Differing Roles of Protein Kinase C-ζ in Disruption of Tight Junction Barrier by Enteropathogenic and Enterohemorrhagic *Escherichia coli*

FAROL L. TOMSON,* ATHANASIA KOUTSOURIS,* V. K. VISWANATHAN,* JERROLD R. TURNER,† SUZANA D. SAVKOVIC,* and GAIL HECHT*,§

*Department of Medicine, Section of Digestive Diseases and Nutrition, University of Illinois at Chicago, Chicago; †Department of Pathology, The University of Chicago, Chicago; and §University of Illinois and Chicago Veterans Affairs Healthcare System, West Side Branch, Chicago, Illinois

Background & Aims: **Enteropathogenic *Escherichia coli* and enterohemorrhagic *E. coli* harbor highly homologous pathogenicity islands yet show key differences in their mechanisms of action. Both disrupt host intestinal epithelial tight junctions, but the effects of enteropathogenic *E. coli* are more profound than those of enterohemorrhagic *E. coli*. The basis for this is not understood. The atypical protein kinase C isoform, protein kinase C-ζ, associates with and regulates the tight junction complex. The aim of this study was to compare the role of protein kinase C-ζ in the disruption of tight junctions after infection with enteropathogenic *E. coli* and enterohemorrhagic *E. coli*.** ***Methods:*** **Model intestinal epithelial monolayers infected by enteropathogenic *E. coli* or enterohemorrhagic *E. coli* were used for these studies.** ***Results:*** **Neither bisindolylmaleimide nor Gö6976, which block several protein kinase C isoforms but not protein kinase C–ζ, protected against the decrease in transepithelial electrical resistance after enteropathogenic *E. coli* infection. Rottlerin at concentrations that block novel and atypical isoforms, including protein kinase C–ζ, significantly attenuated the decrease in transepithelial electrical resistance. The specific inhibitory peptide, myristoylated protein kinase C–ζ pseudosubstrate, also significantly decreased the enteropathogenic *E. coli*–associated decrease in transepithelial electrical resistance and redistribution of tight junction proteins. In contrast to enteropathogenic *E. coli,* the level of protein kinase C–ζ enzyme activity stimulated by enterohemorrhagic *E. coli* was transient and minor, and protein kinase C–ζ inhibition had no effect on the decrease in transepithelial electrical resistance or the redistribution of occludin.** ***Conclusions:*** **The differential regulation of protein kinase C–ζ by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* may in part explain the less profound effect of the latter on the barrier function of tight junctions.**

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) are noninvasive enteric pathogens that cause acute watery diarrhea.[1] The mechanisms by which these pathogens cause disease, however, are not fully defined. Neither EPEC nor EHEC is invasive, but both interact in an intimate fashion with host intestinal epithelial cells via expression of virulence genes housed in their genetically similar pathogenicity islands, called the loci of enterocyte effacement (LEEs). Contained within these loci are genes that encode proteins involved in intimate attachment and type III secretion. One mechanism that contributes to diarrhea associated with EPEC and EHEC infection is disruption of the intestinal epithelial tight junction (TJ) barrier.[2] Although some strains of EHEC, but not EPEC, produce Shiga-like toxins, these toxins have no effect on TJs.[3] One signaling pathway used by both organisms to alter the TJ barrier is myosin light chain kinase (MLCK),[4,5] which phosphorylates the myosin light chain (MLC), thus inducing cytoskeletal contraction, including the perijunctional actomyosin ring. Contraction of this ring in response to both physiological[6] and pathologic[4,5,7] stimuli has been shown to perturb TJ permeability. Inhibition of MLCK with either pharmacological inhibitors[4,5] or cell-permeant inhibitory peptides[8] attenuates the decrease in transepithelial electrical resistance (TER), a physiological measurement of TJ permeability, in response to EPEC and EHEC.

In contrast, several differences in the effects of EPEC and EHEC have been described. For example, although both organisms alter TJs, the disruption inflicted by

Abbreviations used in this paper: A/E, attaching and effacing; BIM, bisindolylmaleimide; EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; LEE, locus of enterocyte effacement; MDCK, Madin-Darby Canine Kidney; MLC, myosin light chain; MLCK, myosin light chain kinase; MOI, multiplicity of infection; PIK, membrane-Permeant inhibitor of MLC kinase; PKC, protein kinase C; TER, transepithelial electrical resistance; Tir, translocated intimin receptor; TJ, tight junction.

0016-5085/04/$30.00
doi:10.1053/j.gastro.2004.06.014

EHEC occurs slower and to a lesser extent than that seen with EPEC.[9] Although the LEEs of EPEC and EHEC are 94% identical, transformation of nonpathogenic *E. coli* HB101 with the LEE of EPEC, but not of EHEC, confers the ability to perturb TJs.[10] Both pathogens insert their own receptor, translocated intimin receptor (Tir), into host cells, but EPEC Tir is subsequently phosphorylated and recruits the adapter molecule Nck, whereas EHEC Tir remains unphosphorylated and does not recruit Nck.[11–13] In addition, the adapter molecules Grb2 and CrkII are present in EPEC-induced attaching and effacing (A/E) lesions but absent from those formed in response to EHEC.[14] These molecules, via protein/protein interactions, may regulate downstream signaling events. Therefore, although EPEC and EHEC are genetically similar, significant differences exist in their pathogenic mechanisms.

TJs are composed of scaffolding proteins, signaling molecules, including protein kinase C (PKC)-ζ, and transmembrane proteins, namely occludin, and claudins, whose extracellular loops form the actual barrier. PKC has long been recognized as a regulator of TJs.[15–17] The number and expression of different PKC isoforms is cell-type specific; however, in epithelial cells, the atypical isoform PKC-ζ was the only isoform found at the TJs of Madin-Darby Canine Kidney (MDCK) and Caco-2 cells.[17] EPEC and EHEC have been shown to activate the conventional PKCs, specifically, PKC-α for EPEC.[4,18] It is interesting to note that the general PKC inhibitor staurosporine, which does not block PKC-ζ, had no protective effect on the perturbation of the TJ barrier by EPEC,[5] yet the staurosporine analogue CGP41251 provided some degree of protection against barrier disruption by EHEC.[4] The aim of this study was to investigate the role of the atypical isoform PKC-ζ in disruption of the TJ barrier by EPEC and EHEC.

Materials and Methods

Cell Culture

T_{84} cells (polarized human intestinal epithelial cells) were grown in a 1:1 (vol/vol) mixture of Dulbecco–Vogt modified Eagle medium (Invitrogen, Carlsbad, CA) and Hams F-12 (Invitrogen) with 6% newborn calf serum (Invitrogen) at 37°C in 5% CO_2.[10,19] Caco-2 cells were grown in high-glucose Dulbecco–Vogt modified Eagle medium supplemented with 10% fetal calf serum (Invitrogen) at 37°C in 5% CO_2.

Growth of Bacteria and Infection of Host Cells

The EPEC strain E2348/69 and the EHEC strain SE806/85-170, a derivative of O157, which does not express Shiga toxins, were used in these studies. Wild-type EPEC or EHEC was grown as previously described.[10] T_{84} or Caco-2 monolayers were infected with EPEC at a multiplicity of infection (MOI) of 100, but for EHEC they were infected at an MOI of 1000 to achieve equivalent numbers of adherent bacteria.[9] After 1 hour, medium was aspirated and replaced. Monolayers were incubated for additional time periods as indicated.[5,20,21]

Protein Kinase C Inhibition

Cells were treated for 1 hour before infection with select PKC inhibitors. The specific activities of these inhibitors on PKC isoforms in T_{84} cells have been published previously.[22] Gö6976 [(12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carazole); Calbiochem, La Jolla, CA] was used at 5 μmol/L. Bisindolylmaleimide [BIM; (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide, HCl; Calbiochem] was used at 5 μmol/L. Rottlerin (mallotoxin; Calbiochem) was used at 6 and 30 μmol/L. Myristoylated PKC-ζ pseudosubstrate (Calbiochem) was used at a concentration of 50 μmol/L. An N-terminal myristoylated pseudosubstrate sequence from PKC-α and PKC-β that is cell-membrane permeant (Calbiochem) was also used to inhibit these specific isoforms (8 μmol/L).

