The Effects of Enterotoxins and Short-Chain Fatty Acids on Water and Electrolyte Fluxes in Ileal and Colonic Loops in vivo in the Rat

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Abstract. The effect of cholera toxin (CT) and Escherichia coli heat-stable enterotoxin (ST) on the ileum and colon was examined in vivo in the rat in an attempt to clarify the effects of enterotoxins on colonic mucosa and to determine if these effects were influenced by short-chain fatty acids (SCFA). Both CT and ST induced similar changes in water and electrolyte fluxes, and the magnitude of these changes in loops of colon was similar to that observed in loops of ileum. The addition of luminal SCFA, acetate, propionate and butyrate did not influence the effect of either toxin in loops of ileum. However, in loops of colon exposed to CT, luminal butyrate (40 mM) largely reversed the effect of CT by converting net water secretion (mean ± SE, -363 ± 154 nl·cm⁻²·min⁻¹) to net water absorption (470 ± 194 nl·cm⁻²·min⁻¹) and by significantly reducing the net secretion of sodium ions. In loops of colon exposed to ST, similar effects were observed although net water secretion (−784 ± 114 nl·cm⁻²·min⁻¹) was only partially reversed by butyrate (−318 ± 102 nl·cm⁻²·min⁻¹). In contrast to butyrate, acetate and propionate did not influence changes in colonic fluxes of water and sodium induced by enterotoxins. Oxidation of butyrate and glucose was observed to be depressed in colonocytes pre-exposed to CT but not to ST. In this model, colonic secretion induced by enterotoxins is similar to that observed in the ileum but differs from ileal secretion in its modulation by luminal butyrate.

Diarrhea caused by cholera and entero-toxigenic Escherichia coli is a major cause of morbidity and childhood mortality in developing countries [1] despite the benefits achieved by oral rehydration solutions. In health, the colon plays a significant role in the conservation of water and sodium ions [2], but the effect of bacterial enterotoxins...
on these absorptive functions continues to evoke debate.

Early studies in humans [3] and in dogs [4] clinically afflicted with cholera suggested that colonic absorption of water was normal. However, subsequent studies using colonic perfusion techniques showed impairment of colonic absorption, sometimes with the induction of net fluid secretion [5-7]. Studies on the effect of *E. coli* heat stable entero-toxin (ST) have also been inconclusive with the demonstration of both normal and impaired absorptive capacity [6, 8].

Short-chain fatty acids (SCFA) are the major respiratory fuel for colonic epithelial cells [9], providing energy for a number of cellular processes including active sodium absorption. SCFA are known to stimulate water and sodium absorption from the colon under normal circumstances [10-12], but their impact in the setting of enterotoxigenic diarrhea is unknown. In this study in rats, we have compared the effects of enterotoxins in loops of colon and ileum and have examined the potential influence of SCFA on fluxes of water and electrolytes. We have also examined the ability of colonic epithelial cells to use butyrate and glucose as respiratory fuels in the presence of these enterotoxins.

**Materials and Methods**

**Animals**

Adult male Sprague-Dawley rats weighing 250-300 g were maintained on pelleted rat chow and water ad libitum in controlled environment rooms and exposed to alternate 12-hour periods of light and dark. The animals were anesthetized by intraperitoneal administration of pentobarbital sodium (6 mg/100 g body weight) and given supplements as necessary. The bowel was exposed through a midline incision and the colon was transected just distal to the cecum and again at the rectum [13]. Incisions were also made to isolate the distal 10 cm of ileum adjacent to the ileo-cecal valve. Teflon cannulas were tied to the proximal ends of the two loops and the luminal contents were flushed out with isotonic NaCl or NaCl plus SCFA solutions warmed to 37 °C. The distal end was then tied, an appropriate solution was introduced into the colon and the loop returned to the abdomen for the period of study.

