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Stimulation of Calcium Uptake and Cyclic GMP Synthesis in Rat Basophilic Leukemia Cells by *Escherichia coli* Heat-Stable Enterotoxin

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The effect of *Escherichia coli* heat-stable (ST) enterotoxin on calcium and cyclic nucleotide metabolism in rat basophilic leukemia cell cultures was investigated. Addition of ST enterotoxin to rat basophilic leukemia cell cultures resulted in dose- and time-dependent stimulation of calcium uptake and elevation of the intracellular cyclic GMP (cGMP) concentration. The effect of ST enterotoxin on calcium uptake ($P < 0.02$) and cGMP synthesis ($P < 0.02$) was demonstrated after 5 and 30 min of incubation at 37°C, respectively. In further studies ST enterotoxin did not enhance calcium release or the intracellular concentration of cyclic AMP. The stimulation of calcium uptake and cGMP synthesis by ST enterotoxin was inhibited by pharmacological and chemical agents which block cellular calcium entry and prostaglandin synthesis. These results demonstrate that ST enterotoxin induces calcium uptake and cGMP synthesis in rat basophilic leukemia cell cultures.

The intestinal secretory activity induced by enterotoxigenic *Escherichia coli* results from the action of heat-labile or heat-stable (ST) enterotoxin on the intestinal epithelium. The heat-labile enterotoxin, like that of *Vibrio cholerae*, activates adenylate cyclase, thus elevating the intracellular concentration of cyclic AMP (cAMP) (6, 9, 16). In contrast, ST enterotoxin activates particulate guanylate cyclase, thus elevating the intracellular concentration of cyclic GMP (cGMP) (12, 15, 23, 27). Guerrant et al. (21) and Rao et al. (29) have demonstrated that the activation of particulate guanylate cyclase by ST enterotoxin is tissue specific. Thus, the toxin does not activate the enzyme in gastric antrum, pancreas, liver, lung, heart, kidney, or cerebral cortex tissue.

Various pharmacological agents that block calcium uptake and prostaglandin synthesis have been used to diminish the secretory response induced by ST enterotoxin (1, 18–21, 25, 35). Abbey and Knoop (1), using infant mice, demonstrated that the secretory response induced by ST enterotoxin was inhibited by chlorpromazine, a calcium antagonist. Guerrant et al. (21), Greenberg et al. (18–20), and Thomas and Knoop (35) have provided further evidence, using animal systems, to implicate a role for calcium or prostaglandins or both in the secretory response induced by ST enterotoxin.

Recent studies in our laboratory have demonstrated the specific interaction of ST enterotoxin with cultured rat basophilic leukemia (RBL) cells (36). The biological response of RBL cells, after the toxin-receptor interaction, was determined by enzymatic isotopic assay for histamine. The induction of histamine release by ST enterotoxin could be inhibited by pharmacological agents that prevent calcium uptake. These results and those of others (10, 11, 21, 24) suggested that calcium and cGMP, antagonists known to influence histamine release (13, 17), may be involved in the action of ST enterotoxin on RBL cells. The current studies were initiated to determine the effect of ST enterotoxin on calcium and cyclic nucleotide metabolism.

MATERIALS AND METHODS

***E. coli* ST enterotoxin.** The ST enterotoxin of *E. coli* was prepared and purified as previously described (35). Protein estimations were determined by the spectrofluorometric method of Bohlen et al. (3), using bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) as a standard. A molecular weight of 2,500 for ST enterotoxin was used for all calculations (26). A toxin concentration of 2×10^{-8} M (=5 ng) was required to cause a gut/remaining body weight ratio of 0.083 in 3-day-old infant mice (15).

