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Elevation of Intracellular Free Calcium Levels in HEp-2 Cells Infected with Enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) are a class of diarrheagenic organisms that induce a characteristic attaching and effacing lesion in enterocytes and various cultured cell lines. Infection of cultured HEp-2 cells by EPEC isolates 2036-80 (serotype O119) and E2348-69 (serotype O127) resulted in significant elevation of intracellular free calcium levels, determined quantitatively with the fluorescent calcium indicator dye 2-{[2-bis (carboxymethyl)amino-5-methylphenoxy]methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline. This effect, which was not observed on infection with non-lesion-forming *E. coli* strains, was inhibited by dantrolene, a drug that prevents calcium mobilization from intracellular stores. Moreover, activated protein kinase C in infected cells was dissociated from cell membranes by a process that was inhibited by cyclosporin A, suggesting involvement of the calcium-dependent protease calpain. A qualitative method for observing intracellular calcium fluxes by fluorescence microscopy with the recently described fluorescein-based indicator fluo-3 was used to screen a collection of well-characterized *E. coli* isolates from patients with infantile enteritis. Increased localized calcium-dependent fluo-3 fluorescence was observed only in HEp-2 cells infected with known lesion-forming EPEC strains. We propose that enhancement of intracellular free calcium levels in enterocytes infected with EPEC would result in formation of the characteristic lesion by calcium-dependent activation of actin-depolymerizing proteins, with eventual loss of absorptive capacity.

Enteropathogenic *Escherichia coli* (EPEC) comprise a small number of O:H serotypes of *E. coli* epidemiologically associated with outbreaks of infantile diarrhea (30). They remain a significant cause of severe and persistent disease in infants, particularly in developing countries. EPEC possess none of the well-defined virulence determinants of other enteric pathogens, such as enterotoxin production or mucosal invasion (21), but their ability to adhere to human small intestinal enterocytes in a characteristic attaching and effacing (AE) manner is considered important in pathogenesis (19, 27, 32, 33). Initial nonintimate attachment of EPEC to enterocytes is followed by localized vesiculation of brush border microvilli and intimate bacterial adherence to the residual plasma membrane. Disruption of the brush border cytoskeleton allows the host cell membrane to distort around an attached bacterium, often in a cuplike pedestal structure (16, 32, 33) containing a dense plaque of short filaments at the cytoplasmic surface of the plasma membrane (19). The fact that a morphologically identical lesion is produced when EPEC attach to cultured cell lines (16) has allowed the identification of these filaments as actin and the development of the rapid and inexpensive fluorescence actin staining (FAS) test for the diagnosis of EPEC (17, 18).

In considering possible mechanisms for these effects, we noted that treatments that perturb the calcium balance of several cell types caused changes reminiscent of those observed in EPEC-infected material (13, 24, 26). In this paper we show that EPEC infection of cultured HEp-2 cells caused significant elevation of intracellular free calcium levels ($[Ca^{2+}]_I$) by release of calcium from inositol 1,4,5-triphosphate-sensitive stores.

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MATERIALS AND METHODS

Bacterial infection of cultured cells. Most EPEC isolates adhere to cultured HEp-2 cells (10); indeed, patterns of bacterial adherence to these and similar cell lines have been used for preliminary categorization of clinical isolates (34). Moreover, EPEC strains that induce the characteristic cytoskeletal distortions and effacement of microvilli in isolated enterocytes also cause pedestal structures on cultured cell surfaces which are detectable in the FAS test (17, 18). In the quantitative experiments described here we used two AE lesion-forming (i.e., FAS test-positive) EPEC strains (17–19), 2036-80 (serotype O119:H6) and E2348-69 (O127:H6), to assess cellular events in infected HEp-2 cells. Two HEp-2-adherent clinical strains that were negative in the FAS test, 469-3 (O21:H^-) and 135 (O86:H?), and a rough laboratory strain, HB101(pMAR15), were included as comparison strains that were unable to form the AE lesion (16–18). HB101(pMAR15) harbors an EPEC-derived plasmid that promotes initial non-intimate adherence to HEp-2 cells (2). Eight other well-characterized AE lesion-forming EPEC strains and two other strains that were negative in the FAS test were also examined by a rapid screening method; these strains, which have all been described previously (3, 18), are listed in Results.