**Toxins**

Purified CT (No. C 3012) was obtained from Sigma Chemicals, USA, dissolved in 1 ml of water and stored at 4 °C. Preliminary studies indicated that a period of at least 150 min was required for the effects of the toxin to become manifest. Because of this latent period, colonic and ileal loops washed out as above were filled with 20 μg of CT in 1 ml of isotonic saline and returned to the abdomen for a period of 150 min. After this period, fluid in the loops was aspirated and 3 ml of a test solution was introduced into each loop. After 10 s, 1 ml of fluid was withdrawn for baseline electrolyte and 51Cr assays leaving 2 ml in the loop for the absorption study.

Purified ST (STa, E8633) was obtained from Sigma Chemicals. This was made up to 1 ml (10 U/μl) with water and stored in aliquots at -20 °C until use. As ST has a relatively rapid effect on fluid and electrolyte fluxes, 375 U of ST were added to 3 ml of the test solution and instilled into the study loop. As noted above, 1 ml was aspirated back into the syringe to give a final amount of 250 U in each loop.

**Test Solutions**

The test solutions used were NaCl (120 mmol/l, adjusted to 300 mosm/kg with mannitol), NaCl/butyrate (Na 120, Cl 80, butyrate 40 mmol/l, 300 mosm/kg), NaCl/acetate (Na 120, Cl 80, acetate 40, 300 mosm/kg) and NaCl/propionate (Na 120, Cl 80, propionate 40, 300 mosm/kg). 51Cr-EDTA (Lucas Heights) was used as a nonabsorbable marker [13].

**Absorption**

At the end of the test period of 40 min, the fluid remaining in the loops was aspirated into a syringe and the animals sacrificed by exsanguination. The ileum and colon were removed, opened along the antimesenteric border and stretched out on a piece of graph paper to calculate the area of intestine. The aspirated fluid was centrifuged immediately at...
2,500 rpm for 5 min at 4 °C. Aliquots of supernatant were withdrawn into syringes which were capped after expulsion of air for analysis of pH and pCO₂. The rest of the solution was stored on ice for electrolyte estimations and ⁵¹Cr analysis.

Concentrations of sodium and potassium were measured on a Coming 480 flame photometer and chloride on a Radiometer CMT 10 chloride titrator. A gamma counter was used for ⁵¹Cr. Bicarbonate concentrations were derived from measured values of pH and pCO₂ on a Coming blood gas analyzer, using the Henderson-Hasselbalch equation. Fluxes of water and electrolytes were then calculated using standard formulae.

Metabolic Studies on Isolated Colonocytes
Colonocytes were isolated from everted rat colon by mechanical dissociation following incubation in a divalent-cation-free medium containing EDTA [9]. Cell suspensions (1 ml) usually representing 5–10 mg dry weight were incubated for 20–60 min in conical flasks equipped with a center well and stoppered with Suba seals. Incubations were carried out at 37 °C in 1 ml Krebs-Henseleit saline containing 2.5% w/v bovine serum albumin, the incubation mixture being present in the flask outside the center well. The radio-labelled substrates, [⁶-¹⁴C]-glucose (5 mM) or n [¹-¹⁴C]-butyrate (10 mM) were added to the incubation mix. ST was added directly to the incubation mix in concentrations of 25 and 50 U/ml. After incubation for 20, 40 or 60 min, 0.5 ml 2 M NaOH was injected into the center well and, immediately afterwards, 0.5 ml of 10% HClO₄ (v/v) was injected into the cell suspension. The flasks were then kept on ice for 3 h, after which 0.1-ml samples of the alkali solution in the center well were taken for counting in a liquid scintillation counter. The incubation mix was neutralized with 20% KOH, and metabolites (lactate in glucose incubations, and acetate in butyrate incubations) were measured enzymatically [14]. Production of ¹⁴CO₂ and metabolites were calculated and expressed as μmol/min/g dry weight.

For studies of the effects of CT, colonic loops were constructed as described above and incubated in vivo for 150 min with 20 μg of CT in 120 mmol/l NaCl. Loops were then removed and everted and colonocyte suspensions were prepared as described above. Control suspensions were obtained by preparing colonocyte suspensions from loops incubated with 1 ml of 120 mmol/l NaCl in the absence of CT.

