₄ monolayers. T₈₄ monolayers were mounted in the Ussing chamber and ST_a was added to the mucosal reservoir at the concentration indicated. Only one addition was made to each

monolayer. The results are the mean ± SE of the peak change in the *I*_{sc} within 35 min after addition of ST_a and were obtained from four experiments at each concentration.

Table I. Unidirectional Na⁺ and Cl⁻ Flux in Response to *E. coli* Heat Stable Enterotoxin (ST_a)

Group	Experimental condition	Flux period	$J_{m \rightarrow s}^{Na}$			$J_{s \rightarrow m}^{Cl}$			I_{sc}	G
			$\mu eq \cdot h^{-1} \cdot cm^{-2}$			$\mu eq \cdot h^{-1} \cdot cm^{-2}$				
I (n = 4)	No addition	I	0.32±0.03	0.27±0.04	0.05±0.03	0.40±0.03	0.43±0.03	-0.03±0.03	0.02±0.00	0.46±0.07
	No addition	II	0.29±0.05	0.27±0.04	0.02±0.02	0.33±0.02	0.36±0.05	-0.03±0.05	0.02±0.00	0.44±0.07
	No addition	III	0.27±0.04	0.31±0.04	-0.04±0.04	0.31±0.02	0.41±0.08	-0.10±0.03	0.02±0.00	0.48±0.06
II (n = 5)	No addition	I	0.28±0.07	0.30±0.05	-0.02±0.06	0.48±0.07	0.44±0.07	0.04±0.11	0.03±0.01	0.54±0.06
	No addition	II	0.40±0.05	0.30±0.04	0.10±0.03	0.46±0.05	0.43±0.05	0.03±0.06	0.04±0.01	0.56±0.08
	ST _a	III	0.30±0.05	0.39±0.05	-0.09±0.06	0.84±0.05 [†]	1.38±0.10 [†]	-0.53±0.07 [†]	0.47±0.04 [†]	0.98±0.15 [†]
III (n = 4)	No addition	I	0.26±0.04	0.20±0.05	0.06±0.04	0.32±0.04	0.34±0.04	-0.02±0.05	0.02±0.00	0.47±0.07
	Bumetanide	II	0.30±0.03	0.38±0.04	-0.08±0.04	0.36±0.05	0.45±0.03	-0.09±0.06	0.03±0.01	0.55±0.05
	Bumetanide plus ST _a	III	0.31±0.05	0.36±0.01	-0.05±0.06	0.41±0.07*	0.59±0.04*	-0.18±0.03*	0.12±0.01 [†] *	0.80±0.05 [†]
IV (n = 5)	No addition	I	0.29±0.06	0.29±0.06	0.00±0.04	0.40±0.03	0.34±0.07	0.06±0.06	0.03±0.00	0.48±0.08
	BaCl ₂	II	0.38±0.05	0.29±0.07	0.08±0.03	0.45±0.05	0.39±0.06	0.06±0.02	0.02±0.00	0.49±0.07
	BaCl ₂ plus ST _a	III	0.28±0.05	0.22±0.06	0.06±0.05	0.79±0.06 [†]	0.85±0.05 [†] *	-0.06±0.05*	0.10±0.01 [†] *	0.69±0.07

Results are expressed as mean±SE. The number of paired monolayers for each experimental group, (n), is indicated in parentheses. Period I is the average of two consecutive 5-min flux periods starting 15 min after mounting and addition of isotope to the Ussing chambers, and ending just before addition of inhibitors (15–25 min). Period II is the average of two consecutive 5-min flux periods starting 10 min after the addition, if made, of bumetanide or BaCl₂ (35–45 min). Period III is the average of two consecutive flux periods, one 5-min period and one 10-min period, starting 25 min after addition of ST_a (70–85 min). The concentration of ST_a was 0.25 μM; bumetanide, 0.1 mM; and BaCl₂, 3 mM. [†] P < 0.05 with Student's unpaired t test as compared to the same period in group I (control). * P < 0.05 by Student's unpaired t test as compared to the same period in group II (ST_a alone).

conductance were reversed (n = 7). Repeat washing tended to increase monolayer's conductance and might cause a lesser degree of reversal in conductance.

Inhibition of ST_a-stimulated Cl⁻ secretion by specific inhibitors of membrane transport. We have previously shown that a loop diuretic, bumetanide, inhibits the basolaterally localized Na⁺,K⁺,Cl⁻ cotransport system in the T₈₄ cell line (9). This transport pathway serves as the Cl⁻ uptake pathway and its inhibition by bumetanide results in a reversal or inhibition of Cl⁻ secretion mediated by cAMP or Ca²⁺ (9, 11, 13). Therefore, bumetanide was used to test the involvement of this cotransport pathway in the Cl⁻ secretory process activated by ST_a.

Serosal addition of 10⁻⁴ M bumetanide inhibited the action of ST_a while mucosal addition has little or no effect. As was the case for VIP- and PGE₁-induced Cl⁻ secretion, bumetanide incompletely inhibited the action of ST_a. At 10⁻⁴ M bumetanide, added 20 min before addition of ST_a, ~ 20% of the peak I_{sc} induced by ST_a persisted (Fig. 2). In the presence of bumetanide, the time course of the response to ST_a showed that the late effect of ST_a, rather than the initial effect, was inhibited. Thus the time course of the response was shorter in duration, reaching a peak within 10 min. Unidirectional Na⁺ and Cl⁻ fluxes in the presence of bumetanide are summarized in Table I, group III. 10⁻⁴ M bumetanide significantly reduced the ST_a-induced increases in serosal to mucosal as well as mucosal to serosal Cl⁻ flux resulting in reduced net Cl⁻ secretion with a corresponding decrease in I_{sc}. Bumetanide, by itself, had no effect on the basal Na⁺ or Cl⁻ fluxes (group III, period II). Bumetanide also reversed the action of ST_a when added after ST_a had elicited a response (data not shown).

