INFECTION AND IMMUNITY, June 1988, p. 1505–1512
0019-9567/88/061505-08$02.00/0

Vol. 56, No. 6

Relationship of Free Intracellular Calcium to the Cytolytic Activity of *Entamoeba histolytica*

JONATHAN I. RAVDIN,[1,2,3]* FRANCE MOREAU,[1] JAMES A. SULLIVAN,[3] WILLIAM A. PETRI, JR.,[1,2,3] AND GERALD L. MANDELL[3]

Divisions of Clinical Pharmacology,[1] Geographic Medicine,[2] and Infectious Diseases,[3] Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received 9 December 1987/Accepted 29 February 1988

Entamoeba histolytica **adherence and destruction of host cells is required for in vivo pathogenicity; amebic in vitro adherence is mediated by a galactose- or *N*-acetyl-D-galactosamine-inhibitable surface lectin (Gal/GalNAc adherence lectin). Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in living amebae and target cells during amebic cytolysis of Chinese hamster ovary (CHO) cells and human polymorphonuclear neutrophils by utilizing the Ca^{2+} probe Fura-2 and computer-enhanced digitized microscopy. Motile *E. histolytica* trophozoites had oscillatory increases in $[Ca^{2+}]_i$ in head or tail regions; however, there was no increase in regional or total amebic $[Ca^{2+}]_i$ upon contact with a target CHO cell. Target CHO cells and polymorphonuclear neutrophils demonstrated marked irreversible increases in $[Ca^{2+}]_i$ within 30 to 300 s following contact by an ameba ($P < 0.01$); increased $[Ca^{2+}]_i$ preceded the occurrence of nonspecific surface membrane permeability and death of the target cell. Target CHO cells contiguous on a monolayer to a cell contacted by an ameba experienced a rapid but reversible rise in $[Ca^{2+}]_i$ ($P < 0.01$) and were not killed. Galactose (40 mg/ml) totally abrogated the rise in target CHO cell $[Ca^{2+}]_i$ that followed contact by amebae ($P < 0.01$); immunoaffinity-purified amebic Gal/GalNAc adherence lectin (0.25 μg/ml) induced a rapid and reversible rise in CHO cell $[Ca^{2+}]_i$ ($P < 0.01$) which was inhibited by galactose. Amebic $[Ca^{2+}]_i$ was not elevated following parasite adherence to target cells; a rapid and substantial rise in target cell $[Ca^{2+}]_i$ occurred which was mediated, at least in part, by the Gal/GalNAc adherence lectin of the parasite and led to the death of target cells.**

Entamoeba histolytica infects 10% of the world population and is the third leading parasitic cause of death (41). The pathogenesis of invasive amebiasis apparently involves attachment of virulent organisms to the intestinal mucus layer, depletion of colonic mucus with disruption of mucosal barriers, and amebic adherence to and cytolysis of host epithelial and inflammatory cells (3, 4, 23, 41). The determination of the biochemical and cellular bases for *E. histolytica* adherence and cytolytic mechanisms should lead to the development of immunologic or pharmacologic means of disease control.

We have learned from in vitro studies of the interaction of axenic *E. histolytica* trophozoites with mammalian and human target cells (24, 25) that amebic adherence to target Chinese hamster ovary (CHO) cells, rat and human colonic epithelia, human erythrocytes and neutrophils, and Chang liver cells is mediated by an amebic surface lectin which is inhibited by galactose or *N*-acetyl-D-galactosamine (Gal/GalNAc adherence lectin) (4, 20, 25, 26, 28, 34). By using carbohydrate affinity chromatography and recognition by adherence-inhibitory mouse monoclonal antibodies, Petri et al. (20) recently isolated the 170-kilodalton *E. histolytica* Gal/GalNAc adherence lectin. The inhibition of amebic adherence by galactose or *N*-acetyl-D-galactosamine abrogates the ability of amebae to lyse target cells (25, 28, 33, 34).

Amebic *in vitro* cytolytic activity is inhibited by the calcium channel blockers bepridil and verapamil, the putative calcium antagonist TMB-8, and by chelation of extracellular Ca^{2+} ions with EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) (27, 31). In addition, the activity of a membrane-associated calcium-dependent phospholipase A enzyme is also required for parasite cytolytic activity (14, 27). These studies suggest that Ca^{2+} ions play a direct role in the *E. histolytica*-induced death of target cells. As parasite cytolytic activity is specifically stimulated by phorbol esters (42a), free intracellular Ca^{2+} ion concentration $[Ca^{2+}]_i$ may also serve as a second messenger regulating parasite cytolytic activity.

We measured $[Ca^{2+}]_i$ during the interaction of individual viable amebae with target CHO cells or human polymorphonuclear neutrophils (PMN), using the fluorescent calcium probe Fura-2. When deesterified within eucaryotic cells, Fura-2 emits calcium-dependent fluorescence when excited at 340 or 380 nm (8), and it has been used to measure $[Ca^{2+}]_i$ in a variety of single-cell systems (42–44). We observed oscillation of $[Ca^{2+}]_i$ in motile trophozoites, but there was no clear association of amebic $[Ca^{2+}]_i$ with parasite cytolytic activity. Upon contact with *E. histolytica* trophozoites, there was a rapid irreversible rise in target cell $[Ca^{2+}]_i$ which followed binding by the amebic Gal/GalNAc adherence lectin and preceded death of the target cell.

MATERIALS AND METHODS

Cultivation of cells and labeling with Fura-2. Axenic *E. histolytica* trophozoites (strain HM1:IMSS; among the most virulent in axenic culture [16, 28]) were grown in TYI-S-33 culture medium with penicillin (100 U/ml) and streptomycin (100 μg/ml) by the method of Diamond et al. (5) and harvested as described earlier (24). Amebae were washed twice in medium 199 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5.7 mM cysteine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and 0.5% bovine serum albumin at pH 6.8 (M199s) (28). Amebae (2×10^6 per ml) were incubated with 50 μM Fura-2 AM (Calbiochem-Behring, LaJolla, Calif.) at 37°C for 1 h, washed, and suspended in M199s with 1% fetal bovine

* Corresponding author.

serum. CHO cells were cultured in F-12 medium as described elsewhere (24) and prepared for study either in suspension by trypsinization (0.25% for 2 min at 37°C) or by growth to confluency as monolayers on glass cover slips (22 by 22 mm). CHO cells were washed twice and suspended in M199s; 2×10^6 CHO cells per ml in suspension or seven confluent cover slips in 3 ml were incubated with Fura-2 AM (5 μM) for 1 h at 37°C with agitation. The CHO cells were washed twice, and then placed in M199s with fetal bovine serum (1.0%). Neutrophils were isolated from heparinized (10 U/ml) peripheral venous blood from normal volunteers by centrifugation in Neutrophil Isolation Medium (Los Alamos Diagnostics) followed by hypotonic lysis of erythrocytes. Neutrophils (4×10^6 per ml) were washed and incubated in M199s containing Fura-2 AM (5 μM) for 1 h at 37°C, washed, and then suspended in M199s with fetal bovine serum (1.0%).

