Neurokinin 1 and 2 Receptors Mediate Cholera Toxin Secretion in Rat Jejunum

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Background & Aims: Substance P, a member of the tachykinin family, is a prosecretory neuropeptide distributed widely throughout the enteric nervous system. Implicated in inflammatory states, its role in enterotoxicogenic water and electrolyte secretion is unclear. We assessed the effect of substance P antagonists and neurokinin receptor antagonists on cholera toxin–, Escherichia coli heat-labile enterotoxin (LT)–, and heat-stable enterotoxin (STa)–induced water secretion in an in vivo rat jejunal perfusion model.

Methods: Anesthetized adult male Wistar rats were pretreated with substance P antagonists (d-Pro2, d-Trp7,9-substance P, 0.1–3.0 mg/kg; or CP 96,345, 0.3–3 mg/kg) or neurokinin (NK)-1 (sendide, 1.0 mg/kg), NK-2 (GR83074, 1.0 mg/kg), or NK-3 ([Trp7,b-Ala8]NKA(4-10), 1.0 mg/kg) receptor antagonists. In a subgroup, extrinsic sensory afferents were ablated by pretreatment with capsaicin. Jejunal perfusion, with a plasma electrolyte solution containing a nonabsorbable marker, was undertaken after exposure to cholera toxin (25 µg), LT (25 µg), STa (200 µg/L), or saline. Results: Cholera toxin–induced water and electrolyte secretion was inhibited by the substance P antagonists and the NK-1 and NK-2 receptor antagonists, but not by the NK-3 receptor antagonist or by pretreatment with capsaicin. Neither LT- nor STa-induced secretions were affected by the pretreatments.

Conclusions: Prosecretory pathways involving NK-1 and NK-2 receptors specifically mediate the actions of cholera toxin in the small intestine.

Vibrio cholerae causes cholera and enterotoxigenic Escherichia coli travelers’ diarrhea by releasing highly potent enterotoxins that induce the small intestinal secretion of water and electrolytes. They do so by stimulating the synthesis of cyclic nucleotides, which are believed to activate intracellular metabolic cascades that result in the opening of apical chloride channels and the onset of secretion. Cholera toxin (CT) and E. coli heat-labile enterotoxin (LT) increase adenosine 3’,5’-cyclic monophosphate levels, whereas E. coli heat-stable enterotoxin (STa) increases guanosine 3’,5’-cyclic monophosphate.

More recently it has become clear that enterotoxins also induce water and electrolyte secretion by activating neural pathways, predominantly located within the enteric nervous system.9–11 A secretomotor reflex arc composed of 3 parts is thought to exist. First, sensory afferents extend from the activated mucosa to the submucosal and myenteric plexuses. Here gating interneurons permit additional neuronal impulses, such as those from the central autonomic neural network, to relay to and modify the reflex arc. Lastly, secretomotor efferents project back to the mucosa to complete the circuit and initiate water and electrolyte transport. Both cholinergic nicotinic and muscarinic receptors have been implicated in these enterotoxin-activated arcs, as have, more recently, noradrenaline, vasoactive intestinal polypeptide (VIP), 5-hydroxytryptamine (5-HT), nitric oxide, and opiates, although much remains unknown about their precise arrangement of these additional neuromodulators.12–20

Substance P (SP) is a member of a group of peptides, each 40–50 residues in size and sharing a common C-terminal sequence, collectively known as tachykinins.21 The 2 other major mammalian tachykinins are neurokinin A (NKA) and neurokinin B (NKB). Tachykinins bind to 3 distinct tachykinin receptors: neurokinin (NK)-1, NK-2, and NK-3. SP can be characterized by its receptor affinity profile, NK-1 > NK-2 > NK-3. For NKA, rank receptor affinity is NK-2 > NK-1 > NK-3, while NKB has high affinity for NK-3. SP is found in abundance within the small intestine, where the major source is the peripheral endings of most extrinsic primary afferents (C-type unmyelinated sensory fibers).22 These neurons have their cell bodies in the dorsal root ganglia and their multimodal peripheral endings in the mucosa. They are selectively sensitive to ablation by

Abbreviations used in this paper: CT, cholera toxin; 5-HT, 5-hydroxytryptamine; LT, Escherichia coli heat-labile enterotoxin; NK, neurokinin; PEG, polyethylene glycol 4000; PES, plasma electrolyte solution; SP, substance P; SP-PTT, d-Pro2, d-Trp7,9-substance P; STa, Escherichia coli heat-stable enterotoxin; VIP, vasoactive intestinal polypeptide.

