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PKCζ participates in activation of inflammatory response induced by enteropathogenic *E. coli*

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Savkovic, Suzana D., Athanasia Koutsouris, and Gail Hecht. PKCζ participates in activation of inflammatory response induced by enteropathogenic *E. coli*. *Am J Physiol Cell Physiol* 285: C512–C521, 2003;10.1152/ajpcell.00444.2002.—We showed previously that enteropathogenic *Escherichia coli* (EPEC) infection of intestinal epithelial cells induces inflammation by activating NF-κB and upregulating IL-8 expression. We also reported that extracellular signal-regulated kinases (ERKs) participate in EPEC-induced NF-κB activation but that other signaling molecules such as PKCζ may be involved. The aim of this study was to determine whether PKCζ is activated by EPEC and to investigate whether it also plays a role in EPEC-associated inflammation. EPEC infection induced the translocation of PKCζ from the cytosol to the membrane and its activation as determined by kinase activity assays. Inhibition of PKCζ by the pharmacological inhibitor rottlerin, the inhibitory myristoylated PKCζ pseudosubstrate (MYR-PKCζ-PS), or transient expression of a nonfunctional PKCζ significantly suppressed EPEC-induced IκBα phosphorylation. Although PKCζ can activate ERK, MYR-PKCζ-PS had no effect on EPEC-induced stimulation of this pathway, suggesting that they are independent events. PKCζ can regulate NF-κB activation by interacting with and activating IκB kinase (IKK). Coimmunoprecipitation studies showed that the association of PKCζ and IKK increased threefold 60 min after infection. Kinase activity assays using immunoprecipitated PKCζ-IKK complexes from infected intestinal epithelial cells and recombinant IκBα as a substrate showed a 2.5-fold increase in IκBα phosphorylation. PKCζ can also regulate NF-κB by serine phosphorylation of the p65 subunit. Serine phosphorylation of p65 was increased after EPEC infection but could not be consistently attenuated by MYR-PKCζ-PS, suggesting that other signaling events may be involved in this particular arm of NF-κB regulation. We speculate that EPEC infection of intestinal epithelial cells activates several signaling pathways including PKCζ and ERK that lead to NF-κB activation, thus ensuring the proinflammatory response.

inflammation; enteropathogenic *Escherichia coli*; nuclear factor-κB; protein kinase Cζ; IκB kinase; extracellular signal-regulated kinase

WE SHOWED PREVIOUSLY (46) that infection of intestinal epithelial cells by enteropathogenic *Escherichia coli* (EPEC) induces the transepithelial migration of acute inflammatory cells that is in part driven by the secretion of IL-8. Expression of EPEC-induced IL-8 is regulated by the transcription factor NF-κB (45), which is harbored in the cytoplasm through its interaction with a class of inhibitory proteins, IκBs (19). NF-κB activation and translocation to the nucleus occur after IκB phosphorylation and subsequent degradation by proteasomes (56, 58). Phosphorylation of IκB is attributed to two kinases, IκB kinase (IKK)α and IKKβ (32, 40). IKKα/β may be activated by various upstream signaling molecules such as NF-κB-induced kinase (NIK) (40, 57), MEK (24, 30, 36), PKC and PKA (48, 50, 55), and increased intracellular calcium (18, 55).

Activation of NF-κB by microbial pathogens uses various upstream cellular signals that result in inflammation. *Helicobacter pylori* (21a) and *Clostridium difficile* toxin A (56a) utilize p38 to stimulate the inflammatory response; *Salmonella typhimurium* utilizes intracellular Ca^{2+} (18); and *Pseudomonas aeruginosa* (25), *S. typhimurium* (20a), and *H. pylori* (24) have all been reported to engage ERK as a means of activating NF-κB. We showed (47) that EPEC activates the ERK pathway in host intestinal epithelial cells, which then participates in IκBα phosphorylation and degradation and subsequent IL-8 expression. Various upstream signal transduction molecules can activate ERK, the best defined of which is the Ras-Raf1-MEK cascade (56b). The ERK pathway, however, can be alternatively activated by atypical PKCs (aPKCs) (20, 26), and aPKCs have been shown to activate NF-κB in a number of model systems (6, 8, 12, 39, 53).

aPKCs (aPKCζ and -λ/ι) are unresponsive to calcium and play important roles in controlling cell growth and survival (6, 15, 35), most likely by activating ERK and NF-κB pathways (8, 9, 12–14, 33, 34, 49). The mechanism of ERK regulation by aPKC requires its interaction with and subsequent activation of MEK, which can then activate ERK, or IKK (6, 7, 32–34, 36, 42). Additionally, PKCζ can independently regulate NF-κB through interaction with and activation of IKK (12, 17, 28, 53) or phosphorylation of the p65 subunit of NF-κB (1, 23, 31).

EPEC has been shown to activate conventional PKCs in host cells (3, 10), but their role in the intestinal epithelial inflammatory response is not known. We

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reported (47) that intracellular calcium is not involved in EPEC-induced inflammation, suggesting that calcium-dependent, conventional PKCs are not involved and thus implicating either novel PKCs or aPKCs. Therefore, the aim of this study was to determine whether PKCζ is activated by infection with EPEC and to investigate its role in EPEC-associated inflammation. In this report, we show that EPEC infection induces the translocation and activation of aPKCζ in target intestinal epithelial cells. PKCζ inhibition with pharmacological inhibitors, a specific inhibitory pseudosubstrate, or expression of a dominant-negative PKCζ significantly attenuated EPEC-induced IκBα phosphorylation, demonstrating its involvement in the proinflammatory signaling cascade. In contrast, PKCζ inhibition had no effect on EPEC activation of ERK, suggesting that these two signaling molecules independently stimulate the inflammatory response. The mechanism by which EPEC-activated PKCζ regulates NF-κB includes activation of IKK. Although the serine phosphorylation of p65 is significantly increased after EPEC infection, inhibition of PKCζ did not consistently attenuate this response, suggesting that other signaling pathways participate in this event. These studies show that epithelium-based signaling pathways evoked by EPEC infection are complex and independently stimulate inflammation, thus guaranteeing this protective response.

