

Prostaglandins I₂ and E₂ Have a Synergistic Role in Rescuing Epithelial Barrier Function in Porcine Ileum

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Abstract

Prostaglandins (PG) are cytoprotective for gastrointestinal epithelium, possibly because they enhance mucosal repair. The objective of the present studies was to assess the role of prostaglandins in intestinal repair. Intestinal mucosa from porcine ileum subjected to 1 h of ischemia was mounted in Ussing chambers. Recovery of normal transepithelial electrical resistance occurred within 2 h, and continued to increase for a further 2 h to a value twice that of control. The latter response was blocked by inhibition of prostaglandin synthesis, and restored by addition of both carbacyclin (an analog of PGI₂) and PGE₂, whereas the addition of each alone had little effect. Histologically, prostaglandins had no effect on epithelial restitution or villous contraction, indicating that elevations in transepithelial resistance were associated with increases in paracellular resistance. Furthermore, prostaglandin-stimulated elevations in resistance were inhibited with cytochalasin D, an agent known to stimulate cytoskeletal contraction. Synergistic elevations in transepithelial resistance, similar to those of carbacyclin and PGE₂, were also noted after treatment with cAMP and A23187 (a calcium ionophore). We conclude that PGE₂ and PGI₂ have a synergistic role in restoration of intestinal barrier function by increasing intracellular cAMP and Ca²⁺, respectively, which in turn signal cytoskeletal-mediated tight junction closure. (*J. Clin. Invest.* 1997. 100:1928–1933.) Key words: intestinal mucosa • ischemia • cAMP • calcium • tight junctions

Introduction

Since the discovery of the cytoprotective function of prostaglandins (PG)¹ in gastric epithelium by Robert et al., there has been a great deal of research on the mechanism whereby prostaglandins protect tissues from various chemical and physical insults (1–4). Prostaglandins have also proven to be important

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1. Abbreviations used in this paper: [Ca²⁺]_i, intracellular calcium concentration; J_{ms}, mucosal-to-serosal flux; PG, prostaglandin.

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in the small intestine, since chronic treatment of human patients with nonsteroidal antiinflammatory agents results in increased small intestinal permeability (5). Theories to explain cytoprotection, such as increased blood flow, have not gained universal acceptance (2). It is possible that the cytoprotective effects of prostaglandins are actually due to enhanced tissue repair, rather than prevention of injury (4). Recent studies have shown that stimulation of epithelial migration (restitution) by growth factors is mediated by prostaglandins, since inhibition of cyclooxygenase prevented growth factor-stimulated restitution, and replacement of prostaglandins restored the migratory process (6). In addition, prostaglandins have been shown to stimulate villous contraction in the small intestine (7), which aids restitution by decreasing villous surface area (8). An effect of prostaglandins on epithelial tight junctions has also been considered, but has received little attention (3). Considering tight junctions control the passage of solutes via the paracellular space, the major route of transepithelial solute passage, physiologic control over tight junctions is of major physiologic importance (9). Although once thought to be inert connections, it has recently been determined that tight junctions are dynamic structures that are regulated by mediators such as γ -interferon, cAMP, and Ca²⁺ (10–12). Since the secretory effects of PGE₂ and PGI₂ in the intestinal tract are mediated via cAMP and Ca²⁺, we postulated an important role for these prostaglandins in regulation of paracellular permeability following ischemic injury.

Methods

Six- to eight-week-old Yorkshire crossbred pigs of either sex were anesthetized, and the ileum was approached via a ventral midline incision. Porcine ileal segments were delineated by ligating the intestinal lumen at 10-cm intervals, and subjected to ischemia by clamping the local mesenteric blood supply. After the desired period of ischemia, the mucosa was stripped from the seromuscular layer in oxygenated (95% O₂/5% CO₂) Ringer's solution, and mounted in 3.14-cm² aperture Ussing chambers, as described in previous studies (13). Tissues were bathed on the serosal and mucosal sides with 10 ml Ringer's solution. The serosal bathing solution contained 10 mM glucose, and was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O₂/5% CO₂) and circulated in water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Resistance (Ω ·cm²) was calculated from the spontaneous PD and short-circuit current (I_{sc}). If the spontaneous PD was between –1.0 and 1.0 mV, tissues were voltage clamped at \pm 100 μ A for 5 s and the PD recorded. Short-circuit current and PD were recorded every 15 min for 4 h. Treatments were added to serosal or mucosal bathing solutions as appropriate before mounting tissues on Ussing chambers. Select tissues were removed at 1-h intervals and at the end of the experiments for routine histologic evaluation (5- μ m cross-sections taken at 300- μ m intervals, and stained with hematoxylin and eosin).

