Effects of cholera toxin on the potential difference and motor responses induced by distension in the rat proximal small intestine in vivo

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Kordasti, Shirin, Maria Sapnara, Evan A. Thomas, Erik Lindstrom, Mikael Forsman, Joel C. Bornstein, and Henrik Sjövall. Effects of cholera toxin on the potential difference and motor responses induced by distension in the rat proximal small intestine in vivo. Am J Physiol Gastrointest Liver Physiol 290: G948–G958, 2006. First published December 15, 2005; doi:10.1152/ajpgi.00267.2005.—Cholera toxin (CT) may induce uncontrolled firing in recurrent networks of secretomotor neurons in the submucous plexus. This hypothesis was tested in chloralose-anesthetized rats in vivo. The secretory reflex response to graded intestinal distension was measured with or without prior exposure to luminal CT. The transmural potential difference (PD) was used as a marker for electrogenic chloride secretion. In controls, distension increased PD, and this response was reduced by the neural blocker tetrodotoxin given serosally and the vasoactive intestinal peptide (VIP) receptor antagonist [4Cl-D-Phe\(^6\),Leu\(^{17}\)]VIP (2 \(\mu\)g·min\(^{-1}\)·kg\(^{-1}\)·iv) but unaffected by the serotonin 5-HT\(_3\) receptor antagonist granisetron, by the nicotinic receptor antagonist hexamethonium, by the muscarinic receptor antagonist atropine, or by the cyclooxygenase inhibitor indomethacin. Basal PD increased significantly with time in CT-exposed segments, an effect blocked by granisetron, by indomethacin, and by [4Cl-D-Phe\(^6\),Leu\(^{17}\)]VIP but not by hexamethonium or atropine. In contrast, once the increased basal PD produced by CT was established, [4Cl-D-Phe\(^6\),Leu\(^{17}\)]VIP and indomethacin had no significant effect, whereas granisetron and hexamethonium markedly depressed basal PD. CT significantly reduced the increase in PD produced by distension, an effect reversed by granisetron, indomethacin, and atropine. CT also activated a specific motility response to distension, repeated cluster contractions, but only in animals pretreated with granisetron, indomethacin, or atropine. These data are compatible with the hypothesis that CT induces uncontrolled activity in submucous secretory networks. Development of this state depends on 5-HT\(_3\) receptors, VIP receptors, and prostaglandin synthesis, whereas its maintenance depends on 5-HT\(_3\) and nicotinic receptors but not VIP receptors. The motility effects of CT (probably reflecting myenteric activity) are partially suppressed via a mechanism involving 5-HT\(_3\) and muscarinic receptors and prostaglandin synthesis.

enteric nervous system; vasoactive intestinal peptide

AT THE LEVEL OF THE ENTEROCYTE, cholera toxin (CT) induces a dramatic increase in electrogenic chloride secretion via irreversible stimulation of cAMP formation (16). However, in vivo, CT may not actually reach the deeper layers of the mucosa where secreting systems are expressed (45, 51), suggesting the involvement of other mechanisms. One mechanism, for which the evidence is very strong, is concomitant activation of the enteric nervous system. Enteric nervous components directly or indirectly involved with CT’s actions are serotonin (5-HT) released from enterochromaffin cells (48), activation of 5-HT\(_3\) receptors (3, 4, 34, 49), activation of intrinsic primary afferent neurons (10), involvement of nicotinic interneurons (6, 19), and a contribution of secretomotor neurons releasing vasoactive intestinal peptide (VIP) (5, 33). These different components have been well documented in vivo, but the exact neurophysiological mechanisms are not well defined, because there is no good in vitro model as yet. The physiological role of this neural system is also unclear.

In a recent study (9), we proposed that hypersecretion may be due to hyperactivity in recurrent networks of submucous secretomotor neurons. We showed that this was feasible using computer simulations of the activity of anatomically realistic networks of such neurons, as long as the networks in turn interact with sensory neural networks in a physiologically realistic fashion. A key prediction of the simulations was that the mechanisms needed to build up network activity do not need to be identical to those that maintain network activity once it reaches its peak. Under some conditions, the recurrent secretomotor neuron networks can even go into a state of uncontrolled firing and no longer respond to removal of the sensory stimulus. We postulate that this state may actually be the neurophysiological equivalent of the neurogenic component of cholera secretion.

The aim of the present study was to test this hypothesis in two ways: 1) by comparing the reactivity of a physiological secretory reflex (evoked by distension) in the absence and presence of CT and 2) by testing the effects of relevant neural blockers on developing and established cholera secretion. A luminal pressure increase induced by distension or intense intestinal motor activity induces an increase in electrogenic secretion, as reflected by an increase in the transmucosal potential difference (PD). This reflex response has been well documented in previous in vitro and in vivo studies (14, 26, 50). In Ussing chambers containing only the submucous plexus and mucosa, distension excites a neuronal pathway that increases short-circuit current via the release of VIP and/or ACh from enteric secretomotor neurons (12, 18, 42). This response