Electrophysiological Studies

TER was measured as previously described.[5,23]

Protein Fractionation, Extraction, and Immunoprecipitation

The cytosolic and membrane fractions were determined by using the procedures described by Song et al.[22] For immunoprecipitation, proteins were extracted,[10] and clarified whole-protein extracts (0.5 mg) were rotated for 2 hours at 4°C with 5.0 μg of anti-occludin or anti–PKC-ζ antibodies, followed by a 2-hour incubation at 4°C with protein A/Sepharose beads. Beads were washed 3 times with extraction buffer, and proteins were eluted by boiling for 10 minutes in sodium dodecyl sulfate sample buffer. Proteins (100 μg) were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting.[24]

Immunofluorescence Microscopy

Control and EHEC/EPEC-infected monolayers of T_{84} and Caco-2 cells were fixed on glass coverslips with 3.7% paraformaldehyde and then permeabilized with 0.2% Triton X-100 for 15 minutes. Cells were incubated with 2.5% bovine serum albumin for 1 hour and then with primary antibody against PKC-ζ, occludin, or zonula occludens-1 for 1 hour followed by rhodamine- or fluorescein isothiocyanate–conjugated secondary antibody for 1 hour. Monolayers were washed and mounted on glass microscope slides with Antifade reagent (Molecular Probes, Eugene, OR). Stained monolayers were visualized and photographed with a Nikon Opti-Phot inverted microscope equipped with the Spot-RT digital imaging system (Diagnostic Instruments, Sterling Heights, MI).

Protein Kinase C-ζ Activity Assays

PKC-ζ activity assays were preformed as previously described.[22]

Statistical Analysis

Data were analyzed with the Student *t* test for independent samples. Differences were considered significant if *P* was ≤0.05.

Results

Inhibition of atypical, but not conventional, PKCs protects against disruption of the TJ barrier by EPEC. EPEC activates the conventional isoform PKC-α,[18] but its pathophysiological role has not been explored. To determine whether conventional calcium-dependent PKC isoforms were involved in the disruption of the TJ as measured by TER, T_{84} monolayers were pretreated with Gö6976, which blocks only the α and βI isoforms. Gö6976 had no effect on the decrease in TER associated with infection by EPEC, as shown in Figure 1*A*. These data suggested that either a novel or an atypical PKC isoform is involved in the loss of barrier function. Staurosporine, a general inhibitor of PKCs (including βII), had no effect on disruption of the TJ barrier after EPEC infection.[5] Staurosporine, however, does not inhibit the atypical isoform PKC-ζ. In fact, BIM, which inhibits conventional PKC isoforms (α and βI), as well as the calcium-independent isoforms PKC-δ and -ϵ, but not -ζ, also afforded no protection against the EPEC-induced resistance decrease (Figure 1*A*). Rottlerin was originally shown to be isospecific for PKC-δ at low concentrations (50% inhibitory concentration, 3–6 μmol/L) but blocks other PKC isoforms (ϵ, ζ, and γ) at higher concentrations.[25] Figure 1*B* shows that high (30 μmol/L), but not low (6 μmol/L), concentrations of rottlerin protected the effect of EPEC on TER. Concentrations >30 μmol/L significantly decreased the TER of control monolayers (data not shown) and therefore could not be used. T_{84} cells have been reported to express only 5 of the 13 known PKC isoforms (α, βII, δ, ϵ, and ζ).[26] Subtractive analysis of the data obtained from these pharmacological inhibitors suggested that PKC-ζ might be involved. We therefore focused on the role of PKC-ζ in EPEC-induced disruption of TJs.

EPEC infection induces PKC-ζ translocation. PKC-ζ has been shown to localize to TJs[16] and to participate in the regulation of TJs.[27] EPEC infection disrupts both the structure and barrier function of TJs,[10,28] but the mechanisms and signaling pathways are not fully defined. We have previously reported that EPEC induces PKC-ζ translocation and activation in intestinal epithelial

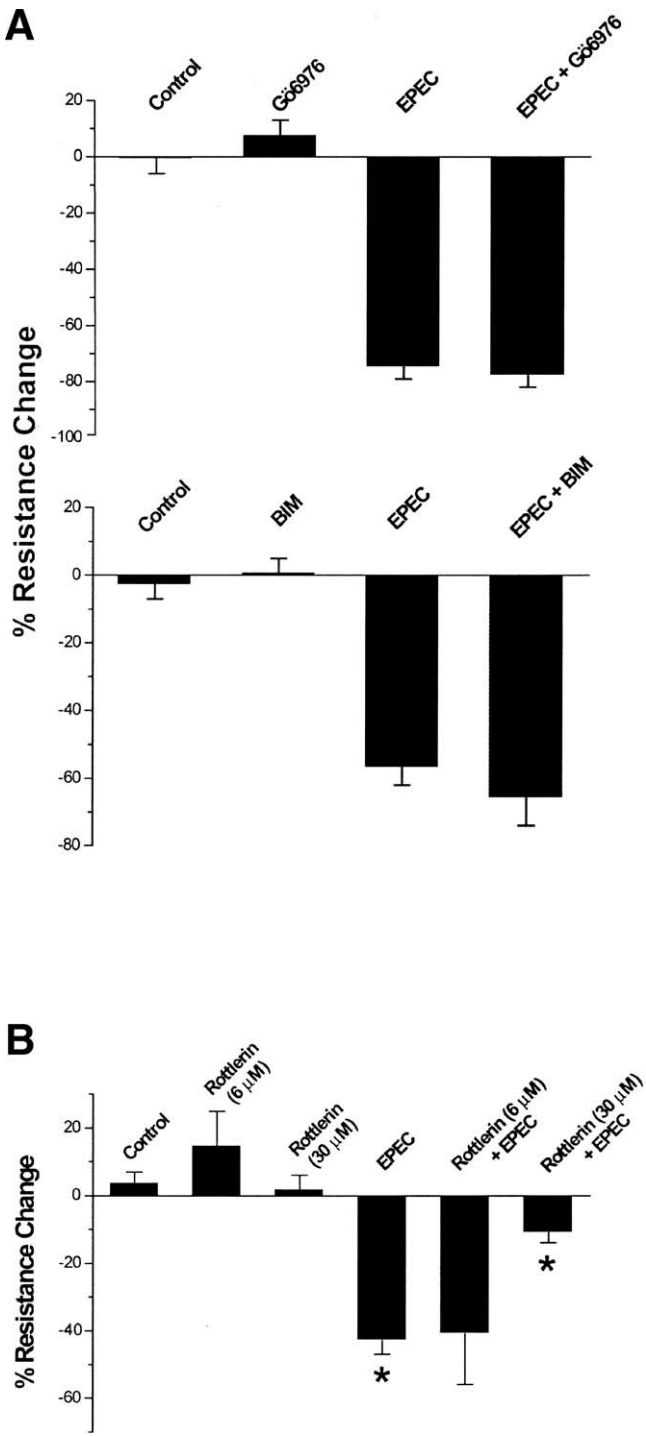

Figure 1. Conventional PKC inhibitors do not prevent EPEC disruption of the TJ barrier. (*A*) Inhibition of conventional PKC isoforms either by Gö6976 or by the general PKC inhibitor bisindolylmaleimide (BIM) was unable to attenuate the decrease in TER resulting from EPEC infection. T_{84} cells grown on Transwell (Corning Incorporated, Corning, NY) filters were infected with EPEC with and without Gö6976 or BIM, and TER was measured after 6 hours. The data represent mean ± SEM for data obtained at 6 hours after infection (n = 6–9 for each group). (*B*) Rottlerin 6 μmol/L had no effect on EPEC-induced decreases in TER, yet 30 μmol/L significantly diminished the decrease in TER resulting from EPEC infection. Data represent mean ± SEM (n = 6–11 for each group; *P* = 0.02).

