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Research Paper

Myosin Light Chain Kinase Inhibition: Correction of Increased Intestinal Epithelial Permeability *In Vitro*

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Purpose. To examine whether myosin light chain kinase (MLCK) inhibitors can reduce intestinal epithelial permeability increases *in vitro*.
Materials and Methods. Isolated rat, mouse and human colonic tissue mucosae and Caco-2 monolayers were exposed to cytochalasin D (cD) and sodium caprate (C_{10}), in the absence and presence of the MLCK inhibitors, ML-9 and D PIK. Transepithelial electrical resistance (TEER) and Papp of [^{14}C]-mannitol or FITC-dextran 4000 (FD-4) were measured. Western blots were used to measure MLC phosphorylation.
Results. Increases in Papp of [^{14}C]-mannitol and decreases in TEER were induced by tight junction openers. These changes were attenuated by ML-9. D-PIK offset the FD-4 Papp increase induced by C_{10} in Caco-2 only, while ML-9 and PIK inhibited MLC directly. cD induced constriction of peri-junctional actin in Caco-2 monolayers, but this was prevented by ML-9. Although mannitol fluxes across colonic mucosae from dextran-sulphate (DSS)-treated mice were higher than control, they were not ameliorated by either ML-9 or PIK *in vitro*.
Conclusions. ML-9 inhibits paracellular permeability increases in several intestinal epithelial models. D-PIK reduced stimulated paracellular fluxes in Caco-2 monolayers, but not in tissue. Pre-established increases were not modified by two MLCK inhibitors in a mouse model of IBD.

KEY WORDS: caco-2 monolayers; cytochalasins; dextran sodium sulphate; inflammatory bowel disease mouse models; mannitol fluxes; myosin light chain kinase; transepithelial electrical resistance.

INTRODUCTION

A common feature of many idiopathic and infectious intestinal diseases is dys-regulated epithelial permeability, which may be either causative or consequential.

Leaky tight junctions in the intestinal epithelium can permit increased paracellular absorption of luminal substances, leading to mucosal inflammation in conditions including Crohn's disease (1) and ulcerative colitis (2). Diarrhoea-associated intestinal fluid secretion is also facilitated by increased intestinal paracellular permeability induced, for example, by Toxin A of *Clostridium difficile* (3), cholera toxin from *Vibrio cholera* (4), and enteropathogenic *Escherichia coli* (EPEC) (5).

Mechanistically, increases in intestinal epithelial tight junction (TJ) permeability are associated with increases in phosphorylation of myosin II regulatory light chain kinase (MLCK)-dependent myosin light chain (MLC) (6,7). Constriction of the peri-junctional ring in response to MLCK-mediated MLC phosphorylation was demonstrated using a Na+-glucose cotransport-induced Caco-2 cell model, where it was shown that increased paracellular permeability mediated by MLC could be stimulated by physiologically-, pharmacologically-, and pathologically-relevant stimuli (reviewed in 8). Na+-glucose co-transporter mediated TJ regulation via MLCK phosphorylation was also demonstrated in isolated hamster (6) and human jejunal epithelium (9). Similar increases in TJ permeability as those stimulated by nutrient-linked Na+ cotransport were also seen when MLCK was activated by an inducible promoter (10).

Drugs aimed at inhibiting MLCK may have potential in repairing intestinal permeability increases. MLCK inhibitors, including serine/threonin protein kinase inhibitors (e.g. ML-7 and ML-9) prevent increases in TJ permeability in Caco-2 monolayers following stimulation by Na+-glucose co-transport (6). Since MLCK is a calmodulin-dependent kinase and is further regulated by protein kinase C (PKC), finding relatively selective inhibitors of MLCK is fraught with difficulty. For example, it is unlikely that ML-7 and ML-9 will be used as therapeutics since they also interact with protein kinase A and PKC within the range of those required for MLCK inhibition (11). However, a membrane permeant nonapeptide inhibitor of MLCK (PIK), reversed MLC phosphorylation, decreased the

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transepithelial electrical resistances (TEER) and prevented the increased mannitol fluxes induced by EPEC, TNF-α and interferon-γ in Caco-2 and T84 cells (12). PIK has an IC_{50} of 29 μM for MLC kinase in Caco-2 and importantly, even though the original peptide is labile (13), it appears to be MLCK-specific (14). Furthermore, PIK was recently shown to enable the correction of T-cell mediated acute diarrhoea in mice, an effect associated with repair of disrupted tight junctions and similar to that seen in healthy MLCK knock-out mice (15). Effects of PIK upon epithelial permeability have yet to be demonstrated in isolated tissue mucosae, nor have they been compared with effects of ML-9.

For this study we selected cytochalasin D (cD) and the medium chain fatty acid, sodium caprate (C_{10}) to increase TJ permeability in Caco-2 monolayers and intestinal tissue (e.g. 16,17). They are therefore suitable candidates to evaluate the effects of MLCK inhibitors on reversing permeability. Since Ma *et al.* (18) showed that cytochalasin-induced increases in paracellular permeability were also mediated by MLC in Caco-2 monolayers, this drug class was therefore suitable for re-investigation with MLCK inhibitors in isolated mucosal tissues. While cytochalasins are tools for pharmacological investigation, mM concentrations of C_{10} are present in marketed dietary supplements and in solid-dose oral drug delivery formulations currently being evaluated in human trials (19). While phospholipase C- and calmodulin inhibitors prevent increases in the flux of paracellular markers across Caco-2 stimulated by C_{10} (20), the role of MLCK in C_{10}-induced TJ regulation has yet to be fully determined.

The potential of MLCK inhibitors to ameliorate already established permeability increases was also examined in excised mouse colonic mucosa from an established dextran sodium sulphate (DSS)-induced model of IBD [reviewed in (21)]. This IBD model induced by the *ad libitum* administration of DSS in water is widely used because of its ease of use and reproducibility. DSS induces colonic inflammation with similarities to human ulcerative colitis, including an increase in mucosal permeability (22). Our aims therefore were firstly to test whether a range of MLCK inhibitors can attenuate the permeability increases induced by selected tight junction openers in colonic tissues from several species including human and, if so, whether MLC and tight junction modifications are involved. A second aim was to extend the study to examine whether the inhibitors could alter the permeability of intestinal tissues isolated from a mouse model of ulcerative colitis.

