Increased Intestinal Permeability in Rats Subjected to
Traumatic Frontal Lobe Percussion Brain Injury

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Running Head: Intestinal permeability and traumatic brain injury

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Abstract:

Background: Dysfunction of the gastrointestinal (GI) tract is a common occurrence following traumatic brain injury (TBI). We hypothesised that increased intestinal permeability may result from a precisely controlled percussion injury to the exposed brains of anaesthetised rats and that such an effect could be assessed in vitro using excised intestinal mucosae mounted in Ussing chambers.

Methods: Following craniotomy over the left medial prefrontal cortex on anaesthetised rats, neurotrauma was produced using a pneumatically-driven impactor on the exposed brain. Control rats were subjected to identical procedures but did not receive an impact. Muscle-stripped rat intestinal ileal and colonic segments were mounted in Ussing chambers within 30 minutes of death. Transepithelial electrical resistance (TEER) and the apparent permeability coefficient (Papp) of $[^{14}\text{C}]$-mannitol were recorded from intestinal tissue for 120 minutes. Histopathology was also carried out to determine any gross morphological changes in the intestine.

Results: Ileal and colonic mucosae showed no differences in TEER in ileum or colon of TBI rats compared to controls. The Papp of mannitol was significantly increased in ilea from rats previously exposed to TBI compared to controls. Histological analysis showed gross changes to 50% of the ileal but not the colonic sections from TBI rats.

Conclusion: TBI results in significantly reduced ileal barrier function, most likely mediated by open tight junctions. For patients with acute head injury, this may have implications for subsequent oral absorption of nutrients. Systemic delivery of luminal endotoxins may contribute to multiple organ failure.

Key words: traumatic brain injury; intestinal permeability, gut-brain axis
INTRODUCTION

A principle function of the gastrointestinal tract (GIT) is to contain normal enteric flora, pathogens, toxins and undigested macromolecules, while simultaneously digesting and absorbing essential nutrients. Permeability restriction is achieved by means of the single continuous sheet of epithelium in each intestinal region, with each epithelial cell interconnected at the apical pole by tight junctions. Additional intestinal impediments to nutrient, drug and pathogen/toxin absorption include enzymatic attack from luminal, brush border and intracellular enzymes. In established diseases of the inflamed bowel (e.g. Crohn’s disease and ulcerative colitis), studies have demonstrated evidence of enhanced paracellular absorption in vivo and in vitro. Such permeability defects in inflammatory bowel disease patients are thought to contribute to the damaging influence of luminal contents including pathogenic and commensal bacteria.

As a consequence of acute stress, increased colonic permeability mediated by corticosteroids, and increased jejunal uptake of large proteins including horse-radish peroxidase have been described in rat intestinal mucosae. A critical role for mast cells was discovered as a mediator of the permeability defect discovered in isolated jejunal mucosae from rats exposed to chronic stress. Furthermore, Saunders et al. showed that isolated mucosae from rats subjected to short-term restraint or following exposure to cold displayed jejunal barrier dysfunction, as indicated by increased conductance and permeability to the paracellular flux markers, mannitol and $^{51}$Cr-EDTA. It has been proposed that stress-induced alteration in intestinal permeability is an initiating event in subsequent chronic intestinal disorders and that the increase in
permeability induced by stress in rats can be prevented by pre-treatment of rats with an intestinotrophic factor, glucagon-like peptide-2 \(^{10}\).

A significant proportion of road traffic accident victims comprise traumatic brain injury (TBI) fatalities and it is the major cause of death in people under the age of 19 in the industrialised world \(^{11,12}\). Dysfunction of the gastrointestinal tract is a common occurrence following TBI \(^{13,14}\). Up to 50% of road-traffic accident victims with acute TBI display intolerance to enteral feedings, which has been correlated with the severity of the brain injury \(^{15}\). Cross-talk between the central nervous system (CNS) and the small intestine is thought to contribute to pathogenesis \(^{16}\). The CNS communicates with the gut via afferent and efferent extrinsic nerves or connections to enteric nerves in the myenteric plexus of the gastrointestinal tract \(^{17}\). It is proposed that TBI sets off a sequence of local and systemic inflammatory and immune-amplified events, which may lead to multiple organ dysfunction syndrome (MODS). Several studies on TBI-related GIT dysfunction have concentrated on physiological aspects on the GIT and have reported increased bleeding \(^{18}\), prolonged gastric emptying \(^{19}\) and proximal intestinal obstruction caused by duodenum compression \(^{20}\).

