

## PKC $\zeta$ participates in activation of inflammatory response induced by enteropathogenic *E. coli*

Suzana D. Savkovic, Athanasia Koutsouris, and Gail Hecht

Section of Digestive Diseases and Nutrition, Department of Medicine, University of Illinois, West Side Department of Veterans Affairs Medical Center, Chicago, Illinois 60612

Submitted 26 September 2002; accepted in final form 17 April 2003

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

inflammation; enteropathogenic *Escherichia coli*; nuclear factor- $\kappa$ B; protein kinase C $\zeta$ ; I $\kappa$ B 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 secre-

tion of IL-8. Expression of EPEC-induced IL-8 is regulated by the transcription factor NF- $\kappa$ B (45), which is harbored in the cytoplasm through its interaction with a class of inhibitory proteins, I $\kappa$ Bs (19). NF- $\kappa$ B activation and translocation to the nucleus occur after I $\kappa$ B phosphorylation and subsequent degradation by proteasomes (56, 58). Phosphorylation of I $\kappa$ B is attributed to two kinases, I $\kappa$ B kinase (IKK) $\alpha$  and IKK $\beta$  (32, 40). IKK $\alpha$ / $\beta$  may be activated by various upstream signaling molecules such as NF- $\kappa$ B-induced kinase (NIK) (40, 57), MEK (24, 30, 36), PKC and PKA (48, 50, 55), and increased intracellular calcium (18, 55).

Activation of NF- $\kappa$ B 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<sup>2+</sup> (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- $\kappa$ B. We showed (47) that EPEC activates the ERK pathway in host intestinal epithelial cells, which then participates in I $\kappa$ B $\alpha$  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- $\kappa$ B in a number of model systems (6, 8, 12, 39, 53).

aPKCs (aPKC $\zeta$  and - $\lambda$ ) are unresponsive to calcium and play important roles in controlling cell growth and survival (6, 15, 35), most likely by activating ERK and NF- $\kappa$ B 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, PKC $\zeta$  can independently regulate NF- $\kappa$ B through interaction with and activation of IKK (12, 17, 28, 53) or phosphorylation of the p65 subunit of NF- $\kappa$ B (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

Address for reprint requests and other correspondence: G. Hecht, Sect. of Digestive Disease and Nutrition, Dept. of Medicine, Univ. of Illinois, 840 South Wood St., CSB Rm. 738A (m/c 716), Chicago, IL 60612 (E-mail: gahecht@uic.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 PKC $\zeta$  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 aPKC $\zeta$  in target intestinal epithelial cells. PKC $\zeta$  inhibition with pharmacological inhibitors, a specific inhibitory pseudosubstrate, or expression of a dominant-negative PKC $\zeta$  significantly attenuated EPEC-induced I $\kappa$ B $\alpha$  phosphorylation, demonstrating its involvement in the proinflammatory signaling cascade. In contrast, PKC $\zeta$  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 PKC $\zeta$  regulates NF- $\kappa$ B includes activation of IKK. Although the serine phosphorylation of p65 is significantly increased after EPEC infection, inhibition of PKC $\zeta$  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- $\kappa$ B, cells were treated for 1 h before infection with select inhibitors. Bisindolylmaleimide (BIM; Calbiochem, La Jolla, CA) at a concentration of 50  $\mu$ M inhibits many PKC isoforms, whereas Gö-6976 (Calbiochem) at a concentration of 10  $\mu$ M inhibits only calcium-dependent PKCs. Rottlerin (Calbiochem) in lower concentrations (3–6  $\mu$ M) selectively inhibits PKC $\delta$ , whereas higher concentrations (30–45  $\mu$ M) also suppress calcium-dependent PKCs. At a concentration of 100  $\mu$ M, rottlerin also blocks PKC $\zeta$ . The myristoylated PKC $\zeta$  pseudosubstrate (MYR-PKC $\zeta$ -PS; Biosource International, Camarillo, CA) suppresses only the PKC $\zeta$  isoform. Intestinal epithelial cells were incubated with MYR-PKC $\zeta$ -PS (20  $\mu$ M) for 1 h before infection with EPEC.

**Plasmids and cell transfection.** The pcDNA3 and pPKC $\zeta$ -KA dominant negative form of PKC $\zeta$  inserted into pcDNA3 vector were kind gifts from Dr. J. Moscat (Univer-

sidad Autonoma, Madrid, Spain). The PKC $\zeta$  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  $4 \times 10^5$  cells/cm<sup>2</sup> in six-well plates containing complete medium. After 72 h, cells were transfected with 1  $\mu$ g 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 PKC $\zeta$  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<sub>3</sub>VO<sub>4</sub>, 1 PMSF, and 1 NaF with 0.5% DOC, 0.1% NP-40, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin). Proteins (100  $\mu$ g) were subjected to SDS-PAGE and then transferred onto 0.45- $\mu$ m 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 I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated I $\kappa$ B $\alpha$  (New England Biolabs, Beverly, MA), PKC $\zeta$ , 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).

