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Phorbol Esters Specifically Enhance the Cytolytic Activity of *Entamoeba histolytica*

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Entamoeba histolytica **causes invasive amebiasis by lysis of host tissue and inflammatory cells. The in vitro cytolysis of target Chinese hamster ovary (CHO) cells by axenic *E. histolytica* trophozoites (strain HM1:IMSS) is a calcium- and phospholipase A-dependent event initiated by the binding to the target cell of the galactose-inhibitable surface lectin of the parasite. We utilized phorbol esters as a probe to determine whether an amebic protein kinase C has a role in the cytolytic event. The addition of phorbol 12-myristate 13-acetate (PMA) at 10^{-6} or 10^{-7} M resulted in a greater than twofold enhancement of amebic killing of target CHO cells over 30 min ($P < 0.01$). Prior exposure of only the amebae, but not the CHO cells, to PMA produced a similar effect ($P < 0.01$). The inactive analog 4-α-phorbol had no effect on amebic killing of CHO cells. The PMA-mediated enhancement of amebic cytolysis persisted for up to 60 min after a 5-min exposure; however, after a 30-min exposure to PMA (10^{-6} M) there was no augmentation of amebic killing of CHO cells. PMA (10^{-6} M) did not promote adherence of parasites to CHO cells but did enhance amebic cytolysis of previously adherent target cells ($P < 0.01$). Sphingosine, a specific inhibitor of protein kinase C, abolished both the PMA-stimulated and the basal cytolytic activity of *E. histolytica*. PMA enhanced CHO cell cytolysis by the less virulent wild-type strain H-303:NIH ($P \leq 0.02$) but did not augment the activity of the less virulent strain H-200:NIH or two avirulent clones of HM1 (L6 and C919). In summary, these experiments with the phorbol esters and sphingosine as probes to modulate the activity of protein kinase C indicate participation of a parasite protein kinase C in the cytolytic activity of virulent, axenic *E. histolytica* trophozoites and thus in the pathogenesis of amebiasis.**

Amebiasis, a cause of worldwide morbidity and mortality, is due to invasion of the colonic mucosa by the enteric protozoan *Entamoeba histolytica*. The in vivo pathogenesis of amebiasis appears to involve parasite adherence to and cytolysis of host intestinal and inflammatory cells, leading to mucosal disruption (27). To study the pathogenesis of amebiasis, we have utilized an in vitro model, the interaction of axenic *E. histolytica* trophozoites with mammalian target cells such as Chinese hamster ovary (CHO) cells or human neutrophils (9, 28, 29, 39). In this model, the first step in killing by amebae is adherence to the target cell, an event mediated by the galactose- or *N*-acetyl-D-galactosamine-inhibitable surface lectin of the parasite (26, 29, 32). The second step is cytolysis of the adherent target cells by the amebae. The cytolytic activity of *E. histolytica* is dependent on extracellular calcium, free cytoplasmic calcium in the ameba, parasite phospholipase A activity, and maintenance of an acid pH in amebic intracellular vesicles (14, 31, 33, 34). The validity of the ameba-CHO cell model has been further established by comparable studies with human erythrocytes, rat and human colonic mucosa, Chang liver cells, isolated rat colonic epithelial cells, and purified rat and human colonic mucins (5, 24, 27, 30, 36).

In the eucaryotic cell membrane, inositol phospholipids can be metabolized through two pathways (2). First, phospholipase A may cleave arachidonic acid from phosphatidylinositol, releasing lysophosphatidylinositol. This pathway may be involved in the cytolysis of target cells by amebae. The second pathway of inositol phospholipid metabolism is initiated when an extracellular signal such as a hormone binds to a specific cell surface receptor, stimulating the activity of phospholipase C, a phosphodiesterase. Phospholipase C, in turn, breaks down membrane inositol phospholipids, releasing diacylglycerol. Diacylglycerol has been shown to increase the affinity of protein kinase C for calcium and, in the presence of phospholipids, particularly phosphatidylserine, leads to full activation of this enzyme with subsequent phosphorylation of protein substrates. Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) or phorbol 12,13-dibutyrate substitute for diacylglycerol and directly stimulate protein kinase C, the phorbol ester receptor (4, 22).