Mucosal-to-serosal fluxes (J_{ms}) were performed by adding ^3H -mannitol ($10 \mu\text{Ci/ml}$) or ^{14}C -inulin ($10 \mu\text{Ci/ml}$) to the serosal bathing solutions once tissues were mounted in Ussing chambers. Time zero samples were taken from the reservoirs after a 15-min equilibration period. After a further 15-min equilibration period, the first flux was initiated over the following 60 min, and performed at 60-min intervals thereafter. Samples were collected in scintillation vials and assessed for β emission (counts/minute). The windows on the scintillation counter were set to avoid counting overlap of β emission from ^3H and ^{14}C .

The elaboration of cAMP and Ca^{2+} in response to prostaglandins was assessed in a porcine jejunal enterocyte line (IPEC-J2) because of the inability to accurately measure the concentration and cellular location of second messengers in native mucosal tissues. We have previously reported on methods used to study this cell line (14). Cells were grown at 37°C in a 5% $\text{CO}_2/95\%$ O_2 air humidified incubator in Dulbecco's modified Eagle's medium with 5% calf serum, 0.1% penicillin-streptomycin, 5 ng/ml EGF, 2.4 mM L-glutamine, and 0.1% of a mixture of 50 mg/liter insulin, 250 mg/liter transferrin, and 250 mg/liter Se. Cells were studied at passage 62, and grown to 100% confluence. Measurements of cAMP were obtained in cells pretreated for 5 min with the phosphodiesterase inhibitor isobutyl methylxanthine (10^{-4} M). Cells were exposed to carbacyclin (10^{-6} M), PGE_2 (10^{-6} M), or both for 15 min, after which cells were washed twice with PBS, and immersed in 6% perchloric acid. After centrifugation and repeated washing, ether extraction was performed three times. Cyclic AMP was measured using RIA (cAMP kit; Biomedical Technologies Inc., Stoughton, MA).

Measurements of $[\text{Ca}^{2+}]_i$ were obtained using a modular fluorimeter system attached to an inverted microscope as previously described (15). IPEC-J2 cells were loaded with fura-2, which was added to the cells at a final concentration of 2×10^{-6} M for 20 min at 37°C . Cells were washed with NaCl Ringers and mounted in a chamber over the objective of the microscope. Fluorescence from cells was acquired alternately at 340 and 380 nm with a photometer and passed to a photomultiplier. A microcomputer averaged the emission intensities collected over 1-s time intervals from each of the excitation wavelengths. This information was used to calculate $[\text{Ca}^{2+}]_i$ using previously described calculations (15).

Results

Tissues subjected to 30, 45, 60, and 120 min of ischemia recovered control levels of resistance when mounted in Ussing chambers according to the severity of mucosal injury: 2-h ischemic tissues required 4 h to recover normal resistance, whereas tissues exposed to 30–60 min recovered normal resistance more rapidly, followed by an elevation in resistance. The elevation in resistance was most apparent in 1-h ischemic tissues, and was blocked by adding 5×10^{-6} M indomethacin to the mucosal and serosal bathing solutions (Fig. 1). Addition of 10^{-6} M carbacyclin to indomethacin-treated ischemic tissues had no effect on transepithelial resistance (i.e., similar to ischemic tissues treated with indomethacin alone), whereas 10^{-6} M 16,16-dimethyl PGE_2 had a limited effect. A combination of carbacyclin and PGE_2 had a striking synergistic effect on resistance (Fig. 2), which not only reversed the indomethacin effect, but within 2 h increased resistance to maximal levels seen in untreated ischemic tissues.

