Mechanisms of Secretory Diarrhea Caused by Bacterial Enterotoxins

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Among the various factors that are known to initiate the pathophysiological events leading to chronic, intractable diarrhea of infancy are intestinal infections by bacteria. Such infections, furthermore, are particularly worrisome in malnourished infants, where they tend to result more often in protracted diarrhea necessitating vigorous and risk-laden supportive treatment. Schematically, bacteria can induce diarrhea by either of the following mechanisms: (a) direct invasion of the intestinal mucosa (either only into the epithelium, e.g., the Shigella group, or also into the submucosa, e.g., the Salmonella group), or (b) release of enterotoxins. Most recently, a third mechanism has been suggested for some E. coli strains, involving only adherence of the microorganism to the brush border membrane of the enterocytes (1-3); however, this mechanism has not yet been found in other species and may therefore not be a property common to several enteric pathogens.

The aim of the present chapter is to focus on the second pathogenetic mechanism, i.e., the release of enterotoxins, a field that has witnessed a rapidly growing body of new information in the last years. It should be noted that a general prerequisite that is necessary for enterotoxin-producing bacteria to produce illness is their capability to adhere to enterocytes and then colonize. Adherence is a property that involves both the microorganism and the surface of the enterocyte. With respect to the microorganism, adherence is mediated by specific heat-labile, plasmid-regulated surface antigens, which have been characterized as K88, K99, and CFA antigens in porcine-specific, bovine-specific, and human-specific strains, respectively. These antigens bind to specific receptors on the animal’s brush border membrane, and such binding insures subsequent proliferation.

All of the enterotoxins produced by bacteria whose mechanism of action has been elucidated are peptides, and these can be grouped according to their chemical characteristics and mechanism of action into two categories: heat-labile stimulators of adenylate cyclase that closely resemble cholera toxin in structure and function and smaller heat-stable compounds that stimulate guanylate cyclase and increase cyclic GMP concentration in intestinal epithelial cells (4). Table
TABLE 1. Enterotoxins with a known mechanism of action

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Increase in cyclic AMP</th>
<th>Increase in cyclic GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio cholerae LT (15)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli LT (32)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli ST (23)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Yersinia enterocolitica ST (24)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Klebsiella ST (25)</td>
<td>-</td>
<td>+</td>
</tr>
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</table>

1 lists all bacteria that elaborate enterotoxins whose mechanism of action is known. We shall focus primarily on the two best-known enterotoxins, the cholera toxin and the heat-stable toxin produced by *Escherichia coli*.

**CHOLERA TOXIN**

The symptoms of cholera result mainly from the production of a protein, choleragen (or cholera toxin), by the microorganism *Vibrio cholerae*. This protein has been purified and characterized (5,6). The events that characterize the action of choleragen on the target cell have been deeply and extensively investigated and are reported in several excellent reviews (7–10).

Choleragen has a molecular weight of 84,000 and is composed of two types of linked subunits: an active fragment, A, and a binding portion, B. Figure 1 depicts how these fragments relate to each other structurally and interact at the cell surface with the specific receptor. The choleragen molecule is composed of one A₁ and one A₂ chain linked through a single disulfide bond and five B peptides. The B subunits of the toxin bind it to the cell surface receptor, the

![Proposed structure of cholera toxin](image.png)

**FIG. 1.** Proposed structure of cholera toxin. The five B subunits bind to oligosaccharide moieties of the ganglioside GM₁, and penetration through the membrane of the active fragment A then follows.
monosialoganglioside $G_{M1}$, by interacting with its oligosaccharide chains, which protrude from the lipid layer of the brush border membrane out into the lumen. Such binding is a specific, high-affinity, saturable process, which results in a close and highly stable contact of the toxin with the cell surface. When all five subunits are occupied, a conformational change in the toxin structure ensues; as a result, hydrophobic portions of the A subunit interact with hydrophobic portions of the brush border membrane. The A component thus separates from the B subunit and penetrates into the membrane. Subsequently, once within the membrane (or in the cytoplasm?), the A subunit splits into its $A_1$ and $A_2$ components, and the $A_1$ peptide initiates a series of intracellular events that eventually lead to the irreversible activation of basolateral membrane-bound enzyme adenylate cyclase.

