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 other product of the breakdown of PIP2 is diacylglycerol, although the production of this labile lipid in response to EPEC has not been demonstrated experimentally.

The ability of EPEC to raise intracellular calcium levels and generate diacylglycerol has led several investigators to propose that EPEC may activate calcium-dependent, phospholipid-dependent protein kinase C (protein kinase C [PKC]) in the host cell (2, 13, 22). Indeed, Baldwin et al. showed that the pattern of phosphorylated bands in EPEC-infected HEp-2 cells resembled that in cells stimulated with phorbol esters, which are known stimulators of PKC (1). The hypothesis that PKC is activated in response to EPEC infection is an attractive one because it provides a plausible mechanism for the watery diarrhea which is a hallmark of EPEC infection. Although tyrosine kinases play a key role in the cytoskeletal rearrangements observed during the formation of the intimate adherence lesion of EPEC, it is dubious whether tyrosine kinases can account for a fluid secretory response (15, 38). In contrast, there is abundant evidence that PKC activation in the intestine triggers a brisk secretion of ions and fluid (6, 7, 42).

We exploited two unique features of the biochemistry of PKC to measure PKC activity in partially purified extracts of EPEC-infected cells. First, PKC is the only known mammalian protein kinase which is stimulated by diacylglycerol in 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 D-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 D-mannose per liter, henceforth referred to as EPEC adherence medium. EPEC adherence medium was pregassed 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. TS4 cells, a colon carcinoma cell line, were grown from 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 800 μl of EPEC adherence medium, and infected with 100 μl of bacterial subculture per well. Adherence was determined by staining with Giemsa 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 TS4 cells or ~2.0 × 10^7 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 Bofinger, Department of Clinical Laboratory Medicine, State University of New York at Buffalo, which were based on the methods of Chen and Murakami (8). After EPEC infection, cell monolayers were rinsed twice with ice-cold PBS and placed on an 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 phosphomethylsulfonyl fluoride, 10 μg of leupeptin per ml, 2 mM diithiothreitol (DTT). For this and all subsequent buffers, phosphomethylsulfonyl fluoride, 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+B+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 90 min, and the supernatant was collected and subjected to DEAE chromatography. DEAE chromatography 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 P5000 Pipetman pipettes (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 to columns. The columns 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 was 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 three eluted fractions were pooled.

Assay for PKC enzymatic activity. PKC was assayed by a modification of the method of Walker and Sandoc (41). Briefly, 30 μl of sample was added in the presence or absence of a phospholipid mixture (consisting of 100 μg of phosphatidylserine per ml and 30 μg dioleoylphosphatidylcholine) for 15 min at 30°C, containing 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 unlabelled ATP, and ~10^15 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 neuroagnin (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 P81 phosphocellulose paper and dropping it into a 500 ml beaker of 75 mM phosphoric acid with stirring. The P81 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 PKCα (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.) at a dilution of 1:20. To reduce background fluorescence, the second antibody was absorbed 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 covered slipped with a glycerol solution (Permoun). 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 Tris 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 2× 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 electroblotted to nitrocellulose, blocked with 5% nonfat dry milk, and probed with the mouse monoclonal antibody against PKCα at a dilution of 1:5,000 for 3 h at RT or overnight at 4°C. Goat anti-mouse antibody conjugated to horseradish peroxidase at a dilution of 1:5,000 in 2% milk was used as the secondary antibody for 1 h at RT. The blot was developed with Luminol chemiluminescence reagents (Kirkegaard & Perry) and exposed to X-ray film (Hyperfilm MP; Amersham) for 10 min. The anti-PKC antibody used recognizes an epitope in the C-terminal half of the enzyme; therefore, it reacts with the intact PKC holoenzyme as well as the proteolyzed, catalytic fragment (PKM). As a positive control, purified rat brain PKC (Calbiochem) was run in an adjacent lane.

