Basolateral potassium (IKCa) channel inhibition prevents increased colonic permeability induced by chemical hypoxia

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Isolation of human colonic crypts. After obtaining informed, written consent, we isolated colonic crypts from four to six endoscopic biopsies removed from the sigmoid colon during routine colonoscopy or flexible sigmoidoscopy in patients being investigated for abdominal pain or iron deficiency anemia, in whom there were no macroscopic mucosal abnormalities and routine histology of the mucosa was subsequently found to be normal. The study was approved by the Leeds Teaching Hospitals Ethics Committee. Biopsies were processed
to provide isolated, intact colonic crypts by a Ca\(^{2+}\) chelation technique, as described previously (2).

**Patch-clamp studies.** Recordings were made from basolateral membrane patches of cells in the mid third of the crypts in the cell-attached and perforated whole cell configurations. Patch pipettes were fabricated from borosilicate glass microhematocrit tubes by using a two-stage pipette puller (Narashige) and had tip resistances of 2–5 MΩ when filled with a solution containing (in mM) 145 KCl, 1.2 CaCl\(_2\), 1.2 MgCl\(_2\), and 10 HEPES. The bathing solution contained (in mM) 4.5 KCl, 140 NaCl, 1.2 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPES, and 5 glucose. Experiments were carried out at room temperature (20–22°C) to maintain cellular viability (35). Single channel and whole cell currents were recorded by use of a patch-clamp amplifier (Axopatch 200B, Axon Instruments). Currents were low-pass filtered at 2 kHz, sampled at 5 kHz, displayed on computer monitor, and saved to hard drive for later analysis using pCLAMP version 9.0 software (Axon Instruments).

Baseline cell-attached recordings were made until steady-state channel activity was observed. Chemical hypoxia was then induced by superfusion of crypts with the bath solution containing 100 μM dinitrophenol (DNP) to inhibit oxidative phosphorylation and 5 mM deoxyglucose (DG) to inhibit glycolysis. Channel open probability (NP\(_o\), where N was the maximum number of channels observed in a patch and P\(_o\) was the single-channel open probability) was calculated by using patch-clamp software (pCLAMP 8.0, Axon Instruments). The effects of metabolic inhibitors on K\(^+\) channel activity were evaluated by comparing steady-state NP\(_o\) values under baseline conditions and following the addition of metabolic inhibitors.

Whole cell K\(^+\) currents were measured after permeabilization of the basolateral membrane to allow electrical access to the cell interior, by using the pore-forming agent amphotericin B in the pipette solution (final concentration 240 μg/ml) (8). Whole cell recordings were obtained while crypts were superfused with bath solution, first in the absence and then in the presence of 100 μM DNP and 5 mM DG. The effects of the specific IK\(_Ca\) channel inhibitors 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) and clotrimazole (CLT) on whole cell K\(^+\) currents were also investigated by adding 100 nM TRAM-34 or 10 μM CLT to the bath solution containing 100 μM DNP and 5 mM DG. Linear regression analysis (Microsoft Excel) was used to determine the whole cell conductance from the slope of the linear portion of the relationship between the whole cell current (I\(_G\)) and the command voltage (V). The reversal potential of the cell was determined from the x-axis intercept of the current-voltage plot.