In the infection by *Vibrio cholerae*, this process is thought to take place mainly in the small intestine, and in both cell populations of enterocytes: the young, undifferentiated crypt cells and the mature cells lining the villi.

As a result of this activation, the intracellular content of cyclic AMP increases, and this induces activation of cyclic nucleotide-dependent protein kinases that phosphorylate brush border and basolateral membrane-bound protein (11). These kinases are ubiquitous membrane-associated proteins that phosphorylate specific brush border and basolateral membrane proteins, the so-called proteins M (in the microvillus, MW 103,000) and B (in the basolateral membrane, MW 113,000), which represent a large portion of the total membrane proteins (11).

The concentrations of cyclic AMP (and cyclic GMP) required for half-maximal phosphorylation are in the range of those normally found in the gut mucosa. These cyclic nucleotide-mediated phosphorylations may well directly induce the ultimate changes in ion transport attributed to cyclic AMP (and illustrated in Fig. 2), although at present this remains speculative. In mature villous cells, cyclic AMP induces a complete block of $Na^+\text{-}Cl^-$ coupled influx across the brush border membrane: this process is the single most important step in the overall absorption of $Na^+$ and $Cl^-$, which is responsible for the absorption of the largest part of water that passively follows the ion pair translocation. Its total inhibition is therefore a major pathophysiological event in the shift of salt and water absorption toward secretion in response to cholera toxin.

Another ion transport change occurs, however, in the crypt cells. These cells are already in a condition of "basal" secretion of anions, namely, $Cl^-$ and $HCO_3^-$, and water (4,12-14); however, this process, under physiological conditions, is masked by the higher rates of electrolyte and water absorption taking place in the mature villous cells. When cyclic AMP levels are increased, this process is stimulated and consistently contributes to the net secretion of water and electrolytes seen under these circumstances (15).

It should be noted that the whole process described takes place in an intestinal mucosa that morphologically and functionally is otherwise entirely normal. Of particular importance is the integrity of the glucose- or amino acid-coupled sodium absorption, a finding that has allowed the use of orally administered
glucose–electrolyte solutions as an effective tool to replace salt and water losses in cholera (7,16). Our demonstration that, in the presence of cyclic AMP, the absorption of dipeptides (which occurs via a highly efficient system, distinct from that used by free amino acids) is also intact (17) further indicates that oral nutrients can be advantageously employed in these circumstances.

**HEAT-STABLE ENTEROTOXINS**

Several diarrheagenic strains of *Escherichia coli* and *Yersinia enterocolitica* elaborate low-molecular-weight, polypeptide, heat-stable enterotoxins (STs). These are molecules that induce small intestinal secretion in a variety of *in vivo* and *in vitro* systems. The system most widely employed for biological assays and quantitation is the suckling mouse: a positive response (not elicited by heat-labile toxins) is manifested by the increase in the gut/body weight ratio 2 to 3 hr after gastric instillation of the toxin (18).

Recently, the existence of a family of ST molecules, rather than a single chemical entity, has been shown by the isolation, purification, and characterization of ST molecules elaborated by *E. coli* strains detected from different host species. In fact, “porcine” ST was found to have a MW of 3,580 (with 33 amino acid residues), whereas a “human” ST was characterized as a smaller molecule, with a MW of 1,972 (and 18 amino acid residues) (19). The latter toxin has recently been sequenced (20) and found to be homologous (with the
exception of two amino acids) with the 18 amino acids at the C-terminal end of the sequence previously predicted by So and McCarthy (21) from the nucleotide sequence of the transposon Tn 1681 that codes for a "bovine" ST. Thus, it appears that the active site of ST molecules is contained in such a sequence.

Effects on Ion Transport and Mechanism of Action

When added in vitro to the mucosal side of rabbit ileum in Ussing chambers, ST induces a dose-dependent increase in the short-circuit current (Isc; this is defined as the current needed to nullify the spontaneous transepithelial potential difference, and its increase is considered a direct, reliable signal of electrogenic anion secretion) (23). At maximal doses, ST also abolishes net Na+ absorption, induces net Cl− secretion, and increases HCO3 secretion (22) (see Fig. 3). These effects (unlike those caused by cholera toxin) take place a few seconds after ST addition and are reversible, inasmuch as they disappear after removal of the toxin (23).