MATERIALS AND METHODS

Cell culture. The intestinal epithelial cell lines T84 and Caco-2 were used for these studies. T84 cells were a generous gift from Dr. Kim Barrett (University of California, San Diego). *Passages 35–55* were used for these studies and were grown in a 1:1 (vol/vol) mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 (Invitrogen, Carlsbad, CA) supplemented with 6% FCS as described previously (29). Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown in high-glucose DMEM medium supplemented with 10% FCS.

Bacterial strains and infection. Overnight cultures of EPEC strain E2948/69, grown in Luria-Bertani broth, were diluted (1:33) in serum- and antibiotic-free tissue culture medium containing 0.5% mannose and grown at 37°C to mid-log growth phase. Monolayers were infected as previously described (46) to yield a multiplicity of infection (MOI) of 100.

Treatment with inhibitors. To define the PKC isoforms involved in activation of NF-κB, cells were treated for 1 h before infection with select inhibitors. Bisindolylmaleimide (BIM; Calbiochem, La Jolla, CA) at a concentration of 50 μM inhibits many PKC isoforms, whereas Gö-6976 (Calbiochem) at a concentration of 10 μM inhibits only calcium-dependent PKCs. Rottlerin (Calbiochem) in lower concentrations (3–6 μM) selectively inhibits PKCδ, whereas higher concentrations (30–45 μM) also suppress calcium-dependent PKCs. At a concentration of 100 μM, rottlerin also blocks PKCζ. The myristoylated PKCζ pseudosubstrate (MYR-PKCζ-PS; Biosource International, Camarillo, CA) suppresses only the PKCζ isoform. Intestinal epithelial cells were incubated with MYR-PKCζ-PS (20 μM) for 1 h before infection with EPEC.

Plasmids and cell transfection. The pcDNA3 and pPKCζ-KA dominant negative form of PKCζ inserted into pcDNA3 vector were kind gifts from Dr. J. Moscat (Universidad Autonoma, Madrid, Spain). The PKCζ mutant was generated by a substitution of lysine-275 for tryptophan and thus lacks a functional catalytic domain (7). Plasmids were amplified in *E. coli* strain JM109 and purified with a Maxi Kit according to the manufacturer's direction (Qiagen, Valencia, CA). Both T84 and Caco-2 cells were plated at a density of 4×10^5 cells/cm^2 in six-well plates containing complete medium. After 72 h, cells were transfected with 1 μg of plasmid by using Lipofectamine Plus (Invitrogen) following the manufacturer's protocol. Cells were infected 48 h later with EPEC as described in *Bacterial strains and infection*.

Immunofluorescent microscopy. Immunofluorescent staining was performed on uninfected and infected monolayers of T84 and Caco-2 cells. Monolayers were fixed with 3% paraformaldehyde pH 7.4 in PBS for 15 min, rinsed with PBS, permeabilized with 0.2% Triton X-100 for 15 min, and blocked in 1% BSA in PBS. Monolayers were incubated with antibodies against PKCζ for 1 h followed by rhodamine-conjugated anti-rabbit IgG antibody for 1 h. After washing, monolayers were mounted with Vectashield (Molecular Probes, Eugene, OR) and assessed with a Nikon Opti-Phot microscope. Images were captured with the Zeiss-RT Digital Imaging System.

Immunoblotting. After infection with EPEC, monolayers were washed and proteins were extracted with RIPA buffer (in mM: 50 NaCl, 50 Tris, pH 7.4, 1 EGTA, 1 Na_3VO_4, 1 PMSF, and 1 NaF with 0.5% DOC, 0.1% NP-40, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Proteins (100 μg) were subjected to SDS-PAGE and then transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking for 1 h at room temperature, membranes were sequentially incubated for 1 h with primary antibody against IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated IκBα (New England Biolabs, Beverly, MA), PKCζ, IKK, or p65 (Santa Cruz Biotechnology). The membranes were washed and incubated with appropriate dilutions of secondary antibodies conjugated by alkaline phosphatase or peroxidase for 1 h at room temperature. Color development was achieved by alkaline phosphatase reaction with nitroblue tetrazolium-5-bromo-4-chloro-indolyl phosphate solution (Zymed, San Francisco, CA) or enhanced chemiluminescence (ECL; Pierce, Rockford, IL).

PKCζ translocation. PKCζ translocation was assessed as previously described (5). Infected cells were washed with *buffer A* (in mM: 25 Tris, pH 7.6, 1 EGTA, 10 NaCl), scraped into *buffer A+* (in mM: 25 Tris, pH 7.6, 1 EGTA, 10 NaCl, and 1 PMSF with 25 μg/ml leupeptin), and homogenized on ice with a Dounce homogenizer. The homogenates were centrifuged at 15,000 *g* for 30 min at 4°C. Supernatants represented the cytosolic fraction. Pellets were solubilized in *buffer A+ X-100* (in mM: 25 Tris, pH 7.6, 1 EGTA, 10 NaCl, and 1 PMSF with 25 μg/ml leupeptin and 1% Triton X-100), homogenized with 10 strokes of the Dounce homogenizer, and centrifuged at 15,000 *g* for 30 min at 4°C. The resulting supernatants represented the membrane fractions. Proteins extracted from both fractions were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblot analysis with PKCζ antibody. Immunoblots were quantitated by densitometric analysis.

