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Research Paper

Mechanisms of action of zinc on rat intestinal epithelial electrogenic ion secretion: insights into its antidiarrhoeal actions

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Abstract

Objectives Zinc is a useful addition to oral rehydration therapy for acute diarrhoea. We have assessed the mechanism of its epithelial antisecretory action when intestinal epithelial tight junctions were pharmacologically opened.

Methods Rat isolated ileal and colonic mucosae were mounted in Ussing chambers and exposed to $ZnSO_4$ (Zn^{2+}) in the presence of secretagogues and inhibition of short circuit current (I_{sc}) was measured.

Key findings Pre-incubation with basolateral but not apical Zn^{2+} reduced I_{sc} stimulated by forskolin, carbachol and A23187. In the presence of the tight junction-opener, cytochalasin D, antisecretory effects of apically-applied Zn^{2+} were enabled in colon and ileum. The apparent permeability coefficient (P_{app}) of Zn^{2+} was increased 1.4- and 2.4-fold across rat ileum and colon, respectively, by cytochalasin D. Basolateral addition of Zn^{2+} also reduced the I_{sc} stimulated by nystatin in rat colon, confirming K channel inhibition. In comparison with other inhibitors, Zn^{2+} was a relatively weak blocker of basolateral K_{ATP} and K_{Ca2+} channels. Exposure of ileum and colon to Zn^{2+} for 60 min had minimal effects on epithelial histology.

Conclusions Antisecretory effects of Zn^{2+} on intestinal epithelia arose in part through nonselective blockade of basolateral K channels, which was enabled when tight junctions were open.

Introduction

Use of oral rehydration solution (ORS) therapy has led to a threefold reduction in child mortality from acute diarrhoea in the 30 years since its introduction.[1] ORS treats the dehydrating symptoms caused by cholera toxin (CT), but it has limited effects on severity and duration of diarrhoea. It stimulates sodium and water re-absorption via the sodium-dependent glucose transporter-1 (SGLT-1) on the apical membrane, which adequately functions despite persistent fluid secretion induced by CT.[2] Field trials proved however, that ORS supplemented with zinc might save a further 400 000 children under the age of five per year.[3] Despite encouraging efficacy data in clinical trials and the support of the World Health Organization, treatment take-up in the Developing World is slow, even though the cost of supplying 20 mg dispersing tablets for a 10-day course is estimated to be just 25 cents.[4] Furthermore, the current lack of detailed knowledge of the mechanism of action of zinc in the intestine seems to limit widespread support for its therapeutic use.[2] For example, aside from zinc's known antibacterial activity in infectious disease, study of its antisecretory effects on intestinal epithelia has produced contradictory results, characterised by species differences, different effects according to the zinc concentration used, and little consensus on the range of intracellular mediators it opposes.[5] In a PubMed search of antisecretory effects of zinc over a 10-year period, we found just 10 papers addressing mechanism(s) of action at the intestinal epithelial cell and tissue level, although there is no shortage of studies describing epithelial zinc transport proteins (e.g. Heitzmann and Warth[6]).

Investigations of zinc's actions have been studied before in isolated rat ileal mucosae, where basolateral addition of $ZnCl_2$ reduced cAMP-mediated electrogenic chloride secretion, but was without effect on that induced by the calcium-generating muscarinic agonist, carbachol.[7] Zinc also blocked the inhibition of short circuit current (I_{sc}) stimulated by 5-hydroxytryptamine (5-HT), vasoactive intestinal peptide (VIP) and carbachol in the isolated small intestine of piglets, an effect requiring high basolateral concentrations.[8,9]

Anti-secretory effects of zinc on forskolin-induced I_{sc} in pig intestine were directly at the epithelium and did not involve neurotransmitter release from the enteric nervous system.[10] Importantly, zinc blocked cAMP-stimulated ^{86}Rb efflux from rat isolated ileal crypt cells, suggesting involvement of K channels.

In Caco-2 monolayers, zinc blocked the secretory electrogenic chloride transport induced by CT (cAMP-mediated), but not that of *Escherichia coli* heat labile enterotoxin (cGMP-mediated).[11] In contrast to the rat ileal study, the same group demonstrated that $ZnCl_2$ reduced calcium-dependent chloride secretion induced by carbachol in Caco-2 monolayers, as well as inhibiting the nitric oxide-dependent secretion induced by interferon-γ.[12] Zinc also increased sodium absorption by activating the apical membrane sodium-hydrogen exchanger (NHE3), in T84 intestinal epithelial monolayers.[2] There is no evidence that it blocks the cystic fibrosis transmembrane regulator, CFTR, or the Na-K-2CI co-transporter or the sodium-potassium ATPase, any of which would also lead to inhibition of electrogenic chloride secretion.

In sum, current data across several in-vitro intestinal models suggest that zinc acts to block cAMP and nitric oxide (NO)-mediated secretion, but not that stimulated by cyclic GMP, and data on interference in calcium-mediated signalling is equivocal. In the absence of effects on intestinal smooth muscle contraction or data to suggest a role in repair of the secreting epithelium, zinc is an anti-secretory agent due to a combination of inhibition of stimulated electrogenic chloride secretion likely via K channel inhibition and by promotion of sodium re-absorption via the NHE3. Our aim was therefore to further examine the antisecretory effects of Zn^{2+} in the form of $ZnSO_4$ in rat ileal and, for the first time, in colonic tissue mucosae mounted in Ussing chambers to generate more specific mechanistic data that would further support clinical use. The adenylate cyclase activator, forskolin was used to drive cAMP-mediated electrogenic chloride secretion and to mimic the effects of cholera toxin A subunit (CTA) activation of G_s.[13] We provide novel data that pharmacological opening of epithelial tight junctions with the established modulator, cytochalasin D, enabled increased zinc permeability and conferred antisecretory action for apically-added concentrations of Zn^{2+}.[14] We have provided new evidence that this effect was mediated primarily by blockade of separate basolateral K channels modulated by cAMP and intracellular calcium. Finally, we confirm that none of these effects were associated with acute cytotoxicity at the concentrations used.

