ELSEVIER

Archives of Medical Research 33 (2002) 389–397

Archives of Medical Research

ORIGINAL ARTICLE

# Signal Transduction in *Entamoeba histolytica* Induced By Interaction with Fibronectin: Presence and Activation of Phosphokinase A and Its Possible Relation to Invasiveness

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Received for publication December 6, 2001; accepted December 6, 2001 (01/221).

*Background.* Interaction of *Entamoeba histolytica* trophozoites with extracellular matrix (ECM) proteins activates signaling pathways through G-protein-coupled receptors. Increments of adenylyl cyclase activity and cAMP produce a striking reorganization of actin into structures that apparently facilitate adhesive, locomotive, and secretory activities. The reorganization of actin is induced by phosphorylation of actin-associated proteins by diverse kinases activated during the signaling process. Although cAMP-dependent kinases have not yet been identified in this parasite, the activation of the adenylyl cyclase route and its effects on particular motility-related functions strongly suggest their presence.

*Methods.* Phosphokinase A (PKA) was detected by phosphorylation of the specific substrate, kemptide, its further activation by cAMP, and its inhibition by H89. The catalytic subunit of the enzyme was identified by immunofluorescence microscopy and by immunoprecipitation. Adhesion and damage to cultured cells were monitored by FN-binding and cytotoxicity assays.

*Results.* A cAMP-dependent kinase activated by effectors and agonists of adenylyl cyclase and also during interaction of trophozoites with fibronectin (FN) was found. The enzyme is associated with small granules in the cytoplasm and upon activation, a fraction of its catalytic subunit with an Mr of 100 kDa was translocated to the nucleus, while another fraction was aggregated into big clusters. Activity and translocation were blocked by H89, a specific inhibitor of PKA. Trophozoites stimulated by dBcAMP or forskolin-formed lamellae and restructured actin, but no significant increase in their adhesion to FN was observed and only showed 10% stimulus in their capacity to damage target cells. Treatment with H89 decreased adhesion to 40% and caused 80% inhibition in cell damage. These amebas showed altered organization of the actin structures induced by dBcAMP or FN.

*Conclusions.* Our results support previous suggestions concerning the participation of PKA in the response elicited by the interaction of *E. histolytica* trophozoites with ECM proteins. They also indicate that adhesion and secretion in conjunction with motile activities are related to invasion processes. 

*Key Words:* Adhesion, cAMP-dependent kinase activity, Cytotoxicity, *Entamoeba histolytica*, Fibronectin, PKA, Signaling.

## Introduction

Invasion of the human host intestinal mucosa and other tissues by trophozoites of the parasite *Entamoeba histolytica* is triggered by yet unknown factors. However, there is accumulating evidence indicating that external stimuli activate signal transduction pathways that trigger some processes associated with invasion and destruction of cells and tissues. Adhesion and protease secretion, as well as locomotion, are known to be stimulated when amebas are in contact with extracellular matrix (ECM) protein substrates or cultured cell monolayers (1–4).

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0188-4409/02 $–see front matter. 
PII S0188-4409(02)00368-5

Two signaling routes have been found to operate in *E. histolytica* trophozoites: the phosphoinositide and the adenylyl cyclase pathways, although not all their components have yet been characterized (5,6). As in other eukaryotes, it was found that activation of these pathways in amebas regulates the reorganization of the actin cytoskeleton through combined effects at several levels in the cell and cross-talk between these pathways (for review, see Reference 7). Several kinase activities related to signal transduction have been reported in this parasite; these activities are or could be associated with secretion and actin organization (5,8–10). As actin organization plays an important role in the regulation of motility-related functions, many of which are relevant for the manifestation of the invasive phenotype, signal transduction processes in *E. histolytica* could be determinant in the interaction with the host. Previous experiments in our group demonstrated the presence of adenylyl cyclase activity coupled to $G_s$ and $G_I$-like proteins that is stimulated after trophozoite-fibronectin (FN) interaction, suggesting the participation of phosphokinase A (PKA)-dependent phosphorylations in signal transduction mechanisms (6). The recent demonstration of cAMP-responsive elements in the amebic actin gene promoter region, which can up-regulate actin mRNA expression, provided additional support to the possibility that PKA phosphorylation of transcription factors regulates the expression of actin (11). Actin levels and actin interactions with PKA or other kinase-phosphorylated proteins could then modulate actin structuration triggered by external stimuli and necessary for motility and secretion.

In this work, we show that *E. histolytica* trophozoites have PKA activity associated with cytoplasmic granules and membranes. Upon stimulation by FN or agonists of PKA, the catalytic subunit of the enzyme, identified as a molecule of 100 kDa, associates with vesicles and granules forming over time big clusters, while another fraction is translocated to the nucleus. PKA activation induces higher levels of phosphorylation, particularly of proteins in the range of 68–200 kDa but without significant difference in adhesion or cell damage. However, inhibition of PKA activity blocks the translocation of the catalytic subunit and has a clear deleterious effect on the dynamics of actin reorganization, rendering amebas defective in adhesion, locomotion, and especially in cell damage.

