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Fibronectin-Induced Intracellular Calcium Rise in *Entamoeba histolytica* Trophozoites: Effect on Adhesion and the Actin Cytoskeleton

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CARBAJAL, M. E., MANNING-CELA, R., PIÑA, A., FRANCO, E., AND MEZA, I. 1996. Fibronectin-induced intracellular calcium rise in *Entamoeba histolytica* trophozoites: Effect on adhesion and the actin cytoskeleton. *Experimental Parasitology* **82,** 11–20. The interaction of *Entamoeba histolytica* trophozoites with fibronectin (FN) promotes adhesion of the protein to the cells and its later degradation by locally released proteases. Binding to FN-covered surfaces induces, in addition, the formation of actin adhesion plates and focal contacts in the amebas. The signaling mechanisms underlying the response to FN are incompletely understood. In this paper we examined the modifications of cytosolic free calcium ($[Ca^{2+}]_i$) induced in the trophozoites by the interaction with FN and their effect on adhesion and the actin cytoskeleton organization. FN produced a sustained rise of $[Ca^{2+}]_i$ that could be correlated to the incremented adhesion to FN-covered surfaces. Further increments in $[Ca^{2+}]_i$ produced by Ca^{2+} ionophores A23187 or ionomycin significantly increased the adhesion of trophozoites, whereas depletion of cytoplasmic Ca^{2+}, by treatment with the ionophores in the absence of external Ca^{2+} or using the chelator BAPTA/AM, blocked it almost completely. To study the role of internal calcium we used the plant lactone thapsigargin, which was found to produce a transient increase of $[Ca^{2+}]_i$ but a low stimulatory effect on adhesion and the organization of actin plates. The shifting of soluble actin to the F-actin form and the stabilization of adhesion plates and focal contacts, seen as results, of the FN stimulus, were positively influenced by rises in $[Ca^{2+}]_i$ and negatively affected by its decrement. Additional evidence for Ca^{2+}-mediated signaling in the response to FN was provided by the poor adhesion and defective actin plate organization observed in trophozoites treated with calmodulin antagonists. The results presented here suggest that FN action is mainly dependent on the influx of external Ca^{2+}.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Entamoeba histolytica;* fibronectin; cytosolic free calcium; adhesion; cytoskeleton; FN, fibronectin; IP3, inositol triphosphate; PKC, protein kinase C; $[Ca^{2+}]_i$, cytosolic free calcium; ECM, extracellular matrix components; CaM, calmodulin; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N′*,*N′*-tetraacetic acid; TG, thapsigargin; TFP, trifluoropiperazine; W7, *N*-6-aminohexyl-5-chloro-naphtalensulfonamide; BSA, bovine serum albumin.

INTRODUCTION

Adhesion of invasive cells to extracellular matrix (ECM) is a first step in the invasion process. The interaction is mediated by cell surface receptors and triggers signals such as the rearrangement of the cytoskeleton to stabilize the interaction and the secretion of proteases to degrade ECM components, thus allowing the cells to deadhere and migrate (reviewed by Aznavoorian and Murphy 1992). Studies *in vitro* have demonstrated that FN, a key ECM component, supports the attachment, spreading, and migration of normal (Carnegie and Cabaca 1991) and transformed cells (Aplin *et al.* 1992), as well as the exocytosis of proteases (Jones and De Clerck 1980; Xie *et al.* 1994). Changes in the cytoskeleton organization, in particular the actin cortical network, play a crucial role in both adhesion to and proteolysis of ECM components. Actin-filament arrays underlie focal contacts that not only maintain the cells adhered but also function as sites of transmembrane communication (Burridge *et al.* 1988; Stamatoglou and Hughes 1992; Lo and Chen 1994). Mounting evidence indicates that transient increases of intracellular Ca^{2+} and activation of several kinases and Ca^{2+}-binding proteins, which in turn modify the actin cytoskeleton and cell motility, are major events triggered by cell

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binding to ECM proteins (Smith *et al.* 1991; Gimound and Aumailley 1992; Schwartz 1993; Bengtsson *et al.* 1993).