In response to TBI in rats, modulation of intestinal function may in part be explained by alterations in plasma and jejunal levels of humoral factors or of enteric peptide neurotransmitters, including vasoactive intestinal peptide (VIP), cholecystokinin and calcitonin-gene-related peptide (CGRP) \(^{21}\). In addition, up-regulated expression of ICAM-1 binding to the jejunum as well as increased NFκ-B expression was detected in rats subsequent to TBI \(^{22}\). Increases in intestinal permeability may permit absorption of bacteria-derived lipo-polysaccharide (LPS)-endotoxin, which in turn
may lead to over-production of local inflammatory mediators, tissue destruction and sepsis. Hang et al. recently demonstrated compromised intestinal histology at 72 hours post-TBI in rats and this was accompanied by increased intestinal permeability in vivo. Furthermore, data denoting altered intestinal permeability has also been reported in human patients with acute brain injury.

Regional intestinal permeability data is lacking and there is considerable debate over the quantitative significance of the effect, its time course, and detail of the mechanism of the cross-talk between brain and gut. Consequently, the purpose of this study was therefore to examine the effects of an acute severe head trauma or TBI on the paracellular permeability, electrophysiological parameters and histological responses of both muscle-stripped ileal and colonic mucosae isolated from anaesthetised rats previously subjected to TBI for a six-hour period. The majority of these brain injuries are directed towards the forehead, analogous to the medial prefrontal cortex (mPFC) in the rat. Therefore, a precise TBI was delivered to the left mPFC of anaesthetised rats in an attempt to part-replicate some of the damage that may be seen in humans.
MATERIALS AND METHODS

Animals

Twenty-two age-matched male Sprague Dawley rats weighing 300-350g were purchased from Harlan Laboratories (UK). Animals were housed under controlled environmental conditions regarding temperature and humidity with a 12-hour light/dark cycle. They had free access to tap water and standard laboratory chow. Rats were maintained in accordance with National Institute of Health guidelines for the care and use of laboratory rats.

Rat model of TBI

The rats were randomly divided into either TBI (n=6) or control (n=6) groups. Following anaesthesia by isoflurane inhalation (4% - 2% in air at a rate of 400 mL/min.), the animals were placed in a stereotaxic frame (David Kopf Instruments, USA) and the head adjusted until the skull between the bregma and lambda was level. Using sterile techniques, a sagittal incision of the scalp was made along the midline from the level of the eyes to the occipital protuberance, exposing the frontal bones. In accordance with previously published methods, a 4mm diameter craniotomy was drilled through the skull over the mPfc, exposing the dura. A cortical contusion injury was performed on the left mPfc using a pneumatically-driven vertical impactor. The device, consisting of a pneumatic cylinder mounted on an adjustable crossbar, was positioned above the left mPfc to provide a single impact by a 3.5mm rounded impactor tip. Air pressure was set at 4 bar, and the depth of penetration determined by zeroing the piston to the cortical surface, withdrawing it, and then lowering it to the required impact deformation. The impact was of 1.2m/s velocity producing a 2.62mm deformation. Anaesthesia was maintained for a further 5 hours and 40
minutes before euthanasia by isoflurane overdose and decapitation. Body temperature was continuously monitored and maintained at 37.5°C using a temperature controlled heating pad (CMA 150 Carnegie Medicine, Sweden). Control rats were treated exactly the same in all respects apart from not receiving an impact to the exposed dura. All live animal procedures were approved by the University College Dublin Animal Research Ethics Committee and carried out under license number B100/3366 from the Irish Department of Health and Children. The current study therefore used post-mortem intestinal tissue from animals that were undergoing TBI as part of a separate CNS project.

