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Effects of theophylline, choleragen and loperamide on rabbit ileal fluid and electrolyte transport *in vitro*

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1 The effects of theophylline and cholera toxin on water and anion movements across rabbit ileum *in vitro* and the reversal of these effects by the opiate action of loperamide have been investigated. Water movement across the mucosal and serosal surfaces of the tissue was measured continuously by a high resolution method.

2 Theophylline caused an increase in short circuit current and reversed the direction of net Cl^- movement, due mainly to a decrease in mucosal-serosal flux. It also caused a rapid, but transient, reversal in the direction of fluid movement across the mucosal surface. Fluid outflow across the serosal surface was decreased but not reversed. Cholera toxin caused a slow inhibition of water movement across both mucosal and serosal surfaces.

3 Theophylline increased the exit rate of ^{77}Br across the mucosal surface and decreased the exit rate of ^{77}Br across the serosal surface. Theophylline increased the exit rate of ^3H-labelled mannitol across the mucosal surface.

4 Loperamide reversed the effects of theophylline and cholera toxin on water flow across the mucosal and serosal surfaces and on net transepithelial Cl^- flux; it also increased the rate of ^{77}Br exit across the serosal surface of theophylline-treated tissue. These effects of loperamide could be reversed by naloxone.

5 The hydraulic conductivity, L_p of the serosal surface was measured directly by determining the osmotic flow generated by low concentrations of polyethylene glycol (mol. wt. 20000 and 90000). Theophylline reduced the L_p by 57%. Loperamide added to theophylline-treated tissues increased the L_p by 340%. This effect was reversed by naloxone.

6 These results indicate that modulation of intestinal smooth muscle tone affects transepithelial ion and water flows *in vitro*. The increase in tone induced by secretagogues increases ion and water reflux via wide shunt channels in the mucosa and thereby reduces net absorption. The increased net fluid and electrolyte absorption induced by loperamide results from the opiate-dependent inhibition of acetylcholine release from intrinsic ganglia which reduces smooth muscle tone and thereby enhances the fluid and electrolyte conductance of the submucosal layers.

Introduction

Opiates, like loperamide, have no effect on the short circuit current increase seen following *in vitro* exposure of small intestine to secretagogues (Dobbins *et al.*, 1980; McKay *et al.*, 1981). Despite this lack of effect on short circuit current, loperamide enhances intestinal net fluid and electrolyte absorption after it has been inhibited by prostaglandins *in vitro* (Coupar, 1978; Beubler & Lembeck, 1980; Hardcastle *et al.*, 1981), or by theophylline in rabbit colon (Baker & Segal, 1981) and also in rat intestine *in vivo* after exposure to cholera toxin (Sandhu *et al.*, 1979).

The mechanism of the opiate-dependent increase in fluid and electrolyte absorption is uncertain. It has been claimed that loperamide prevents the rise in tissue cyclic AMP seen after exposure to prostaglandins (Beubler & Lembeck, 1980); however, opiate-dependent reductions in tissue cyclic nucleotides and anion permeability are not universally observed (Farack *et al.*, 1981).

Neurogenic factors have also been implicated in the control of intestinal transport (Hubel, 1976; Cooke, 1983; Sjövall *et al.*, 1983). These effects have been ascribed to direct interactions with receptors on the enterocyte membranes. This view, at least for opiates, is hard to reconcile with the reported absence of opiate receptor sites on the rat intestinal epithelial cell

membranes (Gaginella *et al.*, 1983).

Nevertheless, the observed correlation between the antisecretory action of antidiarrhoeal drugs in general and opiates in particular, and their antimotility effects (Neuten *et al.*, 1977), is also consistent with the studies by Paton (1953) and Cowie *et al.* 1978), showing that opiates inhibit acetylcholine release from the myenteric plexus of guinea-pig and rabbit ileum.

It has been shown that intestinal fluid absorption is related *in vivo* to smooth muscle tone and interstitial pressure (Granger *et al.*, 1979; Lee, 1983). In this paper we demonstrate directly, by observing changes in ion, mannitol and water movements across the mucosal and serosal surfaces of isolated rabbit ileum that the submucosa exerts a mechanical control on rabbit ileal fluid and electrolyte transport directly.

Our results indicate that the reduction in intestinal fluid absorption in rabbit ileum *in vitro* induced by cholera toxin, or theophylline was, in part, due to increased hydraulic resistance of the serosal barrier to the movements of salts and water, with consequent enhanced reflux via the shunt pathways across the mucosa. Opiates like loperamide reduced this effect by lowering the resistance of the serosal barrier.

Methods

Male New Zealand white rabbits between 2–3 kg were killed with intravenous sodium pentobarbitone and the ileum rapidly removed and washed free of its contents with Krebs-Ringer solution (mM): NaCl 137, KCl 5.7, $CaCl_2$ 1.8, $MgSO_4$ 1, Na_2HPO_4 0.4, $NaHCO_3$ 11.7 at 20°C. The ileum was dissected free of mesentery and longitudinal muscle and then mounted in an Ussing-type chamber. For measurement of water flux the serosal surface was supported by a fine mesh grid and gassed with 95% O_2: 5% CO_2 on the mucosal side only.

Previous studies have shown that such conditions are sufficient to maintain adequate oxygenation and pH control of the ungassed serosal compartment (Naftalin & Tripathi, 1986).

Water flows

The method of monitoring intestinal fluid movement has been fully described (Naftalin & Tripathi, 1985; 1986). Flow of water from the tissue into the serosal solution, J_s, was monitored with a capacitance probe (Wayne Kerr, Bognor Regis), which measures the width of an air gap between the probe surface and the meniscus of the serosal bathing solution. The output was recorded continuously on a chart recorder. This was calibrated for a serosal surface area of 10 cm^2; evaporative losses are constant, so the flow, J_s ($\mu l\ cm^{-2}\ h^{-1}$), may be obtained from the slope of the output signal plotted on a chart recorder (JJ Instruments Ltd, Southampton).