Barium, a K⁺ channel blocker, has been found to inhibit Cl⁻ secretion in a number of epithelia (10, 27, 28). In T₈₄ cells, barium inhibits a K⁺ recycling mechanism on the basolateral membrane. A K⁺ exit mechanism is intimately involved in the Cl⁻ secretory process mediated by cAMP or Ca²⁺ and appears to serve as a site regulated by a number of secretagogues (10,

11, 13, 15). Therefore, barium was used to test whether Cl⁻ secretion induced by ST_a required the same K⁺ recycling mechanism. We found that BaCl₂ added serosally inhibited the increase in I_{sc} induced by ST_a, while mucosal addition had little or no effect. At a concentration of 3 mM, 80% of the effect of ST_a was inhibited (Fig. 2). The results of unidirectional and net Na⁺ and Cl⁻ fluxes, summarized in Table I, group IV, confirm that barium inhibited net Cl⁻ secretion. Similar to bumetanide, barium by itself had no effect on basal I_{sc} or on unidirectional Na⁺ and Cl⁻ fluxes (see group IV, period II). In contrast to bumetanide, barium had little effect on the increase in mucosal to serosal Cl⁻ movements induced by ST_a. Unidirectional Cl⁻ fluxes in both directions were increased by ST_a regardless of the presence of barium, while net Cl⁻ flux was reduced to near zero. The inhibitory effect of barium on the action of ST_a was reversible. Barium also reversed the action of ST_a when added after ST_a had elicited a response (data not shown). Other putative K⁺ channel blockers including apamin (10⁻⁶ M), tetraethylammonium chloride (10⁻² M), and 4-aminopyridine (10⁻² M) did not exhibit an inhibitory effect. Quinidine (3 × 10⁻⁴ M) caused a rise in I_{sc} before exerting an inhibitory action. The results are similar to those observed with secretagogues such as VIP or PGE₁, which increase cellular cAMP levels.

Ouabain, a Na⁺,K⁺-ATPase inhibitor, also inhibited and reversed ST_a-induced I_{sc} when added to the serosal reservoir at a concentration of 10⁻⁴ M. When ouabain was added after ST_a had elicited a response, ~ 80% inhibition was observed 20–25 min after the addition of ouabain and 85 to 90% inhibition at 45 min. Similar results were obtained with ouabain pretreatment (data not shown).

5-Nitro-2-(2-phenylpropylamino)-benzoic acid, an anthracene derivative, is a recently developed potent blocker of the Cl⁻ channel. This Cl⁻ channel blocker, which was tested initially in the thick ascending limb of the loop of Henle (29), is much less effective in the T₈₄ cells requiring 10⁻⁴ M to cause significant inhibition of Cl⁻ secretion. 5-Nitro-2-(2-phenyl-

propylamino)-benzoic acid did inhibit ST_a-induced I_{sc} when added to the mucosal reservoir at a relatively high concentration of 10⁻⁴ M (Fig. 2). This putative Cl⁻ channel blocker also reversed the I_{sc} induced by ST_a when added after ST_a had elicited a response (data not shown). Unfortunately, at the concentration of the agent used in this study, 10⁻⁴ M, the compound partially inhibited the rise in cellular cAMP induced by VIP and the rise in cellular cGMP induced by ST_a (unpublished observations) and caused the results to be inconclusive. Any conclusion related to 5-nitro-2-(2-phenylamino)-benzoic acid's effect in blocking Cl⁻ secretion must remain inconclusive as long as it is unknown as to what extent the inhibition is due to an effect on the Cl⁻ channels or due to an effect on cGMP.

Evidence for ST_a-induced K⁺ efflux on the basolateral membrane, and its similarity to a cAMP-induced effect. To verify the existence of a K⁺ efflux pathway and to test if barium inhibited this process, monolayers were preloaded with ⁸⁶Rb⁺ (as a tracer for K⁺) and mounted in the Ussing chamber. This method allows the measurement of ⁸⁶Rb⁺ efflux across both the apical and basolateral surfaces while Cl⁻ secretion, as reflected by the I_{sc}, is simultaneously monitored (10, 11, 13). The apparent first order rate constants, along with the mean I_{sc} and conductance of the monolayers for each time interval in which additions were made, are shown in Table II. In the basal state, the rate of ⁸⁶Rb⁺ efflux into the mucosal bath was ~ 10 to 20-fold less than that into the serosal bath. The addition of ST_a increased the rate of ⁸⁶Rb⁺ efflux into the serosal bath by approximately two-fold, while the amount of ⁸⁶Rb⁺ efflux into the mucosal bath remained small. The increase in basolateral membrane ⁸⁶Rb⁺ efflux rate was totally inhibited by the prior addition of 3 mM barium to the serosal bathing medium. 3 mM barium had no effect on ⁸⁶Rb⁺ efflux in the

basal state. Net Cl⁻ secretion induced by ST_a, as indicated by the I_{sc}, was also inhibited by barium. The graded dose effect for the inhibition of ST_a-induced I_{sc} by barium is shown in Fig. 4. This effect is identical to that seen with barium inhibition of VIP- or PGE₁-induced I_{sc} (10, 11), but differs from the inhibitory action of barium on A23187- or carbachol-induced I_{sc}. The I_{sc} response of A23187 is more resistant to barium inhibition, while the I_{sc} response of carbachol is totally unaffected to barium (10, 13). It should be noted that at concentrations of 3 mM or above, barium precipitated in the solution and therefore the effective concentration was lower than indicated.

We then quantitated the magnitude of ⁸⁶Rb⁺ efflux induced by a combination of ST_a and carbachol as well as by ST_a and VIP (Table III). An additive effect between ST_a and either VIP or carbachol would confirm that the compounds act via different pathways while the lack of an additive effect would suggest that they act via the same pathway. ⁸⁶Rb⁺ efflux induced by the combination of ST_a and carbachol was additive, while ⁸⁶Rb⁺ efflux induced by the combination of ST_a and VIP was not different from ⁸⁶Rb⁺ efflux induced by ST_a or VIP alone (Table III). These findings suggest the presence of two different types of K⁺ efflux pathways, one activated by ST_a or VIP, and the other activated by carbachol. The former is sensitive to barium as described above and previously (10), while the latter is less so (13). In addition, the increase in I_{sc} induced by the combination of ST_a and carbachol was greater than the predicted additive response (potentiated), while the increase in I_{sc} induced by ST_a and VIP was less than additive and approximated that induced by VIP alone (Table III). At a concentration of VIP (10⁻⁸ M) that gave a maximal response, 10⁻⁶ M ST_a caused no further increase in I_{sc}. Likewise, 10⁻⁸ M VIP added to 10⁻⁶ M ST_a-pretreated monolayers brought the peak I_{sc} from 15.0±1.0 μA/cm² to 26.5±2.5 μA/cm², an I_{sc} level

Table II. ⁸⁶Rb⁺ Efflux in Response to *E. coli* Heat Stable Enterotoxin: Inhibition by Ba²⁺

