Activation of Host Cell Protein Kinase C by Enteropathogenic Escherichia coli

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Enteropathogenic Escherichia coli (EPEC) consists of a group of diarrhea-producing E. coli strains, common in developing countries, which do not produce classical toxins and are not truly invasive. EPEC strains adhere to mammalian cells in an intimate fashion, trigger a localized increase in intracellular calcium levels, and elevate inositol phosphate production. We hypothesized that these mediators could activate host cell protein kinase C (PKC) and tested this idea in vitro with two cultured human cell lines, HeLa cells and T84 cells. Using a recently described subculturing protocol to “induce” or accelerate EPEC adherence, we infected the cells with EPEC at a multiplicity of infection of ~100:1 for 30 to 60 min. Under these conditions, EPEC E2348 increased membrane-bound PKC activity 1.5- to 2.3-fold in HeLa cells and T84 cells, respectively. The increase in membrane-bound PKC activity was accompanied by a decrease in cytosolic PKC activity in EPEC-infected HeLa cells. Nonadherent laboratory E. coli strains such as HB101 and H.S. failed to trigger any consistent change in PKC production, similar to the nonadherent mutant strains derived from E2348, JPN15 (plasmid cured) and CVD206 (eaeA). In addition, immunoblots performed on extracts of T84 cells with a monoclonal antibody against PKC-α showed an increased PKC content in membranes of EPEC-infected cells. Finally, EPEC-infected T84 cells showed a 60% increase in responsiveness to the E. coli heat-stable toxin. We conclude that mediators produced in response to EPEC adherence activate PKC in intestinal and nonintestinal cells.

Enteropathogenic E. coli (EPEC) is an important cause of prolonged, watery diarrhea in infants in developing countries. These organisms show a characteristic, intimate form of adherence to intestinal cells accompanied by effacement of microvilli and actin condensation beneath adherent bacteria. Intimate adherence of EPEC is accompanied by a rapid increase in the phosphorylation of host cell proteins on tyrosine residues by an as yet unidentified protein tyrosine kinase (30, 32). In addition, EPEC triggers an increase in the intracellular calcium concentration in the vicinity of adherent bacteria and causes the production of inositol phosphates, including inositol 1,4,5-trisphosphate (IP3), presumably from the hydrolysis of phosphatidylinositol bisphosphate (PIP2) (4, 16, 19). The presence of phospholipid. Second, after activation, PKC undergoes a major conformational change, causing it to translocate from the cytosol to the membrane-associated fraction of cells. These attributes of the enzyme allowed us to detect an increase in PKC enzymatic activity in EPEC-infected cells of intestinal and nonintestinal origin and to detect an increase in the membrane-associated PKC level by immunoblotting. In addition, we studied the localization of PKC by immunofluorescence in infected cells. Finally, we demonstrated that EPEC infection of T84 cells increases the cyclic GMP (cGMP) response of the cells to E. coli heat-stable enterotoxin (StA), whose receptor is phosphorylated and activated by PKC (9).

MATERIALS AND METHODS

Bacterial culture. The E. coli strains used are listed in Table 1. For use in experiments, an individual colony of each strain, except strain E2348, was picked from a Luria-Bertani (LB) agar plate and used to inoculate an overnight culture of Luria-Bertani broth supplemented with 10 g of t-mannose per liter at 37°C with shaking at 250 rpm. Because of problems of reverision to the nonadherent phenotype with strain E2348 when stored on agar plates, glycerol-treated ~60°C stocks of this strain were thawed freshly for each experiment. The overnight culture was subcultured 1:10 into cell culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium, 15 mM HEPES, 18 mM NaHCO3, 2% heat-inactivated newborn calf serum, and 10 g of t-mannose per liter, henceforth referred to as EPEC adherence medium. EPEC adherence was preassembled with a 5% atmosphere of CO2 in air overnight prior to the subculture. Subcultures were performed at 37°C with shaking for 2 h, at which point the optical densities at 600 nm were ~0.6. By using this subculturing method EPEC E2348 and JCP88 showed cessation of motility and strong spontaneous clumping as reported by Vuopio-Varkila and Schoolnik (40) and exhibited strong localized adherence to eukaryotic cells within 30 min or less (31).