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is abolished by removal of chloride from the medium or by loop diuretics, strongly implying electrogenic chloride secretion as the underlying epithelial transport mechanism (18). The PD increase in response to intense motor activity is also abolished in patients with cystic fibrosis (i.e., with defective CFTR function), again implying active chloride secretion as the final mechanism (2). The secretary and motor responses to distension of the proximal small intestine were studied in anesthetized rats in vivo, and the responses were compared in the absence or presence of CT. As a second test, we also looked for differences in the effects of neural antagonists on developing and manifest cholera secretion, thereby testing the prediction that the induction of hyperactivity and maintenance of hyperactivity are separate processes. The rat was used as a model system because we have a large body of information on the neuropharmacology of cholera secretion in vivo in this particular species. The choice of intestinal segment was based on the availability of quantitative data on the relationship between motor activity, PD, and, importantly, net fluid transport in this particular segment in humans (25, 26).

METHODS

Animals

Experiments were performed in male Sprague-Dawley rats (Mølle- gard, Denmark) weighing 240–400 g. Animals were kept under standardized environmental conditions in animal quarters for at least 7 days before the experiments. Before the experiments, they were fasted overnight with free access to water. The study was approved by the Animal Ethics Committee of Göteborg’s University.

Operative Procedures and Experimental Setup

The general setup and operative procedures have been described previously (46). Briefly, anesthesia was induced by pentobarbital and was maintained by an infusion of chloralose intra-arterially, which also contained buffer to prevent dehydration and acidosis. Arterial pressure was continuously monitored. The abdomen was opened in the midline, and a suitable segment was identified. Each segment used was distal to the pancreaticobiliary duct, had a length of 3–4 cm, and was supplied with a mesentery root containing an artery large enough to supply the segment after ligation. Only one segment was studied in each rat. The intestine was divided between ligatures, and the remaining ligated gut was put back into the abdominal cavity. The segment was soaked and carefully rinsed with warmed saline (37°C). The proximal end was cannulated with a double-lumen catheter containing one piece of polyethylene tubing for fluid administration and pressure measurement (outer diameter: 1.3 mm), and a similar catheter filled with saline agar for transmucosal PD recordings (outer diameter: 2.4 mm). The distal end of the chosen segment was cannulated with a plastic tube that was clamped closed during distension. The segment was filled with saline via the fluid administration catheter, the pressure recording catheter was connected to a pressure transducer (DPT-6000 single-use transducer, Peter von Berg Medizintechnik), and the agar bridge was immersed in a Becker containing saturated KCl and a calomel half cell (REF 402, Radiometer; Copenhagen, Denmark). A similar calomel half cell, immersed in saturated KCl, was connected to another agar bridge placed in the saline-soaked abdominal cavity. After the catheters were placed and a stable PD signal was obtained, the segment was covered with soaked gauze and a thin plastic film to minimize evaporation. The pressure transducers used for recording systemic blood pressure and intestinal luminal pressure were connected to bridge amplifiers on a Grass polygraph (model 7D, Grass Instruments), and the signals were collected in digital form using a Labview program (National Instruments; Austin, TX), at a frequency of 4 Hz. The calomel half cells were connected to a high-impedance bridge amplifier (DVC 1000 current/voltage clamp, World Precision Instruments), and these signals were also sampled at 4 Hz as a third channel with the Labview program.

General structure of the study. The study was divided into three parts. Part 1 involved control experiments in which the effects of distension on PD and motility were measured and the role of enteric neurons was verified using TTX. Animals (n = 18) studied in this component served as controls for all other elements of the study. Part 2 examined the effects of pretreatment with CT on baseline PD (cholera secretion) and on the relationship between distension pressure and transmural PD or motility. Part 3 tested the involvement of different neurotransmitter receptors on the development of cholera secretion and on its maintenance once established as well as examining whether the same receptors were involved in the effects of CT on the secretory and motor components of the distension response.

Protocol for the Study of Responses to Distension

This protocol was followed for all animals except those used for studies of established cholera secretion. After stabilization of the PD and pressure signal, the segment was distended to the desired pressure by injecting saline intraluminally via the perfusion catheter. Pressure was maintained for 5 min, and the segment was then emptied of fluid except for a small amount needed to maintain a PD signal. In all experiments, we followed a standard distension protocol, with 30 min of base line recording, 5 min of distension, 10 min of rest, 5 min of distension, etc. The distension steps were 5, 10, and 20 mmHg.

In preliminary studies, it has been found that repeating the sequence of distensions at 5, 10, and 20 mmHg after completion of the first series of distensions induced a series of responses similar to those seen during the first sequence. Thus it was concluded that responses were reproducible over time within the same animal (data not shown).

The involvement of neurons in the responses to distension was tested using the voltage-dependent Na⁺ channel blocker TTX (n = 8). Because TTX is highly toxic, it was given locally by adding drops (0.1–0.2 ml, 3 μg/ml) onto the serosal layer of the segment 5 min before each distension (7).

