A Novel *In Vitro* Electrophysiological Bioassay for Transport of Loperamide Across Intestinal Epithelia

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INTRODUCTION

The mechanism of action of many common non-anti-microbial anti-diarrhoeal agents such as loperamide at the level of the intestinal cell is not yet established because most research has concentrated on assessment of anti-motility effects on intestinal smooth muscle (1). *In vitro* direct anti-secretory effects of loperamide have been shown in voltage-clamped rat colonic mucosal preparations where secretagogue-stimulated short-circuit currents (Isc) were inhibited (2). Experiments with *in vitro* intestinal smooth muscle indicated that loperamide could also directly inhibit carbachol-induced contractions, evidence in support of an anti-motility effect (3). Whichever mechanism, if any, predominates for non anti-microbial anti-diarrhoeals such as loperamide, adequate GI absorption is necessary for therapeutic effect. *In vivo*, oral uptake of loperamide in normal subjects appears too and slow and inadequate (1–5% in 4 hours) to account for its many effects (4). Furthermore, local accumulation and metabolism of loperamide in the intestinal wall adds complexity to the absorption profile and suggests that it may be secreted back into the lumen (5).

Recently we showed that a similar anti-diarrhoeal agent, the isoquinolone berberine, displayed poor absorption *in vitro* in rat colonic mucosae and T84 human colonic intestinal monolayers where an apparent permeability coefficient (Papp) of the order 10^{-7} cm/s was obtained in each model (3,6) respectively. When added to the basolateral sides of these tissues berberine had a direct anti-secretory effect in rat colon mucosae in response to cyclic nucleotide- and calcium-mediated stimuli (7). Paracellular flux of berberine was induced by pharmacological opening of the tight junctions with cytochalasins, a condition which also conferred apical-side sensitivity to the anti-secretory effects of the agent (3).

We carried out *in vitro* fluxes of loperamide in Caco-2 monolayers with an associated functional electrophysiological anti-secretory assay in rat colonic mucosae, namely the capacity to inhibit both basal and cyclic AMP-stimulated electrogenic chloride secretion. We show that apical side sensitivity can also be conferred to loperamide by opening the epithelial cell tight junctions with low concentrations of cytochalasin D (CD) thereby directly linking an induced paracellular flux to a functional electrophysiological bioassay in a condition which may be similar to compromised cells *in vivo*. Finally we offer a unified hypothesis suggesting action of anti-diarrhoeal agents at a basolaterally-located epithelial K^+ channel, which may serve as a possible common final effector epithelial site of action. This site of action appears only to be accessible under certain conditions in which the intestinal cells are compromised. The study combines transepithelial drug permeation with a funtional bioassay in a way which is pertinent to anti-diarrhoeal drug delivery.

MATERIALS AND METHODS

Materials

Forskolin was obtained from Calbiochem (UK). Loperamide hydrochloride and CD and Orange IV were from Sigma (UK). Transwell Snapwells® were from Costar (Netherlands). The diffusion chambers were from Precision Instruments (Tahoe, USA) and the DVC 1000 Dual Voltage Clamp and electrode system were from World Precision Instruments (WPI), UK. The EVOM voltammeter system was from WPI (UK). The MacLab Data Acquisition System was obtained from AD Instruments (UK). Tissue culture medium was from Gibco (UK).

Isolation of Colonic Mucosae from Rats

Segments of proximal colon from male Wistar rats were isolated and stripped of smooth muscle. The tissue segments were mounted in diffusion chambers with a window area of 0.63 cm^2 and a volume on each side of the tissue of 10 ml. Tissues were bathed in Krebs-Henseleit solution, maintained at 37°C by a heated water jacket and oxygenated with carbogen by a gas-airlift system. Measurements of transepithelial electrical resistance (TEER) and short-circuit current (Isc) were by means of Ag/AgCl voltage and current electrodes connected via agar bridges containing 3 mM KCl/agar gel. The electrodes were attached to a DVC 1000 Dual Voltage Clamp which was linked to a MacLab Data Acquisition System.