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Quantitation of infection. For most experiments, a multiplicity of infection of 100 to 200 bacteria per epithelial cell was desired. To determine the actual number of bacteria added, serial dilutions of the inoculum were made and plated. Preliminary experiments showed that clumps of EPEC bacteria, formed during subculture in EPEC adherence medium, dissociated when diluted into phosphate-buffered saline (PBS) at room temperature (RT). For accuracy of bacterial counts, therefore, an initial inoculum was diluted 100-fold and allowed to stand at RT for at least 10 min before the remainder of the dilutions were made and plated.

Culture of mammalian cell lines and adherence assays. T84 cells, a colon carcinoma cell line, were grown as previously described (10, 11), except that vancomycin (20 μg/ml) and gentamicin (15 μg/ml) were used instead of penicillin and streptomycin.

HELa cells, from human cervical cancer, were obtained from the American Type Culture Collection and grown in a medium consisting of DMEM:F12, 15 mM HEPES, 18 mM NaHCO3, 5% newborn calf serum, 5% fetal bovine serum, and vancomycin and gentamicin at the concentrations noted above.

Before infection, antibiotic-containing medium was removed and the wells were rinsed with sterile PBS and refed with EPEC adherence medium. For studies of adherence by light microscopy or immunofluorescence, cells were grown in four-chamber glass Lab-Tek slides (Nunc, Inter-Medic, Naperville, Ill.) to 60 to 80% confluency, refilled with 400 μl of EPEC adherence medium, and infected with 100 μl of bacterial subculture per well. Adherence was determined by staining with Giemsia or Gram safranin.

For biochemical assays, such as PKC assays, mammalian cells were grown to confluency in 100-mm round dishes, rinsed, and refilled with 10 ml of EPEC adherence medium prior to infection with ~5 ml of bacterial subculture per dish. At confluency, a 100-mm dish contained ~3.2 × 10^7 T84 cells or ~2 × 10^6 HELa cells.

Extraction of protein kinase C from cell monolayers. In general, PKC activity cannot be assayed in crude cell homogenates, because of the abundance of other calcium-stimulated protein kinases and because endogenous cell lipid prevents a detectable stimulation by phospholipid, the hallmark of PKC. For detection of PKC from infected cell monolayers, PKC was extracted and partially purified by procedures developed by Diane Rofinger, Department of Clinical Laboratory Medicine, State University of New York at Buffalo, which were based on the procedures of Cohen and Mancini (5). After EPEC infection, PKC monolayers were rinsed twice with ice-cold PBS and placed on ice. The cells in each 100-mm dish were harvested by being scraped on ice into 2.5 to 3 ml of cell homogenization buffer (buffer A) consisting of 20 mM Tris (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonfluor fluoride, 10 μg of leupeptin per ml, and 2 mM dithiothreitol (DTT). For this and all subsequent buffers, phenylmethylsulfonfluoride, leupeptin, and DTT were added from concentrated stocks just before use. The cells were homogenized on ice with 12 strokes of a Teflon-in-glass homogenizer and then centrifuged at 100,000 × g at 4°C for 45 min. The supernatant from this step was designated the cytosolic fraction and was applied to DEAE minicolumns as described below.

The pellet from the 100,000 × g centrifugation, designated the membrane fraction, was solubilized in 3 ml of a buffer (buffer A + BSA + Triton) consisting of buffer A supplemented with 1% Triton X-100 detergent and 0.6 mg of bovine serum albumin per ml. The membrane pellet was resuspended by trituration successively through 18-, 20-, and 22-gauge needles, followed by tumbling at 4°C for 30 min. The detergent extract was again centrifuged at 100,000 × g for 5 min, and the supernatant was collected and subjected to DEAE chromography.

DEAE chromography was carried out with a 1.2-ml bed volume of DE-52 anion-exchange resin (Whatman, Maidstone, England) in minicolumns made from the large pipette tips intended for the P3000 Pipettman pipetor (Rainin, Woburn, Mass.) plugged with glass wool. Prior to use, the columns were equilibrated with 10 to 15 ml of 20 mM Tris (pH 7.5)–1 mM EGTA–2 mM DTT (equilibration buffer). Experimental samples, in the form of either cytosol or detergent-solubilized membrane fractions (~3 ml), were applied (PKM). The samples were washed with five 3-ml volumes of equilibration buffer. In pilot experiments, no PKC activity was detected in the flowthrough or washes with equilibration buffer. PKC activity eluted with five 0.5-ml aliquots of elution buffer consisting of 20 mM Tris (pH 7.5), 0.3 M NaCl, 1 mM EGTA, and 2 mM DTT. In preliminary experiments, PKC activity was found predominantly in the third, fourth, and fifth 0.5-ml elutions; therefore, in subsequent experiments, these were the eluted fractions collected.