Protocol for Treatment With CT

Pure crystalline CT (20 μg) dissolved in 0.5 ml physiological saline was introduced into the intestinal lumen (6). After 2 h, CT was washed out with isotonic saline. Thereafter, the standard distension protocol was followed to determine the effects of pretreatment with CT on basal PD, the PD response to distension, and motility responses to distension (n = 8). These data were subsequently compared with those from experiments in which various antagonists were administered either before or at the same time as CT and to experiments in which antagonists were administered after basal PD changes produced by CT treatment were fully established.

Roles of Different Endogenous Bioactive Molecules

The roles of various bioactive agents and their receptors were investigated using specific antagonists. To ensure that the routes of administration and dosages of antagonists were appropriate, the only results reported are those obtained using a treatment regime that led to at least one statistically significant difference in the variety of experiments undertaken. Thus any failure to see a statistically significant difference in an element of the study could not be simply ascribed to failure of the relevant drug (or CT) to act on the system.

5-HT₃ acting at 5-HT₃ receptors. In experiments to test the role of 5-HT₃ receptors, a specific antagonist for these receptors, granisetron, was given intravenously (40 μg/kg) at the start of the experiment for control animals (n = 6), just before the administration of CT to examine effects on the induction of cholera secretion (n = 7), or 2 h after CT to examine effects on established CT secretion (n = 6). In the
last set of experiments, CT was incubated in the lumen for 1 h after the operative procedure. Luminal pressure was reduced by opening the distal clamp. This was done to minimize the effect of secretion-induced distension per se. After another hour, granisetron was administered, and changes in PD were examined.

**ACH acting at nicotinic receptors.** The protocol for these experiments was essentially identical to that using granisetron (see **5-HT acting at 5-HT_3_ receptors**) except that the nicotinic receptor antagonist hexamethonium (Hexa) was given as a bolus dose of 10 mg/kg iv and the dose was repeated at 45-min intervals to compensate for breakdown of the drug (46). The number of animals used for the control experiments was seven, whereas eight animals were used to test effects on the induction of cholera secretion and four animals were used to test effects on established cholera secretion.

**Role of prostaglandins.** To test for any potential role for prostaglandins, the prostaglandin synthesis inhibitor indomethacin (Indo) was given (5 mg/kg iv) at the start of the experiment (control, n = 8), together with CT (effects on induction of cholera secretion, etc., n = 8), or 2 h after CT (effects on established cholera secretion, n = 6). The VIP receptor antagonist [4Cl-D-Phe^6^,Leu^17^]VIP was given as an infusion at a rate of 2 μg·min^-1^·kg^-1^, which was started during the equilibration period and continued during the entire experiment, a regime shown by others to block both CT-induced secretion (33) and secretion evoked by exogenous VIP (32). Experiments were undertaken on control animals (n = 6), animals given the antagonist together with CT (induction of CT secretion, n = 6), and animals given the antagonist 2 h after CT treatment (established CT secretion, n = 4). Because the results obtained in the last series of experiments were unexpected, we also checked that [4Cl-D-Phe^6^,Leu^17^]VIP altered PD changes induced by VIP itself, delivered as an infusion (two administrations in one experiment).

**Role of muscarinic ACh receptors.** The involvement of muscarinic receptors was investigated using the muscarinic receptor antagonist atropine given intravenously (0.5 mg/kg) at the start of the experiment. While the effects of atropine are sometimes found to be transient in rats, this protocol sufficed to produce significant changes in responses (see below) 2 h later. A total of six animals was studied in the absence of CT (control), in seven animals atropine was given together with CT (induction of cholera secretion), and six animals were given atropine 2 h after CT treatment (established cholera secretion).

**Sources of Antagonists and CT**

Most drugs (TTX, [4Cl-D-Phe^6^,Leu^17^]VIP, atropine, Indo, and Hexa) were obtained from Sigma-Aldrich Chemical (St. Louis, MI). Granisetron (Kytril, Roche) was bought from Apoteksbolaget. CT was Hexa) were obtained from Sigma-Aldrich Chemical (St. Louis, MI).

**Data Analysis and Statistics**

All raw data were stored as ASCII files on a personal computer. The data processing was done in Matlab.

For each antagonist, the following aspects were analyzed: 1) the effect on the development of cholera secretion, 2) the effect on established cholera secretion, and 3) the effect on the distension response in controls and segments exposed to CT. CT significantly elevated basal PD, so we analyzed the mean PD change induced by distension rather than absolute PD levels during distension. The PD response to distension (“PD response”) was defined as the mean PD during distension minus basal PD (i.e., mean of 2 min before distension). When analyzing the acute effects of drugs on manifest cholera secretion (group 2), the response was calculated as the difference between mean PD 15 min before administration of drug and mean PD during the 30 min after drug administration.