Tissue culture methods. RBL cells were received from Robert Townley, Creighton University School of Medicine, Omaha, Neb. The cells were maintained on Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (K. C. Biologicals, Kansas City, Mo.), 200 U of penicillin G (E. R. Squibb and Sons, Prince-

ton, N.J.), 20 μg of gentamicin (Elkins-Sinn, Inc., Cherry Hills, N.J.), and 1.25 μg of amphotericin B (GIBCO) per ml unless stated otherwise. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

Effect of ST enterotoxin on $^{45}Ca^{2+}$ uptake by RBL cell cultures. Routinely, RBL cells were cultured in glass vials (1.5 by 4.5 cm). Each vial contained a concentration of 2.5×10^5 cells in a final volume of 0.5 ml of tissue culture medium. Cell concentrations were determined with a hemacytometer. To examine the effect of ST enterotoxin on $^{45}Ca^{2+}$ uptake, the cell cultures were washed twice with 1.0 ml of Dulbecco phosphate-buffered saline (DPBS) at pH 7.5. After the cell cultures were washed, they were treated with varied concentrations of ST enterotoxin unless stated otherwise. The enterotoxin was contained in DPBS supplemented with 1.0 mM $CaCl_2$ and 1.0 μCi of $^{45}CaCl_2$ (Amersham Corp., Arlington Heights, Ill.); the final volume was 0.2 ml per vial. $^{45}CaCl_2$ had a specific activity of 5.7×10^2 Ci/mol. The vials were incubated for 30 min at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation, 0.8 ml of ice-cold (4°C) DPBS was added to each vial. The vial was mixed and the contents were aspirated. The cell culture was then thrice washed with 1.0 ml of cold DPBS. The time required for washing was ≈10 s per vial. After the final wash, the cells were disrupted by the addition of 0.1 ml of 0.05 N NaOH. A 3-ml portion of scintillation fluid (Aquasol; New England Nuclear Corp., Boston, Mass.) was then added to each vial, and the amount of radioactivity was determined by liquid scintillation. Counting efficiency was determined by internal standardization. Controls for basal $^{45}Ca^{2+}$ uptake were performed in the absence of enterotoxin. The amount of nonspecific binding to the glass vials was determined by conducting the experiment in the absence of cells. The experiments were completed three times, with triplicate samples used for each experiment. The total concentration of radioisotope (percent recovery) was determined by measuring the amount of radioactivity at each step of the experiment.

In some experiments, the RBL cell cultures were disrupted and the amount of radiolabel in the cell membrane and cytosol was determined. Briefly, the cell cultures were treated for 30 min with 8×10^{-8} M ST enterotoxin and washed as described above. The washed cell cultures were removed from the glass vials with a rubber policeman after the addition of 1.0 ml of ice-cold (4°C) DPBS. The cells were then ruptured with a Dounce homogenizer, and the homogenate was centrifuged at 10,000 × *g* for 1 h. The cell pellet was dissolved in 0.05 N NaOH. The amount of radioactivity in both the supernatant and dissolved cell pellet was determined by liquid scintillation as described above.

Effect of ST enterotoxin on $^{45}Ca^{2+}$ release by RBL cell cultures. Cell cultures were prepared in glass vials as previously stated. Each culture was washed twice with 1.0 ml of DPBS (pH 7.5) and incubated for 90 min at 37°C in the presence of 0.2 ml of DPBS containing 1.0 mM $CaCl_2$ and 1.0 μCi of $^{45}CaCl_2$. After incubation, each vial was washed four times with 1.0 ml of DPBS. The cell cultures were then challenged with 0.2 ml of DPBS containing varied concentrations (range, 2×10^{-8} to 4×10^{-7} M) of ST enterotoxin. The samples were incubated at 37°C for 30 min. After incubation, a volume of 0.1 ml was removed and the level of radioactivity was determined by liquid scintillation as described above. Each sample was performed in triplicate and the experiment was completed twice.

The effect of time of incubation on $^{45}Ca^{2+}$ release by RBL cell cultures was determined. The cell cultures were preloaded with $^{45}Ca^{2+}$ and washed as above. The washed cell cultures were then treated with 2×10^{-7} M ST enterotoxin contained in a final volume of 0.2 ml of DPBS. The samples were incubated at 37°C, and 0.1 ml was removed at time intervals ranging from 2 to 30 min. The amount of radioactivity contained in each sample was determined by liquid scintillation. Counting efficiency and percent radioisotope recovery were determined as noted above. The basal level of $^{45}Ca^{2+}$ release was assayed in the absence of ST enterotoxin.