Assay for PKC enzymatic activity. PKC was assayed by a modification of the method of Wilkerson and Sando (41). Briefly, 30 μl of sample was added in the presence or absence of a phospholipid mixture (consisting of 100 μg of phosphatidylyserine per ml and 30 μg dioctanoyl(glycerol) tetraoleate per ml) in a reaction mixture consisting of 25 mM HEPES (pH 7.8), 0.4 mM CaCl2 (0.1 mM over the concentration of EGTA, carried over from the elution buffer), 6 mM MgCl2, 0.2 mg of histone III-S per ml, 50 μM unlabeled ATP and ~1012 cpm of [γ-32P]-ATP per assay tube (all concentrations represent final concentrations). The assay volume was 100 μl and the reaction was initiated by the addition of a reaction cocktail containing the ATP and the histone substrate. In some experiments, histone was replaced as substrate by 20 μM Selectide, a selective PKC substrate peptide derived from neurogranin (Calbiochem, La Jolla, Calif.). The reaction was allowed to proceed for 5 min at 30°C and terminated by spotting 70 μl of reaction mixture onto a 3- by 5-cm rectangle of Whatman P31 phosphocellulose paper and dropping it into a 500 ml beaker of 75 mM phosphoric acid with stirring. The P31 filters were then washed twice more for 10 to 15 min each in 500 ml of 50 mM NaCl and rinsed once briefly in acetone to aid drying. The dried filters were counted by dry (Cerenkov) counting in a scintillation counter. Lipid-dependent activity was calculated by subtracting the activity measured in the presence of calcium alone from the activity observed in the presence of calcium and phospholipid.

Localization of PKC by immunofluorescence microscopy. Cells grown in Lab-Tek slides were infected with EPEC bacteria subcultured as described above, the infected supernatants were removed by aspiration, and the wells were gently washed twice with PBS. Monolayers were fixed with 3.75% formaldehyde (prepared by depolymerization of paraformaldehyde) in PBS for 5 min, rinsed with PBS, permeabilized with 0.05% Triton X-100 in PBS, and washed five more times with PBS. The monolayers were incubated with 1 μg (a 1:250 dilution) of a mouse monoclonal antibody against PKC-α (Transduction Laboratories, Lexington, Ky.) per ml at 37°C for 1 h. The cells were again rinsed five times with PBS and incubated with a second antibody consisting of fluorescein-conjugated, affinity-purified goat anti-mouse antibody (Kirkegaard & Perry, Gaithersburg, Md.) diluted 1:20. To reduce background fluorescence, the second antibody was adsorbed by incubation with uninfected control cells in a separate well. After these manipulations, the chambers of the Lab-Tek slides were snapped off and the slides were coveredlipped with a glycerol solution (Permount). For examination of the cells, adherent clumps of bacteria were visualized by differential interference contrast (Nomarski) optics with a Nikon Optiphot microscope under ×400 magnification. The same field was then observed under UV illumination for immunofluorescence.

Detection of membrane-bound PKC by immunoblotting. Cells infected with EPEC were again harvested by being scraped in buffer A, homogenized, and subjected to centrifugation as described above. The membrane pellet from one round dish was dissolved in 1 ml of Trits buffer with 1% Triton X-100 and centrifuged at 14,000 × g in an Eppendorf centrifuge to remove insoluble material (cytoskeleton and DNA). This fraction was mixed with an equal volume of 2X Laemmli sodium dodecyl sulfate sample buffer, boiled, and subjected to polyacrylamide gel electrophoresis on a 4 to 12% gradient Tris-glycine minigel (Novex, San Diego, Calif.). The gel was electrophoretically transferred to nitrocellulose and probed with a mouse monoclonal antibody against PKC-α (Transduction Laboratories, Lexington, Ky.) per ml at 37°C for 1 h. The cells were again rinsed five times with PBS and incubated with a second antibody consisting of fluorescein-conjugated, affinity-purified goat anti-mouse antibody (Kirkegaard & Perry, Gaithersburg, Md.) diluted 1:20. To reduce background fluorescence, the second antibody was adsorbed by incubation with uninfected control cells in a separate well. After these manipulations, the chambers of the Lab-Tek slides were snapped off and the slides were coveredlipped with a glycerol solution (Permount). For examination of the cells, adherent clumps of bacteria were visualized by differential interference contrast (Nomarski) optics with a Nikon Optiphot microscope under ×400 magnification. The same field was then observed under UV illumination for immunofluorescence.
Membrane-bound activity

