Cell Death and Differentiation (2005) 12, 115–127

www.nature.com/cdd

Externalization of host cell protein kinase C during enteropathogenic *Escherichia coli* infection

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Received 20.2.04; revised 18.3.04; accepted 30.9.04; published online 03.12.04
Edited by DR Green

Abstract

Enteropathogenic *Escherichia coli* (EPEC) is a common cause of diarrhea in children in developing countries. Protein kinase C (PKC), a serine- and threonine-directed protein kinase, is rapidly activated following EPEC infection and this is accompanied by its translocation to a membrane-bound location where it is tightly bound to phosphatidylserine (PS). EPEC infection causes host cell death, one of whose features is externalization of PS. We hypothesized that externalization of PS would be accompanied by externalization of PKC as well. We report that EPEC infection triggers the externalization of PKC to the outer surface of the host cell. Ecto-PKC remains firmly tethered to the cell but can be released by incubation with peptide or protein substrates for the enzyme. Ecto-PKC is intact and biologically active and able to phosphorylate protein substrates on the surface of the host cell. Phosphorylation of whole EPEC bacteria or EPEC-secreted proteins could not be detected. Externalization of PKC could be reproduced by the combination of an apoptotic stimulus (ultraviolet (UV) irradiation) and phorbol myristate acetate (PMA), a procedure which resulted in externalization of >25% of the total cellular content of PKC-α. In the presence of ATP, ecto-PKC inhibited UV-induced cell shrinkage, membrane blebbing, and propidium iodide uptake but not the activation of caspases 3 and 7. This is the first report that expression of an ecto-protein kinase is altered by a microbial pathogen and the first to note that externalization of PKC can accompany apoptosis.
Cell Death and Differentiation (2005) **12,** 115–127.
doi:10.1038/sj.cdd.4401531
Published online 3 December 2004

Keywords: ecto-protein kinase; ecto-phosphorylation; enteropathogenic *E. coli*; protein kinase C; potassium channels; apoptotic volume decrease; *Escherichia coli*

Abbreviations: PKC, protein kinase C; EPEC, enteropathogenic *Escherichia coli*; PS, phosphatidylserine; PMA, phorbol myristate acetate; UV, ultraviolet; LDH, lactate dehydrogenase; ATP, adenosine 5′-triphosphate

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of watery diarrhea in children in developing countries. Unlike some other types of diarrheagenic *E. coli*, EPEC produces no toxins and the way that it triggers diarrhea has been unclear. EPEC adheres intimately to intestinal cells, causes rearrangement of the host cytoskeleton, activates host signalling pathways including tyrosine kinases, and translocates EPEC-secreted proteins (Esps) into the host cell via its Type III secretion system.

In a previous study, the author and co-workers reported that EPEC triggers an activation of protein kinase C (PKC) in the host cell.[1] Concomitant with this activation, PKC translocates from a cytosolic to a membrane-bound location within the cell. By immunofluorescence staining, PKC-α also was observed to cluster in a loose 'halo' around microcolonies of EPEC bacteria. Subsequently we reported that EPEC induced host cell death with mixed features of apoptosis and necrosis.[2] One of the apoptotic features of EPEC-induced host cell death is externalization of phosphatidylserine (PS). Since PKC, once activated, physically binds to PS on the inner leaflet of the plasma membrane, we wondered if, during EPEC-induced cell death, PKC would become externalized along with PS. Our interest in this possibility increased when we discovered, in a subsequent study, that EPEC causes the release of large amounts of ATP from the host cell.[3] Taken alone, externalization of PKC by EPEC might be considered a mere oddity, but the release of ATP in the same infectious process meant that PKC could act catalytically while in an extracellular location. While the roles of ecto-protein kinases have been studied in other contexts, especially in the brain,[4,5] they have not been studied in regard to microbial pathogenesis. Here, we report that PKC-α is externalized in response to EPEC infection of cultured cells, that ecto-PKC remains intact and biologically active, and it remains tethered to the external surface of the cell unless released by nonphysiologic experimental manipulations.

Results

Initial experiments in T84 cells (a human colon cell line) and HeLa cells (human cervical cancer cell line) failed to detect release of PKC or PKC-like enzymatic activity into the supernatants of infected cells at various times after EPEC infection. Almost 20 years ago, while investigating the presence of ecto-casein kinase activity in certain cell lines, Kubler *et al*.[6] noted that the ecto-casein kinase was tightly associated with the cell surface, but that it could be released by incubation with protein substrates for the enzyme, such as phosvitin or casein itself. Therefore we adopted that strategy and repeated the experiments; at various times after EPEC infection, the medium was changed to physiologic buffer with one of two different PKC substrates, either histone or a selective PKC substrate peptide, and incubation was

continued for an additional 30 min. Then the supernatant medium was transferred to new tubes, [^{32}P]-ATP was added along with other cofactors, and ^{32}P incorporation into substrates was measured. One of the hallmarks of PKC-α is dependence on phospholipid; therefore, assays were performed without and with phosphatidylserine.[7]

When histone (0.1–0.2 g/l) was added to the extraction buffer, PKC-like enzymatic activity was released from EPEC-infected cells into the supernatant (Figure 1a), and this activity was increased compared to uninfected cells (time zero of Figure 1a). In our previous study, intracellular PKC activity increased quickly after EPEC infection, peaked at 30–60 min, and then fell to baseline by 90 min after infection. In contrast, the externalized PKC activity shown in Figure 1a continued to increase gradually up to 2 h after infection and did not begin to fall again until 3 h after infection.

High concentrations of histone in extracellular medium can cause cytotoxicity; therefore, we repeated the procedure using a selective peptide substrate, the PKC Ser^{25}(19–31) substrate peptide. Figure 1b shows that if the substrate peptide was included in the cell wells during the extraction phase, significant amounts of PKC enzymatic activity were released into the supernatant medium (right side of Figure 1b). In contrast, if the substrate was omitted from the cell wells, little PKC activity was detected in the cell supernatants, despite addition of the peptide to the same final concentration (30 μM) in the *in vitro* protein kinase reaction (left side of Figure 1b). The results with this peptide again showed that release of PKC into the supernatant was substrate-dependent.

Figure 1c shows the release of PKC activity by various *E. coli* strains. HB101, a laboratory strain, and JPN15, the plasmid-cured derivative of EPEC strain E2348/69 which fails to adhere, did not trigger significant PKC release above uninfected levels, whereas the wild-type EPEC strain E2348/69 did do so. Other wild-type EPEC strains tested, such as B171-8 and JCP88, also triggered PKC externalization (data not shown).

Figure 1 showed that protein kinase activity was externalized in response to EPEC infection. This protein kinase activity seemed to be PKC based on its dependence on phospholipid (Figure 1a) and its ability to phosphorylate a selective PKC substrate peptide (Figure 1b and c). In order to determine if the externalized kinase activity was authentic PKC, we tested the cell extracts for reactivity with a monoclonal antibody against PKC-α in a Western immunoblot format. Figure 2 shows that after 1 h of EPEC infection, PKC-α did appear in the histone extracts of T84 cells (lanes 3 and 4). EPEC infection in the presence of wortmannin, which augments EPEC-induced cell death,[2] increased the amount of PKC-α released at this early time point (lanes 5 and 6). As in our previous study, histone extracts of *E. coli* bacteria alone did not show a cross-reacting band (lane 7). Treatment of T84 cells with phorbol myristate acetate (PMA) to stimulate PKC also did not result in release of PKC-α into the supernatant