Because protein kinase C is important in the intracellular transduction of many receptor-mediated calcium-dependent signals, we hypothesized that an *E. histolytica* protein kinase C may have a role in the amebic galactose- or *N*-acetyl-D-galactosamine-inhibitable lectin-initiated cytolysis of target CHO cells. Therefore, the purpose of our studies was to investigate whether an amebic protein kinase C participates in the cytolysis of target cells by *E. histolytica*.

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MATERIALS AND METHODS

Parasite and CHO cell maintenance. A cloned population of *E. histolytica* HM1:IMSS (ATCC 30459; American Type Culture Collection, Rockville, Md.) was originally obtained from L. Diamond (National Institutes of Health, Bethesda, Md.) and then maintained in our laboratory. Ameba strains

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H-303:NIH (303) and H-200:NIH (200) were obtained from the American Type Culture Collection. The avirulent clone L6 was derived from strain HM1:IMSS by the use of bromodeoxyuridine-labeled bacteria plus radiation and subsequent screening of clones grown on agar (24, 35). The avirulent clone C919 was isolated by repeated mutagenesis with ethyl methanesulfonate followed by the use of bromodeoxyuridine-labeled bacteria plus radiation as previously described (25, 35). Unless otherwise stated, *E. histolytica* HM1:IMSS was used in all studies. All amebae were grown in TYI-S-33 (tryptic yeast extract, iron, and serum) medium supplemented with 100 U of penicillin (GIBCO Laboratories, Grand Island, N.Y.) per ml and 100 mg of streptomycin sulfate (GIBCO) per ml.

CHO cells are grown routinely in F-12 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO), penicillin (100 U/ml), and streptomycin (100 mg/ml). They were treated with trypsin (0.25% for 2 min), suspended in medium 199 (GIBCO) supplemented with 5.7 mM cysteine, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and 0.5% bovine serum albumin (M199S) with 1% FBS, washed once, and adjusted to a concentration of 2×10^5 cells per tube.

Killing by amebae of target CHO cells. CHO cells were centrifuged with 10^4 amebae in M199S with 1% FBS for 5 min at $150 \times g$ and then incubated for the times indicated below at 37°C. Pellets were dispersed with 0.1 ml of 0.4% trypan blue (Chroma-Gesellschaft; Roboz Surgical Instruments, Washington, D.C.), and the live and dead CHO cells were counted in a hemacytometer chamber. This method of measuring CHO cell viability has been previously established to correlate with release of ^{111}indium oxine from labeled target CHO cells (28). The percent of CHO cells killed by amebae is expressed as the difference in viability observed between CHO cells incubated alone and with amebae present under otherwise identical experimental conditions. Control CHO cells were always greater than 95% viable.

Studies of adherence by amebae to target CHO cells. For adherence studies, amebae (1×10^4) and CHO cells (2×10^5) were suspended in 1 ml of M199S without serum, centrifuged at $150 \times g$ for 5 min, and incubated for 1 to 2 h at 4°C. After incubation, 0.8 ml of the supernatant was removed, and the pellet was suspended by vortexing the tube for 5 s. One drop of the cell suspension was placed on the hemacytometer chamber, and the cover slip was applied. The numbers of amebae that did or did not form rosettes with CHO cells (three or more CHO cells adherent to each ameba) were counted (≥50 amebae were counted per chamber).

Suspension of amebae with adherent CHO cells in dextran solution. Dextran suspension studies were performed as previously described (29). Centrifugation of amebae and CHO cells at $150 \times g$ was followed by incubation in M199S with 1% FBS at 4°C for 1 h. Amebae with adherent CHO cells were suspended in M199S with 1% FBS with 10% dextran (M_r, 500,000; Sigma Chemical Co., St. Louis, Mo.). Suspensions were incubated at 37°C for the desired interval, and CHO cell viability was then determined by exclusion of trypan blue as described above.