Ethical Approval
The study protocol was approved by the Queen Elizabeth Hospital and University of Adelaide Animal Ethics Committee.

Statistics
The Wilcoxon two-sample test for unpaired data was used to test for significant differences.

Results

Studies with CT
Ileal Absorption. As shown in table 1, pretreatment with CT induced net secretion of water and sodium, a significant reduction in net absorption of chloride and a significant increase in net secretion of bicarbonate. These effects were not influenced by the presence of acetate, propionate or butyrate.

Colonic Absorption. Results from identical experiments in colonic loops are shown in figure 1. CT induced a significant reduction in the net absorption of sodium and induced net secretion of water. There was no significant change in the movement of potassium (control -22 ± 2, toxin -27 ± 4 nmol·cm⁻²·min⁻¹) or bicarbonate (control -76 ± 4, toxin -96 ± 12 nmol·cm⁻²·min⁻¹), but net chloride absorption decreased from a mean of 268 ± 24 nmol·cm⁻²·min⁻¹ in NaCl alone to 108 ± 23 nmol·cm⁻²·min⁻¹ (p < 0.005). Acetate and propionate did not significantly improve water or sodium absorption from the colon in the presence of cholera toxin.

Metabolic Studies with Colonocytes. After exposure to CT, colonocytes in vitro showed a significant reduction in the utilization of butyrate and glucose and a significant reduction in the production of lactate (table 2).
Table 1. Net ileal fluxes of water and ions in the absence and presence of CT and in the presence of acetate, propionate and butyrate

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Water</th>
<th>Na</th>
<th>K</th>
<th>Cl</th>
<th>HCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl alone</td>
<td>8</td>
<td>+38±107</td>
<td>+20±17</td>
<td>-11±1</td>
<td>+125±15</td>
<td>-111±12</td>
</tr>
<tr>
<td>CT + NaCl</td>
<td>7</td>
<td>-859±210*</td>
<td>-128±24*</td>
<td>-15±3</td>
<td>+33±8*</td>
<td>-153±20**</td>
</tr>
<tr>
<td>CT + NaCl + acetate</td>
<td>6</td>
<td>-1,473±132</td>
<td>-212±24</td>
<td>-16±1</td>
<td>-24±30</td>
<td>-263±29</td>
</tr>
<tr>
<td>CT + NaCl + propionate</td>
<td>7</td>
<td>-706±77</td>
<td>-104±11</td>
<td>-13±1</td>
<td>+25±6</td>
<td>-237±22</td>
</tr>
<tr>
<td>CT + NaCl + butyrate</td>
<td>8</td>
<td>-687±157</td>
<td>-126±20</td>
<td>-13±1</td>
<td>+16±13</td>
<td>-178±16</td>
</tr>
</tbody>
</table>

+= Net absorption from lumen to serosa; = net secretion into the lumen. All values are expressed as mean ± SE; water in nl·cm⁻²·min⁻¹, and Na, K, Cl and HCO₃ in nmol·cm⁻²·min⁻¹.
* p < 0.01, ** p < 0.05 when results with CT + NaCl were compared with NaCl alone.

Table 2. Utilization of butyrate and glucose by rat colonocytes previously exposed to CT

<table>
<thead>
<tr>
<th></th>
<th>¹⁴CO₂ from butyrate</th>
<th>¹⁴CO₂ from glucose</th>
<th>Acetoacetate production</th>
<th>Lactate production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.54±0.16</td>
<td>1.11±0.09</td>
<td>2.80±0.13</td>
<td>5.23±0.28</td>
</tr>
<tr>
<td>CT</td>
<td>1.12±0.04*</td>
<td>0.63±0.08*</td>
<td>3.71±0.72</td>
<td>3.31±0.60*</td>
</tr>
</tbody>
</table>

All results are expressed as pmol·min⁻¹·g⁻¹ (n = 8).
* p < 0.05 compared to control.

