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Effect of Purified *Escherichia coli* Heat-Stable Enterotoxin on Intestinal Cyclic Nucleotide Metabolism and Fluid Secretion

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Enterotoxigenic *Escherichia coli* cause diarrhea by elaborating two enterotoxins. The large-molecular-weight, heat-labile toxin causes intestinal secretion by stimulating cyclic adenosine 5'-monophosphate production. The mechanism by which the small-molecular-weight, heat-stable enterotoxin induces secretion is unclear. The present study tested the hypothesis that heat-stable enterotoxin induces secretion by altering intestinal cyclic nucleotide concentrations. This was studied in suckling mice by using highly purified *E. coli* heat-stable enterotoxin obtained from a strain pathogenic for humans. At 3 min after administration of this toxin, intestinal cyclic guanosine 5'-monophosphate (GMP) levels were increased 10-fold. Cyclic GMP levels decreased thereafter, but still were greater than control levels at 120 min. Cyclic adenosine 5'-monophosphate levels fell to one-half of control levels at 3 min and remained below control levels for 120 min. When the time course of enterotoxin-induced secretion was compared with changes in cyclic GMP levels, fluid secretion was not evident until 15 to 30 min after enterotoxin administration. Thus, the increase in intestinal cyclic GMP concentration preceded measurable fluid secretion. And finally, administration of the 8-bromo analog of cyclic GMP evoked fluid secretion, the time course of which was similar to that induced by enterotoxin. These, and other data, strongly suggest that *E. coli* heat-stable enterotoxin induces intestinal secretion by increasing intestinal cyclic GMP levels.

Enterotoxigenic *Escherichia coli* elaborate two types of enterotoxins which result in diarrhea in animals and humans (2, 14). One is a large-molecular-weight, heat-labile toxin which has physiological and immunological similarities to cholera toxin (2, 14). The heat-labile toxin induces fluid secretion by stimulating the adenylate cyclase-cyclic adenosine 5'-monophosphate system in the small intestine (2, 14). The other *E. coli* enterotoxin is a small-molecular-weight, heat-stable toxin (ST) (1, 11). The mechanism by which ST induces fluid secretion is uncertain.

The purpose of this study was to test the hypothesis that *E. coli* ST stimulates intestinal fluid secretion by altering intestinal cyclic nucleotide metabolism. Several specific questions were posed. (i) Does purified *E. coli* ST alter intestinal cyclic nucleotide concentrations? (ii) What is the relationship of alterations of cyclic nucleotide concentrations to the time course of fluid secretion? And (iii), do exogenously administered cyclic nucleotides mimic the secretory action of ST?

MATERIALS AND METHODS

Preparation and assay of *E. coli* ST. *E. coli* strain 18D (O42:K86:H37), a strain which was isolated from an infant with diarrhea in Kentucky and which elaborates only ST, was used as the source of ST. The organism was grown in minimal medium, and the toxin elaborated into the culture fluid was purified by sequential ultrafiltration, alcohol extraction, ion-exchange chromatography, and gel filtration chromatography. This procedure results in a 1,500-fold purification of ST, which produces a single band on gel filtration, polyacrylamide gel electrophoresis, and thin-layer chromatography. Amino acid analysis of the toxin demonstrated that all amino acids were present in stoichiometric amounts, indicating that there was no contaminating peptide material (Giannella, Gastroenterology **74**:1124, 1978). Purification was monitored, and ST was quantitated by using the suckling mouse assay as previously described (7). Serial dilutions of ST were inoculated into suckling mice, and the gut-to-carcass ratio was plotted against dilution. One mouse unit was arbitrarily defined as the amount of toxin producing a gut-to-carcass ratio of 0.083 and is approximately 6 ng by weight.

Experimental design. Purified *E. coli* ST or various cyclic nucleotides were injected intragastrically into 3- to 6-day-old suckling mice, the animals were sacrificed, and the intestines were removed at various times as previously described (7). The cyclic nucleotides studied included the following: cyclic guanosine 3',5'-monophosphoric acid, sodium salt; cyclic 8-bromoguanosine 3',5'-monophosphoric acid, sodium salt

(cyclic 8-Br GMP); cyclic adenosine 3',5'-monophosphoric acid, sodium salt; and cyclic 8-bromoadenosine 3',5'-monophosphoric acid, sodium salt (cyclic 8-Br AMP). All were obtained from Sigma Chemical Co., St. Louis, Mo.

Each time point is the mean of at least six individual assays of three mice each. Separate sets of mice were used to quantitate fluid secretion and intestinal cyclic AMP and cyclic GMP concentrations.