Effect of protein kinase C activators or D-sphingosine on amebic cytolytic activity and adherence. Only phorbol esters having a beta-hydroxyl group at position 4 in the parent phorbol molecule bind to and directly activate protein kinase C. The alpha analogs are inactive and do not bind to protein kinase C (4, 21). In our studies, PMA and phorbol 12,13-dibutyrate were used as the active (beta) analogs of the phorbol esters. The inactive parent compound, 4-α-phorbol, was used as the negative control. PMA and phorbol 12,13-dibutyrate were obtained from Consolidated Midline Corp. (Brewster, New York); 4-α-phorbol was from Sigma. Stock solutions of phorbol esters (10^{-3} M) were stored in absolute ethanol at −15°C until use. In some cytolytic assays, PMA was added at time zero to the M199S with the amebae and CHO cells. In other assays, either the amebae or the CHO cells or both were preincubated with various concentrations of phorbol esters, washed once with M199S with 1% FBS, centrifuged for 5 min at $150 \times g$, and then incubated for the cytolytic assays as described above. In the dextran studies, PMA was added to the 10% dextran and the suspension was incubated at 37°C. To determine the effect of D-sphingosine, a specific inhibitor of protein kinase C (10, 19, 40), on basal amebic killing of target CHO cells, amebae were incubated overnight in the presence of 10 μM D-sphingosine (Sigma). The amebae were adjusted to 10^5/ml, and the cytolytic assay was performed as described above. In studies with D-sphingosine, it was added to the assays from a 40 mM stock solution in dimethyl sulfoxide.

TABLE 1. Effect of PMA on the killing of target CHO cells by amebae

Medium	% of CHO cells killed after 30 min (n)[a]
Control[b]	16.7 ± 2.4 (25)
PMA	
10^{-6} M	38.9 ± 2.5[c] (20)
10^{-7} M	31.7 ± 2.3[c] (11)
10^{-8} M	24.5 ± 3.0 (13)

[a] Means ± standard errors of n experiments.
[b] Control is *E. histolytica* trophozoites and CHO cells incubated in control medium for 30 min. For PMA conditions, PMA was added at the beginning of the incubation.
[c] $P < 0.001$ compared with control medium.

Statistics. All results are expressed as a mean ± one standard error of the mean. Either the unpaired or paired Student t test was used to evaluate differences.

RESULTS

Amebic killing of target CHO cells. In our initial experiments, we examined the effect of the most potent phorbol ester, PMA, on the killing of target CHO cells by *E. histolytica* (Table 1). After the 30-min incubation with untreated (control) amebae, 16.7% of CHO cells were killed. However, PMA at 10^{-6} M added at the beginning of the incubation enhanced parasite killing of target CHO cells by greater than twofold ($P < 0.001$). Similarly, PMA at 10^{-7} M augmented CHO cell killing by amebae ($P < 0.0001$).

To determine whether PMA was having an effect on the ameba or on the target CHO cell, we exposed either the *E. histolytica* trophozoites or CHO cells to PMA at 10^{-6} M before the killing assay (Table 2). As in our previous studies, the presence of PMA during the 30-min incubation resulted in a 2.3-fold enhancement of amebic killing of target CHO cells ($P < 0.01$). Amebae exposed to PMA (10^{-6} M) for 5 min exhibited a similar enhanced ability to kill target CHO cells over the next 30 min ($P < 0.01$); prior exposure of CHO cells to PMA (10^{-6} M) had no effect. However, exposure of either the amebae or the CHO cells to 10^{-6} PMA for 30 min did not result in a stimulation of amebic killing of CHO cells (Table 2). Furthermore, exposure of the CHO cells to PMA (10^{-6})

TABLE 2. Effect of preincubation of *E. histolytica* or CHO cells with PMA (10^{-6} M) on killing of target CHO cells by amebae

Conditions[a]	% of CHO cells killed after 30 min (*n*)[b]
Eh + CHO	15.5 ± 4.7 (12)
Eh + CHO + PMA	35.3 ± 3.7[c] (12)
Eh (5 min with PMA) + CHO	38.2 ± 3.7[c] (12)
Eh + CHO (5 min with PMA)	9.3 ± 4.2 (12)
Eh (30 min with PMA) + CHO	20.2 ± 2.9 (6)
Eh + CHO (30 min with PMA)	22.9 ± 6.5 (6)

[a] *E. histolytica* trophozoites (Eh) and CHO cells were incubated in control medium; *E. histolytica* trophozoites plus CHO cells and PMA were incubated in control medium with PMA added at the beginning of the incubation. In each additional condition, either *E. histolytica* trophozoites or CHO cells were incubated in control medium with PMA for the indicated time and washed, and amebic killing of CHO cells was assessed in the 30-min cytolytic assay.
[b] Means ± standard errors of *n* experiments.
[c] $P < 0.01$ compared to *E. histolytica* trophozoites plus CHO cells.

for 5 min did not further augment the killing of CHO cells by control or PMA-stimulated amebae ($n = 8$; data not shown). Under all conditions studied, PMA (10^{-6} M) had no effect on the viability of amebae or CHO cells (always >95% viable). These results indicate that the phorbol-ester-mediated stimulation of *E. histolytica* cytolytic activity was due to an effect on the ameba and not the target CHO cell.