MATERIALS AND METHODS

Tissue Sources

Adult male Wistar rats (250–300g) and C57Bl/6 mice (25–30g) were purchased from the Biomedical Facility at University College Dublin (UCD). Animals were housed under controlled environmental conditions regarding temperature and humidity with a 12:12 hour light/dark cycle. They had free access to tap water and standard laboratory chow. C57BL/6 mice with dextran sodium sulphate (DSS)-induced colitis were kindly donated by Dr. Padraic Fallon (Trinity College Dublin, Ireland). Maintenance and welfare of animals along with approved methods of sacrifice were in accordance with the "Principles of Laboratory Animal Care," (NIH publication number 85–23, revised in 1985).

DSS-Exposed Mice

DSS (35–50,000kDa; MP Biomedicals, OH) was dissolved in the drinking water as a 2.5% solution. Mice were treated over 5days with fresh DSS provided every second day. The mice were checked each day for morbidity and weight was recorded. Pathology was confirmed by weight loss, faecal blood and, upon autopsy, reduced length of colon (22).

Colonic Tissue Dissection and Mounting in Ussing Chambers

Approval for the use of human tissues in this study was obtained from St. Vincent's University Hospital Ethics Committee. Fresh human colonic tissue was collected at the time of surgical resection for malignant or non-inflammatory disease in patients who had not been treated with chemotherapy or radiotherapy. The resected colons were immediately examined by a histopathologist, washed in cold KH buffer and a portion of normal colon taken, remote from any lesion. A section was cut from the margin of the removed portion and histologically-inspected in order to confirm absence of pathology.

Following euthanasia, rodent colons were removed and pinned mucosal surface-down on a dissection board. Colonic tissue dissection and mounting in Ussing chambers were essentially in accordance with previous descriptions for rat (23), mouse (24) and human (25). In brief, colonic mucosae were opened longitudinally along the mesenteric border, cleaned of faecal matter, and carefully stripped of underlying muscle layers by blunt dissection. The exposed epithelial layer and underlying lamina propria was mounted in an Ussing chamber (WPI, UK) with circular window surface areas of $0.63cm^2$ for rat and human colons and $0.13cm^2$ for mouse colon. Tissues were bathed bilaterally with 5ml of Krebs–Henseleit (KH) buffer and were continuously gassed with 95% O_2–5% CO_2 at a pH at 7.4. The potential difference (PD) across the tissue and the required short circuit current (Isc) to maintain a zero PD were monitored using Ag/AgCl electrodes embedded in 3M KCl agar attached to an automated voltage clamp (DVC 4000, WPI, UK). The PD and Isc were recorded using a MacLab® analogue-digital recorder (AD Instruments, UK). Baseline potential difference (mV) and short circuit current ($\mu A.cm^{-2}$) values were recorded following an initial equilibration period of 40min after the tissues were mounted. As an indicator of net active ion transport, Isc, was recorded continuously for the duration of the experiment, while the PD was recorded every 30s for 3s by removing the voltage clamp via an automated timer (Pro-4, WPI, UK). The transepithelial electrical resistance (TEER, $\Omega.cm^2$) was calculated according to Ohm's law. Tissue capacity to respond with an inward Isc to basolateral application of the cholinomimetic secretagogue, carbachol (10μM), was used to confirm tissue viability at the end of experiments (26).

Caco-2 Monolayers

Caco-2 cells (obtained from the European Collection of Animal Cell Cultures, Salisbury, UK) between passages 50–

70 were cultured as monolayers on filters according to previous methods (27). Caco-2 cells were seeded on Transwell® polycarbonate filters (12mm diameter, 3μm pore size) at a density of 5×10^5 cells/cm^2 and incubated in 12-well culture plates with re-feeding every second day. Monolayers were used between day 16 and 21 post-seeding. TEER and potential difference (PD) were measured across monolayers using an Endohm/EVOM ® dual electrode system (World Precision Instruments, UK). Confluent monolayers were required to display stable high resistances (>400 Ω.cm^2). TEER was calculated after subtraction of the resistance value of the filters alone.

Paracellular Fluxes Across *In Vitro* Models

The apical-to-basolateral flux of the probe [^{14}C]-mannitol (Amersham Biosciences, UK) was measured to determine paracellular permeability of colonic mucosae and Caco-2 (28). About 1μCi of [^{14}C]- mannitol was added to the mucosal side of tissue mucosae and allowed to equilibrate for 1min before baseline mucosal (100 μl) and serosal (500 μl) samples were taken. Serosal samples (500 μl) were taken every 20 min for 2 h and replaced with the appropriate non-radioactive buffer. A final mucosal sample (100 μl) was taken at the end of the 2-h experiment. In Caco-2 Transwell® experiments, flux studies were carried out in a humidified incubator on 6-well plates. HBSS/20mM HEPES (pH7.4) instead of KH was used as buffer in Transwell® experiments due to the lack of carbogen gassing. Apical and basolateral volumes of Transwells ® were 0.5 and 1.0 ml respectively. Apical samples of 50 μl were taken from Transwells ® at time zero and 120 min, while 1ml basolateral samples were taken at 20 min intervals for 120 min with replacement by fresh HBSS at each time point. Radioactivity was measured using a liquid scintillation analyser (Packard Tricarb 2900 TR). Fluxes were calculated from the disintegrations/minute (dpm) using the apparent permeability coefficient (P_{app}) equation (28):

$$P_{app}(\mathrm{cm/s}) = \frac{dQ}{dt} \cdot \frac{1}{(A.C_0)}$$

where P_{app} is the apparent permeability coefficient (cm.s^{-1}), dQ/dt, the steady state flux (mol. s^{-1}), A, the surface area of membrane (cm^2) and C_0, the initial concentration in donor chamber (mol. cm^{-3}). In some Caco-2 studies the absorptive Papp of FD-4 was assessed as described previously (29). Samples were taken from the donor chambers by placing the upper compartment into another donor chamber containing fresh HBSS at 40 min. The P_{app} was then calculated as above. FD-4 concentrations were analysed using a fluorescent plate reader (Flurostar Optima, BMG Labtechnologies, Germany) with excitation of 485 and excitation filters of 520nm. The P_{app} ratio were calculated using the equation P_{app} ratio = P_{app} test/ P_{app} control.