<table>
<thead>
<tr>
<th>Fraction and infection</th>
<th>Mean PKC activity ± SD (pmol of $^{32}$P transferred to histone/min/dish)</th>
<th>Change in lipid-dependent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytosolic activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, uninfected</td>
<td>176.1 ± 40.1</td>
<td></td>
</tr>
<tr>
<td>1 μM PMA, 30 min</td>
<td>250.6 ± 14.2</td>
<td>99% decrease</td>
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<tr>
<td>E2348 infected, 1 h</td>
<td>181.6 ± 23.8</td>
<td>34% decrease</td>
</tr>
<tr>
<td>JCP88 infected, 1 h</td>
<td>239.4 ± 31.0</td>
<td>42% decrease</td>
</tr>
<tr>
<td><strong>Membrane-bound activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, uninfected</td>
<td>306.6 ± 14.9</td>
<td></td>
</tr>
<tr>
<td>1 μM PMA, 30 min</td>
<td>236.9 ± 32.3</td>
<td></td>
</tr>
<tr>
<td>E2348 infected, 1 h</td>
<td>368.8 ± 22.4</td>
<td></td>
</tr>
<tr>
<td>JCP88 infected, 1 h</td>
<td>380.6 ± 6.2</td>
<td></td>
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</tbody>
</table>

*Phospholipid-dependent activity is determined arithmetically by subtracting the activity observed in the presence of Ca$^{2+}$ alone from the total kinase activity.

$^b$ Value is not significantly different from uninfected control.

$^c$ $P < 0.001$ compared to uninfected control.

$^d$ $P < 0.01$ compared to uninfected control.

We first examined the effect of EPEC infection on a nonintestinal cell line, HeLa cells. After the cells were infected for 60 min, they were homogenized and separated into cytosolic and membrane-bound fractions by centrifugation and then each fraction was separately subjected to DEAE anion-exchange chromatography. The material eluted from the DEAE resin was assayed for PKC activity in the presence of calcium alone and in the presence of calcium plus phospholipid, which represents PKC activity. Lipid-dependent activity, the strictest definition of PKC activity, was calculated as the difference between the two measured values. Cells were also treated with phorbol esters, which are known stimulators of PKC, for purposes of comparison.

In control uninfected HeLa cells, PKC activity was higher in the cytosolic fractions than in the membrane fractions (Table 2; see Fig. 1, T84 cells). In response to 60 min of infection with EPEC E2348 and JCP88 at a multiplicity of infection of $\geq 200:1$, cytosolic PKC activity fell modestly, while the lipid-dependent portion of PKC activity dropped 34 and 42% for E2348 and JCP88, respectively (Table 2). In contrast, a 30-min exposure to PMA caused cytosolic PKC activity to drop to a level less than 1% of control. In the infected HeLa cells, the lipid-dependent PKC activity in membrane fractions increased 44% for cells infected with E2348 and JCP88, respectively. For comparison, phorbol ester treatment caused membrane-bound PKC activity to increase 4.2-fold.

In other experiments not shown here, results similar to those seen with HeLa cells were also observed in another cell line, HEK-293. In 293 cells, infection with strain E2348 resulted in a 62% decrease in cytosolic PKC activity and a 40% increase in membrane-bound PKC activity ($P < 0.05$ for both differences). In resting, uninfected T84 cells, the distribution of PKC activity differed considerably from that in HeLa cells, with membrane-bound activity greatly exceeding cytosolic activity (Fig. 1). In addition, variables such as the cell confluency and length of time after plating appeared to affect the total PKC activity (compare the ordinates in Fig. 1 to 3). After stimulation with phorbol esters or infection with EPEC bacteria, the already low PKC activity in T84 cell cytosol did not decline significantly (Fig. 1). In membranes, however, total kinase activity and the lipid-dependent portion (strict PKC activity) dramatically increased in response to infection with strain E2348. For example, in six independent experiments, total kinase activity increased by a mean of 1.6-fold in E2348-in-
The release of IP3 from HEp-2 cells (16). HB101 can trigger modest increases (Fig. 2). Dytoc et al. noted that nonadherent see also Fig. 2 and 3). In view of this data, it is of interest that increase occasionally reached statistical significance (Fig. 1; but small increase in membrane-bound PKC activity, and this in-

Fig. 1 to 3), demonstrate that the protein kinase activity we measured in the DEAE eluates represented authentic PKC. The results with the selective PKC substrate, together with the strong stimulatory effect of lipid in all of our assays (Table 1; Fig. 1 to 3), demonstrate that the protein kinase activity we measured in the DEAE eluates represented authentic PKC.