Cyclic nucleotide assays. The effect of ST enterotoxin on intracellular levels of cAMP and cGMP was determined. The RBL cell cultures, contained in 24-well microtiter plates (5×10^5 cells per well), were treated with 8×10^{-8} M ST enterotoxin as described above. At varied time intervals, the cell cultures were washed and lysed by the addition of 0.3 ml of 0.05 M sodium acetate buffer (pH 6.2) containing 0.05 N NaOH. The pH of the resultant sample solution was 12.25. After 15 min at 23°C, 0.3 ml of the same buffer was added and the pH was adjusted to 6.2 with 0.05 N HCl. The samples were then frozen (−20°C) and thawed, and the contents of duplicate wells were transferred to polycarbonate centrifuge tubes (1.6 by 8.0 cm). The tubes were heated in boiling water for 10 min, cooled, and centrifuged at 100,000 × *g* for 1 h at 4°C. The concentrations of cAMP and cGMP in the supernatant were determined by radioimmunoassay (New England Nuclear). Percent recovery and the effect of NaOH treatment and boiling were determined by the addition of ^3H-labeled cAMP or ^3H-labeled cGMP to the cell cultures (1,500 cpm/vial) immediately before cell lysis. The enzymatic and nonenzymatic formations of each cyclic nucleotide were determined by using boiled and nonboiled samples according to the method of Garbers and Murad (14).

Effect of various pharmacological and chemical agents on stimulation of $^{45}Ca^{2+}$ uptake and cGMP synthesis by ST enterotoxin. Stock solutions of calcium ionophore A23187 (Eli Lilly & Co., Indianapolis, Ind.), lanthanum hydrochloride (Fisher Chemical Co., Fair Lawn, N.J.), lodoxamide tromethamine (The Upjohn Co., Kalamazoo, Mich.), diltiazem (Marion Laboratories, Kansas City, Mo.), nifedipine (Pfizer Pharmaceuticals, New York, N.Y.), indomethacin (Merck Sharp & Dohme, West Point, Pa.), and quinacrine hydrochloride (Winthrop Laboratories, New York, N.Y.) were prepared in DPBS (pH 7.5). A stock solution of ρ-bromophenacyl bromide (Sigma Chemical Co.) was prepared in acetone. The stock solutions were prepared daily and diluted with DPBS (pH 7.5) containing 1.0 mM $CaCl_2$ immediately before use. An equal volume (0.1 ml) of the diluted pharmacological or chemical agent was then added to ST enterotoxin, and the solution was used to challenge RBL cell cultures. The final toxin concentration was 4×10^{-8} M. Calcium uptake and cGMP synthesis were determined as described above after 30 and 60 min of incubation, respectively. Dose-response curves were constructed to determine the effect of the pharmacological or chemical agents on basal $^{45}Ca^{2+}$ uptake. In

all cases, a concentration from the linear portion of the curve was used in subsequent studies with ST enterotoxin. The concentration thus determined was also used to study the effect of the pharmacological or chemical agents on cGMP synthesis. Control experiments were completed in the absence of ST enterotoxin.

Statistical methods. Statistical analysis was performed, using Student's *t* test, according to the method of Schefler (32). The data expressed for calcium uptake have been corrected for counting efficiency and converted to concentration values based on the specific activity of the $^{45}CaCl_2$ used. Decay of the radioisotope was determined before each experiment.

RESULTS

Effect of ST enterotoxin on $^{45}Ca^{2+}$ uptake by RBL cell cultures. The addition of varied concentrations of ST enterotoxin to RBL cell cultures resulted in the stimulation of calcium uptake (Fig. 1). As shown, the amount of calcium uptake was directly related to the concentration of ST enterotoxin. Low toxin concentrations ($\simeq 10^{-8}$ M) caused a significant ($P < 0.01$) stimulation of calcium uptake. The optimal stimulation of calcium uptake occurred at a toxin concentration of 2×10^{-7} M. Although higher toxin concentrations (up to 6×10^{-7} M) also caused stimulation, the response was suboptimal. No difference in the ST-mediated response was observed when the cells were cultured in the absence of antibiotics. The amount (0.002%) of radioisotope that bound to the glass vials in the absence of cell cultures was negligible. Routinely, the counting efficiency was 85%. Essentially, all of the radioisotope added was recovered from each experiment.