Cyclic nucleotide assays. The intestine was removed, blotted dry, and immediately homogenized in ice-cold 5% trichloroacetic acid containing a tracer quantity of either ^{3}H-labeled cyclic AMP or ^{3}H-labeled cyclic GMP. The homogenates were centrifuged, and cyclic AMP and cyclic GMP were purified by column chromatography by using Dowex AG50W-X8 resin and a modification of the method of Schultz et al. (15). The resin was obtained from Bio-Rad Laboratories, Richmond, Calif. The column eluates were lyophilized, reconstituted in appropriate buffer, and assayed. Cyclic GMP was assayed by the radioimmunoassay method of Steiner et al. (17), and cyclic AMP was assayed by the protein binding method of Gilman (8). Intestinal protein was measured by the method of Lowry et al., using bovine serum albumin as a standard (12). Cyclic nucleotide concentration is expressed as picomoles per milligram of intestinal protein.

Data presentation and analysis. All data are presented as mean ± standard error of the mean and were evaluated statistically by utilizing the *t* test(16).

RESULTS

Effect of purified *E. coli* ST on intestinal cyclic nucleotide concentrations. As Fig. 1A shows, ST induced a prompt and marked rise in intestinal cyclic GMP concentrations. At 3 min after ST injection, cyclic GMP concentration peaked at a concentration approximately 10-fold greater than control levels. Cyclic GMP concentrations then promptly fell toward control levels but still remained significantly greater than control levels at 120 min.

In contrast, ST resulted in a prompt and sustained fall in intestinal cyclic AMP concentrations. The fall occurred within 3 min and remained significantly below control levels for 120 min.

Expressed in different terms, the ratio of cyclic AMP to cyclic GMP concentrations was initially 14.86, fell to a nadir of 0.64 at 3 min and gradually rose over the ensuing 120 min to 3.03, which is still less than the zero-time or control ratio.

In these experiments, 10 mouse units of ST (approximately 60 ng) was injected into each mouse. This dose was chosen to provide a near-maximal secretory response. This dose of purified ST was not contaminated with either cyclic AMP or cyclic GMP of bacterial origin since an assay of ST resulted in no measurable activity of either cyclic nucleotide.

Furthermore, injection of mice with saline, the vehicle for ST, resulted in no significant change in either cyclic AMP or cyclic GMP concentration (Fig. 2A).

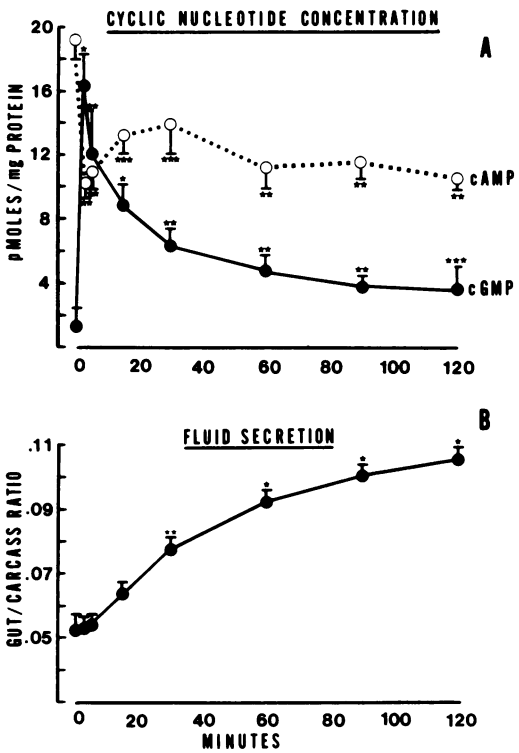

FIG. 1. *Effect of purified E. coli ST on the time course of changes in intestinal cyclic nucleotide concentrations (A) and intestinal fluid secretion (B). *, P ≤ 0.001; **, P ≤ 0.01; ***, P ≤ 0.05.*

Relationship of time course of fluid secretion to alterations in cyclic nucleotide concentrations. As Fig. 1B shows, ST induced fluid secretion at 15 to 30 min after injection. Fluid secretion increased between 30 and 90 min and reached a plateau between 90 and 120 min.

By comparing Fig. 1A and B, it is apparent that the rise in intestinal cyclic GMP concentration and the fall in cyclic AMP concentration were evident as soon as 3 min and thus occurred before measurable fluid secretion.

Effect of exogenously administered cyclic nucleotides on fluid secretion. To further examine the possible role of cyclic nucleotides in ST-induced intestinal secretion, experiments were done to study the effect of cyclic nucleotides on intestinal secretion in suckling mice. Both cyclic AMP and cyclic GMP were studied.