Entamoeba histolytica, an invasive parasite of humans, causes severe damage to the host tissues. Preferential recognition and degradation of ECM proteins by the amebic form or trophozoite has been shown to occur *in vivo* and *in vitro* (Pérez-Tamayo *et al.* 1990; Muñoz *et al.* 1984; Meza and Franco 1988; Talamás-Rohana and Meza 1988; Rosales-Encina *et al.* 1992). Interaction with either soluble FN or FN-covered surfaces is apparently mediated by trophozoite surface peptides with affinity for this protein, some of which have homology with β integrins (Vázquez-Prado and Meza 1992; Talamás-Rohana *et al.* 1992). Recent characterization of the actin adhesion plates formed by the interaction with FN-covered surfaces indicated that these sites of contact contain several elements of a transduction organelle. Besides actin and actin-binding proteins — some of which bind Ca^{2+} and are required to locally organize the cortical cytoskeleton — Ca^{2+}-activated kinases were found to be associated to the plates (Vázquez *et al.* 1995). Studies on the influence of phorbol esters in amebic toxicity (Weikel *et al.* 1988; Ravdin *et al.* 1988), and recent reports on PKC translocation and IP_3 production during the parasite binding to FN (Santiago *et al.* 1994), have provided indicia for the possible participation of calcium in the parasite responses induced by FN. In this work, we present data on the Ca^{2+} homeostasis in *E. histolytica* trophozoites and evidence indicating that the interaction of amebas with FN induces elevation of $[Ca^{2+}]_i$, which in turn could facilitate adhesion and the formation and function of actin plates.

Materials and Methods

Cells. Trophozoites of *E. histolytica*, strain HM1:IMSS, were grown in TYI-33 medium (Diamond *et al.* 1978) with 15% heat-inactivated bovine serum. Cells were harvested in logarithmic phase of growth by gentle agitation of the culture bottles and centrifugation at 250*g*. Pellets were washed with 150 m*M* NaCl, 50 m*M* Tris–HCL, pH 7.2, prepared in glass-distilled, chellex-treated H_2O (TBS) before resuspension for the experiments outlined below.

Purification of FN. FN was purified from human plasma by gelatin–Sepharose affinity chromatography as previously described (Rouslahti *et al.* 1982). Briefly, human plasma was applied to the affinity column and washed extensively with TBS. After an additional wash with 1.0 *M* urea in TBS, FN was eluted with 4.0 *M* urea in TBS and stored at −70°C. Before use FN was extensively dialyzed against TBS.

FN adhesion assay. For the adhesion assays, 96-well plates (Costar Corp., Cambridge, MA) were coated overnight at 4°C with 30 μg/ml of FN in TBS containing 4 *M* urea and 100 m*M* $NaHCO_3$. The plates were washed with TBS to eliminate the urea and then blocked with 1 mg/ml BSA for 2 hr at room temperature. Trophozoites were tested for adhesion in TBS containing 1.0 m/*M*$CaCl_2$ and 0.1% BSA or in TBS/0.1% BSA containing 5 m*M* EGTA. The adhesion experiments were followed for 10 min using 3×10^4 cells/well, in both the presence or the absence of the tested drugs. Specific adhesion is expressed in Tables I and II, as the percentage of adhered cells in relation to the adhesion of untreated control cells. Absolute percentage adhesion was 80% and it was normalized to 100%. Background binding of cells to uncoated control wells (typically < 20% of the binding shown to FN-coated plates) was substracted from the values obtained in the different experiments. Each experiment was made at least in octuplicate. Tests of significance were performed by paired Student *t* tests.

Inhibitors. Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Calmodulin antagonists, TFP, W7, and calmidazolium (compound R24571) were tested at different concentrations in the adhesion experiments and later used at 2.5, 20, and 5 μM, respectively. Calcium ionophores A23187 and ionomycin were used at 1 μM concentrations. Internal Ca^{2+} chelator BAPTA/AM was used at 50 μM. TG was used at 50 n*M*. Stock solutions were prepared in dimethyl sulfoxide and from there diluted with TBS.

Intracellular Ca^{2+} measurements. Resuspended trophozoites (10^6) were washed twice with TBS (passed through chellex 100 columns) and loaded with 20 μM FURA 2/AM and 0.2% pluronic F-127 (Molecular Probes, Eugene, OR) at room temperature in the dark for 1hr in 140 m*M* NaCl, 1.0 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* KCl, 10 m*M* glucose, 10 m*M* Hepes, pH 7.2, supplemented with 0.1% BSA. Loaded cells were washed twice with chellex-treated TBS and resuspended in buffer A (20 m*M* Hepes, pH 7.2, 140 m*M* NaCl, 1.0 m*M* $CaCl_2$) or buffer A without $CaCl_2$. Cells (5×10^6) were transferred to a thermostated fluorometric cuvette containing a magnetic stir bar and maintained at 37°C with gentle agitation. FN, EGTA, and the different drugs were added in the cuvette. Fluorescence values were registered in a Perkin-Elmer LS-50B luminescence spectrometer, programmed to obtain the 340/380 nm ratio. $[Ca^{2+}]_i$ was calculated after substraction of the autofluorescence and corresponding calibration measurements, as indicated by Grynkiewicz *et al.* (1985).

