PI3K signaling is required for prostaglandin-induced mucosal recovery in ischemia-injured porcine ileum

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PI3K signaling is required for prostaglandin-induced mucosal recovery in ischemia-injured porcine ileum. After 45 min of ischemia, villous contraction and epithelial restitution are nearly complete within 60 min of injury, and yet PGs are able to stimulate continued elevations in transepithelial resistance (TER) after 60 min. These elevations in TER are correlated with decreased transmucosal flux of the paracellular probes mannitol and inulin and electron microscopic evidence of closure of paracellular spaces in restituted epithelium (4, 6). Furthermore, PG-induced elevations in TER are inhibited by cytochalasin D (5), an agent that initiates cytoskeletal contraction and opening of tight junctions at the appropriate dosages (14).

The mechanisms by which PGs stimulate closure of paracellular spaces are not fully characterized, although we know that sharp elevations in Cl− secretion precede recovery and that inhibition of Cl− secretion with the loop diuretic bumetanide attenuates mucosal recovery (4). The role of Cl− secretion in recovery of paracellular resistance is unclear, although it is conceivable that this event results in a transmucosal osmotic gradient. Indeed, mucosal osmotic loads have been shown to stimulate elevations in TER in normal guinea pig ileum (12) and recovery of TER in ischemia-injured porcine ileal mucosa (4). We have speculated that initial repair of tight junctions would have to precede their subsequent closure and recovery of TER (6).

PG signaling mechanisms that might result in tight junction repair include their second messengers cAMP and Ca2+ (5), both of which have been shown to alter tight junction structure in Necturus gallbladder (7, 24). Additional signaling intermediates that we have investigated are tyrosine kinases and protein kinase C (6). Although genistein augmented PG-induced mucosal recovery, this did not appear to relate to its ability to inhibit tyrosine kinases, and inhibition of protein ki-
nase C had no effect on PG-stimulated mucosal recovery (6). However, recent evidence suggests that phosphatidylinositol 3-kinase (PI3K) is intimately involved in regulation of tight junction assembly (27) and preferentially binds to specific regions of the transmembrane protein occludin via its p85 regulatory subunit (20). Therefore, in the present study, we sought to provide further evidence for a selective action of PGs on recovery of paracellular resistance and to determine if PI3K plays a role in this reparative process. Our data show that inhibition of PI3K completely inhibits the action of PGs, which is correlated with inhibition of the ability of PGs to restore localization of the tight junction integral membrane protein occludin and the cytoplasmic plaque protein zonula occludens-1 (ZO-1) to interepithelial junctions.

MATERIALS AND METHODS

Experimental animal surgeries. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six- to eight-week-old Yorkshire crossbred pigs of either sex were housed singularly and maintained on a commercial pelleted feed. Pigs were held off feed for 24 h before experimental surgery. General anesthesia was induced with xylazine (1.5 mg/kg im), ketamine (11 mg/kg im), and pentobarbital (15 mg/kg iv) and was maintained with intermittent infusion of pentobarbital (6–8 mg·kg⁻¹·h⁻¹). Pigs were placed on heating pads and ventilated with 100% O₂ via a tracheotomy by using a time-cycled ventilator. The jugular vein and carotid artery were cannulated, and blood gas analysis was performed to confirm normovisceral status. The jugular vein and carotid artery were connected to calomel electrodes, and the PD was measured via a transducer connected to the carotid artery. The ileum was approached via a ventral midline incision. The ileum was delineated by ligating the intestinal lumen at 10-cm intervals. Loops were randomly designated as control or ischemic loops. The latter were subjected to ischemia by clamping the local mesenteric blood supply for 45 min.

