INTRODUCTION

Cholera toxin (CT) causes severe secretory diarrhoea, and acts primarily on enteroocytes. It irreversibly activates the basolateral membrane adenylate cyclase, raising cAMP and stimulating (Cl⁻) secretion (1). Recent in-vivo studies have raised the possibility that the enteric nervous system may play an important role in modulating the secretory activity of CT (2,3,4). The role of myenteric plexus in CT-induced secretion has also been proposed (5).

In the colonic epithelial cell lines, cholinergic neurotransmitters interact with cAMP agonist vasoactive intestinal polypeptide (VIP) to produce a synergistic activation of chloride secretion (6,7). In addition, carbachol potentiates the secretion caused by cGMP-mediated *Escherichia coli* heat-stable toxin in T84 cells (colonic epithelial cell lines) (8).

The aim of this study was to investigate in vitro if the enteric neurotransmitters were involved in the secretory action of cholera toxin.
METHODS AND MATERIALS

Cell studies: HT29-19A colonic adenocarcinoma cell lines were kindly supplied by Dr. C. Laboisse, Institut National de la Sante’et de la Recherche Mèdicale (INSERM), Paris. The cells were cultured in Dulbecco’s modified Eagle medium containing 10% of foetal calf serum and used between passage 38 and 50. For measurements of transepithelial ion transport, the cells were seeded at density of 0.5x10^6 cells/cm^2 onto collagen-coated Millipore filters and cultured for 10 to 14 days (9). Filters were mounted in an Ussing chamber and bathed, on both mucosal and basal surfaces with a bicarbonate-buffered Ringer solution containing 10 mM glucose. Under these conditions, the HT29-19A cell exhibited the following electrical parameters: short-circuit current (Isc) potential difference (PD) and resistance as previously described (10,11). Isc was an used indicator of chloride secretion in this cell line (6). Monolayers were equilibrated for 15-20 minutes after mounting before the addition of agonists.

Determination of cellular cAMP: To measure the cellular cAMP levels, filters were mounted vertically in a heated Perspex block (37 °C) that allowed agonists’ access to both basolateral and apical surfaces (total chamber volume, 1.5 mL). After incubation for 5-30 minutes, the reaction was stopped by rapid washing with an ice-cold medium (less than 5 seconds). Filters were removed into supercooled absolute ethanol and extracted overnight at -70 °C. Extracts were dried under nitrogen, reconstituted in Tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.5) containing EDTA and assayed for cAMP, using a sensitive binding assay as previously described (12).

Tissue studies: The ileum from male Sprague Dawley rats (250 to 300 g) was removed and stripped of its muscle layers. Tissues were mounted in Ussing chambers with their mucosa bathed in Ringer-glucose and the serosa in Ringer-mannitol. The preparations were equilibrated for 30 to 45 minutes prior to the addition of drugs (13).

Electrical measurements: The spontaneous trans-mucosal electrical PD was measured via electrode bridges (containing 3 mol/KCl in 3% (w/v) agar and matched calomel half cells. A high-impedance digital voltmeter Isc was delivered via silver/silver chloride electrodes and 1 mol/L NaCl in 1% (w/v) agar bridges. The electrodes were connected to a voltage clamp for fluid resistance between the PD-sensitive bridges (14).

Tissue resistance was calculated using Ohm’s law by dividing PD by short-circuit current and multiplying by the surface area (0.64 cm^2 for tissues and 1 cm^2 for monolayers) (10).

Chemicals: CT was obtained from Sigma Chemicals Co., St. Louis, MO, USA.

Calculation: All values were expressed as means±SEM of 10 experiments. Statistical comparisons were performed with the Student’s t-tests.
RESULTS

Effects of CCh on CT-induced secretion in HT29-19A monolayers: CT (2 µg/mL) stimulated a progressive increase in short-circuit current across HT29-19A monolayers which peaked 175 minutes after addition. Pretreatment with CCh (100 µM ) 10 minutes prior to addition of CT resulted in a 2-fold stimulation of the CT response. CCh alone elicited a small (4 µA to 5 µA) and a transient increase in short-circuit current (Fig.1). Both effects of CCh were abolished by pretreatment with 100 µM atropine (p<.001) (results not shown).

Potentiation of CT-stimulated cAMP accumulation by CCh in HT29-19A: CT elevated cellular cAMP in HT29-19A cells over a time course similar to that observed for the activation of chloride secretion. CT (2 µg/mL) increased the cAMP levels in the HT29-19A cells from 0.71±0.03 pmol/mg protein to 9.0 pmol in 120 minutes. In the presence of CCh (100 µM) however, CT increased the cAMP levels to 44.89±0.9 pmol/mg, 5-fold the response to CT alone (Fig. 2). CCh alone caused only a minor increase in the cAMP levels (basal: 0.71±0.03; CCh 100 µM: 1.2±0.06 pmol/mg) (p<.001).