Group	Experimental condition	Period	⁸⁶ Rb ⁺ Efflux rate constant			
			Serosal	Mucosal	I _{sc}	G
			<i>h</i>	<i>h</i>	μeq·h ⁻¹ ·cm ⁻²	mS·cm ⁻²
I (n = 4)	No addition	1	0.57±0.01	0.08±0.03	0.02±0.01	1.0±0.2
	No addition	2	0.59±0.04	0.10±0.05	0.02±0.01	0.8±0.1
	No addition	3	0.54±0.10	0.03±0.01	0.02±0.01	0.7±0.1
II (n = 10)	No addition	1	0.58±0.06	0.03±0.01	0.03±0.01	1.0±0.1
	No addition	2	0.67±0.06	0.04±0.01	0.03±0.01	0.8±0.1
	ST _a	3	1.22±0.26* ‡	0.10±0.02* ‡	0.47±0.01* ‡	0.9±0.1 [§]
III (n = 4)	No addition	1	0.57±0.10	0.07±0.01	0.04±0.01	1.1±0.1
	BaCl ₂	2	0.54±0.10	0.04±0.01*	0.07±0.04	0.9±0.1
	ST _a and BaCl ₂	3	0.44±0.12	0.07±0.02	0.11±0.02 [‡]	1.0±0.1 [§]

Rubidium effluxes across the basolateral (serosal) and apical (mucosal) membranes were measured in the Ussing chamber using monolayers preloaded with ⁸⁶Rb⁺. Samples were obtained from the bathing media every 5 or 10 min with appropriate replacement. Results are expressed as mean±SE of the number of experiments, (n), in each group. ST_a, if added, was at 45 min after mounting and BaCl₂ was at 25 min. The concentration of ST_a and BaCl₂ were 0.25 μM and 3 mM, respectively. The efflux period 1 represents the average of two consecutive 5-min intervals before the addition of BaCl₂, if added (15–25 min after mounting). Period 2 represents the average of two consecutive 5-min intervals starting 10 min after the addition of BaCl₂, if added (35–45 min after mounting). Period 3 represents the average of two consecutive periods, one 5-min and one 10-min period, 25 min after the addition of ST_a (70–85 min after mounting). Corresponding short circuit (I_{sc}) which reflects net Cl⁻ secretion and conductance (G) are shown. The monolayers were calculated to contain about 742 nM of K⁺ using ⁸⁶Rb⁺ as a tracer at the beginning of the experiment. * P < 0.05 by Student's paired t test as compared to period I of the same group. ‡ P < 0.05 by Student's unpaired t test as compared to the same period in group I. § P < 0.05 for ΔG between period 3 vs. period 2 as compared to ΔG in group I.

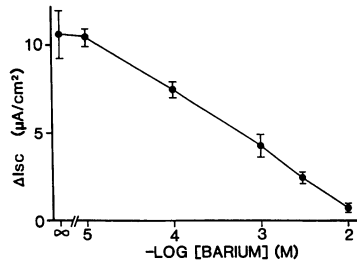


Figure 4. Graded dose effect of barium for inhibition of ST_a -stimulated I_{sc} . I_{sc} reflects net Cl^- secretion across T_{84} monolayers. Varying concentrations of barium were added serosally 15 min after mounting. $0.25 \mu M$ ST_a was added to the mucosal reservoir at 35 min after mounting

following the barium pretreatment. The results are expressed as the peak change in the I_{sc} observed within 35 min after ST_a addition and represent the mean \pm SE of four experiments in each group. Only one concentration of barium was tested in each monolayer. Precipitation of barium was observed at concentrations of 3 and 10 mM. Therefore, the concentration of barium chloride in the solution is overestimated at these concentrations.

similar to that induced by 10^{-8} M VIP alone ($28.0 \pm 1.0 \mu A/cm^2$). The above findings regarding the action of ST_a and carbachol were similar to those reported previously for PGE_1 and carbachol (11) or VIP and A23187 (12).

A complimentary study was carried out with $^{86}Rb^+$ -preloaded monolayers grown on culture dishes as described previously (15). In these studies, $^{86}Rb^+$ efflux was quantitated in the presence of ouabain with or without bumetanide. The study allowed us to determine if the increase in $^{86}Rb^+$ efflux is

dependent on the Na^+, K^+, Cl^- cotransport mechanism and the Na^+, K^+ -ATPase pump. The findings are summarized in Fig. 5. Similar increases in $^{86}Rb^+$ efflux were observed with 2.5×10^{-7} M ST_a in the presence of ouabain alone or in the presence of ouabain and bumetanide. The other words, the bumetanide sensitive effluxes, which represent effluxes of $^{86}Rb^+$ via the Na^+, K^+, Cl^- cotransport carrier, were not affected by ST_a . Therefore, the increase in $^{86}Rb^+$ efflux induced by ST_a must have occurred via another transport pathway, most likely that K^+ channel. Taken together, our results suggest that ST_a activates a K^+ transport pathway which is sensitive to cAMP, but not the one which is sensitive to Ca^{2+} . The results also suggest that the opening of the K^+ transport pathway is an effect of ST_a independent of the Na^+, K^+, Cl^- cotransport pathway and the Na^+, K^+ -ATPase pump.

Uptake studies. Uptake studies were carried out for two purposes. First, $^{36}Cl^-$ uptakes were performed to test whether the Cl^- channel is opened by the ST_a and if so, how the magnitude of this effect compared with that produced by VIP. Second, $^{86}Rb^+$ uptakes were assayed, in the presence of ouabain with or without bumetanide, to reassess whether the Na^+, K^+, Cl^- cotransporter pathway is directly activated by ST_a .