Immunoprecipitation. For immunoprecipitation, cells infected with EPEC were harvested in lysis buffer (in mM: 40 Tris, pH 8.0, 300 NaCl, 6 EDTA, 6 EGTA, 10 β-glycerophosphate, 10 NaF, 10 *p*-nitrophenylphosphate, 1 benzamidine, 2 PMSF, and 1 dithiothreitol with 0.1% Nonidet P-40, 300 μM Na_3VO_4, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Extracts were centrifuged at 15,000 *g* for 15 min, and 1 mg of whole cell lysate was incubated with 10 μg of the

indicated antibody for 1 h at 4°C. Protein A beads (25 μg) were gently rotated for an additional hour at 4°C with the protein-antibody mixture. Immunoprecipitates were washed five times with lysis buffer, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblot analysis with the indicated antibody or used for kinase activity assays.

Kinase activity assays. PKCζ and IKK activity assays were performed as previously described (41). Uninfected control and infected cells were washed with PBS and lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $CaCl_2$, 1% Triton X-100, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin by rocking for 30 min on ice. Extracts were centrifuged at 15,000 *g* for 15 min at 4°C, and 1 mg of protein was incubated with 10 μl of PKCζ antibodies overnight at 4°C. The immune complexes were incubated with 50 μl of protein A beads overnight at 4°C with gentle rotation. Immunoprecipitates were washed seven times with lysis buffer modified to contain 500 mM NaCl and incubated with 2 μg of myelin basic protein (MBP) and 10 μCi of [γ-^{32}P]ATP for 30 min at 37°C in kinase buffer (in mM: 35 Tris, pH 7.5, 10 $MgCl_2$, 5 EGTA, 1 $CaCl_2$, and 1 phenylphosphate) for PKCζ activity assays. Proteins were separated by 20% SDS-PAGE, gels were dried, and MBP was detected as a 20-kDa band by autoradiography.

For IKK activity assays, immunoprecipitates were incubated with 2 μg of glutathione *S*-transferase (GST)-tagged IκBα produced in *E. coli* (Santa Cruz Biotechnology) and 10 μCi of [γ-^{32}P]ATP for 30 min at 37°C in kinase buffer (in mM: 35 Tris, pH 7.5, 10 $MgCl_2$, 5 EGTA, 1 $CaCl_2$, and 1 phenylphosphate). Kinase reaction mixtures were separated by 12% SDS-PAGE, gels were dried, and phosphorylated rIκBα was detected as a 70-kDa band by autoradiography.

Coimmunoprecipitation. Proteins were extracted for coimmunoprecipitation with lysis buffer (in mM: 40 Tris, pH 8.0, 300 NaCl, 6 EDTA, 6 EGTA, 10 β-glycerophosphate, 10 NaF, 10 *p*-nitrophenylphosphate, 1 benzamidine, 2 PMSF, and 1 dithiothreitol with 0.1% Nonidet P-40, 300 μM Na_3VO_4, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). One milligram of whole cell lysate was incubated with ten micrograms of PKCζ, anti-phosphoserine, or p65 antibody (Zymed) and then twenty-five microliters of protein A beads for 2 h at 4°C. Immunoprecipitates were washed five times with lysis buffer. Samples were separated by 12% SDS-PAGE and transferred to membranes, and immunoblot analysis was performed with the appropriate antibody.

Statistical analysis. Data represent means ± SE. Data comparisons were made with either ANOVA or Student's *t*-test. Differences were considered significant at $P \leq 0.05$.

RESULTS

EPEC-induced inflammation in intestinal epithelial cells is regulated by PKC. Others have reported that EPEC infection of host cells activates PKC (3, 10). We showed previously (44) that general PKC inhibitors reduce EPEC-induced IL-8 production in intestinal epithelial cells. PKC activation, depending on the specific isoform, can be calcium dependent or independent. In our model system of cultured intestinal epithelial cells, EPEC infection elevates intracellular calcium; however, calcium is not involved in the associated IL-8 production (47), suggesting that calcium-independent PKC isoforms are responsible. To define which PKC isoforms are involved in EPEC-associated inflammation, we initially used inhibitors for different PKC groups. General PKC inhibition with BIM attenuated EPEC-induced IκBα phosphorylation, as shown in Fig. 1. In contrast, inhibition of only calcium-dependent PKC isoforms with Gö-6976 did not significantly affect this event (Fig. 1), confirming our hypothesis that calcium-dependent PKCs are not involved. These data suggest that novel or atypical isoforms contribute to EPEC-induced inflammation in intestinal epithelial cells.

EPEC infection induces translocation of aPKCζ in intestinal epithelial cells. The atypical isoform PKCζ has been reported to be involved in the activation of NF-κB (12, 17, 28) and ERK (6, 7). Both NF-κB and ERK are activated by EPEC and involved in inflammation in intestinal epithelial cells (11, 47). Therefore, we focused on PKCζ and its role in EPEC-induced inflammation in intestinal epithelial cells. To initially address this issue, we examined the impact of EPEC infection on the localization of PKCζ. Cell lysates from control and infected T84 and Caco-2 monolayers were collected at 30 and 60 min after infection and then fractionated into cytosolic and membrane pools as previously described (5). In uninfected intestinal epithelial cells the majority of PKCζ was found in the cytosol, but after EPEC infection, progressive translocation to the membrane fraction was seen (Fig. 2, *A* and *B*). Immunostaining of control and EPEC-infected T84 cells confirmed that the PKCζ redistributed to the periphery of cells, consistent with translocation to the