**Measurement of colonic permeability.** We obtained sigmoid colon from patients undergoing resection for colonic cancer after first obtaining their informed, written consent. Segments of tissue 10 cm in length were taken at least 10 cm from the cancer, and sheets of mucosa stripped of their underlying serosa and muscle were prepared as described previously (30). Each mucosal sheet was divided into two equal parts and mounted in paired Ussing chambers. Tissues (area 0.5 cm\(^2\)) were bathed on the mucosal side with a solution containing (in mM) 145 KCl, 1.2 KH\(_2\)PO\(_4\), 25 KHCO\(_3\), 2.5 Ca\(^{2+}\) gluconate, 1.2 KH\(_2\)PO\(_4\), 10 Ca\(^{2+}\) methane sulfonate, 1.2 MgSO\(_4\), and 11 glucose, and on the serosal side with a solution containing 113 Na\(^+\) gluconate, 25 NaHCO\(_3\), 5.8 K\(^+\) gluconate, 1.2 KH\(_2\)PO\(_4\), 10 Ca\(^{2+}\) methane sulfonate, 1.2 MgSO\(_4\), and 11 glucose. Both solutions were gassed with 95% O\(_2\) and 5% CO\(_2\) and maintained at 37°C (30). Under open-circuit conditions, transepithelial voltage (V\(_T\)) was monitored with KC1–4% agar bridges, and Ag-AgCl electrodes placed at opposite ends of the chamber were used to pass 2.5-s rectangular current pulses (I = 50 μA) across the tissue. Measurements of V\(_T\), total tissue conductance (G\(_T\) = I/V\(_T\)), and calculated short-circuit current (I\(_sc\) = V\(_T\)/G\(_T\)) were obtained when they had reached constant values. Chemical hypoxia was induced in one of the paired tissues by adding 100 μM DNP to the nongassed mucosal and serosal solutions. The other tissue acted as a "control" tissue and was gassed constantly with 95% O\(_2\) and 5% CO\(_2\).

After V\(_T\) and I\(_sc\) had reached steady-state values, the paracellular permeability of both tissues was determined by the nystatin technique (43). Nystatin (Sigma Chemical, St. Louis, MO; final concentration 500 U/ml) was added to the mucosal solution to permeabilize the apical membrane to monovalent ions, resulting in marked increases in V\(_T\), I\(_sc\), and G\(_T\), which were monitored at 15-s intervals until steady-state values were achieved. Under these experimental conditions, the increased values of V\(_T\) and calculated I\(_sc\) after apical membrane permeabilization reflected current flow as K\(^+\) moved across the basolateral membrane down its concentration gradient. The relationship between G\(_T\) and I\(_sc\) was linear, as described by the equation

\[
G_T = \frac{1}{E_B I} + I_{sc} + G_s
\]

where E\(_B\) was the electromotive force across the basolateral membrane and G\(_s\) was the shunt or paracellular conductance. G\(_s\) was estimated from the y-axis intercept of the linear relationship between G\(_T\) and I\(_sc\) (43).

In separate series of experiments, we investigated the effects of the IK\(_Ca\) channel blockers TRAM-34 and CLT on paracellular permeability during chemical hypoxia using the nystatin permeabilization method described above, having first added 100 nM TRAM-34 or 20–50 μM CLT to the serosal solution of both control tissues (gassed with 95% O\(_2\) and 5% CO\(_2\)) and tissues exposed to 100 μM DNP (nongassed).

**Statistics.** Results are shown as means ± SE. Comparisons were made using Student’s t-test for paired data, P < 0.05 indicating a statistical difference between means.

**RESULTS**

**Effect of chemical hypoxia on IK\(_Ca\) channel activity.** IK\(_Ca\) channels dominate the basolateral membrane conductance of human colonic crypt cells and have previously been characterized in detail (21, 31). Initial experiments were done to determine the response of IK\(_Ca\) channels to chemical hypoxia. In cell-attached basolateral membrane patches on cells in the mid third of crypts (n = 10 crypts from 4 patients), chemical hypoxia induced by adding 100 μM DNP and 5 mM DG stimulated IK\(_Ca\) channel activity (Fig. 1A), NP\(_o\) increasing from 0.58 ± 0.21 to a new steady-state value of 0.81 ± 0.26 within 2–3 min (P = 0.04), and the subsequent washout of DNP and DG resulted in partial reversal of the effect, NP\(_o\) decreasing to 0.70 ± 0.22 (Fig. 1B).