Measurement of cellular $[Ca^{2+}]_i$ with Fura-2. Fura-2-loaded cells (15 μl of a cell suspension or a confluent CHO cell monolayer) were placed on a heated slide (37°C) sealed with paraffin and observed with a microscope (Leitz Orthoplan; Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.) equipped with a 100-W mercury vapor epi-illuminator, quartz optical elements, and a glycerine immersion objective (Nikon 100× UV-CF). Amebae with or without CHO cells or PMN were observed by bright-field microscopy; only a single cell type was loaded with Fura-2 during any study. Fluorescence images were collected with a silicon-intensified target camera (DAGE series 65) and stored for processing with an image processor (Quantex 9210). Cells were excited first at 340 nm, then at 380 nm, and lastly at 360 nm; fluorescence images were separated in time by less than 1.0 s. During studies of amebae with target cells, a precontact observation was always included.

The raw fluorescence image was digitized to a pixel assay (640 by 480 by 8 bits). Point density readings were taken for each image (at 340, 380, and 360 nm), and a visual display of the 340- to 380-nm ratio was produced. We expressed $[Ca^{2+}]_i$ as the 340- to 380-nm ratio to which it is proportional (8). However, $[Ca^{2+}]_i$s in amebae, CHO cells, and PMN were also estimated by calibration by utilizing the following equation (8, 19):

$$[Ca^{2+}]_i = K_d \times \frac{R_{340/380} - R_{min}}{R_{max} - R_{340/380}} \times \frac{I_{380}/I_{360} \text{ of } Ca^{2+} \text{ free}}{I_{380}/I_{360} \text{ of } Ca^{2+} \text{ saturated}}$$

where K_d is the dissociation constant of Fura-2 for Ca^{2+} and is assumed to be 224 nM, and $R_{340/380}$ is the ratio of fluorescence intensity at 340 nm (I_{340}) to that at 380 nm (I_{380}). R_{min} was the value of $R_{340/380}$ obtained by incubation of cells in Hanks balanced salt solution free of Ca^{2+} and Mg^{2+} with EGTA (5 mM) and ionomycin (1 μM), and R_{max} was the value obtained when calcium-saturated cells were studied in Hanks balanced salt solution with $CaCl_2$ (10 mM) and ionomycin (1 to 5 μM) added. Evidence for deesterification and calcium responsiveness of Fura-2 in the cells studied included an increased ratio in response to ionomycin (R_{max} was 1.83, 5.12, and 2.27 for amebae, CHO cells, and PMN, respectively). There was no increased Fura-2 signal observed when amebae or CHO cells were incubated for an additional 15 to 60 min at 37°C postloading as utilized in other studies (45), indicating that there was maximal availability of intracellular deesterified free Fura-2. Fura-2 was uniformly distributed in amebae, CHO cells, or PMN without evidence of intracellular compartmentalization during the period of study.

Purification of Gal/GalNAc adherence lectin of *E. histolytica*. The amebic Gal/GalNAc adherence lectin was purified as reported elsewhere (20); the lectin-specific mouse monoclonal antibody (H8-5) (20, 29) was purified from mouse ascites on a protein A-agarose column (Sigma Chemical Co., St. Louis, Mo.). Purified antibody was coupled to Affigel 10 (Bio-Rad Laboratories, Richmond, Calif.) and extensively washed. Amebae were solubilized in 0.1% Triton X-100–50 mM Tris–10 mM EDTA–150 mM NaCl–50 mM KI (pH 8.0), with insoluble debris removed by centrifugation at 10,000 × *g* for 10 min. After application to the affinity column, the column was washed extensively with the above buffer until no amebic proteins were detected (A_{280}) in the flowthrough. The lectin was eluted from the column with 50 mM glycine (pH 2.5). The pH of the eluate was adjusted to 7.0 with 1.5 M Tris (pH 8.8), and the sample was concentrated in Centricon microconcentrates (Amicon Corp., Lexington, Mass.). Protein was assayed by the method of Bradford (2) prior to use.

Statistics. Data are presented as the means ± standard errors of the means as appropriate; comparisons of significance were made by the unpaired Student *t* test.

RESULTS

Measurement of $[Ca^{2+}]_i$ in *E. histolytica* trophozoites. (i) In motile amebae. Motile Fura-2-loaded *E. histolytica* trophozoites appeared as polarized cells and had uniformly distributed Fura-2 within the cell cytoplasm (Fig. 1). Amebae demonstrated cyclic increases in $[Ca^{2+}]_i$ at the advancing front of cytoplasm (defined as the leading area of 2 by 2 μm) or cytoplasmic tail, in comparison with the average $[Ca^{2+}]_i$ for the whole cell. As demonstrated in Fig. 2 for a representative ameba, front and tail $[Ca^{2+}]_i$s were not simultaneously elevated in comparison with whole cell $[Ca^{2+}]_i$. For 1 random observation in 31 individual motile amebae, there was no difference between mean head, mean tail, or mean whole cell $[Ca^{2+}]_i$ ($R_{340/380}$s were 0.99 ± 0.05, 0.98 ± 0.07, and 0.99 ± 0.06, respectively); this observation was also confirmed in six separate experiments in which individual amebae were studied at 30-s intervals for 300 s. In summary, we noted no sustained pattern of increased amebic $[Ca^{2+}]_i$; rather, we observed oscillation of $[Ca^{2+}]_i$ in relation to the direction of cell movement or pseudopod extension.

(ii) In amebae upon contact with target CHO cells or PMN. Upon contact of Fura-2-loaded *E. histolytica* trophozoites with target CHO cells, there was no increase in amebic whole cell $[Ca^{2+}]_i$ (Fig. 3; $n = 14$). Similar results were observed with target PMN; the mean amebic whole cell $[Ca^{2+}]_i$s at precontact, contact, and 30 or 60 s postcontact were identical ($R_{340/380}$s were 0.92 ± 0.07, 0.89 ± 0.05, and 0.90 ± 0.06, respectively; $n = 22$). Fura-2-loaded amebae were able to kill target CHO cells or neutrophils as well as control amebae (93.5% of target cells killed by Fura-2-loaded amebae compared with that by control amebae; $n = 12$), indicating that there was no decreased amebic cytolytic function due to possible calcium chelation by Fura-2. In addition, there was no increase in $[Ca^{2+}]_i$ in the area (2 by 2 μm) of amebic cytoplasm immediately adjacent to target CHO cells ($n = 14$) or human PMN ($n = 22$) at the time of contact and at 30 or 60 s postcontact.