**PKC $\zeta$  translocation.** PKC $\zeta$  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  $\mu$ g/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  $\mu$ g/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 PKC $\zeta$  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  $\beta$ -glycerophosphate, 10 NaF, 10 *p*-nitrophenylphosphate, 1 benzamidine, 2 PMSF, and 1 dithiothreitol with 0.1% Nonidet P-40, 300  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml pepstatin). Extracts were centrifuged at 15,000 *g* for 15 min, and 1 mg of whole cell lysate was incubated with 10  $\mu$ g of the

indicated antibody for 1 h at 4°C. Protein A beads (25  $\mu$ g) 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.** PKC $\zeta$  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<sub>2</sub>, 1% Triton X-100, 10  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/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  $\mu$ l of PKC $\zeta$  antibodies overnight at 4°C. The immune complexes were incubated with 50  $\mu$ l 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  $\mu$ g of myelin basic protein (MBP) and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP for 30 min at 37°C in kinase buffer (in mM: 35 Tris, pH 7.5, 10 MgCl<sub>2</sub>, 5 EGTA, 1 CaCl<sub>2</sub>, and 1 phenylphosphate) for PKC $\zeta$  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  $\mu$ g of glutathione *S*-transferase (GST)-tagged I $\kappa$ B $\alpha$  produced in *E. coli* (Santa Cruz Biotechnology) and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP for 30 min at 37°C in kinase buffer (in mM: 35 Tris, pH 7.5, 10 MgCl<sub>2</sub>, 5 EGTA, 1 CaCl<sub>2</sub>, and 1 phenylphosphate). Kinase reaction mixtures were separated by 12% SDS-PAGE, gels were dried, and phosphorylated rI $\kappa$ B $\alpha$  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  $\beta$ -glycerophosphate, 10 NaF, 10 *p*-nitrophenylphosphate, 1 benzamide, 2 PMSF, and 1 dithiothreitol with 0.1% Nonidet P-40, 300  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml pepstatin). One milligram of whole cell lysate was incubated with ten micrograms of PKC $\zeta$ , 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  $\pm$  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 I $\kappa$ B $\alpha$  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 aPKC $\zeta$  in intestinal epithelial cells.** The atypical isoform PKC $\zeta$  has been reported to be involved in the activation of NF- $\kappa$ B (12, 17, 28) and ERK (6, 7). Both NF- $\kappa$ B and ERK are activated by EPEC and involved in inflammation in intestinal epithelial cells (11, 47). Therefore, we focused on PKC $\zeta$  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 PKC $\zeta$ . 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 PKC $\zeta$  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 PKC $\zeta$  redistributed to the periphery of cells, consistent with translocation to the

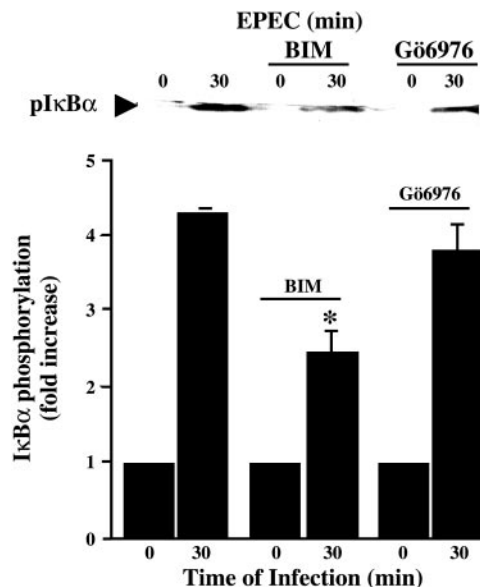


Fig. 1. Enteropathogenic *Escherichia coli* (EPEC)-induced I $\kappa$ B $\alpha$  phosphorylation involves calcium-independent PKCs. T84 cells were preincubated with inhibitors and infected with EPEC for 30 min, and extracted proteins were immunoblotted for phosphorylated I $\kappa$ B $\alpha$ . The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

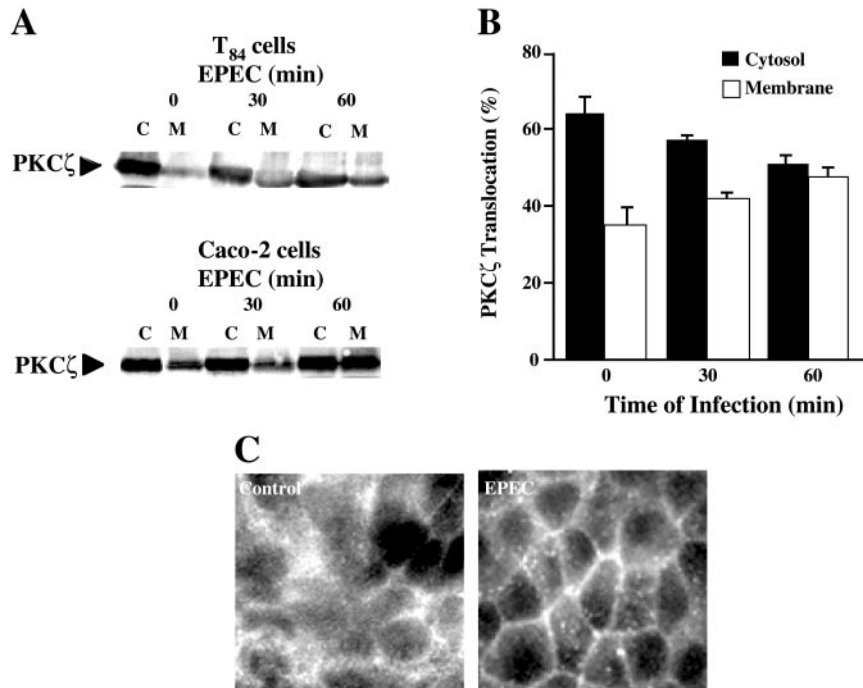


Fig. 2. EPEC infection induces the translocation of PKC $\zeta$  from the cytosol to the membrane of intestinal epithelial cells. **A**: immunoblot analysis of cytosolic (C) and membrane (M) protein fractions from T84 and Caco-2 cells infected with EPEC. Progressive translocation of PKC $\zeta$  is evident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

membrane after infection (Fig. 2C). Similar PKC $\zeta$  translocation was obtained in Caco-2 cells after EPEC infection (data not shown).