To determine the specificity of the PMA stimulation of amebic killing of target CHO cells, we performed studies with the inactive phorbol analog 4-α-phorbol. Neither preincubation of amebae for 5 min with 4-α-phorbol (10^{-6} M) nor the addition of 4-α-phorbol (10^{-6} M) to the assay system at time zero significantly enhanced amebic killing of CHO cells compared with the control without the phorbol esters present (Table 3).

A dose response of a 5-min preincubation of the amebae with PMA on amebic killing of target CHO cells is shown in Fig. 1. There was a narrow concentration range over which PMA enhanced the cytolysis of target CHO cells (5×10^{-6} to 5×10^{-7} M; $P \leq 0.002$). At both higher and lower concentrations of PMA, amebic cytolysis of CHO cells was unchanged compared with that of the control. At all concentrations tested, PMA did not alter parasite viability.

In Table 4, the duration of stimulation of amebic killing of target CHO cells after a 5-min exposure to PMA (10^{-6} M) is shown. The PMA-mediated enhancement of amebic cytolysis of target CHO cells occurred predominantly in the first 30 min after exposure to PMA. Killing of CHO cells after 60- and 120-min incubations was only marginally in-

TABLE 3. Specificity of the effect of PMA on amebic killing of target CHO cells[a]

Conditions	% of CHO cells killed after 30 min (*n*)[b]
Eh + CHO	19.3 ± 3.3 (20)
Eh (5 min with PMA) + CHO	35.9 ± 2.6[c] (20)
Eh (5 min with 4-α-phorbol) + CHO	23.6 ± 6.0[d] (8)
Eh + CHO + 4-α-phorbol	19.2 ± 2.4[d] (12)

[a] The concentrations of both PMA and 4-α-phorbol were 10^{-6} M. Experiments were conducted as described in footnote *a* of Table 2. Eh, *E. histolytica* trophozoites.
[b] Means ± standard errors of *n* experiments.
[c] $P < 0.001$ compared with *E. histolytica* plus CHO cells or conditions with 4-α-phorbol exposure.
[d] Not significant compared with *E. histolytica* plus CHO cells.

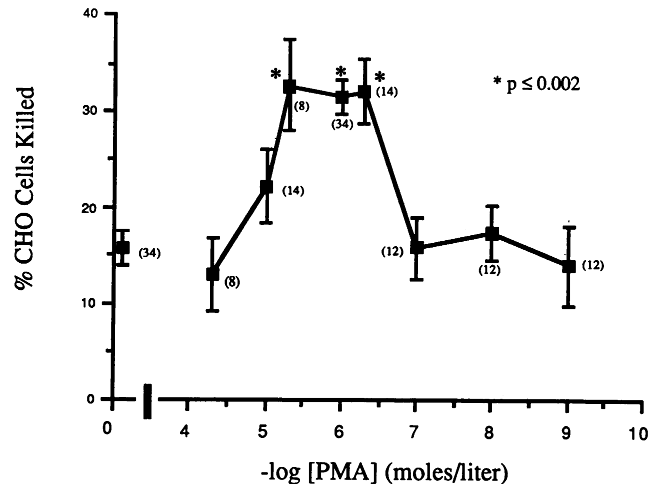

FIG. 1. Dose response of PMA on the killing of CHO cells by amebae. The percentage of CHO cells killed by amebae in control experiments is shown as zero concentration of PMA. Data are expressed as means ± standard errors for (*n*) experiments.

creased when the amebae were preincubated with PMA for 5 min, although statistical significance was achieved at 120 min. To determine how long after preincubation with PMA amebae maintained an augmented ability to kill target CHO cells, amebae were preincubated with PMA (10^{-6} M) for 5 min and washed, and their activity was assessed immediately in the 30-min cytolytic assay or at delayed intervals of 30 and 60 min. In these experiments (data not shown), significant enhancement of amebic killing of CHO cells persisted at 60 min (i.e., 30- min delay plus the 30-min cytolytic assay) after the 5-min preincubation with PMA ($P < 0.001$) but not at 90 min.