## Materials and Methods

*Materials.* All reagents unless otherwise indicated were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA). Forskolin (FK) and N-[2-(p-bromocyanamylamino)ethyl]-5- isoquinolinesulfonamide (H89) were solubilized in dimethyl sulfoxide, and dBcAMP was solubilized in $H_2O$. They were diluted in culture medium to final concentrations of 100 μM, 30 μM, and 5 mM, respectively. The concentrations of dBcAMP and FK were determined as optimal concentrations to induce reorganization of actin and actin gene expression in previous work (12). The $Ce_{50}$ for H89 was established from dose-response curves using concentrations reported in the literature for other cells (13).

*Cells.* Trophozoites of *E. histolytica* strain HM1:IMSS were grown in TY1-33 medium (14) with 10% heat-inactivated bovine serum. Cells were harvested in the logarithmic phase of growth by gentle agitation of the culture bottles and centrifugation at 250 *g*. Pellets were washed with 150 mM NaCl, 50 mM Tris-HCl, pH 7.2 (TBS) before resuspension for the experiments outlined subsequently.

*Purification of fibronectin.* FN was purified from human plasma by gelatin-sepharose affinity chromatography and eluted with 4.0 M urea in TBS as previously described (15). Before use, FN was extensively dialyzed against TBS.

*Adhesion to FN-coated wells.* Trophozoites were resuspended in TY1 medium without serum to deposit $3 \times 10^4$ cells per well in 96-well Costar plastic plates (Cambridge, MA, USA). The wells were previously coated with 30 μg/mL of FN, washed and blocked with 1 mg/mL of bovine serum albumin (BSA). The cells were deposited on the wells and incubated for 20 min at 37°C. Nonadhered cells were washed away by rinsing with TBS, also at 37°C. When indicated, trophozoites were preincubated with dBcAMP, FK, or H89 as indicated in the experiments, then resuspended and added to the wells. Cell adhesion values are expressed as relative to the adhesion of control cells, normalized to 100% after subtraction of the values obtained in wells only covered with BSA.

*Damage to cell monolayers.* Damage was measured after interaction of *E. histolytica* trophozoites with confluent MDCK cell monolayers as previously reported (16). As in the case of the adhesion assays previously mentioned, trophozoites were preincubated with the agonists or the inhibitor of PKA before testing for cell damage.

*Viability assays.* Trophozoites were tested for viability before and after adhesion and cell damage using a mixture of 10 mg/mL of fluorescein diacetate and 30 μg/mL propidium iodide in TBS, pH 7.2 (Molecular Probes, Eugene, OR, USA). Under these conditions, viable cells were not stained with propidium iodide. Viability was >95%.

*Phosphokinase A activity.* Trophozoite extracts were prepared by sonication of $2.5 \times 10^6$ cells in 1.0 mL of 50 mM Tris, pH 7.5, 5 mM EDTA buffer containing a cocktail of proteinase inhibitors (Complete, Amersham, Buckinghamshire, UK) and 50 mM NaF, 100 μM $Na_3VO_4$ as phosphatase inhibitors. PKA activity was measured in the extracts by incorporation of [$\gamma$-$^{32}$P] ATP (sp. activity 5,000 mCi/mmol) into kemptide, a small peptide shown to be a specific substrate for PKA (17). The quantities of 100 μM kemptide,

10 μM cAMP, 10 μM ATP, and 300 nM H89 were established as optimal for activation and inhibition of kemptide phosphorylation. Radioactivity incorporated into kemptide was quantified by liquid scintillation after phosphoric acid precipitation of 10-μL aliquots on phosphocellulose disks.

*Electrophoresis and immunotransference.* Trophozoite cell extracts prepared as previously indicated were precipitated with cold acetone (vol/vol). The precipitate was resuspended in TBS, an aliquot separated for protein determination; the remainder was used for polyacrylamide SDS gel electrophoresis. Fifty micrograms of protein was loaded per lane. After electrophoresis, gels were transferred to nitrocellulose as indicated (18) and blocked with PBS-milk (6%). The PKA monoclonal antibody directed against the catalytic subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 1:500 dilution. After extensive washing with PBS–Tween 0.05%, the second antibody coupled to peroxidase was used at 1:5000 dilution. Bands were visualized with the Amersham ECL kit for chemiluminescence.

*Immunofluorescence and immunoprecipitation of the PKA catalytic subunit.* Trophozoites were adhered to glass coverslips coated with 30 μg/mL of FN or 1 mg/mL BSA, without or after incubation with FK, dBcAMP, or H89 as indicated previously. After fixation with 3.7% formaldehyde for 20 min and permeabilization with freezer-cold acetone, they were stained with the monoclonal antibody to PKA at a dilution of 1:50. A second antibody labeled with FITC (Molecular Probes, Eugene, OR, USA) was used at dilution 1:200 in PBS. Actin was visualized using rhodamine-labeled phalloidin (Molecular Probes). Photographs were taken with a standard Zeiss epifluorescence microscope equipped with a 63× apo-fluorescence objective using 400 ASA films from Kodak. Immunoprecipitation of the PKA catalytic subunit was done with the antibody to PKA using reported conditions for amebic cell extracts (10). Immunoprecipitated pellets were resuspended in gel-electrophoresis buffer for electrophoretic separation and immunoblot analysis.