Electrophysiological Methods Using Rat Colonic Mucosae

Since measurement of Isc changes in response to secretagogues in Caco-2 is highly unpredictable, we chose rat colon tissue for detailed electrophysiological studies. Basal Isc was measured 30 minutes after addition of loperamide (10–50 μM) to either the apical or basolateral side of the tissue. Cyclic-AMP-stimulated Isc, an indirect measure of electrogenic chloride secretion in rat colon, was measured after addition of the adenylate cyclase activator, forskolin, in two concentrations (4 and 10 μM) in the presence and absence of loperamide. CD (2 μg/ml) was present bilaterally for 60 minutes during paired experiments with untreated tissue to test whether tight junction openings could influence the effects of loperamide on the stimulated Isc.

The membrane potential of colonic mucosae were clamped stepwise over a range ±2 mV in order to establish the linear

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Ohmic relationship. During experiments, intermittent voltage clamping every 5 minutes from zero to 0.5 mV for 3 seconds allowed TEER to be calculated from the resulting current change.

Culture of Caco-2 Monolayers

Caco-2 (Passage 30–40, ATCC) were grown in DMEM supplemented with glutamax®, foetal bovine serum (10%), non-essential amino acids (1%), sodium pyruvate (1%), penicillin (50 U/ml) and streptomycin (50 U/ml). Cells were subcultured onto Costar tissue culture-treated 12 mm diameter Snapwell filters of area size 1.13 cm^2 (Catalogue number 3407). Monolayers were used for fluxes at day 20–28, at which point a mean TEER of 300 Ω cm^2 was achieved, as indicated by the EVOM epithelial voltammeter.

Fluxes of Loperamide Across Caco-2

Passive flux measurements across rat colon segments display wide variation by comparison with those obtained across Caco-2. In previous work with T84 colonic epithelial monolayers using berberine (6) we used the monolayers for both electrophysiology and fluxes. Despite the excellent cyclic-AMP-stimulated Isc changes achieved in those experiments, the undifferentiated crypt cell phenotype (8) of T84 would not be expected to act as a good model for drug absorption across absorbing epithelium *in vivo* in comparison to the villous differentiated absorptive cell phenotype of Caco-2.

Loperamide fluxes were determined under sink conditions over 120 minutes in Caco-2 monolayers. Snapwells were mounted on an orbital shaker and maintained in an incubator at 37°C. Experiments were carried out in Hank's Balanced Salt Solution (Gibco catalogue number 14065-031) buffered to pH 7.4 with HEPES (25 mM) with addition of D-glucose (5 mM) and with an osmolarity of 281 mOs/l. Samples were taken from the basolateral side every 30 minutes and the solution replaced with fresh buffer. Analysis of loperamide was by the colorimetric Orange IV assay with spectrophotometric determination at 405 nm (9). In some experiments CD (2 μg/ml) was present on both sides of the monolayers throughout the flux of loperamide.

Papp was determined from the following equation (10),

$$\text{Papp} = \frac{dQ}{dt} \times \frac{V}{A \cdot C_0}$$

where dQ/dt is flux rate (mmoles/min), V is volume of receiver chamber (ml), A is surface area of the monolayer (cm^2) and C_o is the starting concentration (mmoles) on the apical side. The flux rate was calculated from the slope of the regression line of the amount transported over time.

Statistics

Statistic analysis was by means of the unpaired or paired Student's t-test for matched or paired preparations respectively. $P < 0.05$ (2 tailed) was regarded as being statistically significant.

RESULTS AND DISCUSSION

Electrophysiological Effects of Loperamide in Rat Colonic Mucosae

Basal Isc was statistically reduced only when loperamide was present on the basolateral but not the apical side of tissue. Table 1 shows that even 50 μM loperamide on the apical side was without effect on basal Isc by comparison with the effect of 10 μM loperamide administered to the basolateral side.

Basolateral addition of forskolin to rat colon mucosae as expected caused a concentration-dependent increase in Isc, which is accounted for by stimulation of electrogenic chloride secretion in this tissue (11). When 50 μM loperamide was present on the basolateral side of the tissue for 30 minutes, the forskolin-stimulated Isc was reduced ($P < 0.05$). By contrast, using the same protocol except with loperamide added to the apical side of tissue, the cyclic-AMP-stimulated Isc was unaffected. As in the case of the basal current, inhibition of the stimulated current by loperamide only occurred when the agent was added to the basolateral side.

Measurements of TEER decreases in response to loperamide showed that in control rat colon segments the percentage decrease in TEER was 8.2 ± 4.9% by comparison to 33.2 ± 2.9% for CD-treated segments ($P < 0.001$). Previous work showed that CD in low concentrations causes tight junction openings which are non-toxic to cells and which indirectly lead to the increase in flux of paracellular flux of marker molecules (12).

