INFECTION AND IMMUNITY, Aug. 1997, p. 3277–3285 Vol. 65, No. 8
0019-9567/97/$04.00+0

Activation of Host Cell Protein Kinase C by Enteropathogenic *Escherichia coli*

JOHN K. CRANE* AND JENNY S. OH

Division of Infectious Diseases, Department of Medicine, State University of New York at Buffalo, Buffalo, New York 14214

Received 2 December 1996/Returned for modification 9 January 1997/Accepted 23 May 1997

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 (IP_3), presumably from the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) (4, 16, 19). The other product of the breakdown of PIP_2 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 (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 reversion 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 $NaHCO_3$, 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 CO_2 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).

* Corresponding author. Mailing address: Infectious Diseases, Room 109, Biomedical Research Bldg., 3435 Main St., Buffalo, NY 14214. Phone: (716) 829-2676. Fax: (716) 829-2158. E-mail: jcrane@acsu.buffalo.edu.

TABLE 1. *E. coli* strains used in this study

Strain	Description	Serotype	Source	Reference(s)
HB101	Nonadherent, laboratory strain	O:rough	Gibco/BRL	
H.S.	Nonadherent, human fecal flora	O9:H4	J. J. Mathewson	16
E2348 (also called E2348/69)	Classic EPEC strain; localized adherence; EAF^{+a}	O127:H6	J. B. Kaper	23
JPN15	Plasmid-cured derivative of E2348; nonadherent	Same	J. B. Kaper	24
CVD206	*eaeA*-deleted derivative of E2348; slight, nonintimate adherence	Same	J. B. Kaper	12
JCP88	Classic EPEC strain; localized adherence	O119:B14	J. J. Mathewson	33, 34

[a] EAF, EPEC adherence factor.

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 $NaHCO_3$, 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 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 T84 cells or ~2×10^7 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 method of Chen and Murakami (8). After EPEC infection, cell 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 phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, and 2 mM dithiothreitol (DTT). For this and all subsequent buffers, phenylmethylsulfonyl 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+BSA+Triton) consisting of buffer A supplemented with 1% Triton X-100 detergent and 0.1 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 45 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 Pipetteman pipettor (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, and 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 and assayed.

Assay for PKC enzymatic activity. PKC was assayed by a modification of the method of Walker and Sando (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 μM dioctanoylglycerol) to a reaction mixture consisting of 25 mM HEPES (pH 7.8), 0.4 mM $CaCl_2$ (0.1 mM over the concentration of EGTA, carried over from the elution buffer), 6 mM $MgCl_2$, 0.2 mg of histone III-S per ml, 50 μM unlabelled ATP, and ~10^6 cpm of [γ-^{32}P]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 3 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 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.) at a dilution of 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 coverslipped 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 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 2% nonfat dry milk, and probed with the mouse monoclonal antibody against PKC-α 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 1:3,000 in 2% milk was used as the second antibody for 1 h at RT. The blot was developed with Lumiglo 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.

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

Fraction and infection	Mean PKC activity ± SD (pmol of ^{32}P transferred to histone/min/dish)			Change in lipid-dependent activity
	With Ca^{2+} alone	With Ca^{2+} plus phospholipid (total kinase activity)	Phospholipid-dependent portion[a]	
Cytosolic activity				
Control, uninfected	176.1 ± 40.1	557.8 ± 41.3	381.7 ± 57.6	
1 μM PMA, 30 min	250.6 ± 14.2[b]	253.6 ± 18.3[c]	3.0 ± 23.1	99% decrease
E2348 infected, 1 h	181.6 ± 23.8[b]	433.6 ± 26.0[c]	252.0 ± 35.2	34% decrease
JCP88 infected, 1 h	239.4 ± 31.0[b]	459.7 ± 45.5[d]	220.2 ± 55.1	42% decrease
Membrane-bound activity				
Control, uninfected	306.6 ± 14.9	476.3 ± 26.5	169.7 ± 30.4	
1 μM PMA, 30 min	236.9 ± 32.3	957.5 ± 47.4[c]	720.6 ± 57.3	324% increase
E2348 infected, 1 h	368.8 ± 22.4	649.9 ± 16.9[d]	281.1 ± 28.1	66% increase
JCP88 infected, 1 h	380.6 ± 6.2	624.7 ± 52.1[d]	244.1 ± 52.5	44% increase

[a] 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.