*Gelatin-substrate gels.* After culturing amebas in TYI-medium without serum for 30 min in the absence or presence of FN, FK, dBcAMP, or H89, the supernatant (100 μL) was recovered and centrifuged at 250 *g*, and 10-μL aliquots were analyzed for thiol proteinase activities in 1% gelatin/10% polyacrylamide nondenaturing gels as indicated (16).

In vivo *phosphorylation.* For this assay, 400,000 trophozoites were deposited on 2.5-cm plastic wells previously covered with FN or BSA. After 30 min in DMEM without phosphate and serum, the unbound amebas were discarded. New medium was added to each well containing 0.25 mCi of $^{32}$P-orthophosphate (sp. activity 5 mCi/500 μL). dBcAMP, FK, or H89 was added to the amebas and plated on BSA-covered wells. After 2 h, the cells were washed with the same medium containing 10 mM NaF and 500 μM $Na_3VO_4$. Cell extracts were prepared as previously indicated, electrophoresed, and autoradiographies were obtained exposing dried gels to X-ray film.

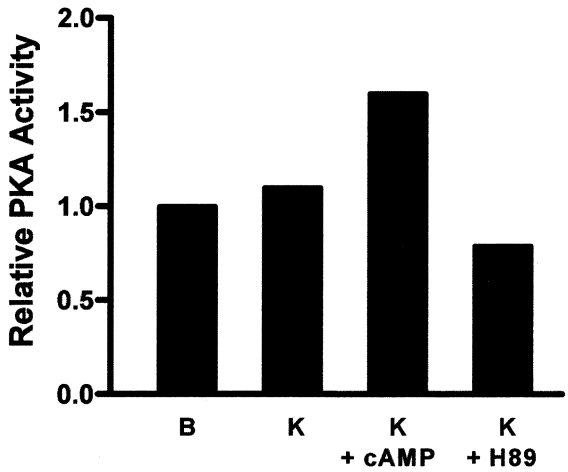


**Figure 1.** PKA activity in cell extracts of *Entamoeba histolytica* trophozoites. Total cell extracts were used as the source of PKA activity measured by phosphorylation of kemptide. (B) Basal activity refers to phosphorylation of proteins in the absence of kemptide and would correspond to all other kinase activities present in the extracts. Addition of 100 μM kemptide (K) and later addition of 10 μM cAMP (K+cAMP) or 300 nM H89 (K+H89) allowed the quantification of PKA activity.

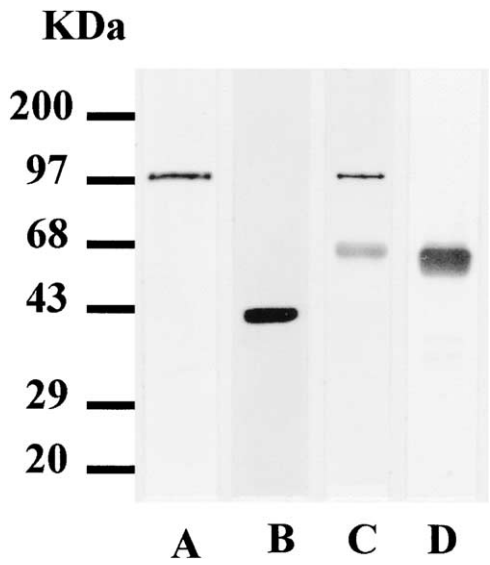


**Figure 2.** Identification of PKA catalytic subunit by immunoblot and immunoprecipitation of cell extracts. SDS-PAGE separated proteins were electrotransferred to Millipore membranes and reacted with the antibody to the catalytic subunit of PKA. *Entamoeba* extract (A), MDCK cell extract (B). Trophozoite cell extracts were immunoprecipitated with the PKA antibody (C) or preimmune serum (D); the pellets were submitted to electrophoresis in 10% SDS-polyacrylamide gels and then transferred to Millipore membranes. Western blots of the immune complexes were done as indicated previously. Molecular weight markers are indicated at left.