The effect of incubation time on the level of calcium uptake by RBL cell cultures treated with ST enterotoxin was examined. A half-maximal effective dose of ST enterotoxin, determined from the data presented in Fig. 1, was used for these studies. The amount of calcium uptake was directly related to increased time of incubation up to 30 min at 37°C (Fig. 2). Although maximum uptake occurred after 30 min of incubation, significant ($P < 0.02$) calcium uptake was detectable soon after toxin addition. As shown (Fig. 2), a characteristic short lag period of about 5 min transpired between the addition of toxin and initiation of significant ($P < 0.02$) calcium uptake.

In further studies, the amount of radiolabeled calcium in the cell membrane and cytosol of control and toxin-treated RBL cell cultures was determined. Approximately 5.0 pmol of the $^{45}Ca^{2+}$ added (1.8 nmol) to the culture system remained cell associated under the conditions tested. In all cases, >95% of the radioactive isotope that was cell associated could be found in the cytosol (Fig. 2). The amount of radioisotope in the cell membrane of control and toxin-treated cells remained constant ($\simeq$4.5%) and was not affected by the time of incubation.

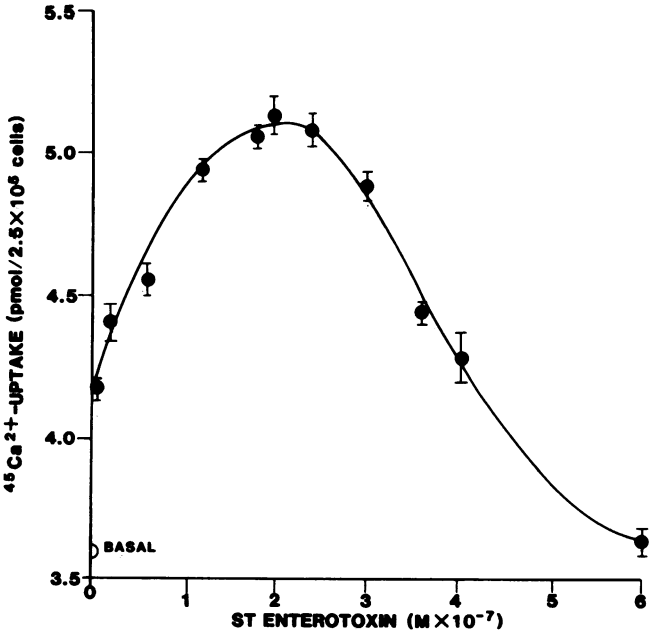

FIG. 1. Effect of *E. coli* ST enterotoxin on $^{45}Ca^{2+}$ uptake by cultured RBL cells. Data represent the mean ± standard deviation (bars). Symbols: basal (○) and toxin-mediated (●) $^{45}Ca^{2+}$ uptake.

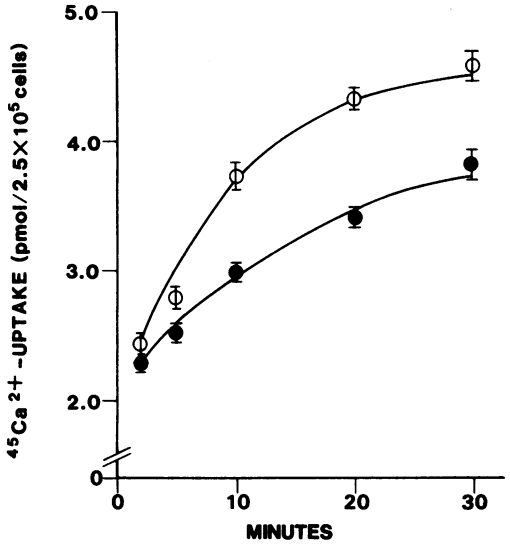

FIG. 2. Time course for $^{45}Ca^{2+}$ uptake induced by *E. coli* ST enterotoxin. Data are the mean ± standard deviation (bars). Symbols: basal (●) and toxin-mediated (○) $^{45}Ca^{2+}$ uptake for each time interval. A concentration of 4×10^{-8} M ST enterotoxin was used.