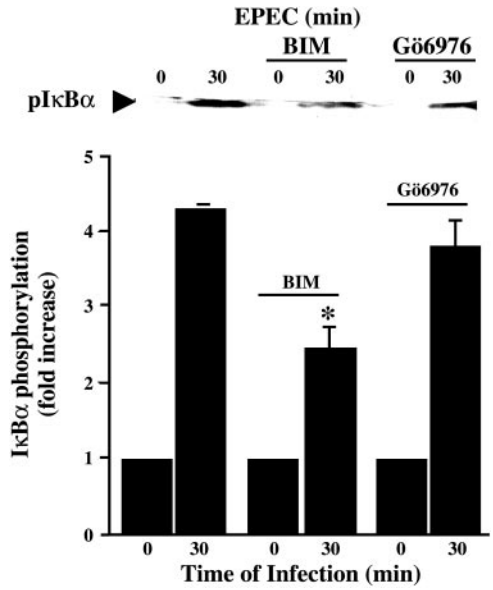

Fig. 1. Enteropathogenic *Escherichia coli* (EPEC)-induced IκBα phosphorylation involves calcium-independent PKCs. T84 cells were preincubated with inhibitors and infected with EPEC for 30 min, and extracted proteins were immunoblotted for phosphorylated IκBα. The representative immunoblot and corresponding bar graph show that the general PKC inhibitor bisindolylmaleimide (BIM) at a concentration of 5 μM significantly attenuates EPEC-induced IκBα phosphorylation. In contrast, inhibition of calcium-dependent PKCs with Gö-6976 (10 μM) had no effect. Densitometric analysis of 3 immunoblots is represented by the bar graph. Data are expressed as mean ± SE fold increases in IκBα phosphorylation compared with control. *Statistically significant difference in EPEC-induced IκBα phosphorylation in the absence vs. the presence of BIM ($P = 0.006$).

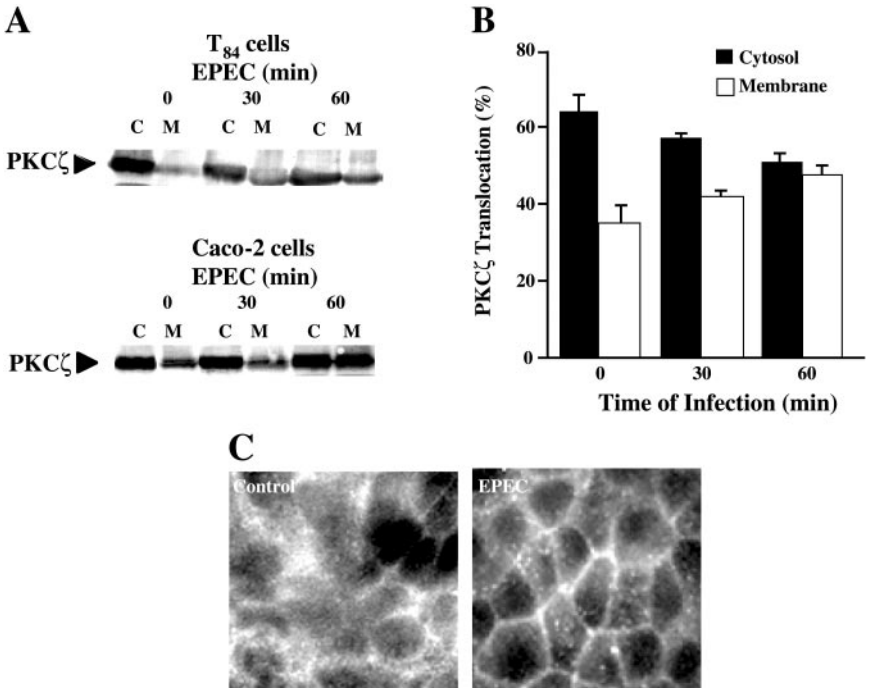

Fig. 2. EPEC infection induces the translocation of PKCζ from the cytosol to the membrane of intestinal epithelial cells. *A*: immunoblot analysis of cytosolic (C) and membrane (M) protein fractions from T84 and Caco-2 cells infected with EPEC. Progressive translocation of PKCζ is evident in both cell lines after EPEC infection. *B*: densitometric analysis of 3 immunoblots is shown as mean ± SE percentages of total PKCζ in the cytosolic and membrane fractions. *Statistically significant difference in the amount of PKCζ in the membrane fraction after EPEC infection compared with control ($P = 0.01$ at 60 min). *C*: immunofluorescence staining of PKCζ in control and EPEC-infected (60 min) T84 cells confirmed the translocation of PKCζ to the membrane of the cells.

membrane after infection (Fig. 2*C*). Similar PKCζ translocation was obtained in Caco-2 cells after EPEC infection (data not shown).

EPEC infection activates PKCζ in intestinal epithelial cells. Translocation of PKCζ from the cytosolic to the membrane fraction after EPEC infection suggests activation of this molecule. To determine whether PKCζ activity is increased after infection, PKCζ kinase activity assays were performed. Figure 3 shows that PKCζ activity, determined as phosphorylation of MBP, increased 1.4 ± 0.2-fold by 15 min after infection, reached a plateau of 2.9 ± 1.2-fold after 30 min, and remained activated for up to 60 min. It should be noted that although PKCζ kinase activity was significantly increased by 15 min after infection, PKCζ translocation did not reach statistical significance until 60 min, although the trend was apparent at 30 min. It is possible that the discrepancy between PKCζ activity and translocation results from the different sensitivities of the techniques used. That is, cell fractionation and immunoblotting used to detect translocation are less sensitive than kinase activity assays that use radiolabeled ATP to measure phosphorylation. Nonetheless, these data show that EPEC infection induces not only the translocation but also the activation of PKCζ in intestinal epithelial cells.