Ussing chamber studies. After the ischemic period, the mucosa was stripped from the seromuscular layer in oxygenated (95% O₂-5% CO₂) Ringer solution and mounted in 3.14-cm³ Ussing chambers after 120 min (peak TER) during three separate experiments. Tissues were bathed in Ringer solution and mounted in 3.14-cm³ Ussing chambers after 120 min (peak TER) during three separate experiments. Tissues were placed in Trump chambers after 120 min (peak TER) during three separate experiments. Tissues were placed in Trump chambers after 120 min (peak TER) during three separate experiments. Tissues were placed in Trump chambers after 120 min (peak TER) during three separate experiments. Tissues were placed in Trump chambers after 120 min (peak TER) during three separate experiments. Tissues were placed in Trump chambers after 120 min (peak TER) during three separate experiments. Tissues were placed in Trump chambers after 120 min (peak TER) during three separate experiments.

Electron and light microscopy. Tissues were taken at 0, 30, 60, 120, and 180 min for routine histological evaluation. Tissues were sectioned (5 μm) and stained with hematoxylin and eosin. For each tissue, three sections were evaluated. Four well-oriented villi were identified in each section. The height of the villus and the width at the midpoint of the villus were obtained by using a light microscope with an ocular micrometer. For height measurements, the base of the villus was defined as the intersection between adjacent villi at the opening of the crypt. For villi in which the height of one side of the villus was disparate from the other side, an average height was recorded. In addition, the height of the epithelial-covered portion of each villus was measured. The surface area of the villus was calculated by using the formula for the surface area of a cylinder. The formula was modified by subtracting the area of the base of the villus and multiplying by a factor accounting for the variable position at which each villus was cross-sectioned. In addition, the formula was modified by a factor that accounted for the hemispherical shape of the upper portion of the villus (1). The percentage of the villous surface area that remained denuded was calculated from the total surface area of the villus and the surface area of the villus covered by epithelium. The percentage of denuded villous surface area was used as an index of epithelial restitution.

In experiments designed to assess epithelial ultrastructure under the influence of PGs, tissues were removed from Ussing chambers after 120 min (peak TER) during three separate experiments. Tissues were placed in Trump’s 4F:1G fixative and prepared for transmission electron microscopy by using standard techniques (8). For each tissue evaluated, five well-oriented interepithelial junctions were evaluated. A calibrated grid was placed over electron micrographs extending from the apical-most aspect of the interepithelial space to 3 μm deep to the apical membrane and 1.5 μm from either side of the apical interepithelial space, so that the entire grid encompassed 9 μm². The number of squares that were occupied by paracellular space within this 9-μm² grid was used to calculate the area of the paracellular space.
Epithelial isolation. Tissues were rinsed with 30 ml of cold CO₂-saturated PBS and subsequently dropped into a tube containing CO₂-saturated citrate phosphate buffer (in mM: 96 NaCl, 1.5 KCl, 27.0 Na citrate, 5.6 KH₂PO₄, and 8.0 Na₂HPO₄). The tube was capped immediately and incubated at 37°C in a water bath for 20 min. The tissue was then transferred to a tube containing CO₂-EDTA buffer (in mM: 137 NaCl, 2.7 KCl, 1.5 KH₂PO₄, 8.0 Na₂HPO₄, 1.5 trisodium EDTA, and 2.5 glucose) and incubated at 37°C in a water bath for 30 min. Tissues were vortexed, after which a histological sample was submitted to check for the degree of epithelial sloughing. Tissues were subsequently centrifuged at 2,000 rpm for 10 min, and the pellet was solubilized in EDTA buffer in preparation for Western blotting.