Histopathologic examination of mucosa immediately after 1 h of ischemia showed epithelial sloughing from the tips of the villi ($17.5 \pm 5.6\%$ of the villous perimeter was denuded). After 4 h in the Ussing chamber, villi were covered by flattened epithelium as a result of restitution (migration of epithelium across denuded villous tips). In addition, these latter tissues demonstrated marked reductions in villous height compared

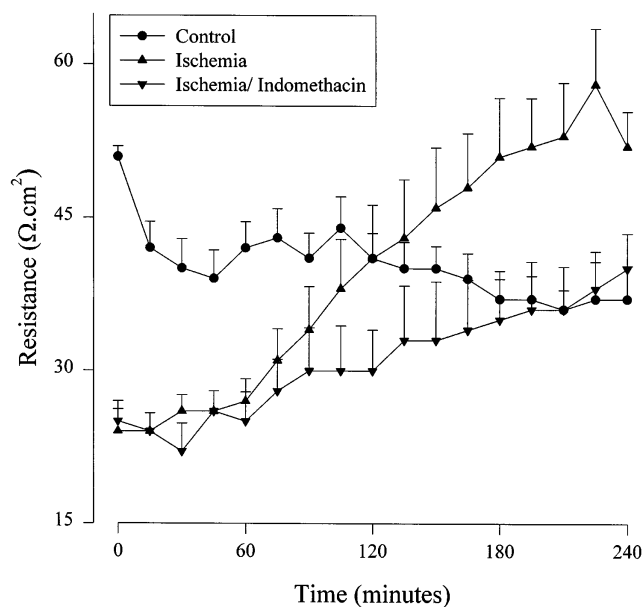


Figure 1. Electrical responses of repairing tissues. Tissues subjected to 1 h of ischemia had markedly reduced resistances at the beginning of the experiment. Control levels of resistance were attained in these tissues after 120 min, after which resistance continued to climb until it was approximately twofold that of control tissue. Addition of indomethacin to mucosal and serosal bathing solutions of injured tissues resulted in slower recovery of resistance, such that resistance only attained control levels of resistance at 240 min. Data points represent the mean \pm SE, $n = 6$.

with control tissues maintained in Ussing chambers for the same length of time ($169.5 \pm 3.3 \mu\text{m}$ vs. $275.0 \pm 12.4 \mu\text{m}$ respectively, $n = 6$). Reductions in villous height were apparently not due to neurally mediated villous contraction, since addition of the neural blocker tetrodotoxin (10^{-7} M) to the serosal surface of tissues had no effect on villous height. Injured tissues treated with indomethacin had similar histologic evidence of villous atrophy (villous height, $180.5 \pm 13.0 \mu\text{m}$, $n = 6$) and had also undergone complete restitution. Addition of carbacyclin and PGE_2 had no apparent effect on epithelial restitution or villous height ($168.2 \pm 4.8 \mu\text{m}$, $n = 6$).

Mucosal-to-serosal flux (J_{ms}) of ^3H -labeled mannitol and ^{14}C -labeled inulin were performed to further investigate changes in electrical resistance. Inulin, which is a polysaccharide of considerable size (11.5 \AA stokes radius), traverses leaky epithelia via the paracellular space ($\sim 9 \text{ \AA}$ stokes radius) (9, 11). Inulin fluxes in injured tissues paralleled changes in resistance. In particular, indomethacin exacerbated elevations in inulin flux demonstrated in ischemic tissues, and addition of carbacyclin and PGE_2 significantly reduced inulin flux (Fig. 3). Flux of the smaller mannitol (4 \AA stokes radius) showed similar trends, except that mannitol fluxes did not return to control levels in repairing tissues by the end of the experiment (J_{ms} mannitol at 240 min: control, 0.15 ± 0.01 ; ischemia/indomethacin, 0.28 ± 0.03 ; ischemia/indomethacin/carbacyclin and PGE_2 , $0.24 \pm 0.04 \mu\text{mol/cm}^2$ per h, $n = 6$).

Considering the remarkable differences between treatments in resistance and mucosal-to-serosal flux of inulin at 60 min (Figs. 2 and 3), tissues were removed after 1 h in the