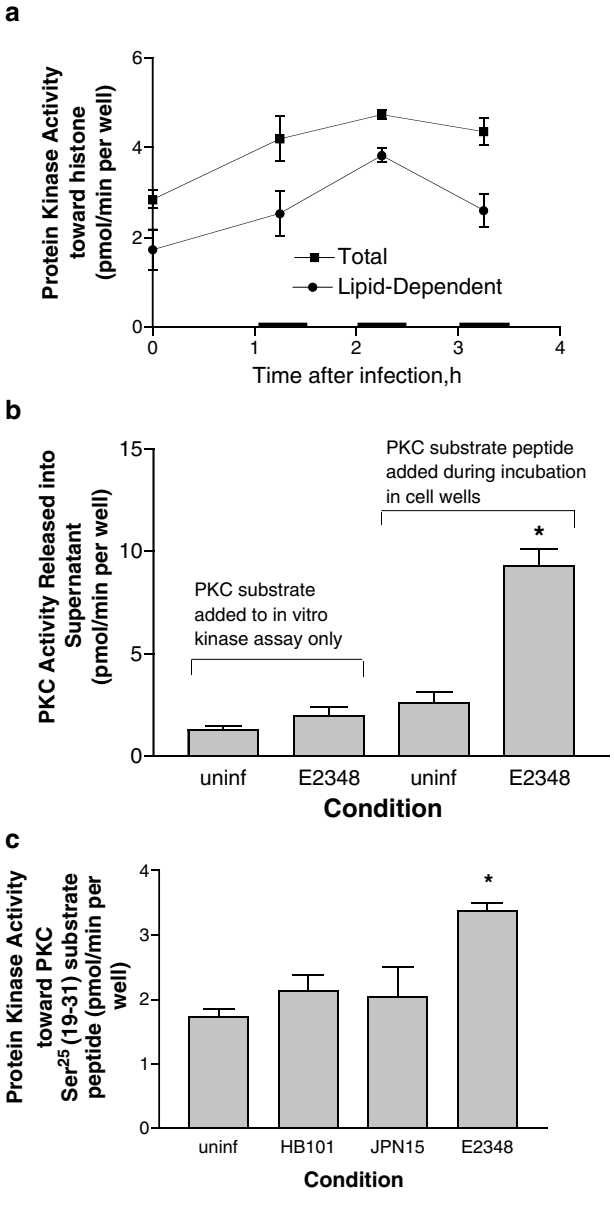

Figure 1 Time course and protein or peptide substrate dependence of the release of protein kinase C activity into T84 cell supernatants. (**a**) T84 cells were infected with EPEC strain E2348/69 for the time indicated on the graph, followed by a 30 min extraction phase in HBSS + 0.2 g/l histone (indicated by the black horizontal bars). Extracts were then assayed for protein kinase activity with and without a mixture of phosphatidylserine and dioctanoyl glycerol. The difference between total protein kinase activity in the presence of lipid and basal activity without lipid is the lipid-dependent portion, an operational definition of classical protein kinase C activity. (**b**) Substrate-dependent release of ecto-PKC activity. T84 cells were infected with E2348 for 2 h, then the medium was changed to either HEPES-buffered saline alone, or buffered saline plus 30 μM PKC Ser^{25}(19–31) substrate peptide and allowed to extract for 30 min more; then, the extracts were assayed for ^{32}P incorporation into the substrate peptide as described in Materials and Methods. Additional substrate peptide was added as needed in the *in vitro* kinase assay to yield a final concentration of 30 μM in all assay conditions. PKC loses much of its lipid dependence when peptide substrates are used; therefore, all assays were carried out in the presence of the lipid mixture and the total protein kinase activity is shown; *, $P < 0.05$ by *t*-test. (**c**) Comparison of wild-type EPEC with other *E. coli* strains in ability to induce PKC externalization. T84 cells were infected for 2 h with the *E. coli* strain indicated, then subjected to extraction in HEPES-buffered saline + 30 μM PKC Ser^{25}(19–31) substrate peptide. As in (**b**), the total protein kinase activity is shown. HB101, a nonadherent, nonpathogenic laboratory *E. coli* strain; JPN15, the plasmid-cured, EAF(−) derivative of EPEC E2348/69. *Significantly increased compared to uninfected control

medium (Figure 2, lane 8). The immunoblot results indicated that the protein kinase externalized in response to EPEC infection was authentic PKC-α.

Since the monoclonal antibody against PKC-α was able to detect PKC after histone extraction in a Western immunoblot format, we tested whether this antibody was capable of detecting PKC while it was still bound to the surface of the cell by immunocytochemistry. This would eliminate the need to add histone, or a PKC substrate peptide, to induce release of PKC into the supernatant medium. In cells which have not been permeabilized, antibodies are capable of recognizing their target only if it is exposed on the cell surface.[8] Figure 3 shows that in uninfected, control HeLa cells (Figure 3a) or cells infected with nonadherent laboratory *E. coli* strain HB101 for 2 h (Figure 3b), there was little or no surface-exposed PKC immunoreactivity by peroxidase stain. In cells infected with wild-type EPEC strain E2348/69 for 2 h, PKC immunoreactivity (brown stain) was seen in cells adhered to by microcolonies of EPEC bacteria (Figure 3c, where bacteria are stained blue and are visible as large clumps). Not all cells adhered to by EPEC showed peroxidase staining. In Figure 3d, HeLa cells were fixed, then permeabilized with methanol before the immunostaining, and all cells showed PKC reactivity throughout the cytoplasm.

As indicated in above, our hypothesis was that EPEC-induced PKC externalization was the result of dual stimuli resulting in PKC activation (accompanied by translocation to the plasma membrane) and in cell death (resulting in surface exposure of PS to which the PKC was bound). In order to test whether this idea was correct, we used the methods already developed in Figures 2 and 3 to determine if we could reproduce the PKC externalization with an apoptotic stimulus (UV irradiation), a chemical activator of PKC (phorbol ester), or both. HeLa cells were again chosen because they undergo more vivid morphological changes after exposure to UV light than do T84 cells, and they were again left unpermeabilized. Figure 4 shows that UV irradiation triggered apoptosis (Figure 4b) as shown by cell shrinkage, marked membrane

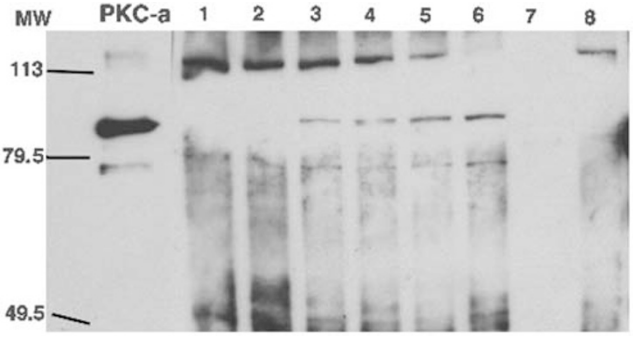

Figure 2 Immunoblot detection of released material as PKC-α. T84 cells were infected with EPEC E2348 with or without 100 nM wortmannin for 1 h, then extracted for 30 min in HBSS + histone. Extracts were subjected to SDS-PAGE, transferred to nitrocellulose, then blotted with a monoclonal Ab against PKC-α. As well-to-well variability is noted in earlier experiments, each unknown condition was run twice, from duplicate wells. For lanes 1–6 and 8, equal volumes of supernatant were loaded per lane, while lane 7 was deliberately overloaded with five times as many *E. coli* bacteria as the normal inoculum. Legend: MW, positions of prestained MW markers; PKC-a, purified PKC-α, diluted 1 : 100; lanes 1 and 2, uninfected control T84 cells; lanes 3 and 4, E2348 infected; lanes 5 and 6, EPEC infected + wortmannin; lane 7, *E. coli* bacteria alone were subjected to the histone extraction procedure, then spun to pellet *E. coli* bacteria, and the supernatant loaded on the gel; lane 8, T84 cells stimulated with 100 nM PMA

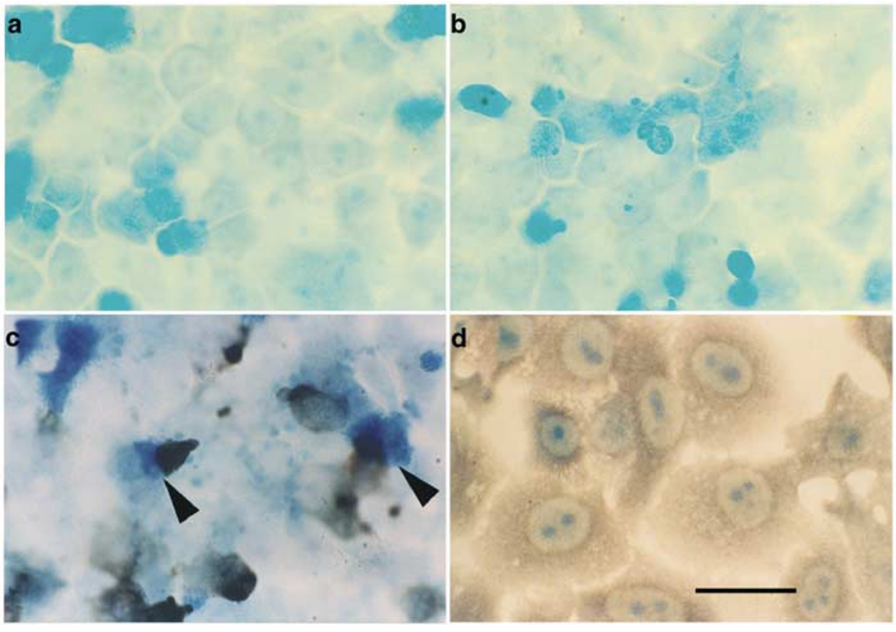

Figure 3 Immunoperoxidase staining of ecto-PKC during EPEC infection. HeLa cells were grown in Lab-Tek chamber slides and subjected to infection for 2 h, or left uninfected as controls. Slides were fixed, but only those in (**d**) were permeabilized. Cells were immunostained as described in Materials and Methods, and counterstained with Giemsa. Photomicrographs were at $\times$ 600. (**a**) Normal uninfected HeLa cells; (**b**) cells infected with HB101, a nonadherent strain. (**c**) Cells infected with wild-type EPEC E2348/69, showing typical localized adherence in large tight clumps of hundreds of bacterial cells (blue microcolonies indicated by black arrowheads). Many but not all host cells in contact with EPEC microcolonies show brown immunostaining. Size bar = 20 μm