Quantification of F-actin. Control and drug-treated trophozoites (10^5) were stained for 20 min at 37°C with 3.3

μM nitrobenzoxadiazole–phallacidin, 1% BSA (NBD phallacidin, Molecular Probes) after fixation with 3.7% glutaraldehyde–1% BSA for 15 min at room temperature and permeabilization with 1% Triton X-100 containing 1% BSA. The bound phallacidin was extracted in 0.40 ml of methanol. Relative fluorescence of the methanol extracts was measured in a fluorescence spectrophotometer at an excitation wavelength of 465 nm and emission wavelength of 535 nm. Results are expressed as relative F-actin content, which was determined by the relative fluorescence intensity (RFI), where RFI = fluorescence intensity of sample/fluorescence intensity of control using 10^5 cells.

Immunofluorescence. Trophozoites adhered to glass coverslips coated with 30 μg/ml of FN were fixed with 3.7% formaldehyde for 10 min after the different treatments, rinsed with PBS, permeabilized with freezer-cold acetone, and stained with rhodamine-labeled phalloidin (Molecular Probes) following the instructions of the manufacturer. Photographs were taken in a standard Zeiss fluorescence microscope using Tri-X film from Kodak.

RESULTS

Effects of FN on $[Ca^{2+}]_i$

To understand the nature of the FN-induced intracellular signaling, we investigated the effect of FN on cytosolic free calcium concentration in trophozoites. As shown in Fig. 1a, $[Ca^{2+}]_i$ raised from basal conditions corresponding to 175 ± 10 to 427 ± 20 n*M* with FN concentrations as low as 5 μg/ml, reaching a plateau at 30 μg/ml. Higher concentrations did not significantly change $[Ca^{2+}]_i$ up to 80 μg/ml, after which it started to decrease gradually. As shown in Fig. 1b, FN at 5 μg/ml also induced a sharp increase in adhesion to covered surfaces. The response reached a plateau at 30 μg/ml and remained with little variation up to 100 μg/ml. The increase of $[Ca^{2+}]_i$ induced by FN could be due to either extracellular Ca^{2+} influx through the plasma membrane or Ca^{2+} release from intracellular stores. To address this issue, we used drugs known to function as regulators of cytoplasmic Ca^{2+} in several eukaryotic cells and monitored the changes in FURA/AM loaded trophozoites. As shown in Fig. 2a, addition of 30 μg/ml of FN to trophozoites produced a sustained increase in $[Ca^{2+}]_i$ from 160 to 450 n*M* that lasted for several minutes. Further addition of ionomycin to trophozoites maintained in the presence of 1.0 m*M* external Ca^{2+} rapidly increased $[Ca^{2+}]_i$ to 1500 n*M*, indicating the operation of ionophore-induced Ca^{2+} channels. In order to explore the role of extracellular Ca^{2+} in the modulation of $[Ca^{2+}]_i$, we tested the effect of FN in cells maintained in the absence of external Ca^{2+}. In these cells the increase in $[Ca^{2+}]_i$

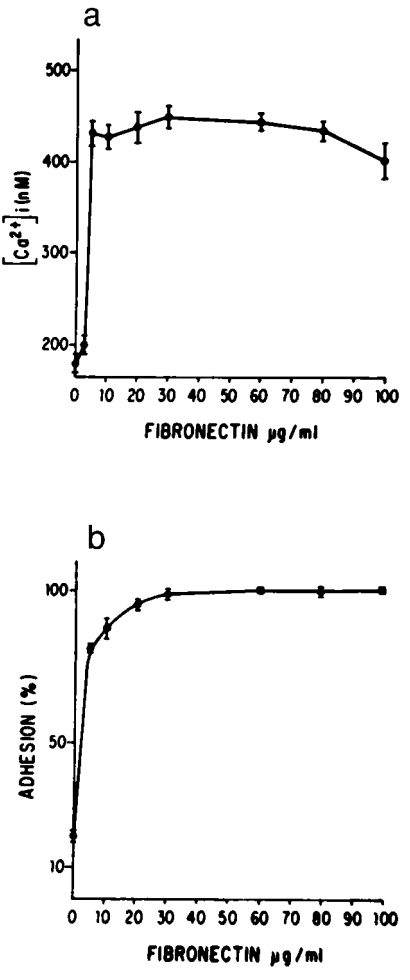

FIG. 1. FN-induced rise in adhesion and $[Ca^{2+}]_i$. (a) Basal levels of cytoplasmic Ca^{2+} were measured in FURA 2/AM loaded trophozoites. Increasing concentrations of FN (0 to 100 μg/ml) were tested and the modifications in $[Ca^{2+}]_i$ registered. Values taken for each point in the graph correspond to 10 min after the addition of FN. Results represent four separate experiments. (b) Adhesion was assayed on FN-coated wells using increasing concentrations of the protein (0 to 100 μg/ml) in the presence of 0.1% BSA. Adhesion values were taken at the maximum adhesion time (10 min). Results represent three separate experiments done by octuplicate.

