SMALL INTESTINE

Intracellular potentiation between two second messenger systems may contribute to cholera toxin induced intestinal secretion in humans

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Background: Cholera toxin (CT) acts on intestinal epithelial cells both directly and indirectly via activation of a secretory neural reflex. The reflex may release acetylcholine as one of its final neurotransmitters. This opens up the possibility of a third mechanism of action for CT, namely a synergistic interaction between two secretagogues acting on different second messenger systems within the epithelial cell.

Aims: To establish evidence for cholinergic innervation to human ileal epithelial cells and to investigate whether CT potentiates the action of acetylcholine on human intestinal epithelial cells.

Methods: Transverse sections of human ileum were examined for mucosal cholinergic nerves and M3 muscarinic receptors using antibodies raised to choline acetyltransferase and M3 receptors. Short circuit current (Isc) responses and ion flux movements were elicited from T_{84} epithelial cell monolayers set up in Ussing chambers.

Results: Immunohistochemistry of native human ileal mucosa revealed the presence of both cholinergic nerves and muscarinic M3 receptors located to the basolateral domain of epithelial cells. Secretory responses of T_{84} cell monolayers to acetylcholine were greatly potentiated in the presence of CT. This effect, substituting forskolin for CT, was mirrored by increases in basolateral ^{86}Rb and apical ^{125}I efflux. Charybdotoxin plus apamin reduced both Isc and ^{86}Rb efflux evoked by acetylcholine, in the presence of forskolin.

Conclusions: Human ileal mucosa receives a direct cholinergic innervation to its epithelial cells. Secretory effects of acetylcholine on epithelial cells are augmented in the presence of CT. Such a synergistic response is dependent on optimum opening of basolateral potassium channels by acetylcholine and apical chloride channels by CT. The interaction may contribute to the mechanism of action of cholera toxin induced secretory diarrhoea.

Vibrio cholerae is known to cause profuse watery diarrhoea primarily through the action of cholera toxin (CT) on the intestinal mucosa. On isolated intestinal mucosal preparations mounted in Ussing chambers, CT increases cAMP levels and short circuit current (Isc).[1] The apparent non-neural nature of this action was confirmed by similar findings using the T_{84} human colonic cell line[2] and by observations that CT elicits secretory responses from human ileal mucosa treated with tetrodotoxin.[3] However, both the magnitude of diarrhoea in cholera and the likelihood that access of the bacterium to intestinal crypts may be restricted has prompted investigations into additional mechanisms of action that may amplify the effects of the toxin.

Convincing evidence that CT activates a secretomotor neural reflex arose from observations that CT induced diarrhoea was reduced by drugs such as local anaesthetics, tetrodotoxin, and hexamethonium.[4] The reflex required the presence of the myenteric plexus[5] and utilised secretomotor neurones, the most prominent of which appear to be those containing vasoactive intestinal polypeptide (VIP) and acetylcholine (ACh).[6 7]

The major body of evidence supports a role for ACh as a putative neurotransmitter directly involved in epithelial cell electrolyte transport and comes from anatomical, functional, and receptor binding studies.[7–9] However, investigations using muscle stripped preparations of rat colon set up in Ussing chambers have demonstrated that ACh also acts indirectly through activation of uncharacterised secretomotor neurones.[10 11] Anatomical studies in the guinea pig and pig have demonstrated cholinergic neurones projecting to the intestinal mucosa.[7 12 13] However, cholinergic neurones could not be demonstrated innervating mucosa of the human colon or ileum.[14 15] This may represent species differences or more likely a false negative (SJ Brookes, personal communication).

In humans therefore, if ACh released by CT exerts a direct action on epithelial cells, then it is possible that there may be a synergistic interaction between the two secretagogues at the level of the enterocyte.[16 17]

Using native human ileal mucosa and human intestinal epithelial cells (T_{84}), we have investigated whether there is cholinergic innervation to human ileal enterocytes and if there is a synergistic interaction between CT and the neurotransmitter ACh. Positive evidence for both would be clinically significant in representing an additional mechanism whereby diarrhoea would be worsened in cholera.

METHODS

Cell culture

T_{84} cells, a human colonic epithelial cell line, were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK) and used between passages 70 and 85. The methods have been described previously.[18] One million or four million cells were seeded onto 12 mm or

Abbreviations: CT, cholera toxin; Isc, short circuit current; IBMX, 3-isobutyl-1-methylxanthine; VIP, vasoactive intestinal polypeptide; PGP, protein gene product; ChAT, choline acetyltransferase; TBS, Tris buffered saline; M3, muscarinic type 3 receptor; TER, transepithelial resistance

24 mm internal diameter collagenised semipermeable membranes, respectively (Costar Snapwell inserts, 0.4 μM pore diameter) and after nine or 11 days the inserts with attached confluent monolayer were used for experimentation.

Isc measurements

Monolayers on 12 mm inserts were placed into a modified Ussing chamber for continuous recording of Isc. Monolayers were bathed on both sides with 10 ml of circulating gassed Kreb's buffer kept at 37°C (2.5 ml for isotope experiments). Electrical measurements were carried out as discussed previously.[18] CT was applied to the apical domain of monolayers unless indicated otherwise. All other drugs were given basolaterally. No monolayer received more than one concentration of a given compound.