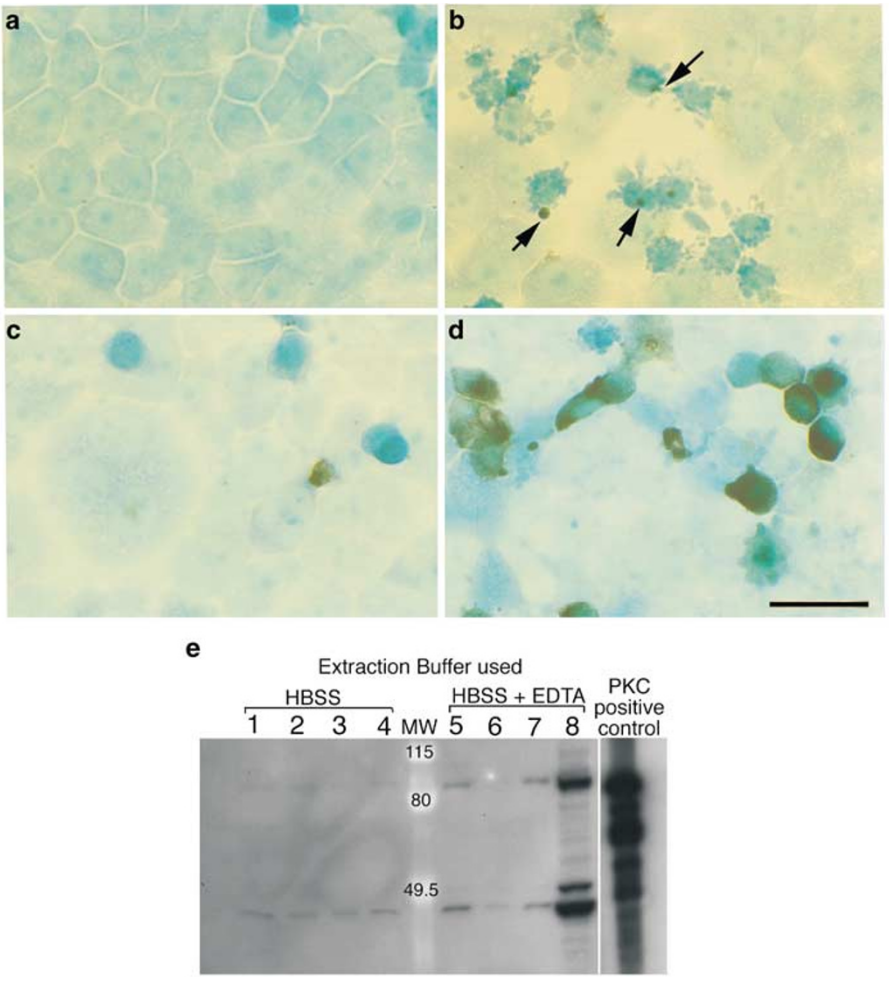

Figure 4 Externalization of PKC-α in cells in response to UV irradiation, phorbol ester treatment, or both. Panels (**a**–**d**) show immunocytochemical staining in HeLa cells as in Figure 3, while panel (**e**) is an immunoblot analysis on T84 cells. In panels (**a**–**d**), HeLa cells in glass Lab-Tek slides were left untreated (**a**), exposed to UV light for 90 s on a transilluminator box (**b**), treated with 0.1 μM PMA (**c**), or UV followed by PMA (**d**). At 2 h after the treatment, cells were fixed but not permeabilized, then immunostained as in Materials and Methods and the legend to Figure 3. In panel (**b**), black arrows show small amounts of ecto-PKC-α localized in apoptotic membrane blebs. Size bar $=$ 20 μm. In panel (**e**), T84 cells were left as untreated controls (lanes 1 and 5), treated with 1 μM phorbol myristate acetate (PMA) alone (lanes 2 and 6), irradiated with UV light on a transilluminator box for 2 min (lanes 3 and 7), or irradiated with UV light, followed by stimulation with 1 μM PMA (lanes 4 and 8). At 2 h after the experimental treatment, the medium was changed to either Hank's balanced salt solution (HBSS) alone, or HBSS supplemented with 12 mM EDTA and the cells were allowed to incubate another 30 min at 37° in this buffer. After 30 min, the supernatants were collected, separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody against PKC-α. MW, prestained MW markers, which left a negative stain in the chemiluminescence blot. Note the presence in lane 8 of an 82 kDa band representing the PKC-α holoenzyme, and a 45 kDa band representing the catalytic fragment, so-called PKM

blebbing, and increased avidity for the Giemsa counterstain (blue). In the UV-exposed, apoptotic cells, PKC immunoreactivity was sparse in amount and localized in the apoptotic membrane blebs (Figure 4b, black arrows). In cells stimulated with PMA, surface-exposed PKC immunoreactivity was similarly limited to an occasional cell (Figure 4c); the frequency of these cells was low ($<$5%) and comparable to the number of cells in the monolayer that are spontaneously undergoing apoptosis. In contrast, in cells exposed to UV light followed by PMA treatment, much larger numbers of cells showed strong surface expression of PKC (Figure 5d). Addition of PMA after UV irradiation also seemed to reduce the number of cells showing overt apoptotic morphology, which is consistent with the known antiapoptotic effects of PKC.[9] HeLa cells labelled with an irrelevant antibody of the same isotype and same concentration as that used in Figures 3 and 4 showed no peroxidase staining (photographs not shown). Figure 4e shows that the externalization of PKC-α seen by immunocytochemistry in HeLa cells was confirmed by the immunoblot method.

In a variation on the method shown in Figure 4, we attempted to use the immunolabelling technique to quantitate the externalization of PKC-α. In these experiments, HeLa cells or T84 cells were again exposed to UV light or PMA or both, fixed, and incubated with anti-PKC-α mAb. However, in the final step instead of using diaminobenzidine (DAB) to generate an insoluble brown stain for microscopy, the soluble peroxidase substrate *o*-phenylenediamine ('OPD') was substituted. The amount of yellow color generated was quantitated by spectroscopy at 492 nm. Figure 5 shows that in both HeLa and T84 cells, the combination of UV and PMA triggered the greatest PKC-α externalization. In both cell lines, greater

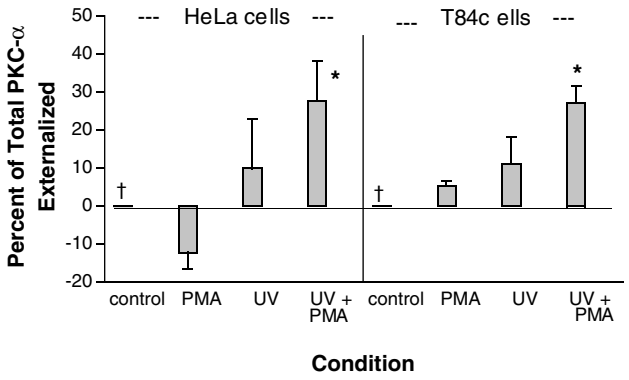

Figure 5 Detection of PKC by quantitative immunodetection. HeLa or T84 cells were grown in 24-well tissue culture plates and subjected to the experimental treatments shown, as described in Materials and Methods. Wells were then fixed 2 h after the treatment but not permeabilized, except for four additional wells per plate that were permeabilized with -20° methanol; the amount of staining in these permeabilized wells was taken to represent the entire content of PKC-α. Immunoreactivity was measured using the soluble colorimetric substrate *o*-phenylenediamine (OPD) and expressed as a percent of the reading in the permeabilized wells according the formula in Materials and Methods. †by definition, control wells have a reading of zero , since the OD_{492} in these wells was subtracted as a blank; *significantly increased compared to the untreated controls by ANOVA; these values were also significant if the raw OD_{492} values were compared. Significant cell loss and detachment was noted in the UV-exposed wells, especially in the HeLa cells, an effect which tends to underestimate the actual amount of PKC exposed in those conditions

than 25% of the total cellular content of PKC-α became surface accessible after exposure to UV + PMA.

We had shown that the PKC activity externalized in response to EPEC infection was active toward histone and a selective PKC substrate peptide, but we also wished to determine if externalized PKC could phosphorylate proteins naturally present in an experimental EPEC infection. First we attempted to determine if ecto-PKC was able to phosphorylate any of the EPEC-secreted proteins (Esps). Our rationale for this experiment was that serine- and threonine-rich domains are found in the N-termini of EspA, EspB, and EspF.[10–12] In addition, the amino-acid sequence of the extreme C-terminus of EspD (RIVSGRV) strongly resembles the consensus site for phosphorylation by PKC of known PKC substrates in the host cell. Initial attempts to detect phosphorylation of EPEC-secreted proteins by ecto-PKC, using labelling with γ-[^{32}P]ATP at various times after EPEC infection, yielded negative or faint, equivocal results. As an even more rigorous test, purified exogenous PKC-α was tested for its ability to phosphorylate the Esps *in vitro*. Figure 6a shows that purified PKC-α did not yield detectable phosphorylation of EPEC-secreted proteins, even under conditions in which histone III-S was strongly phosphorylated (lane 9). In other variations of the experiment shown in Figure 6a, we also observed no phosphorylation of whole EPEC bacterial cells by PKC-α, or of EPEC-secreted proteins when EPEC supernatants were concentrated five-fold (data not shown).