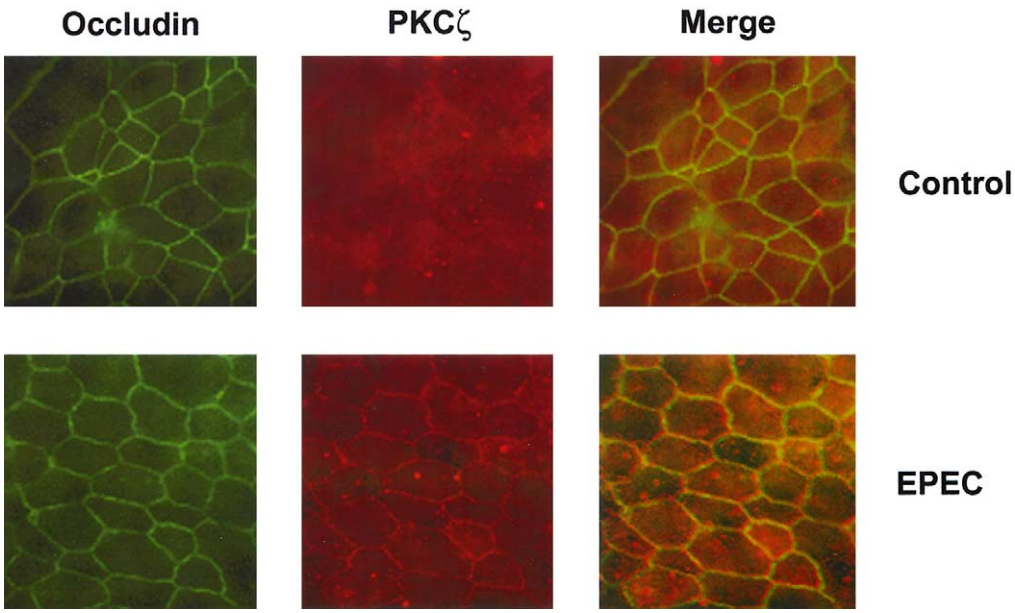

Figure 2. EPEC infection induces PKC-ζ translocation. T_{84} monolayers were infected with EPEC for 1 hour and then dual-stained for PKC-ζ and occludin. Panels in the left column were stained with anti-occludin antibody (*green*), those in the middle column were stained with an anti–PKC-ζ antibody (*red*), and the panels in the right column show the overlay of the 2 images. In control monolayers, occludin is primarily localized to the periphery of the cells, and PKC-ζ is primarily situated in the cytoplasm, with some residing at the membrane. After 1 hour of infection with EPEC, the distribution of occludin was essentially unchanged, but a significant portion of PKC-ζ had translocated to the membrane, as evidenced by its colocalization with occludin (right panel). Similar changes were seen at 30 and 120 minutes after infection (not shown).

cells.[29] To assess whether membrane-associated PKC-ζ colocalized with TJs, dual immunofluorescent staining for PKC-ζ and occludin was performed (Figure 2). In uninfected monolayers, occludin staining is limited to TJs, whereas PKC-ζ is primarily cytoplasmic. Although some PKC-ζ associates with TJs under control conditions,[17] at 1 hour after infection there is a clear shift of PKC-ζ to the periphery of the cell, correlating with membrane translocation.[29] Occludin (green) and PKC-ζ (red) colocalization is shown by the merging of these images (yellow). PKC-ζ has been reported to bind occludin at its coil/coil domain[30] and to regulate its phosphorylation and localization.[31]

To determine whether EPEC infection altered the interaction between occludin and PKC-ζ, coimmunoprecipitation studies were performed. PKC-ζ was immunoprecipitated from uninfected monolayers and those infected with EPEC for 30, 60, and 120 minutes. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then immunoblotted for occludin and PKC-ζ. When expressed as a ratio of immunoprecipitated PKC-ζ, there was no significant increase in the amount of PKC-ζ–associated occludin after EPEC infection (data not shown), thus suggesting that the colocalization seen was not due to direct protein/protein interactions.

PKC-ζ inhibition with myristoylated PKC-ζ pseudosubstrate attenuates the EPEC-induced decrease in TER. Although general pharmacological inhibitors are useful in the study of signaling events, specific inhibitory peptides for individual PKC isoforms have recently become available. To selectively examine the effect of PKC-ζ inhibition on EPEC-induced alteration of the TJ barrier, monolayers were pretreated with a myristoylated PKC-ζ pseudosubstrate. We recently showed that this inhibitory pseudosubstrate significantly reduced the activation of PKC-ζ by EPEC.[29] This inhibitory peptide diminished the translocation of PKC-ζ to the membrane after EPEC infection, as shown in Figure 3*A*.

Incubation of uninfected monolayers with the PKC-ζ inhibitory pseudosubstrate had no effect on TER. In contrast, this inhibitory peptide significantly attenuated the decrease in TER after EPEC infection (Figure 3*B*). Protection was observed at 4 hours after infection, and although the protective trend was still evident at 6 hours, the data were not statistically significant (data not shown). These experiments were also performed in Caco-2 cells and yielded similar results. We have noted that Caco-2 cells have a more rapid response to the effects of EPEC than do T_{84} cells (data not shown). In Caco-2 monolayers, a significant decrease in TER occurred as early as 2 hours ($-21\% \pm 6\%$) and was significantly attenuated by the PKC-ζ pseudosubstrate ($-9\% \pm 6\%$; $P = 0.03$; n = 6–9). No protection was seen at 4 hours, similar to the trend seen in T_{84} cells. To explore the specificity of this response, a myristoylated PKC-α/β