Potentiation of cGMP accumulation in intact T84 cells. Bacteria were grown overnight and subcultured 1:10 into EPEC adherence medium as described 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 experimental conditions were used in duplicate experiments. Some T84 wells were infected with 1 to 2 ml of the E2348 2-h 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 min 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 $NaHCO_3$, 1 g of bovine serum albumin per liter, and 1 mM isobutylmethylxanthine (IBMX; a phosphodiesterase inhibitor). Phorbol-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 accumulation, 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 represent 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 difference as the test of significance.

RESULTS

In our initial attempts to determine whether PKC was activated in response to EPEC infection, we used the traditional procedure of subculturing bacteria in LB broth and then infecting 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 subculturing procedure reported by Vuopio-Varkila and Schoolnik (40) and extended by Rosenshine et al. (31), who showed that subculturing in DMEM was able to induce the adherent phenotype in EPEC strains, allowing strong localized adherence to cells within 30 min of infection. Using this subculturing protocol, EPEC E2348 and JCP88 demonstrated cessation of motility 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 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 ~200:1, 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 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-

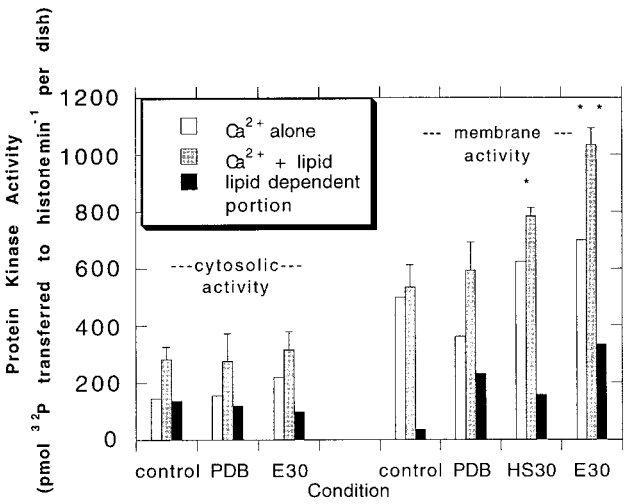

FIG. 1. Effect of EPEC infection on PKC activity in T84 cells. Confluent monolayers of T84 cells were infected with EPEC E2348 or nonadherent strain HS at a ratio of 100:1 for 30 min. The cells were harvested, homogenized, and separated into cytosolic and membrane-bound fractions by centrifugation. Cytosols 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. "Ca^{2+} alone," to the standard reaction mixture plus $CaCl_2$ 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 Ca^{2+} plus lipid and that in the presence of Ca^{2+} 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; *, $P < 0.05$; **, $P < 0.01$.

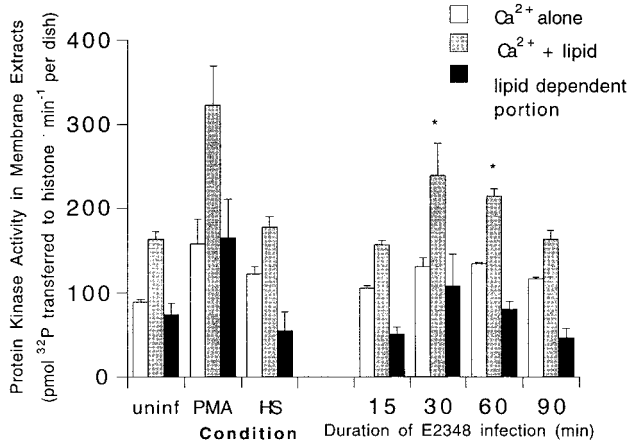

FIG. 2. Time course of PKC activation in T84 cell membrane fractions after EPEC infection. T84 cells were left uninfected (uninf), infected with nonadherent strain HS (HS), treated with 1 μM PMA for 30 min (PMA), or infected with EPEC strain E2348 for different periods, as shown on the right portion 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. *, $P < 0.05$.

fected T84 cells and the lipid-dependent (strict PKC) activity increased by a mean of 2.3-fold in E2348-infected cells.