*EPEC infection activates PKC $\zeta$  in intestinal epithelial cells.* Translocation of PKC $\zeta$  from the cytosolic to the membrane fraction after EPEC infection suggests activation of this molecule. To determine whether PKC $\zeta$  activity is increased after infection, PKC $\zeta$  kinase activity assays were performed. Figure 3 shows that PKC $\zeta$  activity, determined as phosphorylation of MBP, increased 1.4  $\pm$  0.2-fold by 15 min after infection, reached a plateau of 2.9  $\pm$  1.2-fold after 30 min, and remained activated for up to 60 min. It should be noted that although PKC $\zeta$  kinase activity was significantly increased by 15 min after infection, PKC $\zeta$  translocation did not reach statistical significance until 60 min, although the trend was apparent at 30 min. It is possible that the discrepancy between PKC $\zeta$  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 PKC $\zeta$  in intestinal epithelial cells.

*EPEC-activated PKC $\zeta$  is involved in regulation of the NF- $\kappa$ B pathway.* Many investigators using a variety of model systems have reported a role for PKC $\zeta$  in the regulation of NF- $\kappa$ B (12, 28, 39, 53). To define whether PKC $\zeta$  is involved in the EPEC-induced activation of the NF- $\kappa$ B pathway, we used three different approaches. First, we used the pharmacological inhibitor rottlerin, which at high concentrations inhibits PKC $\zeta$ . Figure 4A shows that rottlerin inhibits PKC $\zeta$  translocation in response to EPEC infection. Additionally, the same

concentration of rottlerin significantly suppressed EPEC-induced I $\kappa$ B $\alpha$  phosphorylation (Fig. 4B), 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)

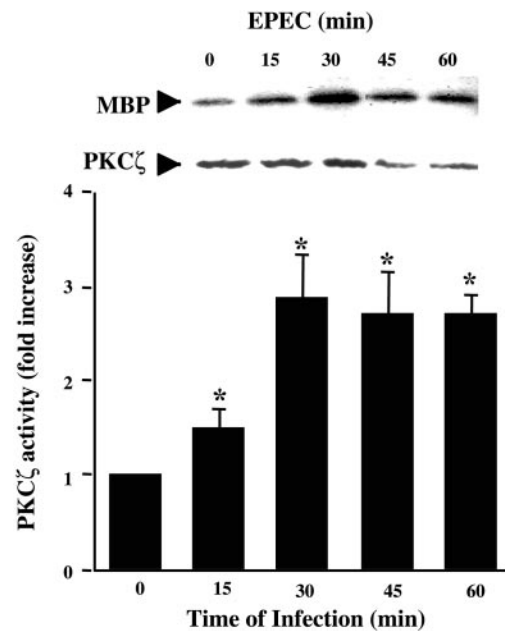


Fig. 3. EPEC infection activates PKC $\zeta$  in intestinal epithelial cells. This representative autoradiogram shows that immunoprecipitated PKC $\zeta$  from EPEC-infected T84 cells phosphorylates myelin basic protein (MBP) in a kinase activity assay. Bar graph represents means  $\pm$  SE of densitometric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

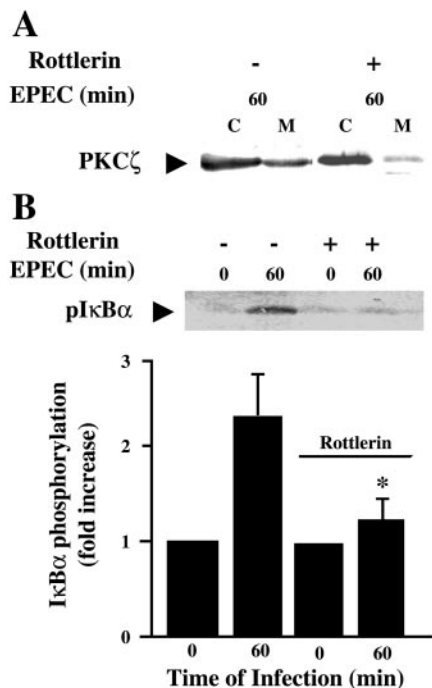


Fig. 4. Inhibition of PKC $\zeta$  with rottlerin attenuates EPEC-induced PKC $\zeta$  translocation and I $\kappa$ B $\alpha$  phosphorylation. *A*: fractionated proteins from T84 cells treated with rottlerin (100  $\mu$ M) and infected with EPEC were separated by SDS-PAGE and immunoblotted for PKC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

suggests that rottlerin decreases intracellular ATP, thus indirectly blocking PKC $\delta$  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- $\kappa$ B activation. For these experiments, we used MYR-PKC $\zeta$ -PS, which binds to the active center of PKC $\zeta$ , thus inhibiting its activity. Figure 5*A* confirms the inhibitory capacity of MYR-PKC $\zeta$ -PS by showing that treatment of cells with this peptide decreases PKC $\zeta$  activity. The EPEC-induced increase in PKC $\zeta$  activity was suppressed  $\sim 40\%$  by MYR-PKC $\zeta$ -PS. Furthermore, inhibition of PKC $\zeta$  with MYR-PKC $\zeta$ -PS significantly attenuated EPEC-induced I $\kappa$ B $\alpha$  phosphorylation (Fig. 5*B*). After 30 min following EPEC infection, I $\kappa$ B $\alpha$  phosphorylation increased  $2.5 \pm 0.1$ -fold above that seen in uninfected controls. Pretreatment with MYR-PKC $\zeta$ -PS diminished this response to  $1.5 \pm 0.3$ -fold above that measured in MYR-PKC $\zeta$ -PS-treated controls. The third approach used in this study was transient transfection of cells with a plasmid containing the PKC $\zeta$  gene harboring a mutation in kinase domain. Although the transfection efficiency of T84 cells is low (43), expression of this nonfunctional PKC $\zeta$  attenuated by  $\sim 30\%$  the phosphorylation of I $\kappa$ B $\alpha$  in response to EPEC (Fig. 5*C*). Similar results were obtained with Caco-2 cells (data not shown).