When CD was added bilaterally to tissue during the test period when loperamide (50 μM) was administered to the apical side, the forskolin-stimulated Isc was statistically inhibited almost to the same level as occurred upon basolateral addition of loperamide in the absence of CD. These results are summarised in Fig. 1. Importantly, CD alone was without effect on forskolin-stimulated Isc (results not shown).

Flux of Loperamide Across Caco-2

The absorptive flux of loperamide was linear and unsaturated over 120 minutes. The maximum flux per hour was on average 1–2% using concentrations of 1 mM and 2 mM on the donor side. The Papp for loperamide was $4.1 \pm 0.5 \times 10^{-6}$ cm/s, a value indicative of poor absorption across Caco-2 if measured against the Papp values for typical low and high permeability reference compounds, such as mannitol (10^{-6} cm/s) and naproxen (7.4×10^{-5} cm/s) respectively. In the presence of 2 μg/ml CD, the Papp of loperamide was statistically increased to $1.2 \pm 0.5 \times 10^{-5}$ cm/s ($P < 0.005$). The pooled basolateral flux of loperamide in presence and absence of CD

Table 1. Basolateral-Side but not Apical-Side Addition of Loperamide Inhibited Basal Isc in Rat Colon

	% Reduction in Basal Isc
10 μM loperamide (B)	35.3 ± 9.9 (8)[a]
50 μM loperamide (B)	87.9 ± 11.0 (7)[b]
10 μM loperamide (A)	1.2 ± 0.7 (6)
50 μM loperamide (A)	15.9 ± 3.9 (6)

Note: Basal Isc was 32.1 ± 2.9 μA cm^{-2} (n = 30). Tissues were equilibrated for 30 minutes in the diffusion chambers. Measurement of basal Isc was made 30 minutes after addition of loperamide or of vehicle control. (A) and (B) correspond to Apical and Basolateral respectively. Values are expressed as a percentage reduction from the basal current in control tissues. N numbers are given in brackets.
[a] $P < 0.05$.
[b] $P < 0.005$.

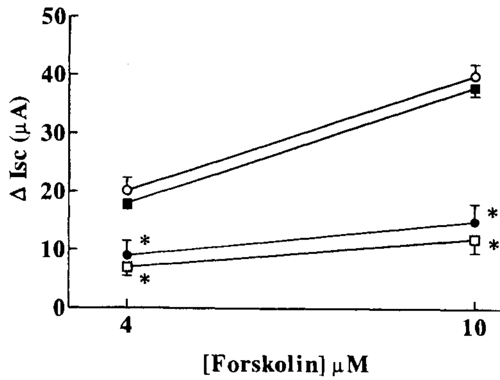

Fig. 1. Cytochalasin D confers apical-side sensitivity to loperamide in rat colonic mucosae. In the presence of 2 μg/ml CD (apical and basolateral), apically-added loperamide (50 μM) inhibited forskolin-stimulated Isc, whereas apically-added loperamide in the absence of CD was without effect. Basolaterally-added loperamide also inhibited the Isc similarly to that caused by apically-added loperamide in the presence of CD. Symbols: (○) control, (■) apically-added loperamide, (●) basolaterally-added loperamide, and (□) apically-added loperamide with CD. * $P < 0.05$ with respect to controls. N = 5–10 for each group.

is shown in Fig. 2. The results show that loperamide flux was increased shortly after exposure of the monolayers to CD. That CD boosts the flux of loperamide may be compared with results from our recent study in which the absorptive flux of berberine was also boosted in rat colonic mucosae (3) and in cultured monolayers of the human colonic epithelial cell line, T84 (6). The CD stimulation of loperamide flux in Caco-2 is consistent with the electrophysiological effects of loperamide in rat colon in which CD conferred apical-side sensitivity.