Measurement of $[Ca^{2+}]_i$ in target CHO cells and PMN. There was a marked rise in $[Ca^{2+}]_i$ in Fura-2-loaded target CHO cells within 30 s postcontact by an *E. histolytica* trophozoite ($P < 0.01$; Fig. 4 and 5). Target CHO cell $[Ca^{2+}]_i$ remained elevated following this lethal hit by an ameba; the

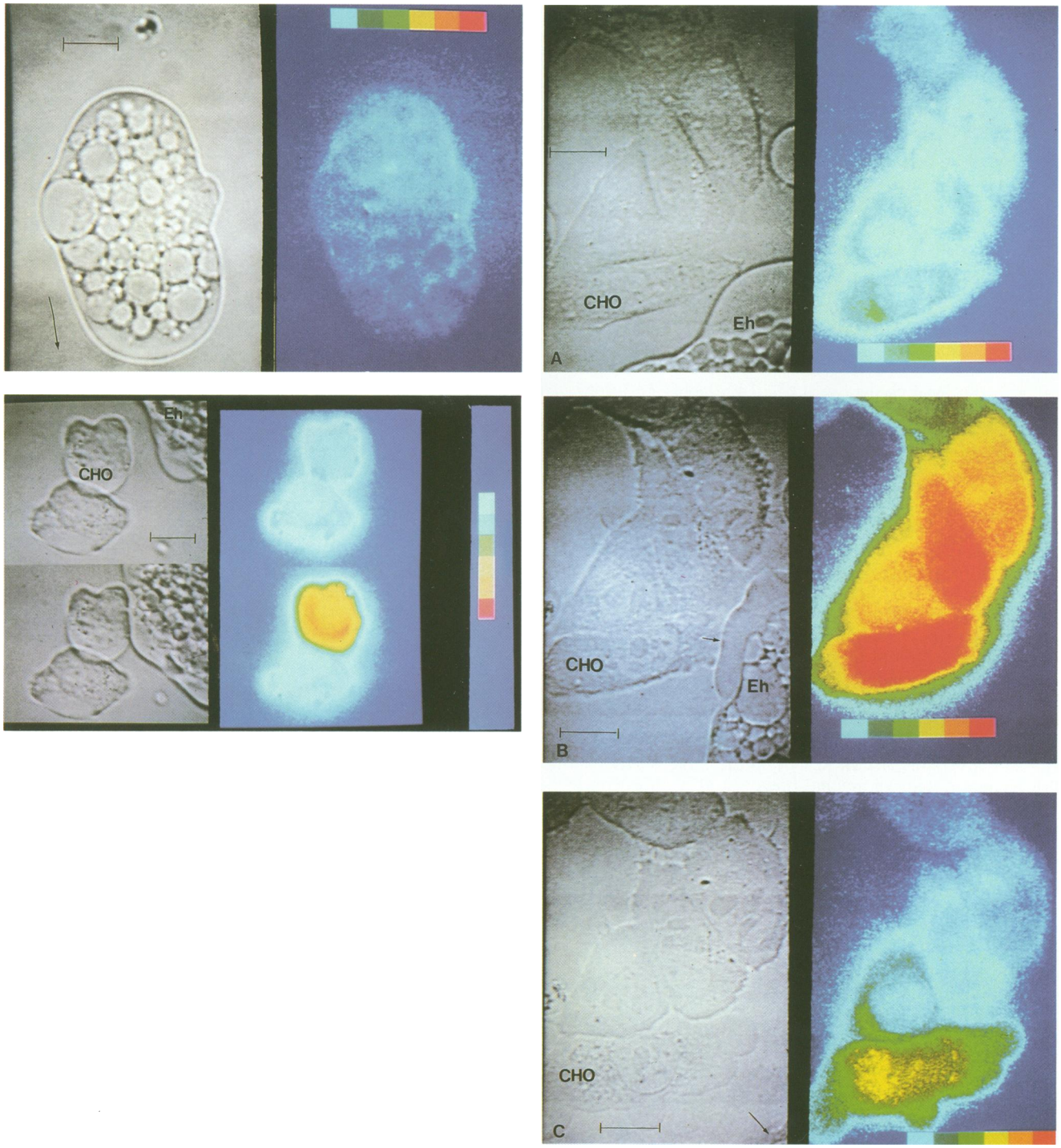

FIG. 1, 4, and 6. (1, upper left) Photomicrograph of phase and digitized $R_{340/380}$ image of a motile Fura-2-loaded *E. histolytica* trophozoite (direction of motion indicated by arrow) at one random point in time. Color bar provides scale from background (dark blue) to a maximal $R_{340/380}$ (red) of Fura-2 fluorescence. There were no net regional increases in $[Ca^{2+}]_i$ observed in relation to the direction of motion of 31 amebae studied randomly at one point in time. Bar, 10 μm. (4, center left) Photomicrograph of phase and digitized $R_{340/380}$ image of a Fura-2-loaded CHO cell precontact (top) and at 30 s postcontact (bottom) with an *E. histolytica* (Eh) trophozoite. The two trypsinized CHO cells were in suspension and not directly contiguous; there was a marked rise in $[Ca^{2+}]_i$ only in the CHO cell in direct contact with the ameba, despite the proximity of the other target cell. Bar, 10 μm. (6, right) Effect of contact by *E. histolytica* trophozoites on the $[Ca^{2+}]_i$ of target CHO cells contiguous on a monolayer. (A) An ameba (Eh) is about to make contact with the lower of four Fura-2-loaded CHO cells. (B) Upon contact with only one CHO cell (arrow), all four CHO cells demonstrate a simultaneous marked rise in $[Ca^{2+}]_i$ (from a mean $R_{340/380}$ of 0.55 to 2.28). (C) At 240 s postcontact, the ameba has moved away (arrow), and only the CHO cell that was in direct contact with the ameba appears to have lost density and to have a persistent irreversible increase in $[Ca^{2+}]_i$ ($R_{340/380}$, 2.28). The contiguous CHO cells continue to appear morphologically viable, with a normal baseline $[Ca^{2+}]_i$ (mean $R_{340/380}$, 0.52) for up to 20 min of observation. Bar, 10 μm.