PKC $\zeta$  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- $\kappa$ B 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 PKC $\zeta$  (7, 12, 16, 17, 28, 53, 56b). Therefore, we questioned whether EPEC activation of ERK was PKC $\zeta$  dependent or independent. To address this, intestinal epithelial cells were preincubated with MYR-PKC $\zeta$ -PS and EPEC-induced ERK phosphorylation was assessed. Although this inhibitory peptide suppressed PKC $\zeta$  kinase activity and attenuated I $\kappa$ B $\alpha$  phosphorylation, it had no effect on EPEC-induced ERK phosphorylation (Fig. 6*A*). These data suggest that PKC $\zeta$  is not involved in ERK activation by EPEC. To further substantiate that ERK activation was independent of PKC $\zeta$ , 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 PKC $\zeta$ . These data suggest, therefore, that the upstream activation of PKC $\zeta$  and ERK is independent but the downstream effects of these pathways converge to stimulate proinflammatory events.

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

The other mechanism by which PKC $\zeta$  can influence NF- $\kappa$ B 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-PKC $\zeta$ -PS, however, did not consistently reduce this response

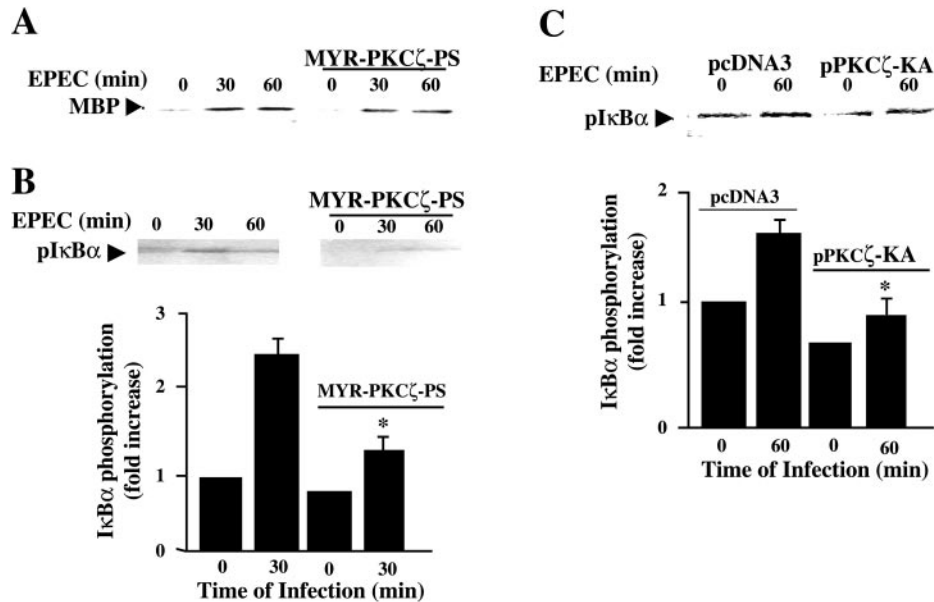


Fig. 5. The inhibitory peptide myristoylated PKC $\zeta$  pseudosubstrate (MYR-PKC $\zeta$ -PS) significantly reduces EPEC-induced PKC $\zeta$  activity and I $\kappa$ B $\alpha$  phosphorylation. **A:** inhibitory MYR-PKC $\zeta$ -PS (20  $\mu$ M) partially suppressed PKC $\zeta$  activity in response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

(Fig. 8C), suggesting that other signaling molecules are likely involved at this level of NF- $\kappa$ B 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- $\kappa$ B (45). The steps preceding EPEC-induced NF- $\kappa$ B activation include the phosphorylation and degrada-

tion of the inhibitory molecule I $\kappa$ B $\alpha$  (47). We also showed (47) that I $\kappa$ B $\alpha$  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)

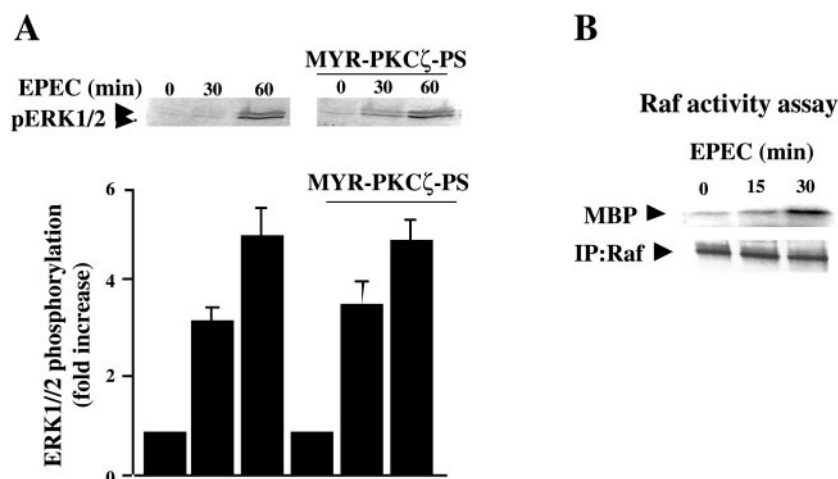


Fig. 6. EPEC-activated ERK in intestinal epithelial cells is PKC $\zeta$  independent. **A:** immunoblot analysis of proteins from control and infected T84 cells treated with MYR-PKC $\zeta$ -PS (20  $\mu$ M) shows that EPEC-activated ERK phosphorylation is independent of PKC $\zeta$ . Densitometric analysis of immunoblots from 3 independent experiments is represented by the bar graph. Data are expressed as mean  $\pm$  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-PKC $\zeta$ -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.