This striking difference implies an entirely different mechanism of action of the two groups of toxins: in fact, no alteration in cyclic AMP concentration can be induced by ST, which instead brings about a rapid (already maximal within 5 min) increase in cyclic GMP, which persists as long as the toxin is

![Figure 3](image-url)  
**FIG. 3.** Net ion fluxes across rabbit ileum in the absence (left panel) and after the addition (right panel) of 15 mouse units/ml of ST. (From Guandalini et al., ref. 21, with permission.)
present. This cyclic nucleotide alteration has been observed for all purified or partially purified STs in which it has been sought [E. coli (23), Yersinia enterocolitica (24), and Klebsiella pneumoniae (25)] and can therefore be assumed to be a common effect of such peptides. Theoretically, it can be attributed either to stimulation of the enzyme responsible for its production, guanylate cyclase, or to the inhibition of those involved in its breakdown, the phosphodiesterases. That the former is the case was conclusively shown by the direct measurement of guanylate cyclase activity (22–24). This enzyme is present in a variety of cells and tissues and exists in two forms: a soluble and a particulate one.

In the small intestine, guanylate cyclase is present in both forms, although to a much greater extent as the particulate enzyme; ST only activates particulate guanylate cyclase, both brush-border and basolateral membrane-associated, and has no effect on the soluble form (26).

It should also be stressed that, again unlike cholera toxin, the toxin shows a unique specificity, activating only intestinal guanylate cyclase: it is in fact without effect on this enzyme from lung, liver, pancreas, and gastric antrum (26). The reasons for this high specificity are at present unclear; it may be speculated, however, that a unique receptor for STs exists on the brush border of the small intestine, which is lacking in other cell membranes.

What is the evidence that cyclic GMP mediates ST effects on ion transport? Although the role of cyclic GMP in intestinal ion transport was, until recently, rather obscure, and some evidence existed in favor of a proabsorptive effect of this substance (27), the bulk of data recently accumulated with ST as a probe of cyclic GMP function makes it possible to substantiate its role as mediator of ST action, mainly on these lines of evidence: (a) the only known change ST induces is the increase in cyclic GMP; (b) there is a close temporal relationship between the ST-induced changes in ion transport and cyclic GMP concentration, both being maximal within 5 min, and both disappearing after toxin removal; (c) cyclic GMP analogs have a secretory effect; and (d) a very close similarity exists between the effects, or lack of effect, of ST and 8-bromo cyclic GMP on the \( I_{sc} \) of different intestinal segments.

Recently, however, a new model has been proposed for ST action that involves not only cyclic GMP but also \( \text{Ca}^{2+} \) ions and the arachidonic acid–prostaglandin cascade. Based on the evidence that drugs inhibiting either \( \text{Ca}^{2+} \) influx into the cells or the synthesis of arachidonic acid also inhibits ST effects (as judged by the suckling mouse assay), Thomas and Knoop (28) have suggested that the initial event following ST binding to the cell surface is opening of \( \text{Ca}^{2+} \) gates; activation of calmodulin then follows, and this, in turn, stimulates the activity of phospholipase A\(_2\), which generates arachidonic acid from membrane triglycerides. Arachidonic acid is then metabolized into the unstable endoperoxides that are then converted into prostaglandins. These hormones finally trigger stimulation of guanylate cyclase and, directly, electrolyte secretion.

Although this model is attractive because it puts together in a common scheme all known intracellular mediators of secretion (cyclic AMP is also to be considered
because prostaglandins certainly also activate adenylate cyclase), it nevertheless suffers from some drawbacks. First, the secretory effect of ST can also take place in the absence of luminal Ca\textsuperscript{2+} (23), and although the absence of Ca\textsuperscript{2+} in the medium may not mean the complete absence of it in the immediate cell surroundings (12), this finding nevertheless speaks against a key role of alterations in Ca\textsuperscript{2+} gating in the enterotoxin action. Furthermore, it is known that the Ca\textsuperscript{2+} ionophore A23187 induces secretion in rabbit ileum by increasing the availability of Ca\textsuperscript{2+} ions without altering intracellular levels of either cyclic AMP (29) or cyclic GMP (P. L. Smith and M. Field, personal communication), a finding that can hardly be in keeping with the suggested Ca\textsuperscript{2+}-calmodulin-arachidonic acid-prostaglandin-cyclic GMP cascade. In addition, increased prostaglandin synthesis would also result in cyclic AMP accumulation, which has been repeatedly shown not to occur after STs. Finally, it should not be forgotten that the activation of guanylate cyclase by ST is established at a constant rate within 1 min of addition of toxin to membranes (26), again a finding hard to reconcile with a rather long series of metabolic events that, according to the proposed model, would have to take place in the enterocyte.

