A Membrane-Permeant Peptide That Inhibits MLC Kinase Restores Barrier Function in In Vitro Models of Intestinal Disease

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Background & Aims: **Maintenance of the mucosal barrier is a critical function of intestinal epithelia. Myosin regulatory light chain (MLC) phosphorylation is a common intermediate in the pathophysiologic regulation of this barrier. The aim of this study was to determine whether a membrane permeant inhibitor of MLC kinase (PIK) could inhibit intracellular MLC kinase and regulate paracellular permeability.** ***Methods:*** **Recombinant MLC and Caco-2 MLC kinase were used for kinase assays. T84 and Caco-2 monolayers were treated with enteropathogenic *Escherichia coli* (EPEC) or tumor necrosis factor (TNF)-α and interferon (IFN)-γ to induce barrier dysfunction.** ***Results:*** **PIK inhibited MLC kinase in vitro and was able to cross cell membranes and concentrate at the perijunctional actomyosin ring. Consistent with these properties, apical addition of PIK reduced intracellular MLC phosphorylation by 22% ± 2%, increased transepithelial resistance (TER) by 50% ± 1%, and decreased paracellular mannitol flux rates by 5.2 ± 0.2-fold. EPEC infection induced TER decreases of 37% ± 6% that were limited to 16% ± 5% by PIK. TNF-α and IFN-γ induced TER decreases of 22% ± 3% that were associated with a 172% ± 1% increase in MLC phosphorylation. Subsequent PIK addition caused MLC phosphorylation to decrease by 25% ± 4% while TER increased to 97% ± 6% of control.** ***Conclusions:*** **PIK can prevent TER defects induced by EPEC and reverse MLC phosphorylation increases and TER decreases induced by TNF-α and IFN-γ. The data also suggest that TNF-α and IFN-γ regulate TER, at least in part, via the perijunctional cytoskeleton. Thus, PIK may be the prototype for a new class of targeted therapeutic agents that can restore barrier function in intestinal disease states.**

Increased intestinal permeability is a common event in gastrointestinal disease. For example, intestinal permeability is increased in patients with active and inactive Crohn's disease and in a significant subset of their first-degree relatives.[1,2] This has led to the hypothesis that increased intestinal permeability precedes the development of Crohn's disease, and this relationship has been documented in at least one patient.[3] Increased intestinal permeability also precedes reactivation of inactive Crohn's disease and is a prognostic marker for disease course.[4–6] Taken together, these data suggest that increased intestinal permeability is an early event in the pathogenesis of Crohn's disease.

Infectious agents can also alter paracellular permeability. These include enteropathogenic *Escherichia coli* (EPEC),[7] enterohemorrhagic *E. coli*,[8] *V. cholerae*,[9,10] *Yersinia*,[11] and *Clostridium difficile*.[12,13] Thus perturbation of intestinal paracellular permeability is a common event in both infectious and idiopathic intestinal diseases.

Little is known of the molecular mechanisms responsible for regulation of intestinal paracellular permeability. However, it is clear that permeability of the intercellular tight junction (TJ) is the rate-limiting step and thus the principal determinant of paracellular permeability. Moreover, phosphorylation of the myosin II regulatory light chain (MLC) is associated with increases in intestinal epithelial TJ permeability.[14–18] To better understand the relationship between MLC phosphorylation and TJ permeability, we evaluated the ability of a short peptide[19] derived from the inhibitory domain of smooth-muscle MLC kinase to cross cell membranes, inhibit intestinal epithelial MLC kinase, and regulate paracellular permeability.

Abbreviations used in this paper: EPEC, enteropathogenic *Escherichia coli*; HBSS, Hank's balanced salt solution; IFN, interferon; MLC, myosin regulatory light chain; PIK, membrane-permeant inhibitor of MLC kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TJ, tight junction; TER, transepithelial resistance; TNF, tumor necrosis factor.

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We also sought to evaluate the ability of this peptide to reverse disease-related increases in TJ permeability. We used 2 models. The first model, EPEC infection of cultured intestinal epithelial cell monolayers, is known to require MLC kinase–mediated phosphorylation for loss of barrier function to occur.[18] As a second model, interferon (IFN)-γ and tumor necrosis factor (TNF)-α were used to increase the permeability of cultured intestinal epithelial monolayers.[20–24] These cytokines are increased in intestinal mucosa of patients with Crohn's disease and, as highlighted by the therapeutic effect of anti–TNF-α antibodies,[25] are intimately related to the pathogenesis of Crohn's disease. Thus the effects of IFN-γ and TNF-α on model intestinal epithelial monolayers may represent a relevant model of altered paracellular permeability in Crohn's disease. However, the specific mediators of this cytokine-dependent loss of barrier function are not known. The data from these studies confirm a critical role for MLC phosphorylation in EPEC-induced opening of the TJ and for the first time also establish MLC phosphorylation as at least one key component of the signaling pathway by which Th1 cytokines alter TJ permeability. Thus this peptide may represent a new class of targeted therapeutic agents that can restore barrier function in intestinal disease states. Moreover, the strategy used here to identify membrane-permeant peptides with specific intracellular targeted function may be of general utility in regulating cellular function.

Materials and Methods

Peptide Synthesis

Peptides were synthesized using an automated Pioneer Peptide Synthesizer (Applied Biosystems, Foster City, CA) on Fmoc-PEG-PS-resin. D-Biotin (Sigma, St. Louis, MO) was incorporated into peptides at the amino terminus using HBTU-HOBt/DIPEA in Me_2SO. Peptide resins were cleaved with a 1-hour exposure of a 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% H_2O solvent mixture. Released peptides were purified by preparative reverse-phase C18 high-performance liquid chromatography, characterized by electrospray ionization mass spectroscopy (Sciex API100; PerkinElmer, Boston, MA) and lyophilized to dryness. The carboxy terminus of all peptides was blocked by amidation.