Bacteria were routinely grown at 37°C without shaking in Luria broth (22) containing 0.2% glucose. HEp-2 cells were cultured as monolayers on 12-mm^2 glass coverslips at 37°C in Dulbecco modified Eagle medium without pyruvate (GIBCO) containing 10% heat-inactivated fetal calf serum (GIBCO) in 5% CO_2 in air. Monolayers were infected by the addition of 10^8 bacteria to 3 ml of Dulbecco modified Eagle medium in 5.5-cm-diameter petri dishes and incubated at 37°C.

Measurement of intracellular free calcium. We used the

TABLE 1. Effect of increasing time of EPEC infection on $[Ca^{2+}]_I$ of cultured HEp-2 cells

Strain	$[Ca^{2+}]_I$ (nM)[a] at the following incubation times (h):				
	0	1	2	3	4
Uninfected	56 ± 2.1		52 ± 2.8		58 ± 2.9
HB101(pMAR15)	50 ± 2.0	51 ± 3.0	51 ± 5.1	59 ± 12.5	54 ± 11.3
2036-80	51 ± 2.4	110 ± 2.0		250 ± 60.3	310 ± 76.0
E2348-69	54 ± 2.0	105 ± 15.0		200 ± 40.0	322 ± 70.2
469-3	53 ± 2.1			62 ± 2.1	
135	52 ± 2.1			65 ± 3.5	

[a] Average values for monolayers in which 80 to 90% of cells were infected with the bacterial strains indicated.

fluorescent Ca^{2+} indicator 2-{[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl}-6-methoxy-8-bis(carboxymethyl) aminoquinoline (quin-2) as a probe of $[Ca^{2+}]_I$. quin-2/AM is an acetoxymethyl ester derivative of quin-2 that, being more lipophilic than the unmodified dye, enters mammalian cells more readily (35). Cellular esterases then remove ester groups to yield free quin-2 acid, trapping it inside cells. quin-2 complexed with Ca^{2+} exhibits maximum fluorescence at 492 nm after excitation at 340 nm, whereas free quin-2 and quin-2 complexed with other divalent cations such as Mn^{2+} and Mg^{2+} show minimal fluorescence at this wavelength (29, 35). All manipulations involving quin-2 were carried out in low light. Optimum loading of HEp-2 monolayers was achieved by incubation in the dark for 1 h at 37°C with 12.5 μM quin-2/AM in Dulbecco modified Eagle medium containing 10% fetal calf serum.

HEp-2 cells were grown to confluence, infected with bacteria as appropriate, and loaded with quin-2 for the last hour of incubation. Single coverslips were placed in 3-ml plastic cuvettes containing 1.5 ml of Hanks buffered salts medium for measurements of Ca^{2+}–quin-2 fluorescence (492 nm) with a Perkin-Elmer 204A dual-wavelength UV spectrophotometer. In freshly loaded cells a fluorescence peak at 430 nm was also observed; this decreased with incubation for a further 15 to 20 min in fresh medium lacking quin-2/AM and probably represents uncleaved ester bound to cell surfaces.

Fluorescence at 492 nm (F) in these conditions depends upon average levels of endogenous free Ca^{2+} in all cells of a monolayer. Treatment of quin-2-loaded cells with 1 μM ionomycin (Calbiochem) caused rapid influx of saturating levels of Ca^{2+} from the external medium, leading to an increase in fluorescence to a maximum (F_{max}), which reflects the efficiency of quin-2 loading. Subsequent addition of excess Mn^{2+} (1 mM) to ionomycin-treated cells caused quenching of intracellular Ca^{2+}–quin-2 fluorescence (F_{min}). Free $[Ca^{2+}]_I$ was calculated from values of F, F_{max}, and F_{min} by using the relationship $[Ca^{2+}]_I = K(F - F_{min})/(F_{max} - F)$, where K is the effective dissociation constant (115 nM at 1 mM Mg^{2+}) of the Ca^{2+}–quin-2 complex (35).