Materials and Methods

Rat intestinal mucosae

Male Wistar rats (250–300 g; Charles River Labs, Margate, UK) were killed by the approved method of stunning, followed by cervical dislocation. We adhered to the UCD AREC policy, 'Regarding the use of post mortem animal tissue in research and research (2007)' (see: http://www.ucd.ie/researchethics/pdf/arec_post_mortem_tissue_policy.pdf). Ileal and colonic segments were removed and placed into freshly oxygenated Krebs-Henseleit buffer (KHB) at pH 7.4 at 37°C. Excised intestinal tissue was dissected and muscle-stripped according to previous methods.[15]

Electrophysiology of intestinal mucosae

Mucosae were pinned between Ussing chamber halves with a circular diameter of 0.63 cm^2, and 5 ml KHB was added bilaterally. KHB was oxygenated using a gas-lift system with 95% O_2/5% CO_2. Each chamber half had a voltage (V) and current (I) Ag/AgCl electrode that were connected to a pre-amplifier (Pre-Amp), and all four Pre-Amps were connected to the voltage clamp apparatus (EVC4000; WPI, Stevenage, UK). The potential difference (PD, mV) was measured across the mucosa in an open circuit configuration. When the voltage was clamped to 0 mV, the short circuit current (I_{sc}, $\mu A/cm^2$) was determined. After 20 min equilibration, tissue transepithelial electrical resistance (TEER, $\Omega.cm^2$) was determined using Ohm's Law. Zn^{2+} in the form of $ZnSO_4$ (40 nM–4 mM) was added to either side of the epithelium before basolateral additions of forskolin (10 μM), the muscarinic agonist carbachol (10 μM) or the calcium ionophore A23187 (10 μM). In some experiments, tissues were pre-incubated bilaterally with the tight junction opener cytochalasin D (8 μM), and I_{sc} responses determined to forskolin and A23187 in the presence of apical Zn^{2+}. After additions, the ΔI_{sc} was recorded as a measure of electrogenic anion secretion across the mucosae. In selected studies, the K^+ channel blockers, $BaSO_4$ (10 mM), tetrapentylammonium (TPeA; 100 μM) or tetraethylammonium (TEA; 10 mM) were added to the basolateral sides of mucosae and compared with effects of Zn^{2+}.

Zinc permeation across colonic mucosae

Zinc concentrations were determined using a QuantiChrom Zinc Assay Kit (BioAssay Systems, Hayward, CA, USA) with a linear detection range of 0.12–10 μM.[16] Zn^{2+} (0.4 mM) was added apically in the presence or absence of cytochalasin D (8 μM) and basolateral samples (200 μl) were taken every 20 min for 120 min. Samples were stored at −20°C in 96-well plates. Absorbance was read at 425 nm after 30-min incubation on a multiplate reader. The apparent permeability coefficient (P_{app}, cm/s) of zinc was determined using the following equation:

$$Papp = \frac{dQ}{dt}\left(\frac{1}{A \times Co}\right)$$

Where dQ/dt is the transport rate across the epithelium (mol/s), A is the surface area (0.63 cm^2) and C_0 is the initial concentration of zinc on the apical-side (mol/ml).

Lactate dehydrogenase release assay

Rat mucosae were mounted in Ussing chambers for 1 h and the apical bathing solution (200 µl) was removed at 0 and 60 min. Samples were mixed with an equal volume of assay substrate solution for 30 min (TOX-7; Sigma-Aldrich, Dublin, Ireland). The reaction was stopped using 0.1 M HCl and the formation of the tetrazolium dye was measured spectrophotometrically at 490 nm in a multiwell plate reader. Percent lactate dehydrogenase (LDH) release was measured relative to Triton X-100 (10% v/v), which released 100% of the enzyme.[17]

Nystatin-permeabilized mucosae

Nystatin forms cation-permeable pores in the apical membrane of polarized epithelia, allowing the apical-to-basolateral K^+ gradient to be observed.[18] The apical side of rat colon was bathed in high-K^+ solution of composition (mM): NaCl 17, $CaCl_2$ 0.3, $MgSO_4$ 3, KH_2PO_4 1.2, K_2HPO_4 2.9, D-glucose 11, K gluconate 120 and HEPES 5. The basolateral side was bathed in low-K^+ solution containing (mM): NaCl 20, Na gluconate 100, K gluconate 5, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2 and D-glucose 11. After equilibration, Zn^{2+} (0.4 mM) was added to either side for 20 min. Nystatin (100 µM in dimethyl sulfoxide (DMSO)) was then added to the apical side and the K^+-dependent ΔI_{sc} observed.

Caco-2 cell culture

Caco-2 cells (passage 55–60; European Collection of Cell Cultures (ECACC)) were grown in 75 cm^2 flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 1% penicillin/streptomycin solution, 1% nonessential amino acids and 1% L-glutamine in a humidified incubator with 95% O_2/5% CO_2, at 37°C. At 70–80% confluence, they were trypsinized with trypsin-EDTA (1 x) and seeded onto rat type I collagen-coated 24-well plates at a density 1×10^6 cells/ml. Cells were used when 95% viability was present, as determined by trypan blue exclusion using a Vi-CELL Series Cell Viability Analyzer (Beckman Coulter, High Wycombe, UK).

CAMP levels in Caco-2 cells and rat colonic mucosae

On day 3 after seeding, Caco-2 cells in 24-well plates were washed with serum-free medium (SFM) and then incubated with fresh SFM, supplemented with phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (1 mM) in a humidified incubator with 95% O_2/5% CO_2, at 37°C for 60 min.[19] Zn^{2+} (0.4 mM) and/or forskolin (10 µM) were added for 15 min and then aspirated. The cells were trypsinized and DMEM was added to inactivate the trypsin. The cells were then centrifuged at 9000*g* for 10 min at 4°C and washed three-times with cold phosphate buffered saline. The pellet was resuspended in cell lysis buffer 5 (1×) at a concentration 1×10^7 cells/ml and stored at −20°C. The lysate was then centrifuged at 600*g* for 10 min at 4°C and the supernatant was stored at −20°C. For colon, mucosae were equilibrated for 45 min before addition of treatments. After 15–20 min, the mucosae were removed from the chamber and snap-frozen in liquid nitrogen and treated as above. Supernatants were tested for cAMP content using a Parameter cyclic AMP ELISA (R&D Systems, Abingdon, UK). The standard curve was linear in the range 4–240 pmol/ml, as measured spectrophotometrically at 450 nm (with 540 nm as a correction) on a multiwell plate reader.