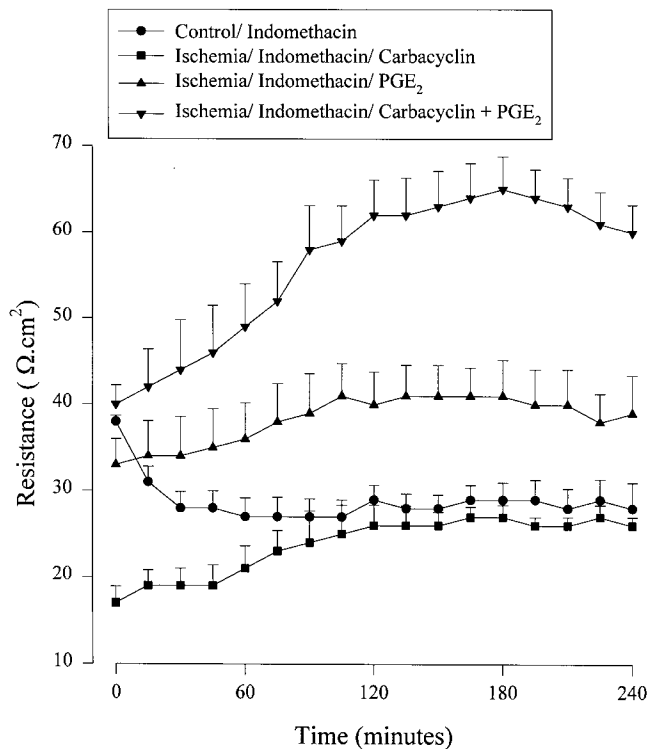


Figure 2. Response of repairing tissues to prostaglandins. The effects of indomethacin on tissues subjected to 1 h of ischemia were reversed by addition of both carbacyclin and PGE₂. Carbacyclin alone had no discernible effect, whereas PGE₂ had a limited effect. Treatment with carbacyclin and PGE₂ resulted in significantly greater elevations in resistance than the additive effects of the individual prostaglandins indicating a synergistic relationship between carbacyclin and PGE₂. Statistical significance was determined by two-way analysis of variance for repeated measures (SigmaStat, version 2.0; Jandel Scientific, San Rafael, CA). Data points represent the mean ± SE, *n* = 6.

Ussing chamber for histologic examination. Restitution was near complete in all tissues, and varied little between treatments (3.4 ± 1.2 , 3.8 ± 0.9 , and $3.5 \pm 0.7\%$ of the villous perimeter was denuded in ischemic, indomethacin-treated ischemic, and prostaglandin/indomethacin-treated ischemic tissues respectively, *n* = 4). Measurements of villous height at 1 h were similar to those taken at 4 h. Taken together, these findings suggested an increase in paracellular resistance as the cause of higher levels of transepithelial resistance in prostaglandin-treated tissues, rather than mechanisms involving villous contraction or epithelial restitution.

Increases in paracellular resistance have been associated with mucosal exposure to osmotic loads (16). Physiologically, increases in mucosal osmotic loads may occur during chloride secretion (13), such as that stimulated by prostaglandins I₂ and E₂ (17). In support of this concept, addition of prostaglandins to injured tissues in the present study resulted in additive but not synergistic increases in I_{sc} (ΔI_{sc} 1 h after addition of carbacyclin, PGE₂ or carbacyclin/PGE₂ to indomethacin-treated injured tissues was -5.5 ± 4.3 , 18.5 ± 4.0 , and 26.5 ± 3.8 $\mu A/cm^2$, respectively, *n* = 6), suggesting that prostaglandin-induced increases in paracellular resistance may be associated with chloride secretion.

We speculated that tight junctions responded to cAMP (10), triggered by PGE₂ receptor-mediated adenylate cyclase activity (17) and release of intracellular calcium (12) triggered by PGI₂-stimulated cholinergic nerves (18). To test this hypothesis, 10^{-5} M 8-bromo-cAMP and 10^{-7} M A23187 (a potent and specific Ca²⁺ ionophore) were added to the serosal or mucosal and serosal bathing solutions of indomethacin-treated tissues, respectively. Similar synergistic elevations in transepithelial resistance between cAMP and Ca²⁺ were noted in injured tissues during the first 60 min of the experiment (Fig. 4). In further experiments, IPEC-J2 cells were exposed to carbacyclin (10^{-6} M), PGE₂ (10^{-6} M), or both to isolate the effects of prostaglandins on intestinal epithelial second messengers. Prostaglandin E₂ triggered a significant increase in cAMP compared with control (18.8 ± 1.4 and 9.7 ± 1.2 pmol/ml, respectively, *n* = 3, *P* < 0.05 [one-way ANOVA]), but the addition of carbacyclin had no further effect (18.9 ± 3.6 pmol/ml), and carbacyclin alone had no effect (14.5 ± 1.4 pmol/ml; *P* > 0.05 versus control).