Measurement of ion efflux

We used previously validated methods in T_{84} cells[19] to measure basal and stimulated cellular effluxes of ^{125}I and ^{86}Rb as a measure of the activities of apical chloride and basolateral potassium channels, respectively. Cells were seeded onto 12 mm inserts as described, and cultured until confluent. Inserts and confluent monolayers were loaded for 180 minutes in medium containing either 2.5 μCi/ml ^{125}I or 1 μCi/ml ^{86}Rb. Inserts were rapidly washed following which 500 μl aliquots of radioactive Kreb's were removed and replaced with the same volume of isotope free Kreb's sequentially at two minute intervals for a 22 minute period from either the apical or basolateral compartments for ^{125}I and ^{86}Rb experiments, respectively. At the end of the experiments, isotope remaining in the monolayer was extracted with 0.1 M NaOH and the extract solution counted with the aliquot samples. For the efflux experiments, data were corrected for sampling volume (0.5 ml from a 2.5 ml reservoir) and previous sampling loss due to buffer replacement. Efflux rate constants were calculated as described by Venglarik and colleagues.[19] Briefly, release of isotope from monolayers (efflux) was calculated as a percentage of the total (tissue and solution) radioactivity present at the beginning of a collection period. Rate constants (r) were calculated by the following equation:

$$r = [\ln(R_2) - \ln(R_1)]/(t_1 - t_2),$$

where R_1 and R_2 are the percentages of counts released in the cell layer at times 1 (t_1) and 2 (t_2), respectively.[19] Experiments were performed with forskolin as a cAMP dependent agonist, because it was not technically possible to use CT within the time frame of the experiments, due to the slow onset of action of the toxin.

cAMP measurements

Cells were cultured as previously described and then seeded onto large 24 mm diameter inserts. When confluent, the inserts were rapidly washed and bathed in pregassed Kreb's at 37°C in a 5% CO_2 atmosphere. 3-Isobutyl-1-methylxanthine (IBMX) 0.1 mM was added to Kreb's at the beginning of each experiment. Agonists or antagonists were then added to the appropriate apical or basal domains. At the end of the experiments, bathing fluid and cells were removed and frozen at −20°C prior to cAMP measurements. cAMP was then quantitated by protein binding assay as previously described by Kapas and colleagues,[20] and expressed as pmol/cm^2 of cell monolayer.

Data analysis

All data are expressed as arithmetic mean (SEM) where n = number of monolayers. Statistical comparisons used the two tailed Student's t test with $p<0.05$ being taken to represent a significant difference.

Drugs and materials

CT was obtained from the Swiss Serum and Vaccine Institute (Berne, Switzerland). ^{125}I and ^{86}Rb were obtained from Amersham Pharmacia Biotech (UK). ACh chloride, barium chloride, cAMP, dimethyl sulphoxide, forskolin, histamine dihydrochloride, hyoscine hydrobromide, IBMX, charybdotoxin, apamin, and vasoactive intestinal peptide were all obtained from Sigma (UK).

Immunohistochemistry

Materials

Formalin fixed paraffin embedded 4 μm transverse sections of ileum, mounted on chrome-alum coated glass slides, were collected from pathology archives. Tissue had been removed from five patients (median age 33 years (range 29–46)) at the time of right hemicolectomy for non-obstructing caecal tumours. A senior histopathologist had reported all ileal specimens as both macro and microscopically normal. Ethics permission for the use of archival material was obtained from the East London Health Authority ethics committee (T/02/027).

Methods

Primary antibodies to protein gene product 9.5 (PGP), a marker of total neural tissue, colocalised with choline acetyltransferase (ChAT), were used to identify cholinergic nerves in the mucosa and submucosa. Anti-M3 muscarinic receptor antibodies were used to localise M3 receptors in the same layers, using serial sections. Slides were deparaffinised using standard techniques. Antigen retrieval was achieved by incubation in 2 μg/ml proteinase K (Promega, Madison, Wisconsin, USA) at pH 7.6 and room temperature for 20 minutes. Sections were then rinsed in Tris buffered saline (TBS) at pH 7.6 and room temperature for five minutes before blocking non-specific binding of the first secondary antibody with 5% horse serum. Anti-PGP and anti-ChAT antibodies were then applied sequentially. Sections were initially incubated with primary anti-PGP antibodies (table 1) at 37°C for 90 minutes. Slides were washed in TBS and incubated with a secondary biotinylated antibody (Vectastain Universal Elite ABC kit, Vector laboratories, Peterborough, UK) at room temperature for 30 minutes and rinsed again in TBS.

A tertiary peroxidase labelled avidin was applied at room temperature for 20 minutes, before developing with a Tyramide Signal Amplification Fluorescein system (NEN Life Science Products, Boston, Massachusetts, USA) for five minutes. Slides were washed with TBS and non-specific binding of the second secondary antibody was blocked using 5% goat serum. Sections were then incubated with primary anti-ChAT antibodies at 4°C for 18 hours and rinsed in TBS. A TRITC-immuno conjugated secondary antibody (Nordic Immunology, the Netherlands) was added for 30 minutes. Slides were finally mounted in Immuno-Mount (Shandon, Pittsburgh, Pennsylvania, USA).