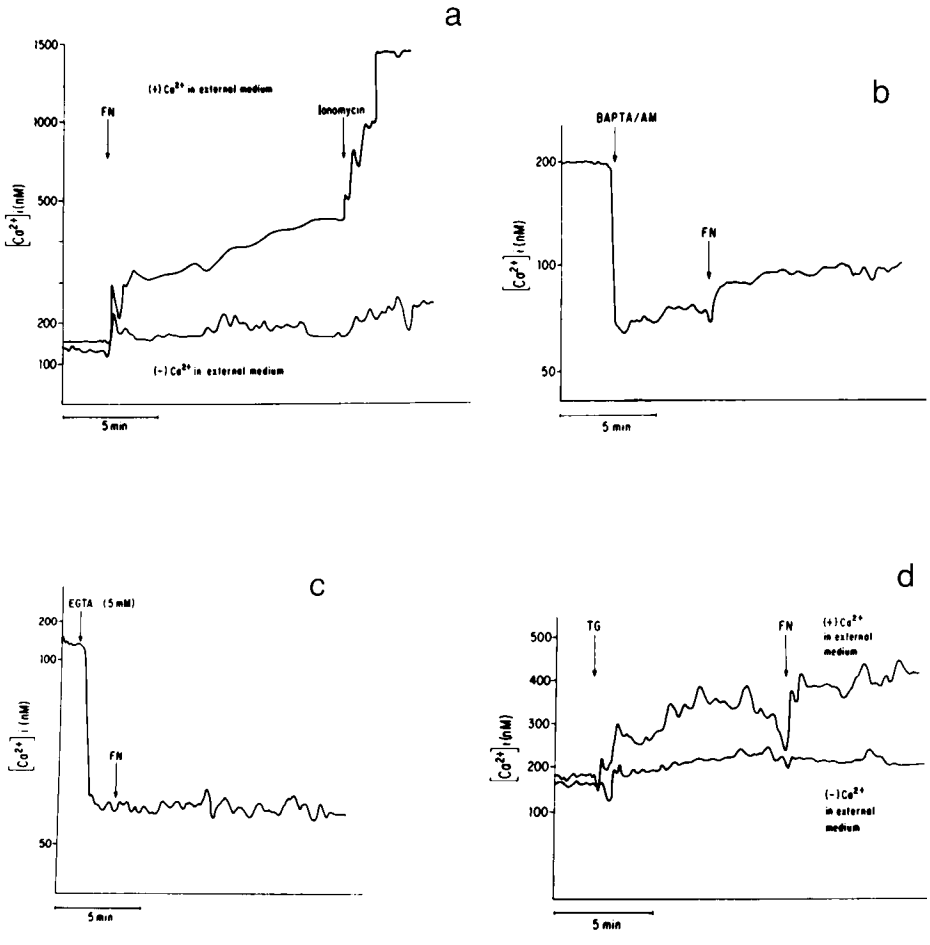

FIG. 2. $[Ca^{2+}]_i$ responses in trophozoites induced by FN. Cells were loaded with FURA 2/AM and FN (final concentration of 30 μg/ml) or the corresponding drugs were added at the times indicated in the graphs. (a) Plots of responses induced by FN in the presence or absence of external Ca^{2+}. Ionomycin (1 μM) was added only to cells maintained in the presence of 1.0 m*M* external Ca^{2+}. (b) $[Ca^{2+}]_i$ elevation induced by FN addition was not significant in the presence of BAPTA/AM. (c) $[Ca^{2+}]_i$ elevation induced by FN addition is blocked in cells treated with EGTA. (d) TG-induced increase in $[Ca^{2+}]_i$ is transient. Addition of FN after 10 min produced a further increase in the presence of external Ca^{2+}. Neither TG nor FN produced a significant increase in $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. Curves are representative of three or more separate experiments.

was not observed (Fig. 2a). Moreover, when we tested the role of intracellular Ca^{2+} in the FN-induced calcium increase, we found that FN addition to cells previously treated with the Ca^{2+} chelator BAPTA/AM—which produced a very rapid drop of basal $[Ca^{2+}]_i$ to a new basal value of about 70 n*M*—had a negligible effect with $[Ca^{2+}]_i$ remaining around 100 n*M* (Fig. 2b). Treatment of trophozoites with 5 m*M* EGTA produced similar effects to those seen with BAPTA/AM rendering cells unable to react to the FN stimulus (Fig. 2c).