Purification of Recombinant Human Intestinal Epithelial Myosin Regulatory Light Chain

MLC was cloned from a T84 intestinal epithelial cell cDNA library (Stratagene, LaJolla, CA) into an inducible expression vector (Novagen, Madison, WI) and transformed into BL21 (DE3) pLysS codon plus *E. coli* (Stratagene). Log phase cultures were induced by the addition of 1 mmol/L isopropyl-β-D-thiogalactopyranoside for 2.5 hours. Cells were lysed (20 mmol/L Tris-HCl, pH 7.5; 10 mmol/L EDTA; 1% triton X-100; and 100 μg/mL lysozyme) and sonicated. Inclusion bodies were isolated by centrifugation and solublized in 25 mmol/L Tris, pH 8.0, 2 mmol/L $MgCl_2$, and 6 M urea. MLC was purified by ion-exchange chromatography over DEAE cellulose (Sigma) with elution over a 50–175-mmol/L NaCl gradient prepared in the solubilization buffer. Fractions were analyzed using the Pierce BCA Protein Assay (Pierce, Rockford, IL) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing purified MLC were pooled, dialyzed to gradually remove the urea, concentrated, and stored in aliquots at −80°C until use.

In Vitro Kinase Assay

Confluent Caco-2 monolayers expressing the 215-kilodalton MLC kinase[16] were used as the source of MLC kinase. After dilution in kinase reaction buffer (20 mmol/L morpholinepropanesulfonic acid, pH 7.4; 2 mmol/L $MgCl_2$; 0.25 mmol/L $CaCl_2$; and 0.2 μmol/L calmodulin), peptides were added to the mixtures, and the reaction was initiated by the addition of γ^{32}P-ATP (ICN, Costa Mesa, CA) and 5 μmol/L recombinant MLC and transferring the reaction tubes from ice to 30°C. Preliminary experiments demonstrated that the conditions used were within the linear range of the assay. MLC phosphorylation was determined by autoradiography of reaction mixtures separated by SDS-PAGE. All experiments were performed at least 3 times with triplicate or greater samples in each experiment.

Cell Culture and Preparation

Caco-2 cells expressing SGLT1 were maintained and monolayers grown on collagen-coated 0.4-μm pore size polycarbonate membrane transwell supports (Corning-Costar, Cambridge, MA) and used 18–25 days after confluence, as described previously.[15] T84 cells were plated on collagen-coated Transwell supports (0.33 cm^2) and grown to confluence, as described previously.[18]

Electrophysiology

Electrophysiologic measurements were made using agar bridges with Ag-AgCl calomel electrodes and a voltage clamp (University of Iowa Bioengineering, Iowa City, IA), as described previously.[15,18] Fixed currents of 50 μA and 25 μA were passed across Caco-2 and T84 monolayers, respectively. Transepithelial resistance (TER) was calculated using Ohm's law, and fluid resistance was subtracted from all values before subsequent analysis. All experiments were performed at least 3 times with triplicate or greater samples in each experiment.

Transepithelial Flux

Snapwell inserts were transferred into Üssing chambers (Physiologic Instruments, San Diego, CA) with Hank's balanced salt solution (HBSS). After 25 minutes of equilibration, ^{3}H-mannitol was added to the apical chamber of each monolayer. Aliquots were removed from the basal chambers at

intervals over 40 minutes to establish a baseline flux rate for each monolayer. Peptide or vehicle was then added to the apical chamber to a final peptide concentration of 330 μmol/L. Aliquots were removed from the basal chambers at 10-minute intervals. All experiments included triplicate or greater samples in each experiment.

Intracellular Myosin Light Chain Phosphorylation

Confluent monolayers were loaded with 100 μCi/mL $H_3{}^{32}PO_4$ (ICN) for 24 hours. Monolayers were then transferred to phosphate-free HBSS, with or without peptides. Cells were harvested and MLC phosphorylation determined by SDS-PAGE and autoradiography, as described previously.[15] Each experiment was performed at least 3 times with duplicate or greater samples in each experiment.

IFN-γ/TNF-α Treatment

Transwells with confluent Caco-2 monolayers were placed into fresh wells with cytokines in the lower chamber for 72 hours. Preliminary experiments demonstrated that the cytokine doses used caused reproducible TER effects that were greater than those caused by each cytokine individually. All experiments were performed at least 3 times with triplicate or greater samples in each experiment.

EPEC Infection

Monolayers were placed in antibiotic-free tissue culture medium containing 0.5% normal calf serum overnight. Groups of monolayers were infected with EPEC at a multiplicity of infection of 500 for 1 hour and washed to remove nonadherent bacteria, resulting in a final multiplicity of infection of 50.[26] Tissue culture medium containing 330 μmol/L peptide was added to the apical chamber reservoir of uninfected control and EPEC-infected monolayers. TER was measured serially for up to 6 hours. Experiments were performed twice with quadruplicate samples in each experiment.

Fluorescence Microscopy

Membrane permeability of biotinylated PIK was determined by fluorescence microscopy using intact Caco-2 monolayers. These monolayers were incubated with 330 μmol/L peptide in HBSS, rinsed to remove extracellular peptide, and fixed with 1% paraformaldehyde. Separate monolayers were then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (to allow detection of surface and intracellular peptide) or were not permeabilized (to allow detection of surface peptide only). Biotinylated peptide was detected by incubation with Alexa 488-conjugated streptavidin (Molecular Probes, Eugene, OR) in phosphate-buffered saline with 1% bovine serum albumin. For triple labeling, monolayers were incubated with peptide, fixed, and permeabilized as described earlier. Monolayers were then incubated with anti-ZO1 antisera (Zymed, San Francisco, CA), washed and incubated with Alexa 488-conjugated streptavidin, Alexa 350-conjugated phalloidin (Molecular Probes), and Alexa 594-conjugated goat anti-rabbit IgG (Molecular Probes). Stained monolayers were mounted in SlowFade reagent (Molecular Probes) and examined by epifluorescence microscopy. Serial images were collected every 0.25 μm over a 15-μm interval. Image stacks were deconvolved using 80 iterations and reconstructed in the *xz*-plane using AutoDeblur and AutoVisualize software, respectively (AutoQuant, Watervliet, NY).