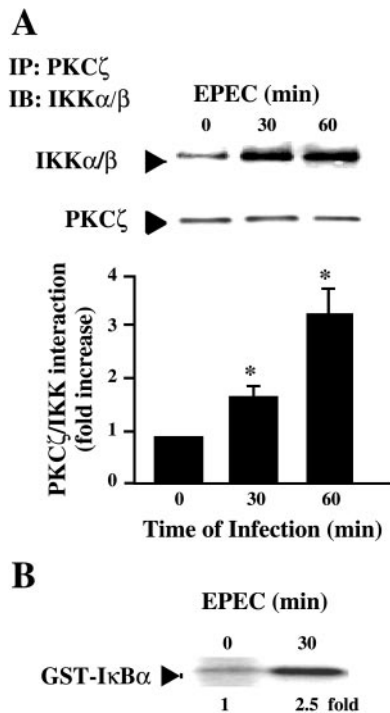


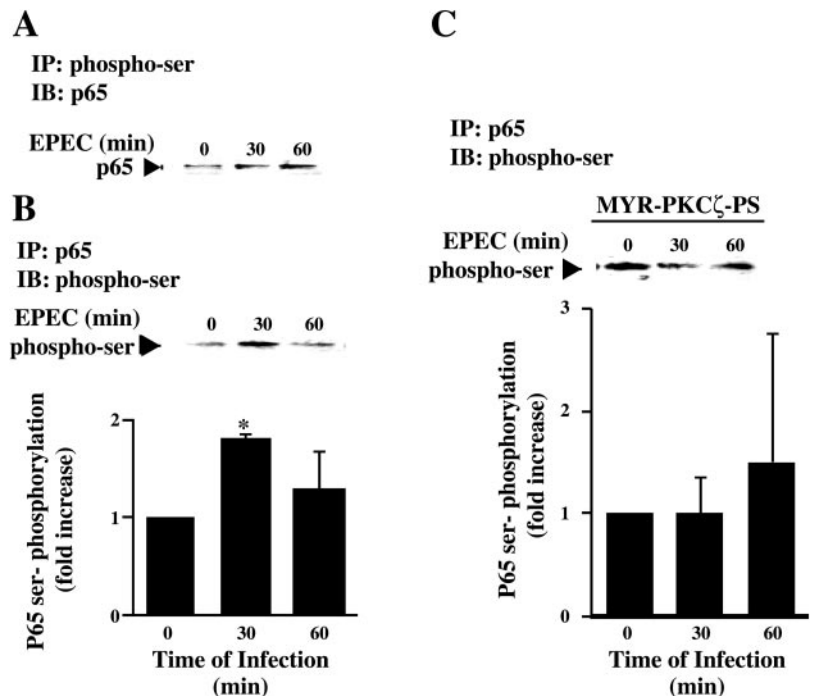
Fig. 7. PKC $\zeta$  interaction with and activation of I $\kappa$ B kinase (IKK) $\alpha/\beta$  increases after EPEC infection. **A**: proteins from uninfected and EPEC-infected T84 cells were immunoprecipitated with antibody against PKC $\zeta$  and immunoblotted (IB) for IKK $\alpha/\beta$ . The representative immunoblot and corresponding densitometric analysis of 3 independent experiments demonstrate an increased association between PKC $\zeta$  and IKK after EPEC infection. \*Significant ( $P < 0.05$ ) increase in EPEC-induced PKC $\zeta$ -IKK association. There was no change in the total amount of PKC $\zeta$  after infection as shown by the PKC $\zeta$  immunoblot. **B**: IKK activity assays using immunoprecipitated PKC $\zeta$ -IKK complexes from T84 cells infected with EPEC and recombinant glutathione *S*-transferase (GST)-I $\kappa$ B $\alpha$  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 PKC $\zeta$ , participate in the EPEC-induced inflammatory response.

It was shown previously that aPKC isoforms play a crucial role in NF- $\kappa$ B 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 PKC $\zeta$  (7, 12, 17) dramatically impairs NF- $\kappa$ B activation. In this report, we show that blocking PKC $\zeta$  with a pharmacological inhibitor, inhibitory pseudosubstrate peptide, or kinase-defective enzyme significantly attenuated EPEC-induced I $\kappa$ B $\alpha$  phosphorylation in intestinal epithelial cells. A role for PKC $\zeta$  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 PKC $\zeta$  in intestinal epithelial cells and that it is involved in the inflammatory cascade.

The mechanisms of NF- $\kappa$ B regulation by PKC $\zeta$  are best defined in response to TNF- $\alpha$ . In this case, PKC $\zeta$  interacts with IKK, thus activating the latter (12, 17, 28, 53). Another kinase that can activate the NF- $\kappa$ B pathway is NIK (40, 57). NIK communicates with the intracellular domain of TNF- $\alpha$  receptors, thus triggering additional downstream signaling events that ultimately activate IKK (27, 40). NIK can also interact directly with IKK $\alpha$  and IKK $\beta$  but phosphorylates and activates only IKK $\alpha$  (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- $\kappa$ B, but it is not significantly altered by MYR-PKC $\zeta$ -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  $\pm$  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-PKC $\zeta$ -PS (20  $\mu$ M) show that EPEC-induced serine phosphorylation of p65 is not consistently reduced by MYR-PKC $\zeta$ -PS. This suggests that other signaling pathways may be involved at in this level of NF- $\kappa$ B regulation. Densitometric analysis of 3 independent immunoblots is represented by the bar graph. Data are expressed as mean  $\pm$  SE fold increases in phosphorylation compared with control.