**Effect of chemical hypoxia on whole cell conductance.** In whole cell perforated basolateral membrane patches (n = 7 crypts from 7 patients), the addition of 100 μM DNP and 5 mM DG increased whole cell conductance from 1.74 ± 0.63 to 3.54 ± 0.47 nS (P = 0.004; Fig. 2C) within 2–3 min (Fig. 3), which was accompanied by a shift in the cell reversal potential from −58 ± 8 to −74 ± 4 mV (P = 0.03) consistent with K\(^+\) channel activation; results from a representative experiment are shown (Fig. 2, A and B). These electrical changes reversed completely after washout of DNP and DG (Fig. 2, B and C).

In further experiments (n = 6 crypts from 6 patients), we studied the effects of the highly specific IK\(_Ca\) channel blocker TRAM-34 (100 nM) on the increase in whole cell conductance induced by DNP and DG. As before (Fig. 2), the addition of DNP and DG increased whole cell conductance from 1.88 ± 0.5 to 5.43 ± 1.2 nS (P = 0.03; Fig. 4C), whereas cells hyperpolarized from −69 ± 4 to −80 ± 1 mV (P = 0.006); results from a representative experiment are shown (Fig. 4, A and B). The subsequent addition of TRAM-34 decreased whole cell conductance from 5.43 ± 1.2 to 2.28 ± 0.7 nS (P = 0.01;
Effect of chemical hypoxia on colonic paracellular permeability.

Under open-circuit conditions, colonic paracellular permeability in pairs of tissue was evaluated by estimating GS from the y-intercept of the linear plot of the relationship between GT and I (30). The reason for this discrepancy is unclear, but under open-circuit conditions part of the transepithelial potential could be dissipated by back-leakage of cations across TJs, resulting in estimates of GS higher than those obtained under short-circuit conditions (where there is no current flow through TJs). This hypothesis was tested in 11 pairs of tissues by estimating GS from transepithelial measurements under short-circuit conditions in the absence and in the presence of DNP. GS in the absence of DNP was 4.3 ± 0.4 mS/cm² and increased twofold (to 8.7 ± 0.9 mS/cm²; P = 0.00025) in the presence of DNP. Using two pairs of tissues from each of three colons, we also studied the effect of DNP on GS under short-circuit and open-circuit conditions. Under short-circuit conditions, estimated values of GS were greater in the presence than in the absence of DNP (12.9 ± 1.8 vs. 4.8 ± 0.3 mS/cm²; P = 0.047) and were lower than those obtained under open-circuit conditions (19.5 ± 1.8 vs. 138 ± 1 mS/cm²; P = 0.013). Taken together, these data indicate that, although estimates of GS under short-circuit conditions were generally lower than those obtained under open-circuit conditions, DNP-induced chemical hypoxia resulted in significant increases in GS in both cases.

Fig. 4C) and depolarized cells from −80 ± 1 to −69 ± 4 mV (P = 0.04).

We also studied the effect of CLT (10 μM), an IKCa channel blocker with potential clinical efficacy. In this set of experiments (n = 7 crypts from 7 patients), the addition of DNP and DG increased whole cell conductance from 0.91 ± 0.2 to 2.65 ± 0.5 nS (P = 0.02; Fig. 5C), whereas the cell hyperpolarized from −55 ± 7 to −74 ± 3 mV (P = 0.01); results from a representative experiment are shown (Fig. 5, A and B). The addition of CLT completely inhibited the DNP and DG-enhanced whole cell conductance (Fig. 3), which decreased from 2.65 ± 0.5 to 0.75 ± 0.15 nS (P = 0.006, Fig. 5C), whereas there was an accompanying cell depolarization from −74 ± 3 to −54 ± 10 mV (P = 0.07).

Effect of chemical hypoxia on colonic paracellular permeability.