Although we used histone as the substrate in most of our protein kinase assays, we also used a selective PKC substrate peptide (Selectide) in some experiments. For example, the membrane-bound protein kinase activity toward Selectide in the presence of calcium plus lipid increased (in units of picomoles per minute per dish) from 139 ± 9 in uninfected T84 cells to 227 ± 17 in cells infected with E2348 for 30 min, compared with 381 ± 91 in cells treated with PMA for 60 min. 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 IP_3 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-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 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).

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-

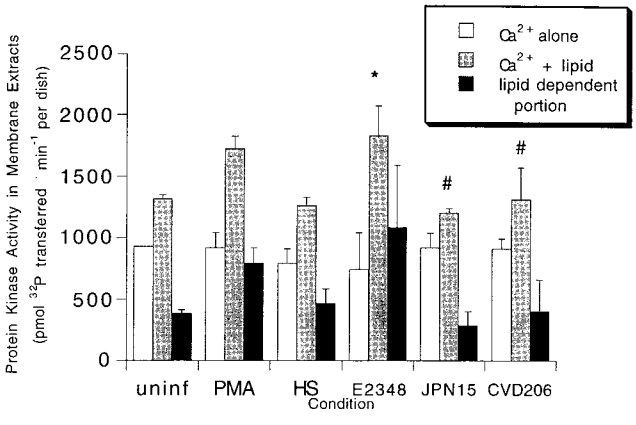

FIG. 3. Strain specificity of the effect of EPEC on PKC in T84 cells. T84 cells were left uninfected (uninf), treated with 1 μM PMA for 30 min (PMA), or infected with either strain HS, E2348, JPN15, or CVD206 for 30 min, and then harvested and processed for the PKC assay as described in Materials and Methods and the legend to Fig. 1. Only the activity in the membrane extracts was determined in this experiment. *, $P < 0.05$; #, not significant.

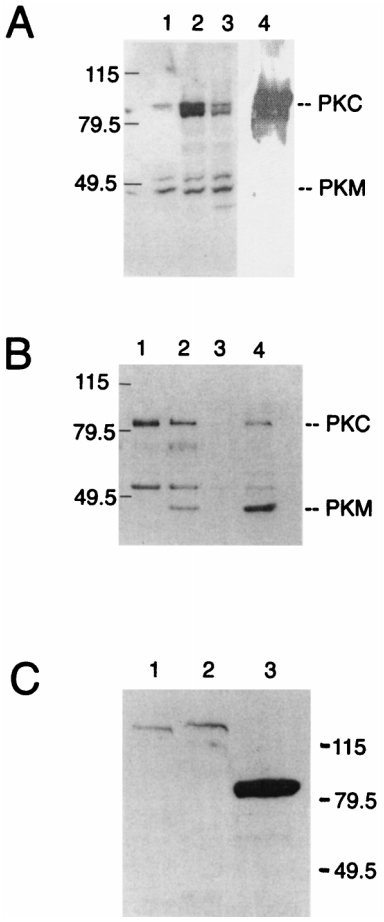

FIG. 4. Detection of EPEC-induced translocation of PKC from cytosol to membrane fractions by immunoblotting. T84 cells or HeLa cells were left uninfected or infected with EPEC E2348 for 60 min and harvested, and the cytosols and membranes prepared as described in Materials and Methods. Cellular fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted 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.