There was no effect of either prostaglandin on [Ca²⁺]_i in IPEC-J2 cells, while 10^{-4} M ATP induced marked elevations in [Ca²⁺]_i (data not shown). We postulated that the inability of prostaglandins to stimulate elevations in [Ca²⁺]_i resulted from the absence of enteric nerves in cell culture, since carbacyclin has been shown to act via cholinergic and VIPergic nerves in porcine intestine (19). Therefore, we applied 10^{-7} M tetrodotoxin to the serosal surface of indomethacin-treated ischemic

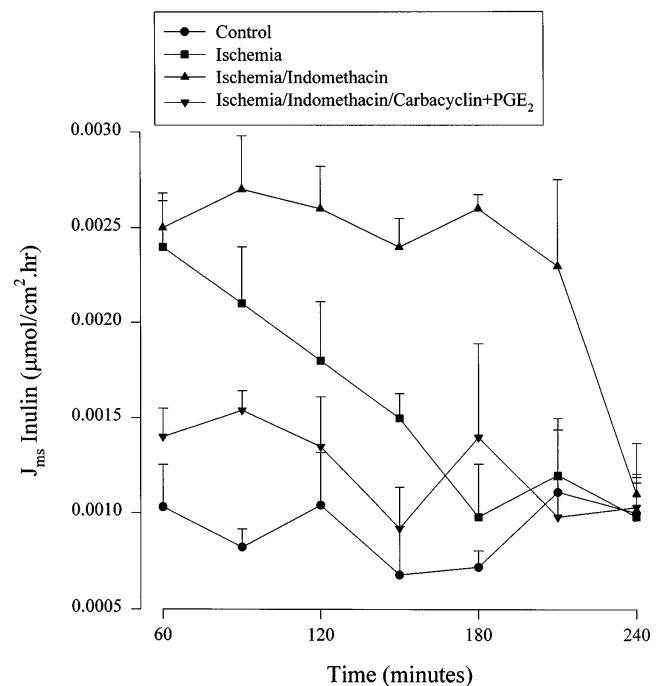


Figure 3. Mucosal-to-serosal flux of inulin across injured tissues. Ischemic tissues recovered normal mucosal-to-serosal flux of ¹⁴C-inulin steadily over 240 min. Treatment with indomethacin significantly increased the flux of inulin during the initial flux periods, and addition of carbacyclin and PGE₂ resulted in significant decreases in ¹⁴C-inulin flux compared with ischemic tissues in the presence of indomethacin alone. Statistical significance was determined by two-way analysis of variance for repeated measures. Data points represent the mean ± SE, *n* = 5.

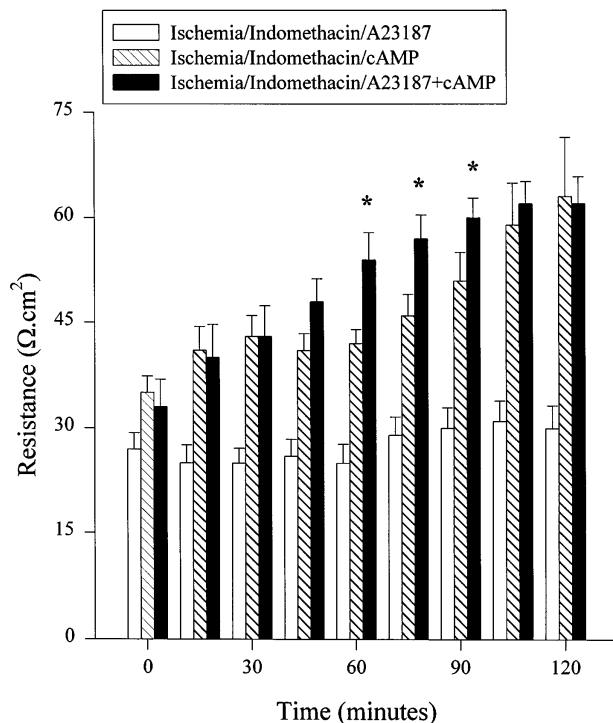


Figure 4. Response of repairing tissues to A23187 and cAMP. Addition of the calcium ionophore A23187 and cAMP to indomethacin-treated ischemic tissues partially duplicated the results of addition of carbacyclin and PGE₂ to similarly treated tissues, suggesting the possibility that the effect of the prostaglandins was mediated through their respective second messengers. Addition of A23187 had no effect on its own (no increase above baseline measurements), whereas cAMP caused marked increases in tissue resistance. Between 45 and 90 min after addition of both A23187 and cAMP, there was a significant synergistic increase in resistance. Statistical significance was determined by two-way analysis of variance. Data points represent the mean \pm SE, $n = 6$. * $P < 0.05$ for A23187/cAMP/indomethacin-treated tissues versus the additive effect of A23187/indomethacin and cAMP/indomethacin-treated tissues.