IKK $\beta$  and have no effect on IKK $\alpha$  (23). Additionally, it was shown recently that IKK can be activated by MEK kinase. However, MEK kinase 1 selectively activates IKK $\beta$  and has no effect on IKK $\alpha$  (36). Hence, there appear to be specific kinase pathways upstream of the different IKKs that control I $\kappa$ B phosphorylation and NF- $\kappa$ B activation. We have shown in this article that PKC $\zeta$  regulates NF- $\kappa$ B through binding to and activating IKK. Thus activated IKK phosphorylates I $\kappa$ B $\alpha$ , triggering its degradation and release of the NF- $\kappa$ B molecule. Recently, another mechanism by which PKC $\zeta$  can regulate NF- $\kappa$ B has been identified, PKC $\zeta$ -dependent phosphorylation of serine residues in the p65 subunit of NF- $\kappa$ B (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 PKC $\zeta$ . These findings suggest that other signaling molecules likely contribute to EPEC-induced p65 phosphorylation. Nonetheless, our data clearly reveal the involvement of PKC $\zeta$  in the upstream activation of NF- $\kappa$ B 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 IKK $\alpha$  and IKK $\beta$  (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 PKC $\zeta$  independent and that the Ras/Raf kinase cascade is responsible instead. Therefore, EPEC activation of the proximal signaling pathways, PKC $\zeta$  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 PKC $\zeta$  in epithelia is the regulation of tight junctions (21, 37, 54). PKC $\zeta$  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 PKC $\zeta$  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.

This work was presented in preliminary form at the annual meeting of the American Gastroenterological Association, Digestive Disease Week 2001 and 2002.

## DISCLOSURES

This work was supported by a Crohn's and Colitis Foundation of America Research Fellowship Award (to S. D. Savkovic), National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-50694 (to G. Hecht), and Merit Review and Research Enhancement Awards from the Department of Veterans Affairs (to G. Hecht).

## REFERENCES

1. Anrather J, Csizmadia V, Soares MP, and Winkler H. Regulation of NF- $\kappa$ B RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells. *J Biol Chem* 274: 13594–13603, 1999.
2. Bain C, Keller R, Collington GK, Trabulsi LR, and Knutton S. Increased levels of intracellular calcium are not required for the formation of attaching and effacing lesions by enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* 66: 3900–3908, 1998.
3. Baldwin TJ, Brooks SF, Knutton S, Manjarrez Hernandez HA, Aitken A, and Williams PH. Protein phosphorylation by protein kinase C in HEp-2 cells infected with enteropathogenic *Escherichia coli*. *Infect Immun* 58: 761–765, 1990.
4. Baldwin TJ, Ward W, Aitken A, Knutton S, and Williams PH. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. *Infect Immun* 59: 1599–1604, 1991.
5. Batlle E, Fabre M, and Garcia de Herreros A. Antipeptide antibodies directed against the C-terminus of protein kinase C zeta (PKC zeta) react with a Ca<sup>2+</sup>- and TPA-sensitive PKC in HT-29 human intestinal epithelial cells. *FEBS Lett* 344: 161–165, 1994.
6. Berra E, Diaz-Meco MT, Dominguez I, Municio MM, Sanz L, Lozano J, Chapkin RS, and Moscat J. Protein kinase C zeta isoform is critical for mitogenic signal transduction. *Cell* 74: 555–563, 1993.
7. Berra E, Diaz-Meco MT, Lozano J, Frutos S, Municio MM, Sanchez P, Sanz L, and Moscat J. Evidence for a role of MEK and MAPK during signal transduction by protein kinase C zeta. *EMBO J* 14: 6157–6163, 1995.
8. Bjorkoy G, Overvatn A, Diaz-Meco MT, Moscat J, and Johansen T. Evidence for a bifurcation of the mitogenic signaling pathway activated by Ras and phosphatidylcholine-hydrolyzing phospholipase C. *J Biol Chem* 270: 21299–21306, 1995.
9. Bjorkoy G, Perander M, Overvatn A, and Johansen T. Reversion of Ras- and phosphatidylcholine-hydrolyzing phospholipase C-mediated transformation of NIH 3T3 cells by a dominant interfering mutant of protein kinase C lambda is accompanied by the loss of constitutive nuclear mitogen-activated protein kinase/extracellular signal-regulated kinase activity. *J Biol Chem* 272: 11557–11565, 1997.
10. Crane JK and Oh JS. Activation of host cell protein kinase C by enteropathogenic *Escherichia coli*. *Infect Immun* 65: 3277–3285, 1997.
11. Czerucka D, Dahan S, Mograbi B, Rossi B, and Rampal P. Implication of mitogen-activated protein kinases in T<sub>84</sub> cell responses to enteropathogenic *Escherichia coli* infection. *Infect Immun* 69: 1298–1305, 2001.
12. Diaz-Meco MT, Berra E, Municio MM, Sanz L, Lozano J, Dominguez I, Diaz-Golpe V, Lain de Lera MT, Alcami J, and Paya CV. A dominant negative protein kinase C zeta