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treatment with capsaicin (8-methyl-N-vanillyl-6-nonenamide). The second major source of SP is the sensory intrinsic neurons of the myenteric plexus. NKA is colocalized with SP in enteric neurons, whereas NKB is not found in the porcine enteric nervous system. Tachykinins exhibit a wide range of biological actions such as the stimulation of inflammatory cells, mast cell degranulation, vasodilatation, and smooth muscle contraction. In addition, SP is a potent small intestinal secretagogue, and like CT, its action can be inhibited by neuronal blockade and VIP antagonists.

The aim of this study was, first, to assess the role of SP in CT-, LT-, and STa-induced water and electrolyte secretion in rat jejunum using 2 SP antagonists, D-Pro², D-Trp⁷⁹, SP (PTT-SP) and CP 96,345. CP 96,345 is a novel nonpeptide SP antagonist that has an inactive enantiomer, CP 96,344. Second, we assessed the effect of ablating C-type unmyelinated sensory fibers with capsaicin on the actions of CT, LT, and STa. Finally, we explored the role of individual neurokinin receptors in CT-induced secretion using specific NK-1, NK-2, and NK-3 receptor antagonists.

Materials and Methods

Male adult Wistar rats (180–220 g body wt) were fasted for 18 hours with free access to water. The rats were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbitone (60 mg/kg plus 15–30-mg/kg interval injections as required). Animals were kept at 37°C using a heat pad. After making a midline abdominal incision, a proximal cannula was placed 5 cm distal to the duodenoojunal junction and a distal cannula 20 cm beyond that point, to create a jejunal loop. The isolated intestinal segment was gently cleared of residual contents and returned to the abdominal cavity, which was then closed. Next, 25 µg CT or 25 µg LT, each in 2 mL of isotonic saline or saline alone, was instilled into the jejunal segments and both canulas were clamped. The enterotoxins remained in the intestinal segments for 2 hours after which intestinal perfused was commenced at a rate of 0.5 mL/min with plasma electrolyte solution (PES) containing 140 mmol/L Na⁺, 4 mmol/L K⁺, 104 mmol/L Cl⁻, and 40 mmol/L HCO₃⁻ to which 5 g polyethylene glycol 4000 (PEG) and 4 µCi/L of [¹⁴C]PEG had been added. In STa experiments, 200 µg/L STa (equivalent to 50,000 mouse units) was additionally added to the PES. Thirty minutes were allowed to elapse to ensure establishment of a steady state after which 3 consecutive collections of the effluent were obtained from the distal cannula. Steady-state condition was shown by less than 5% variation in water movement between consecutive 10-minute collections, and also the values were accepted only if recovery of radioactive PEG was between 95% and 105%. Rats were then killed by an overdose of pentobarbitone, and the dry weight of the perfused segment was obtained after desiccation in an oven at 100°C for 18 hours. The samples were stored at −50°C for up to 48 hours before analysis of net water and electrolyte movement.

Five groups of experiments were performed.

1. Assessment of the effect of SP antagonists and NK-1, NK-2, and NK-3 receptor antagonists on basal water and electrolyte transport. Rats were pretreated, by IP injection, with PTT-SP (3.0 mg/kg), CP 96,345 (3 mg/kg), CP 96,344 (3 mg/kg), the NK-1 receptor antagonist sendide (1.0 mg/kg), the NK-2 receptor antagonist GR83074 (1.0 mg/kg), and the NK-3 receptor antagonist [Trp⁷,βAla⁸]NKA(4-10) (1.0 mg/kg), all in 0.25 mL saline or saline alone.