Statistical Analysis

The Student *t* test for unpaired samples was used to compare results from different experimental treatments. Individual experiments were performed with triplicate or greater samples, and each individual experiment was performed independently 3 or more times.

Results

A Linear Oligopeptide Can Inhibit Caco-2 MLC Kinase In Vitro

In confluent Caco-2 cell monolayers, the 215-kilodalton isoform of MLC kinase[27] accounts for more than 95% of MLC kinase activity.[16] This activity is critical to the regulation of myosin, because phosphorylation of MLC at serine 19 leads to increased actomyosin contraction. The activity of smooth-muscle MLC kinase is regulated by intramolecular interactions between the catalytic domain and the calmodulin-binding inhibitory domain. In the presence of Ca^{++} and calmodulin, this intramolecular interaction is blocked, the inhibition is released, and the enzyme is activated. Consistent with this model, a 22–amino acid peptide from the inhibitory domain of smooth-muscle MLC kinase has been shown to effectively inhibit the catalytic domain of smooth-muscle MLC kinase.[28] More recently, a 9–amino acid oligopeptide, derived from that 22–amino acid peptide sequence, has been identified.[19] This peptide inhibits MLC kinase but does not significantly inhibit calmodulin-dependent protein kinase II or adenosine 3′,5′-cyclic monophosphate–dependent protein kinase.[19] We tested the ability of this oligopeptide to inhibit the larger Caco-2 MLC kinase in vitro. Addition of the oligopeptide caused a dose-dependent inhibition of Caco-2 MLC kinase activity with an IC_{50} of 29 μmol/L (Figure 1). To verify the sequence specificity of MLC kinase inhibition by the oligopeptide, 2 variants were synthesized with *cis*-4-aminocyclohexanecarboxylic acid substituted at position 5 or at positions 4, 5, and 6. As shown previously for inhibition of smooth-muscle MLC kinase by these substituted peptides,[19] we found that the monosubstituted oligopeptide inhibited Caco-2 MLC kinase with a potency similar to the parent oligopeptide, with a 50% inhibitory concentration (IC_{50}) of 36 μmol/L, but that the trisubstituted oligopeptide inhibited Caco-2 MLC

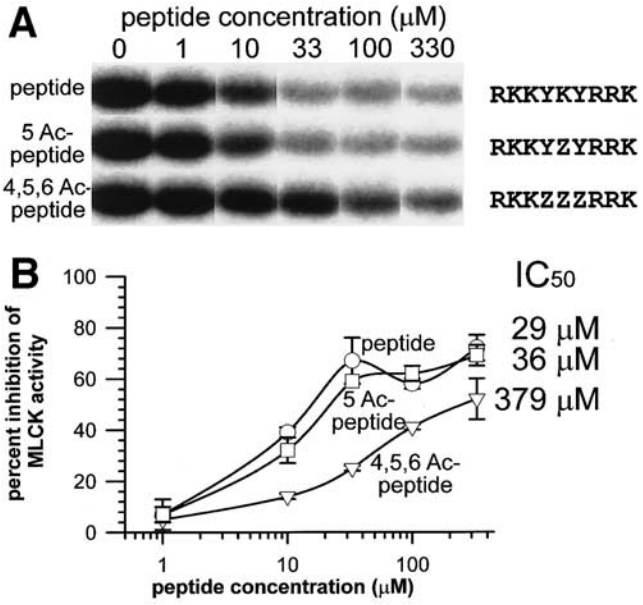


Figure 1. Caco-2 MLC kinase is inhibited by a linear oligopeptide. (*A*) MLC kinase activity was assayed with increasing concentrations of the linear oligopeptide or substituted oligopeptides in an in vitro reaction with recombinant human enterocyte MLC and γ-^{32}P-ATP. Reaction mixtures were separated by SDS-PAGE and MLC phosphorylation assessed by autoradiography. Peptide sequences are shown to the right (Z = *cis*-4-aminocyclohexanecarboxylic acid). (*B*) MLC kinase activity was expressed as γ-^{32}P-ATP incorporation into MLC normalized to γ-^{32}P-ATP incorporation in the absence of oligopeptide. The oligopeptide (○) and monosubstituted oligopeptide (□) had similar MLC kinase inhibitory activity, whereas trisubstituted oligopeptide (▽) had markedly decreased MLC kinase inhibitory activity. The IC_{50} for MLC kinase inhibition by each peptide is shown at the right.

kinase poorly, with an IC_{50} of 379 μmol/L (Figure 1*B*). Thus inhibition of Caco-2 MLC kinase by the oligopeptide is dependent on both peptide sequence and concentration.

