Regulation of Protein Kinase C in Escherichia coli K1 Invasion of Human Brain Microvascular Endothelial Cells*

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Sunil K. Sukumaran and Nemani V. Prasadaraoo‡§

From the Division of Infectious Diseases, Childrens Hospital Los Angeles and ‡Keck School of Medicine, University of Southern California, Los Angeles, California 90027

Escherichia coli is one of the most important pathogens involved in the development of neonatal meningitis in many parts of the world. Traversal of E. coli across the blood-brain barrier is a crucial event in the pathogenesis of E. coli meningitis. Our previous studies have shown that outer membrane protein A (OmpA) expression is necessary in E. coli for a mechanism involving actin filaments in its passage through the endothelial cells. Focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K) have also been activated in host cells during the process of invasion. In an attempt to elucidate the mechanisms leading to actin filament condensation, we have focused our attention on protein kinase C (PKC), an enzyme central to many signaling events, including actin rearrangement. In the current study, specific PKC inhibitors, bisindolmaleimide and a PKC-inhibitory peptide, inhibited E. coli invasion of human brain microvascular endothelial cells (HBMEC) by more than 75% in a dose-dependent manner, indicating a significant role played by this enzyme in the invasion process. Our results further showed that OmpA+ E. coli induces significant activation of PKC in HBMEC as measured by the PepTag nonradioactive assay. In addition, we identified that the PKC isoform activated in E. coli invasion is a member of the conventional family of PKC, PKC-α, which requires calcium for activation. Immunocytochemical studies have indicated that the activated PKC-α is associated with actin condensation beneath the bacterial entry site. Overexpression of a dominant negative mutant of PKC-α in HBMEC abolished the E. coli invasion without significant changes in FAK phosphorylation or PI3K activity patterns. In contrast, in HBMEC overexpressing the mutant forms of either FAK or PI3K, E. coli-induced PKC activation was significantly blocked. Furthermore, our studies showed that activation of PKC-α induces the translocation of myristoylated alanine-rich protein kinase C substrate, an actin cross-linking protein and a substrate for PKC-α, from the membrane to cytosol. This is the first report of FAK- and PI3K-dependent PKC-α activation in bacterial invasion related to cytoskeletal reorganization.

Among the several pathogenic bacteria causing meningitis in neonates, E. coli is the most common. The mortality and morbidity associated with this infection is significantly high, with the case fatality rates ranging from 15 to 40% (1–4). This poor outcome can be attributed to the insufficient understanding of the pathogenesis of this highly prevalent pathogen, which hinders the development of new therapeutic strategies. Traversal of E. coli across the blood-brain barrier is a crucial event in the pathogenesis; however, the mechanisms underlying this process have yet to be clearly elucidated.

Cell culture models have been used to dissect the pathogenic process into discrete events involving one or more virulence associated factors and their interplay with the host cell surface structures (5, 6). Using an in vitro model, human brain microvascular endothelial cell (HBMEC) culture, we have demonstrated that E. coli invasion is a multifactorial process and involves important virulence factors such as S-fimbriae, OmpA, IbeA, and IbeB (7–10). Although the receptors for the OmpA and IbeA on the surface of brain microvascular endothelial cells have been characterized, it is not clear whether these factors act independently or provide synergistic support during invasion (11). Following the initial interaction between the host and E. coli, the bacteria is progressively drawn into the host by a “zipper mechanism,” which utilizes the bacterium-induced actin filaments for bacterial uptake (12). Our previous studies showed that E. coli induces actin condensation beneath the bacterial entry site, which contains highly phosphorylated proteins including focal adhesion kinase (FAK) and paxillin, a cytoskeletal protein associated with FAK (13). FAK, a 125-kDa nonreceptor tyrosine kinase, plays a crucial role in the assembly of signaling complexes, thereby mediating F-actin rearrangements in the cell (14). The activated FAK interacts with the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), p85 for further signaling events that are necessary for actin reorganization (15). However, it is not known which other signaling molecules play a role in E. coli invasion of HBMEC. Here we focused on protein kinase C (PKC), an enzyme central to many signaling events leading to major alterations in the cytoskeletal framework.

PKC is a ubiquitous phospholipid-dependent serine/threonine kinase involved in major signaling events that regulate cellular growth, migration, permeability, proliferation, apoptosis, and a wide variety of biological responses to stimuli (16). Based on their structure, 12 different isoforms of PKC have been identified and are divided into three groups: (i) classical or

The abbreviations used are: HBMEC, human brain microvascular endothelial cells; PKC, protein kinase C; PKC-CAT/KR, dominant negative PKC-α; MARCKS, myristoylated alanine-rich C kinase substrate; FAK, focal adhesion kinase; FRNK, FAK dominant negative mutant; PI3K, phosphatidylinositol 3-kinase; BIM, bisindolmaleimide; Akt, protein kinase B; cfu, colony forming units; E44, spontaneous rifampin-resistant mutant of strain RS218 (018:K1:H7); E91, a derivative of E44 in which ompA was disrupted; CAT, catalytic domain; DAG, diacylglycerol; PKC-WT, wild type PKC; FAK-WT, wild type FAK.

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‡ To whom correspondence should be addressed: Division of Infectious Diseases, MS 51, Childrens Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027. Tel.: 323-669-5465; Fax: 323-660-2661; E-mail: pnemani@chla.usc.edu.

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conventional PKC (α, βI, βII, γ), which are activated by diacylglycerol (DAG) or calcium; (ii) novel PKCs (δ, η, θ, μ, ε, ζ), which can be activated by DAG but not calcium; and (iii) atypical PKCs (ζ, ν, ν), which are not responsive to either DAG or calcium. Unphosphorylated PKC is associated with the membrane, where it is phosphorylated on its activation loop by external stimuli. The immediate consequence of this event is the autophosphorylation of two positions in the carboxyl terminus (C-1 and C-2) of PKC. The phosphorylated enzyme is maintained in its inactive conformation in the cytosol by binding to an autoinhibitory pseudosubstrate. PKC attains its active conformation when DAG recruits the inactive PKC to the membrane in a calcium-dependent manner, where membrane binding provides sufficient energy to disengage the pseudosubstrate, thus exposing the substrate-binding site.