For the first purpose, initial rates of $^{36}Cl^-$ uptakes were determined under conditions that favor the uptake of $^{36}Cl^-$ through Cl^- channel mediated processes. Dose-dependent activation of the apical-localized Cl^- channel by VIP can readily be detected using this method. Similarly, application of ST_a

Table III. $^{86}Rb^+$ Efflux in Response to *E. coli* Heat Stable Enterotoxin: Additive Effect of Carbachol

Group	Experimental condition	Period	$^{86}Rb^+$ Efflux rate constant		I_{sc}	G
			Serosal	Mucosal		
			<i>h</i>		$\mu eq \cdot h^{-1} \cdot cm^{-2}$	$mS \cdot cm^{-2}$
I	No addition ST_a	1	0.65 ± 0.10	0.03 ± 0.01	0.04 ± 0.02	0.66 ± 0.06
		2	$1.21 \pm 0.29^*$	0.09 ± 0.03	$0.37 \pm 0.02^*$	0.75 ± 0.06
		Δ	0.66 ± 0.19	0.06 ± 0.02	0.33 ± 0.02	0.09 ± 0.03
II	No addition Carbachol	1	0.55 ± 0.09	0.03 ± 0.01	0.03 ± 0.01	0.93 ± 0.31
		2	$0.95 \pm 0.10^*$	0.06 ± 0.02	$0.13 \pm 0.02^*$	0.83 ± 0.26
		Δ	0.40 ± 0.03	0.03 ± 0.02	0.10 ± 0.01	-0.10 ± 0.05
III	No addition Carbachol and ST_a	1	0.66 ± 0.07	0.05 ± 0.01	0.05 ± 0.02	0.78 ± 0.21
		2	$1.66 \pm 0.26^*$	0.09 ± 0.01	$1.00 \pm 0.08^*$	$1.08 \pm 0.66^*$
		Δ	1.00 ± 0.20	0.04 ± 0.02	0.95 ± 0.07	0.30 ± 0.07
IV	No addition VIP	1	0.68 ± 0.11	0.05 ± 0.02	0.04 ± 0.02	0.66 ± 0.07
		2	$1.09 \pm 0.13^*$	$0.11 \pm 0.02^*$	$0.62 \pm 0.06^*$	$0.86 \pm 0.06^*$
		Δ	0.41 ± 0.13	0.06 ± 0.02	0.58 ± 0.05	0.20 ± 0.11
V	No addition VIP and ST_a	1	0.67 ± 0.10	0.05 ± 0.01	0.03 ± 0.01	0.69 ± 0.06
		2	$1.29 \pm 0.25^*$	0.11 ± 0.03	$0.75 \pm 0.03^*$	$0.97 \pm 0.06^*$
		Δ	0.62 ± 0.21	0.06 ± 0.02	0.72 ± 0.03	0.28 ± 0.06

Rubidium effluxes across the basolateral (serosal) and apical (mucosal) membranes were measured in the Ussing chamber using monolayers preloaded with $^{86}Rb^+$. Samples were obtained every 5 or 10 min from the bathing media with appropriate replacement. Results are expressed as mean \pm SE of 4 experiments in each group. ST_a , carbachol, and VIP, if added, were at 45 min after mounting. The concentrations of ST_a , carbachol, and VIP were $0.25 \mu M$, 0.1 mM and $0.1 \mu M$, respectively. Previous studies indicate that maximal I_{sc} responses were induced by the compounds at these concentrations except for ST_a which gave a near maximal response (Fig. 2, references 9, 12, 13). The efflux period 1 represents the average of two consecutive 5-min intervals before the addition of any drug (35–45 min after mounting). For comparison, period 2 in all groups represents the average of three consecutive 5-min intervals followed by two 10-min intervals starting 5 min after the addition of drug(s) (50–85 min after mounting). Corresponding short circuit (I_{sc}) and conductance (G) are shown. The monolayers were calculated to contain about 750 nM of $^{86}Rb^+$ at the beginning of the experiment. * $P < 0.05$ by Student's paired *t* test as compared to period 1 of the same group.

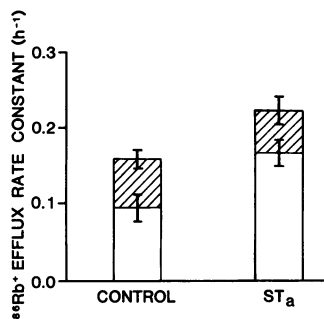


Figure 5. Stimulation of bumetanide-insensitive $^{86}\text{Rb}^+$ efflux by ST_a . Efflux of $^{86}\text{Rb}^+$ from preloaded, ouabain-treated T_{84} cell monolayers attached to 35-mm culture dishes was determined in the presence or absence of 0.25 μM ST_a and/or 0.1 mM bumetanide as described in Methods. Effluxes were stopped after 15- and 30-min intervals. Under these conditions,

$^{86}\text{Rb}^+$ efflux appears to follow first order kinetics. The apparent first order rate constants observed in the presence or absence of ST_a and in the presence (clear portion) or absence (striped plus clear portion) of bumetanide. The results represent the mean \pm SD of three determinations. Under these conditions, 0.25 μM ST_a had no effect on bumetanide sensitive ^{86}Rb efflux, but increased the rate of bumetanide-insensitive ^{86}Rb efflux almost twofold. This increase was similar to, but slightly less than that observed with 10 nM VIP under identical conditions (data not shown).

activated the Cl^- channel in a dose-dependent manner. The dose-response curve was nearly identical to that observed for ST_a 's effect on I_{sc} (data not shown). However, even at saturating doses (1 μM), ST_a required at least 30 min to achieve 90% maximal stimulation of the Cl^- channel (Fig. 6). This contrasts with the action of VIP which gives $\sim 90\%$ maximal stimulation within 5 min. Thus, the action of ST_a is slower than that observed with VIP, agreeing with the rather sluggish response to ST_a in the Ussing chamber. Furthermore, even at supra-maximal doses of ST_a (1 μM) and optimal stimulation times (30–60 min), the extent of Cl^- channel activation by ST_a was only $73 \pm 4\%$ that observed with saturating doses of VIP. These data are similar to the peak I_{sc} values produced by ST_a and VIP in the Ussing chamber.