tissues 30 min before the addition of prostaglandins, and inhibited recovery of transepithelial resistance to the level attained with PGE₂ treatment alone (Fig. 5). Furthermore, 10⁻⁵ M serosal atropine had a similar effect as tetrodotoxin, whereas serosal application of 10⁻⁶ M of the VIP receptor antagonist [Lys¹, Pro^{2,5}, Arg^{3,4}, Tyr⁶]-VIP had no effect. Taken together, these experiments suggest that prostaglandins, most likely carbacyclin, act via cholinergic nerves which would be expected to trigger increases in [Ca²⁺]_i.

To assess the role of the tight junction-cytoskeletal interface in prostaglandin-stimulated increases in resistance, 10⁻⁶ M cytochalasin D, an agent known to stimulate cytoskeletal contraction and opening of tight junctions (20), was added to ischemic tissues treated with indomethacin and prostaglandins. Cytochalasin D abolished previously noted synergistic elevations in transepithelial resistance in response to prostaglandins (Fig. 6). However, cytochalasin D did not lower the resistance of control tissues, suggesting that inhibition of recovery of transepithelial resistance in injured tissues was not a result of a nonspecific toxic effect. In addition, we evaluated cytochalasin

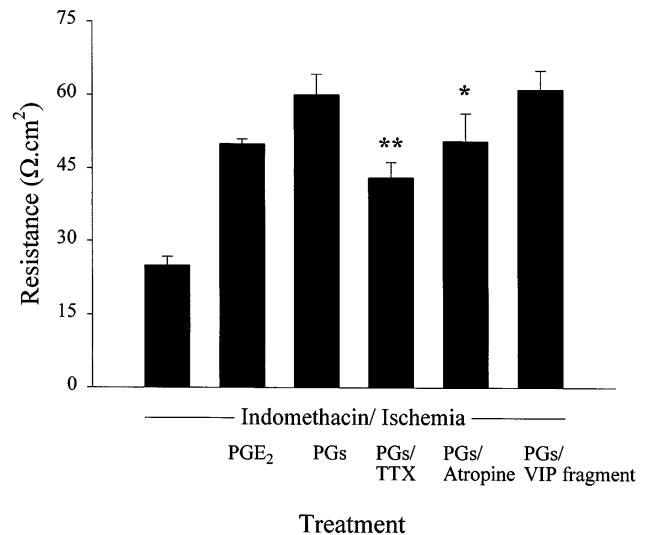


Figure 5. Response of injured tissues to prostaglandins after pretreatment with the neural-inhibitory agents tetrodotoxin, atropine and the VIP antagonist LPAT. Tetrodotoxin and atropine inhibit recovery of resistance at 120 min to levels similar to those of tissues treated with PGE₂ alone, whereas the VIP antagonist had no effect. Statistical significance was determined by two-way analysis of variance. Bars represent the mean \pm SE, $n = 6$. * $P < 0.05$ versus tissues treated with PGE₂ and carbacyclin (PGs).

D-treated tissues for evidence of reduced epithelial restitution, since restitution also depends on a functional cytoskeleton and may be inhibited by cytochalasin D (21). However, ischemic tissues treated with indomethacin and prostaglandins appeared to reconstitute similarly in the presence of cytochalasin