To further study a possible participation of Ca^{2+} release from amebic intracellular stores, $[Ca^{2+}]_i$ was monitored in trophozoites depleted of Ca^{2+} from intracellular pools by treatment with thapsigargin (TG)—known to activate release of Ca^{2+} from intracellular reservoirs by blocking Ca-ATPases in the endoplasmic reticulum. As seen in Fig. 2d, TG induced a tran-

sient increase of $[Ca^{2+}]_i$ from 178 n*M* to an average value of 350 n*M* that lasted almost 10 min in cells maintained in the presence of 1.0 m*M* external Ca^{2+}. FN added to these cells produced an additional increase of $[Ca^{2+}]_i$ to about 400 n*M*. If TG was added to cells in the absence of external Ca^{2+}, the transient increase was not observed and later addition of FN did not modify $[Ca^{2+}]_i$ values (Fig. 2d).

Adhesion to FN Substrates

a. Participation of Ca^{2+}. When trophozoites were incubated onto FN-coated surfaces in TBS containing 1.0 m*M* $CaCl_2$ and 0.1% BSA, the absolute percentage adherence was on average, 80%, while only 18–20% of the total population tested bound to uncoated plastic wells. Maximum adhesion to FN (100% in Table I) occurred rapidly within 5–10 min, so all the adhesion values refer to this time. Preincubation of trophozoites in TBS containing 5 m*M* EGTA produced a 19% decrease in the adhesion to FN that could result from the decrease in $[Ca^{2+}]_i$ to significant lower levels in EGTA-treated cells, as seen in Fig. 2c. The addition of 1 μM Ca^{2+}-ionophores A23187 or ionomycin in the presence of 1.0 m*M* extracellular calcium, produced an additional binding of 46 and 38%, respectively, above that induced by FN alone. On the other hand, if the ionophores were added in medium containing 5.0 m*M* EGTA, the adhesion of the trophozoites preincubated in this medium dropped to 50% or less. As these experiments indicated that modifications of cytoplasmic calcium levels had a strong effect on FN binding, trophozoites were preincubated with BAPTA/AM before the adhesion assay. As also shown in Table I, in BAPTA/AM-loaded trophozoites, preincubated and tested for adhesion in Ca^{2+}-containing medium, adhesion was down to 80%, and it was completely blocked (21%) if the trophozoites were preincubated and tested for adhesion in EGTA-containing medium. These experiments and the $[Ca^{2+}]_i$ measurements made in trophozoites treated with the same compounds, under identical conditions, suggested that amebas have a plasma membrane-regulated exchange of calcium that could be responsible for the rapid increase and depletion of cytoplasmic Ca^{2+}. Preincubation of trophozoites with TG, used to selectively increase cytoplasmic Ca^{2+} levels, produced a slight increase in adhesion (110%) when 1.0 m*M* Ca^{2+} was present in the medium, while in the absence of external Ca^{2+} a significant decrease in adhesion—to 34% of the control—was observed. The light stimulatory effect of this drug on adhesion in the presence of external Ca^{2+} suggests a small participation of internal Ca^{2+} release in the FN-induced modifications. However, as this parasitic ameba does not have a typical endoplasmic reticulum, the effect of TG on "putative" Ca^{2+} reservoirs — represented by the numerous vacuoles — could be different from that reported in other eukaryotic cells and further evidence will be necessary to validate our interpretation. The decrease in adhesion observed in these experiments cannot be ascribed to cell damage or cell death, since viability throughout the experiment was >95%.

TABLE I
Adhesion onto FN-Coated Surfaces

Treatment	FN	Adhesion %
TBS[a]	−	20 ± 2.7
TBS[a]	+	100 ± 4.5
TBS[b]	+	81 ± 4.5
1 μM A23187[a]	+	146 ± 5.1
1 μM A23187[b]	+	50 ± 5.6
1 μM ionomycin[a]	+	138 ± 6.5
1 μM ionomycin[b]	+	40 ± 7.8
50 μM BAPTA/AM[a]	+	80 ± 6.6
50 μM BAPTA/AM[b]	+	21 ± 1.6
50 n*M* Thapsigargin[a]	+	110 ± 6.0
50 n*M* Thapsigargin[b]	+	34 ± 6.4

Note. Adhesion assays were performed plating cells on wells coated with fibronectin (30 μg/ml) or uncoated, both in the presence of 0.1% BSA. Trophozoites were preincubated for 20 min with the indicated drug before the adhesion assay. Results represent experiments done by octuplicate. $P < 0.01$.

[a] TBS containing 1.0 m*M* Ca^{2+}.

[b] TBS containing 5.0 m*M* EGTA.

b. Participation of CaM. Since FN binding apparently induced modifications in cytosolic Ca^{2+} levels and calcium, as a second messenger, activates several participants of signal transduc-

tion pathways such as CaM, we examined the effect of three antagonists with different affinity for this protein on the adhesion to FN. TFP, W7, and calmidazolium showed a negative dose-dependent effect. As shown in Table II, the three antagonists caused inhibition close to 50% at concentrations (20.0, 2.5, and 5.0 μM, respectively) reported by other authors to block exocytic activities in trophozoites that are also part of the response to FN binding (Weikel *et al.* 1988; Muñoz *et al.* 1991). However, as these drugs also inhibit different types of cellular kinases, we cannot rule out for the moment that their effect on adhesion is partially due to PKC inactivation, as recently reported in *E. histolytica* (Santiago *et al.* 1994).