The main aim was to study secretion arising from enteric network behavior in CT-exposed segments. However, it has been reported that CT induces a specific pattern of motor activity, giant migrating contractions (24), so we analyzed the motility changes induced by distension and CT. Quantitative analysis of the pressure signal was done at a distension pressure of 10 mmHg. The intestinal pressure curve during distension exhibited two types of activity: 1) rapid, low-amplitude fluctuations with a frequency between 30 and 50 contractions/min (see RESULTS; Fig. 4A) and 2) sustained pressure increases lasting 10–20 s with amplitudes of 1 mmHg or more that were superimposed on the high-frequency, low-amplitude fluctuations to produce a rise in baseline and much larger overall pressure increases (“cluster contractions”; see RESULTS; Fig. 4B). The frequency and amplitude of the high-frequency, low-amplitude pressure oscillations were analyzed manually by counting the number of oscillations per unit time in time windows of 20–80 s (depending on the quality of the signal). The numbers of cluster contractions were counted manually, and their duration, amplitude, and frequency (if more than one) were calculated manually. Recording cluster contractions had the additional advantage of acting as a “positive control” for a CT effect on the system in cases when secretory effects of the toxin were blocked by antagonists (see below). This was important, because the doses of different antagonists were based on literature data rather than dose-response curves.

**Statistics.** Nonparametric statistics were used whenever possible (n > 4 for paired data). The main reason for this, despite the high total number of animals, was that data from the CT-exposed segments clearly showed nonnormal distributions. When more than two groups were compared, the existence of a significant group difference was confirmed with the Kruskall-Wallis test, and then, if appropriate, individual groups were compared with the Mann-Whitney U-test. Paired data (effects of interventions) were analyzed by the Wilcoxon test. Data are given as box plots, with medians, interquartile ranges, and upper and lower data ranges. A P value of <0.05 was regarded as statistically significant.

**RESULTS**

**Control Responses to Distension and the Effect of TTX**

**PD.** In control experiments, PD increased during distension, with an initial peak followed by an adaptation. The amplitude of the initial peak became more pronounced with increasing pressure (Fig. 1). The mean PD response was approximately linearly related to the distension pressure (Fig. 2).

Serosal TTX abolished the PD response to distension at 5 mmHg and significantly reduced the response at 10 mmHg (Fig. 3). Serosal TTX also depressed the increase in PD produced by 20 mmHg, but this effect just failed to reach statistical significance (P = 0.07). Thus it was concluded that the effects of distension in increasing transmural PD were neurally mediated.

**Motility.** In all animals, low-amplitude, high-frequency oscillations of intestinal pressure (Fig. 4A) were seen during the distensions. These oscillations had a median amplitude of 0.6 mmHg and a median frequency of 40 contractions/min at a distension pressure of 10 mmHg. In 1 of the 18 animals, the high-frequency contractions were accompanied by 2 cluster contractions (Fig. 4B) during the 10-mmHg distension, but cluster contractions were not seen in the other 17 control animals.

Serosal TTX produced a significant increase in the amplitude of the high-frequency pressure oscillations seen during distension (control median 0.6 mmHg and TTX median 2.5 mmHg, P < 0.01; Fig. 4C) without producing any significant
change in their frequency (control 40 contractions/min and TTX 32 contractions/min, \( P < 0.1 \)). No clusters were seen in TTX-treated animals.

**Responses to Distension in Segments Exposed to CT**

*PD.* Two hours after exposure to CT, basal PD was significantly elevated (Fig. 5A). In CT-exposed segments, the magnitude of the PD response to distension was significantly reduced at all pressures studied and was not significantly different from zero (median response: 5 mmHg; control group 0.80 mV and CT group 0.26 mV, \( P < 0.05 \); 10 mmHg: control group 1.45 mV and CT group 0.26 mV, \( P < 0.05 \); and 20 mmHg: control group 2.41 mV and CT group 0.39 mV, \( P < 0.01 \)). The size of this reduction, however, was inconsistent, with two of the eight animals actually responding to a 10-mmHg distension with falls in PD and three others showing increases in PD during similar distensions after CT treatment. That is, the PD response to distension was not in all cases abolished by CT. The variability was not due to the elevated baseline PD after CT, because there was no significant correlation between the magnitude of the PD response to distension and the basal PD (actually a nonsignificant tendency to a larger response with higher basal PD).

*Motility.* CT had no significant effect on the frequency or amplitude of the low-amplitude pressure oscillations during distension. Cluster contractions were seen in two of eight CT-treated preparations, with each preparation exhibiting six such contractions during the 10-mmHg distension (\( P = 0.15 \) vs. the incidence in control experiments).

In summary, CT per se significantly increased basal PD, reduced or abolished the PD response to distension, and had no significant effect on the incidence of clusters during distension.

**Roles of 5-HT\textsubscript{3} Receptors**

*PD.* Granisetron had no significant effect on basal PD in control animals (data not shown) nor did it alter the PD response to distension at any pressure (10-mmHg data shown in Fig. 5C). When granisetron was given together with the toxin, the CT-induced increase in basal PD was blocked (Fig. 5A). When granisetron was administered to segments in which
CT had already induced an elevation in basal PD, there was also a marked and significant decrease in PD (Fig. 5B).

When granisetron was given together with CT early in the experiment, distensions of 5 and 20 mmHg produced increases in PD that were similar to those seen in controls (no CT treatment) and were significantly larger than those with CT alone (in both cases \( P < 0.05 \), data not shown). Pressure increases of 10 mmHg also produced PD increases similar to controls, but this effect failed to reach statistical significance compared with the PD increases seen in the presence of CT alone (\( P = 0.072 \); Fig. 5C).