Effect of protein kinase inhibitors. The PKC(19–31) inhibitor peptide and bisindolylmaleimide I were from Calbiochem. Staurosporine and genistein were from Sigma. The last three inhibitors were prepared as concentrated 2 mM stocks in dimethyl sulfoxide and stored at −20°C.
Membrane-bound activity concentration of 1 as the test of significance. Concepts, Berkeley, Calif.) and with Fisher's protected least significant differ-

Errors and error bars shown in tables, figures, and text repre-

 ration, and the plates were returned to the incubator. At 60 min after STa

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tility as well as the spontaneous clumping previously reported

such as HB101 and HS. Therefore, we adopted the subcultur-

procedure reported by Vuopio-Varkila and Schoolnik (40)

above. T84 cells, grown to confluency in six-well plates, were rinsed once to

remove antibiotics and then refed with 4 ml of EPEC adherence medium. All

experiments were used in duplicate experiments. Some T84 wells

were infected with 1 to 2 ml of the E2348-2h subculture or 0.5 to 1.0 ml of JCP88

subculture with the goal of achieving a multiplicity of infection of 100:1. After 15

to allow bacterial adherence to the cells, the supernatant medium was

aspirated and replaced with 1.5 ml of serum-free medium consisting of DMEM/

F12, 15 mM HEPES, 18 mM NaHCO3, 1g of bovine serum albumin per liter,

and 1 mM isobutylmethylxanthine (IBMX; a phosphodiesterase inhibitor). Phor-

bol-12-myristate-13-acetate (PMA) was added to appropriate wells to a final

concentration of 1 μM. STa was added to 0.1 μM to begin the cGMP accumu-

lation, and the plates were returned to the incubator. At 60 min after STa

addition, the reaction was terminated by the addition of 1.5 ml of 0.2 M HCl. The

acid extracts were frozen at –20°C, and cGMP levels were measured in triplicate

by radioimmunoassay as previously described (10, 11).

Data analysis and presentation. All experiments presented were performed at

least three times. Errors and error bars shown in tables, figures, and text repre-

sent standard deviations. Tests of statistical significance were done by one-way

analysis of variance with the StatView program for the Macintosh (Abacus

Concepts, Berkeley, Calif.) and with Fisher’s protected least significant differ-

ence as the test of significance.

RESULTS

In our initial attempts to determine whether PKC was ac-

tivated in response to EPEC infection, we used the traditional

procedure of subculturing bacteria in LB broth and then in-

fected the epithelial monolayers for 3 h. In these experiments,

PKC activity was markedly decreased in monolayers infected

with all E. coli strains tested, including nonadherent strains

such as HB101 and HS. Therefore, we adopted the subcultur-

ing procedure reported by Vuopio-Varkila and Schoolnik (40)

and extended by Rosenshine et al. (31), who showed that subcultur-

ing in DMEM was able to induce the adherent pheno-

type in EPEC strains, allowing strong localized adherence to

cells within 30 min of infection. Using this subculturing pro-

tocol, EPEC E2348 and JCP88 demonstrated cessation of mo-

tility as well as the spontaneous clumping previously reported

whereas strains HB101 and HS remained actively motile and
did not clump. When these “induced” cultures were used to

infect cells, localized adherence was seen within 15 min and

strong localized adherence was seen within 30 min. With this

protocol, 5 to 10% of the added EPEC bacteria adhered to the

T84 cells and HeLa cells in 30 min. However, since the bacteria

were adhering in large clumps of 25 to 50 bacteria, only about

one of every three to six eukaryotic cells had adherent bacterial

colonies.