For rapid screening of clinical isolates, we used the fluorescein-based calcium fluorophore 9-{4-bis-4(carboxymethyl) amino-3-[2-(2-bis(carboxymethyl)amino-5-methylphenoxy) ethoxy]phenyl}-2,7-dichloro-6-hydroxy-3H-xanthin-3-one (hereafter referred to as fluo-3) (25) as an alternative to quin-2; the advantage of this compound is that it has visible excitation and emission wavelengths (490 and 530 nm, respectively). As with quin-2, the acetoxymethyl ester derivative of fluo-3 (fluo-3/AM) loads readily into mammalian cells and is cleaved by endogenous esterases to yield the free dye (20). Subconfluent HEp-2 monolayers were treated with 50 μM fluo-3/AM for the last 45 min of infection with bacteria, and localized changes of $[Ca^{2+}]_I$ in infected cells were assessed with a Zeiss AXIOPHOT photofluorescence microscope. To minimize fluorescence quenching, all manipulations were carried out in low light.

Determination of membrane-associated PKC. Confluent HEp-2 cell monolayers were infected with bacteria for 3 h at 37°C. Cells were harvested in 1 ml of ice-cold phosphate-buffered saline containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 20 mg each of the protease inhibitors leupeptin and pepstatin A per ml. Cells were ruptured by sonication, and membrane fragments were pelleted at 100,000 × g for 1 h at 4°C. Membrane pellets were suspended by brief sonication on ice in 1 ml of sonication buffer containing 1% Triton X-100 (14), insoluble material was removed by ultracentrifugation as described above, and soluble material was adjusted to a protein concentration of 400 μg/ml. Protein kinase C (PKC) activity was assayed by incorporation of ^{32}P into histone III-S essentially as described previously (14). Briefly, Triton X-100-soluble membrane fractions containing 4 μg of protein were incubated with 400 μg of histone III-S (Sigma) per ml for 15 min at 37°C in the presence of 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in 250 μl of 20 mM Tris-HCl (pH 7.5) buffer containing 5 mM $MgCl_2$, 40 μg of phosphatidylserine (Sigma) per ml, 500 μM $CaCl_2$, and 10 μM [γ-^{32}P]ATP (specific activity, ~110 TBq/mmol; Amersham International). Reactions were terminated by the addition of 250 μl of 1-mg/ml bovine serum albumin and 2 ml of ice-cold 25% trichloroacetic acid. Precipitates were pelleted by centrifugation, dissolved in 250 μl 1 N KOH, precipitated again as described above, and collected on 2.5-cm-diameter Whatman GF/C glass fiber filters. These were extensively washed with cold 25% trichloroacetic acid and then with cold acetone and dried, and the levels of ^{32}P-phosphorylated histone III-S quantified in a Canberra Packard 2000CA Tri-Carb liquid scintillation analyzer.

RESULTS

Effect of EPEC infection on cytosolic free calcium. The level of free Ca^{2+} (as measured with quin-2) in control HEp-2 cell monolayers was typically 50 to 60 nM, similar to levels reported for various other cell lines (35). Infection with strains of classical EPEC O:H serotypes, however, caused significant enhancement in endogenous quin-2 fluorescence of HEp-2 monolayers, indicating elevated $[Ca^{2+}]_I$ in infected cells (Table 1). Note that each individual measurement is an average for all cells in a monolayer; in parallel experiments 80 to 90% of cells in a monolayer were actually infected, so that the figures shown probably underestimate the true $[Ca^{2+}]_I$ of infected cells. Moreover, at points of bacterium-cell contact, local Ca^{2+} concentrations may be very much greater, as indicated by the localized nature of Ca^{2+}-depen-

dent fluo-3 fluorescence in EPEC-infected monolayers (Fig. 1d). The effect was detectable after 1 h, increasing over 4 h to levels some 4 to 6 times higher than those in uninfected HEp-2 cells or in monolayers infected with the laboratory strain *E. coli* HB101(pMAR15). Infection with two clinical strains that do not induce AE lesion formation, 469-3 and 135, caused only marginal enhancement in $[Ca^{2+}]_I$ (Table 1).