Motility. No clusters occurred during distensions in granisetron-treated control animals (\( n = 8 \)). However, in CT-exposed animals pretreated with granisetron, there was a significant increase in the incidence of cluster formation (4 of 7 preparations with 9, 3, 1, and 3 cluster contractions, \( P < 0.01 \) vs. granisetron in control animals).

In summary, blockade of 5-HT3 receptors did not affect basal PD or the PD response to distension in control animals. However, it depressed the CT-induced PD elevation whenever it was administered, prevented the depression of the PD response to distension that is normally seen after CT treatment, and significantly increased the incidence of cluster formation in CT-exposed segments.

**Roles of Nicotinic Receptors**

**PD.** The nicotinic antagonist Hexa had no significant effect on basal PD in control animals. Hexa did not significantly affect the PD response to distension at 5 and 20 mmHg (data not shown) but induced a significant reduction at 10 mmHg (Hexa data shown in Fig. 6C). When administered early in the experiment, Hexa did not affect the rise in basal PD produced by CT (Fig. 6A). However, when given to segments in which CT had already induced an increase in basal PD, the same dose of Hexa produced a marked and sustained fall in PD (Fig. 6B).

Hexa did not significantly alter the magnitude of the PD response to distension in CT-exposed animals, and the effects of distension on PD in the presence of Hexa and CT were virtually identical to those with Hexa alone or CT alone (Fig. 6C).

Motility. In control animals treated with Hexa, we observed one instance of cluster formation in a single preparation. No cluster contractions were observed during distensions in CT-pretreated animals treated with Hexa (not significant vs. CT alone and Hexa alone).

In summary, blockade of nicotinic receptors had no consistent effects on basal PD or the PD response evoked by distension in controls or after treatment with CT. Similarly,
nicotinic blockade did not affect the development of CT-induced PD elevation but fully blocked an established CT-induced increase in PD while having no effect on motility.

Roles of Prostaglandins

**PD.** The inhibitor of prostaglandin synthesis Indo had no significant effect on basal PD (data not shown) or the rise in PD resulting from increased intraluminal pressure in control segments (Indo data in Fig. 7C). When given early in the experiment together with CT, Indo blocked the increase in basal PD induced by the toxin (Fig. 7A), but when given later, it had no significant effect on the established CT-induced rise in PD (Fig. 7B). At all studied pressures, Indo prevented the suppression of the PD increase produced by distension in CT-exposed segments so that distension evoked a normal increase in PD in segments treated with Indo and CT simultaneously (10-mmHg data in Fig. 7C).

**Motility.** Indo did not significantly affect the incidence of clusters during distension in control animals (1 cluster contraction in 2 of 8 preparations). However, in Indo-treated animals, CT significantly increased the incidence of cluster formation during distension (5 of 8 preparations with 6, 5, 6, 8, and 9 cluster contractions, \( P < 0.01 \) vs. Indo in control animals).

In summary, blockade of prostaglandin synthesis did not affect basal PD or the PD response to distension in control animals. However, it prevented the rise in PD produced by CT, blocked the suppression of the PD response to distension by

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**Fig. 5.** Effects of granisetron (Gran), the serotonin (5-HT) 5-HT3 receptor antagonist, on a developing and manifest CT-induced PD rise (A and B) and on the distension-induced PD rise (distension at 10 mmHg; C). Data are given as box plots with medians, upper and lower quartiles, and ranges. In A and B, we compared control vs. CT and CT vs. CT + Gran. In C, we compared control vs. Gran, control vs. CT, and CT vs. CT + Gran. Significant differences are indicated (*\( P < 0.05 \) and **\( P < 0.01 \)) with that shown in C indicating a difference between CT and control.

**Fig. 6.** Effects of hexamethonium (Hexa), the nicotinic receptor antagonist, on a developing and manifest CT-induced PD rise (A and B) and on the distension-induced PD rise (distension at 10 mmHg; C). Data are given as box plots with medians, upper and lower quartiles, and ranges. In A and B, we compared control vs. CT and CT vs. CT + Hexa. In C, we compared control vs. Hexa, control vs. CT, and CT vs. CT + Hexa. Significant differences are indicated (*\( P < 0.05 \) and **\( P < 0.01 \)).

**Fig. 7.** Effects of indomethacin (Indo), the cyclooxygenase inhibitor, on a developing and manifest CT-induced PD rise (A and B) and on the distension-induced PD rise (distension at 10 mmHg; C). Data are given as box plots with medians, upper and lower quartiles, and ranges. In A and B, we compared control vs. CT and CT vs. CT + Indo. In C, we compared control vs. Indo, control vs. CT, and CT vs. CT + Indo. Significant differences are indicated (*\( P < 0.05 \) and **\( P < 0.01 \)).
CT, and induced cluster formation after CT treatment. In contrast, it had no effect on established CT-evoked PD increases.