Gel electrophoresis and Western blotting. Isolated epithelium from control and ischemia-injured mucosa treated with indomethacin (5 μM), indomethacin (5 μM) and PGs (1 μM), or indomethacin (5 μM), PGs (1 μM), and wortmannin (10 nM) and recovered for 120 min in oxygenated Ringer was snap frozen and stored at −70°C before SDS-PAGE. Tissue aliquots were thawed at 4°C and added to 3 ml chilled lysis buffer (0.5 M Tris, 0.5% NP-40, 1 mM 4-nitrophenyl phosphate, 0.04 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 40 μg/ml bestatin, 2 μg/ml aprotinin, 0.54 μg/ml leupeptin, and 0.7 μg/ml pepstatin A) at 4°C. This mixture was homogenized on ice and then centrifuged at 4°C, and the supernatant was saved. Protein analysis of extract aliquots was performed (DC protein assay; Bio-Rad, Hercules, CA). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2x SDS-PAGE sample buffer and boiled for 4 min. Lysates were loaded on a 10% SDS-polyacrylamide gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Science, Birmingham, UK) by using an electroblotting mini-transfer apparatus. Membranes were blocked at room temperature for 60 min in Tris-buffered saline plus 0.05% Tween 20 and 5% dry powdered milk. Membranes were washed and then incubated for 60 min in primary antibody. After being washed, the membranes were incubated for 45 min with horseradish peroxidase-conjugated secondary antibody. After additional washes, the membranes were developed for visualization of protein by the addition of enhanced chemiluminescence reagent (Amersham, Piscataway, NJ). Densitometry was performed by using appropriate software (IP gel; Scanalytics, Fairfax, VA).

Immunofluorescence microscopy. Tissues were fixed in 10% neutral-buffered formalin for 24 h, transferred to 70% ethanol, routinely processed for paraffin embedding, and cut into 5-μM sections. Slides were subsequently deparaffinized and rehydrated. Epitope retrieval was done by boiling the specimens in citrate buffer (pH 6.0) for 10 min, then allowing specimens to cool for 25 min at room temperature. Sections were blocked with 2% BSA and washed with BLOTTO and PBS, after which they were incubated in primary rabbit polyclonal anti-occludin, primary rabbit polyclonal anti-ZO-1, or an isotype control for rabbit primary antibody (negative control) for 1 h on ice. Sections were then incubated with goat anti-rabbit IgG Cy3 conjugate for 30 min in the dark. Sections were mounted, and well-oriented villi were examined with an immunofluorescence microscope.

Data analysis. Data were reported as means ± SE. All data were analyzed by using an ANOVA for repeated measures, except where the peak response was analyzed by using a standard one-way ANOVA or paired t-test (Sigmastat; Jandel Scientific, San Rafael, CA). A Tukey’s test was used to determine differences between treatments following ANOVA. Flux data was subjected to linear regression analysis, and the correlation coefficient (R) was assessed for significance. P < 0.05 was considered significant for all analyses.

RESULTS

Application of 1 μM 16,16-dimethyl-PGE₂ and 1 μM carbacyclin (a stable analog of PGF₂α) to mucosal sheets of porcine ileum injured by 45 min of ischemia and bathed in 5 μM indomethacin resulted in recovery of control levels of TER within 30 min, whereas ischemia-injured tissues exposed to indomethacin alone showed minimal elevations of TER over a 180-min recovery period (Fig. 1A). As we have shown in previous reports, this PG-induced recovery was preceded by a sharp elevation in Isc (Fig. 1B) attributable to secretion of Cl⁻ (4). As in previous studies (6), there was no difference in the histological appearance of repairing tissues treated with indomethacin compared with those additionally treated with PGs (Fig. 2), which was confirmed by showing no significant difference in the degree of Fig. 1. Electrical responses of ischemia-injured tissues treated with indomethacin (indo, 5 μM) and 16,16-dimethyl-PGE₂ and carbacyclin (PGs, 1 μM). A: serosal addition of PGs to ischemia-injured tissues after an initial 30-min equilibration period resulted in rapid recovery of control levels of transepithelial resistance (TER), whereas tissues treated with indomethacin alone had little evidence of recovery. B: elevations in TER were preceded by significant elevations in short-circuit current (Isc), which is associated with Cl⁻ secretion in this tissue. Plotted values represent means ± SE; n = 8. The significance of the elevations in TER and Isc in the presence of PGs was determined by using 2-way ANOVA on repeated measures (P < 0.05).
epithelial restitution (Table 1). In fact, restitution was nearly complete within 60 min, suggesting that the peak effects of PGs between 90 and 120 min were related to events localized to the paracellular space.