Under open-circuit conditions, colonic paracellular permeability in pairs of tissue was evaluated by estimating GS from the y-intercept of the linear plot of the relationship between GT and Isc following the addition of nystatin (43). As shown in a representative experiment (Fig. 6A), addition of nystatin to the mucosal chamber (containing K⁺ gluconate solution) resulted in a marked and sustained increase in VT in the control tissue, whereas the increase in the paired DNP-pretreated tissue was less pronounced. In both cases, the steady-state postnystatin VT reflected K⁺ current across the basolateral membrane (Na⁺ gluconate solution in the serosal chamber). It should be noted that transient deflections in the VT traces (corresponding to the 2.5 s rectangular current pulses) were smaller in the DNP-pretreated tissue than in its paired control, consistent with a DNP-induced increase in GT (and, by inference, GS), as confirmed by the relationship between GT and Isc (Fig. 6B). In six such experiments, pretreatment with DNP resulted in a twofold increase in GS from 5.7 ± 1.1 to 12.8 ± 1.7 mS/cm² (P = 0.005).1

To determine whether IKCa channel blockade attenuates the increase in colonic paracellular permeability induced by chemical hypoxia, a similar experimental protocol was used, except that 50 μM CLT was added to the serosal solution bathing control and DNP-pretreated tissues. In the presence of CLT, the increase in VT following nystatin was similar in the control and DNP-pretreated tissues (Fig. 7A), and CLT prevented the increase in GS evoked by DNP pretreatment (compare Figs. 7B and 6B). In five experiments in which serosal CLT was present

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1 The value of GS in control tissues obtained from transepithelial measurements under open-circuit conditions (5.7 ± 1.1 mS/cm²) was greater than that previously reported in human sigmoid colon by using intracellular microelectrode measurements, also under open-circuit conditions (1.6 ± 0.3 mS/cm²) (30). The reason for this discrepancy is unclear, but under open-circuit conditions part of the transepithelial potential could be dissipated by back-leakage of cations across TJs, resulting in estimates of GS higher than those obtained under short-circuit conditions (where there is no current flow through TJs). This hypothesis was tested in 11 pairs of tissues by estimating GS from transepithelial measurements under short-circuit conditions in the absence and in the presence of DNP. GS in the absence of DNP was 4.3 ± 0.4 mS/cm² and increased twofold (to 8.7 ± 0.9 mS/cm²; P = 0.00025) in the presence of DNP. Using two pairs of tissues from each of three colons, we also studied the effect of DNP on GS under short-circuit and open-circuit conditions. Under short-circuit conditions, estimated values of GS were greater in the presence than in the absence of DNP (12.9 ± 1.8 vs. 4.8 ± 0.3 mS/cm²; P = 0.047) and were lower than those obtained under open-circuit conditions (19.5 ± 1.8 vs. 138 ± 1 mS/cm²; P = 0.013). Taken together, these data indicate that, although estimates of GS under short-circuit conditions were generally lower than those obtained under open-circuit conditions, DNP-induced chemical hypoxia resulted in significant increases in GS in both cases.
throughout, there was only a small and insignificant difference in GS between control and DNP-pretreated tissues (5.2 ± 0.45 and 7.4 ± 1.2 mS/cm², *P* = 0.22). Similar results were obtained with use of 20 μM CLT (*n* = 4, *P* = 0.99). These results demonstrate that IKCa channel blockade by CLT largely prevents the increase in colonic paracellular permeability induced by chemical hypoxia.

**DISCUSSION**

To the best of our knowledge, the present study is the first to report that metabolic stress secondary to chemical hypoxia elicits a rapid increase in IKCa channel activity in the basolateral membranes of native human intestinal epithelial cells. Metabolic stress has previously been shown to activate different types of K⁺ channel in cholangiocytes and hepatocytes (40, 41), vascular cells (22, 34), and airway cells (14). However, the magnitude of the effects we observed in human colonic crypt cells (45% and ~2-fold increases in IKCa channel activity and whole cell K⁺ conductance, respectively) were much smaller than those reported previously in human hepatoma and biliary cell lines, in which there were 40- to 70-fold increases in whole cell K⁺ conductance (40, 41). On the other hand, we observed more sustained responses to chemical hypoxia in human colonic crypt cells compared with those seen in the cell lines. These differences may reflect differences in the inherent transport functions between these cells, since cholangiocytes are primarily secretory (40) whereas colonocytes are primarily absorptive (29). There may also be cell-specific differences between the intracellular regulatory pathways that determine K⁺ channel responses to chemical hypoxia.