The change in tissue volume, J_t was also monitored continuously with an optical lever. This consisted of a lightweight mirror pivoted at its lower edge. The upper edge rested directly against the mucosal surface of the tissue in the bathing solution. Expansion of the tissue caused a forward rotation of the mirror, which changed the angle of reflection of a low power laser beam (Spectraphysics Ltd). The beam displacement was monitored with a pair of photodiodes and the signal displayed on a second channel of the chart recorder. The signal was calibrated for tissue expansion with a micrometer-controlled displacement of the optical lever.

Flow across the mucosal surface of the tissue, J_m was equal to the algebraic sum of J_s and J_t, the tissue volume change cm^{-2} serosal area.

This new approach to measuring intestinal fluid movements has several advantages over measurement of ion movements; net changes in flow are measured directly, rather than by differences in transepithelial ion flux, as determined by tracer movements. This enhances resolution by at least 20 fold. A second advantage is that flows are simultaneously measured across both the mucosal and serosal surfaces. The independent changes observed in flow across the two surfaces following treatment with drugs gives direct information concerning the separate determinants of flow across the mucosal and submucosal surfaces of the tissue.

Experimental protocol

After mounting the tissue and adjusting the probes, which takes between 10–15 min, the tissue flows were monitored for 15–30 min. During this time fluid movement generally increased rapidly to reach a quasi-steady-state (J_m was 15–35 $\mu l\ cm^{-2}\ h^{-1}$ and flow across the serosal border, J_s rose slowly from approximately 5 $\mu l\ cm^{-2}\ h^{-1}$ to between 15–25 $\mu l\ cm^{-2}\ h^{-1}$.

Measurement of the osmotic L_p of the serosal surface

The hydraulic conductance, determined by the osmotic L_p ($cm\ s^{-1}\ cmH_2O^{-1}$) was determined as follows: addition of impermeant macromolecules to the serosal bathing solution induced osmotic flow across the submucosal layers (Naftalin & Tripathi, 1985). The L_p was estimated from the change in ΔJ_s $mosmol^{-1}$, according to the following relationship:

$$\Delta J_s = L_p \times RT\,C$$

where RT is the product of the gas constant and $K° = 24\ cmH_2O\ mosmol^{-1}$ at 36°C. C is the osmolar concentration of polyethylene glycol added to the

serosal bathing Ringer solution, obtained from vapour pressure readings using a Wescor vapour pressure osmometer. The concentrations of polyethylene glycol, molecular weight 20 kDa and 90 kDa were adjusted to give osmotic activities of 2 and 1 mosmol kg^{-1} respectively. Two sizes of polyethylene glycol were used to ensure that no error was introduced because of permeation of smaller macromolecules into the tissue.

Ion flows

Bidirectional transepithelial Cl^- and Br^- fluxes The tissue was mounted in a six-port modified Ussing chamber with an exposed tissue surface, 1.76 cm^{-2} at each of the ports. These chambers are described in detail elsewhere (Naftalin & Holman, 1974). Two to five six-port chambers (12 to 30 pieces of tissue) were used in a single experiment. Each piece of tissue was isolated from its neighbours after mounting. The tissue was maintained at 37°C by water circulation through an integral water jacket and gassed bilaterally with 95% O_2:5% CO_2 to pH 7.2. Comparisons were made between adjacent pieces of tissue.

Simultaneous bidirectional transepithelial fluxes of ^{36}Cl and ^{77}Br were measured by the methods previously described (Naftalin & Simmons, 1979). ^{36}Cl and ^{77}Br activity may be determined simultaneously in a liquid scintillation counter with appropriate window settings. As ^{77}Br is 1.2 fold more permeant to rabbit ileum than ^{36}Cl, a correction was made to normalize the ^{36}Cl and ^{77}Br fluxes. Samples of the bathing solutions were taken for counting at 30 min intervals following addition of isotope.

Measurement of unidirectional anion exit across the mucosal and serosal surfaces of rabbit ileum The tissue was loaded with ^{77}Br from the mucosal, or serosal side for 30 min at 37°C. The isotope loading solution was then replaced with isotope-free Krebs-Ringer solution at 3–5°C and this solution was in turn replaced with pre-warmed 37°C Krebs-Ringer solution. Drugs were added to the incubation solutions at this stage. The rate of tracer exit into the serosal, or mucosal bathing solutions was estimated by replacing the entire bathing solution at 30 min intervals and counting aliquots from each sample period.

Measurement of [^3H]-mannitol steady state retention volume within the tissue and its fractional exit across the mucosal surface

Mannitol is restricted to the extracellular compartments of epithelial tissues (Smulders & Wright, 1971; Biber *et al.*, 1972). However, it does cross the mucosal surface of rabbit ileum via paracellular shunt channels (Naftalin & Tripathi, 1985; 1986), thus the exit rate of mannitol is a measure of the permeability of extracellular channels and the steady state retention of volume of labelled mannitol monitored after the transepithelial flux has reached a stationary state is a measure of the relative permeability of the mucosal and serosal surface of the tissue to mannitol.

Steady state retention volume = Vol. $R_m/(R_m + R_s)$. Where 'Vol' is total extracellular fluid volume = 73 $\mu l\ cm^{-2}$ (serosal area) determined by experiments in which the tissue was equilibrated with label from both sides simultaneously; R_m and R_s are the resistances to flow of mannitol across the extracellular pathways of the mucosal and serosal surfaces (where $R_m = 1/P_m$ and $R_s = 1/P_s$; P(cm s^{-1}) is the permeability of the barriers to mannitol). Thus, the closer the steady state retention volume approaches the extracellular volume, during serosal to mucosal flow of mannitol, the greater is the ratio R_m/R_s.

The tissue was preincubated for a 30 min period at 37°C in Krebs-Ringer solution, loperamide was added to the bathing solution when required during this period. ^3H-labelled mannitol was then added to the serosal bathing solution. Theophylline was also added at this time. After a single 30 min incubation period the mucosal bathing solutions were sampled and bathing solutions were then removed and after washing with Krebs-Ringers solution at 5°C to remove adherent radioisotope, the remaining isotope was extracted from the tissue for 12–14 h, in 0.4% triton X-100. Steady-state retention of [^3H]-mannitol is expressed in $\mu l\ cm^{-2}$ serosal area.