To further explore the possibility that PG-induced changes in TER were paracellular in nature, we measured \( J_{ms} \) of the paracellular probes \([\text{H}]\)mannitol and \([^{14}\text{C}]\)inulin as well as \( J_{sm} \) of \( ^{22}\text{Na}^- \) between 60 and 120 min of the recovery period (when PG-treated tissues reached maximum TER values). Flux of these probes was significantly greater in ischemia-injured tissues treated with indomethacin alone compared with tissues treated additionally with PGs (Fig. 3). We then assessed the correlation between the flux of mannitol or inulin and that of \( ^{22}\text{Na}^- \) as a method of assessing the contribution of changes in paracellular permeability (accounted for by mannitol or inulin flux) to changes in TER (accounted for by \( J_{sm} \) of Na\(^+\)), as previously described (15). We first confirmed that \( J_{sm} \) of Na\(^+\) closely correlated with changes in TER in tissues treated with indomethacin or indomethacin/PGs (\( R = 0.76, P < 0.001 \), data not shown). We subsequently documented a significant and linear correlation between fluxes of the paracellular probes and \( J_{sm} \) of Na\(^+\) (Fig. 4), indicating that changes in resistance were indeed reflective of changes in paracellular permeability.

Although the experiments thus far indicated an action of PGs on the paracellular space, we wanted more direct evidence of the involvement of the paracellular structures in the recovery response. Therefore, we performed a series of experiments in which we added increasing levels of serosal hydrostatic pressure by raising the fluid level of the serosal reservoir. We postulated that this would dilate paracellular spaces and apical tight junctions, thereby nullifying the effects of PGs. Accordingly, there was a pressure-dependent decrease in the PG-induced recovery of TER, with 6 cm serosal pressure nullifying the effects of PGs on injured tissues (Fig. 5). This action was not attributable to disruption of Cl\(^-\) secretion, because there was no significant reduction of \( I_{sc} \) by 6 cm serosal pressure. Tissues taken during peak TER levels in response to PGs showed ultrastructural evidence of closely apposed tight junctions compared with tissues treated with indomethacin alone. Furthermore, tissues subject to 6 cm of serosal pressure in the presence of PGs also showed dilatation of paracellular structures (Fig. 6). These observations were confirmed morphometrically by showing pressure-dependent increases in the area of the paracellular space (Fig. 7). There was no effect of hydrostatic pressure on normal tissues (data not shown), suggesting that hydrostatic pressure selectively affected tissues in the process of recovering paracellular resistance.

In further experiments, we attempted to elucidate some of the mechanisms involved in PG-induced recovery of paracellular resistance. In previous studies, we have suggested that increases in Cl\(^-\) secretion that precede recovery of TER may result in development of an osmotic gradient across the mucosa (4, 6). To test this hypothesis, we applied increasing doses of urea on the mucosal surface of ischemia-injured tissues treated with indomethacin and compared the effects of these treatments with that of the PGs. Accordingly, we noted dose-dependent increases in recovery of TER with mucosal application of urea that peaked with application of 200 mosmol/kgH\(_2\)O (Fig. 8). Application of other osmotic agents to the mucosal surface of tissues, including mannitol (300 mosmol/kgH\(_2\)O) and lactulose (300 mosmol/kgH\(_2\)O), resulted in similar increases in TER in ischemia-injured mucosa (peak TER in response to mannitol, 66 \( \pm \) 4 \( \Omega \cdot \text{cm}^2 \), \( n = 6 \); peak TER in response to lactulose, 65 \( \pm \) 2 \( \Omega \cdot \text{cm}^2 \), \( n = 3 \)). To demonstrate the importance of the direction of the osmotic gradient, we applied 300 mosmol/kgH\(_2\)O urea to the serosal surface of ischemia-injured tissues and saw a reduction rather than an increase in recovery of TER.
We next reasoned that, if PGs were setting up a mucosal-to-serosal osmotic gradient, the effect of the PGs should be reversed with serosal application of urea. In support of this premise, application of 300 mosmol/kgH₂O urea to the serosal surface of recovering tissues...