Studies with ST

Ileal Absorption. The addition of ST to ileal loops resulted in changes in water and electrolyte fluxes similar to those induced by CT. For example, a comparison of loops exposed to NaCl alone with those exposed to ST + NaCl showed that the latter group had net secretion of water (+60±132 vs. -745±92 nl·cm⁻²·min⁻¹, p < 0.005), net secretion of sodium ions (+28±20 vs. -82±12 nmol·cm⁻²·min⁻¹, p < 0.005) and enhanced secretion of bicarbonate ions (-65±12 vs. -178±19 nmol·cm⁻²·min⁻¹, p < 0.005). The addition of SCFA did not significantly alter absorption of water or sodium in the presence of ST, the mean values for water absorption in the presence of acetate, propionate and butyrate being -983±175, -1,278±210 and -996±213 nl·cm⁻²·min⁻¹, respectively.

Colonic Absorption. Sodium ion and water movement in colonic loops exposed to ST and SCFA are shown in figure 2. Loops exposed to ST + NaCl showed a net secretion of sodium ions and water, and this effect was significantly reduced by butyrate but not by
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Fig. 1 Sodium and water movement in loops of colon exposed to NaCl alone (control), NaCl + CT and CT in the presence of acetate, propionate and butyrate. Results are expressed as the mean ± SEM (n = 8). Values above the horizontal axis indicate net absorption from the lumen while values below indicate net secretion into the lumen. * p < 0.005 compared to NaCl control. ** p < 0.005 compared to NaCl + CT.

Fig. 2. Sodium and water movement in loops of colon exposed to NaCl alone (control), NaCl + ST and ST in the presence of acetate, propionate and butyrate. Results are expressed as the mean ± SEM (n = 8). * p < 0.005 compared to NaCl control. ** p < 0.02 compared to NaCl + ST.

acetate or propionate. There was no significant change in the movement of potassium or bicarbonate ions but chloride absorption decreased significantly (p < 0.005) from a mean (± SE) of +293 ± 24 nmol·cm⁻²·min⁻¹ with NaCl alone to -5 ± 11 nmol·cm⁻²·min⁻¹ in the presence of ST. The latter value was not significantly changed by the addition of SCFA, being -26 ± 19, -29 ± 18 and +15 ± 11 nmol·cm⁻²·min⁻¹ in the presence of acetate, propionate and butyrate, respectively.

Metabolic Studies with Colonocytes. Incubation of colonocytes with ST did not result in significant changes in butyrate or glucose utilization (table 3).
Table 3. Utilization of butyrate and glucose by rat colonocytes in the presence of ST

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$CO$_2$ from butyrate</th>
<th>$^{14}$CO$_2$ from glucose</th>
<th>Acetoacetate production</th>
<th>Lactate production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.50 ± 0.07</td>
<td>0.59 ± 0.06</td>
<td>3.56 ± 0.23</td>
<td>5.50 ± 0.31</td>
</tr>
<tr>
<td>ST 25 U/ml</td>
<td>1.39 ± 0.07</td>
<td>0.57 ± 0.04</td>
<td>3.33 ± 0.20</td>
<td>5.32 ± 0.18</td>
</tr>
<tr>
<td>ST 50 U/ml</td>
<td>1.25 ± 0.09</td>
<td>0.59 ± 0.04</td>
<td>3.34 ± 0.29</td>
<td>5.00 ± 0.11</td>
</tr>
</tbody>
</table>

All results are expressed as μmol·min$^{-1}$·g$^{-1}$ (n = 8). Differences not significant.

Discussion

Intestinal secretion in response to CT and ST is mediated by the cyclic nucleotides cAMP and cGMP, respectively [15, 16]. These nucleotides decrease neutral NaCl absorption from villus cells and increase the secretion of chloride ions from crypt cells. Unlike ST which is thought to act mainly through elevated cyclic nucleotide levels, CT has been demonstrated to have a variety of other effects including stimulation of submucosal nerves [17], and increases in the activity of prostaglandins [18] and 5-hydroxytryptamine [19]. Changes in cell metabolism have not been previously documented, but in this study CT was associated with a decrease in the oxidation of both glucose and butyrate. As ST did not induce similar changes, it is possible that the effects of CT on cell metabolism are secondary to some of the various changes described above. In any case, such an effect might contribute to the effects of CT on sodium absorption since many cellular processes, including the activity of Na$^+$.K$^+$.ATPase, use energy derived from oxidative metabolism.