2. Assessment of the effect of SP antagonists on CT-induced secretion of water and electrolytes. Before CT exposure, rats were pretreated with PTT-SP (0.1, 0.3, 1.0, or 3.0 mg/kg) or PTT-SP (3.0 mg/kg) plus the 5-HT₃ antagonist granisetron (75 µg/kg) or granisetron (75 µg/kg) or saline alone. A second group of rats, also exposed to CT, was pretreated with CP 96,345 (0.3, 1, 3, or 10 mg/kg) or CP 96,344 (10 mg/kg) or saline. In addition, some rats were treated with 3.0 mg/kg PTT-SP after CT-induced small intestinal water and electrolyte secretion had been established.

3. Assessment of the effect of NK-1, NK-2, and NK-3 receptor antagonists on CT-induced secretion of water and electrolytes. Before CT exposure, rats were pretreated with either the NK-1 receptor antagonist sendide (0.1, 0.3, or 1.0 mg/kg), the NK-2 receptor antagonist GR83074 (1.0 mg/kg), the NK-3 receptor antagonist [Trp⁷,βAla⁸]NKA(4-10) (1.0 mg/kg), or saline alone. In addition, some rats were treated with 1.0 mg/kg sendide after CT-induced small intestinal water and electrolyte secretion had been established.

4. Assessment of the effect of SP antagonists on LT and STa-induced secretion of water and electrolytes. Before LT or STa exposure, rats were pretreated with 3.0 mg/kg PTT-SP or saline alone. A second group of rats was pretreated with CP 96,345 (3 mg/kg) or CP 96,344 (3 mg/kg) or saline alone before exposure to LT or STa.

5. Assessment of the effect of pretreatment with capsaicin on small intestinal function. Rats were pretreated, in 3 divided, incremental doses, with a total of 50 mg/kg capsaicin or vehicle by IP injection. Ten days later, the effectiveness of nerve depletion was confirmed using the eye-wide response to topical capsaicin (0.1% capsaicin in alcohol). Thereafter, small intestinal net water movement was assessed under basal conditions and after exposure to CT, LT, and STa as described above.

CT was obtained from the Swiss Serum and Vaccine Institute (Berne) and LT and STa from Sigma Chemical Co. The SP antagonist, D-Pro², D-Trp⁷⁹, SP (PTT-SP) and sendide ([Tyr⁶,D-Phe⁷,D-His⁹]SP(6-11)), GR83074, and [Trp⁷,βAla⁸]NKA(4-10) were supplied by Bachem U.K. and the selective 5-HT₃ antagonist granisetron from SmithKline Beecham. CP 96,345 and CP 96,344 were kindly supplied by Central Research Division.
Pfizer Inc. (Groton, CT). Radiolabeled [14C]PEG 4000 was obtained from Amersham International; all other chemicals were supplied by British Drug House.

[14C]PEG concentrations in the effluent were measured in triplicate by liquid scintillation spectroscopy in LKB Wallac Ultra-beta 1210 scintillation counter (Turku, Finland). Sodium concentrations were determined by a flame photometer (Instrument Laboratories 943), and chloride concentrations by a Corning 925 chloride analyzer. The mean of the net fluid and solute movement of the 3 consecutive effluent samples was calculated and expressed as $mL/min/g$ dry intestinal wt, respectively. Positive values denote net absorption and negative values net secretion. Results are expressed as median and interquartile range in each group of animals studied. Differences in net fluid and solute movement with different doses of antagonist were examined using a nonparametric analysis of variance (Kruskal–Wallis test and Scheffes test for within group analysis). Differences between pairs in all other experiments were tested using the Mann–Whitney test.

### Table 1. Effect of SP and NK Receptor Antagonists on Basal Jejunal Water Movement

<table>
<thead>
<tr>
<th>Neurokinin receptor antagonists</th>
<th>Control</th>
<th>PTT-SP</th>
<th>CP96,345</th>
<th>CP96,344</th>
<th>NK-1</th>
<th>NK-2</th>
<th>NK-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net flux</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water ($\mu L/min/g$)</td>
<td>141.7</td>
<td>154.9</td>
<td>151.4</td>
<td>120.1</td>
<td>134.5</td>
<td>120.3</td>
<td>148.6</td>
</tr>
<tr>
<td>Chloride ($\mu mol/min/g$)</td>
<td>1.1</td>
<td>-0.3</td>
<td>1.4</td>
<td>2.0</td>
<td>0.5</td>
<td>3.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Sodium ($\mu mol/min/g$)</td>
<td>18.2</td>
<td>22.2</td>
<td>16.5</td>
<td>17.4</td>
<td>20.6</td>
<td>21.0</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Number of rats: 26 8 6 6 6 6 6