We first examined the effect of EPEC infection on a nonin-

testinal 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-ex-

change 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 def-

inition of PKC activity, was calculated as the difference be-

tween the two measured values. Cells were also treated with

phorbol esters, which are known stimulators of PKC, for pur-

poses 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 100:1, cytosolic PKC activity fell modestly, with the lipid-
dependent portion of PKC activity dropping 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 66 and 44% for cells infected with E2348 and JCP88, respectively. For comparison, phorbol ester treatment caused mem-
brane-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 stimula-

tion 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-

TABLE 2. Effect of EPEC adherence on PKC activity in HeLa cells

<table>
<thead>
<tr>
<th>Fraction and infection</th>
<th>Mean PKC activity ± SD (pmol of 32P transferred to histone/min/dish)</th>
<th>Change in lipid-dependent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Ca&lt;sup&gt;2+&lt;/sup&gt; alone</td>
<td>With Ca&lt;sup&gt;2+&lt;/sup&gt; plus phospholipid (total kinase activity)</td>
</tr>
<tr>
<td>Cytosolic activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, uninfected</td>
<td>176.1 ± 40.1</td>
<td>557.8 ± 41.3</td>
</tr>
<tr>
<td>1 μM PMA, 30 min</td>
<td>250.6 ± 14.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>253.6 ± 18.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>E2348 infected, 1 h</td>
<td>181.6 ± 23.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>433.6 ± 26.0</td>
</tr>
<tr>
<td>JCP88 infected, 1 h</td>
<td>239.4 ± 31.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>459.7 ± 45.5</td>
</tr>
<tr>
<td>Membrane-bound activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, uninfected</td>
<td>306.6 ± 14.9</td>
<td>476.3 ± 26.5</td>
</tr>
<tr>
<td>1 μM PMA, 30 min</td>
<td>236.9 ± 32.3</td>
<td>957.5 ± 47.4&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>E2348 infected, 1 h</td>
<td>368.8 ± 22.4</td>
<td>649.9 ± 16.9&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>JCP88 infected, 1 h</td>
<td>380.6 ± 62.2</td>
<td>624.7 ± 52.1&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Phospholipid-dependent activity is determined arithmetically by subtracting the activity observed in the presence of Ca<sup>2+</sup> alone from the total kinase activity.

<sup>b</sup> Value is not significantly different from uninfected control.

<sup>c</sup> P < 0.05 compared to uninfected control.

<sup>d</sup> P < 0.01 compared to uninfected control.
HB101 can trigger modest increases \(\ldots\); Dytoc et al. noted that nonadherent strains such as \(\ldots\) E. coli \(\ldots\) strains like \(\ldots\) EPEC. 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-directed protein kinases other than PKC are also stimulated in response to EPEC. This is consistent with the work of other investigators, who have provided evidence that calmodulin-dependent protein kinases are stimulated by EPEC (3, 26).

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 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).

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 experiments was to detect the translocation of PKC into T84 cell membrane preparations immunologically.
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 ~60 kDa in their PKC immunoblots made from E. coli with cross-reactivity with PKC-α. Molecular mass markers are shown in kilodaltons. The position of intact, 82-kDa PKC-α and its 45-kDa catalytic fragment (PKM) are indicated. (A) Membrane extracts of T84 cells. Lanes: 1, from uninfected control cells; 2, from cells treated with 1 μM PMA for 30 min; 3, from cells infected with E2348 for 60 min; 4, purified rat brain PKC as a positive control. (B) Extracts of HeLa cells. Lanes 1 and 2 contain cytosols; lanes 3 and 4 contain membrane fractions. Lanes: 1, from uninfected control cells; 2, from cells infected with E2348 for 60 min; 3, from uninfected control cells; 4, from cells infected with E2348 for 60 min. (C) Blot of *E. coli* strains in the absence of mammalian cells. Lanes: 1, E2348 bacteria alone; 2, JCP88 alone; 3, rat brain PKC as a positive control. Lanes 1 and 2 were deliberately overloaded with four times the number of bacteria as were estimated to have adhered to the cells in panel A.