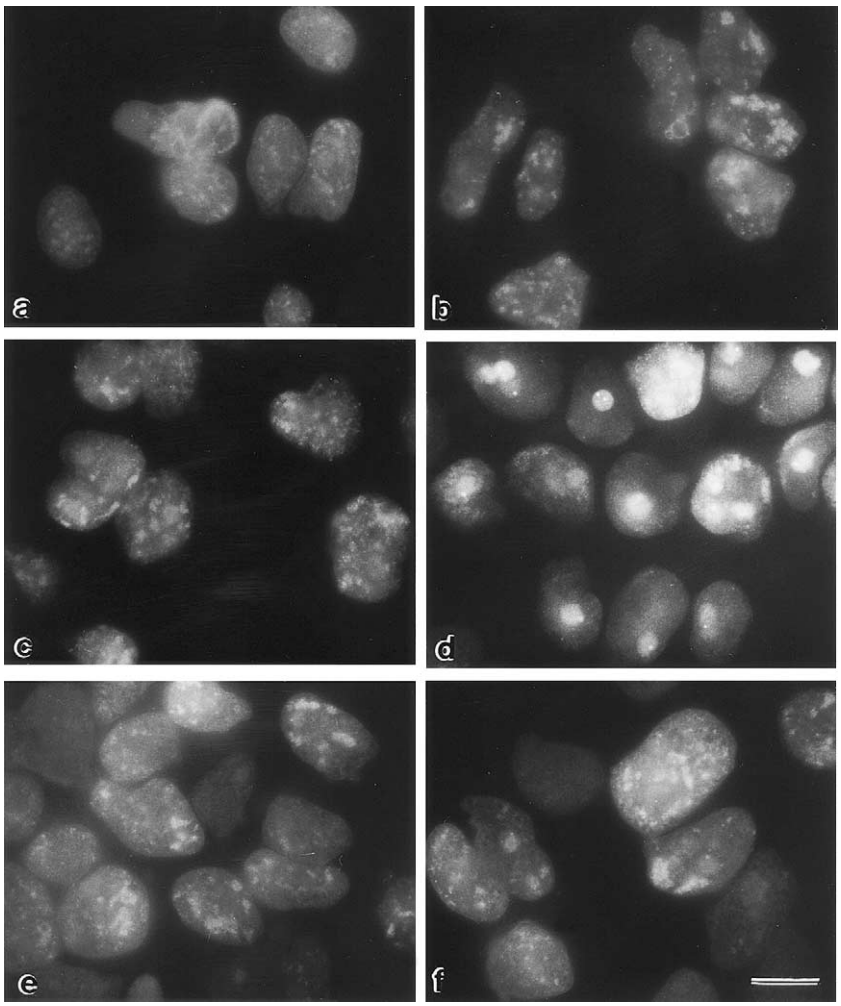


**Figure 3.** Cellular localization of PKA catalytic subunit. Nonstimulated amebas and amebas preincubated with dBcAMP or adhered to FN were fixed and stained with the PKA antibody and then visualized by an FITC-tagged secondary antibody. a: nonstimulated cells; b: cells stimulated with dBcAMP for 30 min; c: cells stimulated with dBcAMP for 1 h. d; cells stimulated with dBcAMP for 4 h; e: cells bound to FN for 30 min, and f: cells bound to FN for 2 h. Bar = 20 μm.

## Results

*Protein kinase A activity in trophozoites.* Enzymatic activity was determined by *in vitro* phosphorylation of the specific PKA substrate kemptide. As seen in Figure 1, basal nonspecific phosphorylation was detected in trophozoite extracts due to the presence of several active kinases (Figure 1B). Specific phosphorylation by PKA was determined after addition of kemptide and the further increment of its phosphorylation by exogenous cAMP added to the assay mixture. The phosphorylation of kemptide without exogenous cAMP (10% above the basal nonspecific phosphorylation value expressed as 1.0 in the figure) indicated that trophozoites have endogenous levels of cAMP that can be used for activation of PKA (Figure 1K). After addition of cAMP (K+cAMP), the 50% increase in phosphorylation attributed to further activation of PKA could be completely inhibited (K+H89) by the highly specific inhibitor H89 that competes with ATP for binding to the enzyme (13). These results indicate that PKA is present in trophozoites and that it can be induced by cAMP.

*Protein kinase A distribution and translocation.* Translocation of the catalytic subunit of PKA is a necessary step in the phosphorylation of the corresponding substrates. Therefore, activation of PKA can be monitored following the movement of the catalytic subunit after activation (19). The apparent conservation of the subunits of PKA among eukaryotes allows the use of polyclonal and monoclonal antibodies that recognize this protein from different sources. Using this advantage, the catalytic subunit of PKA in trophozoite extracts was identified by immunologic procedures. As seen in the immunoblot in Figure 2, a single band of approximately 100 kDa gave a positive reaction to an antibody directed against the catalytic subunit of PKA (Figure 2, lane A). The reaction of the antibody with extracts from MDCK cells was used as positive control in immunoblots