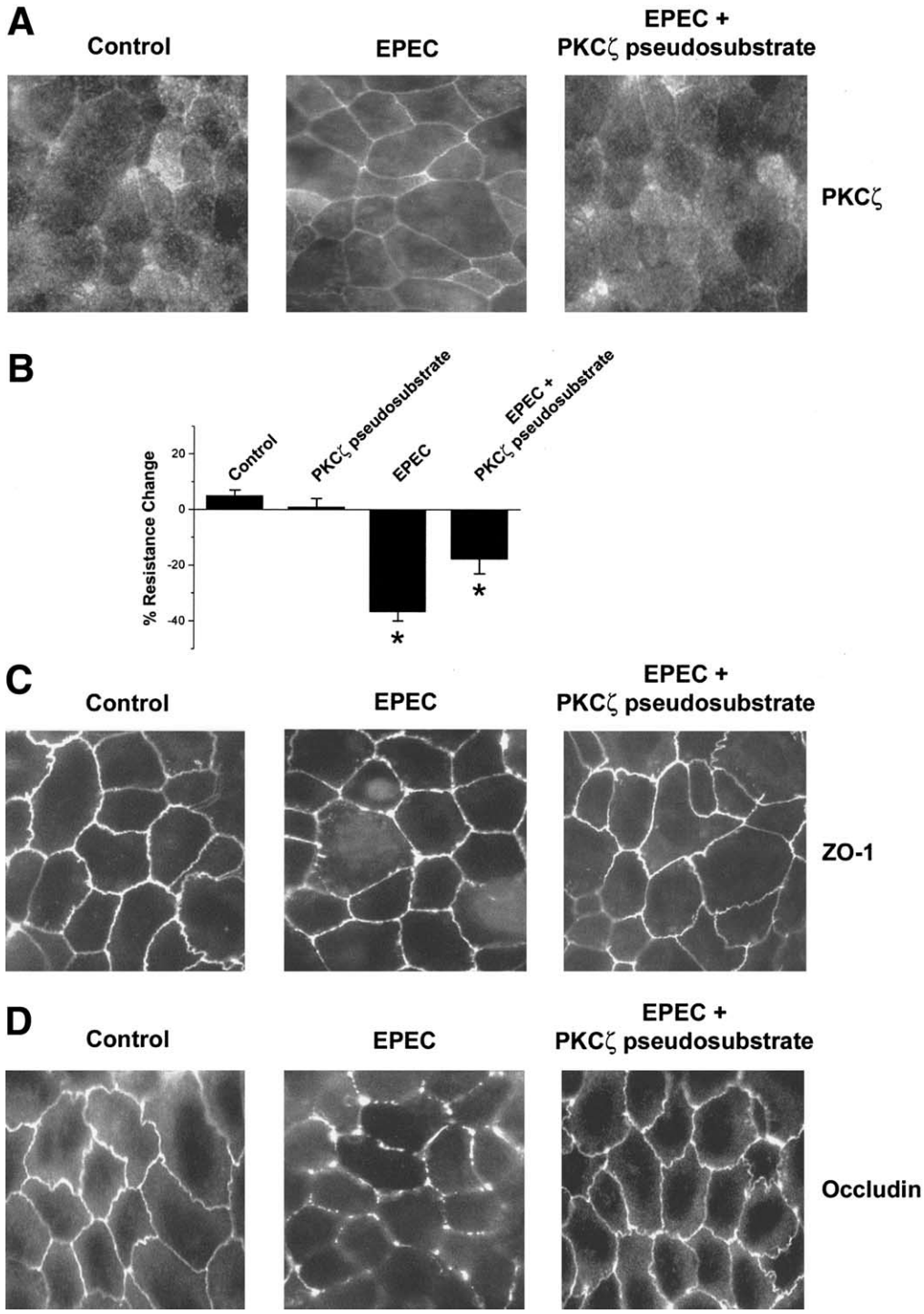

Figure 3. Specific inhibition of PKC-ζ with a myristoylated PKC-ζ pseudosubstrate prevents EPEC-induced translocation of PKC-ζ, a decrease in TER, and relocalization of TJ proteins. (*A*) EPEC-infected T_{84} monolayers in the absence and presence of a PKC-ζ pseudosubstrate were stained for PKC-ζ. In control monolayers, most PKC-ζ was cytoplasmic, although some PKC-ζ can be seen at the membrane. EPEC infection causes most PKC-ζ to shift to the membrane. Treatment with the inhibitory PKC-ζ pseudosubstrate significantly attenuated EPEC-induced PKC-ζ translocation. (*B*) Treatment with PKC-ζ pseudosubstrate before infection with EPEC significantly attenuated the EPEC-induced decrease in TER at 4 hours ($P = 0.02$). The data represent mean ± SEM (n = 6–9 for each group) from 3 separate experiments. (*C*) In uninfected monolayers, zonula occludens (ZO)-1 localizes primarily to the area of the TJ. After EPEC infection, ZO-1 staining is irregular, with beading and small discontinuities. ZO-1 rearrangement is prevented by the PKC-ζ inhibitory pseudosubstrate. (*D*) Similar protection against the redistribution of occludin by EPEC is seen with the PKC-ζ pseudosubstrate.

pseudosubstrate was also tested. In contrast to the protective effect of the PKC-ζ pseudosubstrate, the PKC-α/β pseudosubstrate had no effect on the EPEC-induced decrease in TER (−53% ± 3% vs. −52% ± 5% change in TER for EPEC and EPEC plus PKC-α/β pseudosubstrate, respectively; n = 6; $P = 0.85$). These results indicate that a PKC-ζ–dependent pathway is involved at least in the early phases of barrier disruption associated with EPEC infection.

We previously reported that EPEC alters the structure and function effects of TJs and that these changes correlate temporally.[10] We therefore queried whether inhibition of PKC-ζ prevented the redistribution of zonula occludens-1 or occludin. As shown in Figure 3*C* and *D*, the PKC-ζ pseudosubstrate prevented the redistribution of both zonula occludens-1 and occludin.

EHEC induces translocation but only minimal activation of PKC-ζ. Both EPEC and EHEC increase TJ per-

meability, but the kinetics differ greatly.[9] Because EHEC attaches less efficiently than EPEC to host cells, we increased the MOI for EHEC by 10-fold and thus achieved equivalent numbers of attached bacteria ($1.5 \times 10^7 \pm 4.6 \times 10^6$ vs. $1.3 \times 10^7 \pm 4.4 \times 10^6$ for EHEC and EPEC, respectively, at 6 hours). Despite equal numbers of attached organisms, the decrease in TER by EHEC ($-51\% \pm 0.7\%$) was still significantly less than that associated with EPEC infection ($-88\% \pm 2.3\%$; $P < 0.005$; n = 3). Therefore, the higher MOI was used for EHEC to negate any differences in attachment.

As shown in Figure 4*A*, the level of PKC-ζ remained constant over the course of infection, as was previously published for EPEC.[29] Cell fractionation studies to assess EHEC-induced PKC-ζ translocation[29] showed a trend for PKC-ζ translocation at 1–2 hours after infection, but the data were not statistically significant (Figure 4*B*). When assessed by immunofluorescence, however, EHEC did seem to cause PKC-ζ translocation (Figure 4*C*), as was seen with EPEC. Although immunofluorescence microscopy suggested colocalization with occludin (yellow), coimmunoprecipitation studies for PKC-ζ and occludin showed no increased association (data not shown). The effect of EHEC infection on PKC-ζ activity was determined by performing kinase activity assays with myelin basic protein as a substrate. Only a slight increase in PKC-ζ activity was seen at 30 minutes after infection, and it was not sustained (Figure 4*D*). In contrast, EPEC significantly increased PKC-ζ activity as early as 15 minutes, reaching a peak activation of 3.0-fold at 30 minutes that was sustained for at least 1 hour.[29]

Inhibition of neither conventional PKCs nor PKC-ζ prevents EHEC-induced barrier alterations. As was found for EPEC, neither Gö6976 nor BIM blocked the EHEC-induced decrease in TER (Figure 5*A*). To investigate whether PKC-ζ participated in the barrier disruption by EHEC, T_{84} monolayers were treated with rottlerin or the PKC-ζ pseudosubstrate. In contrast to EPEC, neither of these inhibitors altered the decrease in TER caused by EHEC. In Caco-2 cells, the decrease in TER in response to EHEC was not significant at either 2 or 4 hours, consistent with our observation of slower kinetics.[9] Therefore, the effect of the PKC-ζ pseudosubstrate could not be assessed at these early times. By 6 hours, TER had decreased significantly ($-32\% \pm 2\%$), and the PKC-ζ pseudosubstrate was not protective ($-29\% \pm 12\%$; $P = 0.7$; n = 6–9). This could be due to a lack of effect of the pseudosubstrate at this later time point, as was observed in EPEC-infected T_{84} monolayers, or to the lack of involvement of PKC-ζ in EHEC-induced perturbation of TJs. In support of the latter conclusion, the inhibitory pseudosubstrate also failed to prevent the EHEC-induced changes in occludin in Caco-2 monolayers at 3 hours (Figure 5*B*), thus suggesting that PKC-ζ is not involved in the disruption of TJs by EHEC.