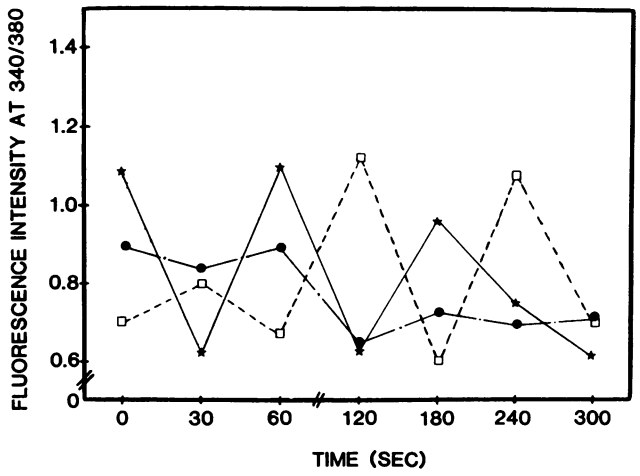

FIG. 2. Regional changes in $[Ca^{2+}]_i$ in a motile *E. histolytica* trophozoite. There were cyclic increases in the leading cytoplasmic edge (★) and tail (□) $[Ca^{2+}]_i$, compared with total cell (●) $[Ca^{2+}]_i$, when observed at 30-s intervals over 300 s in this representative study.

increase in $[Ca^{2+}]_i$ preceded target cell morphologic dissolution (observed at >5 min) or leakage of Fura-2 from the target cell (in comparison with control CHO cells, there was no decrease in Fura-2 fluorescence at 360 nm observed at up to 300 s postcontact; $n = 16$). Target CHO cell $[Ca^{2+}]_i$ was estimated to rise from a mean baseline of 40 nM to a mean maximum of 1,240 nM at 3 min (Fig. 5).

When amebae made contact with a monolayer of target CHO cells, there was a marked rise in $[Ca^{2+}]_i$ in both the target cell contacted by the ameba and those immediately contiguous to the index target cell (Fig. 6A and B). There was an irreversible rise in $[Ca^{2+}]_i$ in the target CHO cell in direct contact with an ameba; however, $[Ca^{2+}]_i$ in contiguous target CHO cells returned to normal baseline values by 240 s (Fig. 6C). In contrast to the dead index target cell, contiguous CHO cells appeared to remain viable (retained Fura-2 dye) and attached to the substrate under subsequent observation for 20 min. This phenomenon was observed in three separate experiments.

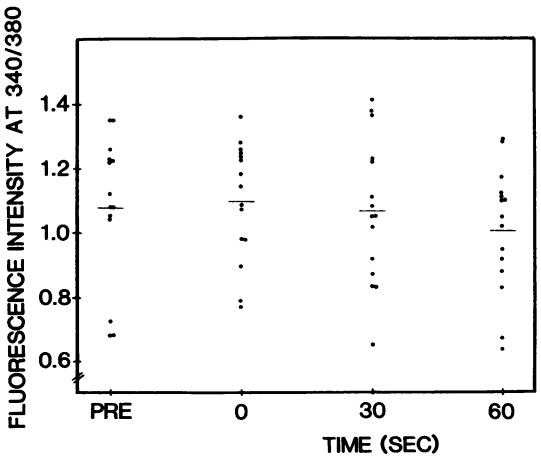

FIG. 3. Lack of effect of contact with target CHO cells on total *E. histolytica* $[Ca^{2+}]_i$. Compared with $[Ca^{2+}]_i$ immediately before contact (PRE), there was no increase in total amebic $[Ca^{2+}]_i$ upon contact with target CHO cells and for the subsequent 60 s ($n = 14$). Amebae were studied for up to 300 s postcontact, with no change in $[Ca^{2+}]_i$ observed (n = 14; data not shown). By using ionomycin calibration (8, 19), mean precontact amebic $[Ca^{2+}]_i$ was estimated to be 150 nm.

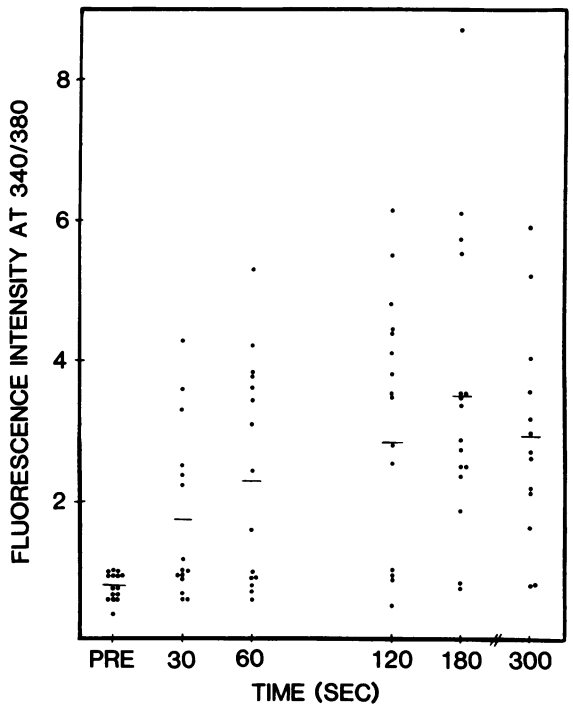

FIG. 5. Effect of contact by *E. histolytica* trophozoites on target CHO cell $[Ca^{2+}]_i$. There was a marked rise in CHO cell $[Ca^{2+}]_i$ at ≥30 s postcontact ($P < 0.01$ compared with precontact [PRE] and with each time period shown; $n \geq 14$ for each time period). Once elevated after amebic contact, $[Ca^{2+}]_i$ never returned to baseline levels in target CHO cells. Only two target cells did not demonstrate an increase in $[Ca^{2+}]_i$ by 300 s. By using ionomycin calibration (8, 19), the estimated mean CHO cell $[Ca^{2+}]_i$s were 40 nM at precontact and 140, 420, 650, 1,240, and 700 nM at 30, 60, 120, 180, and 300 s, respectively.

There was a marked increase in $[Ca^{2+}]_i$ in Fura-2-loaded human PMN following chemotaxis to and contact with an ameba ($P < 0.01$; Fig. 7); mean PMN $[Ca^{2+}]_i$ was estimated to be 1,980 nM at 3 min postcontact with ameba. Only a portion of the increase in neutrophil $[Ca^{2+}]_i$ may be accounted for by endogenous neutrophil mechanisms, as contact of Fura-2-loaded PMN with glutaraldehyde-fixed amebae resulted in an equally rapid but much smaller increment in PMN $[Ca^{2+}]_i$ (Fig. 8).

Effect of galactose on the *E. histolytica*-mediated elevation of target CHO cell $[Ca^{2+}]_i$. Initiation of amebic cytolysis of target CHO cells and neutrophils requires binding by the 170-kilodalton Gal/GalNAc adherence lectin of the parasite (20, 25, 28). In this study, in the presence of galactose (40 mg/ml), contact by an *E. histolytica* trophozoite was not followed by a rise in target CHO cell $[Ca^{2+}]_i$ (Fig. 9); this result was not an osmotic effect, as the contact-mediated increase in target CHO cell $[Ca^{2+}]_i$ occurred in the presence of glucose (40 mg/ml) ($n = 4$).