M3 receptors

M3 receptor epitopes were exposed on serial sections, using microwave for four minutes in citrate buffer (pH 6.6) followed by localisation with anti-M3 antibodies using the method described previously for PGP.

Analysis

Sections were viewed using a Leica epifluorescence microscope (Leica imaging systems Ltd, Cambridge, UK) and appropriate filter blocks. Multiple digital image captures were taken of the mucosa, muscularis mucosa, and submucosa at ×200 magnification, using Leica DC200 software, in order to assess the distribution of staining and the degree of colocalisation of ChAT with PGP.

Table 1 Primary antibodies used for immunohistochemistry

Antibody	Manufacturer	Type	Code	Raised in	Concn	Raised against	Antigen retrieval method
PGP 9.5	Ultraclone UK	Monoclonal	31A3	Mouse	1:400	Human	Proteinase K
ChAT	Schemann, Hannover	Polyclonal	P3/YEB	Rabbit	1:500	Human	Proteinase K
M3	Santa Cruz Biotechnology	Polyclonal	SC-7474	Goat	1:100	Human	Microwave

PGP 9.5, protein gene product 9.5; ChAT, choline acetyltransferase; M3, muscarinic type 3 receptor.

RESULTS

Response of T_{84} monolayers to CT and ACh

Transepithelial resistance (TER) of T_{84} monolayers was 595 (45) Ω/cm^2. Basal Isc was 2 (1) μA/cm^2. CT (0.0001–0.1 μg/ml) applied to the apical domain of T_{84} cell monolayers produced a concentration dependent increase in Isc (fig 1). Time to onset of effect and to peak effect were also dependent on concentration. When given basolaterally, the response to CT (0.1 μg/ml) reached a peak effect more rapidly (54 (4) μA/cm^2 at 90 minutes; n = 5) compared with when given apically (46 (3) μA/cm^2 at 130 minutes; n = 5, $p<0.05$). Compared with an aqueous control, CT decreased TER. For apically applied toxin, TER decreased from 406 (96) Ω/cm^2 to 268 (32) Ω/cm^2 ($p<0.05$, n = 5), and for basolaterally applied toxin, TER decreased from 633 (111) Ω/cm^2 to 348 (48) Ω/cm^2 ($p<0.05$, n = 5). Control values were 566 (41) Ω/cm^2 to 513 (43) Ω/cm^2 ($p>0.05$, n = 5). ACh (0.1–100 μM) applied basolaterally increased Isc from 1 (0) μA/cm^2 (n = 4) to a maximum of 11 (3) μA/cm^2 at 10 μM (n = 6). The responses were sided as apical application of ACh (10 μM) was ineffective (0 (0) μA/cm^2, n = 4).

Interaction of secretagogues

CT and forskolin both potentiated responses to ACh. In the presence of CT, responses of T_{84} cells to ACh were greatly potentiated. Figure 2 shows concentration response curves to ACh after pre-exposure of T_{84} cells to CT (0.01 μg/ml) or H_2O vehicle (100 μl) for 180 minutes. Potentiation of ACh was dependent on the concentration of toxin. Responses to ACh (10 μM) ranged from 104 (9) μA/cm^2 (n = 6) to 213 (11) μA/cm^2 (n = 6), in the presence of 0.0001 and 0.1 μg/ml CT, respectively. Potentiation of ACh was also dependent on the exposure time of cell monolayers to CT. Responses to ACh

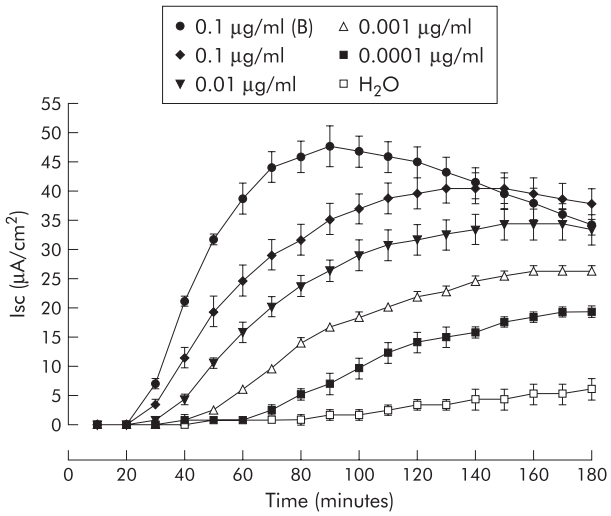

Figure 1 Short circuit current (Isc) responses of T_{84} cells to cholera toxin (CT 0.0001 to 0.1 μg/ml) over 180 minutes. CT was applied to the apical surface unless otherwise indicated, when it was applied to the basolateral surface (B). Isc is expressed as change in current/cm^2 of monolayer. Data are means (SEM) (n = 5).

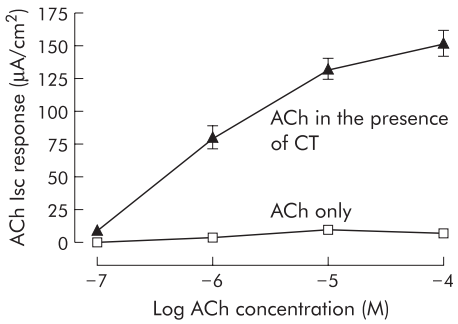

Figure 2 Short circuit current (Isc) responses of T_{84} monolayers to acetylcholine (ACh 0.1 μM–100 μM) given 180 minutes after pre-exposure to cholera toxin (CT 0.01 μg/ml) or vehicle. Responses to ACh in the presence of CT do not include the underlying response to the toxin. Data are means (SEM) (n = 4–6).