In contrast to the lack of phosphorylation of bacterial cells or bacterial proteins by PKC, purified exogenous PKC-α was able to phosphorylate host cell proteins when added to normal, uninfected HeLa cells along with γ-[^{32}P]ATP. In serum-free medium, phosphorylated protein bands were not found in the supernatant medium (Figure 6b, four right lanes),

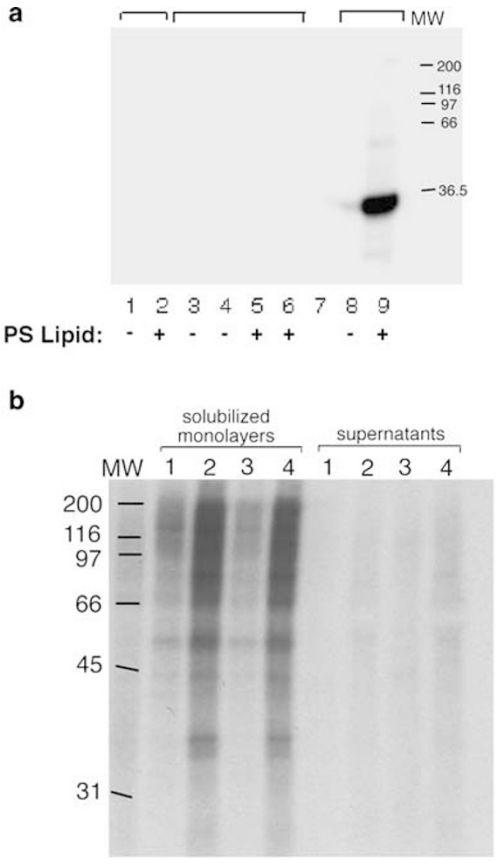

Figure 6 Ability of ecto-PKC to phosphorylate extracellular substrates. (**a**) Autoradiogram showing lack of phosphorylation of EPEC-secreted proteins. EPEC-secreted proteins were collected as described in Materials and Methods from the sterile filtrate of a 2 h subculture of EPEC E2348 (lanes 1 and 2), or a filtrate of the supernatant medium of HeLa cells that had been infected with EPEC for 4 h (lanes 3–6). The phosphorylation reaction in lanes 1 and 2 contained 2.7 μg of EPEC-secreted protein, and those in lanes 3–6 had 15.6 μg of protein; of this, one-fifth was actually loaded on the gel. Lanes 8 and 9 received 10 μg histone III-S as a positive control, but only 0.5 μg was loaded on the gel to prevent overexposure. Each phosphorylation reaction also received 25 ng of purified human recombinant PKC-α and 4 μCi γ-[^{32}P]ATP; conditions marked with a (+) received the lipid mixture of PS and dioctanoylglycerol, while those marked (−) did not. MW, position of regular MW markers, as revealed by Coomassie blue staining. Lane 7 contained prestained MW markers (not shown). (**b**) Normal control HeLa cells in 24-well plates were left untreated (lanes 1–2) or exposed to UV light for 2 min (lanes 3 and 4). After 2 h, tissue culture medium was removed and replaced with 0.2 ml of serum-free medium. To wells 2 and 4 was added 12.5 ng of purified recombinant human PKC-α. All wells received 0.9 μCi γ-[^{32}P]ATP and phosphorylation was allowed to proceed for 10 min at 37° with shaking. After 10 min, supernatants were collected and the HeLa cell monolayer solubilized in SDS sample buffer. The solubilized monolayers and supernatants were separated by SDS-polyacrylamide gel electrophoresis and the radioactive bands were detected by phosphorimager. Lane 1, normal HeLa cells; Lane 2, HeLa cells plus PKC-α; Lane 3, cells exposed to UV light; Lane 4, cells exposed to UV light and treated with PKC-α. MW, position of regular MW markers

but were detectable when the HeLa monolayer was solubilized in detergent (four left lanes). UV irradiation of HeLa cells, to induce surface exposure of PS, did not appear to increase or alter the pattern of phosphorylated bands (Figure 6b, lanes 3 and 4 of each group). Other investigators have shown that phosphorylation resulting from brief incubation with

extracellular γ-[^{32}P]ATP, such at that used in Figure 7b, labels surface-exposed proteins and not intracellular ones.

While the function of ecto-PKC was not initially obvious, preliminary observations seemed to indicate that ecto-PKC might slow or prevent some of the manifestations of apoptosis, as previously suggested in Figure 4d above. A more rigorous examination of the cytoprotective effects of ecto-PKC is shown in Figures 7 and 8, which show that

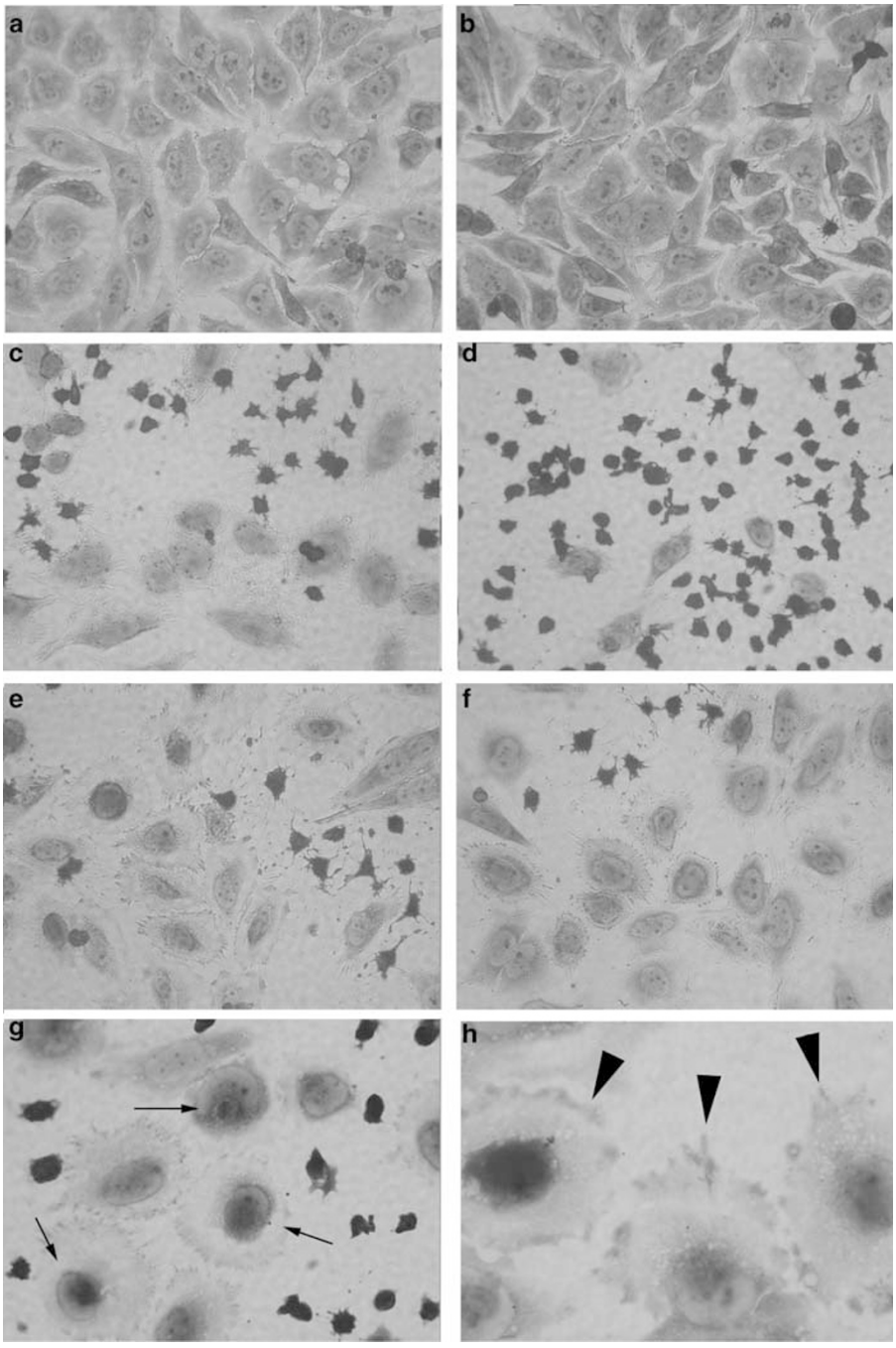

Figure 7 Antiapoptotic effects of extracellular PKC-α after UV irradiation. HeLa cells grown in Lab-Tek chamber slides were UV irradiated for 3 min (panels **c–h**) or left unexposed (panels **a** and **b**). At 2 h after the UV exposure, to some wells were added purified recombinant PKC-α, or 6 μM ATP, or both, and incubation was continued an additional 6 h, then the slides were fixed, stained with Giemsa, and photographed. (**a**) Normal control HeLa cells (not UV exposed); original magnification $\times$ 400; (**b**) HeLa cells treated with 6 μM ATP alone, 400 $\times$ magnification; (**c**) HeLa cells exposed to UV irradiation alone, showing a mixture of apoptotic and nonapoptotic cells; (**d**) HeLa cells treated with UV followed by 6 μM ATP, with a much higher percentage of cells showing apoptosis, $\times$ 400; (**e**) HeLa cells treated with UV followed by PKC-α, $\times$ 400; (**f**) HeLa cells treated with UV, PKC-α, and 6 μM ATP, showing many fewer apoptotic cells than in panel (**d**), $\times$ 400; (**g**) Higher power view of HeLa cells treated with UV, PKC-α, and 6 μM ATP, showing that many cells have undergone nuclear condensation (thin arrows) without showing retraction of the cytoplasm or membrane blebbing, $\times$ 630; (**h**) Higher power view of cells treated with UV, PKC-α, and 6 μM ATP, showing that the cytoplasm is extended, thin, and delicate, with cell margins showing ruffling and filopodia, black arrowheads, $\times$ 1000

addition of exogenous recombinant PKC-α protected HeLa cells from damage induced by UV irradiation in the presence of ATP.