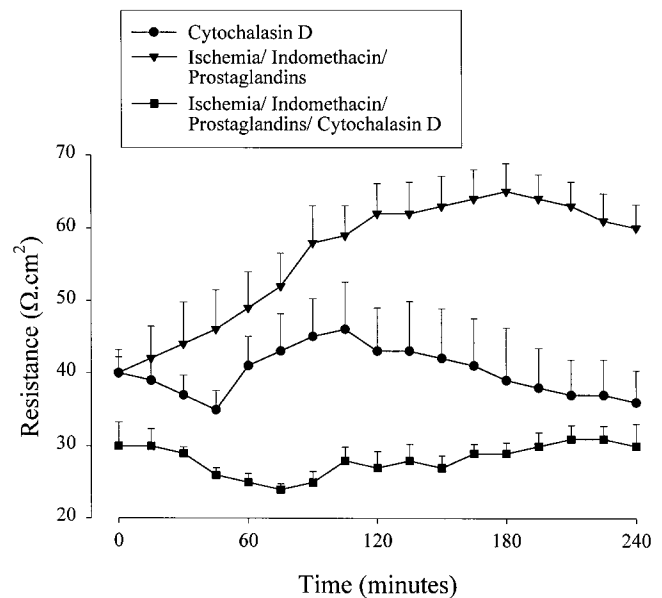


Figure 6. Response of indomethacin/prostaglandin-treated tissues to the actin-binding agent cytochalasin D. Cytochalasin D abolishes prostaglandin-mediated recovery of transepithelial resistance. Data points represent the mean \pm SE, $n = 4$.

D ($3.4 \pm 1.4\%$ of the villous perimeter was denuded in prostaglandin/indomethacin-treated ischemic tissues in the presence of cytochalasin D at 60 min, $n = 4$).

Discussion

These findings have important implications for damaged or leaky epithelia. Prostaglandins have often been assumed to have detrimental effects on tissues as mediators of inflammation (22). In contrast, the present findings suggest that prostaglandins play a salutary role in early restoration of epithelial barrier function. Although repair mechanisms attributed to prostaglandins have previously included enhanced epithelial restitution (6), and subepithelial myofibroblast contraction (7), increased resistance of tight junctions appears to provide an equally important method of rescuing barrier function. The mechanism by which prostaglandins regulate paracellular permeability is unknown, but may include alteration of tight junction structure or functional regulation via second messengers.

Since PGI_2 and PGE_2 reportedly stimulate increases in intracellular Ca^{2+} and cAMP (18), we considered the possibility that prostaglandins regulate tight junctions via second messengers. Synergistic increases in resistance with application of a calcium ionophore and cAMP were consistent with this hypothesis. In addition, PGE_2 directly stimulated increases in cAMP in porcine epithelial cells. While neither PGE_2 nor carbacyclin stimulated increases in $[\text{Ca}^{2+}]_i$ in isolated epithelial cells, the cholinergic nerve inhibitor atropine prevented synergism between PGE_2 and carbacyclin, suggesting that PGI_2 stimulates cholinergic nerves to trigger elevations in $[\text{Ca}^{2+}]_i$. Differences in magnitude of response between the prostaglandins and A23187 and cAMP were attributed to supraphysiologic concentrations of cAMP and Ca^{2+} that may result from treatment of tissues with a Ca^{2+} ionophore (9) and the cell soluble 8-bromo-cAMP.

Previous studies have shown that application of A23187 and cAMP to *Necturus* gallbladder increase transepithelial resistance associated with an increase in the numbers of tight junction filaments (10, 12). However, it is difficult to envision how tight junction strands could accumulate so rapidly. More recent studies suggest that the key site of tight junction regulation is the cytoskeleton-tight junction interface (20, 23–25). The cytoskeleton regulates tight junction resistance by actin fiber contraction (which opens tight junctions) or relaxation (which passively allows tight junctions to close) (20). Our experiments tend to support the hypothesis that prostaglandins, via the second messengers Ca^{2+} and cAMP, trigger closure of tight junctions via the cytoskeleton, since the actin-binding agent cytochalasin D inhibited prostaglandin-stimulated recovery of injured tissues. Previous studies have shown that application of cytochalasin D to epithelial monolayers reduces transepithelial resistance and increases transepithelial mannitol and inulin fluxes without disrupting epithelial monolayers (20). Alterations in transepithelial resistance were attributed to changes in tight junction permeability because of a strong correlation between Na^+ and mannitol fluxes. Our studies were complicated by the nature of the tissue (stripped tissue with secondary villous structure) and the presence of an epithelial defect requiring coverage by epithelial restitution. However, based on histologic evidence of near-maximal villous contraction and epithelial restitution at 60 min regardless

of treatment, we believe that the present studies suggest that prostaglandins induce closure of tight junctions as a mechanism of rescuing epithelial barrier function.

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