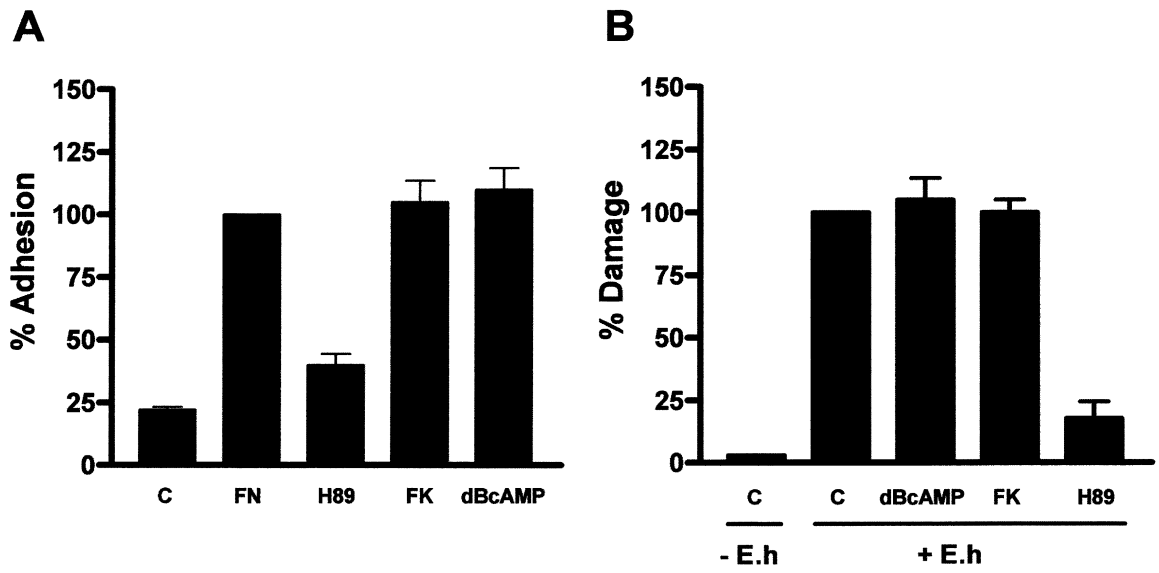


**Figure 4.** Participation of PKA in adhesion to FN and in damage to cultured cells. A: Nonstimulated trophozoites and trophozoites stimulated with dBcAMP, FK, or treated with H89 were deposited on FN-covered plastic wells and allowed to adhere for 20 min. Adhesion to FN in the absence of drugs was normalized to 100%. B: Nonstimulated trophozoites, trophozoites stimulated by dBcAMP, FK, or treated with H89 were added to confluent MDCK monolayers and incubated for 60 min in the presence of the drugs. Wells containing MDCK cells without amebas were used as control for the number of cells plated per well. The values obtained from wells containing nonstimulated amebas were normalized to 100% damage. Results for adhesion represent six separate experiments carried out in octuplicate. Results for cell damage represent two independent experiments done in triplicate; $p < 0.05$ for the two types of experiments.

and immunoprecipitations. The reactive band corresponded to the 41 kDa catalytic subunit of PKA in these cells (Figure 2, lane B). The immunoprecipitate obtained using preimmune serum showed only IgG heavy band (Figure 2, lane D). Immunoprecipitation of cell extracts with the same antibody also showed the band of 100 kDa (Figure 2, lane C). Due to high proteolytic activity of *Entamoeba* extracts, a smaller reactive peptide of approximately 29 kDa was detected in both whole cell and nuclear extracts, particularly after storage of the extracts (data not shown).

Immunostaining of trophozoites with the anti-PKA antibody is depicted in Figure 3. In nonstimulated amebas, the catalytic subunit was localized associated with small granules in the cytoplasm without a particular arrangement and only at several points was the fluorescent label associated with membranes (Figure 3a), indicating that some activated enzyme was present in these cells as also suggested by the basal phosphorylation of kemptide. After 30 min of stimulation with dBcAMP, the label appeared in vacuoles and granular clusters (Figure 3b), and after 1 h the aggregates became bigger and the label began to appear in some nuclei (Figure 3c). After 4 h, the label was clearly localized in the nucleus in a great majority of the cells (Figure 3d). In FN-treated cells, granular aggregates were also seen after 30 min (Figure 3e) and gradual appearance of label in the nuclei was detected after 2 h (Figure 3f). Treatment of trophozoites with FK for periods >2 h also induced translocation of the fluorescent label to the nuclei (data not shown). These results confirmed the presence of PKA in trophozoites and showed a gradual shift of the catalytic subunit in stimulated amebas from small granules to vacuoles and clusters and then to the nucleus.

*PKA participation in adhesion and cell damage.* It has been reported that activation of the adenylyl cyclase route leads to changes in the structure of actin and in the adhesive and motile functions of trophozoites. The possible role of PKA in these processes was analyzed in amebas exposed to activators and inhibitors of PKA prior to being tested for adhesion to FN-coated surfaces or for damage to cultured MDCK cell monolayers. As shown in Figure 4A, FN induced a fivefold stimulation in the adhesion of trophozoites compared to their adhesion to BSA-covered surfaces. Preincubation of trophozoites with dBcAMP or FK produced slight but no significant increases in adhesion to FN. On the other hand, the presence of H89 reduced the adhesion in a concentration-dependent manner in such a way that in amebas preincubated with 30 μM H89 for 3 h adhesion dropped to 40%, indicating that inhibition of PKA activity had a strong negative effect on adhesion to FN. Figure 4B shows the percentage of damage produced to cultured cells by control, dBcAMP, or FK-stimulated amebas and amebas treated with H89. As in the case of adhesion to FN, cAMP and FK induced small nonsignificant increments in cell damage, while H89 inhibited in 80% the destruction of the cells by the trophozoites. These results show that activation of the adenylyl cyclase route and further activation of PKA produce a small increase in adhesion and cell damage; however, direct blockage of the enzyme activity has a very strong effect on both processes.