Light microscopy of intestinal mucosal damage

Rat ileal and colonic mucosa were treated with either apical or basolateral addition of Zn^{2+} (0.4 mM) for 60 min. Mucosae were removed from chambers and placed in 10% buffered formalin for 48 h and subsequently embedded in paraffin wax. Tissue sections (5 µM) were cut on a microtome (Leitz 1512; GMI, Ramsey, MN, USA), mounted on adhesive coated slides, and stained with haematoxylin and eosin (H & E) or Alcian Blue 8GX. The slides were visualized using a light microscope (Labophot-2A; Nikon, Japan) and images taken with high-resolution camera (Micropublisher 3.3 RTV; QImaging, Canada) and Image-Pro Plus version 6.3 software (Media Cybernetics Inc., Pittsburgh, PA, USA).

Statistical analysis

Statistical analysis was carried out using Prism-5 software (GraphPad, San Diego, CA, USA). Kruskal-Wallis nonparametric analysis with Dunn's post-test were used for group comparisons with a range of numbers per group. For other multiple comparisons, two-way analysis of variance with Bonferroni post-analysis was used. Student's unpaired t-test was used to compare P_{app} values. Significance was considered if $P < 0.05$. Results are given as mean ± SEM.

Results

Basolateral $ZnSO_4$ blocked stimulated I_{sc} in rat colon and ileum

Basal I_{sc} was 43.0 ± 3.8 µA/cm^2 ($n = 62$) for rat colonic mucosae, a value within reported ranges.[20] Basolateral forskolin caused a large sustained increase in the change in I_{sc} (ΔI_{sc}). Pretreatment with basolaterally-added Zn^{2+} before forskolin caused a significant decrease in the ΔI_{sc} induced by

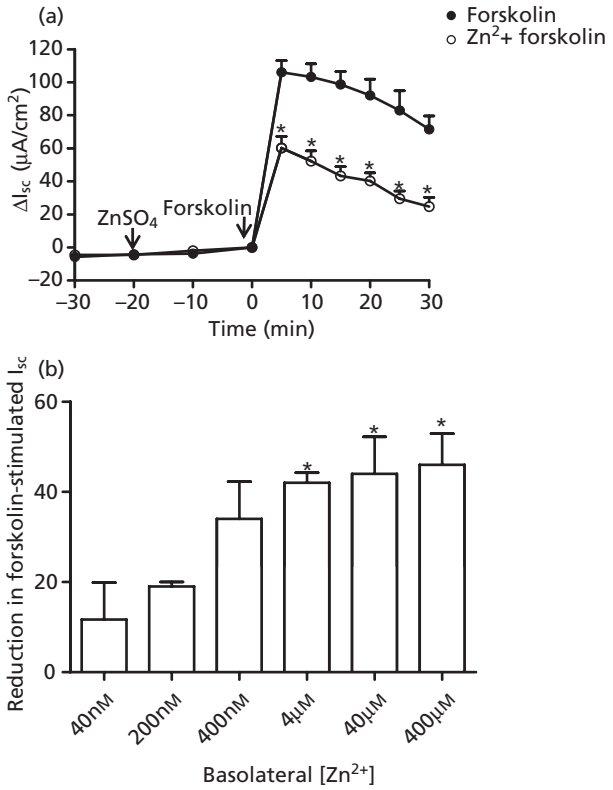

Figure 1 Basolateral Zn^{2+} reduced forskolin-stimulated short circuit current in rat colonic mucosae. (a) Forskolin (10 μM); Zn^{2+} (0.4 mM) and forskolin. ΔI_{sc}, change in short circuit current. $^*P < 0.001$, two-way analysis of variance with Bonferroni's post test. $n = 4$. (b) Zn^{2+} concentration–response for inhibition of forskolin-stimulated I_{sc}, obtained at 30 min after Zn^{2+} addition. The peak height average for forskolin alone was 106 ± 7.1 μA/cm^2; $^*P < 0.05$, by Dunn's multiple comparison post-Kruskal–Wallis test compared with forskolin alone. $n = 3$–5 for each group.

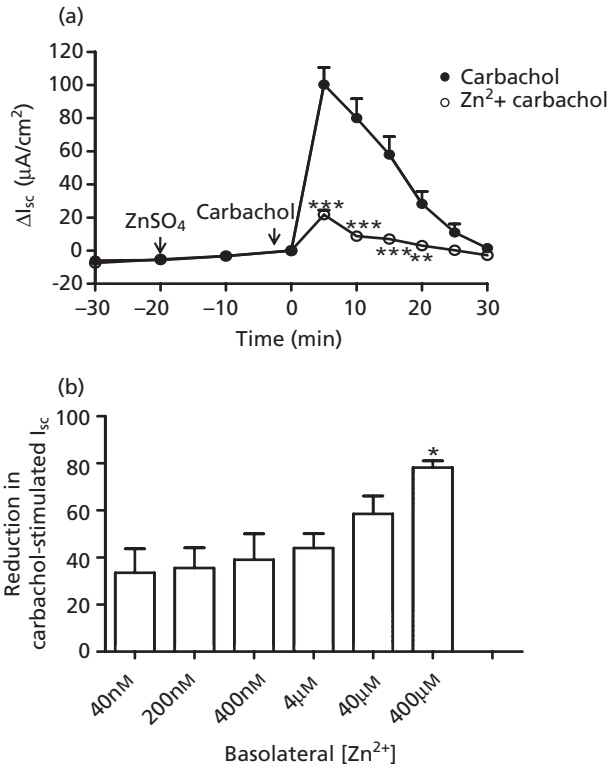

Figure 2 Basolateral Zn^{2+} reduced carbachol-stimulated short circuit current in rat colonic mucosae. (a) Carbachol (10 μM); Zn^{2+} (0.4 mM) and carbachol. ΔI_{sc}, change in short circuit current. $^{**}P < 0.01$, $^{***}P < 0.001$, two-way analysis of variance with Bonferroni's post-test. $n = 4$. (b) Zn^{2+} concentration–response for inhibition of carbachol-stimulated I_{sc}, obtained at 30 min after Zn^{2+} addition. The peak height average for carbachol alone was 92.2 ± 11.3 μA/cm^2. $^*P < 0.05$, by Kruskal–Wallis with Dunn's multiple comparison post-test compared with carbachol. $n = 3$–5 for each group.