Rat epithelial ileal and colonic function: Ussing chamber studies

Rat ileal and colonic segments were immediately removed post-mortem and placed in oxygenated Krebs-Henseleit buffer (KH). They were opened along the mesenteric border, rinsed free of luminal contents and stripped of longitudinal and circular muscle layers and myenteric plexus. Two adjacent pieces of terminal ileum or of distal colon were mounted in Ussing chambers (World Precision Instruments (WPI), UK) with an exposed window surface area of 0.63cm² in accordance with previous descriptions. Epithelial sheets were bathed bilaterally with 5mL of oxygenated KH and maintained at 37°C by a circulating water bath at a pH of 7.4. The buffer contained (mM): NaCl (118), KCl (4.7), 1.2 KH₂PO₄ (1.2), MgSO₄.7H₂O (1.2), glucose (11.1), NaHCO₃ (25) and CaCl₂.2H₂O (2.5). The chambers contained 3% agar bridges in 3M KCl connected to Ag and AgCl voltage and current electrodes. These were used to monitor the potential difference (PD) across the tissue and to supply the required short circuit current (Isc) to maintain zero PD via an automated voltage clamp system (EVC-4000, World Precision Instruments, UK). The responses
were recorded using a MacLab® analogue-digital recorder (AD Instruments, UK). Baseline PD (mV) and $I_{sc}$ ($\mu$A.cm$^{-2}$) values were recorded 15 minutes after the tissues were mounted. $I_{sc}$ was recorded continuously for the duration of the experiment as an indicator of net active ion transport, except for brief interruptions for reading of the PD. The transepithelial electrical resistance (TEER, $\Omega$.cm$^{-2}$) of the tissue, an indicator of ion permeability and tissue viability, was calculated according to Ohm’s law every 20 minutes for 2 hours.

Forskolin (10$\mu$M) (Sigma, UK), an activator of adenylate cyclase and a potent agonist of electrogenic chloride secretion in intestinal tissues, was added to the serosal side of tissues at the end of the experiment to determine electrogenic ion-transporting capacity, as normally reflected by an increase in $I_{sc}$. Changes in $I_{sc}$ ($\Delta I_{sc}$) were also induced by serosal addition of veratridine (10$\mu$M), (Tocris Biosciences, UK). Veratridine opens Na$^+$ channels and prevents their inactivation, thus leading to prolonged membrane depolarisation and an increase in tetrodotoxin-sensitive electrogenic ion secretion in intestinal tissue.

Mucosal-to-serosal permeability of paracellular flux probes was determined by measuring the transepithelial flux of $^{14}$C-mannitol (Amersham Biosciences, UK). $1\mu$Ci of $^{14}$C-mannitol was added to the mucosal side and allowed to equilibrate for 1 minute before baseline mucosal (100$\mu$L) and serosal (500$\mu$L) samples were taken. Serosal samples (500$\mu$L) were taken every 20 minutes for 2 hours and replaced with non-radioactive buffer. A final mucosal sample (100$\mu$L) was taken at the end of the 2-hour experiment. Radioactivity was measured using a liquid scintillation counter.
Fluxes of mannitol were calculated using the apparent permeability coefficient ($P_{app}$) equation and expressed as cm$\cdot$s$^{-1}$.

**Histopathology**

Brain histopathology was carried out following intra-cardial infusion of 10% paraformaldehyde immediately after impact. In addition, after removal of the ileum and colon, a piece of each was preserved in nutrient-buffered 10% formalin for histopathology. Longitudinal sections (7µm thick) of paraffin-embedded mucosae were cut with a microtome, stained with hematoxylin and eosin (H & E) and examined under a light microscope. Samples were randomly allocated, coded, and examined blindly.

**Plasma levels of endotoxin**

Heparinised blood samples for measurement of plasma endotoxin were taken immediately prior to euthanasia. Plasma samples were assayed for endotoxin content by the chromogenic limulus amebocyte lysate (LAL) test, according to manufacturer’s instructions (Cambrex Bio Science, Belgium).

**Statistics**

Statistical analysis was carried out using a one-tailed Wilcoxon test. Results are given as mean ± standard error of the mean (SEM). $P < 0.05$ was considered significant.
RESULTS

Rat brain pathology following TBI

Controlled severe TBI to the rat left mPfc resulted in fragmentation of sub-meningeal cortical parenchyma of the superficial cortex. Diffuse parenchymal pallor (oedema) and multiple foci of haemorrhage were also noted in this region. These changes were accompanied by meningeal rupture and haemorrhage (Fig. 1A, 1B).