To address the second purpose, $^{86}\text{Rb}^+$ uptakes were carried out in the presence or absence of prior stimulation by 1 μM ST_a under conditions designed to optimize $^{86}\text{Rb}^+$ uptake through the $\text{Na}^+, \text{K}^+, \text{Cl}^-$ cotransporter and minimize extrane-

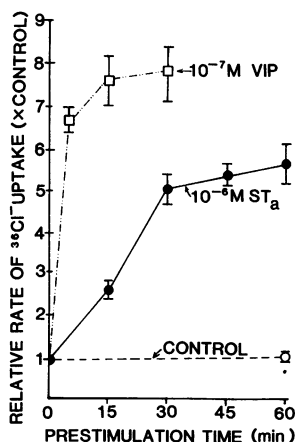


Figure 6. Time course of ST_a activation of Cl^- channel-mediated $^{36}\text{Cl}^-$ uptake. Confluent monolayer cultures of T_{84} cells attached to 35 mm culture dishes were assayed for $^{36}\text{Cl}^-$ uptake as described in Methods. Cells were treated with either 10^{-6} M ST_a (\bullet) or 10^{-8} M VIP (\square), for the times shown, in the KCl preincubation buffer and immediately assayed for $^{36}\text{Cl}^-$ uptake in K gluconate buffer containing 1 $\mu\text{Ci}/\text{ml}$ $^{36}\text{Cl}^-$. The values shown were normalized to control levels of uptake (\circ) and represent the mean \pm SD of triplicate determinations made

after 15 and 30 s of uptake. Control levels of $^{36}\text{Cl}^-$ uptake were 4.46 ± 0.52 nmol/min \cdot mg protein, after taking into account the amount of extracellular Cl^- remaining on the plates (275 nmol Cl^-/mg protein).

ous effects on ion gradients produced by opening of the K^+ and Cl^- channel (Table IV). After a 30-min stimulation, there was no detectable effect on the initial rate of bumetanide-sensitive $^{86}\text{Rb}^+$ uptake. Even though these conditions are suboptimal for uptake via the K^+ channels (15) there was a small but significant increase in the bumetanide-insensitive $^{86}\text{Rb}^+$ uptake which can probably be attributed to $^{86}\text{Rb}^+$ uptake through the K^+ channels. After a 60-min stimulation with ST_a , there was a small 16% decrease in bumetanide-sensitive $^{86}\text{Rb}^+$ uptake. This decrease can probably be attributed to a loss in cell volume due to the efflux of cellular K^+ and Cl^- through the K^+ and Cl^- channels (15, 16). This data agrees with the effect of ST_a on $^{86}\text{Rb}^+$ efflux from ouabain-treated preloaded cells (Fig. 5). Together, these data strongly suggest that ST_a does not directly affect the activity of the $\text{Na}^+, \text{K}^+, \text{Cl}^-$ cotransporter in T_{84} cells. The data do not address a probably indirect activation of the cotransporter through changes in intracellular ion concentrations.

The effect of ST_a on secondary messengers. To further explore the mechanism of action of ST_a , we measured the effect of the enterotoxin on cellular cAMP, cGMP, and free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) in the presence and absence of VIP or carbachol. The results are summarized in Table V. ST_a and carbachol had no effect on cellular cAMP, while VIP increased cAMP. This increase in cAMP by VIP was not altered by ST_a . In contrast, ST_a increased cellular cGMP while VIP and carbachol had no effect. The increase in cGMP by ST_a was not altered by either VIP or carbachol. The time course and graded dose effect of cGMP production and the increase in I_{sc} in response to ST_a are shown in Fig. 7. The changes in I_{sc} correlated well with the changes in cGMP, while cAMP levels did not change, indicating that cGMP did not cross-react in the cAMP assay, or vice versa. In addition, neither VIP nor ST_a increased $[\text{Ca}^{2+}]_i$, nor did they augment carbachol-induced changes in $[\text{Ca}^{2+}]_i$. These results suggest that the action of ST_a is mediated by cGMP alone. The results also suggest that the ability of ST_a or VIP to

Table IV. Effect of ST_a on Bumetanide-sensitive $^{86}\text{Rb}^+$ Uptake

Length of exposure to ST_a	$^{86}\text{Rb}^+$ Uptake		
	Without bumetanide	With 0.1 mM bumetanide	Bumetanide-sensitive (derived)
	nmol/3 min per mg protein		
0	79.5 \pm 2.5	1.3 \pm 0.1	78.2 \pm 2.5
30	82.9 \pm 1.9	1.8 \pm 0.1	81.1 \pm 1.9
60	67.0 \pm 2.0	1.6 \pm 0.0	65.4 \pm 2.0

The effect of ST_a on bumetanide-sensitive $^{86}\text{Rb}^+$ uptake. Confluent monolayer cultures of T_{84} cells were preincubated as described in Methods with 1.0 μM ST_a included in the final preincubation buffer for the duration indicated. The total preincubation time in sucrose buffer was 1.0 h in each case. The initial rate of $^{86}\text{Rb}^+$ uptake was determined in triplicate using 3-min uptake periods in the presence or absence of 0.1 mM bumetanide. The bumetanide-sensitive portion of $^{86}\text{Rb}^+$ uptake decreased significantly only after 60 min exposure to ST_a . Replicate plates were assayed for cGMP content which increased from 0.7 pmol/mg protein to over 300 pmol/mg protein after 30 min exposure to ST_a .

Table V. Measurement of Secondary Messengers in ST_a-Treated T₈₄ Monolayers

Experiment	cGMP Level		Δ[Ca ²⁺] _i
	pmol · mg protein ⁻¹	pmol · mg protein ⁻¹	
Control	<1.8	4.2±1.0	<5.0
0.25 μM ST _a	314.8±50.9*	2.9±1.2	<5.0
0.1 μM VIP	<1.8	458.9±42.5*	<5.0
0.1 mM carbachol	<1.8	3.5±0.6	45.6±5.5*
0.25 μM ST _a + 0.1 μM VIP	378.4±65.0*	493.2±47.8*	<5.0
0.25 μM ST _a + 0.1 mM carbachol	343.5±63.6*	3.1±0.4	51.7±7.5*

Various secretagogues were added alone or in combination at the concentrations indicated. For the cyclic nucleotide measurements, T₈₄ cells were preincubated for 15 min with the indicated agents as described in Methods before the measurement; no addition was made to the control group. The results are expressed as means±SE of five to six experiments in each group. For free cytosolic Ca²⁺ ([Ca²⁺]_i) measurements the monolayers were loaded with Fura-2/AM for 90 min and fluorescence was then measured continuously in the presence of the various secretagogues as described in Methods. Results are expressed as means±SE of the difference between the peak free cytosolic Ca²⁺ value in response to the secretagogue(s) and the baseline value. Baseline values for free cytosolic Ca²⁺ were 62.0±5.1 nM. All experiments were carried out with monolayers on permeable support. Monolayers grown on culture dish also exhibit similar responses to 0.1 μM VIP [427.5±74.4 pmol · mg protein⁻¹ (n = 6)] and 0.25 μM ST_a [288±66.8 pmol · mg protein⁻¹ (n = 3)].

* P < 0.05 by Student's unpaired t test as compared to control.