Enhancement of the number of CHO cells killed by amebae during the first 30 to 60 min after exposure to PMA is consistent with an effect of PMA either to promote amebic adherence to the CHO cells or to promote the subsequent cytolytic event. Therefore, to determine which step was augmented by PMA, we next studied the effect of PMA on the separate events of amebic adherence to and cytolysis of target CHO cells.

Adherence by amebae to target CHO cells. As shown in Table 5, 54.6% of amebae formed ameba-CHO cell rosettes at 4°C. However, neither addition of 10^{-6} M PMA to the assay system at time zero nor a 5-min exposure of amebae or CHO cells or both cells to PMA (10^{-6} M) at 37°C before the 4°C adherence assay altered subsequent ameba-CHO cell rosette formation (Table 5). These results were not altered by incubating either the parasites, the CHO cells, or both cell types for 30 min with PMA at 37°C before assessing rosette formation at 4°C ($n = 5$; data not shown). Last,

TABLE 4. Time course of PMA effect on killing of target CHO cells by amebae

Conditions	% of CHO cells killed[a] after:		
	30 min	60 min	120 min
Control	19.8 ± 4.5	30.5 ± 3.7	51.2 ± 2.8
PMA (10^{-6} M)	30 ± 4.2[b]	34.9 ± 4.1	56.5 ± 3.0[c]

[a] Means ± standard errors of 12 experiments.
[b] $P \leq 0.05$ compared with 30-min control.
[c] $P \leq 0.01$ compared with 120-min control.

TABLE 5. Effect of PMA (10^{-6}) on adherence by amebae to target CHO cells[a]

Conditions	% Rosette formation[b]
Eh + CHO	54.6 ± 1.2
Eh + CHO + PMA	55.2 ± 1.8
Eh (5 min with PMA) + CHO	56.3 ± 1.4
Eh + CHO (5 min with PMA)	57.6 ± 2.2
Eh (5 min with PMA) + CHO (5 min with PMA)	53.5 ± 1.9

[a] Mean ± standard error of 8 experiments.
[b] The adherence assay was done as described in Materials and Methods. Incubations were carried out as described in footnote *a* of Table 2. Eh, *E. histolytica* trophozoites.

exposure of amebae to PMA (10^{-6} M) for 5 min at 37°C did not increase the number of CHO cells adherent to each ameba when compared with controls (n = 10). These data indicate that PMA did not promote adherence of amebae to target CHO cells.

Amebic killing of previously adherent target CHO cells. When amebae and CHO cells are suspended in a 10% solution of high-molecular-weight dextran without prior centrifugation to allow for adherence, they are isolated from one another; no CHO cell death occurs compared with the control of CHO cells alone (29). Thus, interaction of amebae and CHO cells can occur only when they are adherent before suspension in the dextran. For these studies, amebae and CHO cells were pelleted together and incubated at 4°C for 1 h to allow for adherence; the pellet was then suspended in dextran solution at 37°C. PMA (10^{-6} M) added to the dextran suspension significantly enhanced amebic killing of adherent CHO cells from 8.4 ± 3.9% to 24.6 ± 2.6% ($P <$ 0.01) (means ± standard errors for 8 experiments). With PMA (10^{-6} M) in the dextran solution, enhanced amebic cytolysis of adherent CHO cells was also observed at 60 and 120 min ($P \leq 0.02$).