EPEC-activated PKC-ζ and MLCK work through the same pathway to alter TJs. We have previously shown that EPEC in part disrupts the TJ barrier through the activation of MLCK.[5] Having shown that PKC-ζ also participates in this process, we questioned whether these signaling pathways are linked or function independently. To address this question, MLCK was blocked with the cell-permeant inhibitory peptide membrane-Permeant inhibitor of MLC kinase (PIK).[8] As shown in Figure 6*A*, although PIK prevented the redistribution of occludin after EPEC infection, it had no effect on PKC-ζ translocation, thus suggesting either that these 2 signaling pathways work by distinct mechanisms or that the influence of PKC-ζ is via MLCK or MLC, because inhibition of PKC-ζ also protected against the decrease in TER and the redistribution of occludin. We further tested the relationship of these pathways by examining the combined effects of the MLCK and PKC-ζ inhibitors. For these studies, monolayers were treated with PIK alone or with a constant dose of the PKC-ζ pseudosubstrate in the presence of increasing concentrations of PIK (30, 100, and 300 μmol/L) before infection with EPEC. As shown in Figure 6*B*, the addition of PKC-ζ pseudosubstrate to PIK afforded no additional protection compared with PIK alone. These data suggest that PKC-ζ intersects the MLCK pathway at a proximal point, or MLC directly, leading to diminished TER. Previous reports have shown a role for PKC in MLC phosphorylation.[32]

Discussion

A high degree of homology exists between the LEEs of EPEC and EHEC. Encoded on the LEE are proteins comprising and secreted by a type III secretory apparatus.[33] Significant identity exists for genes encoding the structural components of the type III secretory apparatus of EPEC and EHEC.[34] More variability is seen, however, within the secreted and effector molecules, in particular the Tir that serves as a receptor for the outer membrane adhesin, intimin. EPEC Tir contains a C-terminal tyrosine residue (Y474) that is phosphorylated after delivery into host cells and is critical for pedestal formation and recruitment of the adapter molecule Nck and subsequently Wiskott-Aldrich syndrome protein (WASP) and Arp2/3, which nucleate actin. EHEC Tir, in contrast, harbors a serine residue at this site that is not phosphorylated and fails to bind Nck.[11–13] EHEC nonetheless recruits numerous cytoskeletal proteins to the

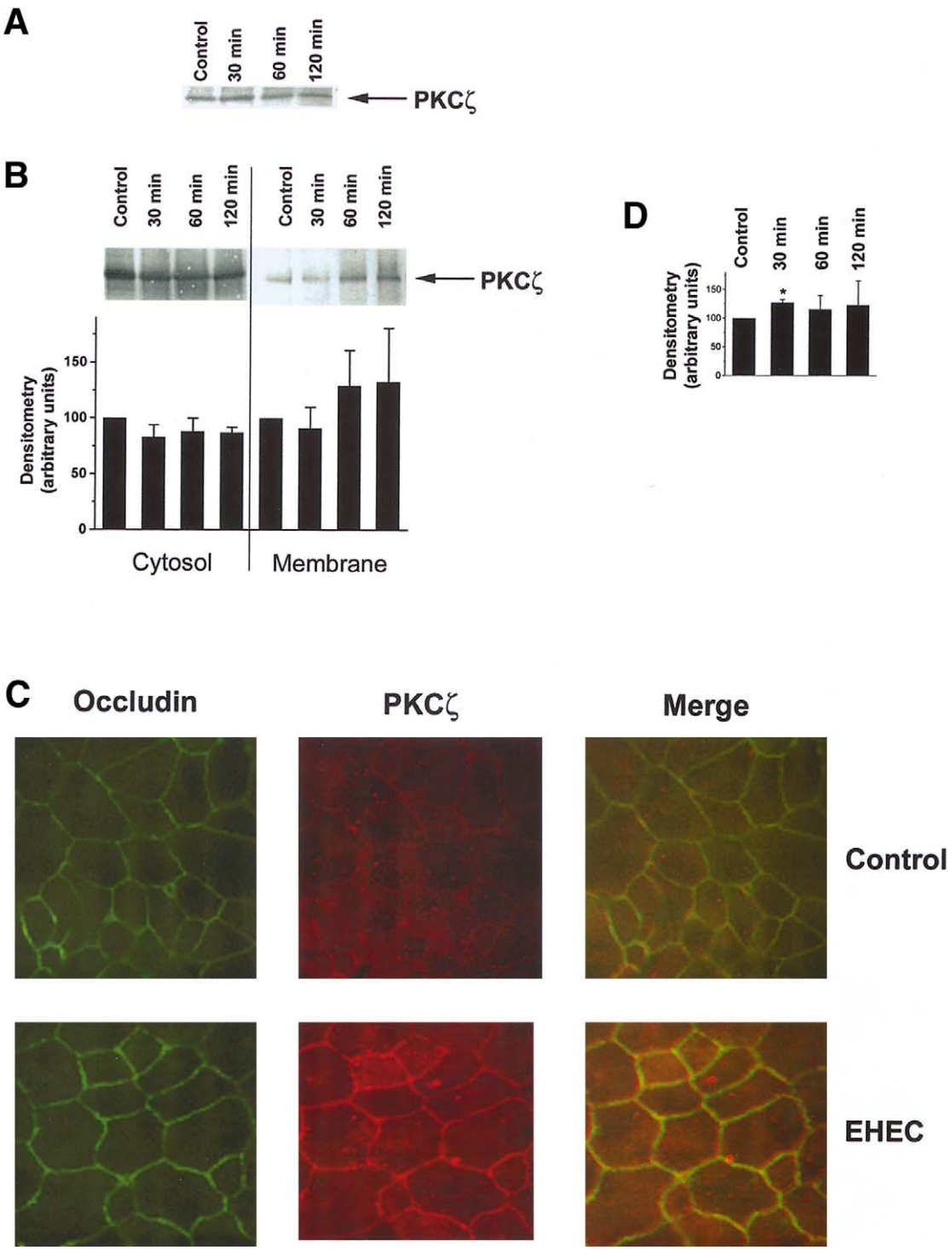

Figure 4. EHEC infection has no effect on PKC-ζ expression and minimal effect on PKC-ζ translocation and activation. (*A*) T_{84} cells were infected with EHEC, and proteins were extracted at the indicated times. As shown in this representative immunoblot, EHEC infection did not alter the level of PKC-ζ expression. (*B*) Cytosolic and membrane extracts from control T_{84} cells and after 30, 60, and 120 minutes of EHEC infection were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted for PKC-ζ. Although a trend toward membrane translocation is suggested, densitometric analysis of 4 independent experiments showed no significant change in the amount of cytosolic or membrane-associated PKC-ζ after EHEC infection. (*C*) EHEC-induced translocation was also assessed by dual-label immunofluorescent staining of occludin and PKC-ζ after 1 hour of EHEC infection. The panels in the left column represent occludin (*green*), the middle column represents PKC-ζ (*red*), and the right panels show the overlays of the 2 images. Occludin is primarily localized to the periphery of the cells (*left panel*), and PKC-ζ is located in the cytoplasm, with some residing at the membrane (*middle panel*). After 1 hour of EHEC infection, the distribution of occludin is unchanged, but PKC-ζ has shifted to the periphery of the cells. The merged images suggest that these 2 proteins localize to similar areas (*right panel*). These changes were also seen at 30 and 120 minutes after infection (not shown). (*D*) Cellular extracts from uninfected monolayers and those infected with EHEC for 30 minutes and 1 and 2 hours were immunoprecipitated for PKC-ζ and reacted with myelin basic protein in the presence of [γ-^{32}P]adenosine triphosphate. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography was performed and analyzed by densitometry. Analysis of 3 independent experiments showed a small but significant increase ($P = 0.01$) in PKC-ζ activity only at 30 minutes after infection.