In some experiments, infection with strain HS produced a small increase in membrane-bound PKC activity, and this increase occasionally reached statistical significance (Fig. 1; but see also Fig. 2 and 3). In view of this data, it is of interest that Dytoc et al. noted that nonadherent E. coli strains such as HB101 can trigger modest increases (~30% over control) in the release of IP3 from HEp-2 cells (16).

Although our primary focus was to determine whether PKC was activated in response to EPEC infection, in our assays we also observed an increase in lipid-independent protein kinase activity toward histone after EPEC infection (Fig. 1, open bars). Thus, our data suggest that serine- and threonine-di-

 strain HS (HS), treated with 1 μM PMA for 30 min (PMA), or infected with EPEC strain E2348 for different periods, as shown on the right portion of the abscissa. In this experiment, only the membrane extracts were processed and assayed for protein kinase activity, as described in Materials and Methods and the legend to Fig. 1. * * * p < 0.05.

Figure 3 shows the strain specificity of the PKC response to bacterial infection. Strain JPN15, a spontaneously occurring EAF plasmid-cured derivative of E2348, and strain CVD206, an eaeA mutant of E2348, did not stimulate PKC. Since strain CVD206 is able to trigger tyrosine phosphorylation of host protein Hp90 but is not able to cause “focusing” of this important membrane target (32), our results suggest that the focusing or clustering of host substrates must be necessary for PKC activation.

In addition to measuring PKC enzymatic activity, we attempted to detect the translocation of PKC into T84 cell membrane preparations immunologically. The purpose of these ex-

still significantly elevated 60 min after infection. This rapid stimulation and fall-off in PKC activity may explain why we and other investigators using the 3-h adherence assay protocol failed to detect a clear-cut increase in PKC activity in EPEC-infected cells compared to uninfected cells (4).

FIG. 3. Strain specificity of the effect of EPEC on PKC in T84 cells. T84 cells were left uninfected (uninf), treated with nonadher-

ent strain HS (HS), treated with 1 μM PMA for 30 min (PMA), or infected with EPEC strain E2348 or different periods, as shown on the right portion of the abscissa. In this experiment, only the membrane extracts were processed and assayed for protein kinase activity, as described in Materials and Methods and the legend to Fig. 1. * * * p < 0.05.

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FIG. 2. Time course of PKC activation in T84 cell membrane fractions after EPEC infection. T84 cells were left uninfected (uninf), infected with nonadher-

ent strain HS (HS), treated with 1 μM PMA for 30 min (PMA), or infected with EPEC strain E2348 for different periods, as shown on the right portion of the abscissa. In this experiment, only the membrane extracts were processed and assayed for protein kinase activity, as described in Materials and Methods and the legend to Fig. 1. * * * p < 0.05.

Histones and membrane extracts were partially purified on DEAE minicolumns as described in Materials and Methods and assayed for protein kinase activity with histone as the substrate. “Ca2+ alone,” to the standard reaction mixture plus CaCl2 added to 0.1 mM above the EGTA concentration; “+ lipid,” 30 μM dioctanoylglycerol plus 100 μg of phosphatidylserine per ml; lipid dependent portion, difference between the activity observed in the presence of Ca2+ plus lipid and that in the presence of Ca2+ alone. PDB, 1 μM phorbol dibutyrate for 15 min; E30, infected with strain E2348 for 30 min; HS30, infected with strain HS for 30 min; *, p < 0.05; **, p < 0.01.

In some experiments, infection with strain HS at a ratio of 100:1 for 30 min. The cells were harvested, homogenized, and separated into cytosolic and membrane-bound fractions by centrifugation. Cy-

focusing or clustering of host substrates must be necessary for PKC activation.

The time course of activation of PKC after EPEC infection was examined next (Fig. 2). When T84 cells were infected with strain E2348 at a multiplicity of infection of 120, membrane-
bound PKC activity peaked at 30 min after infection and was
in membranes from EPEC-infected cells (lane 3). In addition, all the T84 cell membranes demonstrated a 45-kDa band corresponding to PKM, the C-terminal catalytic fragment of PKC. The intensity of PKM appeared to increase somewhat in E2348-infected cells. PKC immunoreactivity was also readily detected in T84 cell cytosols but did not change with PMA treatment or EPEC infection (data not shown).