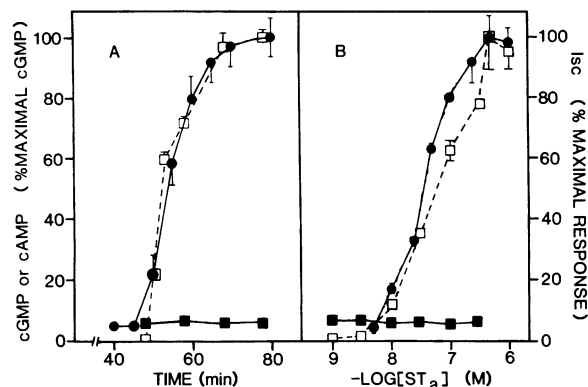


Figure 7. Time course (A) and graded dose effect (B) of *E. coli* heat stable enterotoxin (ST_a) on the changes in cyclic nucleotide concentration (cGMP or cAMP). The changes in I_{sc} reflective of net Cl⁻ secretion (●) shown for comparison as percent maximal response are the same as in Figs. 1 and 2. The concentration of ST_a in the time study was 2.5 × 10⁻⁷ M. Cyclic nucleotide measurements were carried out on T₈₄ monolayers prepared in the same manner as for the Using chamber experiments. The results are expressed as means±SE of at least three experiments for each concentration of ST_a. The maximal responses for the I_{sc} and cGMP in the time study were 13.4±0.9 μA/cm² and 228.7±6.0 pmol/mg protein, respectively. The maximal responses for the I_{sc} and cGMP in the dose, response study were 14.3 μA/cm² and 213.6±1.4 pmol/mg of protein, respectively. Only one concentration of ST_a was tested in each monolayer. The same extract from each monolayer was used to determine both cGMP (□) and cAMP (■) concentrations. cAMP concentrations are expressed relative to the maximal cGMP concentration in each experiment. For the dose response studies, the monolayers were extracted for cGMP and cAMP 35 min after ST_a addition when the I_{sc} response was at its peak.

potentiate carbachol's action on I_{sc} cannot be explained by changes in either [Ca²⁺]_i, cAMP or cGMP alone.

Discussion

In this report, we first demonstrated that T₈₄ monolayers respond to ST_a in a manner similar to that occurring in isolated intestine (1, 5). Having done so, this cell line was used as a model system to study the Cl⁻ secretory mechanism mediated by this enterotoxin and cGMP.

In contrast to other secretagogues which cause Cl⁻ secretion only when applied to the basolateral surface of T₈₄ cells, ST_a was effective only when added to the apical surface. This result suggests that receptors for ST_a are preferentially localized to the apical membrane. This conclusion is supported by receptor binding assays that demonstrate binding of the enterotoxin to apical membrane receptors leading to activation of guanyl cyclase in a dose-dependent manner (30). In this study, both the time course and graded dose effect for Cl⁻ secretion induced by ST_a paralleled the increase in the cellular cGMP level. In addition, there was no change in cAMP or free cytosolic Ca²⁺ in the presence of ST_a. These findings strongly suggest that cGMP is the secondary messenger mediating the Cl⁻ secretory effect of ST_a.

Next, we identified the transport pathways affected by ST_a. Previous studies have defined two sites for hormonal regulation of the Cl⁻ secretory process across T₈₄ monolayers: (a) The basolaterally localized K⁺ channel, which serves to recycle K⁺, and (b) The apically localized Cl⁻ channel, which serves as the Cl⁻ exit pathway. ST_a activates a K⁺ transport pathway on the basolateral membrane and also increases Cl⁻ exit across Cl⁻ channels on the apical membrane. Increased activity of the Na⁺,K⁺-ATPase and the Na⁺,K⁺,Cl⁻ cotransporters, which we believe are secondary, could be inferred by the ability of ouabain and bumetanide to inhibit or reverse the action of this enterotoxin. This pattern of activation by ST_a resembles the mechanism of action of cAMP but is different from that mediated by Ca²⁺. Our discussion will focus on the two regulatory sites, the K⁺ and Cl⁻ channels.

Increased exit of Cl⁻ through the apical Cl⁻ channel was reflected by the electrogenic nature of Cl⁻ secretion as well as the increase in bidirectional Cl⁻ fluxes in the presence of ST_a. Because the occluding junction structure of this model epithelium has a very high resistance (8), we believe that the increased bidirectional Cl⁻ fluxes across T₈₄ monolayers largely result from an increased transcellular movement of Cl⁻. In other words, Cl⁻ flux was a result of an increase in Cl⁻ exit through the Cl⁻ channel on the apical membrane as well as an increase in Cl⁻ uptake by the Na⁺,K⁺,Cl⁻ cotransport carrier across the basolateral membrane. Both pathways must be functional to allow a selective increase in transcellular Cl⁻ fluxes. Either inhibition of the Na⁺,K⁺,Cl⁻ cotransporter by bumetanide or blockage of the Cl⁻ channel reversed the cAMP- or cGMP-mediated mechanisms. It should be noted that blockage of the basolaterally localized K⁺ channel by barium also effectively inhibited net Cl⁻ secretion. Although barium inhibited net Cl⁻ secretion it did not reverse the increase in unidirectional Cl⁻ fluxes induced by cyclic nucleotides as was the case with inhibition of the Cl⁻ channel or the Na⁺,K⁺,Cl⁻ cotransport pathway. This finding suggests that the Cl⁻ channel and the Na⁺,K⁺,Cl⁻ cotransport pathway are regulated independently of the K⁺ channel.

The ST_a -mediated effect on Cl^- transport, although closely resembling the cAMP-mediated mechanism, differed in at least two respects. The time interval required to observe maximal effects of ST_a on either net Cl^- secretion or ^{36}Cl uptake through opening of the Cl^- channels, was longer than that observed with effectors acting through cAMP. Since there was a correlation between the rise in I_{sc} and the increase in cGMP, this result implies that the intracellular mechanisms by which ST_a activates guanylate cyclase are slower than the mechanisms coupling VIP or PGE_1 receptors to adenylate cyclase. The maximal effect of ST_a (10^{-6} M) on either net Cl^- secretion or ^{36}Cl uptake through the Cl^- channels was less than that maximally produced by VIP (10^{-8} M). This result implies that cGMP may be less effective than cAMP in activating the protein kinase(s) responsible for opening the Cl^- channel. Alternatively, it could be argued that cGMP and cAMP open different sets of Cl^- channels. The finding that the combined I_{sc} effect of cGMP and cAMP (ST_a plus VIP) approximated that induced by cAMP (VIP) alone argues against the latter proposal.