periments was twofold: first, to provide an independent method of verifying PKC activation by EPEC, and second, to try to simplify the relatively arduous purification procedure by eliminating the need for the DEAE chromatography steps. 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, and analyzed by Western immunoblotting with a monoclonal antibody directed against PKC-α, the predominant PKC isoenzyme in normal colon and in T84 cells. Figure 4A shows that the 82-kDa PKC-α was detectable in detergent extracts of membranes from control, uninfected T84 cells (lane 1). This reactivity increased markedly in membranes from T84 cells treated with PMA for 1 h (lane 2) and to a lesser extent 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

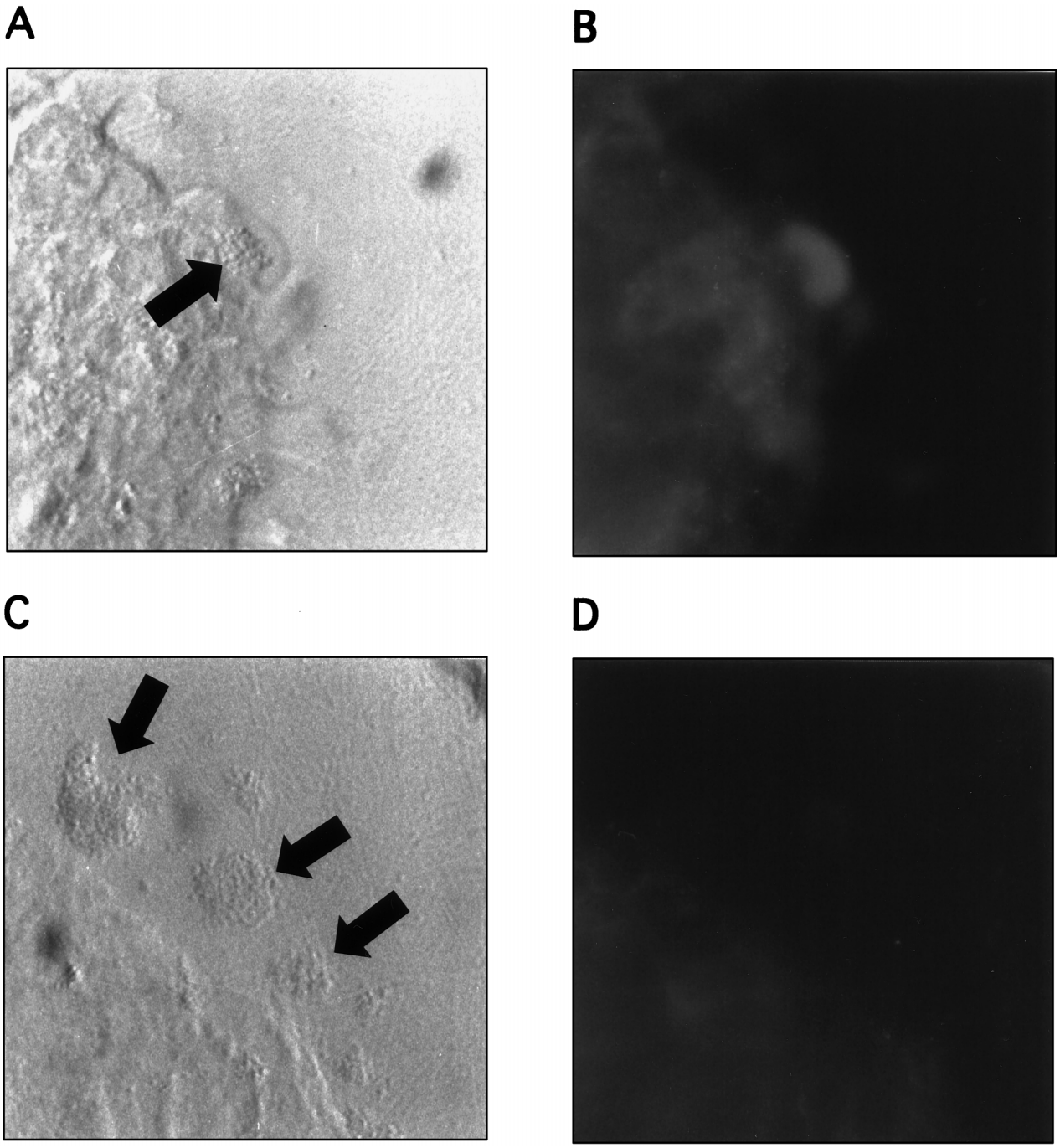

FIG. 5. Colocalization of PKC with adherent EPEC bacteria. T84 cells were infected for 45 min with EPEC E2348, rinsed, fixed, permeabilized, and stained for PKC by immunofluorescence as described in Materials and Methods. The slides were then examined by differential interference contrast microscopy (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.

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

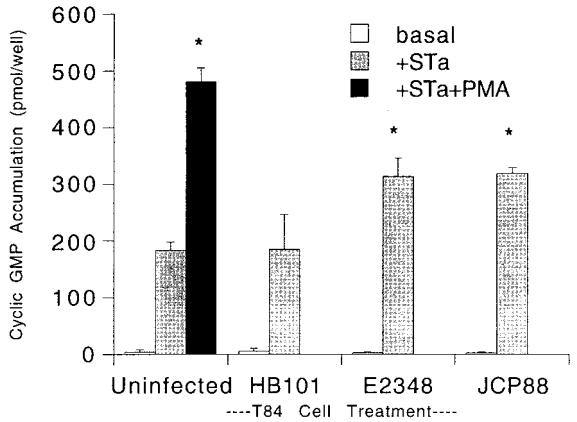

FIG. 6. Effect of EPEC infection on STa-stimulated cGMP levels in T84 cells. T84 cells were either left uninfected or infected with nonadherent strain HB101 or with EPEC E2348 or JCP88 for 15 min, rinsed twice, and refed with medium containing 1 mM isobutylmethylxanthine. The cells were stimulated with 0.1 μM STa alone or STa + 1 μM PMA or left unstimulated (basal), and cGMP was allowed to accumulate for an additional 45 min and then terminated by the addition of HCl. cGMP was determined later by radioimmunoassay. *, statistically significant increase compared to uninfected cells treated with STa alone ($P < 0.05$).