Figure 4B shows PKC-α immunoreactivity in HeLa cell cytosols (lanes 1 and 2) and membranes (lanes 3 and 4). PKC-α immunoreactivity decreased in the cytosol from E2348-infected cells (lane 2) compared to control HeLa cells (lane 1). The HeLa membrane fractions showed the opposite pattern: PKC-α was virtually undetectable in control HeLa cell membranes (lane 3) but became prominent in E2348-infected HeLa cells (lane 4). Again, the intensity of the 45-kDa PKM band increased markedly in infected HeLa cells (Fig. 4B, lanes 2 and 4).

Immunoblots of both the T84 cells and the HeLa cells showed an unidentified band of ~52 kDa. It is unclear whether this band is a larger fragment of PKC or an unrelated protein with cross-reactivity with PKC-α. Fasano et al., in their investigation of the role of PKC in the action of Vibrio cholerae zonula occludens toxin, also observed a prominent cross-reactive band of ~60 kDa in their PKC immunoblots made from rat IEC6 cells (17). In other immunoblot experiments (data not shown), the PKC inhibitors bisindolylmaleimide (1 μM) and sphingosine (10 μM) completely blocked the EPEC-induced translocation of PKC into T84 cell membranes.

Figure 4C shows that EPEC bacteria alone, in the absence of eukaryotic cells, do not show immunological cross-reactivity with the PKC-α monoclonal antibody in the size range of the PKC family, although there was faint reactivity at ~120 kDa in the lanes which were deliberately overloaded with bacteria. These immunological results confirm a large body of biochemical and genetic sequence data indicating that PKCs are not found in E. coli bacteria.

Next, immunofluorescence staining of EPEC-infected monolayers, with PKC-α monoclonal antibody, was used to determine the localization of PKC-α in relation to adherent clumps of bacteria. The purpose of these experiments was to determine whether PKC was redistributed to areas immediately subjacent to adherent clumps of bacteria and whether changes in PKC distribution were confined to the cell containing the bacterial colony or occurred in neighboring cells as well. In meeting these goals, we were only partially successful.

In these experiments, cells grown on Lab-Tek chamber slides were either infected or not infected with EPEC bacteria, fixed, permeabilized with 0.05% Triton X-100, detergent, immunostained with the PKC-α antibody, and visualized with a fluorescein-labelled second antibody. Adherent clumps of bacteria were located with differential interference contrast (Nomarski) optics, and then the same field was observed under UV illumination for fluorescence. In both the HeLa and T84 cells, high background fluorescence was observed in control cells, especially in areas of confluent cell growth and in the perinuclear areas of cells. Variations in the staining technique reduced but did not eliminate this background fluorescence, and it was difficult to distinguish whether this background represented artifactual staining or the actual normal, diffuse resting distribution of PKC-α within cells. In thinner areas of the cell monolayer, however, background staining was less and localized increases in PKC immunofluorescence could be visualized immediately surrounding adherent clumps of EPEC bacteria (Fig. 5A and B). PKC immunofluorescence appeared greatest in a rim or halo around the adherent clumps. This pattern is different from the published appearance of EPEC monolayers...
examined by the fluorescent actin staining technique or with antiphosphotyrosine antibodies, which produce such tightly localized labelling as to give the appearance that the bacteria themselves are labelled (14). In addition, we observed that medium-sized clumps of adherent bacteria (an estimated 25 to 50 bacteria) were associated with the most intense fluorescence with the PKC-α antibody. Very large adherent clumps and small clumps often gave fluorescence only slightly greater than background. This finding may be a consequence of the short-lived activation of PKC observed in the enzymatic assays.

Although adherence to the glass slide is not a characteristic of strain E2348, we did encounter rare clumps on the glass, not in contact with epithelial cells. Clumps of E2348 bacteria alone did not fluoresce (Fig. 5C and D), nor did any E. coli strain

FIG. 5. Colocalization of PKC with adherent EPEC bacteria. T84 cells were infected for 45 min with EPEC E2348, rinsed, fixed, permeabilized, and stained for PKC by immunofluorescence as described in Materials and Methods. The slides were then examined by differential interference contrast microscopy (left-hand panels) to locate adherent EPEC colonies, and then these areas were viewed under UV illumination to visualize PKC (right-hand panels). (A) Colony of E2348 bacteria (arrow); (B) same field shown in panel A, immunofluorescence view; (C) clumps of E2348 bacteria adhering to the glass slide (arrows); (D) same field as in panel C, showing that E2348 bacteria alone do not fluoresce. Original magnifications, ×400 for all panels.
dried on glass slides in the absence of epithelial cells. Strains HB101, HS, and JPN15 showed only rare, scattered adherence to cell monolayers, and these individual bacteria were difficult to visualize with interference contrast optics; no increased PKC fluorescence was observed in the vicinity of these bacteria, however (data not shown).