NOTE. SP antagonist and NK-1, NK-2, and NK-3 receptor antagonists do not effect the basal transport of water and electrolytes. Data are expressed as median and interquartile ranges. Doses: PTT-SP, 2.5 mg/kg IP; CP 96,345, 10 mg/kg IP; CP 96,344, 10 mg/kg IP; NK-1 (sendide), 1 mg/kg IP; NK-2 (GR83074), 1 mg/kg IP; NK-3 ([Trp^7,βAla^8]NKA(4-10)), 1 mg/kg IP.

### Results

#### Effect of SP Antagonists and NK-1, NK-2, and NK-3 Receptor Antagonists on Basal Water and Electrolyte Transport

Basal water and electrolyte movement was unaffected by either of the SP antagonists or the neurokinin receptor antagonists (Table 1).

#### Effect of SP Antagonists on CT-Induced Secretion of Water and Electrolytes

CT induced net jejunal secretion of water and electrolytes. Both pretreatment with PTT-SP (0.3–3.0 mg/kg) and CP 96,345 (3 and 10 mg/kg) significantly reduced CT-induced secretion (Figures 1 and 2; Tables 2 and 3). There was no significant difference in the efficacy of PTT-SP and CP 96,345 in reducing net water and electrolyte secretion. The inactive enantiomer CP 96,344 had no effect on CT-induced secretion. PTT-SP (3.0 mg/kg IP) and granisetron (75 μg/kg IP) in combination were significantly better than their respective monotherapies in reducing net secretion of water and electrolytes.

![Figure 1](image1.png) **Figure 1.** Effect of pretreatment with the SP antagonist PTT-SP (0.1, 0.3, 1.0, and 3.0 mg/kg IP), granisetron (75μg/kg), and PTT-SP (3.0 mg/kg IP) and granisetron (75 μg/kg IP) in combination on CT-induced jejunal net water secretion. Data are expressed in $mL/min/g$ dry wt. Horizontal lines across the bars represents median and interquartile ranges. *P < 0.05 compared with CT (Kruskal–Wallis).

![Figure 2](image2.png) **Figure 2.** Effect of CP 96,345 (0.3, 1.0, 3.0, and 10.0 mg/kg IP) and CP 96,344 (10.0 mg/kg IP) on CT-induced jejunal net water secretion. Data are expressed in $mL/min/g$ dry wt. Horizontal lines across the bars represents median and interquartile ranges. *P < 0.05 compared with CT (Kruskal–Wallis).
mg/kg) had no effect on established CT-induced secretion (CT: water, −101.2 [−74.9 to −146.5]; chloride, −26.5 [−21.4 to −32.2]; sodium, −23.5 [−20.0 to −28.4] [n = 14] vs. CT and PTT-SP: water, −114.6 [−95.0 to −140.2]; chloride, −23.1 [−21.5 to −25.5]; sodium, −26.4 [−20.6 to −31.2] [n = 6]).

No additional antisecretory effect was observed in PTT-SP pretreated rats that were additionally pretreated with granisetron.

Effect of NK-1, NK-2, and NK-3 Receptor Antagonists on CT-Induced Secretion of Water and Electrolytes

Both the NK-1 receptor antagonist sendide and the NK-2 receptor antagonist GR 83,074 inhibited CT-induced jejunal net water and electrolyte secretion (Figure 3; Table 4). By contrast, the NK-3 receptor antagonist had no effect on CT-induced secretion. Sendide, 1.0 mg/kg, had no effect on established secretion (water, −109.6 [−78.8 to −141.3]; chloride, −25.3 [−20.7 to −26.9]; sodium, −24.7 [−21.1 to −33.5]; n = 6).