forskolin (Figure 1a) with a threshold of 0.2 μM, an IC_{50} value of 1.9 μM, and a maximum inhibition of 50% of the induced current with 0.4 mM (Figure 1b). Addition of apical Zn^{2+} in concentrations up to 1 mM were without effect on the subsequent ΔI_{sc} induced by forskolin, with peak values of 97.8 ± 8.2 μA/cm^2 ($n = 5$) detected after 10 min compared with 100.0 ± 7.3 μA/cm^2 ($n = 5$) for forskolin alone. Basolaterally-added Zn^{2+} had no effect on colonic basal I_{sc} (e.g. Figure 1a). Basolateral addition of carbachol also caused significant increases in ΔI_{sc} in colonic mucosae that were also inhibited by prior incubation with basolateral Zn^{2+} (Figure 2a and b). An IC_{50} value of 3.5 μM was obtained for Zn^{2+}-mediated blockade of the carbachol-stimulated I_{sc}, with a maximum of 85% of the carbachol-induced current inhibited by 0.4 mM. Apical Zn^{2+} (1 mM) was without effect on the subsequent carbachol-stimulated ΔI_{sc} with mean values of 43.2 ± 33.9 μA/cm^2 ($n = 3$) compared with 48.6 ± 35.9 μA/cm^2 ($n = 4$) in separate matched mucosae. Zn^{2+} 0.4 mM was therefore used in subsequent studies. Similar results were demonstrated for blockade of the ΔI_{sc} stimulated by A23187 (10 μM) in colon, where peak responses in the presence of basolateral Zn^{2+} (0.4 mM) were reduced from 42.5 ± 3.2 to 0.3 ± 0.3 μA/cm^2, while apical Zn^{2+} was without effect (Figure 3). In ileal tissues displaying a mean basal I_{sc} of 47.9 ± 4.7 μA/cm^2 ($n = 30$), a value within the reported range, pretreatment with basolaterally-added Zn^{2+} inhibited the subsequent peak ΔI_{sc} stimulated by forskolin from 50.2 ± 2.1 to 34.8 ± 4.5 μA/cm^2 ($n = 5, P < 0.05$).[21] By comparison, apical Zn^{2+} was without statistical effect on the ΔI_{sc} stimulated by forskolin in ileum (39.3 ± 1.2 μA/cm^2, $n = 4$).

Cytochalasin D enabled apical Zn^{2+} to inhibit forskolin-stimulated Isc in rat colon and ileum

Rat colonic and ileal mucosae were incubated with apical cytochalasin D (8 μM) and Zn^{2+} (0.4 mM) for 60 min, after

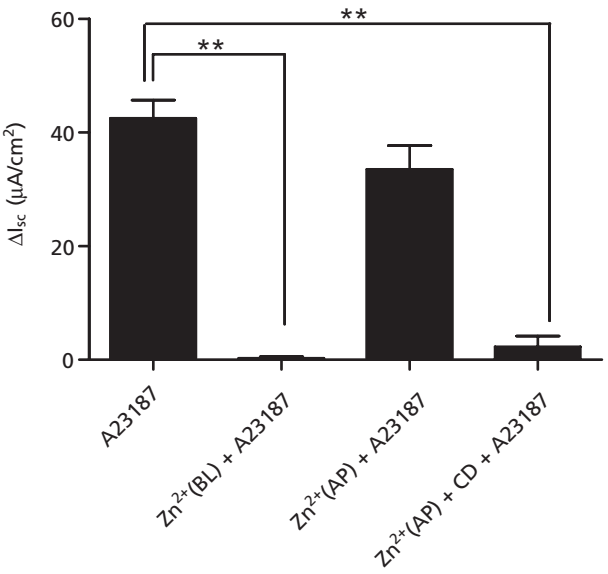

Figure 3 Apical addition of Zn^{2+} abolished the change in short circuit current stimulated by A23187 in rat colonic mucosae in the presence of cytochalasin D (60 min). Following addition of cytochalasin D (CD; 8 μM), apical Zn^{2+} (0.4 mM, AP) blocked the change in short circuit current (ΔI_{sc}) caused by A23187 (10 μM) to a level similar to that achieved with basolateral Zn^{2+} (BL). $n = 4$, $^{**}P < 0.001$, by Kruskal–Wallis with Dunn's multiple comparison post-test compared with A23187 alone. $n = 4$ for each group.

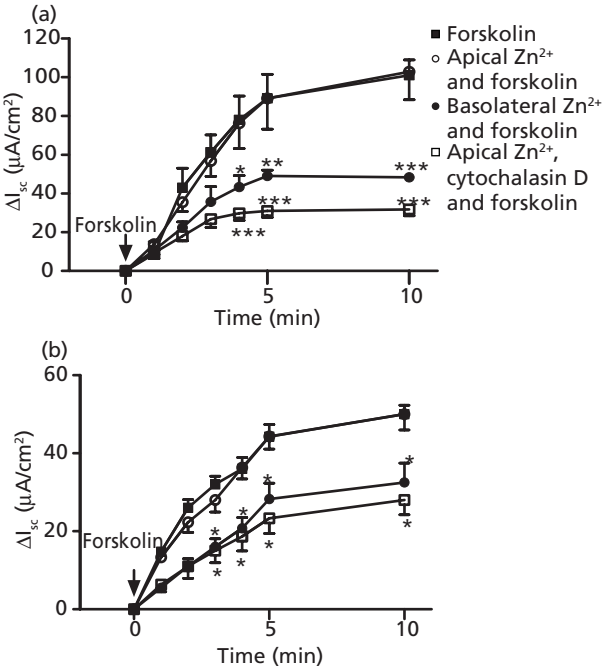

Figure 4 Apical Zn^{2+} reduced forskolin-stimulated short circuit current in the presence of cytochalasin D (60 min). (a) Rat colon: forskolin (10 μM); apical Zn^{2+} (0.4 mM) and forskolin; basolateral Zn^{2+} and forskolin; apical Zn^{2+}, cytochalasin D (8 μM) and forskolin. ΔI_{sc}, change in short circuit current. $n = 4$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with forskolin, using two-way analysis of variance with Bonferroni's post-test. (b) Rat ileum. $^{*}P < 0.001$, compared with forskolin.