Results from this study corroborate previous reports showing secretion of water and electrolytes in the colon after exposure to CT and ST [5-7]. In the colon (but not the ileum) these effects can be partially reversed by the presence of butyrate in concentrations similar to those observed in the rat and human cecum [20-22]. Presently available evidence suggests that butyrate is taken up in the unionized state [23], this process being favored by H$^+$ secretion in the colon [24]. Bicarbonate secretion in the ileum has the opposite effect, probably explaining the lack of effect of SCFA in the ileum. Under resting conditions, sodium uptake in the colon is predominantly electroneutral. According to this model, butyrate (taken up into the cell in the protonated form) facilitates sodium uptake by parallel Na$^+$.H$^+$ and Cl$^-$-butyrate exchanges [23]. There is evidence to suggest that Cl$^-$-butyrate exchange remains intact in the presence of cyclic AMP, unlike Cl$^-$-HCO$_3^-$ exchange which is inhibited [25]. In a way this is analogous to the preservation of glucose and amino acid-linked sodium absorptive pathways in the small intestine in cholera [26]. Fluid losses into the gut are associated with corticoid secretion [27] which may induce amiloride-sensitive sodium channels in colonocytes [28]. Under certain conditions SCFA promote sodium
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absorption through amiloride-inhibitable channels [29]. This may be an additional pathway for butyrate to promote sodium salvage in the colon exposed to enterotoxins.

The reason for a beneficial effect with butyrate in relation to negative responses with acetate and propionate is not clear. Butyrate is far more active in stimulating sodium absorption from the colon than either acetate or propionate [23]. In the present study, all three SCFA were tested at the same concentration (40 mmol/l). While this approximates concentrations of butyrate and propionate found in rat and human cecum [20–22], acetate is usually present in higher concentrations. It is possible that at higher concentrations acetate may also have a beneficial effect on sodium absorption in the colon exposed to enterotoxins. Another possibility is that butyrate, being a preferred fuel for colonic metabolism [9], might better maintain the metabolic integrity of colon epithelial cells or might have a specific effect on the activity of cyclic nucleotides. Indeed, effects of butyrate at more than one site might account for our observation that the influence of butyrate was more marked in the presence of CT than in the presence of ST.

The influence of butyrate on colonic secretion induced by CT and ST may explain some of the apparent discrepancies in results from previous studies. Indirect estimates of colonic absorptive capacity using fecal output and flow across the ileo-cecal valve showed that patients with cholera had near-maximal colonic absorption of water [3, 30]. In contrast, studies using colonic perfusion techniques revealed impaired colonic absorption of water which improved during the convalescent phase [5]. As the perfusion experiments were conducted with relatively high rates of flow in the cecum in fasted patients, it seems possible that these studies induced substantial falls in the luminal concentrations of SCFA [23].

Other indirect evidence also supports the possibility of an effect of SCFA on fecal output in various diarrheal states. For example, children with diarrhea who continue a normal diet (presumably with higher colonic levels of SCFA) have a smaller fecal output than those who change to a liquid diet [31]. Similarly, rice powder has been shown to be superior to glucose in oral rehydration solutions, perhaps because of the presence of amino acids and oligopeptides (which enhance small bowel absorption) as well as polysaccharides capable of generating SCFA in the colon [32, 33]. This assumption that the colon is capable of generating SCFA in the presence of gastroenteritis is supported by studies in transmissible gastroenteritis, a viral illness of pigs. In this disease, despite the entry of profuse secretion from the small bowel, production of SCFA continues to be normal in the colon of adult animals, and improves colonic fluid conservation [34]. If these observations are applicable to man, it may be possible to increase the colonic salvage of fluid and electrolytes in cholera and diarrhea caused by enterotoxigenic E. coli by providing substrates to the colon for generation of luminal SCFA. This might be possible by the dietary addition of unabsorbable starch which, in particular, increases luminal butyrate concentrations in man [35].

References


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