Effect of SP Antagonists on LT- and STa-Induced Secretion of Water and Electrolytes

LT-induced jejunal net water secretion (−67.6 [−89.4 to −33.0]; n = 16) was not inhibited by pretreatment with PTT-SP (−52.8 [−67.7 to −36.3]; n = 10). In the same way, STa-induced secretion (−65.3 [−83.2 to −54.6]; n = 6) was not affected by PTT-SP (−77.4 [−81.9 to −76.2]; n = 5). Net electrolyte movement paralleled net water movement (Table 2).

Neither pretreatment with CP 96,345 or its inactive enantiomer CP 96,344 affected either LT- or STa-induced jejunal net water and electrolyte movement (Figure 4; Table 3).

Effect of Pretreatment With Capsaicin on Small Intestinal Function

There were no differences in net water and electrolyte movement under basal conditions and after CT, CT-Induced Secretion of Water and Electrolytes

Table 2. Effect of PTT-SP on Enterotoxin-Induced Net Sodium and Chloride Secretion in Rat Jejunum

<table>
<thead>
<tr>
<th>Enterotoxin</th>
<th>PTT-SP (mg/kg IP)</th>
<th>Net Na⁺ movement (µmol · min⁻¹ · g⁻¹)</th>
<th>Net Cl⁻ movement (µmol · min⁻¹ · g⁻¹)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>0.1</td>
<td>−23.5 (−20.0/−28.3)</td>
<td>−26.5 (−21.4/−32.2)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>−12.6 (−9.5/−16.1)</td>
<td>−18.5 (−16.5/−19.5)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>−17.1 (−12.8/−19.6)</td>
<td>−17.7 (−14.1/−20.3)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>−7.8 (−6.8/−14.1)</td>
<td>−13.9 (−12.3/−17.5)</td>
<td>7</td>
</tr>
<tr>
<td>LT</td>
<td>3.0</td>
<td>−15.4 (−10.1/−17.4)</td>
<td>−16.8 (−15.8/−18.8)</td>
<td>16</td>
</tr>
<tr>
<td>STa</td>
<td>3.0</td>
<td>−17.4 (−15.4/−18.3)</td>
<td>−16.8 (−15.8/−17.4)</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE. CT-, but not LT- or STa-induced, secretion of electrolytes is inhibited by PTT-SP. Data are expressed as median and interquartile ranges. Net electrolyte movement is expressed as µmol · min⁻¹ · g⁻¹ dry wt.

Effect of CP 96,345 and CP 96,344 on Enterotoxin-Induced Net Sodium and Chloride Secretion in Rat Jejunum

Table 3. Effect of CP 96,345 and CP 96,344 on Enterotoxin-Induced Net Sodium and Chloride Secretion in Rat Jejunum

<table>
<thead>
<tr>
<th>Enterotoxin</th>
<th>Neurokinin antagonist (mg/kg IP)</th>
<th>Na⁺ movement (µmol · min⁻¹ · g⁻¹)</th>
<th>Cl⁻ movement (µmol · min⁻¹ · g⁻¹)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>CP96345 0.3</td>
<td>−19.7 (−17.2/−25.4)</td>
<td>−18.8 (−17.7/−21.0)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>CP96345 1.0</td>
<td>−22.5 (−19.0/−27.9)</td>
<td>−18.1 (−16.4/−20.3)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>CP96345 3.0</td>
<td>−15.8 (−12.9/−20.7)</td>
<td>−16.3 (−13.3/−18.5)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>CP96344 10.0</td>
<td>−15.0 (−14.1/−17.1)</td>
<td>−14.4 (−10.5/−15.2)</td>
<td>8</td>
</tr>
<tr>
<td>LT</td>
<td>CP96345 10.0</td>
<td>−25.4 (−24.1/−26.9)</td>
<td>−19.2 (−10.0/−27.2)</td>
<td>8</td>
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<tr>
<td></td>
<td>CP96345 3.0</td>
<td>−20.6 (−16.7/−23.8)</td>
<td>−14.9 (−13.9/−16.6)</td>
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<td>CP96344 3.0</td>
<td>−24.2 (−19.9/−27.8)</td>
<td>−16.1 (−14.3/−20.2)</td>
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<tr>
<td></td>
<td>CP96344 3.0</td>
<td>−18.5 (−16.4/−21.2)</td>
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<td>6</td>
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<tr>
<td>STa</td>
<td>CP96345 3.0</td>
<td>−22.2 (−20.2/−23.4)</td>
<td>−16.8 (−14.0/−22.5)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>CP96344 3.0</td>
<td>−23.1 (−22.1/−25.4)</td>
<td>−20.9 (−14.3/−25.5)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>CP96344 3.0</td>
<td>−22.8 (−17.5/−24.3)</td>
<td>−16.8 (−12.0/−21.5)</td>
<td>6</td>
</tr>
</tbody>
</table>