Fractional exit rate

The fractional exit of [^3H]-mannitol across the mucosal surface was estimated from the fraction of the total isotope uptake across the serosal surface which was lost across the mucosal surface from the submucosa during the 30 min observation period.

i.e. Fractional rate of loss $h^{-1} = c.p.m_{(\text{lost to mucosal solution})} / \{c.p.m_{(\text{lost to mucosal solution})} + c.p.m_{(\text{accumulated in submucosal})} \times 2\}$

Potential Difference, short circuit current and tissue resistance measurements

All electrical measurements were made with a microprocessor based voltage clamp device (Naftalin & Smith, 1984).

Materials

Purified Cholera toxin, leucine enkephalin, theophylline and all salts were obtained from Sigma, Poole, Dorset. Naloxone was obtained from commercial sources as Narcan, DuPont (U.K.) Ltd Pharmaceuticals, Stevenage, Herts. Loperamide was a gift

from Janssen Pharmaceuticals (UK), Wantage, Oxon. Trifluoperazine was a gift from Smith, Kline and French, Welwyn Garden City, Herts. Polyethylene glycols, molecular weights 20000 and 90000 were obtained from BDH, Poole Dorset.

Radioisotopes

^{36}Cl was obtained from Amersham (U.K). ^{77}Br from the MRC cyclotron, Hammersmith, and ^{3}H labelled mannitol from New England Nuclear (West Germany).

Radioactive counting

All radioisotopes were counted by β emission in a Packard 3320 Tri-Carb liquid scintillation spectrometer. The scintillation counting fluid was composed of 2.5 g diphenyloxazole (PPO), 500 cm^3 toluene, 500 cm^3 Symperonic-X (detergent). This fluid was designed to accept a large aqueous sample (up to 20% by volume).

Results

The effects of theophylline, loperamide on short circuit current

Figure 1a shows that loperamide (10 μM) did not reduce transepithelial short circuit current stimulated by pretreatment with theophylline (2 mM). Also, loperamide when added to control tissue did not alter short circuit current. Exposure of the tissue to naloxone (1 μM) was also without effect. A similar absence of effect on short circuit current was noted in control and theophylline-treated tissue after addition of leucine enkephalin ([Leu]Enk, 1 μM and 10 μM) (Figure 1b) and also morphine (10 μM) (Figure 1c). The effects of trifluoperazine (100 μM) are shown in Figure 1d. As previously noted (Smith & Field, 1980) this drug reduces short circuit current as it inhibits, or reverses the increase in anion conductance across the mucosal border (Ilundain & Naftalin, 1979). These data showing the effect of trifluoperazine were included here to emphasize the absence of effect of opiates. The absence of effect of loperamide on the electrical changes, induced by theophylline is consistent with the opiate-insensitivity of electrical properties of rabbit ileum previously noted (Dobbins *et al.*, 1980; McKay *et al.*, 1981).

The effect of theophylline and loperamide on undirectional ^{36}Cl and ^{77}Br and net anion flux across rabbit ileum in vitro

Theophylline (10 mM) changed net Cl^- absorption (in controls) to net secretion (in treated tissue), mainly by reducing the mucosal-serosal (m-s) Cl^- flux (Table 1). There was only a small effect on the s-m flux.

In the presence of loperamide, theophylline-dependent Cl^- secretion was prevented ($P < 0.01$). A small increase in net Cl^- absorption was observed in control tissues with loperamide present, but just fails to reach the level of statistical significance ($0.1 > P > 0.05$).

This effect of loperamide on theophylline-dependent secretion was reversed by naloxone, (1 μM), i.e. in the presence of both loperamide and naloxone, theophylline induced net Cl^- secretion and also caused a significant reduction in m-s Cl^- flux. These results are similar to those obtained in rabbit colon (Baker & Segal, 1981). They indicate that the absorption enhancing effect of loperamide is due to a specific opiate action.

The effects of theophylline on water flow

In Figure 2a, average water flows across the mucosal and serosal surfaces during the initial period of 60 min incubation in control tissue ($n = 5$) are shown. Flow across the mucosal border, J_m increased rapidly to around 12 $\mu l\,cm^{-2}\,h^{-1}$ and then more slowly to around 20 $\mu l\,cm^{-2}\,h^{-1}$. Flow across the serosal surface was initially about 5 $\mu l\,cm^{-2}\,h^{-1}$ and after approximately 60 min increased towards 10 $\mu l\,cm^{-2}\,h^{-1}$. Extension of the incubation period beyond 1 h (not shown), resulted in an increase in J_s and a gradual decrease in J_m due to increased fluid exit via mucosal shunt channels as interstitial pressure rose (Naftalin & Tripathi, 1985; 1986).

In Figure 2b, the effects of theophylline (10 μM) on fluid movement across the mucosal and serosal borders are shown. After an initial incubation period of 20–30 min, gassed and prewarmed theophylline (10 mM) Krebs-Ringer was exchanged for Krebs-Ringer in both mucosal and serosal bathing solutions. Within 2 min of exposure to theophylline, net flow across the mucosal surface was reversed from 17 to $-10.5 \pm 1.8\,\mu l\,cm^{-2}\,h^{-1}$ ($n = 9$) and flow across the serosal surface was reduced from 9 $\mu l\,cm^{-2}\,h^{-1}$ to 2–3 $\mu l\,cm^{-2}\,h^{-1}$ ($P < 0.001$). Since flow across the serosal surface did not reverse, there was no true net transepithelial secretory flow induced by theophylline. As is the case *in vivo* (Granger *et al.*, 1979) net secretion was observed only across the mucosal layer and not across the whole epithelium. Mucosal flow, J_m returned to zero within 5 min and thereafter, rose slowly to 1–2 $\mu l\,cm^{-2}\,h^{-1}$.

Serosal outflow remained at approximately the same reduced level during the entire period of exposure to theophylline. These results demonstrate the independence of fluid movements across the mucosal and serosal surface.