The Oligopeptide Is Membrane-Permeant and Localizes to the Perijunctional Actomyosin Ring and Tight Junction in Caco-2 Cells

We noted that 7 of 9 amino acids in the oligopeptide are positively charged lysine or arginine residues. This is similar to the protein transduction domain of the HIV-1 TAT protein (Table 1). This protein transduction domain allows proteins to cross cell membranes independent of specific transporters.[29] We thus hypothesized that the oligopeptide might also be membrane permeant. To test this hypothesis, confluent Caco-2 monolayers were incubated with biotinylated oligopeptide. The monolayers were washed to remove excess extracellular biotinylated oligopeptide and fixed. Surface and total (surface plus intracellular) oligopeptide were detected by differential permeabilization of the fixed monolayers such that fluorochrome-conjugated streptavidin was not or was, respectively, able to access cytoplasmic oligopeptide. In fixed nonpermeabilized cells, only a faint fluorescent signal was detected, indicating that surface biotinylated oligopeptide was effectively removed by the wash before fixation (Figure 2*A*). In contrast, permeabilization, to allow the fluorochrome-conjugated streptavidin to access intracellular oligopeptide, resulted in detection of a bright fluorescent ring outlining each cell (Figure 2*B*). Thus the biotinylated oligopeptide is able to cross cell membranes and access the cytoplasm in monolayers of intact living Caco-2 cells. For this reason, we refer to the oligopeptide as PIK, a membrane-permeant inhibitor of MLC kinase.

The *en face xy*-plane images of intracellular PIK (Figure 2*B*) suggest that PIK localized to the perijunctional actomyosin ring after entry into the cell. To evaluate this in detail, Caco-2 monolayers were triple-labeled for biotinylated PIK, F-actin, and the TJ protein ZO-1 (Figure 2*D*, *E*, *F*, *G*, and *H*). Both *xy*-plane images and *xz*-plane serial reconstructions showed that PIK (Figure 2*E*) was localized primarily within the perijunctional actomyosin ring, as defined by phalloidin staining (Figure 2*F*). PIK also partially colocalized with TJs, as defined by ZO-1 (Figure 2*G*). Thus PIK crosses cell membranes and localizes to sites rich in F-actin, such as the perijunctional actomyosin ring.

PIK Decreases Intracellular MLC Phosphorylation and Enhances Barrier Function When Added to Intestinal Epithelial Cell Monolayers

Because PIK inhibits MLC kinase in vitro and also crosses the cell membrane freely, we tested its ability to alter MLC phosphorylation in intact Caco-2 cell monolayers. MLC phosphorylation decreased by 22% ± 2% of control monolayers within 60 minutes after PIK addition ($P < 0.01$; Figure 3*A*). Total MLC content within the cells was unchanged (data not shown). Thus the addition of PIK causes decreases in intracellular MLC phosphorylation, consistent with inhibition of intracellular MLC kinase. Because increased TJ permeability is associated with increased MLC phosphorylation,[15,17,30] we hypothesized that PIK-associated decreases in MLC phosphorylation might decrease TJ permeability. Apical PIK addition resulted in decreased flux of the paracellular