NOTE. Data are expressed as median and interquartile ranges. Net electrolyte movement is expressed as µmol · min⁻¹ · g⁻¹ dry wt.

*p < 0.05 compared with CT; Kruskal–Wallis test.
spike transient potential (STP) into the SP backbone, has been criticized by others because of its poor selectivity and unwanted side effects.

To confirm that our initial findings were genuinely related to specific SP antagonism, we repeated the experiments using CP 96,345 and its inactive enantiomer CP 96,344. CP 96,345 was discovered by Snider et al. in 1991 in radioligand binding assays using “chemical file screening” and represents the first highly selective non-peptide SP antagonist. CP 96,345 but not CP 96,344 had similar efficacy as PTT-SP in inhibiting CT-induced jejunal water and electrolyte secretion. Both CP 96,345 and CP 96,344 have, additionally, been implicated as calcium channel blockers, possessing “verapamil-like” properties. Verapamil has previously been shown to inhibit CT-induced secretion. However, because only CP 96,345 had an antisecretory effect, it is unlikely that CP 96,345 and CP 96,344 are acting as calcium channel blockers in the doses used in this study. The findings thus support the role of SP in the pathogenesis of CT-induced intestinal secretion.

SP is well established as a mediator of a range of inflammatory processes within the intestine. Pothoulakis et al. have shown that CP 96,345 potently inhibits the fluid secretion accompanying Clostridium difficile toxin A–induced inflammatory enteritis. However, they were unable to show that CT-induced fluid secretion was inhibitable by CP 96,345. The discrepancy with our observations may lie in the dose of CT used (our model

Table 4. Effect of Neurokinin Receptor Antagonists on CT-Induced Net Sodium and Chloride Movement in Rat Jejunum

<table>
<thead>
<tr>
<th>Neurokinin antagonist (mg/kg IP)</th>
<th>Net Na⁺ movement (µmol · min⁻¹ · g⁻¹)</th>
<th>Net Cl⁻ movement (µmol · min⁻¹ · g⁻¹)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK-1 0.1</td>
<td>-23.5 (-20.0/-28.3)</td>
<td>-26.5 (-21.4/-32.2)</td>
<td>14</td>
</tr>
<tr>
<td>0.3</td>
<td>-13.2 (-12.4/-15.5)</td>
<td>-7.6 (-6.7/-10.6)</td>
<td>6</td>
</tr>
<tr>
<td>1.0</td>
<td>-12.1 (-9.3/-14.2)</td>
<td>-13.7 (-12.1/-14.9)</td>
<td>8</td>
</tr>
<tr>
<td>NK-2 1.0</td>
<td>-10.9 (-5.9/-21.3)</td>
<td>-15.4 (-11.0/-21.6)</td>
<td>7</td>
</tr>
<tr>
<td>NK-3 1.0</td>
<td>-10.5 (-7.0/-11.1)</td>
<td>-18.2 (-16.5/-20.6)</td>
<td>6</td>
</tr>
<tr>
<td>-24.5 (-19.2/-26.3)</td>
<td>-25.7 (-20.1/-28.9)</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

NOTE. Data are expressed as median and interquartile ranges. NK-1, sendide; NK-2, GR83074; NK-3, [Trp⁷,βAla⁸]NKA(4-10). Net electrolyte movement is expressed as µmol · min⁻¹ · g⁻¹ dry wt.

“P < 0.05 compared with CT; Kruskal–Wallis test.