EPEC-activated PKCζ is involved in regulation of the NF-κB pathway. Many investigators using a variety of model systems have reported a role for PKCζ in the regulation of NF-κB (12, 28, 39, 53). To define whether PKCζ is involved in the EPEC-induced activation of the NF-κB pathway, we used three different approaches. First, we used the pharmacological inhibitor rottlerin, which at high concentrations inhibits PKCζ. Figure 4*A* shows that rottlerin inhibits PKCζ translocation in response to EPEC infection. Additionally, the same concentration of rottlerin significantly suppressed EPEC-induced IκBα phosphorylation (Fig. 4*B*), suggesting the involvement of this isoform in the activation of the inflammatory cascade by this enteric pathogen. However, the mechanisms by which rottlerin blocks PKC are not defined. A recent publication (52)

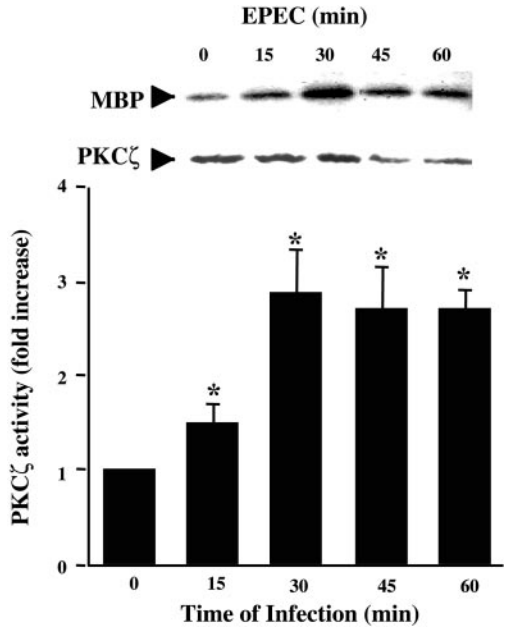

Fig. 3. EPEC infection activates PKCζ in intestinal epithelial cells. This representative autoradiogram shows that immunoprecipitated PKCζ from EPEC-infected T84 cells phosphorylates myelin basic protein (MBP) in a kinase activity assay. Bar graph represents means ± SE of densitometric data from autoradiograms of 4 independent experiments. *Statistically significant difference in PKCζ kinase activity analyzed by ANOVA between control and infected samples ($P = 0.01$ for control vs. 30 min after infection).

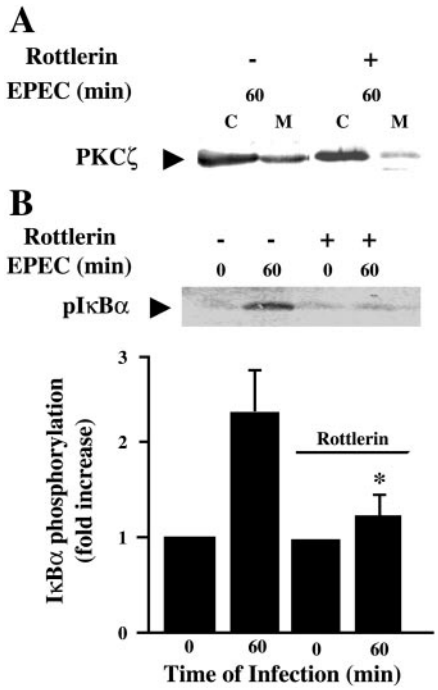

Fig. 4. Inhibition of PKCζ with rottlerin attenuates EPEC-induced PKCζ translocation and IκBα phosphorylation. *A*: fractionated proteins from T84 cells treated with rottlerin (100 μM) and infected with EPEC were separated by SDS-PAGE and immunoblotted for PKCζ. Rottlerin significantly reduced PKCζ translocation. *B*: rottlerin-treated cells were also examined for IκBα phosphorylation by EPEC. The representative immunoblot shows that rottlerin also inhibits EPEC-induced phosphorylation of IκBα. The bar graph represents densitometric analysis of 3 independent immunoblots. *Statistically significant difference in EPEC-induced IκBα phosphorylation in the absence vs. the presence of rottlerin ($P = 0.01$).

suggests that rottlerin decreases intracellular ATP, thus indirectly blocking PKCδ while the inhibitory mechanisms for the other isoforms remain unclear. Therefore, we used a second, more specific approach to define the role of this isoform in NF-κB activation. For these experiments, we used MYR-PKCζ-PS, which binds to the active center of PKCζ, thus inhibiting its activity. Figure 5*A* confirms the inhibitory capacity of MYR-PKCζ-PS by showing that treatment of cells with this peptide decreases PKCζ activity. The EPEC-induced increase in PKCζ activity was suppressed ~40% by MYR-PKCζ-PS. Furthermore, inhibition of PKCζ with MYR-PKCζ-PS significantly attenuated EPEC-induced IκBα phosphorylation (Fig. 5*B*). After 30 min following EPEC infection, IκBα phosphorylation increased 2.5 ± 0.1-fold above that seen in uninfected controls. Pretreatment with MYR-PKCζ-PS diminished this response to 1.5 ± 0.3-fold above that measured in MYR-PKCζ-PS-treated controls. The third approach used in this study was transient transfection of cells with a plasmid containing the PKCζ gene harboring a mutation in kinase domain. Although the transfection efficiency of T84 cells is low (43), expression of this nonfunctional PKCζ attenuated by ~30% the phosphorylation of IκBα in response to EPEC (Fig. 5*C*). Similar results were obtained with Caco-2 cells (data not shown).

PKCζ is not involved in EPEC activation of the ERK pathway in intestinal epithelial cells. We (47) and others (11) reported recently that EPEC infection activates ERK, which is involved in the activation of the NF-κB pathway in intestinal epithelial cells. The best-described pathway leading to ERK activation is the Ras-Raf-MEK-ERK kinase cascade (7, 12, 16, 17, 28, 53). Another possible activator of MEK is PKCζ (7, 12, 16, 17, 28, 53, 56b). Therefore, we questioned whether EPEC activation of ERK was PKCζ dependent or independent. To address this, intestinal epithelial cells were preincubated with MYR-PKCζ-PS and EPEC-induced ERK phosphorylation was assessed. Although this inhibitory peptide suppressed PKCζ kinase activity and attenuated IκBα phosphorylation, it had no effect on EPEC-induced ERK phosphorylation (Fig. 6*A*). These data suggest that PKCζ is not involved in ERK activation by EPEC. To further substantiate that ERK activation was independent of PKCζ, the effect of EPEC infection on an upstream kinase in the ERK pathway, Raf, was investigated. Figure 6*B* shows that EPEC indeed activates Raf, implying that ERK activation is a result of these upstream regulators and not of PKCζ. These data suggest, therefore, that the upstream activation of PKCζ and ERK is independent but the downstream effects of these pathways converge to stimulate proinflammatory events.