Effect of ST enterotoxin on $^{45}Ca^{2+}$ release by RBL cell cultures. The effect of incubation time on calcium release by toxin-treated RBL cell cultures was examined. The optimal concentration of toxin (2×10^{-7} M) that would effect calcium uptake was used for these studies. No significant difference in calcium release by control and toxin-treated RBL cell cultures was detectable under the conditions studied (Fig. 3). Similar experiments with toxin concentrations ranging from 2×10^{-8} to 4×10^{-7} M did not result in a significant difference for calcium release. The concentration of calcium released could not be related to the dose of toxin used when tested at varied intervals (up to 30 min) during the incubation period.

Cyclic nucleotide concentrations of RBL cell cultures treated with ST enterotoxin. The effect of ST enterotoxin on the synthesis of intracellular cyclic nucleotides in RBL cell cultures was determined (Fig. 4). As shown, ST enterotoxin increased ($P < 0.02$) the level of cGMP after 30 min of incubation at 37°C. A significant ($P < 0.01$) increase in cGMP remained 90 min after the addition of ST enterotoxin; longer incubation periods were not tested.

The concentration of cAMP in RBL cell cultures treated with ST enterotoxin was not significantly different from the basal concentration during the first 60 min of incubation at 37°C (Fig. 4). However, the concentration of cAMP was significantly ($P < 0.02$) lower in ST-treated RBL cell cultures after 90 min of incubation. At this time the concentration of cGMP was highest; a 2.7-fold increase above the basal concentration was observed. The results were not affected when the experiment was performed in the absence of antibiotics. Under the conditions specified, a recovery of 97% was obtained for the cyclic nucleotides. No significant enzymatic or nonenzymatic formation of cAMP or cGMP was detected by the methods used.

Effect of pharmacological and chemical agents on stimulation of $^{45}Ca^{2+}$ uptake and cGMP synthesis by ST enterotoxin in RBL cell cultures. In preliminary experiments, the effect of pharmacological and chemical agents on basal $^{45}Ca^{2+}$ uptake was examined in dose-response studies. A concentration of each agent which inhibited basal $^{45}Ca^{2+}$ uptake, as determined by the dose-response curve, was used to determine the ability of these drugs to inhibit the ST-mediated stimulation of $^{45}Ca^{2+}$ uptake and cGMP synthesis (Table 1). Agents which antagonize calcium flux (nifedipine, lodoxamide tromethamine, dil-

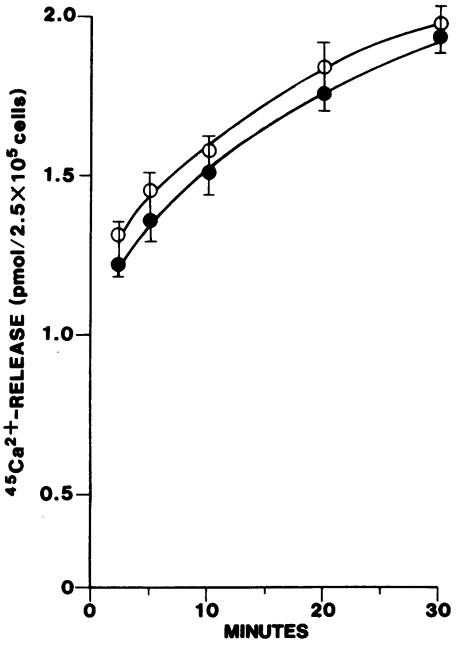

FIG. 3. Effect of time of incubation and *E. coli* ST enterotoxin concentration on $^{45}Ca^{2+}$ release from cultured RBL cells. Each point (open circle) represents the mean ± standard deviation (bars) of values obtained when RBL cell cultures were treated with a toxin concentration of 2×10^{-7} M for the time interval indicated. Basal values are represented by closed circles. Similar results were obtained with toxin concentrations of 2×10^{-8}, 1.2×10^{-7}, 3×10^{-7}, and 4×10^{-7} M. All determinations were completed in triplicate ($n = 3$).