Addition of excess ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (4 mM) to chelate free Ca^{2+} in the incubation medium had no significant effect on EPEC-induced elevation of $[Ca^{2+}]_I$ (Table 2). This, coupled with the fact that determination of maximum fluorescence required the addition of ionomycin, strongly suggests that EPEC infection did not simply cause influx of Ca^{2+} from the medium. An alternative possibility is that additional intracellular free Ca^{2+} is derived from stores within the host cell. To test this, we treated quin-2-loaded HEp-2 monolayers before EPEC infection with 50 μM 1,5-(*p*-nitrophenyl)furfurylidene aminohydantoin (dantrolene), a drug which reportedly traps Ca^{2+} within intracellular stores (11, 36). Bacteria attached and grew normally on dantrolene-treated cells to form microcolonies that were indistinguishable from those on untreated cells, but in this case there was no significant elevation of free $[Ca^{2+}]_I$ over a 4-h incubation period (Table 2).

Quantitative measurement of $[Ca^{2+}]_I$ by this method is time consuming and inappropriate for rapid analysis of clinical isolates. Therefore, to determine the specificity of the effects described above, we developed a screening technique with the newly described calcium fluorophore fluo-3 (20, 25). The advantage of this compound is that its emission spectrum is within the range of normal fluorescence microscopy, so that qualitative changes in $[Ca^{2+}]_I$ in individual infected HEp-2 cells can be visualized directly (Fig. 1). Thus, in addition to strains 2036-80 and E2348-69 tested quantitatively with quin-2 as described above, FAS test-positive clinical isolates 660/79 (serotype O55), BCH2/82 (O111), QEH1/86 (O114), E1621-1 (O119), E57107 (O127a), E57106 (O127ab), 182/83 (O128ac), and E851 (O142) all showed enhanced localized calcium-dependent fluorescence in fluo-3-loaded infected cells similar to that shown in Fig. 1d. On the other hand, FAS test-negative isolates DPO95 (serotype O18ac), 469-3 (O21), 135 (O86), and 444-3 (O?) had no such effect (Fig. 1b).

EPEC-stimulated loss of PKC activity from cell membranes. Membrane fractions of HEp-2 cells contain significant levels of PKC whose activity can only be detected in the presence of TPA or similar activators of PKC (Fig. 2). Prior treatment of HEp-2 cells with TPA resulted in marked enhancement of membrane-associated PKC activity. However, the addition of ionomycin to allow influx of excess Ca^{2+} from the medium resulted in dramatic depletion of activity from the membrane fraction of TPA-treated cells (Fig. 2). Elevation of $[Ca^{2+}]_I$ reportedly activates the neutral serine protease calpain, which cleaves active PKC from the membrane (1, 28). This effect was prevented by cyclosporin A (Fig. 2), a known inhibitor of calpain (38).

We previously reported that EPEC infection of HEp-2 cells stimulates phosphorylation of several protein species that are substrates for PKC (3). However, because EPEC infection also elevates $[Ca^{2+}]_I$ (Table 1), we predict that this activity would not be associated with the membrane fractions of infected cells. HEp-2 cells infected with EPEC strains 2036-80 and E2348-69 did, indeed, show low levels of membrane-associated activity (Fig. 2). On the other hand, high PKC levels were retained in the membrane fraction of 2036-80-infected cells incubated with cyclosporin A.