**Discussion**

CT induces secretion by activating a neural pathway within the enteric nervous system, involving nicotinic sensory afferents and with VIP as the putative neurotransmitter of efferent neurons. Our demonstration that the SP antagonist PTT-SP inhibits CT-induced jejunal water and electrolyte secretion is in keeping with these observations. Brunsson et al. found that an infusion of SP resulted in a secretory state similar to that induced by CT. Both CT-induced and SP-induced secretion were inhibited by tetrodotoxin, hexamethonium, and methionine enkephalin and were associated with release of VIP into mesenteric veins. In an earlier study, however, the same investigators were unable to demonstrate the inhibition of CT-induced secretion by the NK-1 receptor antagonist Spantide II. Only 5 rats were used in their study in which Spantide II (30 µg) was given by close intra-arterial injection after rat jejunal segments had been exposed to CT and secretion had been established. We too were unable to reverse established CT-induced water and electrolyte secretion with either PTT-SP or the NK-1 receptor antagonist sendide. However, these same agents inhibited secretion when given before exposure to CT.

PTT-SP has been described as a potent and specific SP antagonist. However, this type of SP antagonist, a peptide constructed by the insertion of D amino acids into the SP backbone, has been criticized by others because of its poor selectivity and unwanted side effects. To confirm that our initial findings were genuinely related to specific SP antagonism, we repeated the experiments using CP 96,345 and its inactive enantiomer CP 96,344. CP 96,345 was discovered by Snider et al. in 1991 in radioligand binding assays using “chemical file screening” and represents the first highly selective non-peptide SP antagonist. CP 96,345 but not CP 96,344 had similar efficacy as PTT-SP in inhibiting CT-induced jejunal water and electrolyte secretion. Both CP 96,345 and CP 96,344 have, additionally, been implicated as calcium channel blockers, possessing “verapamil-like” properties. Verapamil has previously been shown to inhibit CT-induced secretion. However, because only CP 96,345 had an antisecretory effect, it is unlikely that CP 96,345 and CP 96,344 are acting as calcium channel blockers in the doses used in this study. The findings thus support the role of SP in the pathogenesis of CT-induced intestinal secretion.

SP is well established as a mediator of a range of inflammatory processes within the intestine. Pothoulakis et al. have shown that CP 96,345 potently inhibits the fluid secretion accompanying Clostridium difficile toxin A–induced inflammatory enteritis. However, they were unable to show that CT-induced fluid secretion was inhibitable by CP 96,345. The discrepancy with our observations may lie in the dose of CT used (our model
is designed to induce maximal secretion) or in that Pothoulakis et al. studied closed ileal loops, whereas we performed steady-state jejunal perfusions.

Our findings also suggest that CT acts via both NK-1 and NK-2 receptors. SP is the natural agonist of the NK-1 receptor, and the finding that CT-induced net water and electrolyte secretion is inhibited by the NK-1 receptor antagonist sendide is similar to our observations with PTT-SP and CP 96,345. Sendide is a highly selective NK-1 receptor antagonist, considerably more potent than CP 96,345.35,36 The demonstration that the NK2 antagonist GR 83,074 also inhibits CT-induced secretion is of interest because both NK-1 and NK-2 receptors have been colocalized on neurons within the enteric nervous system.25 GR 83,074 is a high-affinity, high-selectivity linear heptapeptide that prevents binding of the natural NK-2 ligand, NKA.37 Antagonism of the NK-3 receptor, the receptor for NKB, did not inhibit CT-induced secretion. Compared with the availability of NK-1 and NK-2 antagonists, the development of NK-3 antagonists has been limited. [Trp7,βAla8]NKA(4-10) has relatively high affinity for NK-3 receptors but also has some affinity for the NK-1 receptor.38 It may be relevant that the NK-3 receptor has not been identified in the rat enteric nervous system, although submucosal neuronal receptors have been described in the guinea pig by MacNaughton et al.,39 and neurokinin B induces a small intestinal myoelectric response in the rat.40

SP has been identified within intrinsic neurons of the enteric nervous system, particularly the myenteric plexus, and within C-type, unmyelinated extrinsic sensory afferents that project to the spinal cord. It is known that sensory and motor spinal inputs modulate the effects of CT, and our findings suggest that SP mediates CT-induced jejunal water and electrolyte transport by its action within intrinsic neurons of the myenteric plexus. Evidence for this comes from our demonstration that the neurotoxin, capsaicin, which selectively ablates C-type, unmyelinated extrinsic sensory afferents, by depleting them of tachykinins, does not inhibit the secretory activity of CT.