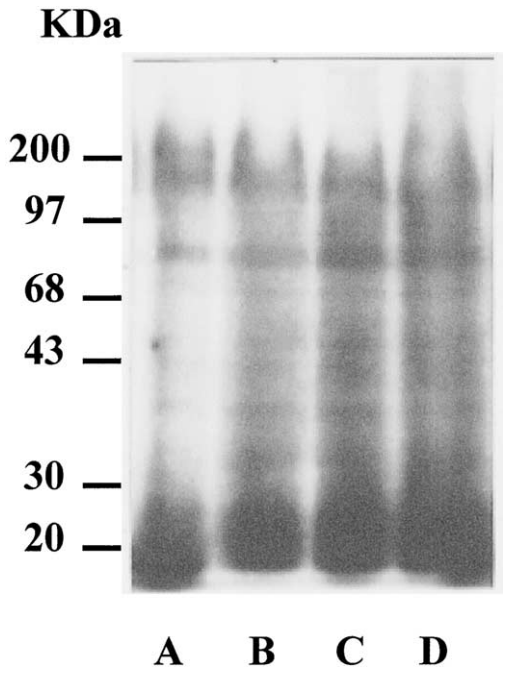


**Figure 5.** Phosphorylation of proteins by activation or inhibition of PKA. Trophozoites were incubated in the presence of $^{32}$P-orthophosphate for 2 h under regular conditions, stimulated by FN, dBcAMP, or inhibited by H89. Total cell extracts were prepared and after electrophoresis, autoradiographs were obtained. Fifty micrograms of protein were loaded per gel. A: control; B: H89; C: dBcAMP and D: FN. Molecular weight markers are indicated at left.

In vivo *phosphorylation.* Phosphorylation due specifically to activation or inhibition of PKA that could explain the effects in adhesion and cell damage was analyzed incubating control and stimulated trophozoites in the presence of $^{32}$P-orthophosphate. As shown in the autoradiographs of Figure 5, stimulation with dBcAMP or FN produced a higher incorporation of label into several proteins, particularly proteins >68 kDa (Figure 5, lanes C and D) when compared with the labeling of proteins in nonstimulated amebas (Figure 5, lane A). However, specific labeling of one or more proteins was not detected. In amebas incubated with H89 (B), incorporation of label was slightly lower than in stimulated amebas. Again a particular effect on particular proteins was not observed. Although these results showed an inductive effect in protein phosphorylation by PKA agonists as well as inhibition by H89, they did not allow identification of PKA substrates. As background protein phosphorylation seems to be high in trophozoites, if PKA substrates are present in low concentrations, than their phosphorylation may not be distinguished above the background level in this type of experiment. Antibodies to known PKA-phosphorylated proteins would be necessary to identify these substrates, as carried out for the focal adhesion protein paxillin in adrenal cortex-derived Y1 cells (20).

*Effect of H89 on actin organization and release of thiol proteinases.* The previously mentioned experiments suggested that inhibition of PKA activity could lead to defects in functions related to adhesion and proteinase secretion. To test this possibility, actin and the catalytic subunit of PKA were visualized in amebas stimulated by FN in the absence or presence of H89. Figure 6 shows actin plates, lamellae, and other actin structures formed in amebas adhered to FN substrates or stimulated by dBcAMP for 2 h (Figures 6a and b, respectively). In cells bound to FN and double-stained with the anti-PKA antibody and rhodamine phalloidin, adhesion plates showed a typical organization (Figure 6c, arrowhead) and the catalytic subunit of PKA was localized in cytoplasmic clusters and in the nucleus of some cells (Figure 6d). In contrast, in amebas bound to FN in the presence of H89, actin adhesion plates and lamellae were poorly organized, and the PKA subunit remained associated with membranous material in the cytoplasm (Figures 6e and f), as if PKA was not being fully activated. These alterations, however, could not explain the strong inhibitory effect of H89 in adhesion, particularly in cell damage.

It has been reported that inhibition of PKA results in blockage of cellular exocytic transport (21). As cysteine proteinases released by trophozoites are thought to be at least partially responsible for cell damage (16,22,23), we tested the secretory activity of amebas analyzing, by gelatin zymograms, the cysteine proteinases released into the culture medium. As shown in Figure 7, culture medium of control amebas (Figure 7, lane A) contained two main cysteine proteinase activities that corresponded to approximately 56 and 37 kDa, and FN-, dBcAMP-, or FK-stimulated amebas (Figure 7, lanes B, C, and D) released to the medium the same two proteinases in slightly higher concentration. Contrary to what we expected, amebas exposed to H89 released higher amounts of the same cysteine proteinases (Figure 7, lane E). Therefore, PKA inhibition by H89 has no deleterious effect on the release of cysteine proteinases, at least during the times analyzed. These results suggest that the presence of cysteine proteinases in the medium is not sufficient to cause severe cell damage. Other factors required, for example, for adhesion and actin structure may also be necessary.