![Image]

**Fig. 3.** Evaluation of the effects of indomethacin and PGs on serosal-to-mucosal fluxes ($J_{sm}$) of $^{22}$Na and mucosal-to-serosal fluxes ($J_{ms}$) of $[^{3}]$Hmannitol and $[^{14}]$Cinulin. Fluxes were commenced 30 min after the addition of PGs and were conducted over a 1-h period. Tissues in the presence of indomethacin (5 μM) and PGs (1 μM) had significantly reduced $J_{sm}$ $^{22}$Na over a 1-h time period compared with tissues treated with indomethacin alone (A). Similar results were obtained for $J_{ms}$ of the paracellular probes $[^{3}]$Hmannitol (B) and $[^{14}]$Cinulin (C). Plotted values represent means ± SE; n = 8. *P < 0.05 vs. indomethacin-treated tissues.

![Image]

**Fig. 4.** Correlation between $J_{sm}$ $^{22}$Na and $J_{ms}$ $[^{3}]$Hmannitol or $J_{ms}$ $[^{14}]$Cinulin in ischemia-injured tissues treated with indomethacin (5 μM) and PGs (1 μM) or indomethacin alone. There was a significant correlation between $J_{sm}$ $^{22}$Na and $J_{ms}$ mannitol (A) or $J_{ms}$ inulin (B), suggesting that changes in TER (which correlate closely with $J_{sm}$ $^{22}$Na) were related to changes in paracellular permeability (as indicated by changes in $J_{ms}$ of the paracellular probes mannitol and inulin). The correlation coefficient (R) and its significance (P values) are indicated adjacent to linear regression plots.

![Image]

**Fig. 5.** Effects of serosal hydrostatic pressure on recovery of TER. A: recovery response of ischemia-injured tissues treated with indomethacin (5 μM) and PGs (1 μM) was marginally reduced by 2–4 cmH₂O of serosal pressure, whereas 6 cmH₂O of hydrostatic pressure fully and significantly inhibited recovery of TER. B: changes in $I_{sc}$ in response to serosal pressure did not appear to be correlated with changes in TER. In particular, 6 cmH₂O caused a small increase in $I_{sc}$ but fully inhibited TER, suggesting that the effects of serosal pressure are related to mechanical effects on the paracellular space rather than alterations in Cl⁻ secretion. Plotted values represent means ± SE; n = 8. The significant reduction in TER in the presence of PGs/6 cmH₂O compared with tissues in the presence of PGs alone was determined by using 2-way ANOVA on repeated measures (P < 0.05).
fully inhibited the action of PGs on injured tissues (Fig. 8).

In previous studies (6), we have postulated that tight junction reassembly would be required to initiate recovery of TER. Because of studies (27) implicating PI3K in tight junction assembly, we were particularly interested in this signaling pathway. Application of the PI3K inhibitor wortmannin (10 nM) completely inhibited PG-induced recovery but had no effect on $I_{sc}$ in PG-treated tissues (Fig. 9). To rule out an effect of
wortmannin on restitution, we calculated the percentage of denuded mucosa during in vitro recovery, as in our initial experiments. Following a 60-min recovery period, there was no significant effect of wortmannin compared with other treatment groups (Table 1), suggesting that wortmannin inhibited paracellular effects of PG addition. However, wortmannin appeared to reduce the small recovery response of ischemia-injured tissues treated with indomethacin alone, suggesting the possibility of nonspecific toxic effects of wortmannin.

Therefore, we also assessed the effects of the alternative PI3K inhibitor LY-294002 (10 \( \mu \)M). This agent fully inhibited recovery of TER in PG-treated tissues. However, LY-294002 did not fully inhibit the PG-stimulated elevations in \( I_{sc} \), which remained significantly elevated compared with tissues treated with indomethacin alone (Fig. 10). LY-294002 appeared to have no effect on TER or \( I_{sc} \) measurements when applied to ischemia-injured tissues treated with indomethacin in the absence of PGs.