Previous reports by Weikel et al. (43), Crane et al. (10), and Levine et al. (25) showed that agents capable of stimulating PKC (phorbol esters, carbachol, and histamine) in intestinal cells potentiated the cellular response to the E. coli heat-stable toxin STA. We also recently showed that PKC is able to phosphorylate and activate the membrane-bound guanylyl cyclase which is the intestinal receptor for STa (9). Therefore, we examined whether EPEC infection of T84 cells would result in an increased cGMP response to STa. Initial experiments showed that infection of the T84 cells markedly decreased STa-stimulated cGMP levels, and this inhibition was seen with nonadherent strains as well as EPEC strains. Sterile culture supernatants of strain HB101 also inhibited STa-stimulated cGMP accumulation, which we attributed to either proteases or esterases in the bacterial culture medium. Consequently, to detect an enhancement of the response to STa, T84 cells were infected for 15 to 30 min, the monolayers were washed to remove nonadherent bacteria, STa was added in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine, and cGMP accumulation was allowed to proceed for an additional 45 to 60 min. By using this method, EPEC E2348 and JCP88 potentiated STa-stimulated cGMP accumulation ~60% compared to control, uninfected cells (Fig. 6). Nonadherent E. coli strains, such as HB101 (Fig. 6) and HS (data not shown) did not potentiate the effect of STa. Our results contrast with the recent work of Philpott et al., who studied T84 cells in the Ussing chamber after EPEC infection (28). After prolonged periods of infection (3 to 18 h) with strain E2348, the chloride secretory response to forskolin, a cAMP agonist, was decreased compared to uninfected cells. Our results show that at earlier time points, EPEC-infected monolayers show increased responsiveness to STa. These results raise the possibility of a pathogenic interaction between EPEC and the cGMP-linked STAs.

Finally, we tested whether protein kinase inhibitors could block the EPEC-induced activation of PKC in T84 cells and whether this blockade had any effect on EPEC adherence to or invasion of host cells (Tables 3 and 4). Addition of staurosporine to intact cells produced relatively little inhibition of the membrane-bound PKC activity (Table 3). This poor inhibition may be due to stripping of the inhibitor during the detergent extraction and DEAE chromatography steps, since re-adding staurosporine during the in vitro enzymatic assay led to 100% inhibition of PKC. Bisindolylmaleimide 1 and PKC(19–31) inhibitor peptide both also substantially inhibited the PKC activity when added in vitro (Table 3). In contrast, treatment of T84 cells with 0.1 to 1 μM staurosporine or 1 to 10 μM bisindolylmaleimide 1 (data not shown) had no effect on the adherence of EPEC E2348 and JCP88 to T84 cells or on their ability to invade T84 cells, as measured by the gentamicin protection assay (Table 4). In contrast genistein, a tyrosine kinase inhibitor, did reduce the number of intracellular bacteria, as previously reported by Rosenshine et al. (30). These results show that PKC activation in host cells, unlike tyrosine kinase activity, is not necessary for the intimate adherence or internalization of EPEC. Instead, PKC activation must occur at a step after the intimate adherence of EPEC to cells and appears to follow an initial burst of tyrosine phosphorylation of substrates.

### DISCUSSION

Recent advances in the pathogenesis of EPEC have included the identification of several key bacterial virulence genes and the host cell cytoskeletal changes involved the formation of the attaching-and-effacing adherence lesion. In addition, tyrosine phosphorylation of host cell protein substrates is a key event in

### TABLE 3. Effect of protein kinase inhibitors on the ability of EPEC E2348 to activate PKC

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Point at which inhibitor added</th>
<th>Inhibitor concn (μM)</th>
<th>% Inhibition^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staurosporine</td>
<td>To intact cells</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>To in vitro kinase assay</td>
<td>2</td>
<td>93</td>
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<td></td>
<td>To intact cells and again in vitro</td>
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<tr>
<td>Bisindolylmaleimide 1</td>
<td>In vitro</td>
<td>2</td>
<td>84</td>
</tr>
</tbody>
</table>

^a Percent inhibition was calculated in membrane-bound fractions by using the lipid-dependent (strict PKC) activity.