## Discussion

Trophozoites of *E. histolytica* interact and destroy ECM components while moving across tissues of the human host (24). Experiments *in vitro* have shown that amebas bind to ECM through surface receptor proteins and activate signal transduction processes (25–27). The cascade of events triggered by the interaction elicits structural (rearrangement of the actin cytoskeleton), biochemical (phosphorylation of proteins, release of proteinases), and gene expression (up-regulation of actin mRNA) changes already described in higher eukaryotes as a result of cell responses to external signaling (28). PKA activity has been identified as a main participant in phosphorylation cascades and activation of transcription factors (29–31). In *E. histolytica,* adenylyl cy-

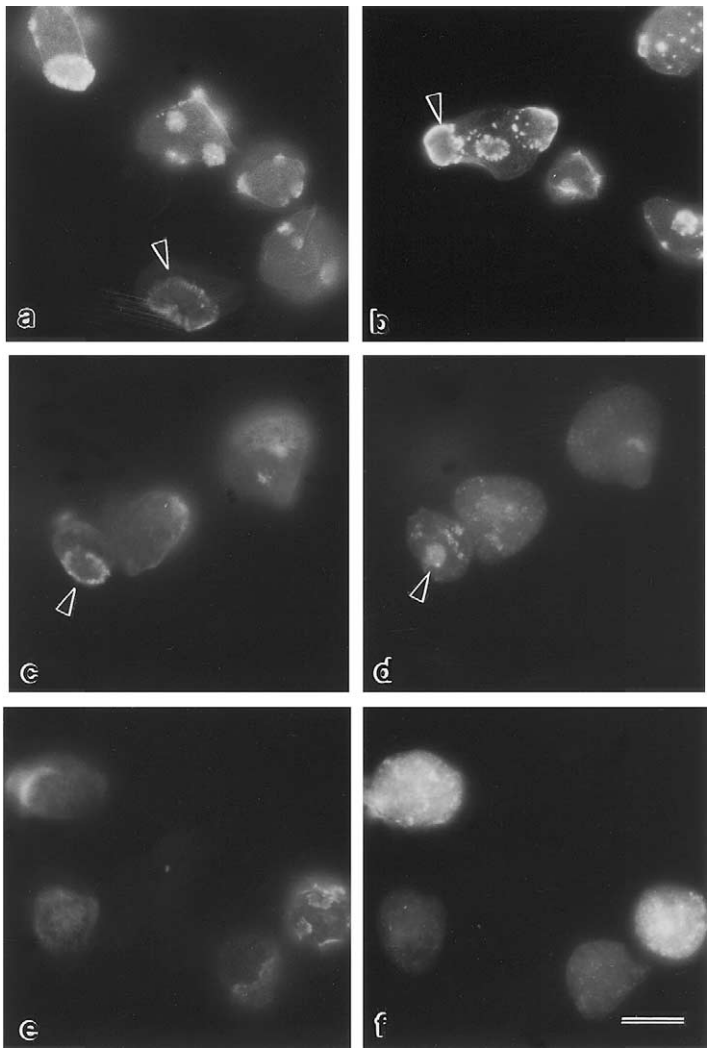


**Figure 6.** Specific effects of H89 on actin structuration; a and b show the actin structures that are induced by the interaction with FN or stimulated by dBcAMP, respectively. Trophozoites bound to FN and double-stained with αPKA and rhodamine phalloidin are shown in Figures c–f. (c) shows an actin adhesion plate (arrowhead) and in the same cell, shown in (d) PKA labeling is seen in the nucleus (also indicated by an arrowhead) and in cytoplasmic clusters. Trophozoites bound to FN after incubation for 60 min with H89 show an irregular, altered structuration of adhesion plates (e) and irregular distribution of the PKA label or association with membranous material (f). Bar = 20 μm.

clase activation coupled to G-proteins could provide the necessary increments of cAMP required to activate phosphorylation of proteins by PKA (6). It has also been shown that in this parasite, cAMP can induce the expression of actin mRNA by activation of CRE-like elements in the actin gene promoter that respond to binding of PKA-phosphorylated transcription factors (11).

Previous work by our group established that increments in cAMP levels by either activation of the adenylyl cyclase or preincubation of the amebas with membrane-permeable dBcAMP induce striking rearrangements of the actin cytoskeleton into aggregates, lamellae, and plates similar to those observed when the amebas interacted with FN (12). These observations, which suggested a functional adenylyl cyclase route in *E. histolytica* trophozoites coupled to actin dynamics, led us to search for the presence of cAMP-dependent kinases to complement the current signal scheme established for this parasite (7).