Myristoylated alanine-rich protein kinase C substrate (MARCKS) is a primary and prominent protein substrate for PKC, which binds calmodulin in a calcium-dependent manner and also binds to phospholipid membrane (17–22). MARCKS has been shown to bind to and cross-link filamentous actin and has been implicated in cellular processes associated with cytoskeletal restructuring, specifically by regulating actin-membrane interaction (23). MARCKS is an acidic protein of 309 amino acids with an apparent molecular mass of 65 kDa. It contains three conserved domains: (i) a basic effector, (ii) phosphorylation site domain, and (iii) a myristoylated amino-terminal domain, which, along with the phosphorylation site domain, helps anchor the protein to the inner surface of the plasma membrane. MARCKS has the capacity to cycle between the membrane and the cytosol. The nonphosphorylated protein associates tightly with the membranes by the cooperative binding energies contributed by the insertion of the myristic acid moiety into the lipid bilayer and by the electrostatic interaction of the basic effector domain with acidic phospholipids. PKC-dependent phosphorylation introduces negative charges into the positively charged effector domain, thereby neutralizing the electrostatic interaction, which results in the displacement of MARCKS from the membrane. In addition to its role in associating MARCKS with the membrane, the phosphorylation domain of the MARCKS protein serves as an important binding site for both F-actin and calmodulin (24). Thus, MARCKS plays a significant role in translating extracellular signals to events associated with actin plasticity and actin plasma membrane interactions like phagocytosis, membrane trafficking, membrane ruffling, and formations of lamellipodia (19, 25).

In this report, we show that PKC-α is autophosphorylated and translocates to the membrane upon *E. coli* invasion of HBMEC. The activated PKC-α co-localizes with actin condensation points induced by invasive *E. coli* at the bacterial entry site. Overexpression of a dominant negative form of PKC-α significantly inhibits the *E. coli* invasion, PKC activation, and its association with actin. In addition, the data suggest that the PKC-α activation is FAK- and PI3K-dependent and probably acts downstream of PI3K. The activation of MARCKS, a substrate for PKC-α, and its translocation to the cytosol from the membrane have been highlighted.

**EXPERIMENTAL PROCEDURES**

**Bacteria—** *E. coli* E44 is a rifampin-resistant mutant of *E. coli* K1 strain RS 218 (serotype O18:K1:H7), which has been isolated from the cerebrospinal fluid of a neonate with meningitis and invades HBMEC in a cell culture model (8). E49 is a noninvasive derivative of E44 that expresses no OmpA, since the ompA gene is disrupted. HB101 (K-12 capsular polysaccharide), a laboratory strain, is noninvasive in HBMEC. All bacteria were grown in brain heart infusion broth with appropriate antibiotics as necessary. All bacterial media were purchased from Difco.

**Materials—** Monoclonal antibodies to various isoforms of PKC were purchased from Cell Signaling Technology Inc. (Beverly, MA). Antibodies to FAK, PKC-α, and phospho-Akt were obtained from Transduction Laboratories (Lexington, KY). Fluorescein isothiocyanate-conjugated secondary antibodies and rhodamine phalloidin were obtained from Molecular Probes, Inc. (Eugene, OR). Cy2-conjugated secondary antibody was obtained from Rockland Immunochemicals (Gilbertsville, PA). Monoclonal anti-actin antibody was obtained from Sigma Chemical Products (Boston, MA). Normal goat serum and the Vectashield mounting medium with 4',6-diamidino-2-phenylindole were obtained from Vector Laboratories Inc. (Burlingame, CA). Monoclonal anti-MARCKS and anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). PKC inhibitors, LY 249002 and its analog LY 305511, phosphol-12-myristate 13-acetate, BIM, and PKC inhibitory peptide were obtained from Calbiochem. The PepTag nonradioactive PKC assay was obtained from Promega (Madison, WI). SuperSignal chemiluminescence reagent was obtained from Pierce. Mammalian expression vectors for PKC were described previously (26). Both wild type and mutant PKC proteins were cloned into pcDNAs, which provides G418 resistance to transfected cells. LipofectAMINE was obtained from Invitrogen. All other chemicals were obtained from Sigma.

**HBMEC Culture Maintenance and Transfections—** HBMEC were isolated and cultured as described earlier (7). HBMEC cultures were routinely grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 10% Nu-serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 units/ml streptomycin (100 μg/ml), 50 μM 2-mercaptoethanol, and vitamins. HBMEC were transfected with mammalian expression vectors using LipofectAMINE. Briefly, DNA-LipofectAMINE in RPMI 1640 was added to 50% confluent HBMEC monolayers. After 6 h of incubation at 37 °C, the cells were washed with RPMI 1640, and complete medium was added. Three days following transfection, HBMEC were transferred to medium containing the antibiotic G418 (400 μg/ml) for 3 weeks, and the antibiotic-resistant colonies were either pooled or separated into individual clones for further analysis.

**E. coli Invasion Assays—** Confuent HBMEC in 24-well plates were incubated with 1 × 10⁹ *E. coli* in experimental medium (1:1 mixture of Ham’s F-12 and M-199 containing 5% heat-inactivated fetal bovine serum) for 90 min at 37 °C. Monolayers were washed three times with RPMI 1640 and incubated in experimental medium containing gentamicin (100 μg/ml) for 1 h to kill extracellular bacteria. The monolayers were washed again and lysed with 0.5% Triton X-100. The intracellular bacteria were enumerated by plating on sheep blood agar plates. In some experiments, HBMEC were pretreated with various inhibitors for 30 min prior to the addition of bacteria. Effects of these inhibitors on HBMEC were assessed by the trypan blue exclusion method, and effects on cellular viability were measured by trypan exclusion.