**Roles of VIP Receptors**

**PD.** The VIP receptor antagonist [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP tended to reduce basal PD in control segments, but this effect was not statistically significant (data not shown). [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP prevented the CT-induced PD rise when given together with the toxin (Fig. 8A) but had no significant effect when given to a segment in which CT had already induced an elevated PD (Fig. 8B). However, [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP profoundly depressed the rise in PD produced by VIP itself (Fig. 9).

In control segments, [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP depressed the increase in PD produced by distension at all pressures tested (Fig. 3). This effect was virtually identical to the depression of the PD response to distension by CT pretreatment, and, unsurprisingly, treatment with [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP and CT together also suppressed the normal PD response (Fig. 8C).

**Motility.** In controls, treatment with [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP appeared to markedly enhance the incidence of cluster contractions during distensions, with such contractions being seen frequently in three of six preparations (9, 9, and 7 clusters, P = 0.01 vs. the incidence in controls).

No cluster contractions were observed CT-pretreated animals in the presence of [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP, even though the latter increased the incidence of these contractions in control animals.

**Roles of Muscarinic Receptors**

**PD.** The muscarinic receptor antagonist atropine had no significant effect on basal PD (data not shown) or on the PD response to distension in control animals (Fig. 10C). Nor did it affect the CT-induced PD rise, whether given early (Fig. 10A) or late in the experiment (Fig. 10B). However, the increase in PD evoked by distension was significantly enhanced by atropine over the response seen with CT alone; indeed, the median increase was greater than that seen in control segments (Fig. 10C).

**Motility.** No cluster contractions were seen during distension in atropine-treated control animals. However, atropine significantly increased the incidence of clusters after CT pretreatment (4 of 6 preparations with 6, 3, 2, and 2 clusters on a 10-mmHg distension, P < 0.01 vs. atropine in control animals).

In summary, the VIP receptor antagonist [4Cl-d-Phe<sub>6</sub>,Leu<sub>17</sub>]VIP had no significant effect on basal PD in control animals but blocked the PD response to distension. The latter action was unchanged in the presence of CT, although the antagonist prevented the rise in basal PD evoked by CT. Once a CT-induced rise in basal PD was established, the VIP antagonist had no effect. On the motility side, this antagonist increased the incidence of cluster contractions during distension in controls but had no significant effect in CT-exposed animals.

**DISCUSSION**

This study has shown that CT has several pharmacologically distinct effects within the proximal small intestine of the rat.
data suggest that these elements are controlled by the immune system rather than being acutely neurally regulated (20, 36, 37). There is also an atropine-sensitive system for transepithelial uptake of large molecules, but this system is too slow to account for the rapid distension-induced electrical responses (44). A permeability mechanism behind the distension response therefore seems exceedingly unlikely.

In the absence of substrates for Na\textsuperscript{+}-solute cotransport in the duodenum, active anion secretion (chloride or HCO\textsubscript{3}\textsuperscript{-}) is the main source of the short-circuit current in the proximal small intestine. Electrogenic chloride secretion occurs through the CFTR. The PD response to sustained pressure increases is abolished in patients with cystic fibrosis, a disease caused by CFTR dysfunction, providing seemingly conclusive evidence for the involvement of this channel in the PD response to distension (2). The CFTR is, however, permeable not only to chloride but also to HCO\textsubscript{3}\textsuperscript{-}. The relative chloride/HCO\textsubscript{3}\textsuperscript{-} conductance of the CFTR is \textasciitilde 4:1 (38). In the proximal duodenum, distension induces a 75\% increase in basal HCO\textsubscript{3}\textsuperscript{-} secretion (40); thus a contribution of increased HCO\textsubscript{3}\textsuperscript{-} secretion through the CFTR to the PD increase evoked by distension cannot be excluded a priori. However, the segment used in the present study was located considerably more distally than that in the cited study (40). This is important, because at least in humans, HCO\textsubscript{3}\textsuperscript{-} secretion decreases very rapidly in the aboral direction and amounts to only 10\% of that in the duodenal bulb in a segment distal to the pancreatobiliary duct, i.e., the approximate level of our test segment (17). This fact, together with the 4:1 chloride/HCO\textsubscript{3}\textsuperscript{-} conductance relationship of the CFTR makes it very unlikely that increased HCO\textsubscript{3}\textsuperscript{-} secretion is the main mechanism behind the PD response in the proximal small intestine. The relative contribution of chloride or HCO\textsubscript{3}\textsuperscript{-} probably depends on the expression of basolateral transporters [Na-K-2Cl cotransporter (NaKCC) or NaHCO\textsubscript{3} cotransporter]. Accordingly, the short-circuit response to distension in the distal colon is abolished by bumetanide (blockade of NaKCC) and by removal of chloride from the medium (18).