Effect of sphingosine on amebic killing of target CHO cells. Sphingosine, a natural component of cellular sphingolipids, has recently been reported to specifically inhibit protein kinase C activity and phorbol dibutyrate binding in a micelle system in vitro (10). Sphingosine has also been demonstrated to inhibit the responses of human platelets, neutrophils, and the promyelocytic leukemic cell line HL-60 to phorbol esters, suggesting a physiologic role for sphingoid bases as negative effectors for protein kinase C (10, 19, 40). Therefore, to further assess the specificity of the PMA-mediated enhancement of amebic cytolysis of target CHO cells, we examined the effect of sphingosine on PMA-stimulated and basal amebic cytolytic activity. When amebae were preincubated with sphingosine for 30 min (Table 6), sphingosine at 30 and 50 μM, but not 20 μM, significantly reduced the PMA-mediated stimulation of amebic cytolytic activity ($P \leq$ 0.006). Because prolonged incubation may be necessary for incorporation of sphingosine into cells (19), the amebae were incubated with 10 μM sphingosine overnight before measurement of basal amebic cytolytic capacity in a 2-h killing assay. In these experiments (n = 10), untreated amebae killed 61.3 ± 2.4% of CHO cells when compared with controls; however, after incubation with sphingosine, amebae only killed 16.8 ± 4.9% of CHO cells ($P < 0.001$). These studies further support the data that the PMA enhancement of amebic cytolytic activity is mediated by an amebic protein kinase C.

Phorbol ester responsiveness of amebic strains or clones of reduced virulence. To determine whether the PMA stimulation of amebic cytolytic activity was related to in vivo virulence, we studied two less virulent wild-type axenic strains, H-303:NIH and H-200:NIH, and two avirulent HM1 clones, L6 and C919 (23, 32). In comparison with HM1:IMSS, H-303:NIH and H-200:NIH have reduced ability to produce liver abscesses in an in vivo model (16, 17) and have reduced cytolytic activity for several target cells (32, 35). Both avirulent clones are deficient in phagocytosis, do not produce liver abscesses in an in vivo model, and are deficient in killing CHO cells and human neutrophils (24, 25, 35). The cytolytic ability of wild-type strain H-303:NIH, but not that of H-200:NIH, was augmented significantly by PMA (10^{-6} M) ($P \leq 0.02$) (Table 7). Although the percent increase in the killing of target CHO cells was similar for both the HM1 and H-303:NIH strains treated with PMA (10.2 ± 0.6% versus 11.3 ± 1.2%, respectively), the killing of CHO cells by HM1 amebae was significantly greater than that by the H-303:NIH amebae (control killing, 1.9 ± 3.9% for H-303:NIH versus 12.5 ± 3.0% for HM1, $P \leq 0.008$; killing in the presence of 10^{-6} M PMA, 13.3 ± 5.0% for H-303:NIH versus 22.7 ± 3.7% for HM1, $P \leq 0.004$). In contrast, both avirulent clones derived from the HM1 strain showed no augmentation of cytolytic capacity in the presence of PMA. These results suggest a relationship between the virulence of the amebic strain or clone and the ability of PMA to enhance parasite cytolytic activity.

TABLE 6. Effect of sphingosine on the PMA-enhanced killing of target CHO cells by amebae[a]

Conditions	% of CHO cells killed after 30 min[b]
Eh + CHO	5.6 ± 3.8
Eh (5 min with PMA)[c] + CHO	26.9 ± 4.0[d]
Eh (5 min with PMA) + CHO + 50 μM sphingosine	5.3 ± 3.0[e]
Eh (5 min with PMA) + CHO + 30 μM sphingosine	13.2 ± 2.7[e]
Eh (5 min with PMA) + CHO + 20 μM sphingosine	26 ± 4.1[d]

[a] For each PMA condition, amebae were preincubated with PMA for 5 min, washed, and then studied in the cytolytic assay. Sphingosine was present throughout the 30-min incubation period where noted. Eh, *E. histolytica* trophozoites.
[b] Means ± standard errors of 12 experiments.
[c] The concentration of PMA was 10^{-6} M.
[d] $P \leq 0.0006$ compared with *E. histolytica* plus CHO cells.
[e] Not significant compared with *E. histolytica* plus CHO cells and $P < 0.01$ compared with *E. histolytica* pretreated for 5 min with PMA plus CHO.

TABLE 7. Effect of PMA (10^{-6} M) on the killing of target CHO cells by amebae of varied in vivo virulence[a]

E. histolytica strain or clone	Increase in % of CHO cells killed 30 min after PMA exposure (n)[b]
HM1:IMSS	10.2 ± 0.6[c] (24)
HM1 clones	
L6	−1.8 ± 1.1 (12)
C919	−1.3 ± 1.2 (12)
H:303:NIH	11.3 ± 1.22[d] (12)
H:200:NIH	5.8 ± 1.7 (12)

[a] Amebae were preincubated with PMA for 5 min, washed, and then studied in the cytolytic assay.
[b] Means ± standard errors of n experiments; data expressed as the increase in percent of CHO cells killed compared with control studies without prior exposure of amebae to PMA.
[c] $P \leq 0.002$ versus HM1:IMSS control (not treated with PMA).
[d] $P \leq 0.02$ versus H:303:NIH control (not treated with PMA).