**Preparation of Cytosolic and Membrane Fractions for PKC Assay**—For detection of PKC activity from the infected monolayers, confluent monolayers of HBMEC grown on collagen-coated dishes (60-mm diameter) were washed with RPMI 1640, and *E. coli* suspended in the experimental medium was added. Following stimulation for different time periods (0, 5, 10, 15, and 30 min) the cells were rinsed twice in ice-cold phosphate-buffered saline and placed on ice. The monolayers were then washed 60-mm dish were harvested by scraping on ice into 2 ml of cell homogenization buffer (buffer A) consisting of 20 mM Tris (pH 7.5), 0.25 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, and 2 mM dithiothreitol. The cells were subjected to mild sonication, and the cell lysates were used for the PepTag assay. To obtain the membrane and cytosolic fractions, the cell lysates in the above buffer were initially centrifuged at 10,000 × g to remove the debris followed by centrifugation at 100,000 × g at 4 °C for 45 min. The supernatant from this step was designated as the cytosolic fraction, and the pellet was designated as the membrane fraction.

**PepTag Assay for Nonradioactive Detection of PKC Activity**—The PepTag assay utilizes a brightly colored, florescent peptide substrate that is highly specific to PKC (according to the manufacturer’s instructions (Promega)). Phosphorylation by PKC changes the net charge of the substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated versions of the substrate to be separated on an agarose (0.8%) gel. The phosphorylated species migrates toward the negative electrode. The phosphorylated and nonphosphorylated bands in the gel were visualized under UV light. HBMEC total cell lysates or membrane proteins (10–25 μg in 10 μl) were incubated with PKC reaction mixture (25 μl) according to the manufacturer’s (Promega) protocol at 30 °C for 30 min. The reactions were stopped by placing the tubes in a boiling water bath. After adding 80% glycerol (1 μl), the samples were loaded onto an agarose gel (0.8% agarose in 50 ml Tris-HCl, pH 8.0). The samples were separated on the agarose gel in the same buffer at
100 V for 15 min, and the bands were visualized under UV light.

Quantitation of PepTag Assay Results by Spectrophotometry—Immediately after photographing the gel (within 10 min), the negatively charged phosphorylated bands were excised using a razor blade, placed in a graduated microcentrifuge tube, and heated at 95 °C until the gel slice melted. The volume of the solution was adjusted to 250 μl with water. The hot agarose solution (175 μl) was added to a separate tube containing 75 μl of gel solubilization solution, 100 μl of glacial acetic acid, and 150 μl of distilled water. The mixture was vortexed and then transferred to a cuvette, and absorbency was read at 570 nm. Using the absorbance, we calculated the number of units of kinase activity in each slice of agarose as per the manufacturer’s instructions (Promega).

Immunoprecipitations and Western Blotting—Total cell lysates of HBMEC exposed to bacteria for varying time periods were centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was collected, and protein content was determined. For immunoprecipitations, 300–500 μg of protein was incubated with the appropriate antibody overnight at 4 °C and then incubated for 1 h with protein A-agarose. The immune complexes were washed four times with cell lysate buffer, and the proteins bound to agarose were eluted in SDS sample buffer for further analysis by Western blotting. Portions of the cell lysates were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, which was then blocked with 5% bovine serum albumin in Tris-buffered saline containing 1% Tween 20 (TBST) for 2 h at room temperature. The blot was then incubated with the primary antibody overnight at 4 °C in 5% bovine serum albumin/TBST. The blot was washed with TBST and further incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the blot was washed four times with TBST for 1 h, developed with SuperSignal chemiluminescence reagent, and exposed to x-ray film to visualize the proteins. The protein bands on the x-ray films were quantitated on a Bio-Rad densitometer using Alpha-imager software (Alpha Innotech Corp., San Francisco, CA).

Immunofluorescence Staining—HBMEC were grown in eight-well chamber slides coated with collagen and infected with E. coli as described above. The monolayers were then washed with phosphate-buffered saline and fixed in 2% paraformaldehyde for 15 min at room temperature. Subsequently, the monolayers were incubated with 5% normal goat serum in phosphate-buffered saline containing 1% Triton X-100 (NGS/PBST) for 30 min and further incubated with primary antibody in NGS/PBST for 1 h at room temperature. The cells were then washed with phosphate-buffered saline and incubated with secondary antibody conjugated to Cy2 and rhodamine phalloidin for 30 min at room temperature. The cells were washed again, the chambers were removed, and the slides were mounted in Vectashield (Vector Laboratories) anti-fade solution containing 4′,6-diamidino-2-phenylindole. Cells were viewed by a Leica (Wetzlar, Germany) DMRA microscope with Plan-apochromat ×40/1.25 NA and ×63/1.4 NA oil immersion objective lenses. Image acquisition was with a SkyVision-2VDS digital CCD (12-bit, 1280 × 1024 pixel) camera in unbinned or 2 × 2-binned mode into EuroFISH software, saved as 16-bit monochrome, and merged as 24-bit RGB TIFF images (Applied Spectral Imaging Inc., Carlsbad, CA).

RESULTS

PKC Inhibitors Block E. coli Invasion—Our previous studies showed that expression of OmpA on E. coli is necessary for effective invasion of HBMEC (8). In addition, PAK activation and its subsequent interaction with P38K have been shown to associate with the actin rearrangements underneath the bacterial entry site and are required for the invasion (13, 15). To further extrapolate the signaling pathways involved in the E. coli invasion, we attempted to identify the role of PKC. Initially, E. coli invasion assays were performed following treatment of the cells with various concentrations of BIM, a specific PKC inhibitor. PAK activation was effective in blocking the invasion of OmpA+ E. coli. E44 in a dose-dependent manner with a 50% inhibitory concentration of 10 μM and 75% inhibition with 20 μM (1.12 × 10^4 ± 0.03 × 10^4 cfu/well for untreated HBMEC versus 0.3 × 10^4 ± 0.05 × 10^4 cfu/well for 20 μM BIM, p < 0.001) (Fig. 1A). However, the total cell-associated bacteria (represented as binding) did not differ between untreated and BIM-treated cells (3.1 × 10^5 ± 0.7 × 10^5 cfu/well for untreated HBMEC versus 3.4 × 10^5 ± 0.5 × 10^5 cfu/well for BIM-treated HBMEC), indicating that the inhibition is not due to inefficient binding of E. coli to HBMEC. In contrast, the OmpA+ E. coli strain, which did not show significant invasion, also showed no inhibition of either total cell-associated bacteria or background invasion (data not shown). Similarly, pretreatment of HBMEC with another PKC-specific inhibitor, a 10-amino acid peptide, which resembles the pseudosubstrate sequence of PKC-α (PKC-IP) and blocks PKC translocation of the activated PKC to the membrane, also showed significant inhibitory effect on E. coli invasion. A dose of 50 μM was able to exert an inhibition of ~50%, while a dose of 100 μM exerted an inhibitory effect of 75% invasion of HBMEC (1.0 × 10^4 ± 0.3 × 10^4 cfu/well for untreated HBMEC versus 0.21 × 10^4 ± 0.1 × 10^4 cfu/well for PKC-IP-treated HBMEC, p < 0.001) (Fig. 1B). We also used RGD and RPDS peptides as control peptides in the invasion assays, which showed no significant effect on the invasion. None of these inhibitors showed either antibacterial effect or cytotoxicity to HBMEC under the conditions employed. These results suggest that the activation of PKC and its translocation to the membrane might be playing a role in E. coli invasion.