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 STs.

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

Inhibitor	Point at which inhibitor added	Inhibitor concn (μM)	% Inhibition[a]
Staurosporine	To intact cells	2	29
	To in vitro kinase assay	2	93
	To intact cells and again in vitro	2	100
PKC(19–31) inhibitor peptide	In vitro	20	35
	In vitro	200	86
Bisindolylmaleimide I	In vitro	2	84

[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. Bisindolymaleimide 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.

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 4. Effect of protein kinase inhibitors on the ability of EPEC E2348 to invade cells[a]

Cell treatment	No. of intracellular bacteria (10^4 CFU/well)	% Invasion (no. invaded/total adherence)
None (control)	1.98 ± 0.3	0.07
0.5 μM staurosporine	2.05 ± 0.6	0.07
200 μM genistein	1.0 ± 0.4	0.04[b]

[a] 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.

[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 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 secretagogs. 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 Vuopio-Varkila and Schoolnik for "inducing" the localized adherent phenotype in EPEC does not necessarily apply to other enteroadherent *E. coli* strains. Therefore, it is still possible that EAggEC, enterohemorrhagic *E. coli* (EHEC), or diffuse-adhering *E. coli* (DAEC) stimulate PKC, albeit with a different time course and culture conditions required for each.

Activation of PKC in intestinal cells may trigger secretion via several pathways. First, PKC, like cAMP-dependent protein kinase, is able to phosphorylate and activate the cystic fibrosis transmembrane regulator, which is the major chloride channel in the brush border of enterocytes (18, 29). PKC also stimulates another plasma membrane Cl^- channel distinct from the cystic fibrosis transmembrane regulator (21).