Initially dose-response curves contrasting the dose of various cyclic nucleotides and intestinal secretion were constructed. These are shown in Fig. 3. The 8-bromo analogs of both cyclic GMP

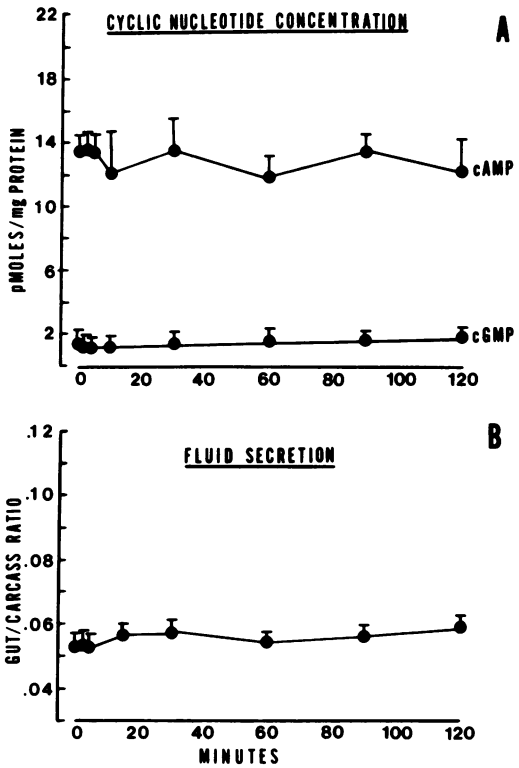

FIG. 2. *Effect of saline on the time course of changes in intestinal cyclic nucleotide concentrations (A) and intestinal fluid secretion (B).*

and cyclic AMP were much more potent secretagogues than the parent compounds.

Cyclic 8-Br GMP and cyclic 8-Br AMP resulted in similar dose-response curves. In subsequent experiments, a dose of 3 μmol of these compounds per mouse was used since this dose seemed to elicit a maximal secretory response.

The time courses of fluid secretion in response to cyclic 8-Br GMP and cyclic 8-Br AMP are shown in Fig. 4. Both nucleotides elicited a similar time course of fluid secretion; i.e., significant fluid secretion was evident after 15 to 30 min and increased to reach a plateau after 90 min.

The patterns of fluid secretion elicited by cyclic 8-Br GMP and cyclic 8-Br AMP are virtually identical to that elicited by ST.

DISCUSSION

Our data demonstrate that in suckling mice purified *E. coli* ST resulted in prompt alterations in intestinal cyclic nucleotide concentrations. As soon as 3 min after exposure to ST, cyclic GMP concentration was increased 10-fold, and cyclic AMP concentration fell to one-half of its control value. These concomitant changes resulted in a profound change in the ratio of cyclic AMP to cyclic GMP concentrations (from 14.86 at zero time to 0.64 at 3 min after toxin exposure). Although cyclic GMP levels fell thereafter, the ratio of cyclic AMP to cyclic GMP activity remained below control levels for 2 h.

These changes in intestinal cyclic GMP concentration are consistent with the findings of Hughes et al. (9) and Field et al. (4), who demonstrated that crude and semipurified preparations of ST, respectively, increased cyclic GMP concentration in rabbit intestines. These authors, however, did not state the cyclic GMP and cyclic AMP concentrations of their toxin preparations, and thus the possible confounding influence of bacterial cyclic nucleotide contamination on secretion cannot be evaluated. While this manuscript was being prepared, Newsome et al. (13) reported that a partially purified preparation of ST of bovine origin which was little contaminated with bacterial cyclic GMP also increased cyclic GMP levels in the intestines of suckling mice. Our findings that a preparation of *E. coli* ST, derived from an *E. coli* strain of human origin, purified to chemical homogeneity, and devoid of any cyclic nucleotide contamination, increased cyclic GMP concentration confirm previous reports. Thus, all observations are similar and demonstrate that *E. coli* ST preparations of varying purity and from *E. coli* strains of diverse origin (pig, calf, human) increase intestinal cyclic GMP concentration.

Our data also demonstrate that ST results in increased concentrations of cyclic GMP before

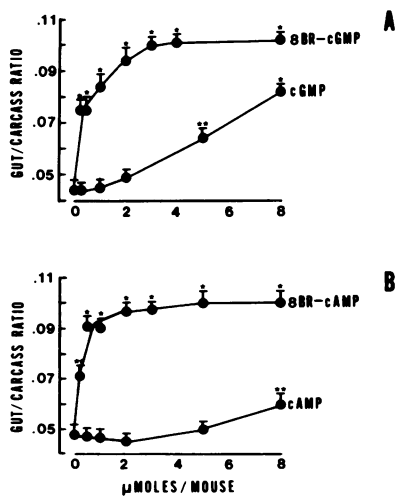

FIG. 3. *Dose response curves of effect of exogenously administered cyclic guanosine nucleotides (A) and cyclic adenosine nucleotides (B) on intestinal fluid secretion. *, $P \leq 0.001$; **, $P \leq 0.01$. Fluid secretion was measured at 30 min.*