E. coli Induces the Activation of PKC in HBMEC—Having demonstrated the importance of PKC activation and its translocation to the membrane in the E. coli invasion, we examined the level of PKC activation in HBMEC exposed to the OmpA+ E. coli strain E44 and to OmpA- E. coli strain E91. HBMEC were treated with the E. coli strains for different time periods, and the total cell lysates were analyzed for PKC activity, based on the ability to phosphorylate the PKC-specific substrate provided in the PepTag assay kit. HBMEC treated with HB101

FIG. 1. Inhibition of E. coli invasion of HBMEC by PKC inhibitors. Confluent monolayers of HBMEC were treated with varying concentrations of either BIM (A) or PKC-inhibitory peptide (B) for 20 min before the addition of the bacteria. The invasion assays were carried out as described under “Experimental Procedures.” The results were expressed as percentages of control HBMEC (11,450 ± 1250 cfu/well). The data represent three independent experiments performed in triplicate. Binding represents the total HBMEC-associated bacteria and invasion indicates intracellular bacteria in A. Error bars, S.D.
were used as a negative control. As shown in Fig. 2A, HBMEC infected with E44 demonstrated significant PKC activity within 10 min, which peaked at 15 min and decreased by 30 min. The increased PKC activity was approximately 4-fold compared with PKC activity in control as estimated by the spectrophotometric method using phosphorylated substrate peptides (0.09 units/ml for untreated cells versus 0.36 units/ml at 15 min for HBMEC treated with E44, p < 0.01) (Fig. 2B). However, cells treated with either E91 or HB101 did not show such an increase in the activity, indicating that the OmpA interaction with HBMEC is important for inducing the signals leading to the activation of PKC. Interestingly, phorbol 12-myristate 13-acetate (positive control)-treated HBMEC lysates showed PKC activation even at 30 min (Fig. 2C), suggesting that E. coli induced PKC activation might be for a short period of time. These results are in agreement with our previous studies of actin rearrangement, which occurs at 15 min postinfection of HBMEC with OmpA+ E. coli. Treating HBMEC with BIM prior to the infection with OmpA+ E. coli completely abolished the PKC activation (Fig. 2C). This corroborates with the results of E. coli invasion of BIM-treated HBMEC.

Activation of Conventional PKC Isoform, PKC-α, and Its Translocation to the Cell Membrane by E. coli—Since OmpA+ E. coli induces significant activation of PKC, we set out to identify which PKC isoform was activated. Among the various isoforms of PKC, the conventional isoform, PKC-α, has been reported to be the major isoform involved in actin rearrangements and also known to be regulated by cytosolic levels of calcium. Thus, we initially treated the HBMEC with EGTA to chelate both extracellular and intracellular calcium before infecting with OmpA+ E. coli (E44) and OmpA- E. coli (E91). PepTag assays for PKC activation showed that EGTA treatment completely abolished the PKC activity induced by E. coli (Fig. 3A), indicating that the PKC isoform could be a conventional isoform, PKC-α. However, to rule out the possibility that EGTA globally affects the phosphorylation of other cytosolic proteins, we examined the phosphorylation of FAK in lysates of EGTA-treated HBMEC infected with E44. In our previous studies, increased phosphorylation of FAK was observed with E44 invasion of HBMEC (13). The lysates were immunoprecipitated with polyclonal anti-FAK antibody, followed by immunoblotting with anti-phosphotyrosine antibody (4G10). The blot was stripped and reprobed with respective PKC antibodies to show equality of loading. The intensity of the phospho-PKC-α bands was quantitated as described under “Experimental Procedures.” The intensities of the bands were expressed as area for each time period (C). The data represent at least three separate experiments. The error bars indicate S.D.
tibody to verify the total FAK loaded in each lane. The data showed that FAK phosphorylation increased up to 15 min postinfection followed by a decline at 30 min, which did not differ between EGTA-treated cells and control untreated HBMEC infected with E44 (Fig. 3A). These results suggest that chelation of Ca²⁺ with EGTA did not affect other steps involved in the invasion process. To further confirm the role of PKC-α, Western blot analysis of the total cell lysate of HBMEC exposed to E44 and E91 at various time points was performed using anti-phosphoantibodies to each of the PKC-α, δ, and γ isoforms. The blots probed with anti-phospho-PKC-δ and anti-phospho-PKC-γ were loaded with 2.5 times more proteins than for anti-phospho-PKC-α to obtain equal intensity of the bands. The results indicated significant increase in the PKC-α phosphorylation when compared with other two isoforms (Fig. 3B). Each blot was stripped and, when reprobed with respective anti-PKC antibodies, showed the presence of equal quantities of protein in each lane. Thus, the absence of reactivity with phospho-PKC-δ and phospho-PKC-γ antibodies is not due to unequal loading of the proteins. The phosphorylation of PKC-α peaked at 15 min postinfection, followed by a decline at 30 min (Fig. 3B). Quantitation of phosphorylated bands by a densitometer showed a 5-fold increase of PKC-α phosphorylation at 10–15 min compared with control cells (Fig. 3C). Phosphorylation of PKC-α decreased by more than 80% at 30 min postinfection. The OmpA–E. coli, in contrast, could not induce a comparable increase in PKC-α phosphorylation.