Table 1. Comparison of PIK and HIV-1 TAT Protein Transduction Domain Sequences

	Sequence
PIK	R K K Y K Y R R K
Protein transduction domain	Y G R K K R R Q R R R

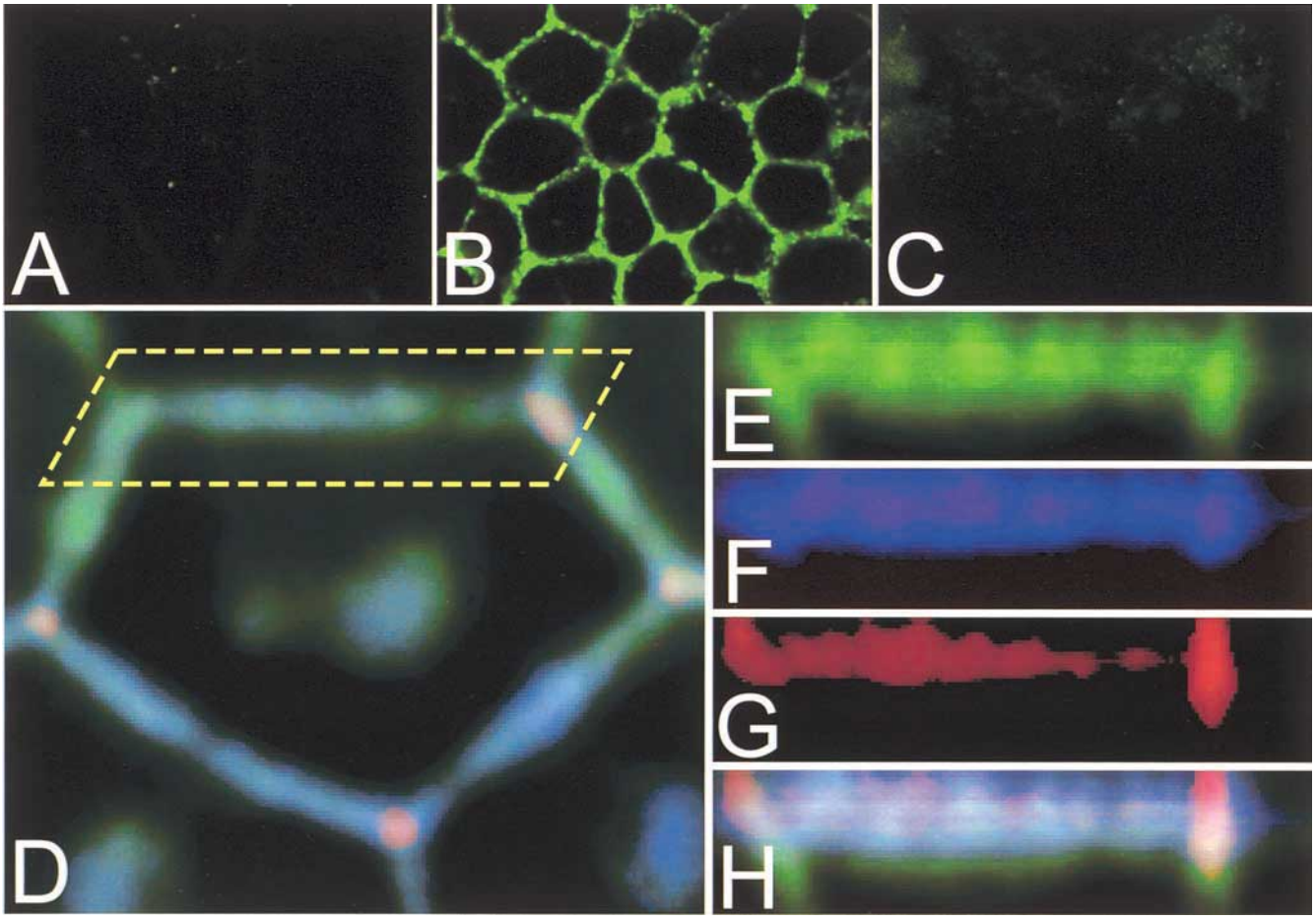


Figure 2. PIK crosses intact cell membranes and localizes to the perijunctional actomyosin ring. Caco-2 monolayers were treated with biotinylated PIK, washed, and fixed. (*A*) Surface (extracellular) biotinylated PIK was detected with Alexa 488-conjugated streptavidin. (*B*) Total (surface and intracellular) biotinylated PIK was detected in monolayers treated with biotinylated PIK, washed, fixed, and then permeabilized to allow Alexa 488-conjugated streptavidin to access intracellular sites. In these cells, a bright circumferential ring outlining each cell was detected. (*C*) Control cells, not treated with biotinylated PIK, were permeabilized and stained with Alexa 488-conjugated streptavidin. No significant fluorescent signal was detected. (*D–H*) Caco-2 monolayers were stained for biotinylated PIK as in *B* (*green*), F-actin (*blue*), and ZO1 (*red*). (*D*) An *en face xy*- plane image at the level of the perijunctional actomyosin ring and TJ shows colocalization of PIK, F-actin, and ZO1. The area indicated (dashed box in *D*) was used to generate *xz*-plane images of PIK (*E*), F-actin (*F*), ZO-1 (*G*), and a composite image (*H*). These images confirm that PIK localizes to the perijunctional actomyosin ring and TJ of Caco-2 cells.

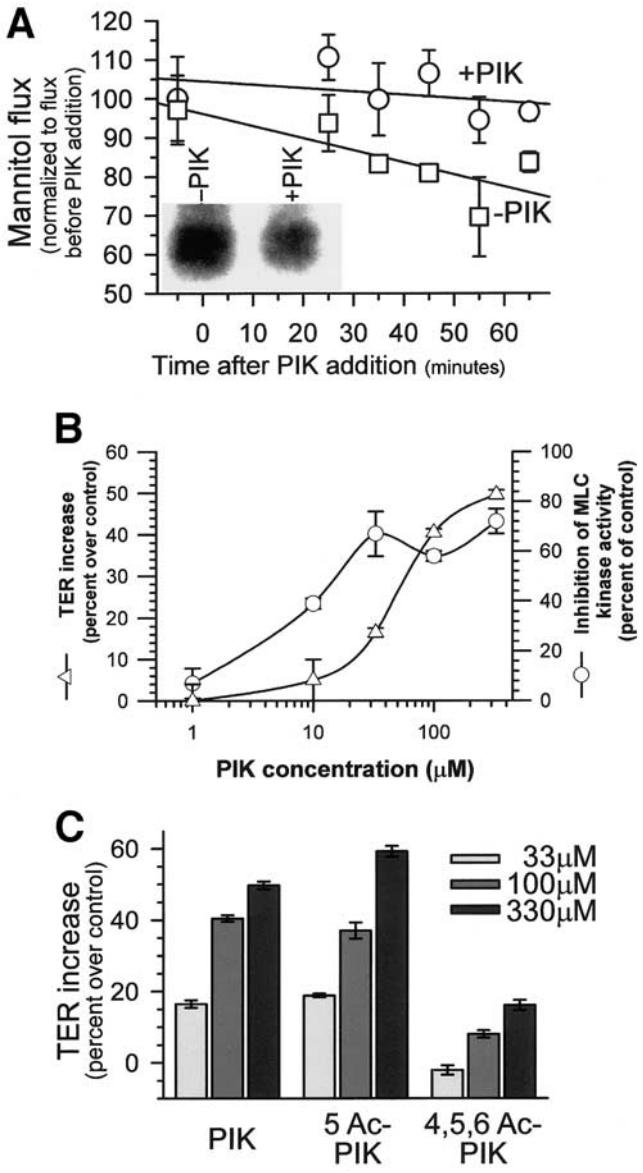
marker mannitol in Caco-2 monolayers (Figure 3*A*), demonstrating that PIK does alter epithelial TJ permeability. TER, a sensitive marker of TJ permeability, was also evaluated. PIK caused rapid increases in TER of Caco-2 monolayers that were sustained for at least 5 hours. Similar TER increases were seen in T84 cell monolayers.

To determine whether inhibition of MLC kinase was the mechanism by which PIK alters TER and paracellular permeability, we performed 2 separate analyses. First, the dose dependence of PIK's ability to increase TER was compared to the dose dependence for PIK-dependent inhibition of MLC kinase in vitro (Figure 3*B*). TER effects increased in parallel with and over the same concentration range as MLC kinase inhibition, suggesting that TER increases are due to MLC kinase inhibition. Second, the effects of PIK with *cis*-4-aminocyclohexanecarboxylic acid substituted at position 5 or at positions 4, 5, and 6 on TER were evaluated. As was true of MLC kinase inhibition in vitro, monosubstituted PIK caused TER increases similar to those induced by PIK (Figure 3*C*). Trisubstituted PIK, which inhibited MLC kinase poorly, was similarly ineffective at increasing TER (Figure 3*C*). Thus the effects of these 3 peptides on TER paralleled their ability to inhibit MLC kinase in vitro. Together with the dose–response data, these data strongly support the conclusion that the effects of PIK on TER are directly related to MLC kinase inhibition.