EPEC-activated PKCζ regulates NF-κB by interacting with and activating IKKα/β. There are two possible mechanisms by which PKCζ can regulate the NF-κB pathway: *1*) through its interaction with and subsequent activation of IKK (12, 28) and *2*) by phosphorylation of the p65 subunit of NF-κB, which increases its transcriptional activity (1, 23, 31). To determine whether PKCζ interacts with IKK after infection with EPEC, coimmunoprecipitation experiments were performed. Figure 7*A* shows that there is a 1.4-fold increase in PKCζ and IKK association at 30 min and a 3-fold increase at 60 min after infection. The only substrate of IKK is IκBα. To determine whether the interaction of PKCζ with IKK in response to EPEC infection led to its activation, immunoprecipitated PKCζ-IKK complexes from uninfected and EPEC-infected intestinal epithelial cells were assayed for kinase activity with recombinant GST-tagged IκBα as a substrate. Figure 7*B* shows that GST-IκBα phosphorylation increased 2.5-fold in T84 cells after 30 min of EPEC infection. These findings suggest that EPEC infection enhances the interaction between IKK and PKCζ and activates IKK, leading to increased phosphorylation of the IKK-specific substrate IκBα.

The other mechanism by which PKCζ can influence NF-κB is through the phosphorylation of several serine residues in the p65 subunit (1, 23, 31). The effect of EPEC infection on p65 serine phosphorylation was therefore examined. Figure 8, *A* and *B*, shows that there is a significant increase in the serine phosphorylation of p65 in intestinal epithelial cells after EPEC infection. Pretreatment of cells with MYR-PKCζ-PS, however, did not consistently reduce this response

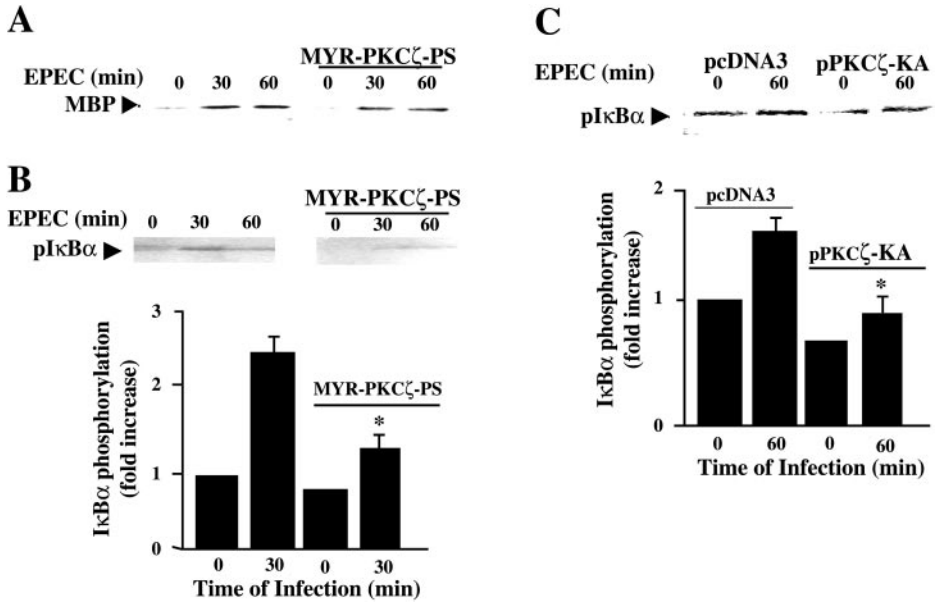

Fig. 5. The inhibitory peptide myristoylated PKCζ pseudosubstrate (MYR-PKCζ-PS) significantly reduces EPEC-induced PKCζ activity and IκBα phosphorylation. *A*: inhibitory MYR-PKCζ-PS (20 μM) partially suppressed PKCζ activity in response to EPEC infection with an in vitro kinase reaction with MBP as the substrate. *B*: MYR-PKCζ-PS significantly decreased EPEC-induced IκBα phosphorylation. The bar graph represents densitometric analysis of 3 independent experiments. *Statistically significant difference ($P = 0.02$). *C*: expression of a dominant-negative PKCζ attenuates EPEC-induced IκBα phosphorylation. Intestinal T84 cells were transiently transfected with the empty pcDNA3 vector or the construct containing the PKCζ gene (pPKCζ-KA) harboring a mutation in the kinase domain. Transfected monolayers were then infected with EPEC. Expression of dominant-negative PKCζ suppressed EPEC-induced phosphorylation of IκBα. The bar graph represents densitometric analysis of 3 separate experiments. *Statistically significant difference in EPEC-induced IκBα phosphorylation in cells transfected with empty vector vs. the nonfunctional kinase ($P = 0.02$).

(Fig. 8*C*), suggesting that other signaling molecules are likely involved at this level of NF-κB regulation.

DISCUSSION

We showed previously (46) that infection of intestinal epithelial cells with EPEC induces the transepithelial migration of acute inflammatory cells. The transmigration of neutrophils is directed by the polarized secretion of IL-8, whose expression is regulated by NF-κB (45). The steps preceding EPEC-induced NF-κB activation include the phosphorylation and degradation of the inhibitory molecule IκBα (47). We also showed (47) that IκBα phosphorylation and degradation are in part regulated by the EPEC activation of ERK. However, additional signaling pathways including PKC are likely involved (44).