(10 μM) varied from 26 (9) μA/cm^2 (n = 6) to 43 (4) μA/cm^2 (n = 6) after 30 minutes of exposure to CT (0.0001 μg/ml and 0.1 μg/ml, respectively). Hyoscine (1 μM given 30 minutes before ACh) abolished responses to ACh (10 μM; n = 6) given 180 minutes after CT (0.01 μg/ml). By itself, hyoscine had no effect on basal Isc or TER. Responses to ACh were also potentiated by forskolin. After five minutes of preincubation with forskolin (10 μM), responses to ACh (50 μM) were 111 (9) μA/cm^2 (n = 6, $p<0.05$). Responses to ACh (50 μM) alone were 10 (1) μA/cm^2 (n = 6).

ACh potentiated forskolin but not CT. In the presence of ACh (10 μM) given 90 seconds before the toxin, the response to CT (0.01 μg/ml) after 180 minutes of exposure to the toxin was 27 (2) μA/cm^2 (n = 6, $p>0.05$). In contrast, responses to forskolin (1.25 μM) after five minutes increased from 32 (2) μA/cm^2 (n = 9) to 108 (11) μA/cm^2 (n = 5, $p<0.05$) when ACh was given 90 seconds before forskolin.

CT also potentiated responses to histamine but not to VIP. In the presence of CT (0.01 μg/ml, 120 minutes of preincubation), responses to histamine (100 μM) increased from 14 (4) μA/cm^2 (n = 6) to 60 (8) μA/cm^2 (n = 6, $p<0.05$) while responses to VIP (0.3 nM) decreased from 51 (4) μA/cm^2 to 28 (4) μA/cm^2 ($p<0.05$, n = 6).

Effect of potassium channel block on secretagogue responses

Barium (5 mM) reduced responses to CT and forskolin but not to ACh (table 2). When barium was given 20 minutes before CT, subsequent responses obtained with ACh were unchanged (table 2). A combination of the highly specific calcium dependent potassium channel blockers charybdotoxin (0.5 μM) and apamin (0.5 μM) reduced responses to ACh in the presence of forskolin (table 2). None of the blockers used had any effect on basal Isc or TER.

Effect of secretagogues on basolateral ^{86}Rb efflux and apical ^{125}I efflux

Forskolin (10 μM) applied to the basolateral surface produced a rapid increase in the rate of ^{125}I efflux which was significantly different from time matched controls. This

Table 2 Effect of the potassium channel blockers barium and charybdotoxin plus apamin on short circuit current (Isc) responses to cholera toxin, forskolin, and acetylcholine in T_{84} cells

	Control Isc response ($\mu A/cm^2$)	Isc response in the presence of barium ($\mu A/cm^2$)	Isc response in the presence of charybdotoxin+apamin ($\mu A/cm^2$)
CT 0.01 μg/ml (n=5)	34 (2)	12 (1)*	ND
ACh 10 μM (n=5)	10 (3)	12 (1)	ND
ACh 10 μM (180 min after CT 0.01 μg/ml; n=5)	133 (8)	137 (26)	ND
Forskolin 10 μM (n=6)	22 (1)	11 (1)*	27 (2)
ACh 50 μM (5 min after forskolin 10 μM; n=6)	112 (9)	ND	41 (3)*

Barium (5 mM) and charybdotoxin (0.5 μM) plus apamin (0.5 μM) were given 20 minutes before secretogogues to the basolateral surface. Acetylcholine (ACh) was given 180 minutes after cholera toxin (CT) and five minutes after forskolin.
Data are means (SEM) (n=6).
*$p<0.05$ compared with controls.
ND, no data.

coincided in time with the initial increase in Isc produced by forskolin. There was no significant change in efflux rate constants with ACh alone (50 μM). ACh (50 μM) applied to the basolateral surface, in the presence of forskolin (10 μM), produced a second increase in ^{125}I efflux. This again was significantly different from time matched controls (fig 3, table 3).

Forskolin (10 μM) alone caused a small delayed but significant increase in ^{86}Rb basolateral efflux compared with time matched controls. This was reduced in the presence of the potassium channel blocker barium. ACh also produced an increase in ^{86}Rb efflux. In the presence of forskolin, ACh produced an additional increase which was significantly greater than that induced by ACh alone (fig 4, table 4). This was reduced by a combination of the calcium activated potassium channel blockers charybdotoxin plus apamin (table 4).