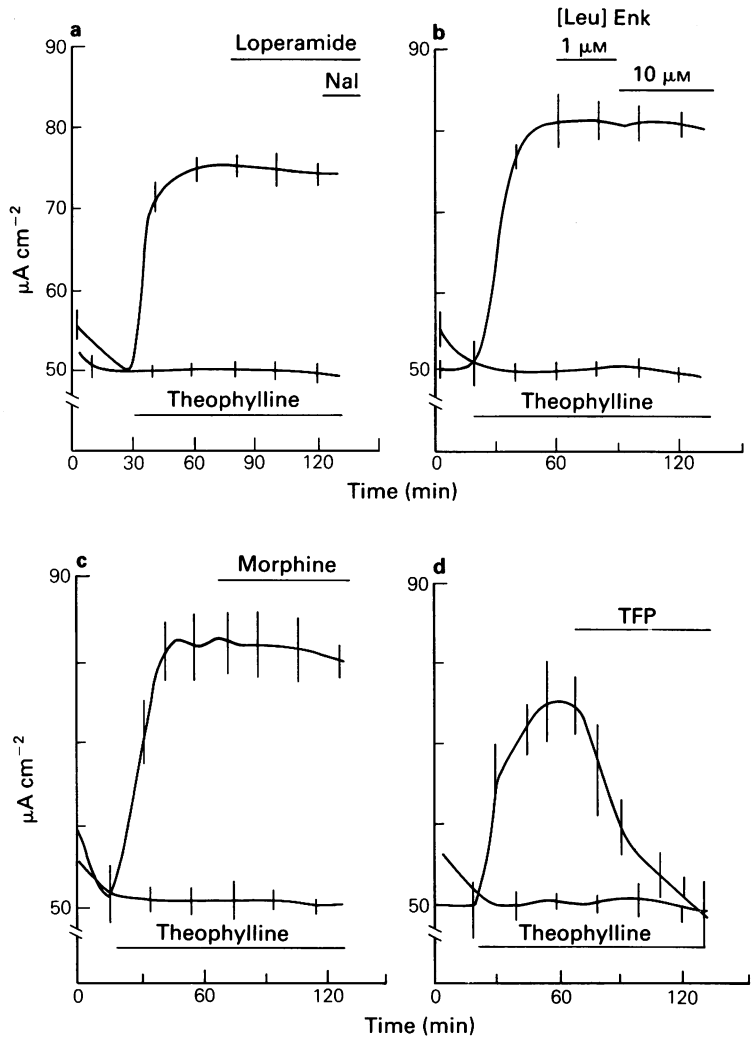

Figure 1 All four panels show the effect of theophylline (2 mM) added to the bathing solution on the short circuit current across rabbit ileum, mounted in a Ussing type chamber. The currents are measured with a digital microprocessor based voltage clamp device. (a) The effects of loperamide (10 μM) and naloxone (1 μM) on the short circuit current; upper trace shows effect on theophylline treated tissue, lower trace on controls. The vertical lines are the standard error of the means of three independent but simultaneous traces. There is no significant effect of the drugs in either case. (b) The effects of leucine enkephalin ([Leu]Enk 1 μM and 10 μM) on short circuit current of control (lower trace) and theophylline (2 mM)-treated tissue. There is no significant effect of the drug in either case. There is no significant effect of the drug on the current. (c) Effects of morphine (10 μM), on control and theophylline-treated tissue. There is no significant effect of the drug on the trace. (d) Effect of trifluoperazine (TFP, 100 μM) in control and theophylline-treated tissue added after 60 min incubation. There is a significant decrease in the secretion current induced by theophylline after exposure to trifluoperazine. These data are included for comparison with the effects of opiates.

Effects of theophylline on water movement in tissue bathed in 20 mM glucose-Krebs-Ringer

In the presence of 20 mM D-glucose, fluid movement across the mucosal surface of rabbit ileum was sustained at a higher rate than in the absence of D-glucose (Figure 3). Flow across the serosal surface was also faster in the presence of D-glucose, as has been previously noted (Smyth & Taylor, 1957; Naftalin & Tripathi, 1986; Lee, 1987).

Table 1 Transepithelial Cl^- fluxes measured with ^{77}Br and ^{36}Cl

	Cl^- *flux* (μmol cm^{-2} h^{-1})		
	J_{ms}	J_{sm}	J_{net}
Control	12.16 ± 0.75 (15)	10.44 ± 0.70 (15)	1.74 ± 0.67 (15)
Loperamide (10 μM)	14.26 ± 1.59 (6)	10.90 ± 1.04 (6)	3.33 ± 1.01 (6)
Theophylline (10 mM)	8.71 ± 0.51 (12)**	10.34 ± 0.96 (12)	− 1.63 ± 0.59 (12)**
Theophylline (10 mM) + loperamide (10 μM)	12.75 ± 1.56 (7)‡‡	11.66 ± 1.49 (7)	1.08 ± 0.89 (7)‡‡
Theophylline + loperamide + naloxone (1 μM)	11.34 ± 1.6 (4)	13.05 ± 0.06 (4)	− 1.76 ± 1.70 (4)

Values are mean ± s.e.mean.
** test vs control and ‡‡test vs theophylline respectively, ($P < 0.01$)