In Vitro Exposure to cD and C_{10}: Effect of MLCK Inhibitors

Following an equilibration period of 40 min, cD (8μM in 1% DMSO, Sigma, UK) was added bilaterally to excised rat, mouse and human colonic mucosae mounted in Ussing chambers or to Caco-2 monolayers (16,19). This concentration of cD induces MLCK activity in Caco-2 (19). For the C_{10} experiments, Ca^{2+}-free KH was used in the apical compartment to prevent precipitation (30). 10–13 mM C_{10} (Sigma, UK) was added to the apical side of tissues at as this concentration is known to open tight junctions and to increase epithelial permeability *in vitro* (28).

A range of MLCK inhibitors, ML-9 (1-(5-Chloronapthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (Calbiochem, UK), ML-7 (1-(5-iodonapthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride, Sigma, UK), D PIK (rkkykyrrk-NH_2) and Dreverse PIK (krrykykkr-NH_2; both American Peptide Company, USA) were assessed in selected experiments for their potential to attenuate absorption enhancer-induced increases in permeability. ML-9 (50μM in 50% ethanol), ML-7 (10 μM in water), D PIK (400μM in water), Dreverse-PIK (330 μM in water) were used at established concentrations known to inhibit MLCK (6,12,31). Following a 40-min equilibration period of tissues and monolayers, inhibitors were added bilaterally to the chambers 20min prior to the addition of absorption promoters. ML-9 and D PIK were also added to mounted colonic mucosae from DSS-treated mice. D PIK and D Reverse-PIK were used in selected studies due to their limited availability.

Western Blot for Myosin Light Chain Phosphorylation

Phosphorylation of myosin light chain (MLC) was used as an indicator of MLC kinase activity. Rodent tissues exposed to cD, C_{10} with and without ML-9 or D PIK were snap- frozen and stored at −80°C until analysis. Caco-2 monolayers exposed to cD and/or ML-9 were washed with PBS, removed from the filters by scraping, transferred to microcentrifuge tubes. Tissues and monolayers were homogenised mechanically and lysed/denatured in sodium dodecyl sulphate (SDS) sample buffer. Following brief sonication the samples were heated to 95–100°C for 5 min, cooled and centrifuged for 5min. The protein lysates were loaded onto a 15% SDS-PAGE gel, together with prestained protein molecular weight markers and gels were run at 100 V for 2 h. Proteins were electrotransferred to nitrocellulose in a semi-dry blotter. Blots were blocked with 5% (*w*/*v*) Marvel in Tris (Sigma, UK) buffered saline (TBS) 0.1% (*v*/*v*) Tween-20 (T; Sigma UK) for 1h at room temperature to prevent non-specific binding of the primary antibody. Blots were washed three times with TBS-T and incubated with rabbit phospho-MLC 2 (Thr18/Ser19) antibody (Cell Signaling Technology, USA; 1:1000 in TBS-T with 5% bovine serum albumin at 4°C with gentle shaking. Four 5-min washes in TBS-T were carried out before incubation with 1:5,000 HRP-conjugated mouse anti-rabbit IgG (Sigma, UK) in blocking buffer for 1h at room temperature. Blots were washed four times with TBS-T before incubation with SuperSignal (Pierce, UK) chemiluminescent solution for 1min with gentle agitation. After draining the membrane of excess luminescent solution it was placed between two sheets of transparent acetate paper, exposed to CL-XPosure™ film (Pierce, UK) and processed in standard developing and fixing chemicals (Kodak Co. USA).

Confocal Microscopy

After pre-treatment of Caco-2 monolayers with combinations of cD, ML-9 and vehicles, they were washed in HBSS,

fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Monolayers were then incubated with FITC-phalloidin (Sigma), washed thoroughly in HBSS and mounted in Vectashield (Vector Laboratories). Cells were examined by confocal laser scanning microscopy using a Leica DM IRB microscope equipped with a 50mW Argon laser and a 1mW HeNe laser (Leica Microsystems). An oil immersion objective lens (×63) was used and imaging parameters were selected to optimize confocal resolution. Cytochemical staining for comparative studies was performed in the same experimental session, and images were obtained using identical imaging parameters.

Statistical Analysis

Results are expressed as mean ± SE of the mean and differences between groups were analysed using two-tailed paired or unpaired Student's *t* test or ANOVA, as appropriate. A *P* value of <0.05 was taken as the minimum level of significance in all cases. Statistical analysis was performed using Prism version 5.0 (GraphPad® software, San Diego, California).

RESULTS

Effects of TJ Opening Agents on TEER and [^{14}C]-Mannitol Papp With and Without MLCK Inhibitors: Rat and Mouse Colon

Basal electrophysiological and flux parameters of isolated colonic mucosae and Caco-2 monolayers are shown in Table I. TEER values were within the range of previous reports for mouse (32), for rat (33), for human (34), and for Caco-2 (35). There was a significant reduction in TEER following addition of 8μM cD in rat colonic mucosae compared to vehicle controls, which was significantly attenuated by pre-treatment with 50μM ML-9 (Fig. 1A). There was a corresponding increase in the permeability of [^{14}C]-mannitol in rat colonic mucosae treated with cD, which was also significantly attenuated by pre-incubation with ML-9 (Fig. 1B). ML-9 (50μM) did not significantly alter TEER or Papp compared to vehicle controls. TEER and Papp data for C_{10} (10mM) displayed a similar pattern to cD in rat colonic mucosae and both types of responses were also attenuated by pre-treatment with ML-9 (Fig. 2A, B). Note that any attempts to observe effects of MLCK inhibitors when added after the induction of a decrease in TEER by tight junction openers in mucosae were unsuccessful because tissue incubation beyond 120 min affected the tissue viability and linearity of the mannitol flux. The decrease in TEER induced by apical addition of 10mM C_{10} was reversible upon washing and re-incubation with KH buffer (Fig. 2C). In mouse colonic mucosae, there were also significant reductions in TEER following treatment with cD and C_{10} compared to vehicle controls (Fig. 3A). Pre-treatment with ML-9, significantly reduced the effects of cD ($P < 0.05$) and C_{10} ($P < 0.05$) on TEER, but only between 60–120min (n = 7–10 in each case). There were significant increases in the Papp of [^{14}C]-mannitol in mouse colons treated with either cD or C_{10} compared to controls and these effects were statistically reduced by ML-9 (Fig. 3B). Importantly, ML-9 did not alter Papp or TEER values *per se* compared to untreated controls. Bilateral addition of D PIK (400μM), however, did not reduce the effects of cD and C_{10} on mannitol permeability across murine tissue and did not alter basal electrophysiological parameters (data not shown).