Epithelial electrophysiology of intestinal mucosae from TBI rats

Baseline TEER, PD and Isc values were stable and similar in ileal or colonic mucosae of TBI rats compared with their respective ileal and colonic controls (Table 1). There were no statistical differences in these parameters over the 2-hour time period in the ilea or colonic mucosae of the TBI rats compared to controls (data not shown).

TBI induced a statistically significant increase (P<0.05) in the absorptive Papp of $^{14}$C-mannitol across the ileum of TBI rats compared to controls (Fig. 2). The mean ileal TBI Papp value was $1.5 \pm 0.3 \times 10^{-6}$ cm.s$^{-1}$ (n=6) and the control ileal value was $9.2 \pm 0.6 \times 10^{-7}$ cm.s$^{-1}$ (n=6), a 1.6 fold increase in permeability. In contrast, there was no difference in the Papp of $^{14}$C-mannitol in the colon of the TBI animals ($2.6 \pm 0.4 \times 10^{-7}$ cm.s$^{-1}$, n=7) compared to controls ($3.0 \pm 0.4 \times 10^{-7}$ cm.s$^{-1}$, n=6).

There was a statistically significant 30% reduction (P<0.05) in the Isc response to veratridine in ileal tissue following a TBI compared to ileal controls. Although there was a slight reduction in the forskolin-stimulated Isc responses in ileal mucosae of TBI animals compared to ileal controls, this was not significant. Furthermore, there was no difference in either the veratridine or forskolin stimulated-Isc responses in the colonic mucosae from the TBI rats compared to colonic controls (Table 2).
Histopathology of ileal and colonic mucosae from TBI and untreated rats

Histological analysis of control ileal segments sampled at the time of death revealed an intact villous and crypt epithelium with finger-like and tongue-shaped villi, while minimal lymphocyte and plasma cell infiltration was noted in the lamina propria. There was no evidence of oedema and there was no significant vascular congestion of villous tips (Fig. 3A). In the TBI group however, 3 out of 6 ileal samples displayed distorted villous structures that appeared to fold over onto one another (Fig. 3B). This was accompanied by mild oedema of the lamina propria. A fourth sample from the TBI group displayed features of villous distortion and mild lamina propria oedema in one section, while a second section appeared to be more like controls. The fifth and sixth sections showed no distortion of villous architecture or evidence of lamina propria oedema. All sections in the TBI ileal group had intact villous and crypt epithelium and vascular congestion of villous tips only occurred in 2 out of 6 samples. There was no significant pathology in colonic tissue from either controls or TBI groups, although mild oedema was noted in the lamina propria of some of the TBI-derived samples.

Blood analysis

There was no difference in the plasma endotoxin levels over the 6 hour time period between the TBI rats and controls. Values in all groups were within the normal plasma range for rat serum endotoxin levels (0.3-0.5 EU/mL).
DISCUSSION

TBI leads to acute disturbances in autonomic nervous system activity resulting in inflammatory responses, metabolic and immune alterations and intolerance to enteric feeding \(^{31,37}\). TBI-associated dysfunction of the GIT, manifesting in symptoms including stress ulcer, gastrointestinal bleeding and motility dysfunction, has been widely reported \(^{18,20}\). Sepsis-induced MODS, the leading cause of mortality following TBI, is thought to be associated with increased gut permeability due to derangement of epithelial tight junctions \(^{32}\). There is some suggestion that bacterial translocation via the leaky bowel may play a subsequent role in the dissemination of MODS through promotion of production of local inflammatory mediators \(^{33}\). In the present *in vitro* study, direct evidence is presented to suggest that decreased ileal permeability accompanied by histological changes follow a specific mPFC-induced TBI performed under anaesthesia within a few hours.