This is not to say, in any event, that intracellular free Ca\textsuperscript{2+} plays no role in the action of ST. Indeed, the action of both cyclic nucleotides may well be mediated, at least in part, by increases in cytosol Ca\textsuperscript{2+} activity (7): intracellular stores of bound Ca\textsuperscript{2+} are large, and cyclic nucleotides may release part of this Ca\textsuperscript{2+}. It has, in fact, been reported (30) that cyclic AMP increases the efflux of radiolabeled Ca\textsuperscript{2+} from colon previously preincubated with the radioisotope. It also is presently unknown how increases in Ca\textsuperscript{2+} activity can induce secretion: a direct effect on protein kinases and stimulation of calmodulin (a ubiquitous protein showing a wide range of metabolic effects, including activation of protein kinases) are both likely guesses.

Another interesting finding in the problem of ST action is the "colon discrepancy": ST activates guanylate cyclase and increases cyclic GMP in the rabbit descending colon, but no secretory change ensues. The cyclic GMP analog 8-bromo cyclic GMP is also without effect, whereas the cyclic AMP analog 8-bromo cyclic AMP evokes an $I_{sc}$ increment (26). Thus, a cyclic AMP-sensitive ion transport system seems to be present in the colon, which lacks sensitivity to cyclic GMP.

From the secretory changes that, in rabbit small intestine, follow ST addition, it can be seen (see Fig. 3) that the net Cl\textsuperscript{-} secretion is definitely less than that induced by cyclic-AMP-related agents. Since the secretory effects of cyclic AMP are the sum (see Fig. 2) of two separate effects that take place in different cell populations, we compared cyclic GMP to cyclic AMP for both effects. It is evident from the data reported in ref. 22 and shown in Fig. 4 that the electrically neutral process of Na\textsuperscript{+}-Cl\textsuperscript{-} absorption is equally well inhibited by a cyclic-AMP-related agent such as theophylline and by ST. In contrast, when the cyclic nucleotide analogs were compared with respect to their effects on $I_{sc}$ (whose increment arises simply because of Cl\textsuperscript{-} and/or HCO\textsubscript{3}\textsuperscript{-} secretion), it appeared
Effects on Nutrient Transport

In cyclic-AMP-related diarrheas, the Na\(^+\)-coupled absorptive processes for glucose and amino acids are intact, thus allowing oral use of nutrient-electrolyte solutions as a therapeutic tool. To investigate if this was also the case for cyclic GMP, we looked at the effects of ST (or 8-bromo cyclic GMP) on the transport of several nutrients \textit{in vitro} in the rabbit ileum (31).

Results indicate that cyclic GMP leaves intact Na\(^+\)-glucose coupled influx over a range of glucose concentration as well as the influx of the amino acids glutamic acid, lysine, phenylalanine, and two dipeptides, glycyl-phenylalanine and glycyl-sarcosine. Also, the net transepithelial absorption of both glucose and phenylalanine was not affected, thus implying that intestinal nutrient handling is unaltered in cyclic-GMP- as in cyclic-AMP-related diarrheas.

To very briefly summarize the \textit{in vivo} and \textit{in vitro} data presently available on ST-induced diarrheas, it appears that such illnesses are of shorter duration and milder, the stimulated intestinal secretion being of lesser magnitude and more rapidly vanishing, than those caused by LT-producing bacteria.

As in the case of cyclic-AMP-related diarrheas, forms caused by cyclic GMP
should respond equally well to the oral administration of nutrient-electrolyte solutions.

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