In healthy HeLa cells not exposed to UV irradiation, low concentrations (6 μM) of ATP were tolerated without inducing cell damage (Figure 7b). UV irradiation induced apoptosis

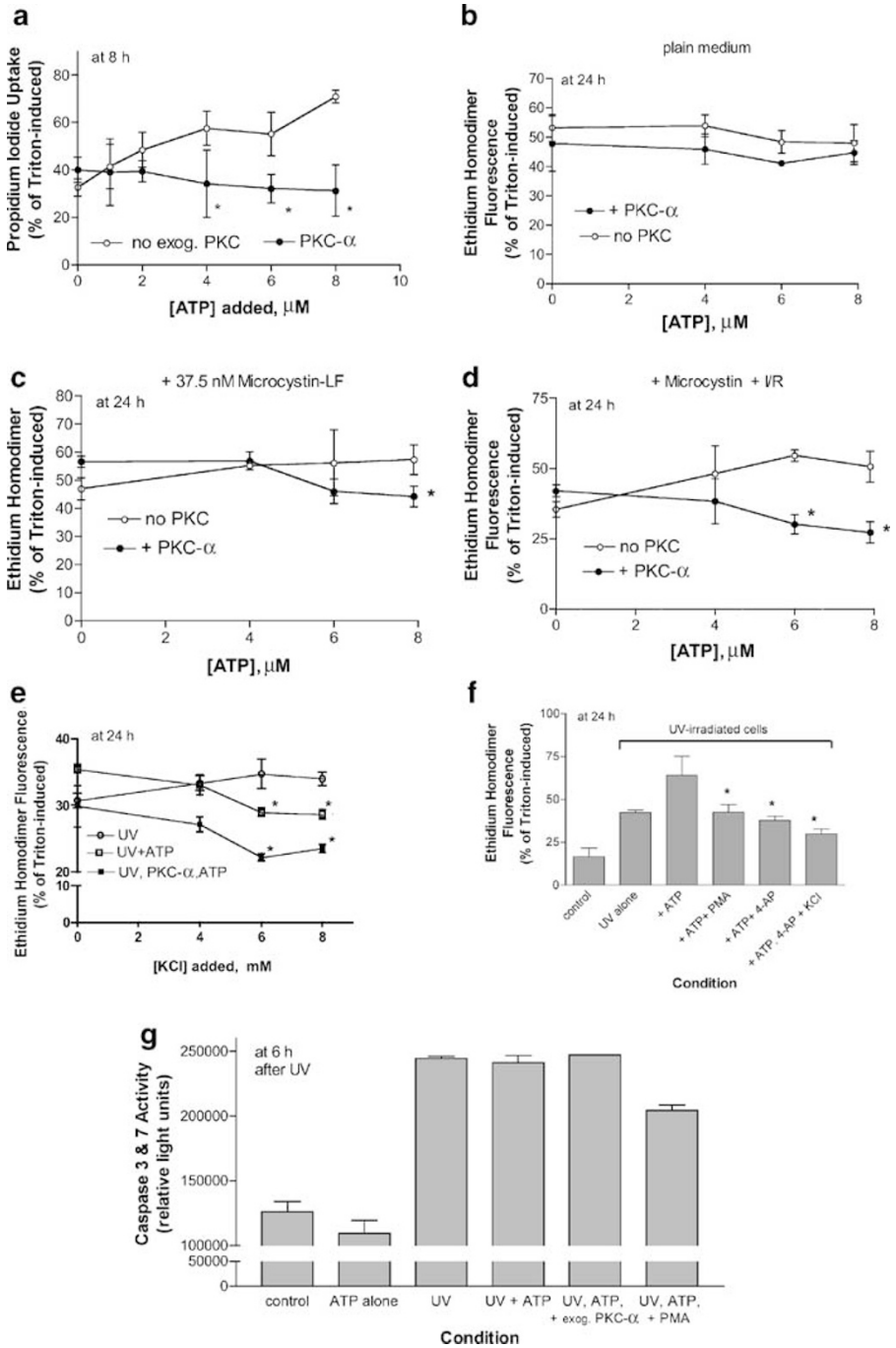

Figure 8 Protection of HeLa cells from UV-induced cell death by extracellular PKC-α and inhibitors of K^+ efflux. HeLa cells were grown to near confluency in 48-well plates, then exposed to UV irradiation for 2.5 min. After 2 h, the medium was changed to phenol red-free RPMI medium with 2 mg/l propidium iodide or ethidium homodimer. Next, purified recombinant PKC-α was added along with the concentration of ATP indicated on the abscissa, and incubation was continued. Propidium iodide fluorescence was read on a fluorescent plate reader 8 h (**a**) or 24 h after the UV exposure. Medium in panels (**c** and **d**) was supplemented with 37.5 nM Microcystin-LF as a protein phosphatase inhibitor. In panels (**d–f**), the medium also contained an ATP regenerating system consisting of 20 mg/l creatine kinase, 5 mM phosphocreatine, and 250 μM α, β-methylene-ADP. In panels (**a–d**), asterisks (*) indicate values that were significantly lower than the corresponding condition without added PKC ($P<0.05$ by *t*-test). In panel (**d**), the points indicated by asterisks were also lower than cells exposed to UV alone, without ATP, by ANOVA. In panel (**e**), asterisks indicated significant protection compared to the same condition without added KCl ($P<0.05$ by ANOVA). In panel (**f**), the concentrations of reagents used were as follows: PMA, 0.5 μM; ATP, 8 μM; 4-aminopyridine (4-AP), 1 mM; and KCl, 8 mM; * indicates protection compared to UV + ATP, $P<0.01$, by ANOVA. In panel (**g**), caspase activity was measured in lysates of HeLa cells using a Caspase-Glo luminescence kit designed to measure caspases 3 and 7

(Figure 7c) and the extent of apoptosis was greatly increased when UV treatment was followed by ATP (Figures 7d and 8a). Protective effects of exogenous recombinant PKC-α were barely discernible in the absence of added ATP (Figure 7e), but in the presence of ATP, PKC-α provided striking protection from UV-induced morphological changes (Figure 7f). In the presence of PKC-α and ATP, many fewer HeLa cells showed cell shrinkage and membrane blebbing (Figure 7, compare panels d and f). Thus, exogenous PKC-α seemed to protect HeLa cells from developing the typical morphological changes from UV damage. However, the cell morphology of the 'protected' HeLa cells was actually noticeably different from that of normal control HeLa cells. For example, the cell shape of the cells protected by exogenous PKC-α and ATP was rounded or oval, compared to the polygonal shape of normal HeLa cells (Figure 7, panels a and f). In addition, HeLa cells protected with exogenous PKC-α often showed nuclear condensation without shrinkage of the cytoplasm and without membrane blebbing (Figure 7g and 7h). Indeed, instead of shrinkage and blebbing, HeLa cells treated with exogenous PKC-α and ATP appeared enlarged and possessed a thin, delicate, extended cytoplasm (Figure 7f–h). In addition, protected cells often had cell margins which were ruffled or had delicate filopodia (Figure 7h), which is not a characteristic of normal HeLa cells (Figure 7, panels a, g, and h). These observations seemed to indicate that exogenous PKC-α was more effective in preventing apoptotic changes in the plasma membrane and cytoplasm than in the nucleus.

The apparent cytoprotective effects of exogenous PKC-α seen in Figure 7 were confirmed using the propidium iodide uptake assay as a quantitative measure of cell death in Figure 8. ATP strongly potentiated UV-induced propidium iodide uptake (Figure 8a, upper curve) and this damage was prevented in the presence of exogenous PKC-α (Figure 8a, lower curve). The protective effects of exogenous PKC-α were not observed when adenosine 5′-(3-thio)triphosphate (ATP-γ-S) or ADP were substituted for ATP (data not shown).

The cytoprotective effects of exogenous PKC-α + ATP in Figures 7 and 8a were observed 8 h after UV irradiation. We increased the duration of the assay to determine how long the cytoprotective effect of ecto-PKC-α would last. In addition, we attempted to distinguish whether the effects of extracellular PKC-α were explained by a trivial mechanism, such as destruction of ATP or phosphorylation of irrelevant substrates, or an actual positive effect upon a target protein. We reasoned that addition of an ATP regenerating system, previously shown to greatly prolong the duration and actions of intact ATP in cultured cell systems,[3] would reduce or abolish the protective effects of extracellular PKC-α if the role of PKC-α was merely to destroy ATP or phosphorylate 'innocent bystander' substrates. Conversely, addition of the ATP regenerator would be expected to increase or prolong the effects of extracellular PKC-α if the kinase were exerting its effects by phosphorylation of a specific target or targets. Similarly, we tested the effects of microcystin, a potent but cell-impermeant protein phosphatase inhibitor.