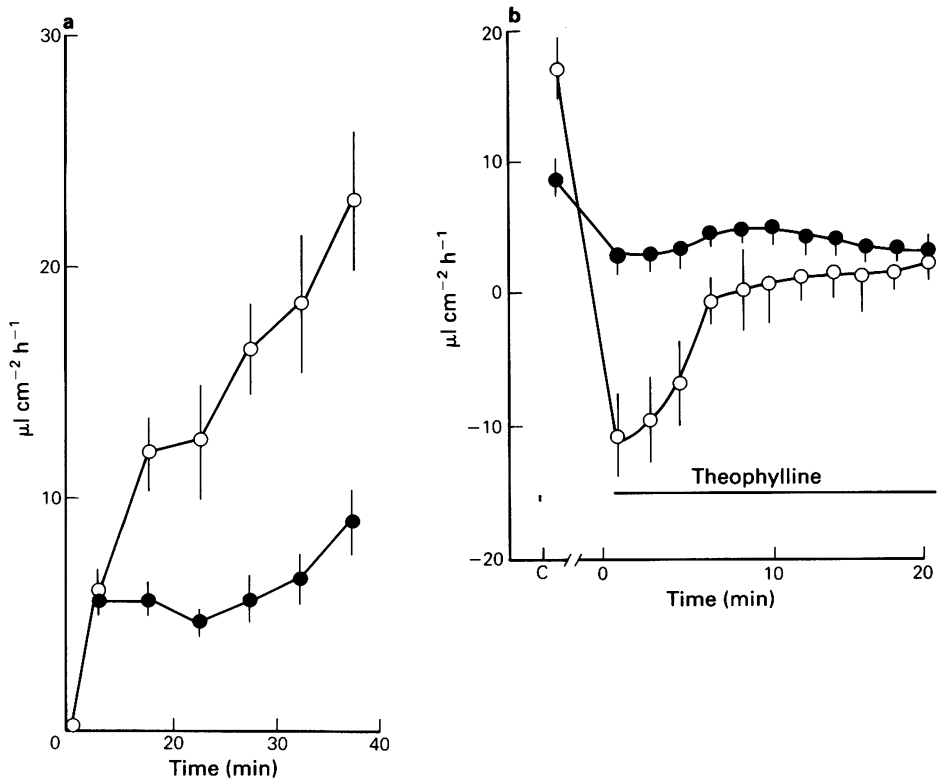

Figure 2 (a) The flow of water measured across the mucosal surface, J_m (○) and serosal surface, J_s (●). Error bars show s.e.mean ($n = 5$). (b) The effect of theophylline (10 μM) added to both mucosal and serosal solutions on water flow rates across the mucosal surface, J_m (○), and serosal surface, J_s (●). Error bars show s.e.mean ($n = 9$).

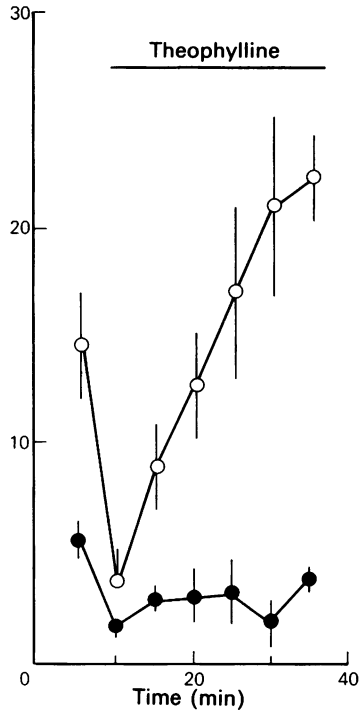

Figure 3 The effect of theophylline (10 mM) added 7 min after the start of incubation on water flow across the mucosal, J_m (○) and serosal surfaces, J_s (●) of tissue bathed in glucose (20 mM) Krebs-Ringer solution. Error bars show s.e.mean ($n = 7$).

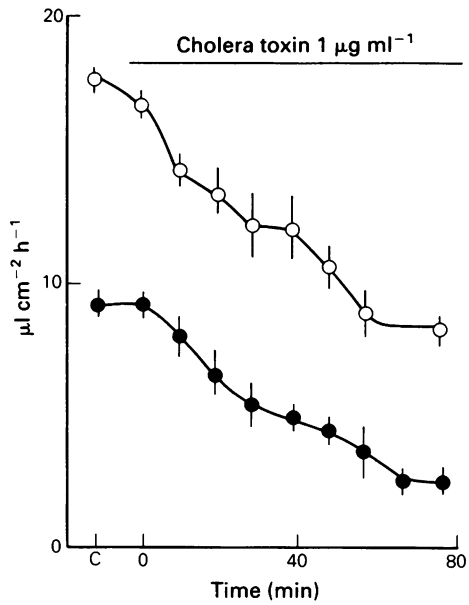

Figure 4 The effect of cholera toxin (1 μg ml^{-1}) added to the mucosal bathing solution on J_m (○) and J_s (●). Error bars show s.e.mean ($n = 10$).

The presence of D-glucose affected the response of the tissue to theophylline. The theophylline-dependent decrease in J_m was insufficient to induce reversal of net flow across the mucosal border and recovery of mucosal inflow to control levels was quick. Outflow across the serosal surface, J_s was reduced immediately by theophylline from 9 ± 1 to $3 \pm 0.5\,\mu l\,cm^{-2}\,h^{-1}$ ($n = 7$) and this decrease is sustained for at least 30 min, during this time J_m recovered to control levels and continued to increase thereafter.

Effects of cholera toxin on water flow

Addition of 1 μg ml^{-1} of purified cholera toxin to the mucosal solution caused a slow, but sustained decrease in water flow across tissues bathed in glucose-free Krebs-Ringer (Figure 4). The time course of the tissue response to cholera toxin was much slower than observed with theophylline. Only after 1 h was the inhibition of mucosal and serosal water flow similar to that seen following exposure to theophylline (10 mM) for 2 min. However, there was no obvious delay in the onset of inhibition of fluid movements. This contrasts with the reported delayed onset of inhibition of ion flux as measured by transepithelial ion fluxes and changes in short circuit current (Field *et al.*, 1972; Powell *et al.*, 1973; Naftalin & Simmons, 1979; Holman *et al.*, 1979). In this series of experiments, mucosal inflow, J_m always exceeded serosal outflow, J_s i.e. the tissue volume increased throughout the duration of the experiment, albeit at a slow rate. Thus, although inflow was inhibited, the tissue is not in a true secretory state.

The decrease in flow across the serosal border without a net reduction in tissue volume must result from a decreased hydraulic conductance of the submucosal (see Discussion).

The effects of loperamide and naloxone on theophylline-dependent changes in water flow

Following a decrease in J_m and J_s induced by theophylline (10 mM), addition of 10 μM loperamide to the serosal bathing solution caused a rapid increase in both J_m and J_s (Table 2). The IC_{50} for the loperamide-dependent increase in J_m was approximately 500 nM (unpublished results, Ahsan & Naftalin).