The opening of K^+ efflux pathway on the basolateral membrane by ST_a has been demonstrated in this study. This process can be blocked by Ba^{2+} , a K^+ channel blocker, but is not sensitive to inhibition by tetraethylammonium chloride, 4-aminopyridine, or apamin. Quinidine, which may release Ca^{2+} from an intracellular store, increased the I_{sc} before exerting an inhibitory action. The sensitivity pattern of this K^+ efflux pathway, including the graded dose effect of barium inhibition, resembles that of a cAMP-sensitive pathway reported earlier for VIP and PGE_1 (11, 15) but differs from that of a Ca^{2+} -mediated mechanism reported for carbachol (13). We have also shown that K^+ efflux induced by ST_a is not additive to that induced by VIP but is additive to that induced by carbachol. Taken together these findings suggest that the same K^+ channel is activated by both cAMP and cGMP. The critical involvement of this K^+ efflux pathway in the Cl^- secretory process is demonstrated by the fact that blockage of this pathway inhibited net Cl^- secretion. The depolarization of the cell, caused by the efflux of K^+ , may be a necessary feature required to drive Cl^- exit across the apical membrane (10).

Recently, inhibition or stimulation of the Na^+, K^+, Cl^- cotransport mechanism by atrial natriuretic factor (ANF) and 8-Br-cGMP but not cAMP has been suggested by other investigators (31–33), thus leading us to investigate this pathway. When Cl^- secretion across the T_{84} monolayer increased, an increased activity of this cotransport pathway can be assumed as it is the Cl^- uptake pathway. We were unable to demonstrate that ST_a caused any direct changes in bumetanide-sensitive $^{86}Rb^+$ efflux or bumetanide-sensitive $^{36}Cl^-$ uptake in the presence of ouabain. Concurrently, we were able to demonstrate an increase in bumetanide-insensitive $^{86}Rb^+$ efflux and bumetanide-insensitive $^{86}Rb^+$ uptake as well as an increase in cellular cGMP by ST_a . The results suggest that the Na^+, K^+, Cl^- cotransport mechanism is not directly activated by ST_a . The presumed increase in its activity requires an active Na^+, K^+ ATPase pump and is probably secondary to the favorable gradient created by K^+ and Cl^- exits, primary regulatory processes activated by ST_a .

Our studies suggest that ST_a -induced Cl^- secretion is mediated by cGMP. Its mechanism of action resembles that of cAMP (VIP and PGE_1) despite the different localization of their receptor-cyclase activation. Table VI summarizes the re-

Table VI. Comparison of cAMP-, cGMP and Ca^{2+} Mediate Secretory Response in T_{84} Cells

Secretory mechanism	Transport pathways regulated
cAMP-mediated	Apical Cl^- channel
(VIP, PGE_1 , high concentration of adenosine)	Basolateral (Ba^{2+} sensitive) K^+ channel. (both presumably cyclic-nucleotide dependent)
cGMP-mediated	Apical Cl^- channel
(ST_a)	Basolateral K^+ channel (Ba^{2+} sensitive) (both presumably cyclic-nucleotide dependent similar to cAMP-mediated response)
Ca^{2+} -mediated (carbachol, histamine)	Basolateral K^+ channel (Ba^{2+} insensitive) presumably Ca^{2+} dependent

All three mechanisms involve the Na^+, K^+, Cl^- cotransport on the basolateral membrane, which serve as the Cl^- uptake mechanism and the Na^+, K^+ ATPase pump. Utilizing $^{36}Cl^-$ uptake and efflux studies, we were unable so far to demonstrate an opening of apical Cl^- channel by Ca^{2+} -mediated response. It is assumed, therefore, that Cl^- was secreted via the Cl^- channels that are randomly open. Patch clamp studies should clarify this point in the future.

sults of our investigation on regulatory mechanism of Cl^- secretion in the T_{84} cell to date. The T_{84} model epithelium may facilitate further investigation of ST_a -related pathobiology.

Acknowledgments

The authors wish to thank Dr. Kim Barrett for her helpful suggestions and her aid in editing the manuscript, Mr. Greg Beuerlein for technical assistance, and Mr. Gary L. Deming for typing the manuscript.

The study was supported by grant AM-28305 from the National Institutes of Health, a grant from the National Ileitis and Colitis Foundation, a grant from the Cystic Fibrosis Foundation and VA Project No. 539-3108-01 from the Veterans Administration. Mr. Patrick A. Huott and Mr. Wilson Liu were supported by Student Summer Research Fellowships from the American Gastroenterological Association. Dr. Kiertisin Dharmasathaphorn is a recipient of a National Institutes of Health Research Career Development Award (AM-01146) and an American Gastroenterological Association/Glaxo Research Scholar Award.

References

- Rao, M. C., S. A. Orellana, M. Field, D. C. Robertson, and R. A. Giannella. 1981. Comparison of the biological actions of three purified heat-stable enterotoxins: effects on ion transport and guanylate cyclase activity in rabbit ileum in vitro. *Infect. Immun.* 33:165–170.
- Giannella, R. A., and K. W. Drake. 1979. Effect of purified *Escherichia coli* heat-stable enterotoxin on intestinal cyclic nucleotide metabolism and fluid secretion. *Infect. Immun.* 24:19–23.
- Spiegel, A. M., P. Gierschik, M. A. Levine, and R. W. Downs, Jr. 1985. Clinical implications of guanine nucleotide-binding proteins as receptor-effector couplers. *N. Engl. J. Med.* 312:24–33.
- Guandalini, S., M. Migliavacca, E. de Campora, and A. Rubino. 1982. Cyclic guanosine monophosphate effects on nutrient and electrolyte transport in rabbit ileum. *Gastroenterology.* 83:15–21.
- Guandalini, S., M. C. Rao, P. L. Smith, and M. Field. 1982.

cGMP modulation of ileal ion transport: *in vitro* effects of *Escherichia coli* heat-stable enterotoxin. *Am. J. Physiol.* 243:G36–G41.

6. Rao, M. C., N. T. Nash, and M. Field. 1984. Differing effects of cGMP and cAMP on ion transport across flounder intestine. *Am. J. Physiol.* 246:C167–C171.

7. Dharmasathaphorn, K., K. G. Mandel, J. A. McRoberts, L. D. Tisdale, H. Masui. 1984. A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am. J. Physiol.* 246:C204–C208.