In these experiments, a crude membrane fraction was prepared from T84 cells, solubilized in Triton X-100, detergent, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blocked to nitrocellulose, and probed with a monoclonal antibody against PKC-α. Molecular mass markers are shown in kilodaltons. The position of intact, 82-kDa PKC-α and its 45-kDa catalytic fragment (PKM) are indicated. (A) Membrane extracts of T84 cells. Lanes: 1, from uninfected control cells; 2, from cells treated with 1 μM PMA for 30 min; 3, from cells infected with E2348 for 60 min; 4, purified rat brain PKC as a positive control. (B) Extracts of HeLa cells. Lanes 1 and 2 contain cytosols; lanes 3 and 4 contain membrane fractions. Lanes: 1, from uninfected control cells; 2, from cells infected with E2348 for 60 min; 3, from uninfected control cells; 4, from cells infected with E2348 for 60 min. (C) Blot of *E. coli* strains in the absence of mammalian cells. Lanes: 1, E2348 bacteria alone; 2, JCP88 alone; 3, rat brain PKC as a positive control. Lanes 1 and 2 were deliberately overloaded with four times the number of bacteria as were estimated to have adhered to the cells in panel A.

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In these experiments, a crude membrane fraction was prepared from T84 cells, solubilized in Triton X-100, detergent, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blocked to nitrocellulose, and probed with a monoclonal antibody against PKC-α. Molecular mass markers are shown in kilodaltons. The position of intact, 82-kDa PKC-α and its 45-kDa catalytic fragment (PKM) are indicated. (A) Membrane extracts of T84 cells. Lanes: 1, from uninfected control cells; 2, from cells treated with 1 μM PMA for 30 min; 3, from cells infected with E2348 for 60 min; 4, purified rat brain PKC as a positive control. (B) Extracts of HeLa cells. Lanes 1 and 2 contain cytosols; lanes 3 and 4 contain membrane fractions. Lanes: 1, from uninfected control cells; 2, from cells infected with E2348 for 60 min; 3, from uninfected control cells; 4, from cells infected with E2348 for 60 min. (C) Blot of *E. coli* strains in the absence of mammalian cells. Lanes: 1, E2348 bacteria alone; 2, JCP88 alone; 3, rat brain PKC as a positive control. Lanes 1 and 2 were deliberately overloaded with four times the number of bacteria as were estimated to have adhered to the cells in panel A.
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 Weikl 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 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 (Fig. 6) and HS (data not shown) had no effect on the 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 in the formation of the attaching-and-effacing adherence lesion. In addition, tyrosine phosphorylation of host cell protein substrates is a key event in

<p>| Table 3. Effect of protein kinase inhibitors on the ability of EPEC E2348 to activate PKC |
|---------------------------------------|---------------------------------|-------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Point at which inhibitor added</th>
<th>Inhibitor concn (mM)</th>
<th>% Inhibitiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staurosporine</td>
<td>To intact cells</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>To in vitro kinase assay</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>To intact cells and</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>again in vitro</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>PKC(19–31) inhibitor</td>
<td>In vitro</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>peptide</td>
<td>In vitro</td>
<td>200</td>
<td>60</td>
</tr>
<tr>
<td>Bisindolylmaleimide I</td>
<td>In vitro</td>
<td>200</td>
<td>60</td>
</tr>
</tbody>
</table>

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

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 I 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 I (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.

**Table 4. Effect of protein kinase inhibitors on the ability of EPEC E2348 to invade cells**

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>No. of intracellular bacteria (10⁴ 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>0.04b</td>
</tr>
</tbody>
</table>

a T84 cells in six-well plates were infected for 2 h with 2.7 × 10⁶ bacteria (multiplicity of infection, 61); 2.8 × 10⁵ bacteria (about 10% of the initial inoculum) adhered per well. Invasion was assessed by the gentamicin protection method.

b 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 lipoprotein-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. Second, 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 medium (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, Weikel 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, enteroaggregative E. coli (EAggEC) 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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