Effect of *E. histolytica* Gal/GalNAc adherence lectin on CHO cell $[Ca^{2+}]_i$. Purified Gal/GalNAc adherence lectin was obtained by monoclonal antibody (H8-5) immunoaffinity chromatography of Triton X-100-solubilized amebae (20). At a sublethal concentration (0.25 μg/ml), the amebic lectin caused a rapid and reversible rise in CHO cell $[Ca^{2+}]_i$ (Fig.

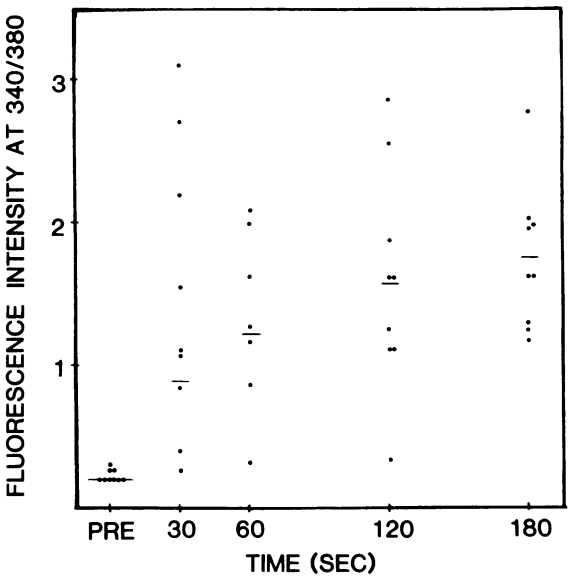

FIG. 7. Effect of contact by *E. histolytica* trophozoites on target neutrophil [Ca^{2+}]$_i$. There was a marked irreversible rise in neutrophil [Ca^{2+}]$_i$ upon contact by an *E. histolytica* trophozoite ($P < 0.01$ at ≥30 s; $n = 9$). By using ionomycin calibration (8, 19), mean neutrophil [Ca^{2+}]$_i$ was estimated to be 80 nM and rose to 360, 680, 1,410, and 1,980 nM at 30, 60, 120, and 180 s, respectively. PRE, Precontact.

10). At up to 60 s, the addition of galactose (40 mg/ml) completely abrogated the elevation in CHO cell [Ca^{2+}]$_i$; glucose (40 mg/ml) had no inhibitory effect (Fig. 10). The lectin-mediated elevation in CHO cell [Ca^{2+}]$_i$ was comparable in rapidity and magnitude to that observed upon contact by viable amebae (Fig. 5). Exposure of CHO cells to ≥0.5 μg

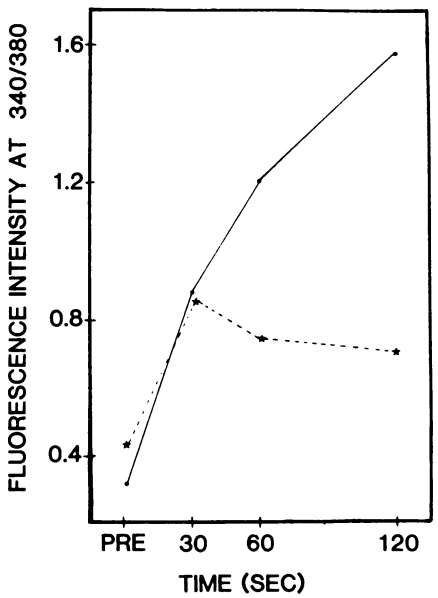

FIG. 8. Increase in neutrophil [Ca^{2+}]$_i$ following contact with glutaraldehyde-fixed *E. histolytica* trophozoites. Compared with contact with viable amebae (●), contact with a fixed ameba (★) resulted in a rise in neutrophil [Ca^{2+}]$_i$ equal to that with viable amebae only at 30 sec, with no further increase observed ($n = 4$). PRE, Precontact.

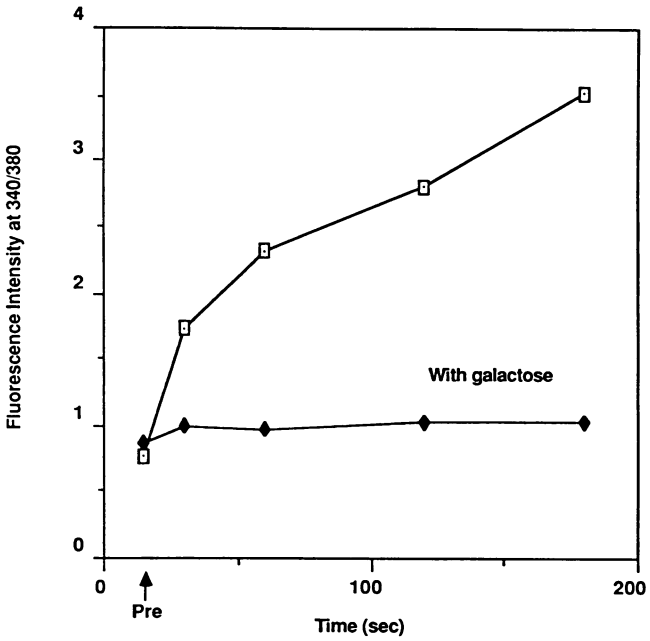

FIG. 9. Effect of galactose on *E. histolytica*-mediated rise in target CHO cell [Ca^{2+}]$_i$. Compared with control medium (□), galactose (40 mg/ml) in medium caused the complete abrogation of the rise of [Ca^{2+}]$_i$ in target CHO cells following contact by amebae ($P < 0.01$ at 30, 60, 120, and 180 s; $n = 6$). In contrast, the addition of glucose (40 mg/ml) had no effect on the *E. histolytica*-mediated rise in target CHO cell [Ca^{2+}]$_i$ ($n = 3$; data not shown). PRE, Precontact.

of the Gal/GalNAc adherence lectin per ml resulted in a galactose-sensitive but irreversible rise in [Ca^{2+}]$_i$ with rapid CHO cell death as evidenced by morphologic dissolution and leakage of Fura-2 at 60 s of incubation ($n = 6$).

DISCUSSION

We demonstrated that motile *E. histolytica* trophozoites had oscillatory regional increases in [Ca^{2+}]$_i$ which were unaltered during and following contact with a target cell. Contact by an ameba induced an irreversible rise in target cell [Ca^{2+}]$_i$. This effect was galactose sensitive, and a comparable increase in CHO cell [Ca^{2+}]$_i$ was observed with the purified *E. histolytica* Gal/GalNAc adherence lectin.