Reorganization of the Actin Cytoskeleton

Amebas do not display a very organized actin cytoskeleton, although it has been recently shown that they have typical actin filaments in the cortical region (Vázquez *et al.* 1995). Binding of trophozoites to FN triggers the formation of actin-containing adhesion plates and focal contacts at the sites of attachment. These structures can be visualized by staining F-actin in the FN-bound trophozoites with fluorescently tagged phalloidin (Talamás-Rohana and Meza 1988). We investigated whether $[Ca^{2+}]_i$ mobilization and Ca^{2+}-dependent activation of CaM, both of which modified the efficiency of adhesion to FN, also influenced the organization of the actin cytoskeleton. As shown in Figs. 3b–3f, preincubation of trophozoites with 1 μM ionomycin, 50 n*M* TG, 50 μM BAPTA/AM, 5 μM calmidazolium, or 20 μM W7 caused modifications on the normal structure of the actin plates—shown in Fig. 3a in control cells adhered to FN. Ionomycin-treated trophozoites maintained well-formed adhesion plates as well as a high number per cell (Fig. 3b), while TG-treated trophozoites (Fig. 3c) showed few organized plates as such, but numerous patches of structured actin associated to the cortical region, which could explain the slightly stimulatory effect of this drug on adhesion to the FN substrate. On the contrary, clearly disrupted plates and remnants of cortically organized actin were seen in amebas treated with BAPTA/AM (Fig. 3d) or treated with the antagonists of CaM, W7, and calmidazolium (Figs. 3e and 3f). In the latter, actin also appeared as bright dots in the cytoplasm. These results indicate that actin polymerization and organization into defined structures are processes very sensitive to Ca^{2+} levels and Ca^{2+} regulation in the cytosol. As alterations in the cytoskeleton organization are reflected on the equilibrium between soluble and structured actin (F-actin), shifts in this equilibrium were monitored by measuring F-actin levels in control cells and in cells stimulated by FN but previously treated with the drugs listed above. The results shown in Table III indicate that F-actin levels rose when the amebas interacted with FN and were modified, accordingly to $[Ca^{2+}]_i$-induced changes of cytoskeletal organization.

TABLE II
Effect of CaM Antagonists on the Adhesion to FN-Coated Surfaces

Treatment	FN	μM	Adhesion %
TBS + 1.0 m*M* $CaCl_2$	+	—	100 ± 2.7
TBS + 1.0 m*M* $CaCl_2$	−	—	20 ± 6.6
W7	+	10.0	80 ± 3.0
	+	20.0	52 ± 5.5
	+	30.0	35 ± 7.0
TFP	+	1.0	60 ± 3.4
	+	2.5	45 ± 4.2
	+	5.0	15 ± 6.5
Calmidazolium	+	2.5	75 ± 13.0
	+	5.0	61 ± 15.0
	+	10.0	36 ± 24.0

Note. Adhesion was measured after preincubation of the trophozoites for 20 min in the presence of each drug and plating onto FN-coated or uncoated wells as indicated under Materials and Methods. Adhesion values represent experiments done in octuplicate. $P < 0.01$.

DISCUSSION

Signal transduction coupled to adhesion to specific substrates can lead to alterations in $[Ca^{2+}]_i$, cell shape, cell metabolism, and gene expression (reviewed by Burridge *et al.* 1988; Schwartz 1993). It is well documented that trophozoites of the parasite *E. histolytica* adhere to ECM proteins, in particular to FN—either