With regard to the role of HCO\textsubscript{3}\textsuperscript{-} in the CT response, Tantisira et al. (47) reported that CT induced a marked increase in HCO\textsubscript{3}\textsuperscript{-} secretion in the rat jejunum in vivo. However, in contrast to the full inhibition of a manifest CT-induced PD increase in the present study, Hexa only inhibited HCO\textsubscript{3}\textsuperscript{-} secretion by about 20–25\%. This observation is inconsistent with the idea that CT reduced the PD response by inducing HCO\textsubscript{3}\textsuperscript{-} secretion that then “competes” with chloride within the CFTR. If this had been the case, a larger, not a smaller, effect of Hexa on HCO\textsubscript{3}\textsuperscript{-} secretion than on PD would have been expected. Our interpretation of the PD signal is thus that it mainly reflects electrogenic chloride secretion through the CFTR. To sort out these issues in detail, the present experiments will have to be repeated with a pH stat setup.

**CT Effects on Basal Secretion**

It has previously been reported that nicotinic receptors are essential for CT-induced secretion (6), and we confirmed this finding provided that hypersecretion had been established as a result of prior treatment with the toxin. On the other hand, Hexa did not prevent the induction of hypersecretion when administered at the same dose at the same time as the CT. Thus

**Cellular Mechanisms Behind the PD Response to Distension and CT**

The interpretation of the PD signal is of course crucial when analyzing these data. Theoretically, a PD increase can, according to Ohm’s law, be due to either an increase in epithelial resistance or increased net current. There is no evidence for any sustained permeability effects of duodenal distension at moderate pressures (15, 40). Similarly, CT has only very modest effects on jejunal permeability in rats (43). Admittedly, recent progress in permeability research has revealed a great complexity of the tight junctions between mucosal enterocytes, including the existence of regulatory elements, but available...
nicotinic blockade has differential effects: being able to block the enhanced secretion but not to prevent the mechanism leading to the ongoing enhancement. The reverse applied to the VIP receptor antagonist [4Cl-d-Phe6,Leu17]VIP, which prevented the induction of the CT-induced hypersecretion but had no effect once hypersecretion was established. Both these two observations challenge the currently accepted view of the mechanism by which CT acts to produce secretion.

The present results indicate that induction of the hypersecretion and its maintenance are two separate mechanisms sharing some common features (e.g., involvement of 5-HT3 receptors). We (9) have recently reported that an anatomically realistic model of the submucous plexus can account for such a divergence in mechanism. This model predicts that the induction of hypersecretion depends on positive feedback within recurrent excitatory networks of VIP neurons and the intrinsic sensory neurons of the submucosa. CT and both heat-labile and heat-stable Escherichia coli enterotoxin produce hypersecretion via activation of VIP neurons, and it is usually thought that this is due to release of VIP from secretomotor neurons (1, 5, 33). However, the VIP secretomotor neurons also form recurrent excitatory networks within the submucous plexus (39), and the failure of [4Cl-d-Phe6,Leu17]VIP to block already established CT-evoked secretion suggests that VIP may act within these networks rather than solely on enterocytes. In in vivo experiments, VIP evokes secretion both indirectly via activation of neurons and directly via an action on the mucosa (23). Furthermore, VIP has been reported to induce slow depolarizations in submucous neurons that were presumably VIP secretomotor neurons (27). Thus activation of the VIP secretomotor neurons would be expected to excite both mucosal secretion and the recurrent network of VIP neurons. The present results suggest that this may be via different receptors with the neural receptor being more sensitive to [4Cl-d-Phe6,Leu17]VIP than the mucosal receptor. Indeed, in a preparation of the isolated rat jejunal mucosa, [4Cl-d-Phe6,Leu17]VIP failed to block VIP-induced anion secretion (11). On the other hand, it did block VIP-induced jejunal secretion in vivo (32). Two distinct classes of VIP receptor have been cloned: VPAC1 and VPAC2. In humans, VPAC1 is abundantly expressed in the crypts and submucous plexus, whereas VPAC2 are expressed (interestingly) in neuroendocrine cells and in smooth muscle and blood vessels (41). It has also recently been shown that a VPAC1 receptor blocker, PG-97-269, blocks VIP-induced, field stimulation-induced, and CT-induced secretion in vitro and in vivo (1), suggesting a major role for VPAC1 in secretomotor regulation.

The finding that nicotinic receptors are not required for the induction of CT-evoked secretion is also compatible with our modelling study (9). In our model, hyperactivity in the recurrent networks is enhanced by fast synaptic transmission of the type mediated by nicotinic receptors but is not dependent on it. However, once the positive feedback within the circuit is sufficient to produce sustained firing, this can be maintained in the absence of the slow depolarizing potentials mediated by VIP, if intrinsic sensory neurons can communicate with VIP secretomotor neurons via fast synaptic transmission like that mediated by ACh acting at nicotinic receptors (9).

**Effects of CT on Secretion Evoked by Increased Intraluminal Pressure**

Increasing intraluminal pressure produces a marked increase in transmural PD, indicating that there is an increase in electrogenic chloride secretion. This effect is mediated by neural activity, because it is markedly depressed by TTX, which prevents the opening of voltage-dependent sodium channels and hence blocks most neural activity. It clearly requires activation of VIP receptors, because the increase in PD resulting from a pressure increase is prevented by [4Cl-d-Phe6,Leu17]VIP. By analogy with the effects of this antagonist on CT-evoked secretion, it is possible that the antagonist partially acts by blocking transmission between neurons rather than directly modifying secretomotor transmission, but our data do not allow us to discriminate between these mechanisms.