In addition to stimulation of Cl^- channels, activation of PKC appears to be involved in the abnormalities of tight junctions seen with EPEC infection. Prolonged incubation of cell monolayers with EPEC results in decreased transepithelial electrical resistance and increased permeability to sodium and mannitol, abnormalities which are due to changes in tight junctions (28, 39). Similar changes are observed in monolayers treated with phorbol esters for prolonged periods (20). Interestingly, Fasano et al. studied the mechanism of the *V. cholerae* zonula occludens toxin and found evidence that it acted via stimulation of PKC (17). Thus, in addition to stimulating chloride secretion directly, PKC appears to cause alterations in tight junctions and intestinal permeability, which may perpetuate the diarrhea seen in EPEC infections.

The potentiation of the action of STa by EPEC (Fig. 6) has several implications. First, it demonstrates that the increased PKC activity we observed in cell membrane extracts does have functional consequences in the intact cell. Second, this interaction may have clinical significance in cases of dual infections with enterotoxigenic *E. coli* (ETEC) and EPEC, which is not uncommon in studies of children with diarrhea in developing countries (27). Finally, the recently characterized enteroaggregative heat-stable toxin (EAST1) acts via the same guanylyl cyclase receptor as STa (36). Savarino et al. have gone on to show that many *E. coli* strains other than those classified as enteroaggregative possess the gene encoding EAST1 (37). In that study, all 75 EHEC strains, 41% of EAggEC strains, and 22% of EPEC strains tested hybridized with the *astA* gene probe. If these strains actually produce biologically significant amounts of EAST, some EPEC strains and other strains with EPEC-like adherence could act via PKC to sensitize the host to their own toxin. The ability of *V. cholerae* to upregulate host receptors for cholera toxin is an often-cited example of this phenomenon (35).

In summary, we have confirmed experimentally what had been predicted previously by other investigators, namely, that PKC is activated in host cells in response to adherence of EPEC. Additional work is needed to determine whether PKC activation is a trait shared by other enteroadherent *E. coli* strains or by other species of bacteria which show EPEC-like intimate adherence.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (R29 DK 49410) and from Research for Health in Erie County.

We thank John J. ('Jeff') Mathewson, University of Texas Health Sciences Center, Houston, for providing strains and teaching us how to perform the adherence assays. We thank Diane Bofinger, State University of New York at Buffalo, and Julianne J. Sando, University of Virginia, for help in setting up the PKC assays. Finally, we thank Donald C. Robertson, University of Idaho, for providing high-quality STa.