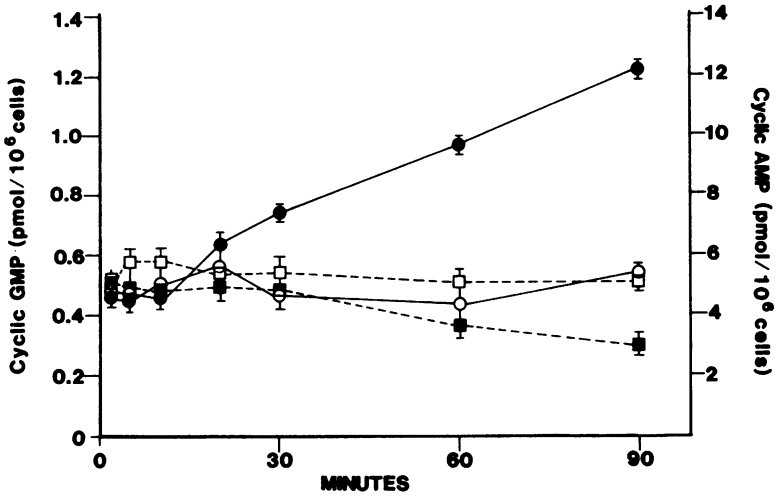

FIG. 4. Effect of *E. coli* ST enterotoxin on cAMP and cGMP concentrations in cultured RBL cells. Data are the mean ± standard deviation (bars). Symbols: cAMP and cGMP concentrations for basal (□, ○) and toxin-treated (■, ●) cell cultures, respectively. A concentration of 8×10^{-8} M ST enterotoxin was used.

tiazem, and lanthanum hydrochloride) caused a significant ($P < 0.02$) inhibition of ST-mediated $^{45}Ca^{2+}$ uptake in RBL cell cultures. Agents which block prostaglandin synthesis (indomethacin and quinacrine hydrochloride) also caused inhibition of the ST-mediated response. No significant effect of ST enterotoxin on calcium uptake induced by ionophore A23187 was observed after the 30-min incubation period. Longer periods of incubation (up to 60 min) in the presence of ionophore A23187 alone resulted in further calcium uptake, as indicated by preliminary dose-response curves.

In further studies, the calcium antagonists lodoxamide tromethamine and diltiazem caused significant ($P < 0.02$ and $P < 0.02$, respectively) inhibition of ST-mediated cGMP synthesis (Table 1). Pharmacological agents that inhibit prostaglandin synthesis (indomethacin and quinacrine hydrochloride) also caused significant ($P < 0.02$ and $P < 0.02$, respectively) inhibition of the ST-mediated response. The chemical agent p-

TABLE 1. Effect of pharmacological and chemical agents on stimulation of $^{45}Ca^{2+}$ uptake and cGMP synthesis by ST enterotoxin in RBL cell cultures[a]

Drug [concn (M)]	$^{45}Ca^{2+}$ uptake (pmol/2.5 × 10^5 cells)[b]		cGMP concn (pmol/10^6 cells)[c]	
	+ST	+ST	+ST	−ST
Diltiazem (10^{-5})	2.53	2.39	0.45	0.45
Lanthanum hydrochloride (10^{-8})	2.84	2.72	NT	NT
Lodoxamide tromethamine (10^{-6})	2.71	2.71	0.44	0.44
Nifedipine (10^{-6})	2.32	2.23	NT	NT
Indomethacin (10^{-5})	2.50	2.51	0.72	0.41
p-Bromophenacyl bromide (10^{-5})	2.12	3.32	0.47	0.53
Quinacrine hydrochloride (10^{-4})	2.12	1.87	0.41	0.49
Ionophore A23187 (10^{-5})	7.63	9.00	NT	NT
Buffer	4.44	3.55	1.02	0.45

[a] Data represent the mean ± standard deviation ($n = 3$). NT, Not tested. A final toxin concentration of 4×10^{-8} M was used for all determinations. See text for details.

[b] In $^{45}Ca^{2+}$ uptake experiments, no significant difference was obtained between cell cultures treated with the respective drug and toxin (+ST) and cultures treated with the drug alone (−ST). The effect of drug (+ST or −ST) was significantly ($P < 0.05$) different from that of buffer and toxin or buffer only, respectively.

[c] In cGMP determinations, no significant difference was observed when the experiments were performed in the presence or absence of the drug alone. The cGMP concentration in the presence of toxin alone (+ST, buffer) was significantly ($P < 0.05$) different from all other values.

bromophenacyl bromide, known to antagonize prostaglandin synthesis via the inhibition of phospholipase A_2 (38), also caused inhibition of the ST-mediated response.