Figure 8, panels b–d, shows the effects of exogenous PKC-α in HeLa cells studied 24 h after UV exposure. In Figure 8b, exogenous PKC-α provided no protection against UV-induced cell death at 24 h when cells were kept in the usual assay medium. In the presence of 37.5 nM microcystin-LF, however, protection by PKC-α was seen at the highest ATP concentration tested (8 μM, Figure 8c). In the presence of both the inhibitor-regenerator and microcystin-LF, protection by PKC-α was even more dramatic (Figure 8d). In Figure 8d, cell death in the presence of PKC-α + ATP was not just lower than in cells receiving no PKC-α (upper curve), but also significantly lower than in cells treated with UV alone (filled circle, *y*-intercept). Figure 8 suggests that in a setting where ATP release is ongoing, such as EPEC infection, extracellular PKC-α could provide protection for a period that is long compared to the life of the enterocyte. The enhancing effects of the ATP regenerator and microcystin, together with the peculiar morphology of cells protected by exogenous PKC-α (Figure 7f–h), suggest that PKC-α is exerting its effects by a positive effect on the cells, and not just by destroying ATP via irrelevant phosphorylation.

The most obvious visible effect of externalized PKC and exogenously added PKC-α was to inhibit cell shrinkage in response to an apoptotic stimulus (Figures 4d and 7f). Cell shrinkage during apoptosis, termed the apoptotic volume decrease (AVD), has been shown to be mediated by efflux of K^+ ions via potassium channels. Activation of these potassium channels has been observed in many cell types, including epithelial cells, and in response to UV irradiation.[13–17] This suggested that K^+ efflux was involved in cell shrinkage in UV-irradiated HeLa cells, and that extracellular PKC might act by inhibiting K^+ efflux. Figure 8e shows that addition of KCl to the extracellular culture medium did inhibit UV-induced cell death in HeLa cells in the presence of ATP. Figure 8f shows that addition of 1 mM 4-aminopyridine, a potassium channel blocker, inhibited cell death induced by UV + ATP treatment approximately as well as did addition of the phorbol ester, PMA. In other experiments, 30 nM of the selective potassium channel blocker ergtoxin also protected HeLa cells from death in response to UV + ATP (data not shown). Cells protected by ergtoxin and 4-aminopyridine also showed the peculiar morphology seen in cells protected by exogenous PKC-α, namely condensed nuclei but extended, ruffled cytoplasm (micrographs not shown, similar to Figure 7, panels f–h). These findings suggest that ecto-PKC inhibits cell shrinkage by inhibiting potassium channels.

The data shown in Figures 7 and 8 suggested that ecto-PKC, in the presence of extracellular ATP, blocked cell shrinkage, membrane blebbing, and reduced uptake of ethidium homodimer, all of which are attributes of the plasma membrane. To assess whether ecto-PKC also affected intracellular, cytosolic events associated with cell death, we performed assays of caspase activity in HeLa cells protected with exogenous purified PKC-α. Results of an assay for caspases 3 and 7 are shown in Figure 8g; as expected, UV exposure of HeLa cells greatly increased caspase activity compared to control cells. Cells treated with UV plus ATP, however, did not show increased caspase activity compared to those exposed to UV alone. Second, cells protected by exogenous PKC-α along with UV + ATP showed no decrease in caspase activity compared to UV alone, or UV + ATP. Cells protected by addition of PMA did show a decrease in caspase activity compared to UV alone, but PMA activates intracellular PKC as well as externalized PKC and this may account for the

modest decrease in caspase activity. The findings of Figure 8g, that is, noninhibition of caspases by ecto-PKC, were confirmed by immunostaining of cells for caspase-cleaved cleaved cytokeratin using the M30 antibody (data not shown). M30 antibody immunostaining showed that cells 'protected' by PKC-α after treatment with UV + ATP nevertheless reacted strongly with the M30 antibody, again showing that cytoplasmic events associated with apoptosis were not blocked even though cell shrinkage and blebbing was prevented. The assays of propidium iodide uptake (Figure 8a–f), caspase activity (Figure 8g), observations of cell morphology (Figure 7), and M30 staining (photographs not shown) together indicate that ecto-PKC differentially inhibits death events related to the plasma membrane without much effect on other intracellular events of apoptosis.

We previously reported that EPEC adherence to UV-exposed HeLa cells was increased compared to control HeLa cells,[2] and this was confirmed and extended by others.[18–20] Figure 9a shows again that EPEC adherence to UV-irradiated HeLa cells was increased over control, and the increase in adherence was maintained in cells also treated with PKC-α, ATP, or both. Although the quantitative effect of exogenous PKC-α and ATP was not impressive, the pattern of EPEC adherence observed in the HeLa cells treated with PKC-α and ATP was strikingly changed from the typical, tightly clumped EPEC microcolonies in control cells ('localized adherence', Figure 9b) to a much looser, more 'spread out' adherence pattern (Figure 9c). HeLa cells treated with UV + PKC-α + ATP showed an even more dramatic change in adherence to a pattern resembling diffuse adherence (Figure 9d). Treatment of HeLa cells with ATP alone or PKC-α alone did not trigger the conversion to a diffuse adherence pattern (photographs not shown). The tendency of EPEC to change its adherence from tight to loose pattern has been noted before, at late times after infection (4–6 h), and was associated with changes in the expression of the EPEC bundle-forming pilus.[21,22] Those changes in adherence and pilus expression were interpretted as being the result of EPECs own internal genetic program during infection. The results shown in Figure 9 suggest that the physiologic status of the host cell also plays a role in the adherence pattern seen; it seems likely that changes in the host cell plasma membrane, or soluble mediators released from the host such as ATP, could provide feedback signals to the EPEC bacterial cell and contribute to changes in the pili noted by others.

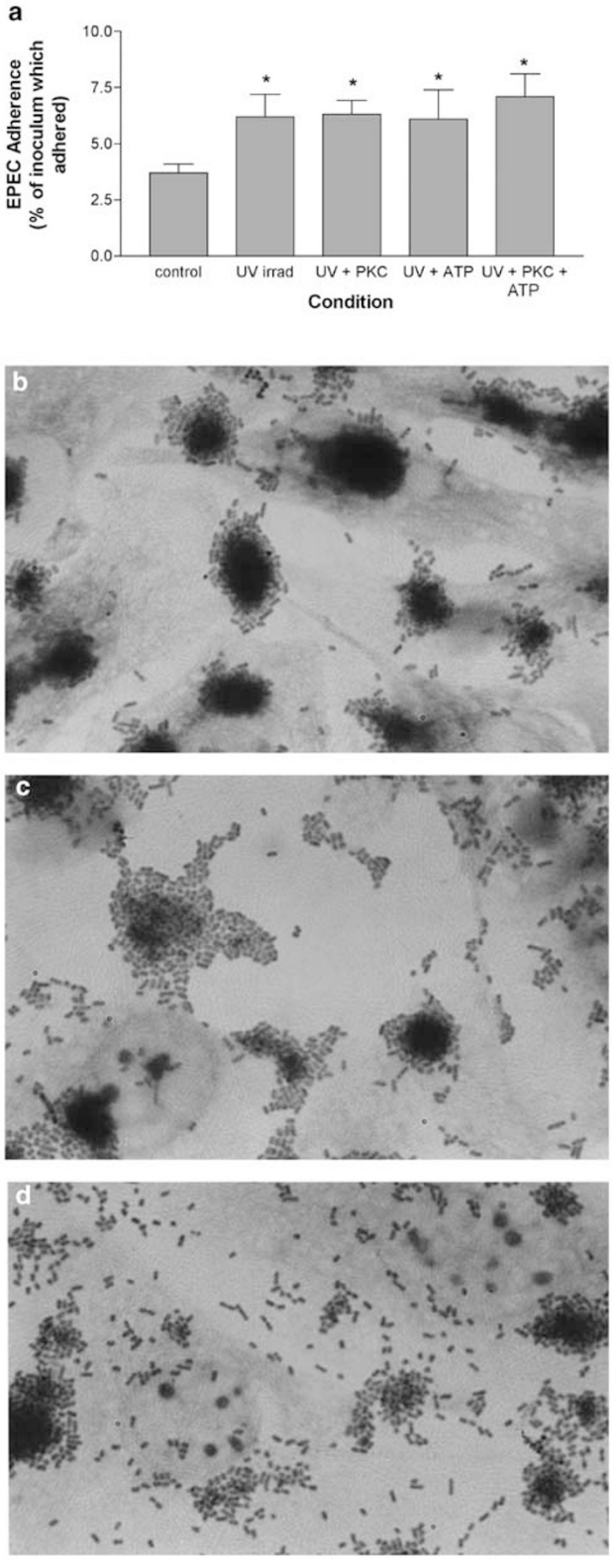

Figure 9 Effect of ecto-PKC and ATP on EPEC adherence. HeLa cells were grown in 24-well plates (A) or in Lab-Tek chamber slides (**b–d**). In panel (**a**), quantitative adherence of wild-type EPEC strain E2348/69 was measured using radiolabelled bacteria as described in Materials and Methods. In panel (**a**), cells were UV irradiated and returned to the incubator for 2 h, then 2 μM ATP or 40 ng PKC-α was added per well, as indicated in the figure labels. Cells were infected with EPEC and adherence was allowed to proceed for 1 h, then cells were rinsed and radioactive counts determined. *Significantly increased compared to control HeLa cells. In panels (**b–d**), HeLa cells were left unexposed to UV (**b** and **c**) or exposed to UV irradiation for 2 min (**d**). After 2 h, recombinant human PKC-α and 2 μM ATP were added to some chambers, cells were infected with EPEC strain E2348/69, and incubation was continued for 3 more hours, at which times the slides were fixed and stained for photography at $\times$ 600 magnification. (**b**) EPEC adherence to normal control HeLa cells, showing typical, tightly clumped EPEC microcolonies. Cells treated with UV alone, ATP alone, or PKC-α alone (not pictured) demonstrated tight localized adherence similar to that seen in panel (**a**). (**c**) HeLa cells treated with PKC-α + ATP, but not UV irradiated. (**d**) Cells irradiated with UV followed by PKC-α + ATP