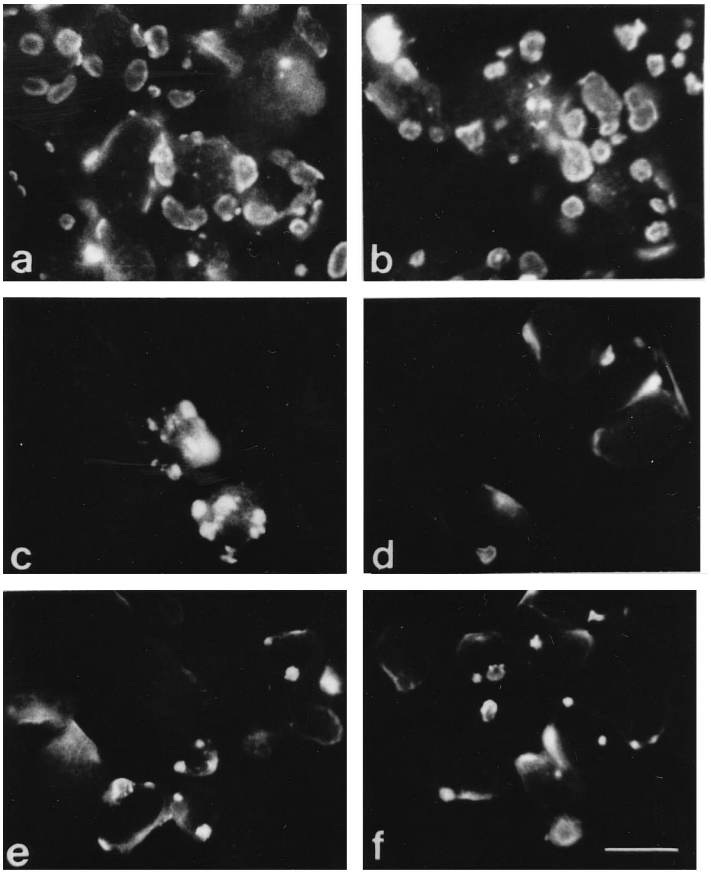

FIG. 3. Adhesion plates and actin organization in FN-stimulated trophozoites. Cells were preincubated 10 min with the indicated drugs and then allowed to adhere to FN-coated surfaces. Actin was visualized with rhodamine–phalloidin. Control (a), 1 μM ionomycin (b), 50 nM TG (c), 50 μM BAPTA/AM (d), 20 μM W7 (e), 5 μM calmidazolium (f) Bar, 20 μm.

soluble or on solid surfaces—through one or several specific FN binding proteins, and that the interaction elicits several structural and metabolic responses, suggesting the activation of signal transduction pathways as described in other eukaryotic cells (Talamás-Rohana and Meza 1988; Vázquez-Prado and Meza 1992; Talamás-Rohana *et al.* 1992; Santiago *et al.* 1994; Vázquez *et al.* 1995). In this work we have analyzed if FN binding induces cytosolic calcium modifications that could initiate Ca^{2+}-mediated signaling leading to adhesion and the reorganization of the actin cytoskeleton.

We selected conditions reported for loading amebas with Ca^{2+} indicators (Ravdin *et al.* 1988), and also those known to modify $[Ca^{2+}]_i$ in other eukaryotic cells, to study Ca^{2+} homeostasis in *E. histolytica* trophozoites. Direct measurements of $[Ca^{2+}]_i$ were performed in FURA 2/AM loaded amebas monitoring the changes in a luminiscence spectrometer. We found that FN, when added to the trophozoites, induced a rapid increase in $[Ca^{2+}]_i$ that reached a plateau at 30 μg/ml. Higher concentrations of FN did not produce significant modifications in $[Ca^{2+}]_i$, nor did it change the duration of the stimulus. Adhesion of trophozoites to FN-covered surfaces followed similar kinetics when the same concentrations of FN were tested, suggesting a correlation between the two processes. If our

TABLE III
Relative F-actin Levels in Trophozoites Stimulated with FN

Treatment	FN	Relative F-actin content
TBS + 1.0 m*M* $CaCl_2$	−	1.00 ± 0.01
TBS + 1.0 m*M* $CaCl_2$	+	1.49 ± 0.15
Ionomycin	+	1.62 ± 0.04
TG	+	1.25 ± 0.08
BAPTA/AM	+	0.90 ± 0.04
W7	+	0.87 ± 0.02
Calmidazolium	+	1.01 ± 0.08

Note. Trophozoites were incubated with the different drugs added to TBS containing 1.0 m*M* $CaCl_2$ for 10 min before the stimulation with FN. The results are expressed as relative F-actin content (RFI sample/RFI of control, where RFI is the relative fluorescence intensity per 1×10^5 cells). In all cases $P < 0.002$. It refers to the comparison to the FN+control.

hypothesis is correct, modifications in $[Ca^{2+}]_i$ would be reflected in a more or a less efficient adhesion to FN. As shown in Table I preincubation of trophozoites in EGTA-containing TBS decreased the adhesion to FN. On the contrary, two Ca^{2+} ionophores tested induced a 38 and 46% increase in adhesion which could be completely reverted when the trophozoites were incubated with the ionophores in Ca^{2+}-free medium. Furthermore, the Ca^{2+} chelator BAPTA/AM also produced a significant decrease in $[Ca^{2+}]_i$ levels that again could be correlated with the lower efficiency in adhesion shown by amebas in the presence of this drug and, in particular, with the lower values observed in the absence of external Ca^{2+}.