Effects of TJ Openers on TEER and [^{14}C]-Mannitol Papp With and Without MLCK Inhibitors: Human Colon and Caco-2

In human colonic mucosae, cD (8μM) caused a significant reduction in TEER, associated with an increase in the Papp to mannitol (Fig. 4A). The increase in permeability was evident within 20min of addition of cD. ML-9 (50μM) attenuated the cD-induced decrease in TEER and increase in the Papp of mannitol in human colonic mucosa (Fig. 4B). In Caco-2, cD also caused a significant reduction in TEER and the associated increase in mannitol Papp (Fig. 5A, B). ML-9 (15μM) offset the reduction in TEER in Caco-2 monolayers by approximately 20% and the increase in the Papp of mannitol by approximately 50% (Fig. 5). Higher concentrations of ML-9 were not used in Caco-2 since they reduced TEER and increased the Papp *per se* and they are also not specific for MLCK.

C_{10} induced a 3-fold increase in the Papp of FD-4 across Caco-2 monolayers. Pre-incubation with Dreverse PIK (330μM) and ML-7 (10μM) attenuated subsequent increases in the Papp of FD-4 induced by C_{10}. The C_{10}-induced FD-4 Papp obtained over 12 to 40min was significantly inhibited by both Dreverse PIK and ML-7 to levels of 45 ± 22 and 38 ± 9% of the C_{10}-induced maximum Papp increase respectively ($P < 0.01$ in each case, ANOVA, data not shown). These results demonstrate that Dreverse PIK effects on Caco-2 Papp of FD-4 are comparable to those of ML-7, which have been shown in a previous study to inhibit permeability effects on C_{10} over the same period (29). C_{10} was not examined in intact human colonic sheets due to paucity of tissue supply.

The increase in permeability induced by cD in Caco-2 was associated with constriction of peri-junctional actin, which forms focal aggregates around the cell circumference associated with the perijunctional region (Fig. 6A, B).

Table I. Basal Parameters in the *In Vitro* Colonic Models

Model	TEER (Ω.cm^2)	Basal Isc (μA.cm^{-2})	Papp (×10^{-6} cm.s^{-1})	ΔIsc CCh (μA.cm^{-2})
Mouse	59±3 (45)	129±12 (45)	8.0±2.5 (12)	28±3 (17)
Rat	127±6 (65)	77±6 (65)	4.8±0.3 (12)	117±13 (19)
Human	57±25 (24)	53±18 (24)	1.0±0.8 (24)	N/A
Caco-2	763±287 (160)	N/A	0.3±0.1 (48)	N/A

Papp Absorptive flux of [^{14}C]-mannitol. Numbers in brackets refer to n numbers. *N/A* Not recorded. About 10 μM CCh was added at the end of most tissue incubations.

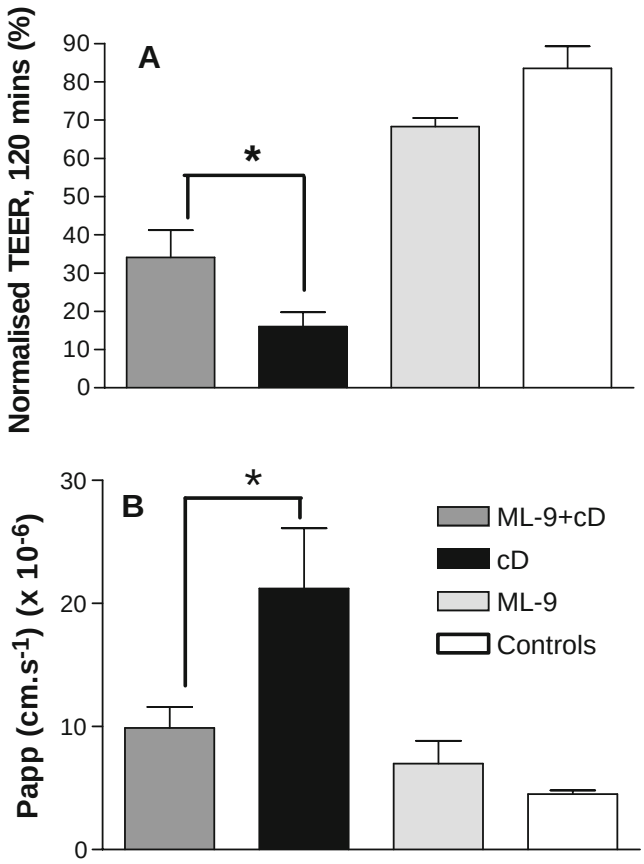

Fig. 1. **A** TEER and **B** Papp of [^{14}C]-mannitol across rat colonic mucosae exposed to ML-9 (50 μM) and cD (8 μM) compared to cD alone. TEER was normalised to zero over the test period in this and subsequent figures. Controls received vehicles in same sequence. N= 6 in all groups; *P<0.05, paired Student's t test.

Confocal microscopy of FITC-phalloidin labeled monolayers pre-treated with ML-9 prior to cD revealed a continuous apical actin ring around the cell periphery similar to that of untreated monolayers. In contrast to control monolayers however actin fragmentation was present in the centre of cells (Fig. 6C).