- subspecies blocks NF-kappa B activation. *Mol Cell Biol* 13: 4770–4775, 1993.
13. **Diaz-Meco MT, Dominguez I, Sanz L, Dent P, Lozano J, Municio MM, Berra E, Hay RT, Sturgill TW, and Moscat J.** zeta PKC induces phosphorylation and inactivation of I kappa B-alpha in vitro. *EMBO J* 13: 2842–2848, 1994.
  14. **Diaz-Meco MT, Lozano J, Municio MM, Berra E, Frutos S, Sanz L, and Moscat J.** Evidence for the in vitro and in vivo interaction of Ras with protein kinase C zeta. *J Biol Chem* 269: 31706–31710, 1994.
  15. **Diaz-Meco MT, Municio MM, Frutos S, Sanchez P, Lozano J, Sanz L, and Moscat J.** The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell* 86: 777–786, 1996.
  16. **Dominguez I, Sanz L, Arenzana-Seisdedos F, Diaz-Meco MT, Virelizier JL, and Moscat J.** Inhibition of protein kinase C zeta subspecies blocks the activation of an NF-kappa B-like activity in *Xenopus laevis* oocytes. *Mol Cell Biol* 13: 1290–1295, 1993.
  17. **Folgueira L, McElhinny JA, Bren GD, MacMorran WS, Diaz-Meco MT, Moscat J, and Paya CV.** Protein kinase C-zeta mediates NF-kappa B activation in human immunodeficiency virus-infected monocytes. *J Virol* 70: 223–231, 1996.
  18. **Gewirtz AT, Rao AS, Simon PO Jr, Merlin D, Carnes D, Madara JL, and Neish AS.** *Salmonella typhimurium* induces epithelial IL-8 expression via Ca<sup>2+</sup>-mediated activation of the NF-kappaB pathway. *J Clin Invest* 105: 79–92, 2000.
  19. **Ghosh S and Baltimore D.** Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* 344: 678–682, 1990.
  20. **Guthridge CJ, Eiden D, Arend WP, Gutierrez-Hartmann A, and Smith MF Jr.** Lipopolysaccharide and Raf-1 kinase regulate secretory interleukin-1 receptor antagonist gene expression by mutually antagonistic mechanisms. *Mol Cell Biol* 17: 1118–1128, 1997.
  - 20a. **Hobbie S, Chen LM, Davis RJ, and Galan JE.** Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol* 159: 5550–5559, 1997.
  21. **Izumi Y, Hirose T, Tamai Y, Hirai S, Nagashima Y, Fujimoto T, Tabuse Y, Kempfues KJ, and 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* 143: 95–106, 1998.
  - 21a. **Keates S, Keates AC, Warny M, Peek RM Jr, Murray PG, and Kelly CP.** Differential activation of mitogen-activated protein kinases in AGS gastric epithelial cells by cag+ and cag- *Helicobacter pylori*. *J Immunol* 163: 5552–5559, 1999.
  22. **Koutsouris A, Savkovic SD, and Hecht G.** PKC $\zeta$  is activated in intestinal epithelial cells infected by enteropathogenic *E. coli* and participates in the alteration of tight junction (Abstract). *Gastroenterology* 122: A-56, 2002.
  23. **Lallena MJ, Diaz-Meco MT, Bren G, Paya CV, and Moscat J.** Activation of IkappaB kinase beta by protein kinase C isoforms. *Mol Cell Biol* 19: 2180–2188, 1999.
  24. **Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, and Landvatter SW.** A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372: 739–746, 1994.
  25. **Li JD, Feng W, Gallup M, Kim JH, Gum J, Kim Y, and Basbaum C.** Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells. *Proc Natl Acad Sci USA* 95: 5718–5723, 1998.
  26. **Liao DF, Monia B, Dean N, and Berk BC.** Protein kinase C-zeta mediates angiotensin II activation of ERK1/2 in vascular smooth muscle cells. *J Biol Chem* 272: 6146–6150, 1997.
  27. **Ling L, Cao Z, and Goeddel DV.** NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. *Proc Natl Acad Sci USA* 95: 3792–3797, 1998.
  28. **Lozano J, Berra E, Municio MM, Diaz-Meco MT, Dominguez I, Sanz L, and Moscat J.** Protein kinase C zeta isoform is critical for kappa B-dependent promoter activation by sphingomyelinase. *J Biol Chem* 269: 19200–19202, 1994.
  29. **Madara JL, Stafford J, Dharmasathaphorn K, and Carlson S.** Structural analysis of a human intestinal epithelial cell line. *Gastroenterology* 92: 1133–1145, 1987.
  30. **Malinin NL, Boldin MP, Kovalenko AV, and Wallach D.** MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature* 385: 540–544, 1997.
  31. **Martin AG, San-Antonio B, and Fresno M.** Regulation of nuclear factor kappa B transactivation. Implication of phosphatidylinositol 3-kinase and protein kinase C zeta in c-Rel activation by tumor necrosis factor alpha. *J Biol Chem* 276: 15840–15849, 2001.
  32. **Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, and Rao A.** IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* 278: 860–866, 1997.
  33. **Monick MM, Carter AB, Flaherty DM, Peterson MW, and Hunninghake GW.** Protein kinase C zeta plays a central role in activation of the p42/44 mitogen-activated protein kinase by endotoxin in alveolar macrophages. *J Immunol* 165: 4632–4639, 2000.
  34. **Monick MM, Carter AB, Gudmundsson G, Mallampalli R, Powers LS, and Hunninghake GW.** A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide-stimulated human alveolar macrophages. *J Immunol* 162: 3005–3012, 1999.
  35. **Moscat J and Diaz-Meco MT.** The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. *EMBO Rep* 1: 399–403, 2000.
  36. **Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, and Okumura K.** Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase-1. *Proc Natl Acad Sci USA* 95: 3537–3542, 1998.
  37. **Nusrat A, Chen JA, Foley CS, Liang TW, Tom J, Cromwell M, Quan C, and Mrsny RJ.** The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. *J Biol Chem* 275: 29816–29822, 2000.
  38. **Procyk KJ, Rippe MR, Testi R, Hoffmann F, Parker PJ, and Baccarini M.** Distinct mechanisms target stress and extracellular signal-activated kinase 1 and Jun N-terminal kinase during infection of macrophages with *Salmonella*. *J Immunol* 163: 4924–4930, 1999.
  39. **Rahman A, Anwar KN, and Malik AB.** Protein kinase C- $\zeta$  mediates TNF- $\alpha$ -induced ICAM-1 gene transcription in endothelial cells. *Am J Physiol Cell Physiol* 279: C906–C914, 2000.
  40. **Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, and Rothe M.** Identification and characterization of an IkappaB kinase. *Cell* 90: 373–383, 1997.
  41. **Rust C, Karnitz LM, Paya CV, Moscat J, Simari RD, and Gores GJ.** The bile acid taurochenodeoxycholate activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. *J Biol Chem* 275: 20210–20216, 2000.
  42. **Sajan MP, Standaert ML, Bandyopadhyay G, Quon MJ, Burke TR Jr, and Farese RV.** Protein kinase C-zeta and phosphoinositide-dependent protein kinase-1 are required for insulin-induced activation of ERK in rat adipocytes. *J Biol Chem* 274: 30495–30500, 1999.
  - 42a. **Sakurai H, Chiba H, Miyoshi H, Sugita T, and Toriumi W.** IkB kinases phosphorylate NF- $\kappa$ B p65 subunit on serine 536 in the transactivation domain. *J Biol Chem* 274: 30353–30356, 1999.
  43. **Savkovic S, Koutsouris A, Wu G, and Hecht G.** Infection of intestinal epithelial cells with bacterial pathogens decreases luciferase activity: ramifications for reporter gene studies. *Bio-techniques* 29: 514–522, 2000.
  44. **Savkovic S, Koutsouris A, and Hecht G.** Activation of NF- $\kappa$ B and IL-8 gene expression in response to enteropathogenic *E. coli* infection involves reactive oxygen intermediates and protein kinases (Abstract). *Gastroenterology* 112: A401, 1997.
  45. **Savkovic S, Koutsouris A, and Hecht G.** Activation of NF- $\kappa$ B in intestinal epithelial cells by enteropathogenic *Escherichia coli*. *Am J Physiol Cell Physiol* 273: C1160–C1167, 1997.