Basal electrophysiological parameters, PD and Isc were not different in the TBI ileal and colonic groups compared to matched controls, suggesting that TBI does not affect electrogenic ion secretion across the ileum or colon. In addition, the epithelial barrier to passive movement of ions, as reflected by electrical resistance measurements was not different in either the ilea or colon of TBI rats compared to controls, indicated by the finding that the TEER values in the ileal and colonic mucosae were similar between matched groups. The relationship between TEER and flux of paracellular markers is complex in epithelial tissues that are regarded from an electrical standpoint as moderately leaky, since a significant portion of overall TEER is contributed by transcellular resistors. While there is evidence of an inverse relationship between TEER and paracellular flux of small molecular weight tracers in electrically-tight
epithelia (e.g. Caco-2 monolayers) \(^{24}\) , other studies have shown that TEER and paracellular fluxes across electrically-leakier epithelia are not always related \(^{33}\). Direct measurement of flux remains the definitive marker of permeability.

In rat intestinal mucosae, stimulated Isc is largely accounted for by the electrogenic chloride secretion \(^{36}\). The Isc response to forskolin in TBI ileum was unimpaired, most likely because it directly activates adenylate cyclase. In contrast, the magnitude of the Isc response to veratridine was reduced by 30% in TBI ilea versus controls suggests partial impairment of neural responsiveness in the small intestine, since at least part of the veratridine-stimulated Isc is mediated through neuronally-released neurotransmitter release \(^{39}\). Overall, the Isc data suggest that TBI animals retain the capacity for basal and cyclic-AMP electrogenic transepithelial secretion of chloride across intestinal tissue up to 6 hours after the impact.

The increased Papp of the paracellular marker, mannitol, across ileal but not colonic mucosae of TBI rats compared to controls suggests that TBI results in a reduction of the intestinal barrier to passive movement of small hydrophilic substances. The basal Papp values of mannitol across control tissues were similar to previous reports for rat ileal \(^{37}\) and colonic mucosae \(^{38}\). Although increased fluxes of paracellular markers are an accepted surrogate for pharmacologically-induced epithelial tight junction openings, as indicated by confocal microscopy techniques in rat ileum \(^{39}\), the histological methods use in this study were designed only to assess gross changes in mucosal structure. A 1.6 fold increase in mannitol flux across the ilea of TBI rats is similar to increases seen in partially compromised Caco-2 monolayers \(^{39}\), yet it is a much lower increase than the 20-30 fold value detected in circumstances where
epithelial integrity and viability is destroyed, for example in the presence of high concentrations of excipients and solvents. In ileal tissue from 50% of the TBI rats, there was lamina propria oedema with dilation of lymphatics coupled with prominent mononuclear cell infiltration of lamina propria and a widening and stunting of villous structures. Importantly, this phenotype was not seen in colonic mucosae from TBI rats, indicating that it is not simply a generalised multiple organ failure at the time points examined. Taken together, ilea from TBI rats display a significant increase in paracellular permeability accompanied by a compromised structure evident in a proportion of tissues.

A recent in vivo study on the jejunum of TBI rats also demonstrated increased permeability to a paracellular flux marker, which was accompanied by overt damage to the mucosal architecture. Significant increases in urinary lactulose:mannitol ratios were detected at 12 hours after TBI, whereas the current in vitro study suggests that increased permeability can be detected in ileum in vitro as early as 6 hours. Hang et al also found maximal histological damage in the rat small intestine between 24 and 72 hours after TBI. Similar to the current study, loss of epithelia from villous tips was apparent at 3 hours. Taken together, permeability increases to mannitol in the ileum are likely to precede anticipated intestinal damage. When overt damage becomes apparent, pathogen absorption and sepsis may result. In TBI rats from another study, addition of enriched immuno-nutrients to an early enteral feeding protocol appeared to protect the mucosa against TBI-induced atrophy. In sum, it seems that differences in intestinal permeability and histopathological results between research groups, although broadly consistent, may be due to different TBI...
protocols, as well as the likelihood of more significant effects being seen at later time points post TBI.