PIK Protects Intestinal Epithelial Cells From Barrier Defects After EPEC Infection

The effects of EPEC infection on intestinal epithelial TJ permeability have been characterized in detail

and are at least partially dependent on the increased MLC phosphorylation that also accompanies EPEC infection.[18] Thus, to test the effect of PIK on a disease-related intestinal barrier defect known to be related to MLC phosphorylation, PIK was added to monolayers 1 hour after infection with EPEC. This time point represents an early stage of infection before the development of barrier defects. PIK prevented 57% of the loss of TER 4 hours after infection ($P < 0.01$; Figure 4*A*). As infection progressed, PIK was less effective, since PIK prevented 30% of the TER decrease 6 hours after infection ($P = 0.05$). This may reflect degradation of PIK or a decreased ability of PIK to prevent barrier defects in more advanced epithelial injury. Thus PIK is effective at preventing the loss of TER induced by EPEC, consistent with the proposed effect of PIK on intestinal epithelial MLC kinase.



Barrier Loss Induced by IFN-γ and TNF-α Is Associated With Increased MLC Phosphorylation and Can Be Reversed by PIK

IFN-γ increases intestinal epithelial TJ permeability,[20] an effect that can be potentiated by TNF-α.[23,24] Preliminary experiments confirmed this synergy between IFN-γ and TNF-α and demonstrated that treatment of Caco-2 monolayers with 100 U/mL IFN-γ and 100 ng/mL TNF-α for 72 hours led to only a small (2.9 ± 0.3-fold) increase in apoptosis but induced a 22% (±3%) decrease in TER ($P < 0.01$; Figure 4*B*). Treatment of Caco-2 monolayers with IFN-γ and TNF-α was also associated with a 172% ± 1% increase in MLC phosphorylation ($P < 0.01$; Figure 4*B*). Addition of PIK to monolayers treated with IFN-γ and TNF-α caused a 25% ± 4% decrease in MLC phosphorylation ($P < 0.03$; Figure 4*B*) and caused TER to return to 97% ± 6% of control monolayers ($P < 0.02$ vs. IFN-γ and TNF-α–treated monolayers not exposed to PIK; Figure 4*B*). PIK

Figure 3. PIK reduces intracellular MLC phosphorylation and regulates paracellular permeability. (*A*) ^{3}H-mannitol flux was evaluated in Caco-2 monolayers mounted in modified Üssing chambers. ^{3}H-mannitol flux for each monolayer was normalized to ^{3}H-mannitol flux before PIK addition. After equilibration and determination of baseline ^{3}H-mannitol flux, 330 μM PIK (○) or vehicle (□) was added to the apical chamber of each monolayer. Addition of PIK to the monolayers caused progressive decreases in ^{3}H-mannitol flux. (*Inset*) ^{32}P-loaded Caco-2 monolayers were incubated with or without apical addition of 330 μmol/L PIK. After 60 minutes, monolayers were harvested and lysates separated by SDS-PAGE. Autoradiographs of the 20-kilodalton MLC band demonstrate decreased MLC phosphorylation after PIK addition. (*B*) Dose dependence of TER responses to PIK parallels MLC kinase inhibition in vitro. PIK was added to the apical chamber of Caco-2 monolayers at 1 μmol/L, 10 μmol/L, 33 μmol/L, 100 μmol/L, and 330 μmol/L. TER values after 60 minutes were normalized to the TER of control monolayers incubated with vehicle only. TER increases are plotted on the left side *y*-axis and parallel the concentration dependence of PIK-mediated MLC kinase inhibition (right side *y*-axis, data from Figure 1 included for comparison). The TER of control monolayers in this experiment was 185 ± 7 Ω cm^2. (*C*) TER responses to substituted PIK derivatives parallel MLC kinase inhibition in vitro. PIK and the 2 substituted derivatives, 5Ac-PIK and 4,5,6Ac-PIK, were each added to the apical chamber of Caco-2 monolayers at 33 μmol/L, 100 μmol/L, and 330 μmol/L. TER values after 60 minutes were normalized to the TER of control monolayers incubated with vehicle only. The monosubstituted 5Ac-PIK increased TER with a potency similar to that of PIK. In contrast, the trisubstituted 4,5,6Ac-PIK was at least an order of magnitude less potent than PIK and monosubstituted PIK in inducing TER increases. This parallels the potency of PIK and the substituted oligopeptides in inhibition of Caco-2 MLC kinase in vitro, suggesting that PIK's ability to increase TER of Caco-2 monolayers is directly related to MLC kinase inhibition. The TER of control monolayers in this experiment was 179 ± 3 Ω cm^2.