DISCUSSION

Transient elevation of $[Ca^{2+}]_I$ in brush border cells, for instance, by treatment with hormones or Ca^{2+} ionophores, causes breakdown of cytoskeletal actin in the microvillus core and eventual loss of microvilli by membrane vesiculation (13). Morphological changes in intestinal brush borders characteristic of the EPEC-induced AE lesion bear a striking similarity to these effects (19, 27, 32, 33), suggesting that they too may result from enhanced $[Ca^{2+}]_I$ levels in infected cells. We have used HEp-2 cells as a model for bacterial infection; EPEC adhere to these cells to produce an AE lesion, detectable in the FAS test (17, 18), that is morphologically similar to that seen in infected gut tissue (16). Infection of HEp-2 monolayers with FAS test-positive EPEC strains significantly enhanced $[Ca^{2+}]_I$, as measured by increased Ca^{2+}-dependent fluorescence of intracellular quin-2. Moreover, fluorescence microscopic analysis of EPEC-infected cells loaded with fluo-3 indicated that Ca^{2+}-dependent fluorescence was particularly intense in the region of adherent bacterial microcolonies. Because of the discrete nature of lesion formation, therefore, EPEC-induced enhancement of $[Ca^{2+}]_I$ at sites of bacterial attachment is, at least transiently, much greater than the concentrations measured with quin-2.

In intestinal epithelial cells, increased $[Ca^{2+}]_I$ activates depolymerization and inhibits bundling of actin by the Ca^{2+}-dependent microvillus protein villin, causing loss of integrity through breakdown of actin in the microvillus core (5, 6). Nonintestinal cells such as HEp-2 cells contain the related Ca^{2+}-dependent actin-severing protein gelsolin (28). At high $[Ca^{2+}]_I$, gelsolin and villin associate with and cleave F actin nonproteolytically between G-actin monomers (9, 23, 39, 40). They remain associated with the ends of cleaved fragments, forming a cap that prevents elongation of filaments by further addition of actin monomers. As locally high Ca^{2+} levels are dispersed, binding of the cap to phosphatidylinositol 4,5-diphosphate in the membrane results in dissociation of gelsolin or villin from the filament ends, facilitating an explosive burst of actin polymerization (23, 39). The presence of high concentrations of F actin at sites of bacterial attachment to HEp-2 cells, as observed in the FAS test (17, 18), may therefore be due to de novo actin polymerization at nucleation sites created by dissociation of gelsolin.

Some hormones release stored intracellular Ca^{2+} to a free cytosolic form by activation of a signal transduction pathway in which a distinct receptor-coupled phospholipase C generates the second messenger, inositol 1,4,5-triphosphate (IP_3) (4, 13, 15, 37). It is likely that a similar mechanism operates in EPEC-infected HEp-2 cells, since enhancement of $[Ca^{2+}]_I$ was abolished by treatment with dantrolene, a drug which is reported to prevent release of Ca^{2+} from IP_3-sensitive stores (11, 36). IP_3-controlled Ca^{2+} release is normally transient because of rapid down regulation of receptor-coupled events and degradation of signal molecules (7, 8, 31). The fact that $[Ca^{2+}]_I$ in EPEC-infected cells continued to increase over 4 h is probably a reflection of the dynamic and localized nature of EPEC infection, in which bacterial growth and attachment occur continuously throughout the incubation period. Attempts to synchronize stimulation by the addition of *E. coli* extracts or culture supernatants to HEp-2 cells have so far failed to reproduce the phenomenon. Moreover, enhanced production of IP_3 in EPEC-infected HEp-2 cells is difficult to