Discussion

The data presented here show that protein kinase C becomes externalized in response to EPEC infection. Once externalized the PKC remains firmly tethered to the external surface of the cell and is not released into the supernant medium except by nonphysiologic methods such as addition of polypeptide substrates (Figure 1) or high concentrations of EDTA (Figure 4e). The tight surface binding of PKC contrasts with other cytosolic proteins released during host cell killing by enteric pathogens, such as LDH, which are released into the supernatant medium. Together with other features of the process, the surface localization of PKC argues that the mechanism of its externalization is different from that of LDH release (see discussion below).

Externalized PKC remains intact, biologically active, and responsive to stimulation by phospholipid (Figure 1). In previous work, we showed that conventional, intracellular PKC is activated in response to EPEC infection, but that its stimulation was brief, and, once activated, intracellular PKC-α was quickly cleaved by intracellular proteases to yield the 45 kDa catalytic fragment (so-called PKM). Our results here (Figure 1a) showed that ecto-PKC activity continued to increase up to 2 h after infection, a time at which intracellular PKC activity was decreasing. The results of the present study suggest that, in addition to cleavage by proteases, externalization of PKC contributes to the short-lived duration of intracellular PKC activity following EPEC infection.

In order to define a mechanism of PKC externalization, we attempted to reproduce the PKC externalization observed with EPEC infection by a combination of two other interventions: UV irradiation as an apoptosis-inducing stimulus, and phorbol ester as a PKC activator. Figures 4 and 5 showed that UV irradiation alone and PMA treatment alone were ineffective or at most very weak stimulators of PKC externalization. In contrast, the combination of UV light followed by PMA was very effective in inducing PKC externalization in a high percentage of cells. Quantitative immunostaining (Figure 5) estimated the amount of PKC-α externalized by the UV + PMA treatment to be as much as 25–30% of the entire cell monolayer's content of PKC-α. The ability to trigger PKC externalization by UV + PMA is particularly significant because neither UV irradiation, nor UV + PMA, triggers LDH release from the host cell.[3] Since PKC externalization proceeds under conditions in which no LDH release occurs (UV + PMA), the mechanisms must be different. Based on what is known about the biochemistry of PKC and of PS externalization during apoptosis, we hypothesize that, once activated, PKC accompanies PS as it translocates from the internal leaflet to the external leaflet of the cell plasma membrane; this redistribution of PKC occurs without release of cytoplasmic contents (Figures 1 and 3).

Further definition of the exact mechanism by which PKC becomes externalized during EPEC infection is complicated by the lack of understanding of how PS is externalized in apoptosis. Some of the other hallmarks of apoptosis, such as membrane blebbing and nuclear condensation, can be ascribed to caspase-mediated cleavage of specific target proteins, such as gelsolin and nuclear lamins, respectively.[23] In contrast, the literature on PS externalization is contradictory regarding whether PS externalization is the result of inhibition of a membrane aminophospholipid translocase, or to activation of an aminophospholipid 'scramblase'.[24] Similarly, there are contradictory reports as to whether PS externalization is caspase-dependent, caspase-independent, or triggered by noncaspase proteases such as calpains.[25] Furthermore, surface PS expression can be increased transiently by Chlamydia infection without triggering apoptosis.[26] An additional question unanswered by the present research is whether PKC is externalized one PKC molecule at a time, or if large aggregates of PKC bound to PS are externalized all at once. Our previous work showed that intracellular PKC clustered around EPEC microcolonies, and our current work shows that the externalized PKC remains tightly clustered in a similar way (Figure 3c), tempting us to speculate that aggregates or 'rafts' of PKC and PS may flip to the extracellular leaflet simultaneously.

Epithelial cells contain substrate proteins capable of being phosphorylated by extracellular PKC-α in the presence of extracellular ATP (Figure 6b). Since EPEC infection releases substantial amounts of ATP from the host cell,[3] it seems reasonable to believe that PKC can continue to act catalytically once externalized following EPEC infection. However, the concentration of extracellular ATP is likely to be the rate-limiting factor for PKC-mediated phosphorylation in our system (EPEC infection of intestinal cells) as well as in other organs and tissues.

Our work indicates that ecto-PKC, whether it arrives on the cell surface by externalization (Figures 3–5), or is added exogenously, inhibits the full morphological expression of apoptosis, especially the changes in the plasma membrane. We and others have noted that EPEC bacteria activate several antiapoptotic pathways inside the host cell, including PI 3-kinase, tyrosine kinases, NF-κB, MAP kinase, and conventional, intracellular PKC.[1,27–29] EPEC bacteria bind directly to lipids exposed on the cell surface early in apoptosis[18] and this results in increased amount and higher affinity of EPEC adhesion. Initiating apoptosis while preventing its completion may be a mechanism that EPEC uses to increase and prolong its adherence to epithelial cells (Figure 9a).

The main effects of ecto-PKC-α on cell death processes are inhibition of cell shrinkage (Figure 7f–g) and propidium iodide uptake (Figure 8), apparently mediated by inhibition of K^+ efflux. The protective effects of ecto-PKC are mimicked by agents that inhibit K^+ efflux, such as increased extracellular potassium and the K^+ channel blockers 4-aminopyridine and ergtoxin (Figure 8e and f, and data not shown). Voltage-dependent potassium channels of the HERG (human ether-a-go-go related gene) family are expressed in HeLa cells,[30] activated by apoptotic stimuli[16,17] and mediate apoptotic volume decrease.[13] Presumably, ecto-PKC could inhibit K^+ channel activity by directly phosphorylating the pore-forming α-subunit, the regulatory β-subunit, K^+ channel interacting proteins (KCIPs), or other membrane proteins which influence K^+ channel function.

In summary, we have shown that significant amounts of PKC-α are externalized in response to EPEC infection, and this represents the first report that expression of an

ecto-protein kinase is altered in response to a microbial infection. In the past, the amount of ecto-protein kinase activity of a cell was felt to be a fixed trait of the cell or tissue, influenced by the organ of origin, and whether the cell was normal or of cancerous origin. Our results, however, show that expression of ecto-PKC is susceptible to manipulation not only by a bacterial infection but also by the combination of an apoptotic stimulus (UV irradiation) and a phorbol ester. Again, this is the first report linking increased expression of ecto-PKC to apoptosis or to any type of cell death. Many microbes are capable of inducing apoptosis in host cells, and a smaller group of microbial pathogens can trigger ATP release from the host.[3,31] EPEC is in the subset of pathogens that trigger both apoptosis and ATP release, which means that EPEC may be uniquely suited to exploit ecto-protein kinases on the host cell surface from its extracellular position. In this context, the role of extracellular ATP and ecto-protein kinases, including ecto-PKC, should receive more attention in the future.

Materials and Methods

Reagents

The following reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA.): ATP, histone III-S, phosphatidylserine, phorbol myristate acetate, DAB, *o*-phenylenediamine, EDTA, and Giemsa stain. Dioctanolyglycerol (DOG) was from Molecular Probes (Eugene, OR, USA) and wortmannin was from Alexis (San Diego, CA, USA). The PKC Ser^{25}(19–31) substrate peptide and the PKC (19–31) inhibitor peptide were from Quality Controlled Biochemicals (QCB, Hopkinton, Mass; now Biosource International). Reagents for development of immunoblots by chemiluminescence were in the Lumiglo kit from Kierkegard & Perry (Gaithersburg, MD, USA).

Purified PKC was recombinant human PKC-α from Cytoskeleton, Inc. (Denver, CO, USA). A 1 : 100 dilution of this PKC preparation was also used as a positive control for PKC immunoblots. Hanks' Balanced Salt Solution (HBSS) was from Gibco/BRL and was the preparation lacking calcium and magnesium. Antibody against PKC-α was from Transduction Laboratories (Lexington, KY, USA, now BD Biosciences) and the M30 antibody was from Roche Applied Sciences (Indianapolis, IN, USA).