The blunted PD response to distension in CT-exposed segments is in fact exactly what one expects in a situation with uncontrolled firing in VIP neuron networks. As might be expected from the results in the absence of CT, this inhibition was not reversed by [4Cl-d-Phe6,Leu17]VIP. However, it is reversed by other agents that prevent induction of secretion by CT, notably the 5-HT3 antagonist granisetron and the prostaglandin synthesis antagonist Indo. These agents are thus able to restore the reactivity of the network, which is again compatible with our basic hypothesis.

Atropine prevented the CT-induced inhibition of PD increases evoked by distension, despite having no effect of its own on either enhanced basal secretion caused by CT or the PD increase evoked by distension. This (somewhat surprising) observation has two implications. First, it corroborates that the depression of pressure-induced PD increases by CT is not simply due to the increased basal PD, by reducing the scope for further PD increases. Second, it suggests that CT is acting to release ACh somewhere within the enteric neural circuits and that this is then acting to either directly or indirectly inhibit the neural pathway that couples intraluminal pressure and PD. Alternatively, CT may act to desensitize neural VIP receptors, thereby mimicking the effect of [4Cl-d-Phe6,Leu17]VIP, and atropine may act to enhance transmission in the pathway that directly excites VIP neurons. This enhanced release, however, might only have a substantial effect on the secretion evoked by distension when the facilitatory effect of VIP is removed. Muscarinic receptors have been implicated in both presynaptic inhibition and postsynaptic excitation in intracellular studies of myenteric and submucous neurons (13, 28, 31, 35). The functional classes of neurons showing these responses have not been identified. Thus whether CT excites an inhibitory pathway or inhibits an excitatory pathway via muscarinic receptors remains to be determined.

**Effects of CT on Motility Evoked by Increased Intraluminal Pressure**

Two qualitatively distinct types of motility were observed during distensions in this study. In all preparations, distension enhanced the amplitude of high-frequency contractions (30–40 contractions/min). The amplitude of these contractions was markedly enhanced by blocking neural activity with TTX, suggesting that they were probably due to the intrinsic pace-
maker activity of the intestinal smooth muscle, the slow waves. The enhancement by TTX implies that distension evoked a substantial inhibition of the intestinal smooth muscle, presumably by exciting inhibitory reflex pathways. Alternatively, TTX might be depressing a tonic inhibitory drive to the muscle (52). Interestingly, atropine also enhanced these high-frequency contractions, although to a lesser extent than TTX. This suggests the involvement of muscarinic receptors in inhibitory pathways to the muscle.

In control animals, distension evoked a quite different contractile pattern, cluster contractions, although these were very rare. A notable effect of CT was to increase the likelihood of observing cluster contractions and the number of clusters evoked when such contractions were observed. This phenomenon was most prominent, and only reached statistical significance, after blockade of prostaglandin synthesis, 5-HT3 receptors, or muscarinic receptors. Thus the same antagonists that reverse the inhibition of distension-evoked secretion by CT also enhance motor patterns induced by CT. This observation clearly indicates that CT can excite more than one pathway within the enteric nervous system, because both Indo and granisetron blocked the induction of hypersecretion by this toxin while enhancing its motility effects. Indeed, these results imply that the conventional idea that CT exerts its effect on enteric neurons by releasing 5-HT from enterochromaffin cells to excite enteric neurons via 5-HT3 receptors can only be partially true at best, with the motility pathway clearly operating via a different mechanism. An alternative explanation is that the mechanism by which CT operates is indeed via the release of 5-HT but that this acts on enteric neurons via another 5-HT receptor (21), thus exciting separately secretion and motility pathways that employ 5-HT3 receptors within the neural circuitry itself. Synaptic potentials mediated by 5-HT3 receptors have been identified in submucosal neurons (30), consistent with a role for these receptors in secretomotor pathways, whereas studies of motor pathways in the myenteric plexus have also implicated 5-HT3 receptors in transmission within excitatory pathways supplying the circular muscle (29).

In summary, this study provides evidence that the conventional view as to how CT acts to alter intestinal behavior requires significant modification or reevaluation. The mechanisms for building up an increased secretion (more formally, an increased PD) seem to be different from those required to maintain it, at least in the case of fast transmission via nicotinic synapses. The clear effect of granisetron on both developing and manifest secretion suggests a key role for 5-HT3 receptors, but the dissociation of the secretory and motility effects of blocking these receptors implies multiple sites at which these receptors play a role. Our results are compatible with the hypothesis that CT induces uncontrolled firing in submucous plexus networks, due to massive input from sensory neuron networks. Once this state is established, it is maintained by nicotinic receptors and 5-HT3 receptors but becomes independent of prostaglandin synthesis. Pharmacological restoration of controlled network activity is a potential new target for treatment of diarrheal disease. Accordingly, we (22) have recently shown that both granisetron and [4Cl-d-Phe6,Leu17]VIP, when given together with the virus, ameliorated the clinical course (diarrheal symptoms) of rotavirus enteritis in mice.

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