This was similar to the concentration of loperamide required to reverse the theophylline effect on anion flux across the mucosal border (unpublished results, Ahsan & Naftalin). Both these effects of loperamide were reversed by naloxone (1 μM), indicating that the

Table 2 Effects of theophylline, loperamide and naloxone on water flow across the mucosal surface J_m and across the serosal surface J_s ($\mu l\,cm^{-2}\,h^{-1}$)

Condition	J_m	J_s
Control	18.3 ± 1.7 (16)	10.0 ± 1.0 (16)
Theophylline (10 mM)	3.1 ± 1.1 (15)***	3.3 ± 0.8 (12)**
Theophylline + loperamide (1 μM)	13.3 ± 1.3 (10)‡‡	7.1 ± 1.4 (9)‡‡
Theophylline + loperamide (10 μM)	15.0 ± 1.5 (6)‡‡	8.0 ± 0.8 (4)‡‡
Theophylline + loperamide + naloxone (1 μM)	6.9 ± 1.4 (4)**	5.0 ± 1.1 (4)**

, * Indicate significant difference (Student's unpaired t test) between test and control ($P<0.01$ and 0.001 respectively).
‡‡ Indicates significant difference (Student's unpaired t test) between test and theophylline-treated tissues ($P<0.01$).

antisecretory effect of loperamide *in vitro*, like its action *in vivo*, is via opiate receptors.

Effects of loperamide on cholera toxin-dependent inhibition of water absorption

Loperamide (10 μM), when added to the serosal bathing fluid of tissue previously incubated with cholera toxin (1 μg ml^{-1}) for 1 h induced a rapid increase in both J_m and J_s ($P<0.01$) ($n=4$) (Table 3). When loperamide was added to control tissue an increased flow across the serosal border was occasionally observed.

Direct measurement of ^{77}Br exit across mucosal and serosal surfaces of rabbit ileum in vitro

Theophylline increased ^{77}Br loss across the mucosal surface, ($P<0.01$) and reduced loss across the serosal surface, ($P<0.01$) (Table 4). Loperamide did not alter theophylline-stimulated ^{77}Br loss across the mucosal surface, which remained significantly above that seen in control tissue, ($P<0.05$). However, loperamide increased ^{77}Br exit across the serosal surface of tissues exposed to theophylline, ($P<0.01$). Naloxone prevented the loperamide-dependent increase in ^{77}Br across the serosal surface of theophylline-treated tissue.

These results corroborate the water flux data shown in Figures 2, 3 and 4 and in Tables 3 and 4.

Table 3 Effect of cholera toxin, (1 μg ml^{-1}) added to the mucosa solution on J_m and J_s fluxes, ($\mu l\,cm^{-2}\,h^{-1}$) were measured after 85 min exposure to toxin; fluxes with loperamide measured after 10 min of exposure of the toxin-treated tissue to the drug

	J_m	J_s
Control	13.3 ± 0.8 (4)	6.3 ± 1.1 (4)
Cholera toxin	3.3 ± 1.0 (4)**	3.3 ± 1.1 (4)**
Cholera toxin + loperamide (1 μM)	7.9 ± 0.9 (4)‡‡	6.6 ± 0.9 (4)‡‡

** and ‡‡ indicate significance of difference (paired Student's t test) between condition and control and test and cholera toxin-treated tissue respectively, ($p<0.01$).

Table 4 Rate constants of ^{77}Br exit across the mucosal and serosal surfaces of rabbit ileum

	Exit rate (h^{-1})	
	Mucosal exit rate	*Serosal exit rate*
Control	0.31 ± 0.02 (16)	0.20 ± 0.01 (20)
Theophylline (10 mM)	0.39 ± 0.01 (20)**	0.13 ± 0.01 (20)**
Loperamide (10 μM)	0.30 ± 0.02 (16)	0.19 ± 0.01 (16)
Theophylline + loperamide	0.37 ± 0.02 (17)*	0.19 ± 0.01 (17)‡‡
Theophylline + loperamide + naloxone (1 μM)	0.42 ± 0.04 (12)*	0.14 ± 0.01 (12)***

Values are mean ± s.e.mean; number of independent estimates shown in parentheses.
*, ** and *** test vs control ($P<0.05$, 0.01 and 0.001) respectively; ‡, ‡‡ test vs theophylline ($P<0.05$ and 0.01) respectively.

Steady state tissue retention and fractional exit of [^{3}H]-mannitol across the mucosal surface

Theophylline caused a significant increase in fractional exit rate of mannitol across the mucosal surface (Table 5). This theophylline-dependent increase was not significantly affected by loperamide (not shown). This demonstrated directly that there is a theophylline-dependent increase in extracellular flow across the mucosal surface. Table 5 also shows the effect of theophylline and loperamide (10 μM) on the steady-state tissue retention volume of [^{3}H]-mannitol, measured during serosa-mucosal flow. Loperamide had no significant effect in controls, but reversed the theophylline-dependent decrease in tissue retention. Naloxone prevented this effect.

Table 5 Rate of fractional exit of [^{3}H]-mannitol and steady state retention volume of [^{3}H]-mannitol measured during unidirectional flux from serosal to mucosal bathing solutions

	Rate of fractional exit (h^{-1})	*Steady state retention volume* ($\mu l\,cm^{-2}$)
Control	0.18 ± 0.01 (70)	25.0 ± 0.92 (67)
Loperamide (10 μM)	—	28.0 ± 2.26 (14)
Theophylline (10 mM)	0.29 ± 0.02 (51)***	14.8 ± 0.9 (48)***
Theophylline + loperamide	—	23.9 ± 1.8 (31)‡‡
Theophylline + loperamide + naloxone (1 μM)	—	16.5 ± 2.1 (7)*
Total extracellular space (bilateral)		73.0 ± 8.71 (4)

Values are mean ± s.e.mean; number of independent estimates in parentheses.
* and *** indicate significant difference between test and control ($P<0.05$ and <0.001, respectively).
‡‡ Significance of difference between test and theophylline (10 mM) treated ($P<0.01$).