Effect of CT and forskolin on cAMP levels

CT (0.01 μg/ml) and forskolin (1.25 μM) caused a time dependent increase in cAMP production in T_{84} cell monolayers. The effect of forskolin was significant after 10 minutes whereas CT took 120 minutes to reach significance (fig 5). Barium (5 mM) given 20 minutes before CT (0.01 μg/ml) had no effect on cAMP production by the toxin (fig 6) despite a considerable reduction in the Isc response (table 2). Barium alone has previously been shown to have no effect on basal cAMP levels.[21] cAMP production was also unaffected by the presence of ACh (10 μM) given after 180 minutes pre-

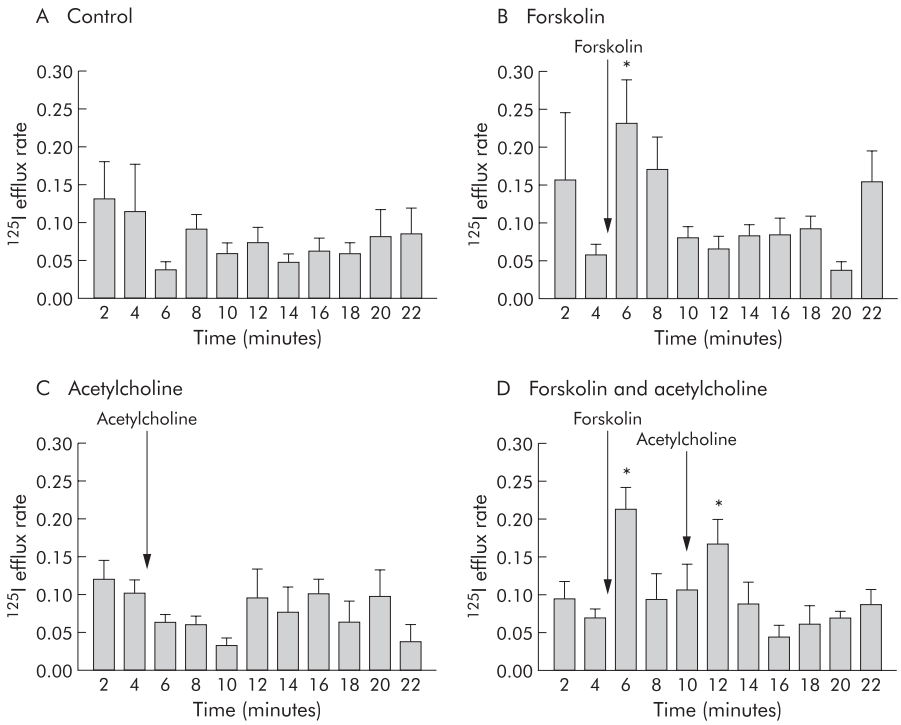

Figure 3 Effect of forskolin (B), acetylcholine alone (C), and acetylcholine in the presence of forskolin (D) on efflux of ^{125}I from T_{84} cells. Acetylcholine (50 μM) was given five minutes after forskolin (10 μM) to the basolateral surface. The Y axis values are efflux rate constants (r/min). *$p<0.05$ compared with time matched controls (A). Data are means (SEM) (n=6).

Table 3 Effect of forskolin, acetylcholine, and acetylcholine in the presence of forskolin on efflux of ^{125}I from T_{84} cells

Treatment	Basal efflux rate	Peak efflux rate
None (n = 9)	0.115 (0.076)	0.092 (0.024)
Forskolin (n = 9)	0.057 (0.014)	0.231 (0.057)*
ACh (n = 7)	0.102 (0.018)	0.063 (0.011)
ACh in the presence of forskolin (n = 9)	0.069 (0.012)	0.166 (0.033)*

Acetylcholine (ACh 50 μM) was given five minutes after forskolin (10 μM) to the basolateral surface. All values are apparent efflux rate constants (r/min), as described in the methods.
Data are means (SEM) (n = 9).
*$p<0.05$ compared with time matched basal controls.

exposure to the toxin; ACh (10 μM) by itself had no effect on cAMP levels (fig 6).

Distribution of cholinergic nerves and M3 receptors in human ileal mucosa

A high proportion of nerve fibres stained positively for ChAT (fig 7A, 7B) in all layers of the captured fields. The highest density of nerves within the mucosa was located towards the luminal aspect of the lamina propria associated, on serial sections, with M3 receptor immunoreactivity adjacent to the basolateral domain of enterocytes (fig 7C). A high density of both ChAT positive nerves and M3 receptors were found in the muscularis mucosa. Nerve fibres and cell bodies stained positively for both PGP and ChAT in the submucosal plexus, with M3 receptors localised to submucosal blood vessels.

DISCUSSION

Acute infectious diarrhoea constitutes a worldwide health problem and continues to cause significant morbidity and mortality. The volume of diarrhoea produced in cholera is greater than most other types of diarrhoea and this is consistent with the proposal that a synergistic mechanism may play a role in the pathogenesis of the acute severe watery diarrhoea produced by *Vibrio cholerae*. Animal studies have demonstrated cholinergic nerves projecting to the mucosa.[7 12 13] Cholinergic neurones have also been demonstrated around the crypts and extending up the villi of the human small intestine.[22 23] However, both of these investigations used cholinesterase and acetylcholinesterase rather than choline acetyltransferase (ChAT) as markers for cholinergic neurones. Cholinesterase is not considered as reliable a marker for cholinergic neurones as ChAT as it can be associated with non-cholinergic neurones.[24] In addition, functional studies support a secretory role for ACh in human and animal tissue.[25–27] Using immunohistochemical staining of normal human ileum resection specimens, we have shown ChAT containing neurones and M3 muscarinic receptors within the submucosa and adjacent to the basolateral domain of epithelial cells. Such findings have not been described previously in the human small intestine. It is probable that CT releases ACh directly onto epithelial cells given the convincing evidence that the toxin activates a secretomotor neural reflex,[4 5 28] and that stimulation of nerve fibres in human ileal mucosa cause atropine sensitive secretory responses.[25]