Addition of a highly specific substrate for PKA to trophozoite extracts under conditions that favored the enzyme's affinity for ATP resulted in its phosphorylation, indicating the presence of a basal PKA activity that could be increased by addition of exogenous cAMP. The induced activity could be reversed using the specific inhibitor H89 that competes with ATP in binding to the enzyme. The presence of PKA was corroborated using a monoclonal antibody that recognizes the catalytic subunit of this enzyme in several eukaryotic cells. A single protein band of 100 kDa was identified by immunoblot and immunoprecipitation of amebic extracts as corresponding to the catalytic subunit of PKA in *E.*

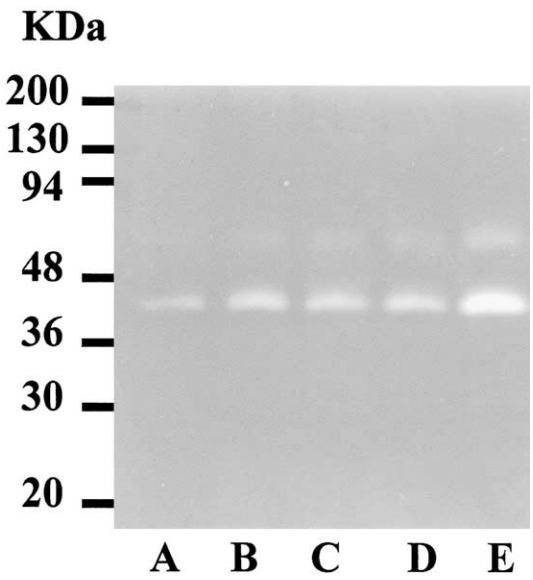


**Figure 7.** Zymograms of culture medium from nonstimulated trophozoites (A), and from trophozoites stimulated with FN (B), FK (C), dBcAMP (D), or treated with H89 (E). Ten-microliter aliquots were loaded per lane. Molecular weight markers are indicated at left.

*histolytica*. In other cells, the catalytic subunit has a lower molecular weight but in lower eukaryotes, the regulatory and the catalytic subunits show special features (32). We found that *E. histolytica* PKA activity could not be competed for by PKI, a small peptide utilized as a pseudosubstrate (17). This indicates a different affinity of the amebic PKA for this substrate and partially explains the specific conditions needed for the phosphorylation of kemptide in the extracts. Similar results have been reported for PKA from *Giardia* (33). The catalytic subunit of the amebic PKA was localized in the cytoplasm associated with small granules and membranous material; however, after exposure to either FN, dBcAMP, or FK the fluorescent label became associated with granular-vesicular aggregates and with time was translocated to the nuclei in many cells, indicating its movement to the sites of active phosphorylation (19).

PKA-dependent phosphorylation of actin and proteins involved in actin organization has been reported (20,34,35). Furthermore, PKA phosphorylation of transcription factors involved in actin expression is well documented (31,36). Interaction of amebas with FN induces activation of PKA through the adenylyl cyclase signaling pathway, but other signaling paths are known to be activated after interaction with FN and there is cross-talk between them. Although at present we cannot identify specifically PKA-phosphorylated proteins, when the amebas were treated with the specific inhibitor of PKA, H89; there was a strong inhibition of binding to FN substrates or damage to cell monolayers, indicating that adhesion, locomotion, and possibly secretion of specific molecules were impaired. These amebas, when analyzed for actin adhesion plates and lamellae formed by the activation of signaling routes, showed poorly organized actin structures and no apparent translocation of the PKA catalytic subunit after 2-h stimulation with FN.

The present data show there is PKA activity in *E. histolytica* trophozoites that can be further stimulated by interaction with FN. Over time, activation of PKA causes the translocation of the catalytic subunit of the enzyme to the nucleus. In other cellular systems, it is known that PKA phosphorylates transcription factors such as CREBs are involved in actin gene expression (30). PKA-phosphorylated proteins also participate in the organization, stability, and function of actin filaments (34,35,37,38) and in exocytic transport (21). Therefore, during activation of the adenylyl cyclase pathway PKA phosphorylation-dephosphorylation cycles could favor dynamic changes of actin-containing organelles and actin functions required for motile functions. The activation of the phosphoinositide-signaling route in amebas has been shown to promote the formation of adhesive structures important in adhesion and degradation of FN substrates. We show now that activation of PKA is necessary for the structuring of actin. The formed structures could be related to adhesion, secretion, and locomotion and in this way complement the effect on actin organization due to activation of the phosphoinositide pathway, as observed in other eukaryotic cells (38). The results presented here corroborate our previous data, suggesting the presence of adenylyl cyclase effectors in *E. histolytica*, and provide additional evidence for the operation of signal transduction mechanisms in this parasite in close relationship with invasive behavior.

## Acknowledgments

We thank A. Trejo for maintenance of *E. histolytica* cultures and photographic work, M.C. Domínguez and C. Márquez for technical assistance, and D. Moreno for help in preparing the manuscript. This work was partially supported by grant 34981-N from CONACYT (México).

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