Taylor et al. (40) studied aequorin luminescence in the free-living ameba *Chaos carolinensis* and noted that continuous luminescence was maximum at the tail, with spontaneous pulses occurring primarily in the anterior half of the cells. In addition, Pollack (22) observed the precipitation of alizarin at sites of pseudopod extension in *Amoeba dubia* and *Amoeba proteus*. Recently, pituitary cell [Ca^{2+}]$_i$ has been found to oscillate concurrently with spontaneous action potentials (38); in addition, the sustained rise in [Ca^{2+}]$_i$ in hormonally stimulated hepatocytes and murine lymphocytes exposed to anti-immunoglobulin is now resolvable into repetitive oscillations between resting and transient peak levels in the micromolar range (46, 47). The oscillatory increases in *E. histolytica* [Ca^{2+}]$_i$ may be related to parasite motility. Chemotaxis occurs in *E. histolytica* (1); one could speculate that the oscillation of [Ca^{2+}]$_i$ may have a role in parasite-directed motility.

We hypothesized that [Ca^{2+}]$_i$ acts as a second messenger initiating amebic cytolytic activity. However, in contrast to

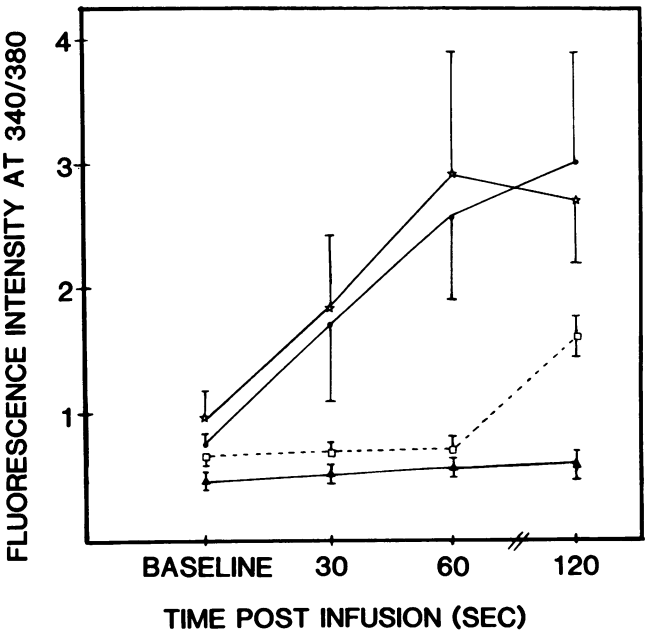

FIG. 10. Effect of purified *E. histolytica* Gal/GalNAc adherence lectin on CHO cell $[Ca^{2+}]_i$. Lectin (0.25 μg/ml) obtained by immunoaffinity chromatography with H8-5 was added in control medium (☆) or medium containing glucose (40 mg/ml) (●) or galactose (40 mg/ml) (□). Results for control medium alone without lectin (▲) are also shown. There was a marked rise in CHO cell $[Ca^{2+}]_i$ at >30 s ($P < 0.01$ versus baseline) equal to that seen with contact by whole amebae (mean maximum $R_{340/380}$, 2.90). Glucose had no effect on the lectin-mediated rise in CHO cell $[Ca^{2+}]_i$ ($P < 0.01$ at ≥30 s compared with the baseline). Galactose completely abrogated the rise in CHO cell $[Ca^{2+}]_i$ until 120 s. The elevation in CHO cell $[Ca^{2+}]_i$ persisted for the 300-s observation, as did inhibition by galactose (data not shown).

recent studies with cytotoxic T lymphocytes (21), our study revealed no increase in amebic $[Ca^{2+}]_i$ upon contact with a target cell, despite our use of a system sensitive enough to detect regional $[Ca^{2+}]_i$ changes in a much smaller cell, the PMN (19, 35). Monocyte and neutrophil phagocytoses can occur without increases in $[Ca^{2+}]_i$ (13, 17), a fact indicating calcium-independent cellular mechanisms or changes in $[Ca^{2+}]_i$ below the level of detection by the methods utilized. We also cannot rule out undescribed effects of the amebic intracellular environment on Fura-2.

The *E. histolytica*-induced increase in target CHO cell $[Ca^{2+}]_i$ precedes cell death as indicated by morphology, determination of Fura-2 leakage, trypan blue uptake, and previous studies of [^{111}In]indium oxine release (24, 25). Cyanide and iodoacetate treatment of hepatocytes results in bleb formation or loss of cellular viability in the absence of a preceding rise in $[Ca^{2+}]_i$ (12). Therefore, is the *E. histolytica*-induced rise in target cell $[Ca^{2+}]_i$ an epiphenomenon, or does it contribute to the death of the target cell? We believe the latter is true; calcium channel blockers or extracellular calcium chelators prevent target cell death after parasite adherence has occurred (27, 31). Cytotoxic T cells also induce a rise in target cell $[Ca^{2+}]_i$ which precedes release of ^{51}Cr and Fura-2 dye (21). That elevation in target cell $[Ca^{2+}]_i$ can directly contribute to cell death has been demonstrated for a wide range of cytotoxic agents (6, 7, 18, 36, 37). There is substantial evidence that cell death induced by the calcium ionophore A23187 or hypoxia results directly from increased $[Ca^{2+}]_i$ (6, 7). In vitro, the lethal effects on hepatocytes of galactosamine, CCl_4, and phalloidin depend on the presence of extracellular calcium ions; yet there is no evidence that the interaction of these cytotoxins with the cells depends on calcium (6, 7, 11, 36). The cytolethal effect of a sustained high $[Ca^{2+}]_i$ may be due to the activation of cellular proteases and phospholipases (18, 37).

A reversible nonlethal elevation in target cell $[Ca^{2+}]_i$ occurred in CHO cells contiguous on a monolayer to one contacted by amebae, indicating that brief elevations of $[Ca^{2+}]_i$ without direct contact by the ameba is not sufficient to cause death of the target cell (24, 25). Amebae exert a lethal hit after a very brief period of adherence (24, 25); appropriate delivery of the *E. histolytica* Gal/GalNAc adherence lectin molecule may be sufficient to account for target cell death. Immunoaffinity-purified lectin elevated target CHO cell $[Ca^{2+}]_i$ in a galactose-specific manner; sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography of *E. histolytica* proteins metabolically labeled with [^{35}S]methionine showed that the immunoaffinity-purified lectin has a 170-kilodalton subunit and two lower-molecular-weight subunits of approximately 34 and 38 kilodaltons (20; W. A. Petri and J. I. Ravdin, unpublished observations). Plant lectins such as concanavalin A and phytohemagglutinin have been shown to induce transient increases in $[Ca^{2+}]_i$ but only by a modest 1.5- to 2-fold (9, 10, 39). Mitogen-induced increases in $[Ca^{2+}]_i$ are apparently due to mobilization from intracellular pools (9). The magnitude of elevation in CHO cell $[Ca^{2+}]_i$ induced by the Gal/GalNAc adherence lectin suggests that the elevation is not simply a manifestation of cross-linking and capping of CHO cell surface receptors, but that it results from a cytotoxic activity. Given the findings with many diverse cytotoxins, the effect of the Gal/GalNaC adherence lectin on target CHO cell $[Ca^{2+}]_i$ does not necessarily indicate that it has ionophore properties.