8. Madara, J., and K. Dharmasathaphorn. 1985. Occluding junction structure-function relationships in a cultured human colonic cell monolayer. *J. Cell Biol.* 101:2124–2133.

9. Dharmasathaphorn, K., J. A. McRoberts, H. Masui, and K. G. Mandel. 1985. Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line. Direct participation of a basolaterally localized Na⁺,K⁺,Cl⁻ cotransport system. *J. Clin. Invest.* 75:462–471.

10. Mandel, K. G., J. A. McRoberts, G. Beuerlein, E. S. Foster, and K. Dharmasathaphorn. 1986. Ba⁺⁺ inhibition of VIP and A23187 stimulated Cl⁻ secretion by T₈₄ cell monolayers. *Am. J. Physiol.* 250:C486–C494.

11. Weymer, A., P. Huott, W. Liu, J. A. McRoberts, and K. Dharmasathaphorn. 1985. Chloride secretory mechanism induced by prostaglandin E₁ in a colonic epithelial cell line. *J. Clin. Invest.* 76:1828–1836.

12. Cartwright, C. A., J. A. McRoberts, K. G. Mandel, and K. Dharmasathaphorn. 1985. Synergistic action of cyclic AMP and calcium mediated chloride secretion in a colonic epithelial cell line. *J. Clin. Invest.* 76:1837–1842.

13. Dharmasathaphorn, K., and S. J. Pandol. 1986. Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. *J. Clin. Invest.* 77:348–354.

14. Dharmasathaphorn, K., P. Huott, C. A. Cartwright, J. A. McRoberts, K. G. Mandel, and G. Beuerlein. 1986. Inhibition of cAMP and ATP levels by quinidine in a human colonic epithelial cell line. *Am. J. Physiol.* 250:G806–G813.

15. McRoberts, J. A., G. Beuerlein, and K. Dharmasathaphorn. 1985. Cyclic AMP and Ca⁺⁺ activated K⁺ transport in a human colonic epithelial cell line. *J. Biol. Chem.* 260:14163–14172.

16. Mandel, K. G., K. Dharmasathaphorn, and J. A. McRoberts. 1986. Characterization of a cyclic AMP-activated Cl⁻ transport pathway in the apical membrane of a human colonic epithelial cell line. *J. Biol. Chem.* 261:704–712.

17. Ammon, H., and K. Dharmasathaphorn. 1986. Mechanism of action of bile salts on colonic Cl⁻ secretion: a study based on a cultured epithelial model. *Clin. Res.* 34:436a. (Abstr.)

18. Wasserman, S., P. Huott, K. Barrett, G. Beuerlein, M. Kagnoff, and K. Dharmasathaphorn. 1988. Immune-related intestinal Cl⁻ secretion: I. Effect of histamine on the T₈₄ cell line. *Am. J. Physiol.* 254:C53–C62.

19. Garty, H., B. Rudy, and S. J. D. Karlish. 1983. A simple and

sensitive procedures for measuring isotopic fluxes through ion-specific channels in heterogeneous populations of membrane vesicles. *J. Biol. Chem.* 258:13094–13099.

20. Tsien, R. Y., T. J. Rink, and M. Poenie. 1985. Measurement of cytosolic free Ca²⁺ in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell. Calcium.* 6:145–157.

21. Poenie, M., J. Alderton, R. Tsien, and R. A. Steinhardt. 1985. Changes of free calcium levels with stages of the cell division cycle. *Nature (Lond.)* 315:147–149.

22. Staples, S. J., S. E. Asher, and R. A. Giannella. 1980. Purification and characterization of heat-stable enterotoxin produced by a strain of *E. coli* pathogenic for man. *J. Biol. Chem.* 255:4716–4721.

23. Thompson, M. R., M. Luttrell, G. Overmann, and R. A. Giannella. 1985. Biological and immunological characteristics of ¹²⁵I-4 Tyr and -18 Tyr *Escherichia coli* heat-stable enterotoxin species purified by high-performance liquid chromatography. *Anal. Biochem.* 148:26–36.

24. Thompson, M. R., and R. A. Giannella. 1985. Revised amino acid sequence for a heat-stable enterotoxin produced by an *Escherichia coli* strain (18D) that is pathogenic for humans. *Infect. Immun.* 47:834–836.

25. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Sixth edition. Iowa State University Press, Ames, IA.

26. Giannella, R. A., P. Huott, and K. Dharmasathaphorn. 1987. Reversal of *E. coli* heat-stable enterotoxin-induced secretion and guanylate cyclase activation by anti-ST monoclonal antibody. *Gastroenterology.* 92:1403. (Abstr.)

27. McLennan, W. L., T. E. Machen, and T. Zeuthen. 1980. Ba²⁺ inhibition of electrogenic Cl⁻ secretion *in vitro* frog and piglet gastric mucosa. *Am. J. Physiol.* 239:G151–G160.

28. Rangachari, P. K. 1975. Ba⁺⁺ on the resting frog stomach: effects on electrical and secretory parameters. *Am. J. Physiol.* 228:511–517.

29. Wangemann, P., M. Wittner, A. DiStefano, H. C. Englert, H. J. Lang, E. Schlatter, and R. Greger. 1986. Cl⁻ channel blockers in the thick ascending limb of the loop of Henle. Structure activity relationship. *Pfluegers Arch.* 407(Suppl. 2):S128–141.

30. Guarino, A., M. Cohen, M. Thompson, K. Dharmasathaphorn, and R. Giannella. 1987. T₈₄ cell receptor binding and guanyl cyclase activation by *E. coli* heat-stable toxin. *Am. J. Physiol.* 250:G775–G780.

31. O'Grade, S. M., M. Field, N. T. Nash, and M. C. Rao. 1985. Atrial natriuretic factor inhibits Na-K-Cl cotransport in teleost intestine. *Am. J. Physiol.* 249:C531–C534.

32. Rao, M. C., and N. T. Nash. 1986. 8-Br-Cyclic AMP (Br-cA) does not affect Na/K/Cl cotransport in the flounder intestine. *Fed. Proc.* 45:890 (Abstr.)

33. O'Donnell, M. E., and N. E. Owen. 1986. Atrial natriuretic factor (ANF) stimulated Na/K/Cl cotransport in vascular smooth muscle cells (VSMC). *Fed. Proc.* 45:653. (Abstr.)