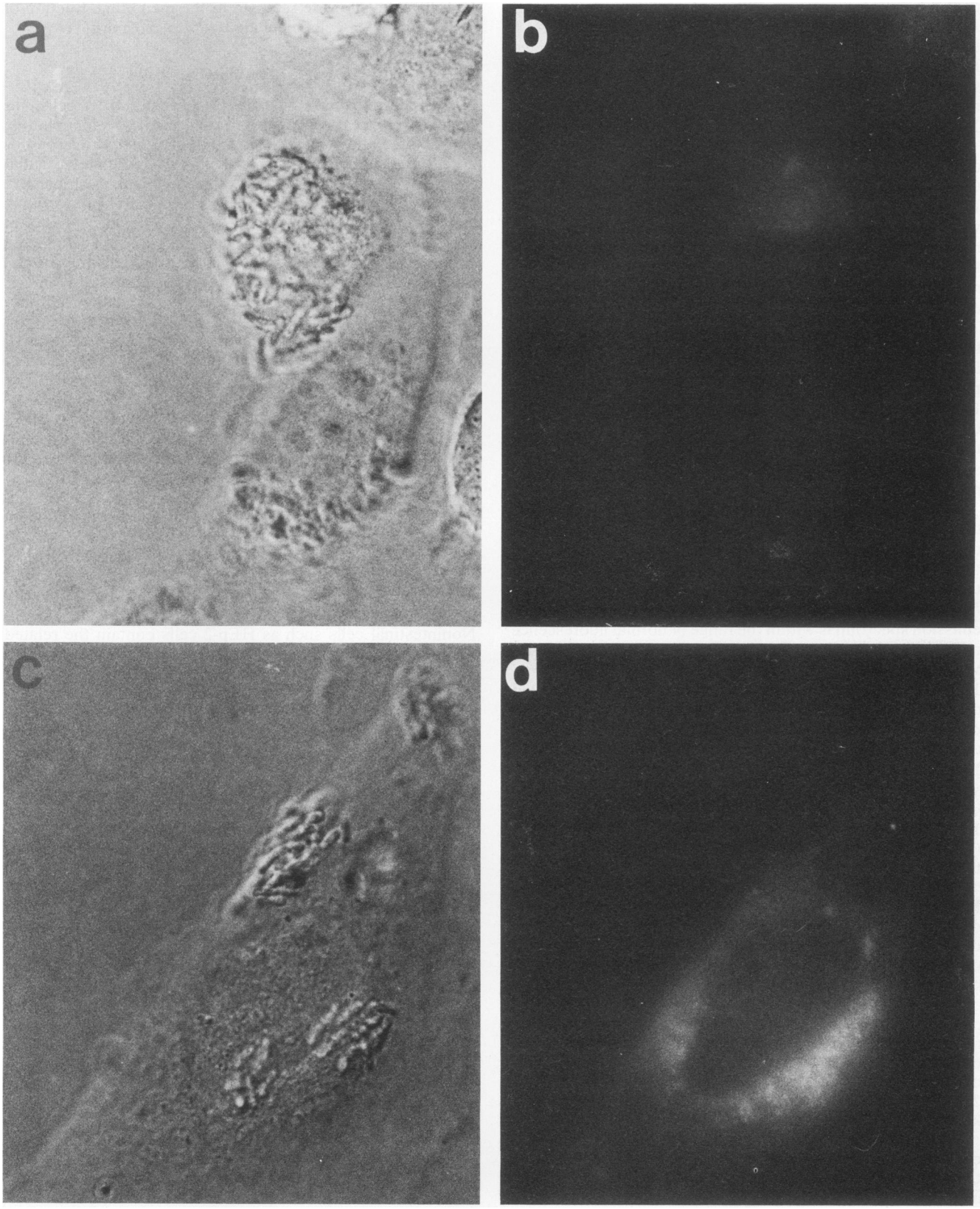

FIG. 1. Fluorescence microscopic analysis of localized calcium fluxes in fluo-3-loaded cultured cells. HEp-2 cell monolayers at approximately 50% confluence were infected with the FAS test-negative strain *E. coli* 135 (a and b) or with EPEC strain 2036-80 (c and d) at 37°C for a total of 3 h as described in the text; fluo-3/AM (50 μM) was added for the final 45 min of the infection period. Monolayers were washed thoroughly by immersing coverslips in fluo-3-free medium and mounted in fresh medium. Identical fields were photographed by phase-contrast (a and c) and fluorescence (b and d) microscopy.