In the present study, we have shown that elimination of extracellular Ca^{2+}, by using a Ca^{2+}-free solution or by the use of the Ca^{2+} chellator EGTA, prevented the FN-induced increase in intracellular Ca^{2+}, therefore suggesting that external Ca^{2+} influx is an important mechanism in the FN-stimulated signal, and that cells depleted of internal calcium cannot respond to the FN stimulus. Unfortunately, nothing is known yet about Ca^{2+} transport, Ca^{2+} channels in the plasma membrane of amebas, or their mechanisms of operation that could help us to understand this part of the process.

To test if Ca^{2+} is also released from internal Ca^{2+} stores by the effect of FN binding, we used TG, a drug known to produce the release of Ca^{2+} from the endoplasmic reticulum by blockage of Ca-ATPases (Jones and Sharpe 1994). The transient increase in $[Ca^{2+}]_i$ produced by TG indicated that, although internal Ca^{2+} release is caused by the drug, addition of FN still induces the influx of external Ca^{2+} further supporting our hypothesis that extracellular Ca^{2+} is critical for the FN effect on $[Ca^{2+}]_i$. The increase of $[Ca^{2+}]_i$ produced by TG is abolished in the absence of external Ca^{2+}, suggesting rapid depletion of cytosolic calcium possibly through Ca^{2+} channels in the plasma membrane. This could also explain the low adhesion observed in cells treated with TG in calcium-free medium.

Variations in cytoplasmic Ca^{2+} have a regulatory effect on Ca^{2+}-activated proteins, known to participate in the cytoskeleton organization (Hinrichsen 1993). CaM and PKC have been identified in *E. histolytica* trophozoites and it has been reported that their activity is blocked by antagonists such as W7, TFP, H7, and staurosporine, with inhibitory effects on adhesion and proteolytic activities of the amebas (Muñoz *et al.* 1991; Santiago *et al.* 1994). It has also been shown that release of IP_3 and translation of activated PKC to membrane–cytoskeleton fractions, where the trophozoite adhesion complexes assemble, occur shortly after FN binding (Santiago *et al.* 1994; Vázquez *et al.* 1995). The decrement in trophozoite adhesion caused by three CaM inhibitors, with different affinity for the protein, and the concomitant disorganization of the actin cytoskeleton further support that Ca^{2+}-activated proteins participate in the organization of the actin cortical network to stabilize cytoskeleton structures needed for the trophozoite interactions. The decrease in adhesion capability, that occurs with the disorganization of the actin plates caused by blockage of the Ca^{2+}-activated proteins could in turn result in the diminution of the trophozoite proteolytic activities and phagocytosis, rendering amebas with attenuated virulence.

Calcium-mediated activation of proteins in-

volved in actin polymerization–depolymerization cycles and actin network organization is known to regulate shape, locomotion, binding, and movements of many cells. Trophozoites depleted of internal calcium or treated with antagonists of CaM cannot form or maintain well organized actin adhesion plates and focal adhesions. In these cells polymerized actin appears as dots and/or polarized patches in apposition to the cell membrane. On the contrary, FN and drugs that act as inducers of cytosolic calcium rise produced not only increments in polymerized actin but elicited its organization into defined cytoskeletal structures.

A physiological role of intracellular calcium and CaM in the organization of actin microfilaments has been reported for different cells (Sasaki *et al.* 1987; Hinrichsen 1993). Calcium interaction with CaM and calcium-dependent PKC isoforms induces the activation of several proteins as CaM-dependent kinases, and Ca^{2+}- or Ca^{2+}/CaM-dependent actin binding proteins. Actin filaments interact with several types of Ca^{2+}-sensitive actin-binding proteins (ABPS), which confer Ca^{2+} sensitivity to the filaments and actin network structures. Recently, ABPs such as α actinin, myosin II, tropomyosin, myosin I, and proteins associated to actin plates such as vinculin, have been identified in *E. histolytica* (Bailey *et al.* 1992; Rahim *et al.* 1993; Vázquez *et al.* 1995). An increase in PKC phosphorylation of proteins associated to the cytoskeleton fraction—in response to binding to FN—has also been reported, as well as the presence of other kinases such as $pp125^{FAK}$ in adhesion plates (Santiago *et al.* 1994; Vázquez *et al.* 1995). ABPs of *E. histolytica* could be kinase substrates during the FN-adhesion process and participate in the assembly of the actin cytoskeleton at plates and focal contacts.

The results of the present study show that trophozoites of *E. histolytica* have mechanisms for regulating $[Ca^{2+}]_i$ that are similar to those found in other eukaryotic cells and that intracellular calcium and CaM are candidate FN-receptor-activated messengers with potential to regulate, directly or indirectly, cytoskeletal structure and, consequently, trophozoite adhesion and motility. Thus, FN may play and important role in the accumulation, distribution, and behavior of trophozoites at sites of tissue injury, promoting tissue invasion. Understanding the role of Ca^{2+} in the interactions between trophozoites and the extracellular matrix *in vivo* would be an important way of controlling tissue damage by this parasite.

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