REFERENCES

1. **Baldwin, T., S. Brooks, S. Knutton, H. Manjarrez-Hernandez, A. Aitken, and P. Williams.** 1990. Protein phosphorylation by protein kinase C in HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect. Immun. **56:**761–765.
2. **Baldwin, T., S. Knutton, R. Haigh, P. Williams, H. Palmer, A. Aitken, and S. Borriello.** 1996. Hijacking host cell signal transduction mechanisms during infection with enteropathogenic *Escherichia coli*. Biochem. Soc. Trans. **24:**552–558.
3. **Baldwin, T., M. Lee-Delaunay, S. Knutton, and P. Williams.** 1993. Calcium-calmodulin dependence of actin accretion and lethality in cultured HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect. Immun. **61:**760–763.
4. **Baldwin, T., W. A. Aitken, S. Knutton, and P. Williams.** 1991. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect. Immun. **59:**1599–1604.
5. **Berger, S., K. Rowan, H. Morrison, and H. Ziltener.** 1996. Identification of a bacterial inhibitor of protein kinases. J. Biol. Chem. **271:**23431–23437.
6. **Beubler, E., and A. Schirgi-Degen.** 1993. Stimulation of enterocyte protein kinase C by laxatives in vitro. J. Pharm. Pharmacol. **45:**59–62.
7. **Chang, E., N.-S. Wang, and M. Rao.** 1985. Phorbol ester stimulation of active anion secretion in intestine. Am. J. Physiol. **249:**C356–C361.
8. **Chen, S., and K. Murakami.** 1992. Synergistic activation of type III protein kinase C by cis-fatty acid and diacylglycerol. Biochem. J. **282:**33–39.
9. **Crane, J., and K. Shanks.** 1996. Phosphorylation and activation of the intestinal guanylyl cyclase receptor for *Escherichia coli* heat-stable toxin by protein kinase C. Mol. Cell. Biochem. **165:**111–120.
10. **Crane, J. K., L. L. Burrell, C. S. Weikel, and R. L. Guerrant.** 1990. Carbachol mimics phorbol esters in its ability to enhance cyclic GMP production by STa, the heat-stable toxin of *Escherichia coli*. FEBS Lett. **274:**199–202.
11. **Crane, J. K., M. S. Wehner, E. Bolen, J. J. Sando, J. Linden, R. L. Guerrant, and C. L. Sears.** 1992. Regulation of intestinal guanylate cyclase by the heat-stable enterotoxin of *E. coli* (STa) and protein kinase C. Infect. Immun. **60:**5004–5012.
12. **Donnenberg, M., and J. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
13. **Donnenberg, M., and J. Kaper.** 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. **60:**3953–3961.
14. **Donnenberg, M., and J. Nataro.** 1995. Methods for studying adhesion of diarrheagenic *Escherichia coli*. Methods Enzymol. **253:**324–336.
15. **Donowitz, M., J. Montgomery, M. Walker, and M. Cohen.** 1994. Brush-border tyrosine phosphorylation stimulates ileal neutral NaCl absorption and brush-border Na^+/H^+ exchange. Am. J. Physiol. **266:**G647–G656.
16. **Dytoc, M., L. Fedorko, and P. Sherman.** 1994. Signal transduction in human epithelial cells infected with attaching and effacing *Escherichia coli* in vitro. Gastroenterology **106:**1150–1161.
17. **Fasano, A., C. Fiorentini, G. Donelli, S. Uzzau, J. Kaper, K. Margaretten, X. Ding, S. Guandalini, L. Comstock, and S. Goldblum.** 1995. Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. J. Clin. Invest. **96:**710–720.
18. **Fondacaro, J., and L. Henderson.** 1985. Evidence for protein kinase C as a regulator of intestinal electrolyte transport. Am. J. Physiol. **249:**G422–G426.
19. **Foubister, V., I. Rosenshine, and B. Finlay.** 1994. A diarrheal pathogen, enteropathogenic *Escherichia coli* (EPEC), triggers a flux of inositol phosphates in infected epithelial cells. J. Exp. Med. **179:**993–998.
20. **Hecht, G., B. Robinson, and A. Koutsouris.** 1994. Reversible disassembly of an intestinal epithelial monolayer by prolonged exposure to phorbol ester. Am. J. Physiol. **266:**G214–G221.
21. **Koumi, S., R. Sato, and T. Aramaki.** 1995. Activation of the plasma membrane chloride channel by protein kinase C in isolated guinea pig hepatocytes. J. Physiol. **487:**379–394.
22. **Law, D.** 1994. Adhesion and its role in the virulence of enteropathogenic *Escherichia coli*. Clin. Microbiol. Rev. **7:**152–173.