DISCUSSION

The role of calcium in a variety of cellular functions, including the regulation of enzymatic activities and release of secretory products, has been demonstrated (7, 8, 30, 31, 33, 34). In mast cells and basophils, calcium uptake and cGMP synthesis have been shown to activate the release of histamine from membrane-limited granules (2, 13, 17).

Previous studies in our laboratory have demonstrated the specific binding and release of histamine from RBL cell cultures by ST enterotoxin (36). The current investigations were initiated to determine whether the action of ST enterotoxin on the release of histamine could be related to specific changes in calcium and cGMP levels.

The present studies have demonstrated an ST-mediated increase in cellular calcium uptake. The induction process was time dependent and could be inhibited by calcium antagonists. The addition of ST enterotoxin, at concentrations of 2×10^{-8} to 6×10^{-7} M, to RBL cell cultures resulted in a biphasic response curve for calcium uptake. This observation may be related to regulation of the calcium channel by calcium ion. Other studies on calcium ion transport, carried out on a variety of cell types, have suggested that a rise in calcium concentration within the cell leads to an inactivation of the membrane channel, and thus an inhibition of ion uptake (4, 22).

In further studies, no significant effect of varied concentrations of ST enterotoxin on calcium release could be detected. These observations demonstrate that toxin-mediated calcium uptake is an actual stimulatory event rather than an event related to the inhibition of calcium release.

Diltiazem, lanthanum hydrochloride, lodoxamide tromethamine, and nifedipine blocked the ability of ST enterotoxin to stimulate calcium uptake. These agents are known calcium antagonists and thus serve as important components in the definition of calcium channels. Inhibition by these agents suggests that ST-mediated responses may result in the stimulation of calcium uptake.

Indomethacin, p-bromophenacyl bromide, and quinacrine hydrochloride, prostaglandin antagonists, also caused inhibition of the ST-mediated response for calcium uptake. Although these agents are known to antagonize prostaglandin synthesis, their ability to inhibit calcium uptake has been investigated. Indomethacin and quinacrine hydrochloride, in addition to some local anesthetics, act as calcium antagonists (28, 37). These observations suggest that our results with these agents may be related to an inhibition of calcium uptake and not to an effect on prostaglandin synthesis. Further studies will be required to assess the effect of ST enterotoxin on prostaglandin metabolism.

Several studies have demonstrated that the effect of ST enterotoxin on particulate guanylate cyclase is specific for intestinal tissues (21, 29). In vitro studies on the addition of ST enterotoxin to rabbit and rat intestinal mucosal homogenates has been demonstrated to cause 1.7- and 2.5-fold increases in particulate guanylate cyclase activity in the presence of Mn^{2+}; 3- and 10-fold increases were observed in the presence of Mg^{2+} (21). The in vivo effect of ST enterotoxin on intestinal tissue has been shown to result in 1.8-fold (distal colon) and 10-fold (ileum) elevations of cGMP (29). The current study indicates that ST enterotoxin causes a 2.3-fold elevation of cGMP concentration in RBL cell cultures. Whether this observation is related to the stimulation of particulate or soluble guanylate cyclase activity is not known.

The observed time kinetics for calcium uptake suggest that calcium influx may precede cGMP synthesis. Although these results agree with several observations that receptor-mediated stimulation involves the influx of calcium into the cell cytoplasm followed by the elevation of cGMP concentration (2, 4, 5), other studies have suggested that the enhancement of calcium uptake may be regulated by guanylate cyclase activation (8). Since we have suggested that optimal histamine release by ST enterotoxin occurs after 60 min (36), it would appear that a sequence of events involving calcium uptake and cGMP synthesis occurs before mediator release.

Whereas various hormones and toxins cause an elevation of cGMP concentration, the precise events between ligand-receptor interaction and guanylate cyclase activation remain unknown. Further, the crucial roles of calcium and cGMP in stimulus-secretion coupling appear complex. Additional studies will be required to determine the nature and sequence of events involved in the induction of a secretory response by ST enterotoxin.

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