which forskolin was added basolaterally and the resulting ΔI_{sc} calculated (Figure 4a and b). Using this protocol, apical addition of Zn^{2+} now induced a decrease in forskolin-stimulated ΔI_{sc} in colon and ileum, similar to that induced by basolaterally-added $ZnSO_4$ in the absence of cytochalasin D. Importantly, there was no effect of either apical Zn^{2+} in the absence of cytochalasin D on forskolin-stimulated ΔI_{sc} in either tissue type (Figure 4). Similarly, apical addition of Zn^{2+} in the presence of cytochalasin D also prevented the ΔI_{sc} stimulated by A23187 in rat colon (Figure 3), similar to the inhibition induced by basolateral Zn^{2+}. Cytochalasin D treatment had very little effect on stimulated ΔI_{sc} itself. For example, in separate studies, the forskolin-stimulated ΔI_{sc} in ileum in the presence of cytochalasin D was 48.7 ± 4.7 μA/cm² compared with control values of 50.2 ± 2.1 μA/cm² ($n = 6$, not significant). The data suggested that cytochalasin D enabled apically-added Zn^{2+} to penetrate epithelial tight junctions to block the I_{sc} stimulating effects of forskolin and A23187 via actions on the basolateral membrane.

Cytochalasin D increased permeation of zinc across rat colon and ileum

Cytochalasin D caused a 2.4- and 1.4-fold increase in the P_{app} of Zn^{2+} across rat colonic and ileal mucosae, respectively (Table 1). After 120 min, the basolateral side concentration of

Table 1 Effects of cytochalasin D on P_{app} values of Zn^{2+} across rat intestinal mucosae

Treatment	P_{app} (×10^{-5} cm/s)	Basolateral [zinc] (μM)
Ileum		
Control	1.1 ± 0.2	50.5 ± 0.6
Cytochalasin D-treated	1.5 ± 0.2**	69.2 ± 1.5**
Colon		
Control	0.4 ± 0.1	21.3 ± 0.3
Cytochalasin D-treated	0.9 ± 0.1**	43.4 ± 0.8**

$^{**}P < 0.01$ by Student's unpaired *t*-test ($n = 4$–5 in each group). P_{app}, apparent permeability coefficient. Cytochalasin D (8 μM, bilateral); Zn^{2+} (0.4 mM, apical) added 20 min later.

Zn^{2+} in colonic mucosae was 43.4 ± 0.8 and 21.3 ± 0.3 μM in the presence and absence of cytochalasin D, respectively. After 120 min, the basolateral side concentration of Zn^{2+} in ileal mucosae was 69.2 ± 1.5 and 50.5 ± 0.6 μM. In each case, the donor-side apical concentration was 0.4 mM Zn^{2+}, so the basal percentage permeating in the period was 2.1% for colon and 5.1% for ileum. Even though micromolar basolateral concentrations of Zn^{2+} were achieved over 120 min in both tissues, the rate of flux was still too slow to inhibit forskolin-stimulated ΔI_{sc} compared with basolateral additions. The P_{app}

values of 10^{-5} cm/s in rat intestinal tissue correlated with those of quite well absorbed passive oral molecules in humans.[22]

Zn^{2+} reduced the intracellular cAMP induced by forskolin in Caco-2 and rat colonic mucosae

Basolateral addition of Zn^{2+} inhibited electrogenic Cl^- secretion induced by 8-bromoadenosine 3,5-cyclic monophosphate in rat ileum.[5] Forskolin (10 μM) caused a significant increase in cAMP after 15 min in both Caco-2 cells and colonic mucosae ($P < 0.001$). Zn^{2+} (0.4 mM) significantly decreased basal levels of intracellular cAMP, as well as those induced by subsequent exposure to forskolin in Caco-2 cells and rat colonic mucosae (Figure 5).

Zn^{2+} had direct inhibitory effects on basolateral K channels

Rat colonic mucosae were permeabilized with nystatin (100 μM), allowing K^+ transport to the basolateral side to be measured directly as an increase in I_{sc}. Basolateral- but not apical-side pre-incubation of tissue with Zn^{2+} caused a significant reduction in the stimulated ΔI_{sc} response to nystatin, suggesting that Zn^{2+} must have been present on the basolateral side to inhibit the basolateral K^+ channels responsible for maintaining the K-dependent current (Figure 6). Forskolin-stimulated I_{sc} is known to be part-generated by activation of cAMP-regulated K channels, while that stimulated by calcium-mediated signalling is regulated by inwardly rectifying calcium-activated K channels, so we compared the inhibitory capacity of known K channel blockers in rat colonic mucosae.[23,24] Pretreatment with the cAMP-regulated K channel blocker, $BaCl_2$, inhibited the forskolin-but not the A23187-stimulated I_{sc}. By comparison Zn^{2+} blocked both. This profile was similar to that of the nonspecific K channel blocker, TPeA (Table 2). When Zn^{2+}, TEA or $BaCl_2$ were added at the plateau of the response to forskolin however, only TPeA reduced the current (by approximately 25%).

Zn^{2+} was not cytotoxic to rat colonic mucosae

A potential criticism of the anti-secretory effects ascribed to Zn^{2+} in these studies is that they might have arisen indirectly due to cytotoxicity. This was not the case as very little LDH was released to the apical side from mucosae exposed to either apical or basolateral Zn^{2+} (0.4 mM; 4.3 ± 0.8% and 3.1 ± 1.0% release, respectively), to bilateral additions of cytochalasin D (8 μM, 4.2 ± 0.3% release) or to the combination of Zn^{2+} and cytochalasin D (8 μM, 3.0 ± 1.0% release) compared with the maximal release induced by Triton-X-100 (10%) over 60 min. H & E staining of rat colon after 60 min exposure to Zn^{2+} and or CD did not reveal any gross changes compared with control tissue (Figure 7). Similar histological data was obtained using rat ileum exposed to the same agents over 60 min, with no differences seen between control, cytochalasin D, Zn^{2+}, or the combination in treated mucosae (Figure 8). It is important to point out that muscle-stripped ileal tissue is not as robust as colonic tissue when mounted in Ussing chambers.