### TABLE 4. Effect of protein kinase inhibitors on the ability of EPEC E2348 to invade cells^b^

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>No. of intracellular bacteria (10^7 CFU/well)</th>
<th>% Invasion (no. invaded/total adherence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.98 ± 0.3</td>
<td>0.07</td>
</tr>
<tr>
<td>0.5 μM staurosporine</td>
<td>2.05 ± 0.6</td>
<td>0.07</td>
</tr>
<tr>
<td>200 μM genistein</td>
<td>1.0 ± 0.4</td>
<td>0.04^a</td>
</tr>
</tbody>
</table>

^b T84 cells in six-well plates were infected for 2 h with 2.7 × 10^8 bacteria (multiplicity of infection, 61); 2.8 × 10^7 bacteria (about 10% of the initial inoculum) adhered per well. Invasion was assessed by the gentamicin protection method.

^a P < 0.05.
the development of intimate adherence. However, neither the cytoskeletal rearrangements associated with intimate adherence nor the tyrosine phosphorylation events provide a plausible explanation for the severe watery diarrhea seen with EPEC infection (38). Here we have shown that EPEC adherence to human cell lines triggers the activation of PKC. Although the magnitude of the PKC stimulation we observed was modest (a 1.5- to 2-fold increase in total kinase activity, and a 2- to 2.5-fold increase in the lipid-dependent activity), there are three reasons why we believe that our results are biologically significant. First, our assay technique may actually underestimate the degree of activation of PKC occurring in the host cell. For example, the localized nature of the adherence of these strains and the multiplicity of bacterial infection we used results in only about one of every three to six host cells being adhered to by a bacterial microcolony. In addition, the activated form of PKC is highly susceptible to proteolytic degradation; although protease inhibitors were included in the homogenization buffer, no protease inhibitors were present during the period of bacterial infection. The prominent and rapid appearance of PKM, the proteolytic fragment of PKC, on immunoblots (Fig. 4B) is strong evidence that proteolysis of PKC is taking place under these assay conditions. Third, Berger et al. recently showed that E. coli bacteria, including EPEC strains, express an enzyme which acts as an inhibitor of mammalian protein kinases in vitro (5). All three of these factors tend to limit our ability to measure PKC activity and may lead to its underestimation in our assays. Second, the degree of PKC activation we measured compares favorably with that reported in the literature for known intestinal secretagogues. Beubler and Schirgi-Degen, for example, found that ricinoleic acid, the active ingredient in castor oil, stimulated PKC activity approximately threefold in rat intestinal cells (6). Therefore, the magnitude of PKC activation actually measured here in response to EPEC infection is close to that seen with a known, highly potent intestinal secretory stimulus.

An unexpected finding in this work was the rapid onset and offset of PKC stimulation (Fig. 2). Using a high inoculum of EPEC bacteria in which the adherent phenotype had been “induced” by subculturing in tissue culture media (40), PKC activation peaked at 30 min after infection and was still detectable 60 min after infection. The short duration of PKC activation which we observed, however, does not mean that PKC is irrelevant in the prolonged diarrheal illness characteristic of EPEC infection. First, in actual clinical EPEC infection, rounds of bacterial replication and infection of new intestinal cells are continuing. Second, PKC-mediated phosphorylation events may cause alterations in intestinal cell function which persist long after PKC enzymatic activity has subsided. For example, Weikle et al. showed that phorbol esters increase the cGMP response of T84 cells to E. coli STa. In their work, the increased cellular sensitivity to STa persisted as long as 18 h after an application of phorbol ester, even though PKC activity falls to below baseline (downregulation) by this time (43). Crane and Shanks recently showed that the PKC-mediated phosphorylation of the STa receptor/guanylyl cyclase in T84 cells is surprisingly long-lasting even in the absence of any phosphatase inhibitors (9).

We have not yet been able to determine whether other enteroadherent strains of E. coli share with EPEC the ability to stimulate PKC. For example, in preliminary experiments, enterohemorrhagic E. coli (EHEC) 221 failed to produce an increase in membrane-bound PKC activity at 30 min. However, the 221 strain subcultured in EPEC adherence medium failed to show spontaneous clumping and accelerated adherence like in the EPEC strains tested. In other words, the method of

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REFERENCES