Western Blot for MLC Phosphorylation: Rat and Mouse Colon

An increase in MLC phosphorylation was detected in rodent tissues following treatment with either cD or C_{10} compared to vehicle controls (Fig. 7A, B). Furthermore, in both rat and mouse colonic mucosae there was an attenuation of the cD- and C_{10}-induced increase in MLC phosphorylation in the preparations pre-treated with ML-9 (50 μM), although ML-9 alone had no effect on phosphorylation status. Analysis to determine the effect of D PIK (400 μM) on rat and mouse colonic mucosa, showed a decrease in MLC phosphorylation following PIK exposure. Thus, PIK interacts with MLC but does not demonstrate a functional effect in the functional assays in these test systems. In Caco-2 monolayers, as was seen previously by Ma *et al.* (18), cD also resulted in increased MLCK activity after 20 minutes exposure and this activity was attenuated by pre-treating the monolayers with 15 μM ML-9 (data not shown).

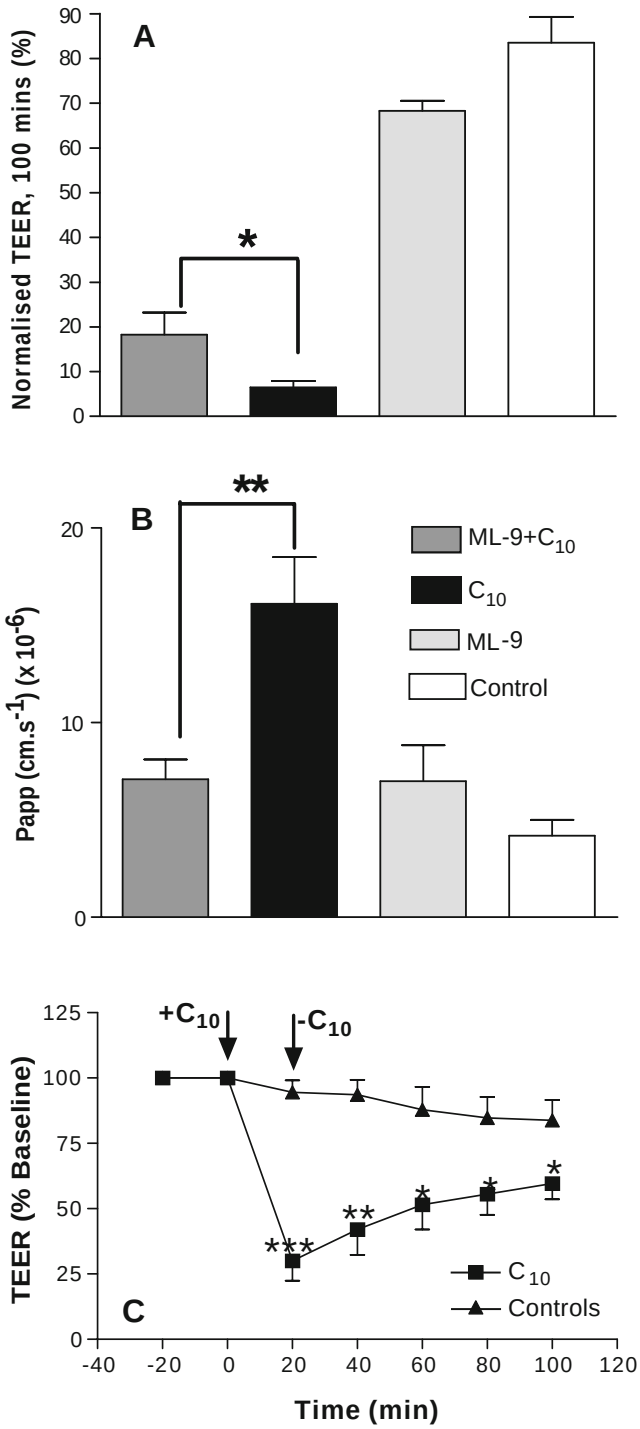

Fig. 2. **A** TEER and **B** Papp of [^{14}C]-mannitol across rat colonic mucosae exposed to ML-9 (50 μM) and C_{10} (10 mM) compared to C_{10} alone. Controls received vehicles in same sequence. **C** Reversibility of C_{10} effect on TEER. N=6 in all groups; *P<0.05, **P<0.005, ***P<0.0005, paired Student's t tests.

Effect of MLCK Inhibitors on DSS-Exposed Mouse Colonic Mucosae

DSS induced shortening of the mouse colon from 6.3 ± 0.2 mm to 5.0 ± 0.1 mm (n = 8, P < 0.0005 *versus* controls). The disease activity index score (36) in the murine intestinal

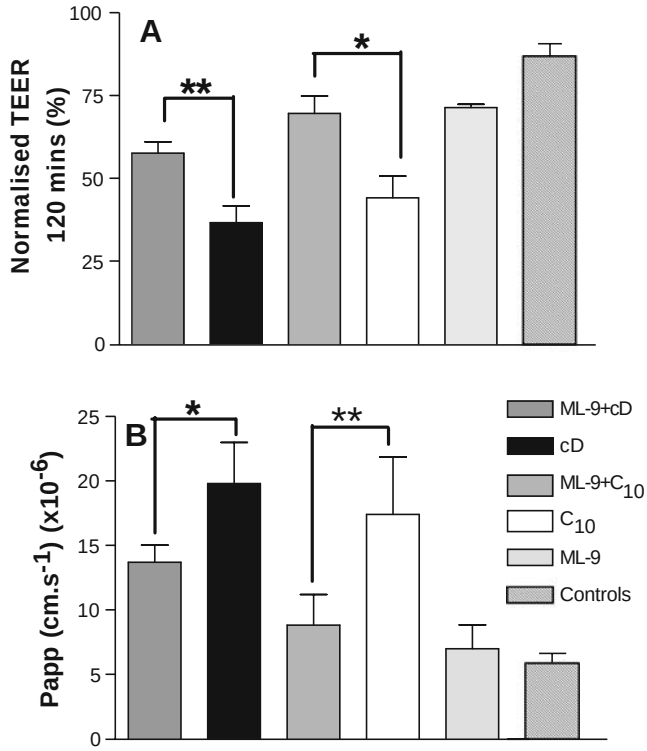

Fig. 3. **A** TEER across mouse colonic mucosae exposed to cD (8 μM) and C_{10} (10 mM) in presence and absence of ML-9. N=7–10; $^{**}P<0.005$, $^{*}P<0.05$, paired Student's t test. **B** Papp of [^{14}C]-mannitol across rat colonic mucosae exposed to cD or C_{10} in the presence and absence of ML-9. $^{*}P<0.05$, $^{**}P<0.01$ paired Student's t tests comparing effects of cD and C_{10} in the presence and absence of ML-9.