The ability of K⁺ channel activity to regulate intestinal permeability has been demonstrated previously in cultured monolayers of T84 human colonic adenocarcinoma cells and
airway epithelia (1, 44). Other ion channels also appear to modulate intestinal permeability, since the ability of ischemia to increase the permeability of porcine ileum was largely reversed by adding the ClC-2 (Cl⁻/H⁺) channel agonist lubiprostone to the mucosal surface (23). In addition, the Na⁺/H⁺ exchanger isoform 2 (NHE2) has been implicated in the regulation of intestinal barrier function, the mucosal application of HOE-694 (a specific NHE2 inhibitor) to ischemic tissue resulting in rapid and significant increases in transepithelial resistance (24).

Fig. 4. 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) inhibits increase in whole cell conductance induced by chemical hypoxia. A: representative experiment showing effect of 100 μM DNP and 5 mM DG, and subsequent addition of 100 nM TRAM-34, on whole cell currents. B: current-voltage relationship using data from A, showing that DNP and DG increased whole cell conductance and hyperpolarized the cell membrane, whereas these effects were completely inhibited by TRAM-34. C: summary of effect of DNP and DG, and subsequent addition of 100 nM TRAM-34, on whole cell conductance (n = 6; *P = 0.03 compared with control, **P = 0.01 compared with DNP and DG alone).

Fig. 5. CLT inhibits increase in whole cell conductance induced by chemical hypoxia. A: representative experiment showing effect of 100 μM DNP and 5 mM DG, and subsequent addition of 10 μM CLT, on whole cell currents. B: current-voltage relationship using data from A, showing that DNP and DG increased whole cell conductance and hyperpolarized the cell membrane, whereas these effects were completely inhibited by CLT. C: summary of effect of DNP and DG, and subsequent addition of 10 μM CLT, on whole cell conductance (n = 7; *P = 0.02 compared with control, **P = 0.006 compared with DNP and DG alone).
The studies with the nystatin-permeabilized human colonic sheets indicate that chemical hypoxia resulted in an increase in paracellular permeability, as judged by the increase in estimated $G_S$. Furthermore, the hypoxia-induced increase in $G_S$ was almost completely prevented by serosally applied CLT, an inhibitor of IKCa channels. It should be emphasized that in the presence of a serosally directed $K^+/H^+$ gradient, transepithelial but not microelectrode estimates of $G_S$ will be influenced by an electromotive force in the paracellular shunt pathway if this is $K^+$ selective (43). However, in nystatin-treated rabbit distal colon, there was no significant difference between the transepithelial and microelectrode estimates of $G_S$ in the presence of a serosally directed $K^+$ gradient (43). Furthermore, similar studies performed in human distal colon indicated identical transepithelial and microelectrode estimates of $G_S$ (1.6 ± 0.2 and 1.6 ± 0.3 mS/cm^2, respectively) (30). These two sets of observations indicate that the paracellular shunt pathway of colonic epithelium has no significant $K^+$ selectivity and therefore there would be no significant paracellular electromotive force under the experimental conditions used in the present studies.

Fig. 6. Chemical hypoxia increases paracellular conductance ($G_S$). A: representative experiment showing increases in transepithelial voltage ($V_T$) after nystatin-induced apical membrane permeabilization in control and paired DNP-treated colon. B: plots of total tissue conductance ($G_T$) against short-circuit current ($I_{sc}$; corrected for tissue area) obtained from A, the y-intercepts providing estimates of $G_S$. C: summary of effect of DNP on $G_S$ ($n = 6$ pairs of tissue, *$P = 0.005$ compared with control).