Other investigators have reported that EPEC infection activates conventional, calcium-dependent PKC isoforms in host cells (3, 10). The role of these isoforms in EPEC pathogenesis is, however, unknown. Although the issue of EPEC-induced elevation of intracellular calcium is controversial (2–4), we demonstrated (47)

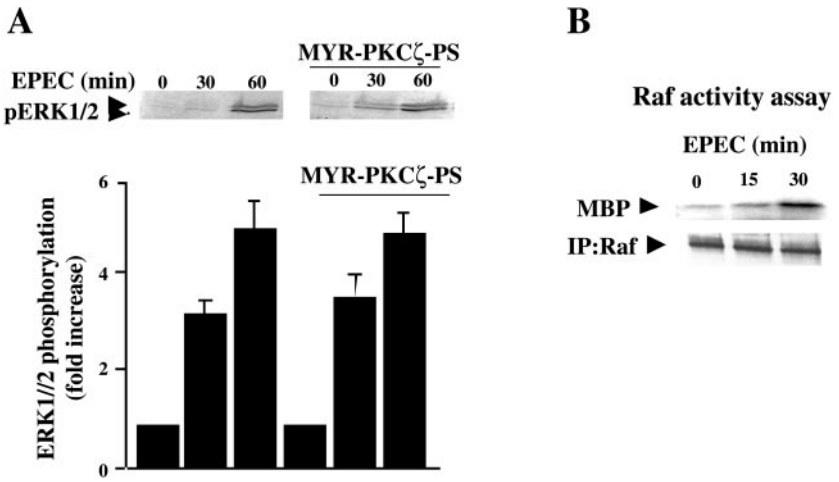

Fig. 6. EPEC-activated ERK in intestinal epithelial cells is PKCζ independent. *A*: immunoblot analysis of proteins from control and infected T84 cells treated with MYR-PKCζ-PS (20 μM) shows that EPEC-activated ERK phosphorylation is independent of PKCζ. Densitometric analysis of immunoblots from 3 independent experiments is represented by the bar graph. Data are expressed as mean ± SE fold increases in ERK phosphorylation compared with control. There were no significant differences in the level of ERK phosphorylation in cells treated with inhibitory MYR-PKCζ-PS compared with controls. *B*: EPEC infection activates Raf kinase from intestinal epithelial cells. The autoradiogram shows that immunoprecipitated Raf (IP:Raf) from EPEC-infected T84 cells phosphorylates MBP, indicating increased kinase activity assay, whereas the immunoblot (*bottom*) shows that the amount of total Raf remains unchanged.

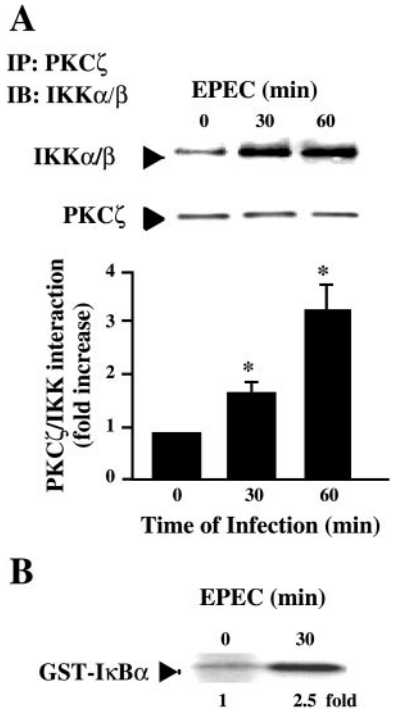

Fig. 7. PKCζ interaction with and activation of IκB kinase (IKK)α/β increases after EPEC infection. *A*: proteins from uninfected and EPEC-infected T84 cells were immunoprecipitated with antibody against PKCζ and immunoblotted (IB) for IKKα/β. The representative immunoblot and corresponding densitometric analysis of 3 independent experiments demonstrate an increased association between PKCζ and IKK after EPEC infection. *Significant ($P < 0.05$) increase in EPEC-induced PKCζ-IKK association. There was no change in the total amount of PKCζ after infection as shown by the PKCζ immunoblot. *B*: IKK activity assays using immunoprecipitated PKCζ-IKK complexes from T84 cells infected with EPEC and recombinant glutathione *S*-transferase (GST)-IκBα as a substrate showed a 2.5-fold increase in the phosphorylation of this IKK-specific substrate.

that EPEC elevates intracellular calcium in T84 cells. We found (47), however, that increased intracellular calcium was not involved in the inflammatory response, suggesting that conventional PKCs do not contribute to this process. We show here instead that calcium-independent PKCs, specifically PKCζ, participate in the EPEC-induced inflammatory response.

It was shown previously that aPKC isoforms play a crucial role in NF-κB activation (7, 12, 16). Thus the blockade of aPKCs with pseudosubstrate peptide inhibitors (16), antisense oligonucleotides (16, 17), or transfection of dominant negative mutants of PKCζ (7, 12, 17) dramatically impairs NF-κB activation. In this report, we show that blocking PKCζ with a pharmacological inhibitor, inhibitory pseudosubstrate peptide, or kinase-defective enzyme significantly attenuated EPEC-induced IκBα phosphorylation in intestinal epithelial cells. A role for PKCζ in a pathogen-associated host response was recently demonstrated in the *Salmonella*-induced stress signaling pathway in macrophages (38). Here, however, we demonstrate for the first time that an enteric pathogen activates PKCζ in intestinal epithelial cells and that it is involved in the inflammatory cascade.