A model thought to induce a similar secretory pathway to CT is mucosal stroking. Like CT, mucosal stroking causes enterochromaffin cells to degranulate and release 5-HT.41 The 5-HT activates receptors on cholinergic neurons in the submucosal plexus that costain for SP and project to cholinergic interneurons both in the myenteric and submucosal plexuses. The concept of SP acting on neurons activated by 5-HT release is attractive because it is consistent with the observations made in this study (Figure 6).

Neither PTT-SP, CP 96,345, nor CP 96,344 inhibited the effects of LT or STa. STa is structurally and functionally distinct from CT. It binds to and activates guanylate cyclase, causing an increase in intracellular levels of cyclic guanosine monophosphate.7,8 Although STa, at least in part, induces small intestinal water and electrolyte secretion through the enteric nervous system, it is not inhibited by 5-HT antagonists.9 Thorboll et al.42 have been unable to inhibit STa-induced secretion in the rat using the NK-1 receptor antagonist CP 99,994.

LT, by contrast, bears a remarkable structural similarity to CT. Both bind to the GM1 receptor and, by a complex mechanism, irreversibly activate adenylate cyclase to increase intracellular levels of cyclic adenosine monophosphate.2–6,10–12 Both activate the enteric nervous system and, as such, SP antagonists might have

![Figure 4](image-url) Effect of pretreatment with CP 96,345 (CP5; 10.0 mg/kg IP) and CP 96,344 (CP4; 10.0 mg/kg IP) on LT- and STa-induced jejunal net water secretion. Data are expressed in μL·min⁻¹·g⁻¹ dry wt. Horizontal lines across the bars represents median and interquartile ranges.

![Figure 5](image-url) Effect of pretreatment with capsaicin (50 mg/kg; solid bars) or vehicle (open bars) on basal, CT-, LT-, and STa-induced net water movement. Data are expressed in μL·min⁻¹·g⁻¹ dry wt. Horizontal lines across the bars represents median and interquartile ranges.
been expected to have similar effects on the water and electrolyte movement they induce. We have recently observed, however, that LT, unlike CT, fails to recruit the neurotransmitter and secretagogue 5-HT and to activate its neural secretory pathway. It appears that CT alone mediates the release of 5-HT from enterochromaffin cells, which then activates the mucosal nerve terminals of sensory afferents by binding to 5-HT_{3} or 5-HT_{1P} bearing sensory afferent neurons that project to the myenteric plexus. These costain for acetylcholine (AChT) and SP. Antagonism of either of these neurotransmitters inhibits CT-induced water and electrolyte secretion. Extrinsic sensory afferents, an additional site of action of SP antagonists, are not involved in this process because capsaicin pretreatment, which selectively ablates these pathways, has no effect. LT and STa induce secretion by alternative pathways.

Figure 6. Neuronally mediated enterotoxigenic secretion. CT induces enterochromaffin cells to release 5-HT into the small intestinal lumen and subepithelial tissues. 5-HT_{3} receptors regulate this process. The released 5-HT induces water and electrolyte secretion via mucosal 5-HT_{2} receptors and 5-HT_{3} and/or 5-HT_{1P} bearing sensory afferent neurons that project to the myenteric plexus. These costain for acetylcholine (AChT) and SP. Antagonism of either of these neurotransmitters inhibits CT-induced water and electrolyte secretion. Extrinsic sensory afferents, an additional site of action of SP antagonists, are not involved in this process because capsaicin pretreatment, which selectively ablates these pathways, has no effect. LT and STa induce secretion by alternative pathways.

In conclusion, while CT characteristically induces a noninflammatory secretory state, the evidence from this study implicates the tachykinin family in its mechanism of action. Both NK-1 and NK-2 receptors are involved in the secretory process, possibly by mediating the afferent pathway of enteric neuronal secretion activated by 5-HT release. These findings are specific to CT, because LT- and STa-induced secretion are not inhibited by PTT-SP or CP 96,345, and support earlier observations that particular secretory pathways are activated by CT.

References