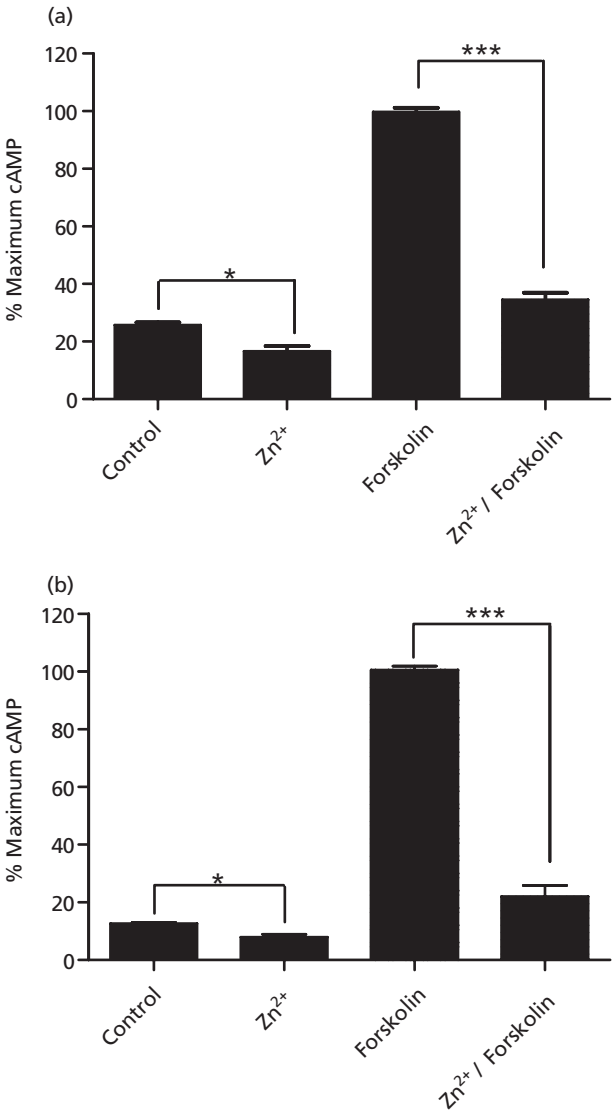

Figure 5 Zn^{2+} reduced basal- and forskolin-stimulated cAMP concentrations in intestinal epithelia. (a) Caco-2 cells; (b) rat colonic mucosae. Zn^{2+} (0.4 mM) was applied to Caco-2 on wells or basolaterally to mucosae for 15 min before forskolin. Forskolin induced maximal levels of 26.1 ± 0.9 and 21.4 ± 0.5 pmol/mg in Caco-2 cells and mucosae in the presence of 3-isobutyl-1-methyl-xanthine, respectively. $n = 3$, $^*P < 0.05$; $^{***}P < 0.01$ by two-way analysis of variance with Bonferroni post-test analysis.

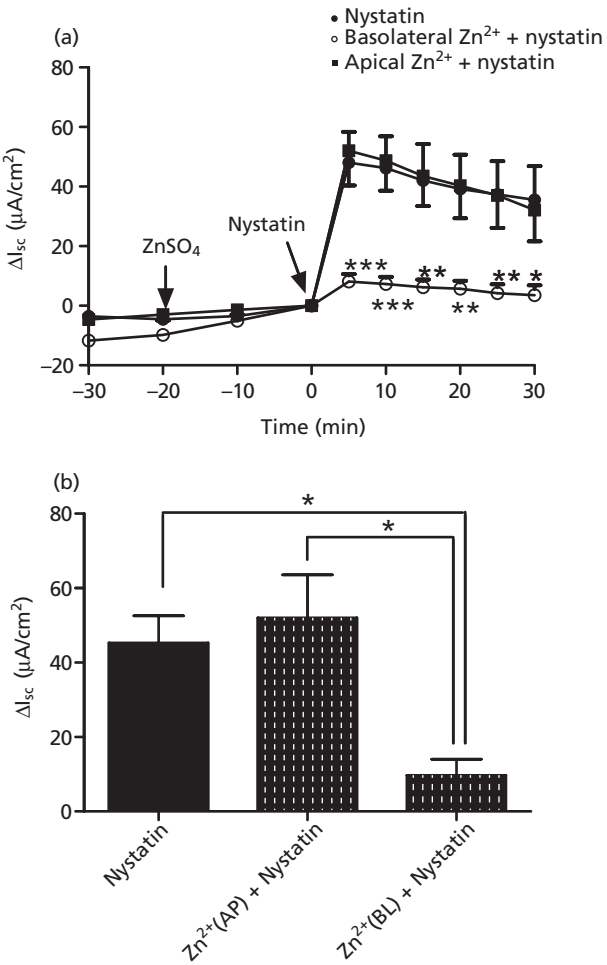

Figure 6 Basolateral Zn^{2+} reduced nystatin-stimulated short circuit current in rat colonic mucosae. (a) Nystatin (100 μM); basolateral Zn^{2+} (0.4 mM) and nystatin; or apical Zn^{2+} and nystatin; $^{**}P < 0.01$ by two-way analysis of variance with Bonferroni post-test analysis compared with basolateral Zn^{2+} /nystatin-stimulated change in short circuit current (ΔI_{sc}) at 30 min. (b) n = 3–5, $^{*}P < 0.05$ $^{*}P < 0.05$, compared with basolateral Zn^{2+} /nystatin-stimulated ΔI_{sc} at 30 min.

Discussion

We modelled the cAMP-regulated electrogenic chloride secretion process used by CTA in intestinal epithelia *in vitro* by using adenylate cyclase activation by forskolin. The CTA1 polypeptide chain catalyses ADP ribosylation of the regulatory component of adenylate cyclase to induce fluid secretion.[25] The model permits investigation of sidedness of anti-secretory effects, the importance of apical-to-basolateral flux, as well as the combined interactions with epithelial channels and pumps. Previously we had investigated the epithelial mechanism of action of the antidiarrhoeal agents, loperamide and berberine, and concluded that inhibition of basolateral K channels was common to a range of antidiarrhoeal agents.[26,27] Moreover, both agents required significant permeation for efficacy, a condition that pertains in secretory diarrhoea, and we now extend these epithelial findings to Zn^{2+}.[28]

Table 2 Effects of K channel blockers on forskolin- and A23187-stimulated ΔI_{sc} in rat colonic mucosae

Treatment	ΔI_{sc} (μA/cm²)
Forskolin (10 μM)	105.5 ± 7.4 (5)
Zn^{2+} (0.4 mM) + forskolin	61.1 ± 4.9* (8)
$BaCl_2$ (10 mM) + forskolin	52.3 ± 12.9* (4)
TPeA (0.1 mM) + forskolin	8.7 ± 2.0** (3)
TEA (10 mM) + forskolin	31.0 ± 7.0** (3)
A23187 (10 μM)	42.5 ± 3.2 (4)
Zn^{2+} + A23187	0.3 ± 0.3** (4)
$BaCl_2$ + A23187	39.8 ± 7.2 (4)
TPeA + A23187	2.0 ± 0.7** (4)
TEA + A23187	ND

$^{**}P < 0.01$, $^{*}P < 0.05$, Kruskal–Wallis non-parametric test followed by Dunn's post analysis. TPeA, tetrapentylammonium 100 μM; TEA, tetraethylammonium 10 mM. Numbers of mucosae are given in brackets. Inhibitors were added 20 min before either forskolin or A23187 and the resulting change in short circuit current (ΔI_{sc}) compared with control responses. ND, not determined.