The mechanisms of NF-κB regulation by PKCζ are best defined in response to TNF-α. In this case, PKCζ interacts with IKK, thus activating the latter (12, 17, 28, 53). Another kinase that can activate the NF-κB pathway is NIK (40, 57). NIK communicates with the intracellular domain of TNF-α receptors, thus triggering additional downstream signaling events that ultimately activate IKK (27, 40). NIK can also interact directly with IKKα and IKKβ but phosphorylates and activates only IKKα (27, 40, 57). The aPKCs can also bind to both IKKs but, in contrast to NIK, activate only

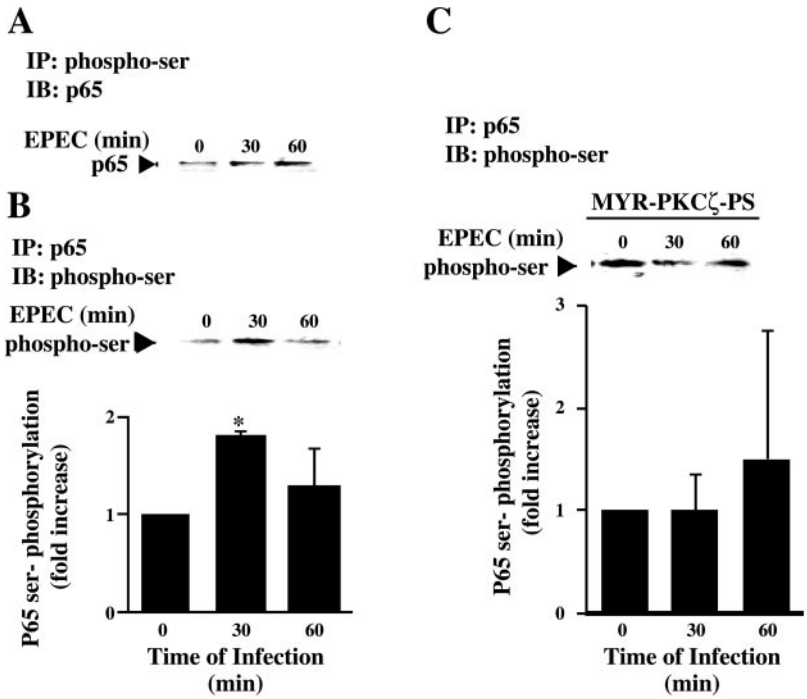

Fig. 8. EPEC infection increases the serine phosphorylation of the p65 subunit of NF-κB, but it is not significantly altered by MYR-PKCζ-PS. Proteins from uninfected and EPEC-infected T84 cells were immunoprecipitated with antibody against phosphorylated serine (phospho-ser) and immunoblotted for p65 (*A*) or immunoprecipitated with p65 and immunoblotted for phosphorylated serine (*B*). EPEC infection significantly enhanced the serine phosphorylation of p65 as shown by the representative immunoblot and corresponding densitometric analysis. Densitometric analysis of immunoblots from 3 independent experiments is represented by the bar graph. Data are expressed as mean ± SE fold increases in phosphorylation compared with control. *Significant difference in EPEC-induced phosphorylation compared with control ($P = 0.02$). *C*: immunoblots of proteins from control or infected T84 cells treated with MYR-PKCζ-PS (20 μM) show that EPEC-induced serine phosphorylation of p65 is not consistently reduced by MYR-PKCζ-PS. This suggests that other signaling pathways may be involved at in this level of NF-κB regulation. Densitometric analysis of 3 independent immunoblots is represented by the bar graph. Data are expressed as mean ± SE fold increases in phosphorylation compared with control.

IKKβ and have no effect on IKKα (23). Additionally, it was shown recently that IKK can be activated by MEK kinase. However, MEK kinase 1 selectively activates IKKβ and has no effect on IKKα (36). Hence, there appear to be specific kinase pathways upstream of the different IKKs that control IκB phosphorylation and NF-κB activation. We have shown in this article that PKCζ regulates NF-κB through binding to and activating IKK. Thus activated IKK phosphorylates IκBα, triggering its degradation and release of the NF-κB molecule. Recently, another mechanism by which PKCζ can regulate NF-κB has been identified, PKCζ-dependent phosphorylation of serine residues in the p65 subunit of NF-κB (1, 23, 31). Other signaling molecules can directly phosphorylate p65, including IKK (42a), Ras (1), and the p38 pathway (55a). We show here that EPEC infection triggers the serine phosphorylation of p65 but could not consistently demonstrate attenuation by inhibition of PKCζ. These findings suggest that other signaling molecules likely contribute to EPEC-induced p65 phosphorylation. Nonetheless, our data clearly reveal the involvement of PKCζ in the upstream activation of NF-κB in intestinal epithelial cells after EPEC infection.

Another level of PKC involvement in inflammation-related signaling is via its effect on ERK. aPKCs have been shown to activate the ERK signaling pathway through Raf-independent mechanisms by interacting with and activating MEK, which then activates both IKKα and IKKβ (20, 26, 33, 34, 39). Although the upstream signaling pathway by which EPEC activates ERK in intestinal epithelial cells is not fully defined, our data suggest that activation of ERK is PKCζ independent and that the Ras/Raf kinase cascade is responsible instead. Therefore, EPEC activation of the proximal signaling pathways, PKCζ and ERK, is independent but converges downstream to ensure stimulation of the proinflammatory response. A variety of pathways likely control the EPEC-induced increase in p65 serine phosphorylation.

An additional role for PKCζ in epithelia is the regulation of tight junctions (21, 37, 54). PKCζ interacts with a number of tight junction-associated proteins including PAR-6 (21, 54) and occludin (37). We reported previously (51) that the localization of tight junction proteins is disrupted after EPEC infection, as is intestinal epithelial barrier function. New data from our laboratory suggest that EPEC-activated PKCζ also participates in the perturbation of barrier function (22), suggesting that this particular signaling molecule, in contrast to MAP kinases (11, 47), is involved in two major physiological effects, inflammation and barrier function.

Over the past several years, a variety of signaling pathways activated in host cells by pathogenic bacteria have been described. Different pathogens can activate different signaling pathways, and a single pathogen can activate a variety of signaling cascades in host cells. The end points of these pathogen-activated signaling networks in host cells include perturbation of physiological processes such as inflammation, barrier function, ion secretion, and cell survival. Additionally, it is clear that a single signaling molecule may be involved in controlling different physiological events. Continued efforts focusing on the elucidation of these pathogen-activated signaling networks and their downstream impact on specific physiological responses will increase our understanding of microbial pathogenesis.

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DISCLOSURES

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