Table 6 The effects of theophylline (10 mM), loperamide (5 μM) and naloxone (1 μM) on the hydraulic permeability, L_p of the serosal surface of rabbit isolated ileum, determined from the change in water flow across the serosal surface after addition of polyethylene glycol (molecular weight 20000 or 90000) to the serosal bathing solution

	$L_p \times 10^9$ ($cms^{-1}\,cmH_2O^{-1}$)	*n*
Condition		
Control	36.0 ± 3.1	11
Theophylline	15.5 ± 2.3	5***
Theophylline + loperamide (5 μM)	52.7 ± 8.4	8§§§
Theophylline + loperamide + naloxone (1 μM)	18.8 ± 5.1	2‡

*** Indicates significance of difference between test and control ($P<0.001$).
§§§ Indicates significance of difference between test and theophylline ($P<0.001$).
‡ Indicates significance of difference between test and theophylline + loperamide (5 μM) ($P<0.01$) (Student's impaired *t* test).

Effect of theophylline and loperamide on the osmotic permeability of the submucosa

The results showing that there was a theophylline and cholera toxin-dependent reduction in fluid outflow across the serosal surface without a concurrent decrease in submucosal volume and the theophylline-dependent decrease in ^{77}Br exit across the serosal surface, indicate that the hydraulic and ion conductance of the submucosal tissue are decreased by theophylline. To test this hypothesis directly, the serosal hydraulic conductance was measured by measuring the osmotic L_p of the serosal surface with high molecular weight polyethylene glycol (see Methods).

Table 6 shows the estimated osmotic L_ps measured following addition of either 2 mosmol kg^{-1} polyethylene glycol 20000, or 1 mosmol kg^{-1} polyethylene glycol 90000. As there was no significant difference between the L_ps obtained with either macromolecule, the combined data are shown.

Theophylline caused a significant decrease in L_p of the serosal surface. The L_p obtained with loperamide and theophylline together was significantly greater than that observed even in controls. Naloxone reversed this loperamide-dependent increase in L_p.

These changes are more than sufficient to account for the theophylline-dependent decrease in transepithelial ion flux and water flux and the opiate-dependent reversal of theophylline and cholera toxin action.

Discussion

The consensus view of the rise in short circuit current following exposure to intestinal secretagogues is that it is associated with a net anion secretion due to increased anion conductance (Field *et al.*, 1972; Powell *et al.*, 1973; Nellans *et al.*, 1973; Naftalin & Simmons, 1979). The absence of any effect of loperamide, or morphine, or leucine-enkephalin on the theophylline-induced 'secretory potential' (Figure 1) is difficult to reconcile with the view that alterations in enterocyte metabolism and membrane permeability to ions are the sole explanation for altered ion and water transport.

Effects of loperamide on calmodulin and cyclic AMP metabolism

Anticalmodulin drugs, like trifluoperazine, prevent intestinal secretion (Ilundain & Naftalin, 1979; Smith & Field, 1980) by preventing the Ca^{2+}-dependent increase in anion conductance across the mucosal border. Some reports show that loperamide interacts with calmodulin and hence, suggest that it affects intestinal secretion in a similar way to trifluoperazine, (Merrit *et al.*, 1982; Zavecz *et al.*, 1982). We have confirmed that loperamide binds to purified calmodulin and also inhibits calmodulin-dependent phosphodiesterase activity. However, this effect of loperamide was not inhibited by naloxone, (Ilundain, Naftalin, Popp & Sandhu; unpublished results). Furthermore, we have confirmed (Ilundain *et al.*, unpublished results) that loperamide does not affect cyclic AMP levels in rat intestine exposed to cholera toxin, (Farack *et al.*, 1981).

The results in this paper (Table 2 and Figure 1) indicate that loperamide did not reduce the theophylline-dependent increase in anion conductance, or permeability of the mucosal border. It therefore seems very improbable that the naloxone-sensitive antisecretory action of loperamide, or other opiates *in vitro*, is primarily mediated via effects on enterocyte calmodulin, or cyclic AMP metabolism.

Evidence for submucosal action of theophylline, cholera toxin and loperamide

Transepithelial intestinal ion and water flow *in vitro* is due to flow across both the mucosal and submucosal, or 'serosal' of layers. These can be regarded as hydraulic resistances in series. Net flow across the mucosal layer has been characterized as the resultant flow across three parallel conductances, namely: a low conductance transcellular route, which has a low hydraulic conductance and does not permit passive flows of even low molecular weight solutes; a low conductance paracellular route, which permits osmotic flow generated by low molecular weight solutes, like NaCl and sucrose and a high conductance paracellular shunt pathway, across which high rates of osmotic flow may be generated by high, but not low, molecular weight solutes. The flows across the wide shunt pathway are not ion-selective and hence are electrically silent (Naftalin & Tripathi, 1985; 1986). The serosal borders of rabbit and rat intestine behave as a membrane with low conductance homogeneous porosity (mean pore radius of 6.5 nm) (Naftalin & Tripathi, 1985; Fromm *et al.*, 1985). The hydraulic conductance of the serosal surface is in the range $1 - 5 \times 10^{-8}\,cms^{-1}\,cmH_2O^{-1}$ (Table 6). Thus, in the absence of a colloid osmotic pressure gradient across the serosal surface, net fluid flow from the submucosa to serosal bathing solution is determined solely by the interstitial pressure, P_i and L_p of the serosal surface.

$$J_s = L_p.P_i$$

The interstitial pressure, P_i is inversely related to the volume elasticity, K (distensibility or tone) of the submucosa and the fractional distension, V/V° of the submucosa relative to the resting level, where V° is the resting volume of the submucosa: i.e. $P_i = V/(K.V^\circ)$.