23. **Levine, M., D. Nalin, R. Hornick, E. Bergquist, D. Waterman, C. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins and are not invasive. Lancet **i:**1119–1122.
24. **Levine, M., J. Nataro, H. Karch, M. Baldini, J. Kaper, R. Black, M. Clements, and A. O'Brien.** 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152:**550–559.
25. **Levine, S. A., M. Donowitz, A. J. M. Watson, G. W. G. Sharp, J. K. Crane, and C. S. Weikel.** 1991. Characterization of the synergistic interaction of *Escherichia coli* heat-stable toxin and carbachol. Am. J. Physiol. **261:**G592–G601.
26. **Manjarrez-Hernandez, H., T. J. Baldwin, A. Aitken, S. Knutton, and P. Williams.** 1992. Intestinal epithelial cell protein phosphorylation in enteropathogenic *Escherichia coli* diarrhoea. Lancet **339:**521–523.
27. **Mathewson, J., R. Oberhelman, H. DuPont, F. de la Cabada, and E. Vasquez-Garibay.** 1987. Enteroadherent *Escherichia coli* as a cause of diarrhea among children in Mexico. J. Clin. Microbiol. **25:**1917–1919.
28. **Philpott, D., D. McKay, P. Sherman, and M. Perdue.** 1996. Infection of T84 cells with enteropathogenic *Escherichia coli* alters barrier and transport functions. Am. J. Physiol. **270:**G634–G645.
29. **Picciotto, M., J. Cohn, G. Bertuzzi, P. Greengard, and A. Nairn.** 1992. Phosphorylation of the cystic fibrosis transmembrane conductance regulator. J. Biol. Chem. **267:**12742–12752.
30. **Rosenshine, I., M. Donnenberg, J. Kaper, and B. Finlay.** 1992. Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. EMBO J. **11:**3551–3560.
31. **Rosenshine, I., S. Ruschkowski, and B. Finlay.** 1996. Expression of attaching/effacing activity by enteropathogenic *Escherichia coli* depends on growth phase, temperature, and protein synthesis upon contact with epithelial cells. Infect. Immun. **64:**966–973.
32. **Rosenshine, I., S. Ruschkowski, M. Stein, D. Reinscheid, S. Mills, and B. Finlay.** 1996. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. EMBO J. **15:**2613–2624.
33. **Rothbaum, R., A. McAdams, R. Giannella, and J. Partin.** 1982. A clinicopathologic study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. Gastroenterology **83:**441–454.
34. **Rothbaum, R., J. Partin, K. Saalfield, and A. McAdams.** 1983. An ultrastructural study of enteropathogenic *Escherichia coli* infection in human infants. Ultrastruct. Pathol. **4:**291–304.
35. **Salyers, A., and D. Whitt.** 1994. Bacterial pathogenesis: a molecular approach, p. 146. ASM Press, Washington, D.C.
36. **Savarino, S., A. Fasano, D. Robertson, and M. Levine.** 1991. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. J. Clin. Invest. **87:**1450–1455.
37. **Savarino, S., A. McVeigh, J. Watson, A. Cravioto, J. Molina, P. Echeverria, M. Bhan, M. Levine, and A. Fasano.** 1996. Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli*. J. Infect. Dis. **173:**1019–1022.
38. **Sears, C., and J. Kaper.** 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol. Rev. **60:**167–215.
39. **Spitz, J., R. Yuhan, A. Koutsouris, C. Blatt, J. Alverdy, and G. Hecht.** 1995. Enteropathogenic *Escherichia coli* adherence to intestinal epithelial monolayers diminishes barrier function. Am. J. Physiol. **268:**G374–G379.
40. **Vuopio-Varkila, J., and G. Schoolnik.** 1991. Localized adherence by enteropathogenic *Escherichia coli* is an inducible phenotype associated with the expression of new outer membrane proteins. J. Exp. Med. **174:**1167–1177.
41. **Walker, J. M., and J. J. Sando.** 1988. Activation of protein kinase C by short chain phosphatidylcholines. J. Biol. Chem. **263:**4537–4540.
42. **Weikel, C. S., J. J. Sando, and R. L. Guerrant.** 1985. Stimulation of porcine jejunal ion secretion in vivo by protein kinase C activators. J. Clin. Invest. **76:**2430–2435.
43. **Weikel, C. S., C. L. Spann, C. P. Chambers, J. K. Crane, J. Linden, and E. L. Hewlett.** 1990. Phorbol esters enhance the cyclic GMP response of T84 cells to the heat-stable enterotoxin of *Escherichia coli* (STa). Infect. Immun. **58:**1402–1407.

Editor: A. O'Brien