Zn^{2+} required epithelial basolateral side access to reduce the ΔI_{sc} stimulated by forskolin and A23187, and this was enabled by increasing paracellular flux with cytochalasin D, an established tight junction opener used previously to confer sensitivity to apically-applied antidiarrhoeal agents.[27] Even though cytochalasin D did not effect histology and there was no evidence of a transcellular action, there is a possibility that its enabling effect on apically-added Zn^{2+} arose in part from additional mechanisms not specific to tight junctions. It would be erroneous however, to conclude that oral Zn^{2+} requires open tight junctions to allow efficacy, since basal P_{app} values in both tissues even in the absence of cytochalasin D were high and resulting concentrations were higher than the IC_{50} values required for inhibition of forskolin-stimulated ΔI_{sc}. While the basolateral micomolar concentrations of Zn^{2+} arising from apical addition might be expected to inhibit I_{sc} in the absence of cytochalasin D, they did not. The fact that such levels were reached on the basolateral side at 120 min may reflect slow permeation, however a time course could not be carried out due to issues of assay sensitivity. In contrast, cytochalasin D acts quickly on tight junctions, perhaps explaining why anti-secretory effects were conferred to apical Zn^{2+} under those conditions. Another puzzle was that Zn^{2+} (and Ba^{2+}) added on the plateau of the forskolin-stimulated I_{sc} were without effect; the most likely explanation was that the K channel blockade was most effective when present in advance of dramatic electrogenic secretion. Use of lower concentrations of secretagogues in the 0.1–1 μM range to stimulate a reduced I_{sc} plateau might allow effects of basolateral Zn^{2+} to be subsequently detected.

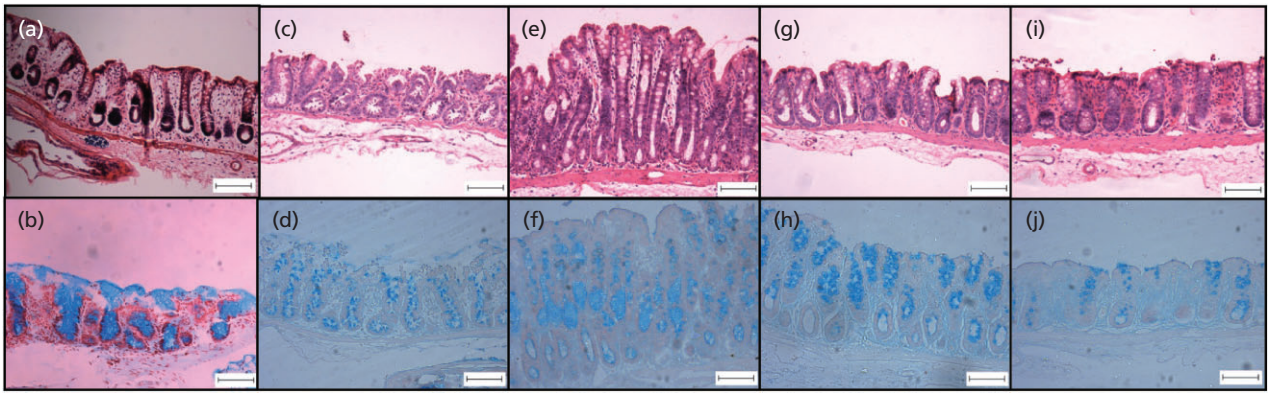

Figure 7 Haematoxylin and eosin and Alcian Blue stained light micrographs of rat colonic mucosae mounted in Ussing chambers for 60 min. (a, b) Control, haematoxylin and eosin and Alcian Blue, respectively; (c, d) basolateral Zn^{2+} (0.4 mM): (e, f) apical Zn^{2+}; (g, h) apical Zn^{2+} and cytochalasin D (8 μM); (i, j) cytochalasin D. Bar = 10 μm.

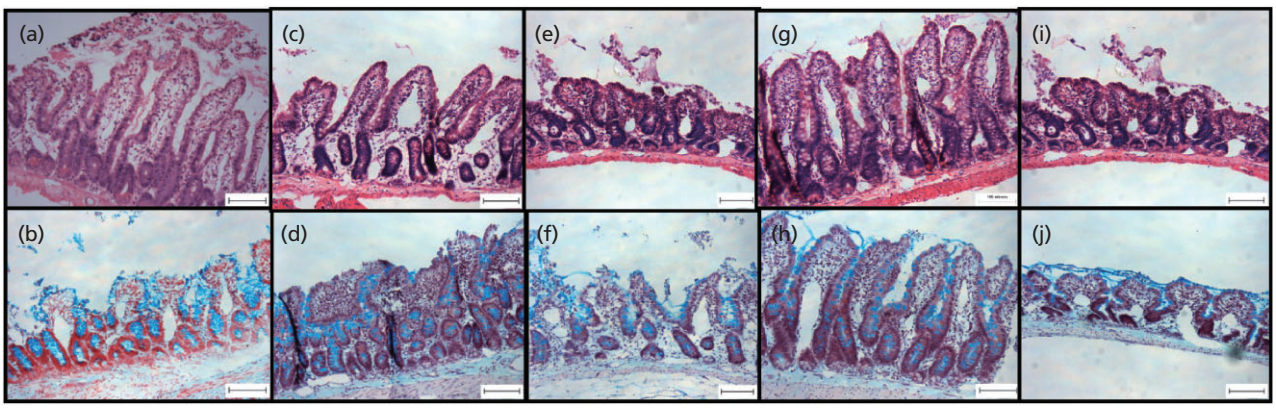

Figure 8 Haematoxylin and eosin and Alcian Blue stained light micrographs of rat ileal mucosae mounted in Ussing chambers for 60 min. (a, b) Control, haematoxylin and eosin and Alcian Blue, respectively; (c, d) basolateral Zn^{2+} (0.4 mM): (e, f) apical Zn^{2+}; (g, h) apical Zn^{2+} and cytochalasin D (8 μM); (i, j) cytochalasin D. Bar = 10 μm.