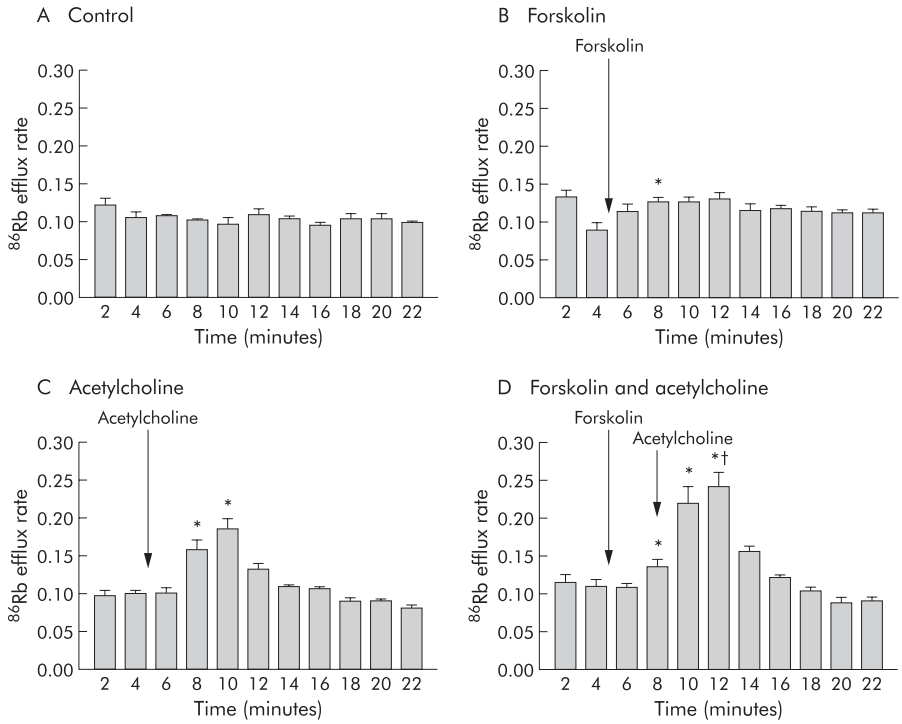

Figure 4 Effect of forskolin (B), acetylcholine alone (C), and acetylcholine in the presence of forskolin (D) on efflux of ^{86}Rb from T_{84} cells. Acetylcholine (50 μM) was given five minutes after forskolin (10 μM) to the basolateral surface. The Y axis values are efflux rate constants (r/min). *$p<0.05$ compared with time matched controls (A). †$p<0.05$ compared with peak ^{86}Rb efflux induced by acetylcholine alone. Data are means (SEM) (n = 6).

Table 4 Effect of the potassium channel inhibitors barium and charybdotoxin plus apamin on forskolin and acetylcholine induced efflux of ^{86}Rb from T_{84} cells

Treatment	Basal efflux rate	Peak efflux rate	Peak efflux in the presence of barium	Peak efflux in the presence of charybdotoxin plus apamin
None (n=6)	0.104 (0.007)	0.107 (0.002)	0.099 (0.005)	0.103 (0.004)
Forskolin (n=6)	0.089 (0.010)	0.127 (0.006)*	0.107 (0.005)†	0.121 (0.003)
ACh (n=6)	0.100 (0.004)	0.186 (0.014)*	ND	ND
ACh in the presence of forskolin (n=6)	0.110 (0.008)	0.242 (0.018)*‡	ND	0.170 (0.012)†

Acetylcholine (ACh 50 μM) was given five minutes after forskolin (10 μM) to the basolateral surface. Barium (5 mM) and charybdotoxin (0.5 μM) plus apamin (0.5 μM) were given 20 minutes before agonists. All values are apparent efflux rate constants (r/min), as described in the methods.
Data are means (SEM) (n=6).
*$p<0.05$ compared with time matched basal controls. †$p<0.05$ compared with peak efflux rates in the absence of antagonists. ‡$p<0.05$ compared with peak efflux induced by ACh alone.

T_{84} colonic epithelial cells show a high degree of sensitivity to CT. Time to onset of Isc response as well as peak effect were concentration dependent. Increases in Isc to basolateral application of the toxin were unexpected as receptors for CT were originally shown to be located on the apical membrane of enterocytes.[29] As basolaterally applied toxin achieved a peak effect more rapidly than apically applied toxin, it was considered unlikely that the toxin was crossing the monolayer. A more likely explanation would be that the CT receptor, a monosialoganglioside, is not restricted to the apical domain of enterocytes, which is not surprising for such a ubiquitous cell component.[30] The earlier peak effect of basolaterally applied CT may be due to the location of adenylate cyclase on the basolateral domain of the enterocyte,[31] in which case less time may be spent in intracellular trafficking of the toxin subunit. Alternatively, there may be more toxin ganglioside receptors on the basolateral membrane.[32]

In contrast with investigations using native tissue preparations,[33 34] CT decreased the TER of T_{84} monolayers. Although increased responses to ACh in the presence of CT were seen after 180 minutes, we cannot exclude the possibility of tissue damage, especially as no index of cellular integrity was utilised.