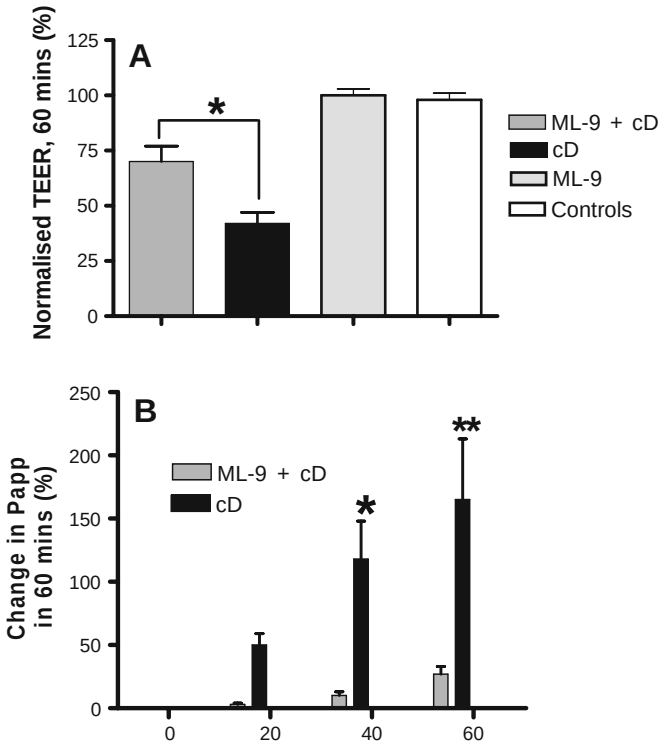

Fig. 4. Effects of ML-9 on cD-induced changes in human colonic mucosae. **A** ML-9 (50 μM) attenuated effects of cD (8 μM) on TEER, normalised to time zero. $^{*}P<0.0001$ (ANOVA). **B** ML-9 offset cD-induced increases in mannitol Papp. $^{*}P<0.01$; $^{**}P<0.001$ (ANOVA).

tract from DSS-treated animals was between 2 and 3, indicative of widespread inflammation, cell infiltration and tissue damage (data not shown). There were significant changes in TEER, Isc and Papp of [^{14}C]-mannitol across colonic mucosae from DSS-treated mice compared to controls (Table II). TEER was decreased by 10% while the Papp was increased twofold. The capacity to generate an inward Isc to carbachol was maintained in the DSS-treated tissues. Therefore, the inflamed mucosae retained ion transport capacity and only displayed moderately increased permeability. Following treatment of excised DSS treated mouse colonic mucosa with ML-9 (50 μM) or D PIK (400 μM) for 120 minutes *in vitro*, there was no significant difference in TEER or the Papp of mannitol compared to untreated DSS tissue. The Papp values were, for DSS-treated: $9.8\pm0.9\times10^{-6}$ cm.s^{-1}; for DSS-treated followed by ML-9: $12.5\pm1.9\times10^{-6}$ cm.s^{-1}; and for DSS-treated followed by PIK: $8.2\pm0.5\times10^{-6}$ cm.s^{-1}. Thus, there is no evidence that MLCK inhibitors can reverse the permeability increase induced in colonic tissue by DSS.

DISCUSSION

There is considerable interest in MLCK inhibitors as potential therapeutics for diseases associated with increased epithelial permeability including Crohn's disease, ulcerative colitis and immune-mediated intestinal diarrhoea. In a murine model of T cell-activated acute diarrhoea, treatment with D PIK in micromolar concentrations prevented intestinal tight junction disruption and ameliorated protein loss and diarrhoea (15). That this effect was mediated by MLCK was suggested by demonstration of decreased MLC phosphorylation in the presence of the inhibitor and by the absence of

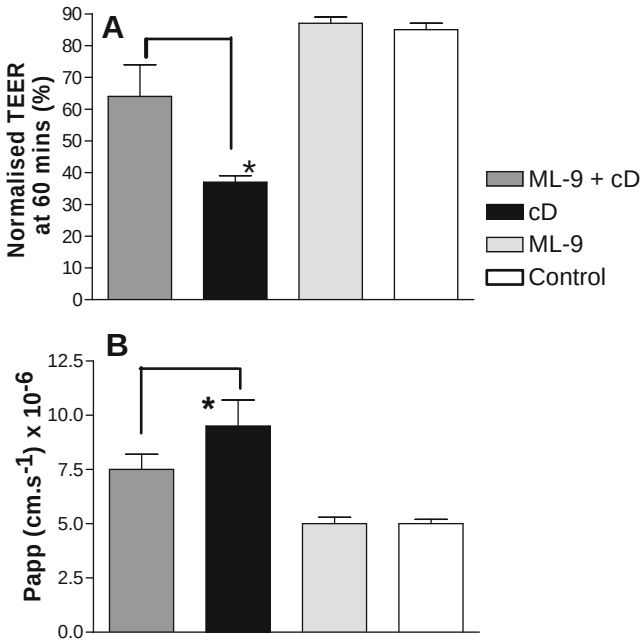

Fig. 5. Effects of ML-9 on cD-induced changes in Caco-2 monolayers. **A** ML-9 (15 μM) attenuated effects of cD (8 μM) on TEER, normalised to time zero. $^{**}P<0.005$, unpaired Student's t test). **B** ML-9 offset cD-induced increases in mannitol Papp. $^{*}P<0.05$; $^{***}P<0.0001$, ANOVA).