Since we have postulated that PG-induced \( \text{Cl}^- \) secretion sets up an osmotic gradient that is in turn responsible for at least part of the recovery of paracellular resistance, we wanted to determine whether the PI3K

Fig. 7. Area of the paracellular space in the region of the tight junction based on electron microscopic images. The area of the paracellular space was dramatically reduced in tissues treated with indomethacin (5 \( \mu \)M) and PGs (1 \( \mu \)M) compared with tissues treated with indomethacin alone. Application of 2–4 cmH\(_2\)O serosal pressure had no significant effect on area of the paracellular space compared with tissues treated with indomethacin and PGs, but 6 cmH\(_2\)O hydrostatic pressure caused a significant elevation in the area of the paracellular space. Plotted values represent means ± SE; \( n = 8 \). *\( P < 0.05 \) vs. ischemia/indomethacin tissues. †\( P < 0.05 \) vs. all other treatment groups, as determined by 1-way ANOVA and a post-hoc Tukey’s test.

Fig. 8. Electrical responses of tissues subjected to osmotic loads of urea. Dose-dependent increases in TER were noted in response to 100–200 mosmol/kgH\(_2\)O urea on the mucosal surface of tissues, whereas 300 mosmol/kgH\(_2\)O urea appeared to have no further effect. Conversely, serosal application of 300 mosmol/kgH\(_2\)O reduced TER below levels of tissues treated with indomethacin alone and fully inhibited recovery of tissues in response to PGs, suggesting that the orientation of osmotic gradients in recovering tissue is critical. Plotted values represent means ± SE; \( n = 8 \). The significant increases in TER in the presence of 200–300 mosmol/kgH\(_2\)O mucosal urea and the significant inhibitory effect of 300 mosmol/kgH\(_2\)O serosal urea were determined by using 2-way ANOVA on repeated measures \(( P < 0.05 )\).

Fig. 9. Electrical responses of tissues to inhibition of phosphatidylinositol 3-kinase (PI3K). A: pretreatment with the PI3K inhibitor wortmannin (10 nM) fully inhibited recovery of TER in tissues treated with indomethacin (5 \( \mu \)M) and PGs (1 \( \mu \)M). B: \( I_{sc} \) in tissues treated with indomethacin and PGs was no different from \( I_{sc} \) levels in tissues additionally treated with wortmannin, suggesting that inhibition of TER did not relate to blockade of \( I_{sc} \). Significant inhibitory effect of wortmannin on tissues treated with indomethacin and PGs was determined by using 2-way ANOVA on repeated measures \(( P < 0.05 )\).
inhibitor LY-294002 would also inhibit urea-stimulated recovery. Therefore, tissues were treated with 200 mosmol/kgH₂O urea on the mucosal surface in the presence or absence of LY-294002 (10 μM). The PI3K inhibitor LY-294002 inhibited the effect of urea (Fig. 11), suggesting that PI3K signaling is required for osmotic load-stimulated recovery of TER, similar to that of PG-induced recovery. Similar results were obtained in tissues pretreated with wortmannin (data not shown).