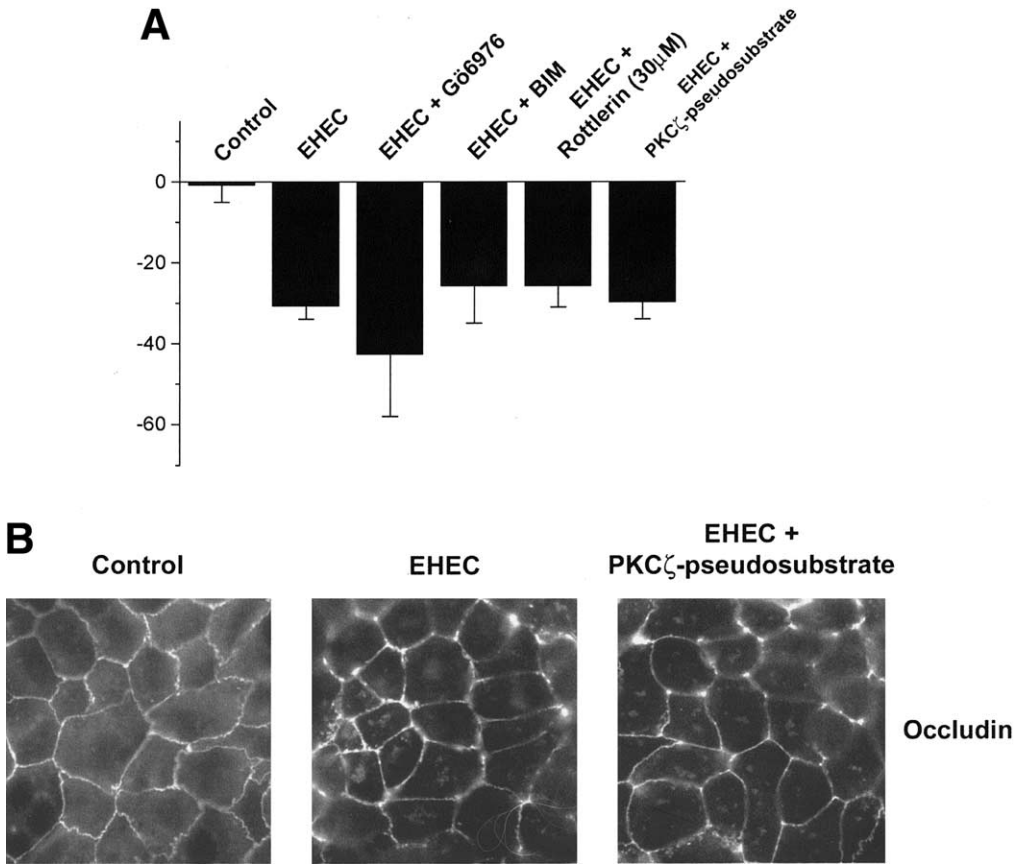

Figure 5. Inhibition of PKC does not alter the EHEC-induced decrease in TER or the alterations in occludin localization. (*A*) T_{84} monolayers were pretreated with Gö6976, bisindolylmaleimide (BIM), rottlerin, or PKC-ζ pseudosubstrate. Neither general inhibitors of PKC nor specific PKC-ζ inhibitors protected against the EHEC-induced decrease in TER. Data represent mean ± SEM (n = 6–9 for each group; $P > 0.05$). (*B*) PKC-ζ pseudosubstrate did not prevent changes in occludin after 3 hours of EHEC infection.

A/E lesion and induces pedestal formation, presumably through the use of an accessory factor possibly outside of the LEE. This is supported by the fact that transformation of the LEE of EPEC,[35] but not EHEC,[36] into K12 nonpathogenic *E. coli* conferred the ability to form A/E lesions in cultured cells, again indicating a role in EHEC for non-LEE factors in this process. The variations between EPEC and EHEC suggest that different signaling pathways may be activated in host cells after infection by the 2 pathogens. Supporting this possibility is the finding that EHEC, in contrast to EPEC, fails to recruit 2 adapter molecules to the A/E lesion: Grb2 and CrkII.[14] These proteins contain SH2 and SH3 domains that mediate protein/protein interactions and thus influence signaling events. The ultimate effect of these differences has not been defined but could account for some of the variability in physiological responses by the intestinal epithelium to these similar pathogens.

Adherence of EHEC to intestinal cells is less than that of EPEC. We were able to overcome this variable by increasing the MOI of EHEC. Despite equal numbers of adherent organisms, EHEC still had a lesser effect than EPEC on TJ permeability. We therefore compared the effect of these related pathogens on signaling pathways that could potentially regulate the TJ barrier. The role of PKC-ζ in TJ physiology is a topic currently under intense investigation. Several studies have shown a role for PKC-ζ in the formation, but not the maintenance, of TJs.[15,16] Inhibition of atypical PKCs with either pharmacological inhibitors or expression of dominant negative constructs retarded the reformation of the TJ barrier and of cell polarity after their disruption by calcium switch.[15,37] In contrast, these perturbations had no effect on the barrier function or the cell polarity of established monolayers. Our data confirm the latter finding by showing that PKC-ζ inhibition with either enzyme inhibitors or the myristoylated PKC-ζ pseudosubstrate had no effect on the TER of intestinal epithelial monolayers. It is interesting to note that, however, inhibition of PKC-ζ protected against disruption of the TJ barrier after EPEC, but not EHEC, infection. The lack of a protective response in EHEC-infected monolayers, as opposed to significant protection in those infected with EPEC, may be explained by the difference in the degree of enzyme activation.

Although it is possible that other PKC isoforms may also be involved in EPEC pathogenesis, T_{84} cells have been reported to express only 5 of the 13 identified isoforms (α, βII, δ, ϵ, and ζ).[26] Although PKC inhibitors are not entirely selective, previous publications regarding