Several studies have demonstrated that autophosphorylated PKC-α migrates to cell membrane for further signaling events (16). Thus, to examine whether the PKC-α activated by invading E. coli also translocates to membrane, HBMEC membrane fractions from cells infected with either E44 or E91 were prepared. PepTag analysis of the membrane fractions treated with E44 displayed significant activation of PKC similar to that observed using whole cell lysates. However, the activation was immediate within 5 min of infection and slightly greater at both 10 and 15 min postinfection when compared with the levels observed in whole cell lysates. We also observed some PKC activity even at 30 min, although it was greatly reduced compared with the levels at 15 min (Fig. 4A). The increase in PKC activity at 15 min was 6.5-fold greater than the activity at 0 min. However, the activity was reduced significantly to the level of untreated HBMEC by 30 min postinfection (Fig. 4B). The OmpA–E. coli infected HBMEC membrane fractions showed no activity at any time point. The HBMEC membrane fractions were also subjected to immunoprecipitation with anti-PKC-α antibody followed by Western blotting with anti-phospho-PKC-α. The blots were stripped and reprobed with anti-PKC-α antibodies. As shown in Fig. 4C, significant levels of phospho-PKC-α were observed in the membrane fractions at 10–15 min compared with control (0 min). In contrast, E91-infected HBMEC membrane fractions showed no such translocation, indicating that only invasive E. coli could elicit translocation of activated PKC-α to the membrane. Immunoblotting with anti-PKC-α antibody revealed the presence of similar levels of PKC-α present in all fractions. The PKC-α associated with E91-infected membrane fractions represents inactive unphosphorylated PKC-α present in normal HBMEC. However, the PKC-α present in E44-infected HBMEC membrane fractions at 10 and 15 min postinfection is phosphorylated and active PKC-α. Again, densitometric analysis of the phospho-PKC-α bands indicated a 5-fold increase in the phosphorylation levels of PKC-α at 15 min, which reduced to basal levels by 30 min postinfection with E44 (Fig. 4D).

In addition, immunocytochemistry was carried out on HBMEC infected with OmpA+E. coli to examine the localization of activated PKC-α in relation to actin condensation. The uninfected HBMEC showed long actin fibers at the apical surface of the cell (Fig. 5B), whereas in HBMEC infected with E44, actin was accumulated beneath a group of bacteria (Fig. 5F). Despite the presence of several bacteria attached to the cell surface, actin condensation was observed only at particular bacterial attachment sites. Our previous results show that only those bacteria entering into HBMEC could elicit actin rearrangements, and the present observation is a representation of that phenomenon. Other bacteria, however, still in the mode of attachment do not induce the actin condensation. Interestingly, staining of the control cells with anti-phospho-PKC-α antibody showed a punctate pattern, which indicates distribution of PKC throughout the cell. The HBMEC infected with E44 revealed that activated PKC-α accumulates at the actin condensation sites (Fig. 5G). Visualization of actin (red) and PKC-α (green) using dual filters showed that PKC-α is associated with actin condensation (yellow; Fig. 5H) underneath the bacteria. In contrast, staining of infected HBMEC with either anti-phospho-PKC-δ or anti-phospho-PKC-δ antibodies showed no association of PKC-δ or PKC-γ with actin condensation sites (Fig. 5, I–L; only PKC-γ stained cells have been shown as representative images). These results demonstrate that OmpA-expressing E. coli induces activation of PKC-α autophosphorylation followed by its translocation to the membrane of HBMEC at the site of actin condensation. The time frame of PKC-α

**Fig. 4.** E. coli invasion of HBMEC induces translocation of PKC-α to the membrane. HBMEC membrane fractions, obtained after infecting monolayers with either OmpA+E. coli, E44 or OmpA–E. coli, E91, for the indicated periods of time, were subjected to the PepTag assay (A). The amount of phosphorylated peptide in each band was estimated by a spectrophotometric assay, converted into PKC activity, and expressed as nmol/min/ml (B). Portions of the membrane fraction (200 μg) were subjected to immunoprecipitation with monoclonal anti-PKC-α antibody followed by Western blotting with polyclonal anti-phospho-PKC-α antibody. The blot was stripped and blotted with anti-PKC-α antibody (C). The intensity of the phospho-PKC bands was determined by densitometry and represented as the area of the bands obtained from three separate experiments (D). The error bars represent S.D.
activation correlates well with data obtained for actin rearrangement, in which condensation occurs within 10–15 min postinfection.

Overexpression of a Dominant Negative Mutant of PKC-α Blocks E. coli Invasion of HBMEC—To further confirm the role of PKC-α in E. coli invasion, HBMEC were transfected with mammalian expression vectors containing hemagglutinin-tagged PKC-WT and PKC-CAT/KR. The resulting cell lines were designated as PKC-WT/HBMEC and PKC-CAT/KR/HBMEC, which confer G418 resistance. The PKC-CAT/KR construct encodes a truncated protein in which the catalytic domain (CAT) containing amino acids 326–672 of PKC is preserved with a point mutation that abolishes the ATP binding ability, while the regulatory N-terminal domain is deleted (26). The PKC-CAT/KR construct has been shown to dominantly inhibit PKC-α activation. HBMEC transfected with pcDNA3 vector alone were used as a control. Western blot analysis of cell lysates from transfected HBMEC with anti-PKC-α antibody showed greater levels of PKC-α protein in PKC-WT/HBMEC when compared with either nontransfected or pcDNA3-transfected HBMEC (Fig. 6A). PKC-CAT/KR/HBMEC lysates, however, revealed the presence of an ~50-kDa truncated PKC-α protein in addition to lower levels of native PKC-α. The blot was also probed with actin antibody to verify