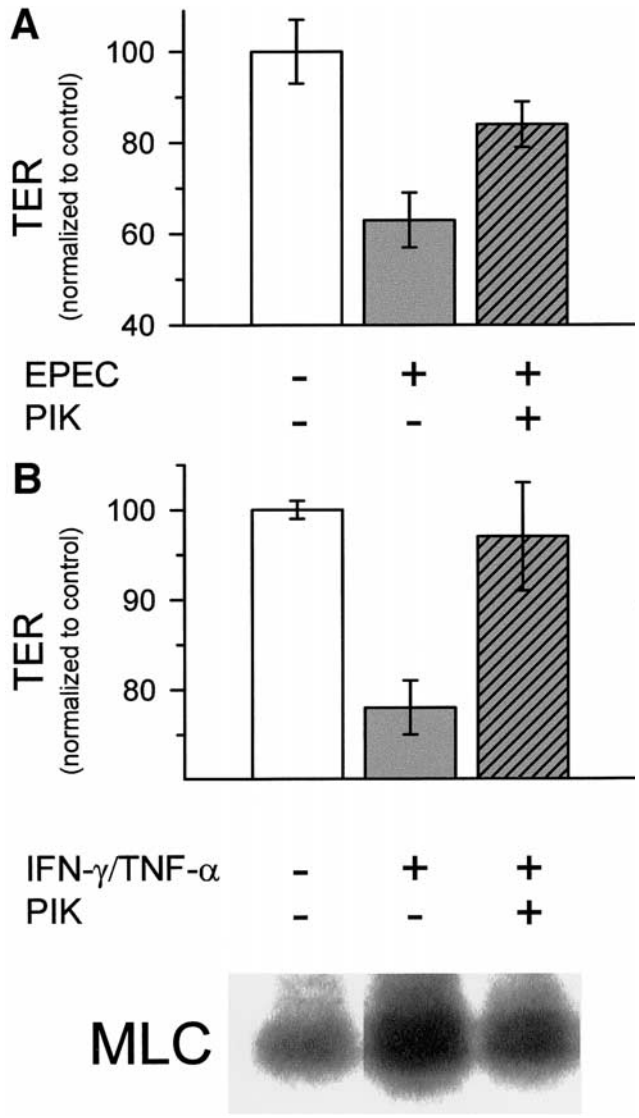


Figure 4. Effects of PIK on barrier function and MLC phosphorylation in EPEC-infected and IFN-γ and TNF-α–treated monolayers. (*A*) PIK can partially prevent EPEC-associated TER decreases. T84 monolayers were infected with EPEC for 4 hours and 330 μmol/L PIK was added to the apical chamber, as indicated, 1 hour after infection. EPEC infection caused a 37% ± 6% fall in TER. When PIK was added, this fall was limited to 16% ± 5% ($P < 0.01$). The TER of control monolayers in this experiment was 752 ± 68 Ω cm^2. (*B*) Caco-2 monolayers were treated with 100 U/mL IFN-γ and 100 ng/mL TNF-α for 72 hours. Relative to control monolayers, IFN-γ and TNF-α treatment induced a 22% ± 3% decrease in TER. Addition of 330 μmol/L apical PIK caused TER of IFN-γ and TNF-α–treated monolayers to increase to 97% ± 6% of control monolayers not treated with IFN-γ and TNF-α. In parallel, ^{32}P-loaded monolayers were harvested and MLC phosphorylation analyzed by SDS-PAGE and autoradiography. Treatment with TNF-α and IFN-γ caused a 172% ± 1% increase in MLC phosphorylation. On treatment with PIK, MLC phosphorylation was decreased by 25% ± 4%. The TER of control monolayers in this experiment was 170 ± 6 Ω cm^2.

treatment had no significant effect on apoptotic or mitotic rates in either cytokine-treated or control monolayers (data not shown). These results suggest that although PIK does not completely reverse cytokine-induced increases in MLC phosphorylation, the partial normalization of MLC phosphorylation is sufficient for restoration of TER.

Discussion

Intestinal paracellular permeability is precisely regulated by the epithelial TJ.[31,32] Although the mechanisms by which TJ permeability is regulated are incompletely defined, MLC within the perijunctional actomyosin ring does appear to be a critical regulator of TJ permeability.[17,33] To better understand the details of actomyosin-dependent TJ regulation, we sought to identify a specific agent capable of inhibiting epithelial MLC kinase. We chose an oligopeptide derived from the inhibitory domain of smooth-muscle MLC kinase.[19] Smooth-muscle MLC kinase is smaller than the ~215-kilodalton Caco-2 MLC kinase,[16] but the sequences of smooth-muscle MLC kinase and epithelial MLC kinase are highly conserved within the catalytic and inhibitory domains (J.R. Turner, unpublished observations, March, 2000). Previous work has shown that the oligopeptide inhibits calmodulin-dependent kinase II only at 4000-fold higher concentrations and does not appreciably inhibit adenosine 3′,5′-cyclic monophosphate–dependent protein kinase, suggesting that the peptide is a highly specific inhibitor of MLC kinase with limited effects on other kinases.[19] Data from in vitro kinase assays with excess calmodulin showed that the oligopeptide inhibited Caco-2 MLC kinase and that the conformational characteristics of the oligopeptide required for effective inhibition of Caco-2 MLC kinase were similar to the requirements previously established for inhibition of smooth-muscle MLC kinase.[19] The efficacy and specificity of the oligopeptide likely reflect its mechanism of action. The inhibitory domain of MLC kinase is thought to interact directly with the catalytic domain, thus inhibiting activity of the enzyme (Figure 5*A*). When calmodulin binds to the inhibitory domain the intramolecular interaction between the inhibitory and catalytic domains is released, and the MLC kinase is activated (Figure 5*B*). Thus, even in the presence of calmodulin, the oligopeptide is able to prevent MLC phosphorylation, presumably through binding to the catalytic domain of MLC kinase (Figure 5*C*).