Isc responses of T_{84} cells to ACh were dramatically increased in the presence of CT. Such potentiation was dependent on both the concentration and time of exposure of cells to the toxin. The Isc response to CT alone was shown to be associated with a time dependent increase in cAMP levels, an observation which is supported by earlier work where the authors also demonstrated a close correlation between Isc response and cAMP levels.[2] It is likely that the magnitude of potentiation is dependent on cAMP levels and the results with forskolin would support such a conclusion. Higher concentrations of ACh were used with forskolin as these data were taken from radioisotopic efflux experiments where we wished to maximise the effects of secretagogues on ion efflux. The converse situation (that is, potentiation of cAMP dependent secretagogues by ACh) could only be demonstrated when using forskolin. This was probably due to the short lived effects of ACh disappearing by the time CT had initiated a significant increase in cAMP. Forskolin was shown to elevate cAMP levels within 10 minutes whereas a significant increase induced by CT was not shown until 120 minutes. The synergism described seems to be operating between secretagogues activating different second messenger pathways. Thus histamine, which in common with ACh is a calcium dependent secretagogue,[35] was potentiated by prostaglandin E_1 and *Escherichia coli* heat stable toxin, while VIP was not potentiated by CT. The interaction between CT and forskolin was also not additive.[3]

Understanding the nature of the synergistic interaction is complicated by the uncertainty regarding the mechanism of action of calcium dependent secretagogues. It is generally agreed that such compounds open basolateral potassium channels which hyperpolarise the cell and increase the electrical driving force for apical chloride secretion.[36] However, there is controversy as to whether they open apical chloride channels. For instance, in cystic fibrosis intestine there is a failure of both cAMP and calcium dependent secretagogues to elicit chloride secretion yet this disease only affects the cAMP activated chloride channel.[37] Similarly, Reenstra[38] could not demonstrate carbachol induced chloride conductance in apical membranes of T_{84} cell monolayers. It

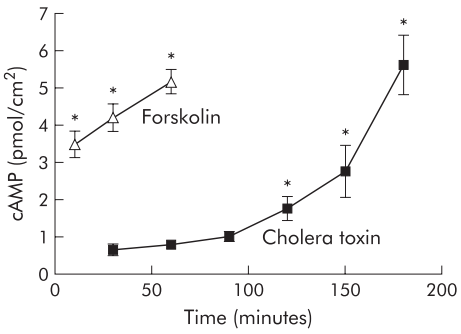

Figure 5 Measurement of cAMP production in T_{84} cells. Time course of the effect of cholera toxin (0.01 μg/ml) and forskolin (1.25 μM) on cAMP production was measured over 180 minutes and 60 minutes, respectively. cAMP levels are expressed as total nucleotide/cm^2 of monolayer. Data are means (SEM) (n=6). *$p<0.05$ compared with controls.

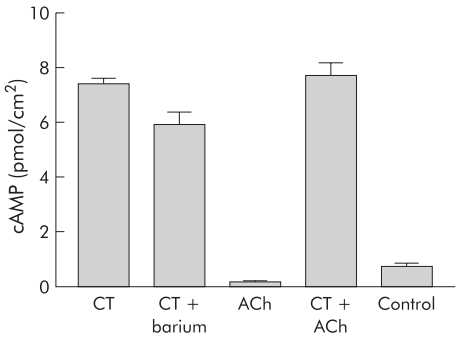

Figure 6 Stimulation of cAMP production in T_{84} cells. cAMP levels (pmol/cm^2 of monolayer) were measured at 180 minutes after exposure to cholera toxin (CT) or 3-isobutyl-1-methylxanthine (IBMX, controls), and at 30 minutes after exposure to acetylcholine (ACh). When given in combination, ACh was added to CT after 150 minutes and cAMP levels were measured at 180 minutes. Barium (5 mM) was given 20 minutes before CT (0.01 μg/ml). Data are means (SEM) (n=6).