**Fig. 5.** Association of PKC-α at bacterial entry sites along with actin and overexpression of the PKC-CAT/KR mutant inhibits the accumulation of actin. HBMEC, uninfected and nontransfected were used as controls (A–D) to show the normal pattern of staining for actin and phospho-PKC-α. HBMEC, either nontransfected (E–H or I–L), or PKC-CAT/KR-transfected (M–P), were treated with OmpA+ E. coli for 15 min. All HBMEC were fixed and then stained with either rhodamine phalloidin (B, F, J, and N) or anti-phospho-PKC-α antibody (C, G, and O) or anti-phospho-PKC-γ antibody (K). The bacteria were visualized using transmitted light optics with blue filter (A, E, I, and M). The cells were also visualized with dual filter mode for both red and green fluorescence (D, H, L, and P). The confocal images were assembled and labeled using Adobe Photoshop software version 6.0. The arrows indicate locations of bacteria or accumulation of either actin or PKC isoforms.
were prepared and subjected to the PepTag assay. OmpA infected HBMEC as controls. As shown in Fig. 6 invasion assays along with vector alone-transfected and untransfected HBMEC as controls. As shown in Fig. 6B, the invasion of OmpA+ E. coli strain E44 was blocked by 80% in HBMEC expressing PKC-CAT/KR, whereas no significant differences were observed in other HBMEC (1.1 × 10^4 ± 0.25 × 10^4 cfu/well with vector alone-transfected HBMEC versus 0.22 × 10^4 ± 0.05 × 10^4 cfu/well with PKC-CAT/KR/HBMEC, p < 0.001). These data clearly support the role of PKC-α in E. coli invasion of HBMEC.

Overexpression of a Dominant Negative Form of PKC-α in HBMEC Reduces E. coli-induced PKC Activation and Actin Condensation but Not FAK Phosphorylation or PI3K Activation—Since E. coli invasion was blocked by overexpression of the PKC-α mutant, we next examined whether PKC activation was inhibited in HBMEC PKC-CAT/KR/HBMEC by PepTag assay. Both PKC-WT- and PKC-CAT/KR-transfected HBMEC were stimulated with E44 for different periods of time. The cell lysates were assayed for PKC activity. PKC-WT/HBMEC showed PKC activity similar to that of untreated HBMEC, with peak activation at 15 min (Fig. 6C). In contrast, E. coli induced PKC activity was completely abolished in PKC-CAT/KR/HBMEC at all time points. These results are in agreement with the results of invasion assays, suggesting that PKC-α activation is necessary for efficient E. coli invasion of HBMEC. In addition, immunocytochemical studies were carried out on PKC-CAT/KR/HBMEC infected with E44 to examine the actin and PKC staining patterns. The PKC-CAT/KR/HBMEC showed groups of bacteria adhered to the surface (Fig. 5M); however, no actin accumulation beneath the bacteria was observed (Fig. 5N). Similarly, no phospho-PKC staining associated with actin was seen, although the pattern showed punctate staining similar to that of control HBMEC (Fig. 5, O and P). Interestingly, the actin pattern in uninfected PKC-CAT/KR/HBMEC is similar to that of untransfected HBMEC (similar to Fig. 5B), indicating that the absence of E. coli invasion is not due to gross cytoskeletal changes at the apical surface of HBMEC. These results suggest that PKC-α activity is necessary for E. coli-induced actin rearrangement in HBMEC.

Our previous studies have suggested that E. coli-induced FAK phosphorylation is also necessary for actin condensation (13). Thus, to examine whether FAK phosphorylation is affected in PKC-CAT/KR/HBMEC, HBMEC lysates infected with E44 for different periods of time were subjected to immunoprecipitation and immunoblotting as described above. The results revealed that the phosphorylation pattern of FAK did not differ significantly between nontransfected and PKC-CAT/KR-transfected HBMEC (Fig. 6D). FAK phosphorylation peaked at 15 min and declined by 30 min post-infection, similar to the results obtained in our previous studies (13). Immunoblotting with FAK antibody showed that immunoprecipitates contained equal amounts of FAK in all samples. These results are in agreement with the results obtained from EGTA-treated HBMEC lysates, suggesting that the PKC activation could be operating either via a pathway independent of FAK or downstream of FAK activation.

Moreover, our previous studies also showed that E. coli induces activation of PI3K and its interaction with FAK during invasion (15). PI3K activation in HBMEC was measured in terms of Akt activation, a product generated by PI3K and recruited to membrane. Thus, to examine the effect of overexpression of dominant negative mutant form of PKC-α on PI3K activation, HBMEC and PKC-CAT/KR/HBMEC cell lysates were immunoprecipitated with anti-Akt antibody. The resulting immunocomplexes were subjected to Western blotting with either anti-phospho-Akt antibody. The blot was stripped and reprobed with either anti-FAK or anti-Akt antibodies. Portions of the cell lysates were immunoprecipitated with anti-Akt antibody. The results obtained in our previous studies (13). Immunoblotting with FAK antibody revealed that immunoprecipitates contained equal amounts of FAK in all samples. These results are in agreement with the results obtained from EGTA-treated HBMEC lysates, suggesting that the PKC activation could be operating either via a pathway independent of FAK or downstream of FAK activation.
We next examined whether blocking FAK activation inhibits PKC activation. FAK wild type (FAK-WT) - and FRNK (FAK-related nonkinase)-transfected HBMEC were infected with E44, and the cell lysates were analyzed for PKC activity. FRNK has been shown to negatively regulate the function of FAK (14). Our previous studies showed that overexpression of FRNK in HBMEC blocked the E. coli-induced activation of FAK and actin rearrangements (12), thus blocking the invasion of HBMEC by E. coli. PepTag assays for PKC activity in FAK-WT/ HBMEC lysates revealed that the activity is similar to that of control HBMEC (Fig. 6E), whereas FRNK/ HBMEC showed no activation at any time point. Taken together, these data indicate that PKC activation is downstream of FAK and PI3K activation.

PKC-α Activation Is Dependent on PI3K—The first event in the regulation of PKC-α is phosphorylation of the activation loop within the catalytic domain (16). The upstream kinase for the activation loop of PKC is 3-phosphoinositide-dependent kinase-1 (PDK1), whose activity is dependent on the activation of PI3K (27). FAK activation induced by E. coli also triggers the activation of PI3K and its association with PKC via an auto-phosphorylation site (Tyr-397) of FAK (14). Thus, we speculated that down-regulation of PI3K activity could block E. coli-induced activation of PKC. To examine this possibility, HBMEC were pretreated with a PI3K-specific inhibitor, LY 294002, and its inactive analog, LY303511, prior to the addition of E. coli. The LY-treated HBMEC lysates were prepared and subjected to PepTag assay. The LY294002-treated HBMEC showed prominent inhibition of E. coli-induced activation of PKC at every time period, whereas treatment with the inactive analog LY303511 showed no inhibitory effect on PKC activation (Fig. 7A). Taken together, these data indicate that PKC activation is downstream of FAK and PI3K activation.