We also showed the oligopeptide is a PIK, in that it crosses cell membranes efficiently and localizes at the perijunctional actomyosin ring and at TJs within confluent intestinal epithelial monolayers. PIK's ability to cross cell membranes is likely due to sequence similarity between PIK and the protein transduction domain of the HIV-1 TAT protein, although the precise mechanism by

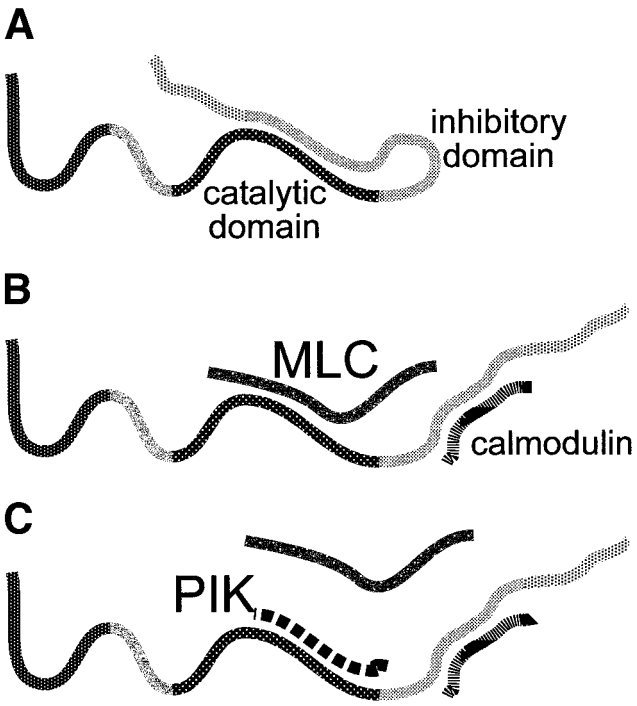


Figure 5. Model of PIK action. (*A*) The inhibitory domain of MLC kinase interacts directly with the catalytic domain, thus inhibiting the enzyme. The unique amino terminal domain present in endothelial and intestinal epithelial MLC kinase but not in smooth- muscle MLC kinase. The carboxyterminal KRP domain also encodes an independently expressed kinase-related protein previously considered to be a marker of smooth-muscle differentiation. (*B*) Calmodulin binds to the inhibitory domain, releasing the interaction between the inhibitory and catalytic domains, and activating MLC kinase. (*C*) PIK binds to the MLC kinase catalytic domain and inactivates the enzyme, even in the presence of calmodulin.

which this domain allows proteins to cross membranes is not clear.[29] The localization of PIK to the perijunctional actomyosin ring is likely due to the unique amino terminal sequence of epithelial MLC kinase (J.R. Turner, unpublished observations, March, 2000), which is shared by endothelial MLC kinase and mediates direct binding to F-actin.[27,34]

Addition of PIK to intact Caco-2 monolayers caused decreases in intracellular MLC phosphorylation, consistent with inhibition of intracellular MLC kinase, and also caused marked increases in TER with corresponding decreases in mannitol flux. The ability of substituted variants of PIK to increase TER correlated directly with the ability of substituted oligopeptides to inhibit Caco-2 MLC kinase in vitro. Thus all of the available data support the conclusion that PIK regulates epithelial TJ permeability via inhibition of MLC kinase. However, it should be noted that the diminished potency of trisubstituted PIK in eliciting TER increases could also be due to poor membrane permeability of this peptide.

Increased intestinal permeability is associated with a broad range of intestinal diseases.[1,6,7,12,35,36] Thus we sought to test PIK's ability to reverse such disease-related permeability increases. As one experimental model, cultured intestinal epithelial monolayers were infected with EPEC. PIK was able to partially prevent the permeability defects that occur following EPEC infection. Because EPEC-induced permeability defects are prevented by MLC kinase inhibitors, these data also support the conclusion that PIK increases barrier function by inhibition of MLC kinase.

Intestinal epithelial monolayers were also treated with IFN-γ and TNF-α. Such treatment increases the permeability of cultured monolayers in a model that is particularly relevant to Crohn's disease.[20–24] The use of these cytokines in combination is synergistic, perhaps because each has been reported to up-regulate expression of the surface receptor for the other.[22,24] This combination of IFN-γ and TNF-α allowed the use of lower doses of each agent and, as a result, apoptotic rates were only modestly increased in IFN-γ and TNF-α–treated monolayers. This contrasts with reported increases in both MLC phosphorylation and apoptosis in human endothelial cells[37] and Madin Darby canine kidney cells[38] treated with TNF-α. The present data show that in intestinal epithelial monolayers, PIK is able to reverse permeability increases (TER decreases) induced by IFN-γ and TNF-α. This suggests that one mechanism by which IFN-γ and TNF-α decrease intestinal epithelial barrier function is via the observed increases in MLC phosphorylation. This in contrast to reports that TNF-α–induced permeability increases in endothelial paracellular permeability are independent of MLC phosphorylation.[37] Of note, a variety of other cellular processes can be altered by IFN-γ and TNF-α treatment. Some of these (e.g., Cl^- secretion) may be regulated by actomyosin function. Thus, although we have focused on the ability of PIK to restore defects in barrier function, it is possible that PIK has additional effects on intestinal epithelia mediated by the cytoskeleton.

PIK was very effective at correcting or preventing mild permeability defects. However, greater degrees of damage (i.e., >50% decreases in TER) induced by higher IFN-γ and TNF-α doses were not reversible with PIK. This may reflect the contribution of additional mechanisms activated by IFN-γ and TNF-α, such as decreased expression of the TJ proteins ZO-1 and occludin.[39,40] Similar decreases in TJ protein content were noted in our studies, but were not corrected by PIK. Similarly, the effect of PIK, although still present, was attenuated 6 hours after EPEC infection, when both

localization and phosphorylation of the TJ protein occludin are altered.[41] Alternatively, we cannot exclude that changing environments, either intracellular or extracellular, might reduce the stability and effectiveness of PIK, because this peptide may be susceptible to proteolytic cleavage.

In summary, we have identified a novel oligopeptide, PIK, that inhibits MLC kinase in vitro, is membrane permeant, effects decreases in intracellular MLC phosphorylation, and causes reversible increases in TER. PIK is also able to reverse permeability defects induced in 2 separate models of human intestinal disease and has allowed the identification of MLC phosphorylation as a significant mediator of IFN-γ and TNF-α–induced permeability defects. We speculate that PIK may serve as a prototype for a new class of therapeutic agents that restore intestinal barrier function after noncytolytic epithelial injury.

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