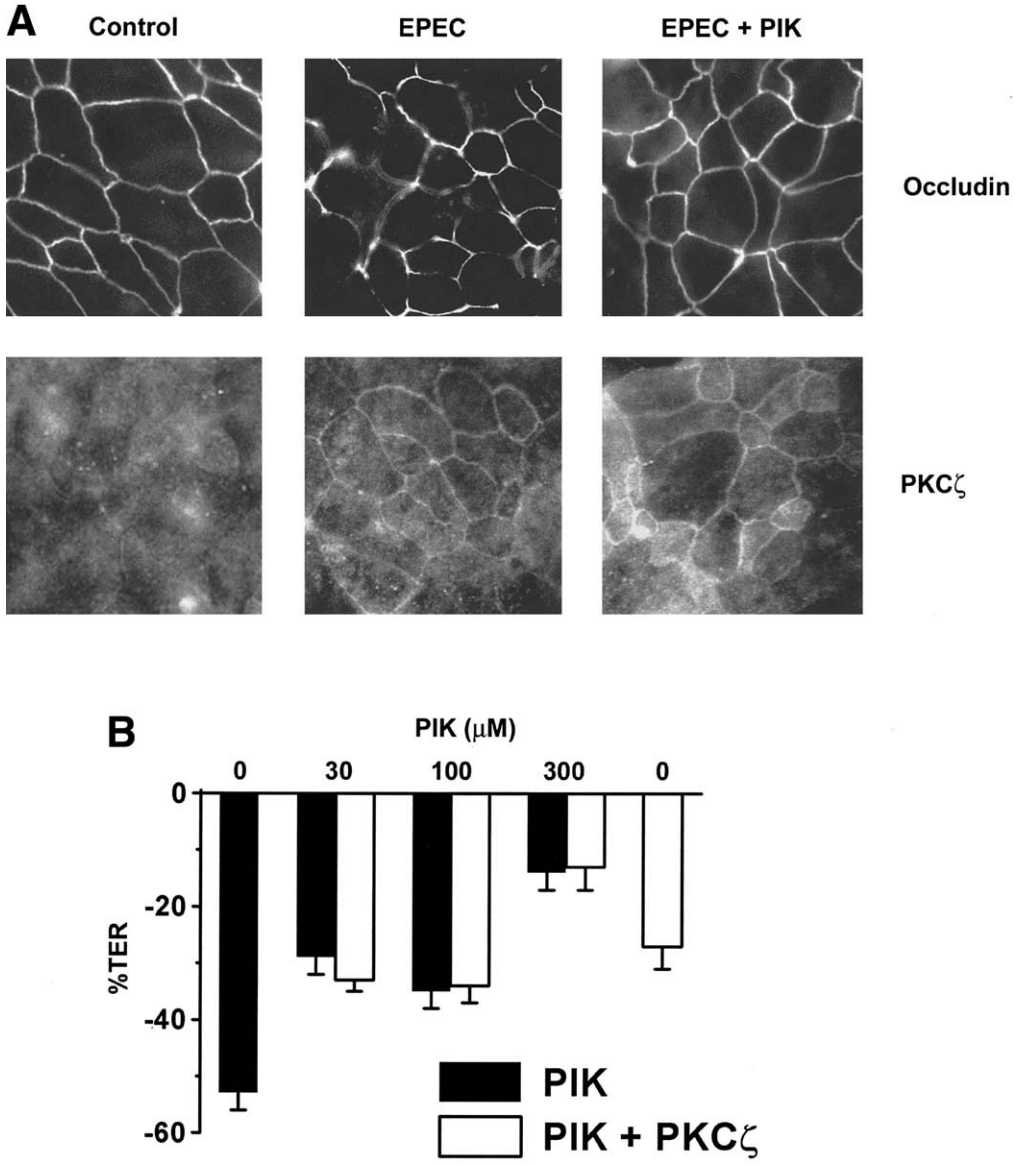

Figure 6. (*A*) Inhibition of MLCK with PIK protected against EPEC-induced changes in occludin but not PKC-ζ translocation. Monolayers were infected with EPEC in the absence and presence of the MLCK inhibitor PIK and stained for occludin and PKC-ζ. PIK inhibited the rearrangement of occludin after EPEC infection. In contrast, PIK did not alter EPEC-induced translocation of PKC-ζ, thus suggesting that these EPEC-activated signaling pathways independently affect TJs. (*B*) The inhibition of both MLCK and PKC-ζ had no additive protection against the EPEC-induced decrease in TER over MLCK inhibition alone. Monolayers were treated with either PIK alone (30, 100, or 300 μmol/L) or together with PKC-ζ pseudosubstrate (50 μmol/L), and TER was measured after 4 hours of EPEC infection. Similarly, the effects of 30 and 100 μmol/L PIK plus PKC-ζ pseudosubstrate, as compared with PKC-ζ pseudosubstrate alone, were not significant ($P = 0.21$ and 0.22, respectively; n = 5–6). These data suggest that these signaling molecules act through the same pathway.

the profile of the inhibitors used in T_{84} cells provide a firm foundation for our conclusions.[22,26] The protective effects of the PKC-ζ inhibitory pseudosubstrate and the lack of protection by the PKC-α/β pseudosubstrate provide the strongest support for our argument that PKC-ζ is essential for barrier disruption due to EPEC infection.

The exact way in which protection is rendered is not known, but it is interesting to point out that overexpression of active PKC-λ, which can act as a PKC-ζ substitute, disrupted the cellular polarity of confluent MDCK monolayers.[15] Corresponding TER data were not provided. We have recently published that EPEC also disrupts cell polarity, which may contribute to pathogenesis by allowing the EPEC adhesin, intimin, access to a cellular binding partner, β1 integrin, which is typically restricted to the basolateral domain.[38] Together these data suggest that atypical PKCs may have dual roles with regard to TJ function and cell polarity. Although PKC-ζ has been found to associate with the TJ transmembrane protein occludin,[30] neither EPEC nor EHEC infection significantly enhanced the association of these proteins. In addition to occludin, PKC-ζ has been shown to complex in mammalian epithelial cells (MDCK) with atypical PKC isotype-specific interacting protein (ASIP)/partitioning-defective protein (PAR-3), PAR-6,[37] and 14-3-3,[39] which is crucial for the establishment of cell polarity. The effects of EPEC on this polarity complex have not been examined.

Having shown that EPEC disrupts both the structure and the function of intestinal epithelial TJs, our laboratory has focused on understanding the mechanisms by which this pathophysiological event occurs. On the basis

of the data presented here and in previous studies, we are able to construct a temporal sequence with which EPEC-initiated events occur. Of course, one caveat is that these studies were performed with cell lines and not animal models. We believe that the relevance of this in vitro model system is supported by the fact that the cell lines are of human origin and are intestinal epithelia. Both EPEC and EHEC are human pathogens, and as a result, ideal animal models do not exist. In addition, other A/E family members, such as the mouse pathogen *Citrobacter rodentium,* show significant differences in infection compared with EPEC (data not shown); therefore, findings obtained in *C. rodentium*–infected mice cannot simply be extrapolated to EPEC and EHEC infection of humans. Nonetheless, in our in vitro model of EPEC infection, A/E lesion formation occurs within 15–30 minutes, followed by the activation of signaling pathways.[24] One early event triggered by an unidentified signal is the recruitment of the membrane/cytoskeleton linker protein, ezrin, to A/E lesions. Ezrin is threonine phosphorylated and activated in a type III secretion system–dependent manner and then participates in the cross-talk between EPEC and host cells that results in disruption of the TJ barrier, as shown by overexpression of dominant negative ezrin.[24] Although PKC-ζ activity increases at 15 minutes after infection,[29] ezrin has not been identified as a PKC-ζ substrate. PKC-α, however, can phosphorylate ezrin.[40] Between 1 and 2 hours after infection, MLC is also phosphorylated,[5] an event indicative of perijunctional actomyosin ring contraction, one mechanism that increases TJ permeability.[6] Inhibition of MLCK affords partial protection against EPEC-induced barrier disruption.[5,8] PKC-ζ inhibition has similar effects. The finding that inhibition of MLCK did not block the translocation of PKC-ζ after EPEC infection suggests either that these 2 pathways independently affect the TJ barrier or that PKC-ζ acts proximally in the MLCK pathway. The studies examining the effect of blocking both pathways suggest that the latter explanation is the case. The interplay between PKC and MLC phosphorylation is complex[32]: direct and indirect interactions have been described. Activation of PKC by phorbol esters has been shown to influence the MLCK/MLC pathway at several levels. PKC can phosphorylate MLC at sites distinct from MLCK; phosphorylate MLCK, reducing its affinity for calmodulin; and phosphorylate myosin Ser/Thr phosphatase 1, leading to inhibition of its activity.[32] Further complicating the dissection of these interactions is that they are cell specific. Nevertheless, our data suggest that EPEC-activated PKC-ζ and MLCK participate in the same signaling cascade that eventually leads to disruption of TJ structure and function.

In contrast to EPEC, EHEC has only minimal effects on PKC-ζ activity but, like EPEC, stimulates MLCK, which participates in perturbation of the TJ barrier.[4] Whether the lack of involvement of PKC-ζ in EHEC-induced changes in TJs is at least in part responsible for the slower kinetics as compared with EPEC is not known. Because PKC-ζ has also been shown to participate in activation of the inflammatory response induced by EPEC,[29] it is possible that PKC-ζ plays some role in the inflammatory response associated with EHEC. Continued comparisons of the related pathogens EPEC and EHEC will undoubtedly uncover additional variations in their mechanisms of pathogenesis.

References

1. Frankel G, Phillips AD, Rosenshine I, Dougan G, Kaper JB, Knutton S. Enteropathogenic and enterohaemorrhagic *Escherichia coli:* more subversive elements. Mol Microbiol 1998;30:911–921.
2. Hecht G. Microbes and microbial toxins: paradigms for microbial-mucosal interactions. VII. Enteropathogenic *Escherichia coli:* physiological alterations from an extracellular position. Am J Physiol Gastrointest Liver Physiol 2001;281:G1–G7.
3. Acheson DW, Moore R, De Breucker S, Lincicome L, Jacewicz M, Skutelsky E, Keusch GT. Translocation of Shiga toxin across polarized intestinal cells in tissue culture. Infect Immun 1996; 64:3294–3300.
4. Philpott DJ, McKay DM, Mak W, Perdue MH, Sherman PM. Signal transduction pathways involved in enterohemorrhagic *Escherichia coli*-induced alterations in T84 epithelial permeability. Infect Immun 1998;66:1680–1687.
5. Yuhan R, Koutsouris A, Savkovic SD, Hecht G. Enteropathogenic *Escherichia coli*-induced myosin light chain phosphorylation alters intestinal epithelial permeability. Gastroenterology 1997; 113:1873–1882.
6. Turner JR, Rill BK, Carlson SL, Carnes D, Kerner R, Mrsny RJ, Madara JL. Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. Am J Physiol 1997;273:C1378–C1385.
7. Scott KG, Meddings JB, Kirk DR, Lees-Miller SP, Buret AG. Intestinal infection with *Giardia spp.* reduces epithelial barrier function in a myosin light chain kinase-dependent fashion. Gastroenterology 2002;123:1179–1190.
8. Zolotarevsky Y, Hecht G, Koutsouris A, Gonzalez DE, Quan C, Tom J, Mrsny RJ, Turner JR. A membrane-permeant peptide that inhibits MLC kinase restores barrier function in in vitro models of intestinal disease. Gastroenterology 2002;123:163–172.
9. Viswanathan VK, Koutsouris A, Lukic S, Pilkinton M, Simonovic I, Simonovic M, Hecht G. Comparative analysis of EspF from enteropathogenic and enterohemorrhagic *Escherichia coli* in alteration of epithelial barrier function. Infect Immun 2004;72:3218–3227.
10. Simonovic I, Rosenberg J, Koutsouris A, Hecht G. Enteropathogenic *Escherichia coli* dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. Cell Microbiol 2000; 2:305–315.
11. DeVinney R, Stein M, Reinscheid D, Abe A, Ruschkowski S, Finlay BB. Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. Infect Immun 1999;67:2389–2398.
12. Kenny B. Phosphorylation of tyrosine 474 of the enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. Mol Microbiol 1999;31:1229–1241.