FIG. 7. Inhibition of PKC activity by LY-294002 and in HBMEC overexpressing PI3K mutants. A, HBMEC monolayers were pretreated with either LY-294002 or its inactive analog, LY-303511, before the addition of OmpA+ E. coli. The cell lysates were then subjected to the PepTag assay. B, HBMEC transfected with either Δp85 or Δp110 were infected with E44 for different periods of time, and cell lysates were prepared and then subjected to the PepTag assay.

Inhibition of PKC activity by LY-294002 and in HBMEC overexpressing PI3K mutants. A, HBMEC monolayers were pretreated with either LY-294002 or its inactive analog, LY-303511, before the addition of OmpA+ E. coli. The cell lysates were then subjected to the PepTag assay. B, HBMEC transfected with either Δp85 or Δp110 were infected with E44 for different periods of time, and cell lysates were prepared and then subjected to the PepTag assay.

Fig. 8. Translocation of MARCKS from HBMEC membranes to cytosol. A, nontransfected HBMEC and PKC-CAT/KR/ HBMEC were infected with E44 for different periods of time, and cytosolic and membrane fractions were prepared and then subjected to immunoblotting with anti-MARCKS antibody. The fractions from nontransfected HBMEC infected with E91 were used as control. The MARCKS band intensities from HBMEC infected with E44 (B) and E91 (C) were determined by a densitometer and expressed as area. (Fig. 7B). These results confirm the observations made in LY-treated HBMEC indicating that the activation of PI3K is necessary for the activation of PKC, which in turn may be important for efficient E. coli invasion of HBMEC.

MARCKS, a Substrate of Active PKC-α, Translocates from Membrane to Cytosol in E. coli Invasion of HBMEC—Among the substrates for PKC-α, MARCKS is a membrane-associated substrate known for cross-linking actin filaments (17). It has long been used as a marker for PKC-α activation. It is reported to disassociate itself from the membrane upon phosphorylation by PKC, which subsequently migrates to the cytosol. Thus, we examined whether PKC-α activation induces the translocation of MARCKS by preparing cytosolic and membrane fractions of HBMEC treated with E44 followed by immunoblotting with anti-MARCKS antibody. As shown in Fig. 8A, the results indicate that the density of the MARCKS band on Western blot increased up to 15 min postinfection in cytosolic fractions and stayed at the same level of phosphorylation even at 30 min. The intensity of MARCKS in the membrane fractions showed a corresponding decrease with time, suggesting that the MARCKS translocated from the membrane to cytosol in OmpA+ E. coli-treated HBMEC. Analysis of MARCKS bands by a densitometer showed a 3-fold increase in the intensity of protein bands from 0 to 30 min in cytosolic fractions of HBMEC infected with E44 (Fig. 8B). In contrast, E91-treated HBMEC showed neither decrease of MARCKS in the membrane fractions nor increase in the cytosolic fraction (Fig. 8A and C). In addition, cytosolic and membrane fractions of PKC-CAT/KR/ HBMEC were also subjected to immu-
nobilting to verify whether overexpression of PKC-CAT/KR mutant blocked the MARCKS translocation. The data showed that MARCKS translocation from membrane to cytosol did not occur in these cells despite infection with E44 (Fig. 8A), suggesting that PKC–α activation is needed for MARCKS translocation, which probably is required for actin rearrangements induced by invasive E. coli.

**DISCUSSION**

Bacterial pathogens have been reported to subvert host cell machinery to gain entry into host cells. This often involves the engagement of integrin receptors through which the bacteria exploit the preexisting signaling pathways to invade. E. coli that causes meningitis also manipulates the HBMEC cytoskeleton for the purpose of invasion (12). Interestingly, our present and previous studies have indicated that E. coli invasion might be via the receptors other than integrins. We recently identified a 95-kDa HBMEC receptor that interacts with OmpA. Over-expression of the OmpA receptor in Chinese hamster ovary cells (generally nonsusceptible for infection) made these cells susceptible for E. coli invasion, highlighting the role of the 95-kDa receptor in invasion. Thus, a novel receptor-mediated invasion is involved in E. coli uptake by HBMEC. In an attempt to identify the signal transduction pathways involved in these events, we have shown that the activation of FAK and its interaction with PI3K play a crucial role in E. coli invasion of HBMEC (13, 15). The connecting signaling molecules between FAK/PI3K and actin filaments to generate gross cytoskeletal modifications, however, have not been clearly elucidated. In this paper, we focused on PKC, a versatile relay station of intracellular signal transduction pathways.

Inhibition of E. coli invasion by PKC-specific inhibitors, BIM and PKC inhibitory peptide, is the first evidence for a significant role of PKC in invasion. Concurrent to these results, we also observed increased activation of PKC in HBMEC during the process of invasion with a spike in activity between 10 and 15 min postinfection. The magnitude of the PKC activation by E. coli was 3.5-fold, which is slightly lower than that of phorbol 12-myristate 13-acetate-induced activation of PKC (4–5-fold increase). The onset and offset of PKC stimulation is rapid in HBMEC infected with OmpA+ E. coli, which corresponds to the time of FAK and PI3K activation as well as actin condensation. Several isoforms of PKC (viz. conventional (calcium- and DAG-dependent), novel (only DAG-dependent), and atypical (neither calcium- nor DAG-dependent) have been documented in the literature (16). Since the E. coli-induced activation of PKC is calcium-dependent, it is reasonable to assume a significant role played by the conventional isoform, PKC-α, that plays a major role in actin reorganization. Western blot analysis indicates that the isoform involved is indeed PKC-α, a 78-kDa protein present in an inactive unphosphorylated form in the membrane. Upon phosphorylation by PKD1 on the activation loop, PKC-α migrates to cytosol, where it resides in an inactive form as its active site is occupied by a pseudosubstrate. The presence of significant levels of cytosolic calcium facilitates the membrane translocation of the inactive PKC-α. Upon binding to DAG, PKC-α releases the pseudosubstrate, exposing the active site, thus becoming active. The presence of significant PKC-α activity in the membrane fractions of HBMEC infected with OmpA+ E. coli is in agreement with the above concept. Additionally, a significant decrease in E. coli invasion of HBMEC by the PKC inhibitory peptide, which prevents the translocation of PKC to the membrane further demonstrates the necessity of membrane-associated active PKC in the invasion process. Results of immunofluorescence staining of E. coli-infected HBMEC presented herein indicate that the PKC-α is associated with actin beneath the invading bacteria. It is possible that PKC-α accumulation is adjacent to or associated with phagocytic cups modulating the activity of actin cross-linking proteins (25). In addition, PKC-α has also been known to bind directly to the actin filaments leading to severe alterations in the cytoskeletal framework of the cell (28). Interestingly, the bacteria merely attached to HBMEC could not elicit either actin condensation or PKC-α localization to bacterial entry site unlike enteropathogenic E. coli, where adherence to the host cell itself triggers the activation of PKC (29).