With a serosal L_p of $3.6 \times 10^{-8}\,cms^{-1}\,cmH_2O^{-1}$ (Table 6), the interstitial pressure required to generate a flow across the serosal surface of $5\,\mu l\,cm^{-2}\,h^{-1}$ is approximately 40 cmH_2O. If the interstitial pressure is maintained when theophylline reduces the L_p to $1.5 \times 10^{-8}\,cm\,s^{-1}\,cmH_2O^{-1}$ (Table 6), then J_s will fall to approximately $2\,\mu l\,cm^{-2}\,h^{-1}$, as was observed (Figure 2).

Following addition of loperamide, the serosal L_p increased by more than threefold to $5.3 \times 10 \times 10^{-8}$ $cms^{-1}\,cmH_2O^{-1}$ (Table 6), but serosal outflow increased only by approximately twofold to $10\mu lcm^{-2}\,h^{-1}$ (Table 3). This small increase in J_s relative to the increase in serosal L_p indicates that loperamide also induced a decrease in interstitial pressure (from 40 cmH_2O to 25 cmH_2O). This fall in interstitial pressure is consistent with an increase in submucosal distensibility from 1.5×10^{-3} to $4 \times 10^{-3}\,cmH_2O^{-1}$.

Other experimental results indicating that theophylline and cholera toxin affect net fluid and electrolyte flow by altering the conductance of the submucosal layers of the *in vitro* preparation are: (a) theophylline and cholera toxin decreased fluid movement across the serosal border, even when the tissue volume was increasing; i.e. $J_m > J_s$ (Figures 2, 3). This apparent separation between the effect of the secretagogue on J_m and J_s was particularly obvious in tissues bathed in Krebs-Ringer containing 20 mM D-glucose and is consistent with the observation that raised plasma glucose in rats perfused *in vivo* increases absorption (Lee, 1987). (b) There was a theophylline-dependent decrease in ^{77}Br exit rate across the serosal surface but a theophylline-dependent increase across the mucosal surface (Table 4). (c) All of the effects of theophylline on serosal flow were reversed by the opiate action of loperamide (Tables 1, 2, 3 and 4).

Effects of secretagogue action on fluid and electrolyte movements across the mucosal surface

Within 2 min after addition of theophylline, J_m decreased by $27\,\mu l\,cm^{-2}\,h^{-1}$ (Table 3). However, in tissues exposed previously, either to cholera toxin or theophylline, addition of loperamide to the serosal bathing solution was followed within 5 min by an increase in J_m of $10\,\mu l\,cm^{-2}\,h^{-1}$ (Tables 3 and 4).

Because J_m changed concurrently with J_s and J_s is determined solely by mechanical factors within the

submucosa, it may be deduced that the changes in J_m are, at least partially, related to interstitial pressure changes.

Furthermore, as the theophylline-dependent decrease in J_m and J_s and the loperamide-dependent increase in J_m and J_s both take place prior to any significant change in tissue volume, these changes are likely to be due to altered distensibility of the submucosa. The theophylline-dependent decrease in J_m is consistent with increased fluid reflux via the wide shunt channel caused by increased interstitial pressure. The loperamide-dependent increase in J_m and concurrent small increase in J_s are consistent with an increase in distensibility, with consequent decrease in interstitial pressure.

The hydraulic conductance of the mucosal shunt pathway in rabbit ileum *in vitro* is 2×10^{-7} cms^{-1} cm H_2O^{-1} (Naftalin & Tripathi, 1985; 1986). Hence a theophylline-dependent decrease in distensibility from 2.5×10^{-3} to 1.5×10^{-3} cmH_2O^{-1} would increase interstitial pressure without any immediate change in interstitial volume and so would increase fluid backflux via the wide mucosal shunt pathway from 25 to 50 μl cm^{-2} h^{-1}. This increased reflux is sufficient to account for the entire transient net fluid secretion observed. The increase in the net rate of fluid absorption across the mucosal surface, J_m following application of loperamide to the serosal bathing solution is consistent with the increase in submucosal distensibility from 1.5×10^{-3} to 4×10^{-3} cmH_2O^{-1} and a decreased fluid reflux via the wide shunt channels.

Unambiguous experimental evidence corroborating the view that theophylline induces fluid reflux via mucosal shunt channels is the theophylline-dependent increase in mannitol efflux across the mucosal surface (Table 5). The observed theophylline-dependent increase in anion efflux across the mucosal surface, (Table 4) could be due both to an increase in transcellular anion permeability and paracellular flux.

The reduction in $J_{m\text{-}s}$ Cl flux following exposure to theophylline (Table 1) has been ascribed to inhibition of an electroneutral uptake of NaCl (Nellans *et al.*, 1973; Frizzell *et al.*, 1979). An alternative suggestion that the apparent theophylline-dependent decrease in J_{ms} is due to enhanced reflux of isotope via mucosal shunt pathways was made because of the estimated theophylline-dependent increase in Cl permeability of the mucosal surface, (Naftalin & Simmons, 1979; Ilundain & Naftalin, 1979).

A cyclic AMP induced increase in Cl conductance in the apical membrane of *Necturus* gallbadder epithelium has been observed using electrophysiological methods (Petersen & Reuss, 1983).

This view is corroborated by the following results: (a) the rate of exit of ^{77}Br from theophylline-treated tissue is enhanced (Table 4); (b) the rate of exit of mannitol across the mucosal surface seen in the presence of theophylline is increased (Table 5).

Relationship of in vitro *to* in vivo *action of secretagogues and opiates*

The relationship of the actions of secretagogues and opiates on the conductance of the submucosal tissues of rabbit ileum *in vitro* to their actions *in vivo* is uncertain. The fact that the submucosal compartment is drained by lymphatics and capillaries means that interstitial pressure will be much less *in vivo* than *in vitro* and hence the backflux component via the mucosal shunt will also be less. The extent of this backflux in *in vivo*, control and secreting tissues is uncertain. Nevertheless, it has been shown that fluid movements *in vivo* are related to smooth muscle tone and interstitial pressure, (Granger *et al.*, 1979; Lee, 1983). A recent study by Lee (1987) has shown that submucosal factors have a large influence on fluid absorption in rat intestine *in vivo*.

It is clear that more detailed studies on the action of opiates and secretagogues *in vivo* are required to understand how these agents affect fluid and electrolyte absorption when circulation is intact.

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