Effects of CCh pretreatment on CT-induced secretion in rat ileal mucosa: Initial studies suggested that CCh was unable to potentiate any CT response in the rat intestine in vitro. Tissues, treated with CT alone and with CT following pre-treatment with 10 µM CCh, exhibited an increase in Isc (also at 200 minutes, CT 19.7±4.5 vs CT+CCh 16.5±4.0) (Fig. 3).
**Effects of CCh on CT responses in indomethacin-treated ileal mucosa:** Tissues, pretreated with 10 μM indomethacin to inhibit endogenous prostaglandins, exhibited a markedly lower basal Isc compared to non-indomethacin-treated tissues (9.3±2.1 μA/64 cm² vs 27.2±4.1, p< 0.01). Under these conditions (Fig. 4), CCh pretreatment induced a significant (p<0.01) potentiation of the CT response. This rise was similar in time course and magnitude to that observed in HT29-19A monolayers (Isc CCh+CT: 45±1.6 vs CT alone 25±1.2; p<0.01).
DISCUSSION

In the present study, CCh caused a synergistic response to the CT action both in colonic cell lines (HT29-19A) and rat ileal tissues. Colonic cells, HT29-19A, appeared to be a good model for studying CT-induced secretion. CT significantly increased the Isc and PD at doses from 0.001 µg/mL to 2 µg/mL (data not shown). The cell lines are free of neuronal elements, and are, therefore, ideal for studying the interaction between CT and cellular receptors of neurotransmitters (data not shown). CCh was used as neurotransmitter on the cell lines to examine the effects of muscarinic activity on the action of CT (15). The results clearly showed that pretreatment with CCh enhanced the CT response in terms of both rate of Isc and peak increase in Isc after the addition of CT. This is not an additive effect, since the small Isc response to CCh was transient, and had almost returned to basal before the CT response began. This represents an amplification of the CT by CCh. Similar potentiation has been demonstrated in the T84 cell line with CCh in conjunction with *E. coli* enterotoxin which raised the cellular cGMP (8,16) and the cAMP agonists like PGE2/VIP (16,9).

The interesting fact is that the effects of CCh on CT are longer lasting than its own effect on basal Isc. CCh potentiated the response to CT despite the fact that the time from the addition of CCh to the initiation of the CT Isc response was 40 to 45 minutes. Therefore, it seems that the potentiating effect of CCh in the HT29-19A cells lasts longer than its ability to activate chloride secretion. This suggests that the effects of CCh may be mediated through cellular pathways. It was further confirmed in the HT29-19A cell lines that there was a huge rise of cAMP (5-fold) in response to CCh and CT.

The mechanism by which carbachol potentiates the secretory response to cAMP agonists is not fully understood. Ca²⁺ agonists potentiate cAMP (7,10). In the present study, CCh+CT raised the level of cAMP 5 folds compared to CT alone.

There is an evidence that a range of Ca²⁺ mobilizing agonists potentiates forskolin and VIP-stimulated cAMP production in the HT29-19A cells by a mechanism mediated by both increases in intracellular Ca²⁺ and activation of protein kinase C (9).

However, in T84 cells, CCh potentiates dibutyryl cAMP-mediated secretion, suggesting a site of potentiation distal to cAMP production (9). This may be a second mechanism of potentiation which has been suggested to act through a cooperative interaction between apical Cl⁻ and basolateral K⁺ channel with little effect on apical Cl⁻ channel alone (6).

The additional stimulation of K⁺ secretion from the basolateral membrane when both cAMP and Ca²⁺ are added together is suggested to cause a dramatic increase in the driving force for Cl⁻. It is not known whether this mechanism also operates in the HT29-19A cells, although preliminary observations suggest that these cells have a Ca²⁺-activated basolateral K⁺ conductance (17). Protein kinase C has been shown to play an important role in regulating transport in both T84 and HT29-19A cells (10,18). It is possible that the prolonged response of CCh could be mediated through protein kinase C. Previous studies have shown that protein kinase C activation has multiple effects on T84 monolayers, including stimulation of non-receptor agonists as forskolin (10).

Further studies are needed to define the role of different parts of the Ca²⁺ pathways (Ca²⁺/protein kinase C) in mediating the potentiation of the CT response in HT29-19A monolayers.

The synergism in colonic epithelial cell lines was also shown in rat ileal tissues, which are composed of variety of cells (goblet cells, paneth cells, etc).
Isolated mucosa: The potentiating action of CCh on the CT response in the isolated mucosa was revealed only when production of endogenous prostaglandins was blocked by indomethacin. One hypothesis to explain it is that, during isolation and mounting the ileum, there is production of endogenous PGE2 from subepithelial tissues (12). This PGE2 can increase epithelial cAMP, and stimulate basal ion secretion which would explain the higher Isc in the absence of indomethacin (19). Under these conditions, the partial stimulation of the cAMP-dependent secretory pathway before CCh addition could reduce or abolish the ability of CCh to further stimulate secretion when CT is added. When the endogenous synthesis of PGE2 is suppressed, basal Isc is lower, and CCh is then able to elicit a potentiating response with CT. In the cell system where there is no subepithelial tissues, there is no appreciable production of PGE2 and, therefore, CCh-induced potentiation would not be suppressed. There has been recent speculation that the full secretory response of CT depends on release of PGE2 (17). Yet, in the present study, although indomethacin ‘unmasked’ the potentiation of CT by CCh, the actual rise of CT alone in tissues was not inhibited by indomethacin. These observations further support the finding that prostaglandins are not mediators of the intestinal response by CT or CT-induced elevation of cAMP (12,13).

Further studies are required to confirm the involvement of the enteric nervous system in modulating the secretory response in isolated mucosal preparations. Cassuto and his colleagues have demonstrated that cholinergic pathways may be of clinical relevance to understand why some patients exhibit different sensitivity to CT (2,3,4).

Recently, HT29-19A has shown that G proteins are involved in the mechanism of secretion (21,22). Further studies are required to locate the role of G proteins in the cholinergic potentiation of cholera toxin.
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REFERENCES