Human oral bioavailability of dietary zinc under nondiarrhoeal conditions ranges from 36–71%.[29] It is likely that facilitated oral absorption of zinc in secretory diarrhoea compensates for the 13% lower serum levels of serum zinc seen in afflicted children.[30] The inverse relationship between dose and bioavailability suggests that zinc permeation of enterocytes is not simply due to passive paracellular flux. In diarrhoea, upregulated Zn^{2+} transporters compensate as Zn^{2+} enters small intestinal epithelia via the *Zip4* transporter on the apical membrane, and then exits via *ZnT1* and/or *Zip5* on the basolateral membrane.[31] Expression of *Zip4* on enterocytes is increased in dietary Zn^{2+} deficiency and is decreased when the diet is replete.[32] Zn^{2+} therefore permeates intestinal epithelia predominantly by transcellular receptor-mediated uptake via transporters in series and this might account for the relatively high basal P_{app} in untreated ileal and colonic tissues. The effective Zn^{2+} concentration used in Ussing chambers was 0.4 mM in the 5-ml bath and this could be related to that in ORS, where a daily dose of 20 mg $ZnSO_4$ for cholera treatment is typical.[3–5] The fluid volume of the jejunum and ileum is estimated as 120–350 ml and the colon at 1.2–1.5 l depending on whether fasting or fed, and so the concentration of Zn^{2+} used *in vitro* was of the same order as that exposed to different regions of the intestine *in vivo*.[33]

Zn^{2+} seems indiscriminate in its antisecretory actions and is effective against a range of intracellular mediators of signalling, but comparisons between studies are still problematic. For example, while Zn^{2+} attenuated forskolin- and carbachol-stimulated electrogenic chloride secretion in piglet small intestine at low concentrations, data from rat ileum showed that 8-bromoadenosine cAMP blocked secretion across rat ileum with a very high IC_{50} of 0.43 mM, and there was no blockade of secretion stimulated by carbachol.[7,9,10] In contrast, Zn^{2+} blocked carbachol-mediated secretion in Caco-2

monolayers.[12] In Caco-2 cells, Zn^{2+} also blocked secretion caused by CT, effects that were ascribed to an associated decrease in cAMP levels.[11] We confirmed that Zn^{2+} reduced the level of cAMP production elicited by forskolin in Caco-2 cells and rat colonic tissue, suggesting that its mode of action encompasses both K channel blockade as well as interference in cAMP production. Zn^{2+} also attenuated production of intracellular calcium and of γ-interferon-stimulated production of NO in Caco-2, a reasonable conclusion is that, apart from cGMP, the main intracellular mediators responsible for secretion are blocked at the epithelial level by Zn^{2+}, depending on concentration, gut region and species.[12]

That blockade of cAMP-stimulated basolateral K channels might be responsible for Zn^{2+}-mediated I_{sc} inhibition was first suggested by blockade of ^{86}Rb efflux from rat isolated ileal epithelial cells.[7] Our data confirmed basolateral K channel blockade by Zn^{2+}, as nystatin converted the I_{sc} to a K-dependent electrogenic current, inhibitable by basolateral zinc. High concentrations of Zn^{2+} inhibited both cAMP- and calcium-mediated I_{sc}, a pattern similar to the nonspecific K channel blocker, TPeA. In contrast, Ba^{2+} inhibition of I_{sc} was restricted to that stimulated by forskolin, but not A23187. Recently there have been attempts to classify more accurately and standardize nomenclature for K channels in intestinal epithelia.[6] In colonocytes and small intestinal enterocytes from rat and human tissue, sustained cAMP-stimulated chloride secretion is reliant on basolateral voltage-gated KCNE3/KCNQ1 channels to drive the electrochemical gradient for sustained Cl efflux via CFTR. In contrast, carbachol-stimulated I_{sc} is regulated by basolateral calcium-activated KCNN4 channels, but the resulting I_{sc} is not sustained in the absence of co-stimulation by cAMP since calcium does not directly regulate CFTR. In terms of blocking the secretion induced by forskolin, Zn^{2+} therefore appeared to block mainly basolateral KCNE3/KCNQ1 channels, but it also seemed to have additional effects on basolateral KCNN4 channels. To definitively prove this will require patch clamping of intestinal epithelial cells in the presence of blockers that are more selective and potent than those currently available.

We have addressed the potential issue that cytotoxicity of Zn^{2+} may have confounded interpretation of the data. Although Zn^{2+} has a long history of safe use in man, nano- and microparticulate formats of Zn^{2+} can induce lethargy, vomiting and diarrhoea as well as tissue pathology in mice following repeated doses of 5 g/kg for 14 days, a feature ascribed to the particulate format.[34,35] On the contrary, dietary Zn^{2+} may protect against intestinal inflammation.[36] High concentrations of Zn^{2+} on the apical or basolateral side of rat intestinal tissue did not affect histology, nor did it result in an increase in LDH release. This is not surprising since Zn^{2+}-supplemented ORS at a dose of 20 mg/day for 14 days in children older than six months is approved as a safe adjunct therapy.[37]

Conclusions

We have provided evidence that intestinal antisecretory effects of Zn^{2+} required basolateral-side access in rat ileum and colon and were mediated predominantly by low potency nonspecific K channel blockade. Increased paracellular permeability appeared to further enable access to the basolateral membrane, and such conditions may pertain in secretory diarrhoea. Since fluxes across rat ileum and colon produced high basal P_{app} values, transcellular passage via channels was the predominant permeating process. In sum, its intestinal antidiarrhoeal effects encompassed blockade of basolateral cAMP-regulated KCNE3/KCNQ1- and calcium-regulated KCNN4 channels, as well as activation of apical NHE3. It also reduced bacterial toxin-induced increases in levels of cAMP, NO, and calcium in intestinal epithelia. It was noncytotoxic *in vitro* when basolateral and apical sides of intestinal tissue were exposed to high concentrations. This data needs to be confirmed in isolated human tissue mucosae.

Declarations

Conflict of interest

None of the authors have any conflicts of interest to disclose.

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