DISCUSSION

The killing of target cells by *E. histolytica* trophozoites involves two sequential steps: adherence followed by extracellular cytolysis. Amebae then ingest the dead adherent target cells (28, 29). Previous studies have demonstrated that extracellular calcium and an intracellular calcium ion flux are vital for amebic adherence and cytolytic events (31, 34). The present report demonstrates a dramatic and specific effect of phorbol esters on the killing of target CHO cells by amebae, suggesting a regulatory role for protein kinase C in *E. histolytica* cytolytic activity. Phorbol esters did not promote amebic adherence, but rather their effects were limited to enhancing the cytolysis event which follows parasite adherence. This conclusion is supported by the studies showing that a brief preincubation of the amebae with PMA resulted in enhanced cytolysis of the target CHO cells by the amebae. Exposure of the CHO cells to PMA had no effect on their susceptibility to lysis by *E. histolytica*. Since phorbol esters are known to rapidly bind to and activate protein kinase C, these results are consistent with an *E. histolytica* protein kinase C-mediated event (4, 22). Although, it is unlikely that PMA was removed from the amebae by washing (3), additional experiments with phorbol dibutyrate (10^{-6} M), which binds less avidly to protein kinase C (21, 37) and can be removed by washing, confirmed that a brief incubation of the amebae with this phorbol ester also enhanced killing of the target CHO cells significantly in the 30-min cytolytic assay (data not shown). These results support the hypothesis that the phorbol esters enhance cytolysis by activating an amebic protein kinase C. In addition, sphingosine, a specific inhibitor of protein kinase C, reduced the ability of unstimulated amebae to kill target CHO cells, suggesting a role for an amebic protein kinase C in the natural galactose- or *N*-acetyl-D-galactosamine-inhibitable lectin-mediated cytolysis of target cells by amebae. Although protein kinase C has been shown to be ubiquitous, occurring in various tissues and various phyla of the animal kingdom (13), to our knowledge this is the first demonstration of a potential biologic role for protein kinase C in a protozoan that is pathogenic for humans.

The enhancing effect of PMA on cytolysis of target CHO cells by ameba occurred in a narrow dose range. Although in many systems phorbol esters exert significant effects at nanomolar concentrations, in vivo or in systems in which large numbers of cells are necessary higher concentrations of phorbol esters have been required (8, 39). Because amebic trophozoites are large organisms ($\geq$20 μm) which rapidly turn over their plasma membrane, it is possible that the higher concentrations required for an effect in our studies is due to the greater membrane surface area of the amebae, enhancing nonspecific binding of the phorbol esters due to lipid partitioning. Alternatively, amebae may metabolize the phorbol esters via esterases as has been described in other systems (23, 38). One other possible explanation for the dose response noted in our studies is the phospholipid composition of the amebae. Aley et al. (1) reported that 48% of the phospholipid composition of the whole amebae was phosphatidylcholine, 15% was ceramide aminoethyl phosphonate, and 17% was phosphatidylethanolamine. However, when a highly enriched plasma membrane preparation was obtained from axenically propagated *E. histolytica*, it was found to be enriched in both ceramide aminoethyl phosphonate (38%) and phosphatidylethanolamine (32%), with comparatively less phosphatidylcholine (12%). Recent studies by several investigators (10, 19, 40) indicated specific inhibition of protein kinase C-mediated biologic events and binding of the phorbol esters to protein kinase C in the presence of sphingosine. In addition, sphingosine inhibited both basal and PMA-enhanced cytolysis of CHO cells by amebae in our assay system. Ceramide is composed of sphingosine and a fatty acid, suggesting that the abundance of this sphingolipid in the amebic plasma membrane may necessitate higher concentrations of the phorbol esters to exhibit a biologic effect. Further studies will be necessary to evaluate these possibilities.