In additional experiments assessing PI3K-mediated events, we sought to further define the role of PGs and PI3K inhibitors on select components of the tight junction, since it is this structure that is largely responsible for regulating paracellular permeability (13). Therefore, we assessed the tissue expression of the tight junction transmembrane proteins occludin and claudin-5 after 120 min of recovery in the presence of PGs and wortmannin. We used a technique to isolate epithelial cells from remaining mucosal elements to be sure that we were not detecting tight junction proteins from other tissues such as endothelium. Microscopic studies confirmed complete epithelial separation from mucosal villi after the isolation procedure (data not shown). Indomethacin appeared to reduce the expression of claudin-5 in ischemia-injured mucosal epithelium, but there was no apparent effect of any of our treatments on occludin expression (Fig. 12). However, in further studies using immunofluorescence microscopy, we noted differences in the distribution of claudin in the various treatment groups (Fig. 13). In particular, we noted interepithelial localization of occludin labeling in control epithelium that was disrupted in ischemia-injured tissue bathed in indomethacin (5 μM) for 120 min. Treatment with PGs (1 μM) appeared to...
PGE2 stimulates contraction of villi in normal mucosa (9) and that the cyclooxygenase inhibitor piroxicam suppresses epithelial migration stimulated by growth factors in cultured intestinal epithelial cells (29). However, we have not found any evidence for an effect of PGs on either villous contraction or epithelial migration in ischemia-injured porcine ileal mucosa. For example, tissues exposed to 45 min of ischemia have histological evidence of a complete epithelial monolayer after 60 min of in vitro recovery in tissues regardless of whether they are treated with indomethacin alone or indomethacin and exogenous PGs (Fig. 2). Nonetheless, PGs stimulate significant elevations in TER and reductions in permeability to mannitol and inulin, leading us to focus on potential effects of PGs on paracellular structures. The present studies provide further evidence for an effect of PGs on the paracellular space. For example, $J_{\text{m}}$ of Na$^+$ (which reflects changes in TER) significantly correlated with $J_{\text{m}}$ of the paracellular probes inulin and mannitol. Furthermore, quantitation of the dimensions of the junctional region of the paracellular space revealed significant reductions in the area of this space in response to PGs, whereas serosal hydrostatic pressure significantly increased the area of the paracellular space and inhibited the actions of PGs on recovery of TER.

Since tight junctions largely regulate paracellular permeability, it is likely that at least a component of the action of PGs is directed at these structures. Immunofluorescence of occludin and ZO-1 would tend to support this conclusion, since localization of these tight junction proteins to the region of the interepithelial junctions was associated with peak TER in response to PGs. However, it is also possible that PGs have an effect on the subjunctional paracellular space, the collapse of which might be responsible for a component of the recovery of TER. The importance of the proximity of epithelial lateral membranes has previously been shown to influence measurements of TER (10, 11), and the experiments with serosal pressure support the idea that dilating the paracellular space reduces the ability

**DISCUSSION**

Mechanisms believed to be critical for recovery of injured epithelium include restitution (18, 21) and, in the case of small intestinal mucosa, villous contraction (19). Restitution is a broad term that denotes recovery of an intact monolayer of epithelium across a previously denuded region of the mucosa (25). Thus restitution may be broken down into epithelial migratory events and tight junction resealing events. PGs have not been extensively linked to villous contraction or epithelial migration, although there is evidence that PGE$_2$ stimulates contraction of villi in normal mucosa.

Fig. 13. Immunofluorescence microscopic evaluation of ischemia-injured tissues for occludin. A: normal mucosa has evidence of accumulation of occludin at the lateral membrane of cells, particularly toward the apical surface of the epithelium where interepithelial junctions reside (arrows). B: ischemia-injured mucosa following a 120-min in vitro recovery period in the presence of indomethacin (5 $\mu$M). Note the disorganized appearance of occludin fluorescence, with a lack of accumulation of occludin at the region of the interepithelial junctions. C: tissues treated with PGs (1 $\mu$M) have a pattern of occludin fluorescence that resembles that of normal tissues (arrows). D: tissues pretreated with indomethacin and wortmannin (10 nM) and subsequently treated with PGs have poorly organized occludin fluorescence similar to that of tissues treated with indomethacin alone. Bar = 5 $\mu$m.

restore the normal interepithelial junctional distribution of occludin, whereas pretreatment of tissues with wortmannin (10 nM) inhibited the ability of PGs to restore occludin distribution. To seek further evidence of tight junction structural restoration in the presence of PGs, we also performed immunofluorescence experiments to assess the localization of the tight junction cytoplasmic plaque protein ZO-1. These experiments revealed highly selective localization of ZO-1 to the tight junction in control tissues and ischemia-injured tissues exposed to PGs (1 $\mu$M). In contrast, ischemia-injured tissues recovered in the presence of indomethacin (5 $\mu$M) alone or with wortmannin (10 nM) had evidence of diffuse staining in the apical region of recovering epithelial cells (Fig. 14).