13. Gruenheid S, DeVinney R, Bladt F, Goosney D, Gelkop S, Gish GD, Pawson T, Finlay BB. Enteropathogenic *E. coli* Tir binds Nck to initiate actin pedestal formation in host cells. Nat Cell Biol 2001;3:856–859.
14. Goosney DL, DeVinney R, Finlay BB. Recruitment of cytoskeletal and signaling proteins to enteropathogenic and enterohemorrhagic *Escherichia coli* pedestals. Infect Immun 2001;69:3315–3322.
15. Suzuki A, Yamanaka T, Hirose T, Manabe N, Mizuno K, Shimizu M, Akimoto K, Izumi Y, Ohnishi T, Ohno S. Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures. J Cell Biol 2001;152:1183–1196.
16. Stuart RO, Nigam SK. Regulated assembly of tight junctions by protein kinase C. Proc Natl Acad Sci U S A 1995;92:6072–6076.
17. Dodane V, Kachar B. Identification of isoforms of G proteins and PKC that colocalize with tight junctions. J Membr Biol 1996;149:199–209.
18. Crane JK, Oh JS. Activation of host cell protein kinase C by enteropathogenic *Escherichia coli*. Infect Immun 1997;65:3277–3285.
19. Madara JL, Stafford J, Dharmsathaphorn K, Carlson S. Structural analysis of a human intestinal epithelial cell line. Gastroenterology 1987;92:1133–1145.
20. Savkovic SD, Koutsouris A, Hecht G. Activation of NF-kappaB in intestinal epithelial cells by enteropathogenic *Escherichia coli*. Am J Physiol 1997;273:C1160–C1167.
21. Hecht G, Marrero JA, Danilkovich A, Matkowskyj KA, Savkovic SD, Koutsouris A, Benya RV. Pathogenic *Escherichia coli* increase Cl^- secretion from intestinal epithelia by upregulating galanin-1 receptor expression. J Clin Invest 1999;104:253–262.
22. Song JC, Hanson CM, Tsai V, Farokhzad OC, Lotz M, Matthews JB. Regulation of epithelial transport and barrier function by distinct protein kinase C isoforms. Am J Physiol Cell Physiol 2001;281:C649–C661.
23. Madara JL, Colgan SP, Nusrat A, Delp C, Parkos CA. A simple approach to measurement of electrical parameters of cultured epithelial monolayers: use in assessing neutrophil-epithelial monolayers. J Tissue Cult Methods 1992;14:209–216.
24. Simonovic I, Arpin M, Koutsouris A, Falk-Krzesinski HJ, Hecht G. Enteropathogenic *Escherichia coli* activates ezrin, which participates in disruption of tight junction barrier function. Infect Immun 2001;69:5679–5688.
25. Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F. Rottlerin, a novel protein kinase inhibitor. Biochem Biophys Res Commun 1994;199:93–98.
26. Silva M, Song C, Nadeau WJ, Matthews JB, McCormick BA. *Salmonella typhimurium* SipA-induced neutrophil transepithelial migration: involvement of a PKC-α-dependent signal transduction pathway. Am J Physiol Gastrointest Liver Physiol 2004;286:G1024–G1031.
27. Izumi Y, Hirose T, Tamai Y, Hirai S, Nagashima Y, Fujimoto T, Tabuse Y, Kemphues KJ, Ohno S. An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of *Caenorhabditis elegans* polarity protein PAR-3. J Cell Biol 1998;143:95–106.
28. Spitz J, Yuhan R, Koutsouris A, Blatt C, Alverdy J, Hecht G. Enteropathogenic *Escherichia coli* adherence to intestinal epithelial monolayers diminishes barrier function. Am J Physiol 1995;268:G374–G379.
29. Savkovic SD, Koutsouris A, Hecht G. PKCζ participates in activation of inflammatory response induced by enteropathogenic *E. coli*. Am J Physiol Cell Physiol 2003;285:C512–C521.
30. Nusrat A, Chen JA, Foley CS, Liang TW, Tom J, Cromwell M, Quan C, Mrsny RJ. The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. J Biol Chem 2000;275:29816–29822.
31. Andreeva AY, Krause E, Muller EC, Blasig IE, Utepbergenov DI. Protein kinase C regulates the phosphorylation and cellular localization of occludin. J Biol Chem 2001;276:38480–38486.
32. Bogatcheva NV, Verin AD, Wang P, Birukova AA, Birukov KG, Mirzopoyazova T, Adyshev DM, Chiang ET, Crow MT, Garcia JG. Phorbol esters increase MLC phosphorylation and actin remodeling in bovine lung endothelium without increased contraction. Am J Physiol Lung Cell Mol Physiol 2003;285:L415–L426.
33. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci U S A 1995;92:1664–1668.
34. Perna NT, Mayhew GF, Posfai G, Elliott S, Donnenberg MS, Kaper JB, Blattner FR. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun 1998;66:3810–3817.
35. McDaniel TK, Kaper JB. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. Mol Microbiol 1997;23:399–407.
36. Elliott SJ, Yu J, Kaper JB. The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* O157:H7 is unable to confer the attaching and effacing phenotype upon *E. coli* K-12. Infect Immun 1999;67:4260–4263.
37. Yamanaka T, Horikoshi Y, Suzuki A, Sugiyama Y, Kitamura K, Maniwa R, Nagai Y, Yamashita A, Hirose T, Ishikawa H, Ohno S. PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. Genes Cells 2001;6:721–731.
38. Muza-Moons MM, Koutsouris A, Hecht G. Disruption of cell polarity by enteropathogenic *Escherichia coli* enables basolateral membrane proteins to migrate apically and to potentiate physiological consequences. Infect Immun 2003;71:7069–7078.
39. Hurd TW, Fan S, Liu C-J, Kweon HK, Hakansson K, Margolis B. Phosphorylation-dependent binding of 14-3-3 to the polarity protein Par3 regulates cell polarity in mammalian epithelia. Curr Biol 2003;13:2082–2090.
40. Ng T, Parsons M, Hughes WE, Monypenny J, Zicha D, Gautreau A, Arpin M, Gschmeissner S, Verveer PJ, Bastiaens PIH, Parker PJ. Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. EMBO J 2001;20:2723–2741.

Received June 17, 2003. Accepted June 10, 2004.

Address requests for reprints to: Gail Hecht, M.D., University of Illinois, Department of Medicine, Section of Digestive Diseases and Nutrition, 840 South Wood Street, CSB Room 738A (m/c 716), Chicago, Illinois 60612. e-mail: gahecht@uic.edu; fax: (312) 996-5103.

Presented in part at the Digestive Disease Week American Gastroenterological Association annual meeting in May 2002.

Supported by grants from the National Institutes of Health (DK50694 and DK58964 to G.H.; DK63030 to V.K.V.; and DK61931 to J.R.T.) and by Merit Review and Research Enhancement Awards from the Department of Veterans Affairs (to G.H.). S.D.S. was supported by a Research Fellowship Award from the Crohn's and Colitis Foundation of America.