We observed that a 30-min preincubation of the amebae with PMA did not enhance subsequent killing of target CHO cells, but inclusion of PMA in the cytolytic assay enhanced amebic cytolysis. Several possibilities may explain these findings. First, prolonged exposure of the amebae with PMA may lead to down-regulation of protein kinase C, preventing a subsequent biologic effect. Alternatively, prolonged exposure of amebae to the phorbol esters may lead to the phosphorylation of additional amebic proteins, resulting in no net promotion of target cell killing, similar to observations in S49 lymphoma cells (12). In biologic systems, a positive signal is frequently followed by immediate negative feedback regulation. In several cell types, recent evidence suggests that protein kinase C exerts negative feedback control on an initiating stimulus (22). Thus, activation of protein kinase C in amebae may initially promote cytolysis but may subsequently lead to negative feedback regulation of this biologic event. Because killing of CHO cells in the cytolytic assay is cumulative, inclusion of PMA throughout the assay could appear to enhance the cytolysis of CHO cells by amebae despite the initiation of negative feedback regulation or down-regulation of protein kinase C. It is also possible that the phorbol esters may be rapidly and sufficiently metabolized (23, 38) after a brief exposure but not after prolonged exposure of the amebae to the phorbol esters to limit their effects to the promotion of the cytolytic event.

Activation of protein kinase C has been associated with a variety of biologic effects in other cell types such as hormone or enzyme release, changes in the function of ion channels, or alterations in receptor function (7, 22). These effects of protein kinase C are believed to be mediated via the phosphorylation of one or more protein substrates. The exact mechanism by which protein kinase C is involved in the basal or enhanced cytolysis of target cells by amebic trophozoites remains unclear. Prior studies have indicated that the surface of *E. histolytica* plays an important role in the contact-dependent lysis of mammalian cells (26, 28, 29). We hypothesize that this galactose- or *N*-acetyl-D-galactosamine-inhibitable lectin-mediated, contact-dependent event may stimulate phosphatidylinositol turnover releasing inositol-1,4,5-trisphosphate and diacylglycerol. Phosphatidylinositol, inositol-1,4,5-trisphosphate, and diacylglycerol have been detected in amebae (1; J. I. Ravdin and C. F. Murphy, unpublished observations), consistent with this hypothesis. The release of diacylglycerol will directly activate protein kinase C and may also enhance the activity of the amebic phospholipase A, leading to the release of arachidonic acid (6). Arachidonic acid and its metabolites have also been shown to activate protein kinase C (11, 18, 20). Previous studies have demonstrated that amebic phospholipase A activity is required for cytolysis of target cells by *E. histolytica* (14, 31). All pharmacologic antagonists of amebic phospholipase A enzyme activity studied inhibited killing of target CHO cells by amebae. CHO cells were also directly lysed by low concentrations of lysophosphatidylcholine, which is released from phosphatidylcholine by phospholi-

pase A enzymes. Release of intracellular calcium by inositol-1,4,5-trisphosphate in the amebae would increase the activity of the calcium-dependent amebic phospholipase A enzyme, which is membrane bound, generating cytotoxic lysocompounds. In addition, release of intracellular calcium should augment the calcium-dependent delivery of intracellular acidic pH vesicles (14, 31, 32a, 33) containing the calcium-independent form of the amebic phospholipase A or an amebic ionophore protein (15, 41) to the cell surface, again possibly contributing to lysis of the target cell membrane. Further studies are necessary to delineate the exact sequence of events which occurs after amebic contact with the target cell and the subsequent cytolytic event.

Two lines of evidence presented in this report suggest that an *E. histolytica* protein kinase C participates in the initiation of the parasite's cytolytic event: first, the cytolytic capacity of the amebae was inhibited by sphingosine, a specific inhibitor of protein kinase C; second, the cytolytic capacity of the amebae was specifically enhanced by the phorbol esters, potent activators of protein kinase C. The potential importance of protein kinase C to the virulence of *E. histolytica* is further supported by our observations with less virulent amebic strains or avirulent clones. Most striking was the absolute inability of PMA to enhance cytolysis in avirulent clones of the HM1 amebae. Future studies will be needed to definitively demonstrate an *E. histolytica* protein kinase C and the phosphorylation of specific parasite proteins by this enzyme. The effects of phorbol esters on *E. histolytica* cytolytic activity suggest that protein kinase C substrates have a role in this event and thus contribute to the pathogenesis of invasive amebiasis.

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