Fig. 3 shows a working model of how non-antibiotic anti-diarrhoeal agents such as loperamide and berberine may act in the intestine. Under normal conditions, fluxes of these agents are very poor, reflected in the lack of an anti-secretory effect by apical but not basolateral addition of either agent. In the presence of CD the induced apical-side sensitivity to the anti-secretory action of these agents imply that inhibition of a basolateral K^+ channel may occur under conditions in which paracellular flux of the anti-diarrhoeals are permitted. In turn, blockade

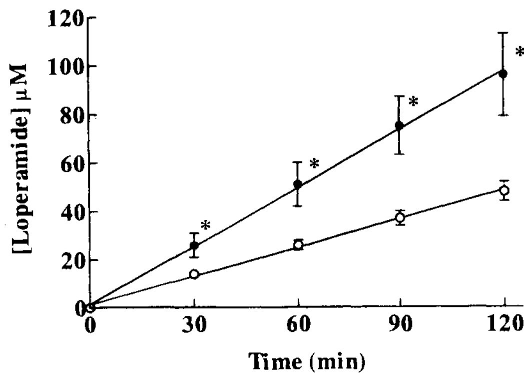

Fig. 2. Absorptive flux of loperamide across Caco-2 monolayers in the presence (●) and absence (○) of 2 μg/ml CD (apical and basolateral). * $P < 0.03$ in all cases. N = 5 for the loperamide group and N = 6 for the loperamide with CD group. The mean starting concentration for loperamide on the donor apical side for each group was 1 mM.

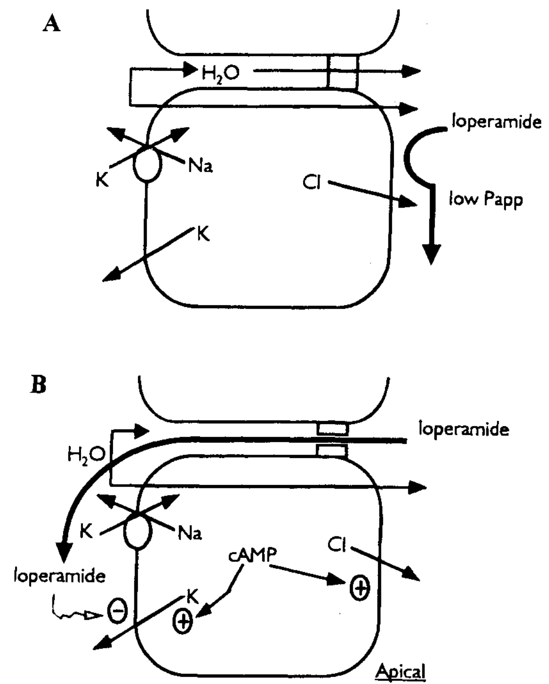

Fig. 3. Working model of the delivery and anti-secretory effect of loperamide at the basolateral side of intestinal epithelia. In A, under normal conditions loperamide is quite poorly absorbed. In B, under pharmacological conditions similar to those expected in secretory diarrhoea, loperamide is absorbed at least in part through the open tight junctions. Loperamide then acts directly at basolateral K^+ channels to directly inhibit cyclic-AMP induced electrogenic chloride secretion which is directly linked to water efflux.

of basolaterally-located K^+ channels interferes with the electrochemical gradient for electrogenic chloride secretion via the CFTR chloride channel on the apical membrane. Because K^+ cannot exit the cell across the basolateral membrane, the $Na^+/K^+/2Cl^-$ cotransporter is not able to carry Cl^- into the cell across the same membrane against its electrochemical gradient due to unavailability of K^+, and therefore there is a reduction in the amount of Cl^- available to complete the secretion process by moving out of the cell across the apical membrane down an electrochemical gradient. Further indirect evidence in support of the model is that berberine was shown to inhibit stimulated ^{86}Rb efflux from monolayers of T84 (unpublished results). The only other potential sites for interference by loperamide and berberine with electrogenic chloride secretion from the basolateral side would be direct inhibition of the $Na^+/K^+/2Cl^-$ cotransporter or a reduction in intracellular calcium or cyclic nucleotides, interactions for which to our knowledge there is little evidence. A direct association between chloride and water efflux in crypts is long-established (8) thus supporting a link between inhibition of electrogenic chloride secretion and diarrhoea.

Overall, the results show that loperamide has a direct anti-secretory effect on intestinal tissue, but such an effect is seen only when open epithelial tight junctions permit paracellular flux of the agents to the basolateral side, a condition not unlike what would be expected in compromised tissue (3). The apical-side sensitivity associated with increased loperamide flux induced by CD in rat colon and Caco-2 respectively suggest that this pharma-

cological model may be an adequate model for paracellular drug absorption across tissue undergoing secretory diarrhoea.

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