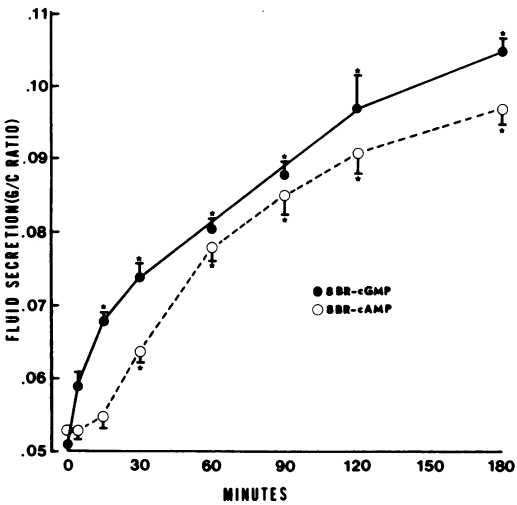

FIG. 4. *Effect of exogenously administered cyclic 8-Br GMP (8BR-cGMP) and cyclic 8-Br AMP (8BR-cAMP) (3 μmol/mouse) on time course of fluid secretion. *, $P \leq 0.001$.*

measurable intestinal secretion; i.e., cyclic GMP concentrations were 10-fold greater than baseline levels at 3 min, whereas measurable fluid secretion did not become evident until 15 to 30 min. These relative changes with time are consistent with the hypothesis that ST-induced increases in intestinal cyclic GMP concentration may mediate the secretory effects of ST. Of the three previously mentioned studies (4, 9, 13), only Field et al. (4) examined the relative changes of cyclic GMP and secretion with time. However, the earliest time point at which cyclic GMP was measured was at 5 min although an increase in short circuit current (a reflection of secretion) had occurred at 30 s. Therefore, their data are inconclusive regarding the relative changes of these two parameters with time.

A third criterion for a cause and effect relationship between ST-induced increases in intestinal cyclic GMP concentration and induction of fluid secretion was also examined in the present study. Exogenously administered cyclic 8-Br GMP resulted in fluid secretion, and the time course of this secretion was virtually identical to that induced by ST. Similar findings were reported by Hughes et al. (9).

Exogenously administered cyclic 8-Br AMP also evoked intestinal secretion. This suggests that the secretory pathway stimulated by cyclic AMP is present in this animal model. Although suckling mice can respond to increased cyclic AMP concentrations with intestinal secretion, this does not seem to be the mechanism by which ST causes secretion, since in ST-treated animals intestinal cyclic AMP concentrations were not increased and, in fact, were significantly reduced. Finally, although it is conceivable that administered cyclic 8-Br GMP could cause secretion by inhibiting cyclic AMP phosphodiesterase and thus increasing cyclic AMP levels, Hughes et al. (9) demonstrated that intestinal cyclic AMP levels are not increased after administration of cyclic 8-Br GMP.

The mechanism by which *E. coli* ST results in increased cyclic GMP concentration is unclear, although the data of Field et al. (4) strongly suggest that ST does so by stimulating intestinal guanylate cyclase activity and not by altering cyclic GMP phosphodiesterase activity.

The role of increased concentrations of intestinal cyclic GMP on intestinal fluid and electrolyte transport is unclear at present. This derives from seemingly contradictory findings reported in the literature; i.e., application of a-adrenergic agonists to in vitro preparations of intestine result in increased cyclic GMP levels and increased sodium and chloride absorption (3, 5), and application of cholinergic agonists, which are known to increase intestinal cyclic GMP concentrations (3), stimulate secretion (10). These observations may be explained by the existence of separate pools of cyclic GMP within intestinal cells, only one of which is involved in secretion. Physiological evidence for the existence of functionally separate pools of cyclic AMP within intestinal cells has been reported (6). Although the hypothesis of separate functional pools of cyclic GMP is totally speculative at present, it is supported by findings that, although a-adrenergic antagonists block the cyclic GMP-stimulating effects and absorptive effects of epinephrine on the intestine (3), a-adrenergic antagonists do not block the secretory effect of *E. coli* ST (J. M. Hughes, F. Murad, and R. L. Guerrant, Clin. Res. **26**:524, 1978).

Thus, our observations that (i) chemically homogenous, highly purified *E. coli* ST (free of either cyclic AMP or cyclic GMP) markedly increases cyclic GMP concentration in the intestine, (ii) the increase in cyclic GMP concentration precedes fluid secretion, and (iii) exogenously administered cyclic 8-Br GMP mimics the fluid secretion induced by ST and the results of the studies by Hughes et al. (9), Field et al. (4), and Newsome et al. (13) strongly support the hypothesis that *E. coli* ST causes intestinal secretion by increasing intestinal cyclic GMP levels.

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