Specific cytokines and neurotransmitters that may be involved in inducing damage to the ileal mucosa in the TBI rats remain to be fully elucidated. While levels of brain interleukin-1β and interleukin-10 are increased in TBI rats, no increased concentrations were seen in plasma or liver. Levels of VIP, CCK and CGRP have also been measured in the plasma and jejunal tissue of rats exposed to TBI. The clearest pattern was a gradual increase in CCK plasma and jejunal levels up to 72 hours following TBI. While CGRP was increased in plasma up to 72 hours post TBI, this was not correlated with jejunal levels. Furthermore, other regulating molecules including nuclear factor kappa B and intercellular adhesion molecule-1, appear to be increased in jejunal tissue following TBI. Increases in bacterial endotoxin in the plasma of TBI rats compared to controls have also been detected in vivo, with a maximal and statistically significant increase at 72 hours. Under normal circumstances, the intestinal mucosal barrier limits the systemic delivery of microorganisms that normally reside in the gut lumen. Abnormalities in this barrier may allow bacteria or their products such as endotoxin to cross the mucosa and gain access to visceral organs via the lymph or bloodstream. However, analysis of the plasma from the TBI rats in the current study showed no differences in endotoxin levels compared to controls; one interpretation is that the sampling point was considerably earlier than in previous reports. It will be interesting to examining whether differing profiles of enteric micro-organisms will produce different outcomes in respect of bacteraemia and survival rates post TBI.
In conclusion, TBI may lead to an initial increase in ileal permeability to paracellular flux markers within 6 hours. Permeability changes most likely precede anticipated gross damage to the epithelium at later time points. This cascade of functional and structural changes may partially explain intolerance to enteral feeding that is commonly observed in patients following a TBI and furthermore, it may pave the way for bacteria to cross the small intestine leading to complications such as sepsis and multiple organ failure. If this is the case, then early intervention to close tight junctions using pharmacological approaches may restore normal function.
REFERENCES


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ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. A. Contusion following a TBI to the left medial pre-frontal cortex of an isoflurane-anaesthetised rat following severe TBI (arrow). B. Sectioned H & E-stained brain showing deformed structure separating from main brain tissue (arrow). Note the diffuse oedema and foci of haemorrhage. Horizontal bar = 250 μm.

Fig. 2. Papp values of 14C-mannitol in the mucosal-to-serosal direction across ileal and colonic tissue. N = 6 in each group. * P< 0.05, for ilea following TBI with respect to ileal controls.

Fig. 3. H&E staining of rat ileum (original magnification 10x) immediately following euthanasia. A. Controls showing no significant morphological abnormality. B. TBI showing evidence of diffuse oedema and elongation of villi, which appear to flop over onto one another (arrows). Horizontal bar = 100 μm.
Table 1. Baseline TEER, PD and Isc responses in rat ileal and colonic mucosae following a TBI compared to controls.

<table>
<thead>
<tr>
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<th>TEER (Ω cm²)</th>
<th>PD (mV)</th>
<th>Isc (µA/cm²)</th>
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<tr>
<td></td>
<td>Ileum</td>
<td>Colon</td>
<td>Ileum</td>
</tr>
<tr>
<td>Control (n=6)</td>
<td>64.0±6.8 84.0±19.2</td>
<td>-2.2±1.1 -7.9±2.2</td>
<td>49.1±4.0 90.5±12.8</td>
</tr>
<tr>
<td>TBI (n=6)</td>
<td>61.6±6.1 56.3±13.8</td>
<td>-1.8±1.0 -6.6±1.9</td>
<td>46.2±3.5 115.9±14.0</td>
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Table 2. Changes in $\Delta I_{sc}$ ($\Delta I_{sc}, \mu A$) responses to direct and indirect secretagogue stimulation in rat ileum and colon mucosae following a TBI.

<table>
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<tr>
<th></th>
<th>Veratridine</th>
<th>Forskolin</th>
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<tr>
<td></td>
<td>Ileum</td>
<td>Colon</td>
</tr>
<tr>
<td>Controls (n=6)</td>
<td>39.0 ± 3.0</td>
<td>84.1 ± 14.9</td>
</tr>
<tr>
<td>TBI (n=8)</td>
<td>27.6 ± 4.4 *</td>
<td>74.2 ± 5.2</td>
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Values given are net changes in $I_{sc}$. The concentrations of both agents were 10 $\mu$M, added to the basolateral side. *P<0.05. N=4-6 in each group.