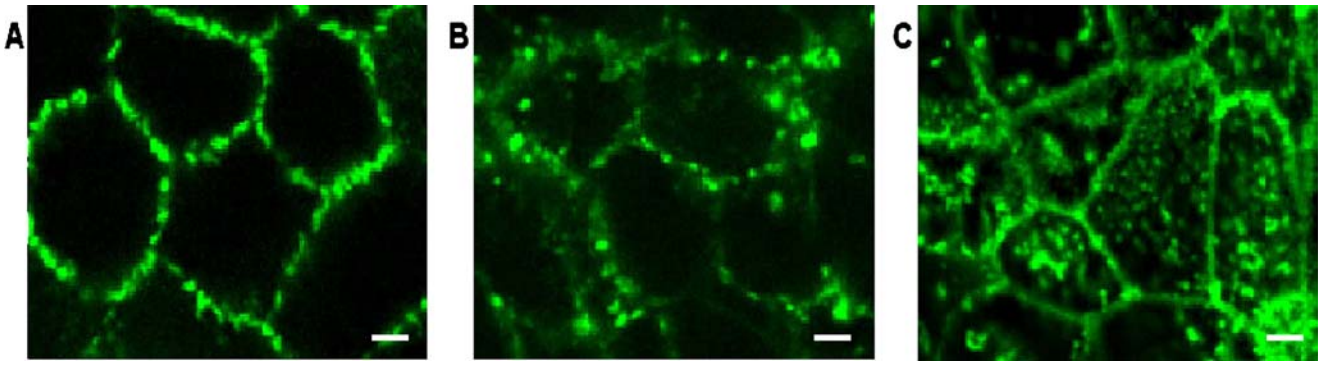

Fig. 6. Confocal microscopy from Caco-2 monolayers demonstrating the effects of ML-9 on cD-induced changes to the actin cytoskeleton. **A** Untreated. **B** Exposure to cD (8 μM). **C** Pre-exposure to ML-9 (15 uM) followed by cD. *Horizontal bars*=1 μm

diarrhoea in MLC knock-out mice. Since PIK is regarded as having increased selectivity for MLCK than ML-9 or ML-7 (11), we tested the effects of selected agents in a range of *in vitro* colonic models from different species and in addition, used them in the DSS model of ulcerative colitis in which increased permeability is a feature. Our work thus extends the epithelial literature on MLCK inhibitors from Caco-2 monolayers into isolated intestinal tissues and importantly, the effects on human colonic mucosae are reported here for the first time.

cD decreased TEER and increased mannitol flux at similar concentrations in all four intestinal epithelial models, in keeping with previous cD data in rat colon (16), pig intestine (37) and MDCK monolayers (38). cD appears to affect epithelial TJ actin filaments in two distinct stages: a rapid energy- independent severing of actin filaments into smaller fragments followed by reorganisation of actin fragments to form cytoskeleton clumps by an energy- dependent process (39). A similar effect has been described in Caco-2 cells in which an another member of the cytochalasin family, cytochalasin B, caused fragmentation of actin filaments followed by late phase actin clump formation (19). Our

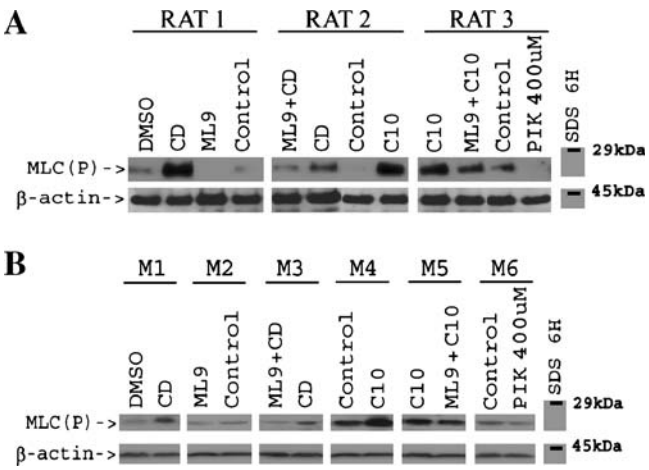

Fig. 7. Western blot analysis for MLC phosphorylation. **A** Rat colonic mucosae. **B** Mouse colonic mucosae. Rodent tissues were exposed to cD (8 μM) and C10 (10 mM) and to the MLCK inhibitors, ML-9 (50 μM) and PIK (400 μM). House-keeper expression for β-actin was used as a further control in rodent tissue. *M1–M6* Mouse 1–6.

Caco-2 confocal data further indicates that cytochalasins produce perijunctional actin aggregation associated with contraction of the enterocyte brush border, in accordance with its established function as an actin depolymerizer.

It is likely that cD-induced actin fragmentation produces MLCK activation, which in turn triggers actin-myosin interaction leading to morphological disruption of junctional proteins and a functional increase in TJ permeability. cD treatment resulted in increased phosphorylation of myosin regulatory light chain (MLC) in rat and mouse colon and Caco-2 as demonstrated by immunoblot analysis. The importance of MLCK in TJ regulation was demonstrated by the ability of the MLCK inhibitor, ML-9, to inhibit cD-induced changes in TEER and paracellular permeability. ML-9 also reduced the concentration of phosphorylated MLC in Caco-2 cell lysates and reduced the phosphorylation of MLC in response to cD treatment. Treatment of Caco-2 monolayers and colonic mucosae with ML-9 had no effect on basal TJ permeability and did not alter basal barrier function. However, ML-9 significantly attenuated subsequent cD induced increases in TJ permeability and inhibited the formation of perijunctional actin clumps. Previously, Ma *et al.* showed that the cytochalasin B-induced increase in TJ permeability was mediated at least in part by MLCK in Caco-2 (18). The current study indicates that this mechanism for cytochalasins is shared in rodent and human colonic mucosae.