Fig. 7. CLT inhibits increase in paracellular conductance ($G_S$) induced by chemical hypoxia. A: representative experiment showing increases in $V_T$ after nystatin-induced apical membrane permeabilization in control and paired DNP-treated colon in the presence of 50 μM CLT. B: plots of $G_T$ against $I_{sc}$ (corrected for tissue area) obtained from A, the y-intercepts providing estimates of $G_S$. CLT inhibited the DNP-induced increase in $G_S$ shown in Fig. 6. C: summary showing that CLT largely prevented the increase in $G_S$ induced by DNP ($n = 5$ pairs of tissue, $P = 0.22$ compared with control).
study. Thus our data provide strong evidence that chemical hypoxia increases paracellular conductance (or permeability) and presumably decreases TJ integrity, through a mechanism involving IK_{Ca} channel activation. Although the exact relationship between chemical hypoxia, IK_{Ca} channel activity and TJ integrity is unclear, it is possible that TJ conformation in native human colonic epithelium changes in response to cell membrane hyperpolarization following the activation of IK_{Ca} channels. However, any link between cell membrane potential and TJ integrity may be tissue specific, given that cell membrane depolarization activated the Rhok signaling pathway leading to MLC phosphorylation in LLC-PK1 kidney tubular cells and MLC phosphorylation increased TJ permeability in malignantly transformed Caco-2 intestinal monolayers. Additional studies are therefore required to determine whether cell membrane potential and MLC phosphorylation have independent roles in TJ regulation during chemical hypoxia in human intestinal epithelia.

In the context of the present study, it is important to distinguish between two entirely separate types of intestinal barrier dysfunction (11). The first involves translocation of particulate antigens (including bacteria) via the transcellular route. The second involves an increase in paracellular permeability (as elicited by chemical hypoxia in our experiments), which raises the question of whether such a change could lead to increased bacterial migration across the intestinal epithelium. This possibility is supported by several studies; for example, Salmonella typhi Ty2 crossed Caco-2 enterocyte monolayers via the paracellular route, whereas other Salmonella crossed transcellularly (18). In addition, Clostridium difficile toxin A and toxin B both changed cytoskeletal actin, decreased transepithelial resistance, and increased paracellular migration of Salmonella typhimurium, Escherichia coli, and Proteus mirabilis across Caco-2 monolayers (9). The importance of enhanced paracellular access as a possible route for bacterial invasion of the intestinal epithelium has also been highlighted by studies in human colonic T84 cell monolayers, which showed that apically applied Shigella flexneri moved across monolayers via paracellular pathways, but only after they had been opened by the migration of basally applied polymorphonuclear leukocytes (28).

From a clinical standpoint, it is noteworthy that CLT inhibited both the increase in IK_{Ca} channel activity and the increase in paracellular conductance elicited by chemical hypoxia in human colon. This raises the possibility that IK_{Ca} channel blockade may be a novel and effective therapeutic approach for limiting increased intestinal permeability secondary to intraoperative intestinal ischemia. CLT is commonly used to treat systemic and cutaneous fungal infections and is relatively free of side effects. It has also been shown to act as an antisickling agent by inhibiting erythrocyte IK_{Ca} channels in healthy volunteers and in patients with sickle cell disease (4–6). However, parenteral administration of CLT in the surgical setting may have unpredictable and possibly hazardous effects, particularly on liver function (6). On the other hand, somatostatin peptides produce rapid, substantial inhibition of basolateral IK_{Ca} channels in human colonic crypt cells (32), and parenteral octreotide (a synthetic analog of somatostatin) is already widely used to control secretory diarrhea (12). Octreotide may therefore be worthy of evaluation as a safe and convenient means of limiting or preventing ischemia-induced increases in intestinal permeability during major liver resection.