Overexpression of a dominant negative mutant of PKC-α (PKC-CAT/KR) in HBMEC showed significant inhibition of E. coli invasion of HBMEC, which results are in concurrence with the studies using inhibitors of PKC. Despite the binding of E. coli to PKC-CAT/KR/HBMEC, no accumulation of actin was observed in HBMEC underneath the groups of bacteria. Thus, the inhibition of PKC-α significantly reduced the actin rearrangements induced by E. coli. The dominant negative mutant form of PKC-CAT/KR has been shown to compete with PKC-α for activation (26), and it is presumed that phosphorylated PKC-CAT/KR stays in the cytosol in an inactive state. The PKC-CAT/KR could not translocate to the membrane due to lack of the pseudosubstrate-binding site. However, no abnormalities in the basal and apical surface stress fibers of these cells when compared with control HBMEC were observed, suggesting that the inhibition of PKC activation is not a phenomenon of HBMEC malfunction. This is also evident from the data of normal phosphorylation levels of FAK and activity of PI3K (as assessed by Akt phosphorylation) in PKC-CAT/KR/HBMEC. Our previous studies have shown that FAK may be upstream of PI3K, since FAK dominant negative mutant over-expression in HBMEC blocked the PI3K activation. In contrast, no inhibition of FAK phosphorylation in HBMEC overexpressing PI3K dominant negative mutants was observed. Thus, taking the present results into consideration, we speculate that PKC-α activation could be downstream of both FAK and PI3K.

Interestingly, HBMEC overexpressing either dominant negative FAK or PI3K mutants did not exhibit PKC activity upon infection with E. coli. This provides further support for a role of FAK and PI3K in the activation of PKC-α. Upon activation of PI3K, the catalytic subunit p110 catalyzes the formation of phosphatidylinositol 3,4,5-trisphosphate (27). Binding of phosphatidylinositol 3,4,5-trisphosphate to pleckstrin homology domains of various cellular and cytoskeletal proteins mediates membrane recruitment of several kinases, including PKC (27). PKC has been reported to form a complex with PKC, which subsequently phosphorylates the activation loop of most members of the PKC family. The effect of PKD1 is PI3K-dependent, since previous studies have shown inhibition of PKD1 by LY294002 (27). Our studies showed significant inhibition of PKC activation in HBMEC pretreated with LY294002 followed by exposure to E. coli, providing additional evidence that the E. coli-induced PKC activation is PI3K-dependent. Activation of PKC has also been shown in EPEC infection by purifying PKC from infected cells followed by γ32P incorporation assays (30). However, PKC-α co-localization with actin has yet to be established. In these studies, PKC-α activation is attributed to a decrease in transepithelial resistance due to abnormalities in tight junctions. N. gonorrhoeae invasion of HeLa cells has also been shown to depend on heparan sulfate proteoglycan induced activation of PKC (31). However, these studies were performed using the inhibitors of PKC, but not by showing the direct activation of PKC. Thus, the PopTag assays used in our study will provide a valuable tool to examine the PKC activity from total cell lysates without purification of PKC.

MARCKS is a PKC substrate that cycles on and off membranes by a mechanism termed the myristoyl-electrostatic
switch (32, 33). While at the membrane, MARCKS binding sequesters acidic phospholipids, including phosphatidyl 4,5-diphosphate (34). MARCKS also binds and cross-links actin, an activity that is regulated by PKC-dependent phosphorylation and calcium-calmodulin (18). In this study, we report for the first time the activation of MARCKS in E. coli invasion. The presence of MARCKS in the cytosol of HBMEC infected with OmpA + E. coli, when compared with HBMEC infected with OmpA – E. coli, is an indication of OmpA-mediated induction of a pathway leading to MARCKS phosphorylation and subsequent migration to the cytosol. Interestingly, the activity of MARCKS was observed in cytosol even at 30 min postinfection, which is in contrast to PKC-α activity that showed a decline by 30 min. It is possible that the activated MARCKS resides in cytosol for longer times to facilitate the actin rearrangements for efficient invasion. This observation is in concurrence to our previous data that the completion of the invasion process takes ~45 min (12). Although there were no reports for MARCKS activation associated with actin condensation in the bacterial entry process until now, MARCKS is shown to modulate actin dynamics in cell migration studies (19, 23). The phosphorylation domain of MARCKS has two actin-binding sites, which, upon phosphorylation of MARCKS, lose site-specific affinity to actin (35). The loss of bound MARCKS by phosphorylation could release actin molecules for reorganization. Thus, the migration of MARCKS induced by E. coli might result in marked cytoskeletal rearrangements, facilitating the ingestion of E. coli by HBMEC.

In summary, the findings presented in this study indicate that the OmpA-mediated invasion of E. coli into HBMEC induces (i) activation of PKC-α within a short period of time, (ii) activation of MARCKS, a substrate for PKC-α, and (iii) association of PKC-α with actin condensation. Identification of the further role of PKC-α activated by E. coli during invasion may provide clues for blood-brain barrier dysfunction, a significant pathological condition that exists in E. coli meningitis.

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