Similar data to that of cD was achieved for the established tight junction opener, C_{10}, in rat and mouse colonic mucosae. The agent was recently shown to increase permeability to paracellular flux markers across human intestinal biopsies mounted in Ussing chambers (40) and part of the mechanism in these tissues involves disassembly of perijunctional filamentous actin (41). Biochemical events leading to tight junction dilatation induced by C_{10} in Caco-2 appear to be mediated in part through phospholipase C-dependent inositol triphosphate/diacylglycerol pathways and are associated with redistribution of tight junctional ZO-1 and occludin (42). MLCK was also shown to play a role in what are classed as longer term effects of the fatty acid on permeability in Caco-2 since increases in mannitol Papp were partially blocked by the MLCK inhibitor, ML-7, between 12–60 minutes (42), although no supporting biochemical data on MLC phosphorylation was provided. Our data supports that hypothesis by showing that ML-9 both inhibited C_{10}-induced

Table II. Basal Parameters in Colonic Mucosae from DSS-Treated Mice Compared to Tissues Obtained from Control Mice

Model	TEER ($\Omega.cm^2$)	Basal Isc ($\mu A.cm^{-2}$)	Papp ($\times 10^{-6} cm.s^{-1}$)	ΔIsc CCh ($\mu A.cm^{-2}$)
DSS mice	53.5±3.0* (22)	91.6±13.3* (22)	9.9±1.5 ** (11)	30±3 (5)
Control mice	58.8±2.7 (22)	129.6±12.4 (22)	5.1±1.2 (11)	28±3 (17)

Mice were fed 2.5% DSS for 5 days. Papp: absorptive flux of [^{14}C]-mannitol. Numbers in brackets are n numbers.
*$P<0.05$
**$P<0.0001$, unpaired Student's *t* tests.

increase in mannitol fluxes and attenuated C_{10}-induced MLCK phosphorylation in rat and mouse colonic tissue. It is likely in fact that many tight junction openers use a range of mechanisms including that of MLCK pathways, while as a corollary, it is known that sodium-glucose cotransport (9), enteropathogenic *E. coli* (EPEC) (12) and anti-CD3 induced TNF-α (15) also activate intestinal epithelial MLCK leading to increased paracellular transport.

ML-9 inhibited the functional changes in epithelial permeability and electrophysiology induced by cD and C_{10}, accompanied by reductions in MLCK phosphorylation. The stable nonapeptide, D PIK (13), however, was without effect on TEER and Papp of [^{14}C]-mannitol in rodent mucosae even though it was biochemically active according to the MLC Western blot data. This is in contrast to established functional effects of PIK on the prevention of TEER decreases and mannitol permeability increases in response to EPEC and Type 1 cytokines in Caco-2 (12), as well as on preventing water flux in a murine model of immune-related diarrhoea (15). Indeed, we confirmed that pre-incubation of Caco-2 monolayers with Dreverse PIK or ML-7 prevented the C_{10}-induced increase in the Papp of FD-4 over 12–40 min.

There are a number of other possible explanations as to why D PIK was not effective in the isolated tissue test systems compared to the effects of D PIK and ML-9 in Caco-2. Firstly, since a non-specific MLCK inhibitor (ML-9) with additional actions on protein kinase A and calmodulin-dependent protein kinase II was effective in rodent tissues, perhaps the more specific action of D PIK on MLCK is not sufficient to prevent the changes in permeability induced by cD and C_{10}. Secondly, although PIK is membrane permeable in Caco-2 monolayers as evidenced by co-localisation of biotinylated PIK with the perijunctional actinomyosin ring (15), at the concentrations used it may not be as accessible to the junctions of intestinal tissue mucosae mounted in well-mixed Ussing chambers, as distinct from the tissue homogenates used for Western blotting. Still, since PIK was able to prevent anti-CD3 mediated tight junction reorganisation and diarrhoea in mice (15), this seems a less likely explanation. Thirdly, it is possible that the degree of tight junction challenge offered by cD and C_{10} in tissue overpowered the protection that can be offered by PIK, although not by ML-9. The concentrations of the tight junction openers were the lowest required to yield a significant increase in mannitol flux, whereas the concentration of D PIK (or Dreverse PIK) was of the same order as that used in previous Caco-2 functional and biochemical studies (12,13,15), above which specificity for MLCK would be lost.

We attempted to further assess the potential of D PIK in the DSS-mouse model of IBD. Tissues from mice exposed to DSS *in vivo* displayed lower TEER and higher mannitol Papp values than controls, consistent with the inflamed histology. Neither ML-9 nor PIK could correct these permeability and TEER changes *in vitro*, perhaps due to the severity of the DSS challenge, although this acute model of colitis is nevertheless widely used to evaluate novel therapeutic agents (43). Perhaps pretreatment with the MLCK inhibitors *in vivo* may have greater efficacy in preventing (rather than reversing) permeability changes induced by DSS raising the possibility of therapeutic benefit in maintenance of remission. This was indeed the case for the D PIK prevention of barrier dysfunction in mice caused by T cell activation (15). Even though we did not have enough material to dose the mice with MLCK inhibitors on a daily basis, it was still important to attempt to show a direct *in vitro* effect of PIK on an established intestinal permeability increase. In attempting to reverse established permeability increases induced by cD and C_{10} with the MLCK inhibitors, the experimental design was constrained by the confounding issue of tissue viability beyond 120 minutes. Finally, in this comparative study examining tight junction permeability in human Caco-2 monolayers as well as in human and rodent intestinal mucosae, it is apparent overall that, while the two selected junctional openers acted consistently in all the models in part through MLC, the two MLCK inhibitors did not have the same capacity to reverse pharmacologically-stimulated tight junction openings across all of the models. In addition, they were ineffective in reversing the pre-established permeability seen in tissue derived from IBD-like mice. Comparative studies of this type therefore allow rigorous testing of mechanistic hypotheses across species.

CONCLUSIONS

We used a selected battery of models from cells in culture through rodent models of disease processes to human tissues *in vitro*. The rational for such an approach was to compare, side by side, specific data sets and to use the more readily available platforms (cell monolayers) to inform the selection of experimental designs for the rodent and human studies. The non-specific MLCK inhibitor, ML-9, prevented functional changes in TEER and paracellular permeability induced by selected tight junction openers in mouse, rat, Caco-2 and human colonic tissue *in vitro* models. cD and C_{10} induce paracellular permeability changes in intestinal tissues in part via the MLC pathway. In contrast to Caco-2 permeability studies, the novel and more specific inhibitor, D PIK, was not effective in the functional assays in rodent tissue even though it was biochemically active. ML-7, ML-9, PIK (12) and a stable analogue can, however, prevent induced permeability increases across Caco-2 monolayers. Neither ML-9 nor D PIK was able to reverse established permeability increases in colonic tissue isolated from mice conferred with IBD-like symptoms.

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