Lynch et al. (15) and Young et al. (49) described an amebic ionophore protein of 13- to 15-kilodalton size, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which is apparently packaged in dense intracellular aggregates (32, 48). The amebic pore-forming protein induces lipid vesicles or planar bilayers to become leaky to Na^+ and K^+ ions and, to a lesser extent, to Ca^{2+} ions (15, 19, 32, 48) and can depolarize erythrocytes (48). Although the 13-kilodalton amebic ionophore protein is a possible mediator of the *E. histolytica*-induced rise in target cell $[Ca^{2+}]_i$, it is unlikely to be responsible for our observations with the purified Gal/GalNAc adherence lectin. This is due to the specificity of the monoclonal antibody used in purification, the lack of low-molecular-weight proteins (<30 kilodaltons) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified lectin (20), and the galactose-specific inhibition observed. However, at this time, we cannot rule out the possibility that the Gal/GalNAc adherence lectin contains the pore-forming ionophore as a subunit.

E. histolytica cytolytic activity requires binding by the amebic Gal/GalNAc adherence lectin (20, 25, 28), maintenance of an acid pH in amebic endocytic vesicles (30), amebic microfilament function (25, 28), and activity of a membrane-associated calcium-dependent phospholipase A enzyme (14, 27) and can be stimulated by the addition of phorbol esters (42a). The present report indicates that $[Ca^{2+}]_i$ was not an important second messenger in the initiation of amebic cytolytic activity, but that oscillatory increases in $[Ca^{2+}]_i$ may be required for parasite motility, chemotaxis, and normal cytoskeletal function (40). A marked increase in target cell $[Ca^{2+}]_i$ rapidly followed ame-

bic contact with binding by the Gal/GalNAc adherence lectin. Increased [Ca^{2+}]$_i$ appeared to be contributory to death of the target cell; however, diverse mechanisms could lead to this final pathway. The amebic Gal/GalNAc adherence lectin molecule may be solely responsible for amebic in vitro cytolytic activity; other parasite factors may only be important in relation to the optimum presentation and delivery of this lectin cytotoxin. Alternatively, additional amebic toxins, such as the low-molecular-weight ionophore protein (15, 49), acid optimal lysosomal enzymes (30), or phospholipases (14) may also be directly contributory.

ACKNOWLEDGMENTS

We thank Daniel Sawyer and Teizo Murata for their advice during the performance of these studies and Holly Gilbert and Cynthia Kogut for secretarial assistance.

This work was supported by Public Health Service grants AI-18841 to J.I.R. and AI-09504 to G.L.M. and training grant AI-07046 from the National Institute of Allergy and Infectious Diseases and a grant from the Lucille P. Markey Charitable Trust to W.A.P. W.A.P. is a Lucille P. Markey Scholar. The Division of Geographic Medicine was supported in part by the Rockefeller Foundation.

LITERATURE CITED

1. **Bailey, G. B., G. J. Leitch, and D. B. Day.** 1985. Chemotaxis by *Entamoeba histolytica*. J. Protozool. **32**:3241.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Chadee, K., and E. Meerovitch.** 1984. The Mongolian gerbil (*Meriones unguiculatus*) as an experimental host for *Entamoeba histolytica*. Am. J. Trop. Med. Hyg. **33**:47–54.
4. **Chadee, K., W. A. Petri, D. J. Innes, and J. I. Ravdin.** 1987. Rat and human colonic mucins bind to and inhibit the adherence lectin of *Entamoeba histolytica*. J. Clin. Invest. **80**:1238–1244.
5. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. **72**:431–432.
6. **Farber, J. L.** 1981. The role of calcium in cell death. Life Sci. **29**:1289–1295.
7. **Farber, J. L.** 1982. Membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. Lab. Invest. **47**: 114–123.
8. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260**:3440–3450.
9. **Hesketh, T. R., J. P. Moore, J. D. H. Morris, M. V. Taylor, J. Rogers, G. A. Smith, and J. C. Metcalfe.** 1985. A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. Nature (London) **313**:481–484.
10. **Hesketh, T. R., G. A. Smith, J. P. Moore, M. V. Taylor, and J. C. Metcalfe.** 1983. Free cytoplasmic calcium concentration and the mitogenic stimulation of lymphocytes. J. Biol. Chem. **258**:4876–4882.
11. **Kane, A. B., E. E. Young, F. A. X. Schanne, and J. L. Farber.** 1980. Calcium dependence of phalloidin-induced liver cell death. Proc. Natl. Acad. Sci. USA **77**:1177.
12. **Lemasters, J. J., J. DiGuiseppi, A. L. Nieminen, and B. Herman.** 1987. Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. Nature (London) **325**:78–81.
13. **Lew, D. P., T. Anderson, J. Hed, F. DiVirgilio, T. Pozzan, and O. Stendahl.** 1985. Ca^{2+}-dependent and Ca^{2+}-independent phagocytosis in human neutrophils. Nature (London) **315**: 509–512.
14. **Long-Krug, S. A., R. M. Hysmith, K. J. Fischer, and J. I. Ravdin.** 1985. The phospholipase A enzymes of *Entamoeba histolytica*: description and subcellular localization. J. Infect. Dis. **152**:536–541.
15. **Lynch, E. C., I. M. Rosenberg, and C. Gitler.** 1982. An ion-channel forming protein produced by *Entamoeba histolytica*. EMBO J. **1**:801–804.
16. **Mattern, C. F. T., D. B. Keister, and P. A. Caspar.** 1978. Experimental amebiasis. III. A rapid *in vitro* assay for virulence of *Entamoeba histolytica*. Am. J. Trop. Med. Hyg. **27**:882–887.
17. **McNeil, P. L., J. A. Swanson, S. D. Wright, S. C. Silverstein, and D. L. Taylor.** 1986. Fc-receptor-mediated phagocytosis occurs in macrophages without an increase in average [Ca^{++}]$_i$. J. Cell Biol. **102**:1586–1592.
18. **Meldrum, B. S.** 1986. Cell damage in epilepsy and the role of calcium in cytotoxicity. Adv. Neurol. **44**:849–855.
19. **Murata, T., J. A. Sullivan, D. W. Sawyer, and G. L. Mandell.** 1987. Influence of type and opsonization of ingested particle on intracellular free calcium distribution and superoxide production by human neutrophils. Infect. Immun. **55**:1784–1791.
20. **Petri, W. A., R. D. Smith, P. H. Schlesinger, and J. I. Ravdin.** 1987. Isolation of the galactose-binding lectin which mediates the *in vitro* adherence of *Entamoeba histolytica*. J. Clin. Invest. **80**:1245–1254.
21. **Poenie, M., R. Y. Tsien, and A.-M. Schmitt-Verhulst.** 1987. Sequential activation and lethal hit measured by [Ca^{2+}]$_i$ in individual cytolytic T cells and targets. EMBO J. **6**:2223–2232.
22. **Pollack, H.** 1928. Microsurgical studies in cell physiology. VI. Calcium ions in living protoplasm. J. Gen. Physiol. **11**:539–545.
23. **Ravdin, J. I.** 1986. Pathogenesis of disease with *Entamoeba histolytica*. Studies of adherence toxins and contact-dependent cytolysis. Rev. Infect. Dis. **8**:247–260.
24. **Ravdin, J. I., B. Y. Croft, and R. L. Guerrant.** 1980. Cytopathogenic mechanisms of *Entamoeba histolytica*. J. Exp. Med. **152**:377–390.
25. **Ravdin, J. I., and R. L. Guerrant.** 1981. The role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*. Study with mammalian tissue culture cells and human erythrocytes. J. Clin. Invest. **68**:1305–1313.
26. **Ravdin, J. I., J. E. John, L. I. Johnston, D. J. Innes, and R. L. Guerrant.** 1985. Adherence of *Entamoeba histolytica* trophozoites to rat and human colonic mucosa. Infect. Immun. **48**: 292–297.
27. **Ravdin, J. I., C. F. Murphy, R. L. Guerrant, and S. A. Long-Krug.** 1985. Effect of calcium and phospholipase A antagonists on the cytopathogenicity of *Entamoeba histolytica*. J. Infect. Dis. **152**:542–549.
28. **Ravdin, J. I., C. F. Murphy, R. A. Salata, R. L. Guerrant, and E. L. Hewlett.** 1985. N-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*. I. Partial purification and relation to amebic virulence *in vitro*. J. Infect. Dis. **151**: 804–815.
29. **Ravdin, J. I., W. A. Petri, C. F. Murphy, and R. D. Smith.** 1986. Production of mouse monoclonal antibodies which inhibit *in vitro* adherence of *Entamoeba histolytica* trophozoites. Infect. Immun. **53**:1–5.
30. **Ravdin, J. I., P. H. Schlesinger, C. F. Murphy, I. Y. Gluzman, and D. J. Krogstad.** 1986. Acid intracellular vesicles and the cytolysis of mammalian target cells by *Entamoeba histolytica* trophozoites. J. Protozool. **33**:478–486.
31. **Ravdin, J. I., N. Sperelakis, and R. L. Guerrant.** 1982. Effect of ion channel inhibitors on the cytopathogenicity of *Entamoeba histolytica*. J. Infect. Dis. **146**:335–339.
32. **Rosenberg, I., and C. Gitler.** 1985. Subcellular fractionation of amoebapore and plasma membrane components of *Entamoeba histolytica* using self-generating percoll gradients. Mol. Biochem. Parasitol. **14**:231–248.
33. **Salata, R. A., R. D. Pearson, and J. I. Ravdin.** 1985. Interaction of human leukocytes with *Entamoeba histolytica*: killing of virulent amebae by the activated macrophage. J. Clin. Invest. **76**:491–499.
34. **Salata, R. A., and J. I. Ravdin.** 1986. The interaction of human neutrophils and *Entamoeba histolytica* increases cytopathogenicity for liver cell monolayers. J. Infect. Dis. **154**:19–26.
35. **Sawyer, D. W., J. A. Sullivan, and G. L. Mandell.** 1985. Intracellular free calcium localization in neutrophils during phagocytosis. Science **230**:663–666.
36. **Schanne, F. A. X., A. B. Kane, E. E. Young, and J. L. Farber.**