46. **Savkovic SD, Koutsouris A, and Hecht G.** Attachment of a non-invasive enteric pathogen, enteropathogenic *Escherichia coli*, to cultured human intestinal epithelial monolayers induces transmigration of neutrophils. *Infect Immun* 64: 4480–4487, 1996.
47. **Savkovic SD, Ramaswamy A, Koutsouris A, and Hecht G.** EPEC-activated ERK1/2 participate in inflammatory response but not tight junction barrier disruption. *Am J Physiol Gastrointest Liver Physiol* 281: G890–G898, 2001.
48. **Schmid RM and Adler G.** NF-kappaB/Rel/IkappaB: implications in gastrointestinal diseases. *Gastroenterology* 118: 1208–1228, 2000.
49. **Schonwasser DC, Marais RM, Marshall CJ, and Parker PJ.** Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isoforms. *Mol Cell Biol* 18: 790–798, 1998.
50. **Schouten GJ, Vertegaal AC, Whiteside ST, Israel A, Toebes M, Dorsman JC, van der Eb AJ, and Zantema A.** IkappaB alpha is a target for the mitogen-activated 90 kDa ribosomal S6 kinase. *EMBO J* 16: 3133–3144, 1997.
51. **Simonovic I, Rosenberg J, Koutsouris A, and Hecht G.** Enteropathogenic *Escherichia coli* dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. *Cell Microbiol* 2: 305–315, 2000.
52. **Soltoff SP.** Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cdelta tyrosine phosphorylation. *J Biol Chem* 276: 37986–37992, 2001.
53. **Sontag E, Sontag JM, and Garcia A.** Protein phosphatase 2A is a critical regulator of protein kinase C zeta signaling targeted by SV40 small t to promote cell growth and NF-kappaB activation. *EMBO J* 16: 5662–5671, 1997.
54. **Stuart RO and Nigam SK.** Regulated assembly of tight junctions by protein kinase C. *Proc Natl Acad Sci USA* 92: 6072–6076, 1995.
55. **Tando Y, Algul H, Wagner M, Weidenbach H, Adler G, and Schmid RM.** Caerulein-induced NF-kB/Rel activation requires both Ca<sup>2+</sup> and protein kinase C as messengers. *Am J Physiol Gastrointest Liver Physiol* 277: G678–G686, 1999.
- 55a. **Vanden BW, Plaisance S, Boone B, De Bosscher MK, Schmitz L, Fiers W, and Haegeman G.** p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-B p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* 273: 3285–3290, 1998.
56. **Verma IM and Stevenson J.** IkappaB kinase: beginning, not the end. *Proc Natl Acad Sci USA* 94: 11758–11760, 1997.
- 56a. **Warny M, Keates AC, Keates S, Castagliuolo I, Zacks JK, Aboudola S, Qamar A, Pothoulakis C, LaMont JT, and Kelly CP.** p38 MAP kinase activation by *Clostridium difficile* toxin A mediates monocyte necrosis, IL-8 production, and enteritis. *J Clin Invest* 105: 1147–1156, 2000.
- 56b. **Widmann C, Gibson M, Jarpe B, and Johnson L.** Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 79: 143–180, 1999.
57. **Woronicz JD, Gao X, Cao Z, Rothe M, and Goeddel DV.** IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science* 278: 866–869, 1997.
58. **Zabel U and Baeuerle PA.** Purified human I kappa B can rapidly dissociate the complex of the NF-kappa B transcription factor with its cognate DNA. *Cell* 61: 255–265, 1990.