TABLE 2. Effect of EGTA and dantrolene on $[Ca^{2+}]_I$ elevation in EPEC-infected HEp-2 cells

Strain	$[Ca^{2+}]_I$ after 3 h of incubation[a]		
	No treatment	EGTA (4 mM)	Dantrolene (50 μM)
2036-80	230 ± 50	225 ± 36	57 ± 2
E2348-69	252 ± 66	238 ± 42	65 ± 3
HB101(pMAR15)	61 ± 8	61 ± 8	70 ± 10

[a] Values for monolayers in which 80 to 90% of cells were infected with the bacterial strains indicated.

measure due to substantial turnover of inositol compounds during these extended incubation periods.

For a variety of technical reasons, we have not been able to demonstrate directly that similar cellular perturbations occur in enterocytes as a consequence of EPEC infection. However, our data based on experiments with HEp-2 cells as a convenient model clearly suggest an attractive hypothesis for EPEC diarrheagenesis in which both severe loss of absorptive capacity of the intestinal mucosa and enhanced secretion of electrolytes and fluid into the gut lumen are independently stimulated by two second messengers generated by hydrolysis of phosphatidylinositol 4,5-diphosphate in the membrane of infected cells. We speculate that IP_3, by stimulating release of stored $[Ca^{2+}]_I$, inflicts gross histological damage on colonized mucosal surfaces through activation of actin-severing proteins in the cytoskeleton. Microvilli are destroyed by vesiculation, and bacteria then attach directly to the plasma membrane, causing the characteristic surface distortions of the AE lesion observed in the FAS test (17, 18). The other second messenger, diacylglycerol, activates Ca^{2+}- and phospholipid-dependent PKC (4), an enzyme that is pivotal in the control of normal ion fluxes across mammalian cell membranes (12). We previously proposed that prolonged stimulation of PKC activity in the course of an EPEC infection may perturb the system sufficiently to cause exaggerated ion efflux from the intestinal mucosa (3), perhaps by phosphorylation of specific ion transport proteins (12). The data presented in this paper indicate that concomitant elevation of $[Ca^{2+}]_I$ in response to IP_3 production in infected cells triggers proteolytic cleavage by calpain of membrane-associated PKC to a constitutive (i.e., independent of Ca^{2+} and phospholipids) cytosolic activity, protein kinase M (1, 28). The relative importance of protein kinase C and protein kinase M in the diarrheagenic mechanism of EPEC remains to be determined.

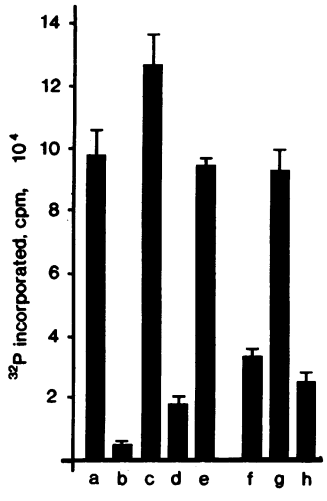

FIG. 2. Calpain-induced loss of PKC from membrane fractions of EPEC-infected HEp-2 cells. Incorporation of ^{32}P into histone III-S in vitro stimulated by membrane fractions of HEp-2 cells in the presence (a) or absence (b) of 100 nM TPA. Incorporation of ^{32}P into histone III-S in the presence of 100 nM TPA was used as an indicator of PKC activity in membrane fractions prepared from HEp-2 cells treated for 1 h at 37°C with 50 nM TPA (c), 50 nM TPA and 1 μM ionomycin (d), or 50 nM TPA, 1 μM ionomycin, and 10 μM cyclosporin A (e) or from cells infected for 3 h with EPEC strain 2036-80 (f), strain 2036-80 in the presence of 10 μM cyclosporin A (g), or EPEC strain E2348-69 (h). Error bars indicate standard deviations from the means of data from at least three independent experiments.

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