1979. Calcium dependence of toxic cell death: a final common pathway. Science **206**:700–702.

37. **Schlaepfer, W. W., and L. A. Freeman.** 1980. Calcium-dependent degradation of mammalian neurofilaments by soluble tissue factors from rat spinal cord. Neuroscience **5**:2305–2314.
38. **Schlegel, W., B. P. Winiger, P. Mollard, P. Vacher, F. Wuarin, G. R. Zahnd, C. B. Wollheim, and B. Dufy.** 1987. Oscillations of cytosolic Ca^{2+} in pituitary cells due to action potentials. Nature (London) **329**:719–721.
39. **Scully, S. P., G. B. Segel, and M. A. Lichtman.** 1984. Calcium exchange and ionized cytoplasmic calcium in resting and activated human monocytes. J. Clin. Invest. **74**:589–599.
40. **Taylor, D. L., J. R. Blinks, and G. Reynolds.** 1980. Contractile basis of ameboid movement. VIII. Aequorin luminescence during ameboid movement, endocytosis, and capping. J. Cell Biol. **86**:599–607.
41. **Tsutsumi, V., R. Mena-Lopez, F. Anaya-Velazquez, and A. Martinez-Palomo.** 1984. Cellular basis of experimental amebic liver abscess formation. Am. J. Pathol. **117**:81–91.
42. **Walsh, J. A.** 1986. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. Rev. Infect. Dis. **8**:228–238.

42a. **Weikel, C. S., C. F. Murphy, E. Orozco, and J. I. Ravdin.** 1988. Phorbol esters specifically enhance the cytolytic activity of *Entamoeba histolytica*. Infect. Immun. **56**:1485–1491.

43. **Wier, W. G., M. B. Cannell, J. R. Berlin, E. Marban, and W. J. Lederer.** 1987. Cellular and subcellular heterogeneity of $[Ca^{++}]_i$ in single heart cells revealed by Fura-2. Science **235**:325–327.
44. **William, D. A., K. E. Fogarty, R. Y. Tsien, and F. S. Fay.** 1985. Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. Nature (London) **318**:558–562.
45. **Williams, D. A., P. L. Becker, and F. S. Fay.** 1987. Regional changes in calcium underlying contraction of single smooth muscle cells. Science **235**:260–265.
46. **Wilson, H. A., D. Greenblatt, M. Poenie, F. D. Finkelman, and R. Y. Tsien.** 1987. Crosslinkage of B lymphocyte surface immunoglobulin by anti-Ig or antigen induces prolonged oscillation of intracellular ionized calcium. J. Exp. Med. **166**:601–606.
47. **Woods, N. M., K. S. R. Cuthbertson, and P. H. Cobbold.** 1986. Repetitive transient rises in cytoplasmic free calcium in hormone-stimulated hepatocytes. Nature (London) **319**:600–602.
48. **Young, J. D. E., and Z. A. Cohn.** 1985. Molecular mechanisms of cytotoxicity mediated by *Entamoeba histolytica*: characterization of a pore-forming protein (PFP). J. Cell Biol. **29**:299–308.
49. **Young, J. D. E., T. M. Young, L. P. Lu, J. C. Unkeless, and Z. A. Cohn.** 1982. Characterization of a membrane pore-forming protein from *Entamoeba histolytica*. J. Exp. Med. **156**: 1677–1690.