Bacterial strains used were as described in recent publications.[2,3] Infections were carried out after overnight culture and a 2 h subculture in DMEM-based 'EPEC Adherence Medium' as described.[2] The same volume of bacterial inoculum was used for both cell lines; however, since T84 cells are twice as dense as HeLa cells at confluency, the multiplicity of infection was 100 : 1 for T84 cells and 200 : 1 for HeLa cells.

Protein kinase assays

The protein kinase assay consisted of three phases. In the first phase, T84 or HeLa cells grown in 24-well plates were subjected to an experimental manipulation, such as EPEC infection, or exposure to UV light and then incubation was continued for 1–3 h, as described in the figure legends. At the end of the incubation, the medium was removed as replaced with 0.25 ml medium (usually HBSS, occasionally phosphate-buffered saline, PBS) supplemented with histone III-S (0.1–0.2 mg/ml), 30 μM PKC Ser^{25}(19–31) substrate peptide, or 12 mM EDTA and incubation was continued for 30 min more. This second phase is referred to as the 'extraction phase'. Aliquots of the supernatant obtained from the extraction were collected and kept on ice and used for protein kinase assay on the same day, or frozen and used for Western immunoblots at a later date. The third phase of the assay was the *in vitro* protein kinase assay itself. The protein kinase assay was carried in a manner similar to that previously described.[1] Briefly, the reaction was carried out in 100 μl final volume at 30°C for 6 min in glass test tubes in a shaking water bath. To each tube on ice was added 50 μl of the supernatant from the extraction phase, and histone or PKC Ser^{25}(19–31) substrate peptide as needed to yield final concentrations of 0.2 mg/ml histone, or 30 μM substrate peptide. Supernatant extracts were assayed without and with 10 μl of a 10 $\times$ lipid mixture of phosphatidylserine and diacylglycerol in order to measure lipid dependence of the kinase activity.[7] $CaCl_2$ was only added in cases where EDTA was used in the extraction buffer, to 0.1 mM over the EDTA concentration. If needed, 25 mM HEPES was added to achieve a final volume of 100 μl, and then the reaction was initiated by addition of a 10 $\times$ 'reaction cocktail' containing γ-[^{32}P]ATP, unlabelled ATP, and $MgCl_2$ in 25 mM HEPES, and transferring the tubes from ice to the water bath. Final concentrations of cofactors were as described.[1] After the incubation, 70 μl aliquots were spotted on P81 phosphocellulose filters, washed, and counted as described.[1] In some experiments, 20 μl of remaining liquid was mixed with 10 μl of 3 $\times$ Laemmli SDS sample buffer, frozen, and analyzed by SDS-PAGE and autoradiography.

Induction of apoptosis by UV irradiation

HeLa cells were grown in glass Lab-Tek chambers slides for photography and in 24-well plates for quantitative analyses. Glass slides were exposed to 1–3 min of UV irradiation and plastic 24-well plates for 2–3 min on a UV transilluminator box (Chromato-Vue TM-20, UVP, Inc., San Gabriel, CA, USA) as described.[32,33]

PKC immunoblots and immunocytochemistry

SDS-PAGE was carried out using precast minigels of 10 or 12% acrylamide from Novex (now Invitrogen). Proteins were transferred to nitrocellulose using standard methods in a Bio-Rad Trans-Blot apparatus, blocked, and probed with mAb against PKC-α at a dilution of 1 : 5000 as described.[1] After three PBS washes, the second antibody-conjugate was added. As second antibody, we used goat anti-mouse IgG_{2b} conjugated to peroxidase, from Roche Molecular Biochemicals, at a dilution of 1 : 3000, for 1 h at room temperature, followed by washes and development using chemiluminescence.

For immunocytochemical staining of cell monolayers, the same first and second antibodies were used but at higher concentrations. HeLa cells were grown in Lab-Tek chamber slides and then subjected to the experimental manipulation. After 2 h, the cells were fixed with 2% glutaraldehyde–0.1% cacodylate for 10 min. Cells were not permeabilized except where stated (e.g., Figure 4d, with $-20°$ methanol), then blocked with 1% BSA in PBS for 15 min. After three brief PBS rinses, cells were incubated with the mAb against PKC-α at a dilution of 1 : 200 in PBS-BSA for 1–1.5 h, rinsed again three times with PBS, then incubated with the second antibody-conjugate at 1 : 250 for 1 h. After three more PBS rinses, the color was developed by the addition of 0.67 mg/ml DAB plus 0.024% H_2O_2 in Tris-buffered saline. Color development was allowed to proceed for 10–15 min while being monitored in a low-power inverted microscope. Color development was stopped by rinsing in deionized water. Then slides were counterstained with Giemsa stain for 10–15 min, rinsed, and allowed to dry. Then slides were observed and photographed at 600 $\times$ magnification under oil.

Quantitative immunodetection of ecto-PKC

Experiments to quantititate ecto-PKC expression by immunostaining were carried out with cells grown in 24-well plates, and conditions were done in quadruplicate. As a positive control, four wells on each plate were permeabilized with ice-cold methanol prior to immunostaining but the remaining wells were left unpermeablized. UV irradiation was for 105 s as mentioned above and the PMA concentration was 4×10^{-7} M. After exposure to UV $\pm$ PMA, cells were fixed as mentioned above, blocked with PBS-BSA, then incubated with the same first and second antibodies as above for immunocytochemistry, and at the same concentrations. Since background immunoreactivity was greater in this method, however, an additional wash of PBS–0.1% Tween was added after each antibody incubation. After the second antibody incubation and washes, color development was with 0.4 mg/ml 'OPD' using premeasured tablets in phosphate-citrate buffer (Sigma cat. no. P4922) and yellow color development was monitored visually while incubation was continued at RT on a shaking platform. When the methanol-permeabilized wells showed strong yellow color (usually about 10 min), 200 μl of each well was transferred to a well of a 96-well plate that had previously been loaded with 50 μl of 3 M HCl, which stops further color development and changes the color to red. The 96-well plate was read at 492 nm on an ELISA reader. The amount of ecto-PKC expression in unknown wells, as a percent of the positive control, was calculated according to the following formula:

$((OD_{492}$ in unknown$)-(OD_{492}$ in untreated control wells$))/((OD_{492}$ in methanol wells$)-(OD_{492}$ in untreated control wells$)) \times 100$. Since the reading in the normal control wells is subtracted as a blank, the control wells show a reading of zero by definition.

Cytoprotection experiments with exogenous PKC-α and ATP

HeLa cells were grown in collagen-coated glass Lab-Tek chamber slides for photography (Figure 8) or in 48-well plates for propidium iodide uptake assays (Figure 9). At 2 h after UV irradiation, ATP or ATP + PKC-α was added and incubation was allowed to continue another 6 h; then slides were fixed and stained for microscopy, or read in the fluorescent plate reader as described.[2] Wells of 48-well plates (0.64 cm^2) received 25 ng of purified recombinant PKC-α (Cytoskeleton, Inc., Denver, CO, USA) and the 2.0 cm^2 wells of Lab-Tek slides received 40 ng PKC-α.

Caspase assay

Activity of caspases 3 and 7 was measured using a Caspase-Glo assay kit from Promega (Madison, WI, USA). HeLa cells were grown to confluency in 96-well plates, then changed into 100 μl of optically clear medium containing the ATP-regenerating system (creatine kinase, phosphocreatine, and α,β-methylene-ADP) and were subjected to the experimental treatment indicated on the graph. ATP and exogenous PKC-α were added 2 h after UV irradiation. At 6 h after the UV irradiation, the Caspase-Glo reagent (lysis buffer with caspase substrate) was added and incubation continued at RT for 1 h. Then the samples were transferred to an opaque 96-well plate and read on the luminometer.

M30 antibody assay

The M30 antibody reacts with an epitope of cytokeratin-18 which is exposed after caspase cleavage. Cells were grown on collagen-coated glass Lab-Tek chamber slides, and M30 antibody staining was carried out according to the manufacture's instructions and as previously described.[33] A monoclonal antibody of the same isotype as M30 (mAb against PKC-β, which is not expressed in epithelial cells) was used as the isotype control (photographs not shown).

Quantitative adherence assays

Adherence assays were performed using EPEC strain E2348/69, which had been labelled with [^{3}H]thymidine overnight as described.[33] HeLa cells were exposed to UV light, then 2 h later 2 μM ATP or 20 ng/well PKC-α were added as shown in the figure, then labelled bacteria were added. To minimize the contribution of ATP released by the bacteria, adherence was determined 1 h after bacteria were added.

Data analysis and presentation

Error bars shown in graphs are all standard deviations. Significance was tested by ANOVA using Graphpad InStat software for the MacIntosh computer.

Acknowledgements

This work was supported by a grant from Research for Health in Erie County (to JKC) and by bridge funds from the Department of Medicine, University at Buffalo. Grant RO1 AI050652, from NIAID, NIH to JKC was used to support the later phases of this project. We thank Dr Julianne Sando, University of Virginia, for helpful discussions in the early phases of this project, and Amanda J Golner and Ruth A Olson for excellent technical assistance.

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