**Fig. 14.** Immunofluorescence microscopic evaluation of ischemia-injured tissues for zonula occludens-1 (ZO-1). Normal mucosa has ZO-1 exclusively localized to the region of the tight junction (A), whereas ischemia-injured mucosa exposed to indomethacin (5 $\mu$M) for 120 min has evidence of diffuse ZO-1 fluorescence at the apical region of recovering epithelial cells (B). Treatment of ischemia-injured tissues with indomethacin and PGs (1 $\mu$M) for 120 min restores localization of ZO-1 to the tight junctions (C), an effect that is inhibited by pretreatment of tissues with wortmannin (D). Bar = 5 $\mu$m.
of PGs to stimulate recovery of TER. However, electron micrographs also showed evidence of dilation of the tight junction in response to serosal pressure, making it difficult to separate the effects of this maneuver on the paracellular space and the tight junction. Similarly, ischemia-injured tissues treated with indomethacin alone had dilated tight junctions and paracellular spaces, whereas those treated with PGs had closely apposed tight junctions and paracellular spaces. However, it is likely that tight junction resealing precedes collapse of the subjacent paracellular space because the continued presence of a dilated tight junction would allow extracellular fluid to enter the paracellular space.

Mechanisms of tight junction resealing following ischemia have not been fully characterized. First, it is likely that tight junctions have to reassemble following ischemic injury, since ischemia or associated ATP depletion disrupts tight junction integrity (16, 17). Reassembly of tight junctions following events such as ATP depletion involves localization of integral membrane proteins such as occludin to the apical-most aspect of the lateral epithelial membrane, along with colocalization of cytoplasmic proteins such as ZO-1 (28). Similarly, studies utilizing a calcium switch (chelation and subsequent repletion of calcium) to disrupt and allow recovery of tight junctions documented the critical role of integral membrane proteins in orchestrating reassembly of tight junctions (22). Although we do not know if these same mechanisms are responsible for PG-stimulated recovery of TER, we do have evidence on immunofluorescence that PGs restore the distribution of the tight junction integral membrane protein occludin and the cytoplasmic plaque protein ZO-1 to the region of the interepithelial junction. However, there was no difference in the expression of occludin in response to PG treatment, suggesting that PGs stimulate movement of preexisting occludin and ZO-1 dispersed throughout the cell during ischemic injury to the interepithelial junction during recovery.

In the present study, we were able to use PI3K inhibitors to functionally separate changes in 

$I_{sc}$ and TER, both of which are stimulated by PGs. We know from previous studies that inhibition of $I_{sc}$ and the associated secretion of Cl$^{-}$ largely blocks the action of PGs on recovery of TER (4). However, it now appears that PI3K-mediated events are critical for recovery of TER despite the continued presence of elevations in $I_{sc}$. Inhibitors of PI3K also blocked recovery of TER in response to mucosal osmotic loads of urea. Together, this data suggests that PI3K-mediated events are downstream of mechanisms resulting in a mucosal-toserosal osmotic gradients, including mucosal urea and PG-induced Cl$^{-}$ secretion (proposed model shown in Fig. 15). However, as an alternate possibility, it is also conceivable that PGs require both elevations in $I_{sc}$ and intact PI3K signaling to stimulate recovery of TER. As far as the specific mechanisms involved in PI3K-sensitive recovery of TER, this will have to await further study. However, previous studies demonstrating preferential binding of the p38 regulatory domain of PI3K to occludin (20) and a role for PI3K in junctional actin rearrangement (23) suggest potential important functions of this enzyme in tight junction resealing.

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