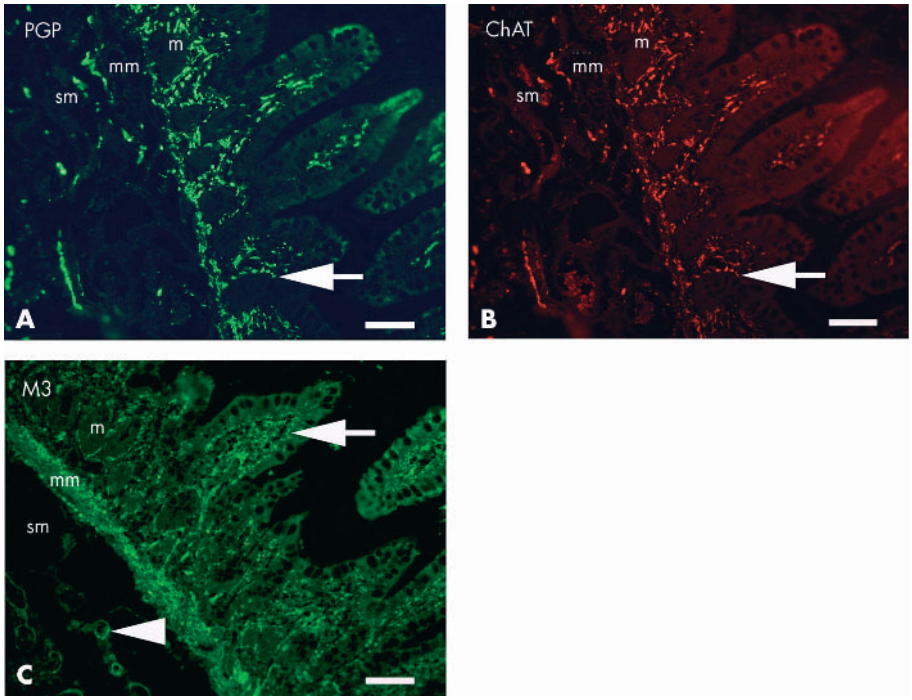

Figure 7 Co-localisation (arrow) of protein gene product (PGP) (A) and choline acetyltransferase (ChAT) (B) in nerve fibres within the mucosa (m), muscularis mucosa (mm), and submucosa (sm) of normal ileum, with a high proportion of fibres staining positively for ChAT. The majority of fibres within the mucosa were located in the lamina propria, adjacent to the basolateral domain of enterocytes. Muscarinic M3 receptors (C) were localised, in serial sections, to the basolateral domain of enterocytes (arrow), muscularis mucosa, and blood vessels (arrowhead) of the submucosa. Bars represent 200 μm.

was therefore proposed that the chloride conductance initiated by calcium dependent secretagogues was through constitutively open cAMP activated chloride channels.[37] In contrast with these findings, Fuller and Benos have isolated and cloned a membrane protein from tracheal epithelium which is associated with a novel calcium activated chloride conductance when heterologously expressed.[39] A porcine homologue has since been cloned and is expressed in porcine enterocytes.[40] Moreover, Barrett and colleagues[41] have produced evidence for an apical calcium activated chloride conductance which is both positively and negatively regulated by calcium dependent agonists. The negative regulation (that is, inhibition) is through generation of inositol tetrakisphosphate and would explain the short duration of action of ACh.

Potentiation of ACh by CT may occur at the level of the second messengers. For example, cAMP could inhibit the generation or actions of the negative regulator of calcium dependent secretion, inositol tetrakisphosphate,[42 43] and thus relieve ACh of its autoinhibitory actions. However, the observation that even potentiated responses to ACh still remain very brief make such a mechanism unlikely. The cause of the synergism is more likely to result from optimum timing of ion channel opening.[16] In the presence of CT the principal apical membrane chloride channel is open, thus allowing unhindered secretion of chloride ions when the driving force for such secretion is increased by opening of ACh activated basolateral potassium channels. This proposed mechanism of action has been supported in this study both by the characteristics of rubidium and iodine efflux during potentiation and also by the use of selective potassium channel inhibitors acting on calcium dependent potassium channels.

Forskolin rapidly increased the rate of apical ^{125}I efflux from T_{84} cells. ACh alone did not have any effect on ^{125}I efflux, in keeping with previous published data,[38] although in the present investigation this may reflect the small responses in short circuit current which is an indication of chloride ion secretion. In the presence of forskolin, the Isc response to ACh was greatly potentiated and this coincided with a significant increase in ^{125}I efflux. This suggests that the potentiation is associated with enhanced apical chloride secretion. Separately, both forskolin and ACh induce increases in basolateral ^{86}Rb efflux. When given in the presence of forskolin, ACh induced ^{86}Rb efflux was increased further, which supports the role of basolateral potassium channels in this mechanism. In T_{84} cells, the potassium channel blocker barium inhibits secretory responses to cAMP but not to calcium dependent secretagogues, by blocking separate and specific basolateral potassium channels.[36 44] This was confirmed in the present investigation where barium attenuated responses to CT and forskolin, but not to ACh. This attenuated Isc response to forskolin was associated with a decrease in the rate of ^{86}Rb efflux, with no effect on cAMP levels (using CT), confirming the role of basolateral potassium channels. Moreover, we demonstrated that barium had no effect on potentiation of ACh by CT despite attenuating short circuit responses to CT. To assess the role of calcium dependent potassium channels we investigated the effect of selective inhibition of such channels on the synergistic action of forskolin and ACh. A combination of charybdotoxin and apamin[45] had no effect on forskolin Isc responses and ^{86}Rb efflux but greatly attenuated the potentiated Isc responses and ^{86}Rb efflux to ACh in the presence of forskolin. These results therefore suggest that the synergism between ACh and CT is dependent on cAMP activated apical chloride channels and calcium activated barium insensitive basolateral potassium channels. The cAMP dependent barium sensitive basolateral potassium channels are unlikely to play a significant role. By itself, barium does not appear to affect cAMP levels in enterocytes.[21]

In summary, the present investigation demonstrates a marked potentiation of the secretory actions of ACh by CT in human epithelial cells. Such an interaction may contribute to the overall secretory effects of the toxin as it has previously been shown to activate enteric secretomotor reflexes. In humans, these reflexes probably utilise ACh as a neurotransmitter at the neuroepithelial junction as we have shown that both cholinergic nerves and muscarinic M3 receptors are located at the basolateral domain of human ileal epithelial cells.

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