Brief Definitive Report

Cytosolic-free Calcium Elevation in *Trypanosoma cruzi* Is Required for Cell Invasion

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Summary

To replicate, the trypomastigote form of *Trypanosoma cruzi* must invade host cells. Since a role for Ca^{2+} in the process of cell invasion by several intracellular parasites has been postulated, changes in the intracellular Ca^{2+} concentration in *T. cruzi* trypomastigotes and in tissue culture L_6E_9 myoblasts during their interaction were studied at the single cell level using digital imaging fluorescence microscopy or in cell suspensions by fluorescence spectrophotometry. An increase in cytosolic Ca^{2+} in *T. cruzi* trypomastigotes was detected at the single cell level after association of the parasites with the myoblasts. Ca^{2+} mobilization in the host cells was also detected upon contact with trypomastigotes either at the single cell level or in cells grown in coverslips and exposed to suspensions of trypomastigotes. Pretreatment of the parasites with the Ca^{2+} chelators quin 2 (50 μM) or bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA, 50 μM) decreased the trypomastigotes' association to myoblasts by ~40 and 63%, respectively, thus indicating that an increase in intracellular Ca^{2+} concentration in the parasites is required for cell invasion in addition to Ca^{2+} mobilization in the host cells.

Trypanosoma cruzi is a protozoan parasite that infects a wide variety of vertebrates and is the agent responsible for Chagas' disease in humans. *T. cruzi* is an obligatory intracellular parasite. Current evidence indicates that *T. cruzi* penetrates cultured mammalian cells by a unique mechanism distinct from phagocytosis (1–8). Host cell lysosomes cluster underneath the parasite's attachment site at the plasma membrane, and lysosomal fusion occurs at very early stages of the invasion process (7, 8). Immediately after invasion, the parasite is found inside a vacuole in both phagocytic (macrophages) and nonphagocytic cells such as fibroblasts, epithelial, endothelial, muscle, and nerve cells. Soon after entry (within 1–2 h), trypomastigotes escape from the vacuole into the cytosol and transform into the roundish amastigotes, which can replicate by binary fission (1).

Since its discovery in 1808 by Humphry Davy, the calcium ion has been found to be critically involved in an increasing number of vital cell functions, including growth and differentiation, motility and contractility, endocytosis, exocytosis and secretion, and regulation of intermediary metabolism (9). Recent observations showed that *T. cruzi* trypomastigotes or their isolated membranes induce repetitive cytosolic-free Ca^{2+} transients in individual normal rat kidney fibroblasts, in a pertussis toxin–sensitive manner (10) and it has been postulated that a trypomastigote membrane factor triggers cytosolic-free Ca^{2+} transients in host cells through a G-protein–coupled pathway (10). Cytosolic-free Ca^{2+} transients may be required for local rearrangement of the cortical actin cytoskeleton allowing lysosome access to the plasma membrane, and lysosome fusion at the site of trypanosome entry (10). Although a role of Ca^{2+} in the process of cell invasion by different parasites such as *T. cruzi* (11–13), *Plasmodium falciparum* (14), and *Leishmania donovani* (15) had been postulated before on the basis of the observation of an increase in cytosolic Ca^{2+} concentration in the host cells after prolonged periods of intracellular presence of parasites, only recent reports (10, 16) have described an early Ca^{2+} signal triggered by parasites.

In this study we demonstrate that in addition to an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the host cells, an increase in cytosolic Ca^{2+} also occurs in *T. cruzi* trypomastigotes after their association to the host cells and that buffering increases in cytosolic Ca^{2+} of trypomastigotes by $[Ca^{2+}]_i$ chelators results in an inhibition of cellular invasion.

Materials and Methods

Culture Methods. *T. cruzi* trypomastigotes (Y strain) were obtained from the culture medium of L_6E_9 myoblasts by a modification of the method of Schmatz and Murray (17) as we have described before (18). The final concentration of trypomastigotes was determined using a Neubauer chamber. The contamination of trypomastigotes with amastigotes and intermediate forms was always <5%. L_6E_9 myoblasts were grown as described before (19).

Chemicals. EGTA, potassium cyanide (KCN), and Dulbecco's PBS were purchased from Sigma Chemical Co. (St. Louis, MO). The tetraacetoxymethyl esters of fura 2 (1-[2-(5-carboxyoxazol-2-

yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid), BAPTA (bis)-*o*-aminophenoxy)-ethane-*N,N,N',N'*,-tetraacetic acid), and quin 2 (2-{[2-bis(carboxymethyl)-amino-5-methylphenoxyl]-methyl}-6-methoxy-8-bis(carboxymethyl)-aminoquinoline), fura 2 AM, BAPTA AM, and quin 2 AM, respectively, were from Molecular Probes, Inc. (Eugene, OR). All other reagents were analytical grade.

Digital Imaging Fluorescence Microscopy. Loading of trypomastigotes with fura 2 was as described before (18). Cells (15 μl of trypomastigotes suspension) were placed on a slide and observed with a microscope (Leitz Orthoplan) equipped with a 150-W xenon arc epi-illuminator, quartz optical elements, and a glycerine immersion objective (100× UV; all from Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, NJ). Loading of L_6E_9 myoblasts (grown in coverslips for 2 d, four coverslips per petri dish) was at 37°C for 30 min in the presence of 3 μM fura 2. After loading, the cells were washed three times with Dulbecco's PBS. Culture medium containing 5 × 10^7 trypomastigotes (loaded or not with fura 2) per petri dish were added to loaded or unloaded myoblasts, and the cells were incubated at 37°C in a CO_2 incubator (95% air/5% CO_2) until analysis. A confluent monolayer of myoblasts in a coverslip was placed on a slide and observed with the microscope. Fluorescence images were collected with a system consisting of a cooled camera head (model CH250; Photometrics Ltd., Tucson, AZ) with a charge-coupled device chip (CCD type KAF 1400; Eastman Kodak, Rochester, NY, 1,348 × 1,035 pixels), an electronic unit (model CE 200 A equipped with a 50-Hz 16-bit A/D converter) and a controller board (model NU 200; both from Photometrics Ltd.). Images were acquired and evaluated by a software package (IPLab, Signal Analytics, Vienna, VA) on a Macintosh IIc computer (Apple Computer, Inc., Cupertino, CA). Cells were excited first at 340 nm, then at 380 nm; fluorescence images were separated in time by <1 min. The raw fluorescence image was digitized to a pixel assay. Point density readings were taken for each image, and a visual display of the 340–380-nm ratio was produced. Ratios of fluorescence with excitation at 340 and 380 nm were calibrated by comparison to a standard curve of fluorescence ratios of a set of solutions of 25 μM fura 2 (free acid) dissolved in calcium/EGTA buffer solutions. The Ca^{2+} concentration of these solutions was calculated employing an iterative computer program as described before (18).

Buffering of Cytosolic Ca^{2+}. To buffer elevations in cytosolic Ca^{2+}, purified trypomastigotes were washed twice in a buffer containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 mM Hepes, pH 7.0 (buffer A), adjusted to 5 × 10^7 cells per ml, and preloaded with the Ca^{2+} chelators quin 2 or BAPTA (20). Cells were loaded with 50 μM quin 2 AM or the same concentration of BAPTA for 1 h in a water bath at 37°C with mild agitation in the buffer described above with the addition of 1.5% sucrose (18). Subsequently, the cells were washed twice with ice-cold buffer A to remove extracellular dye. Cells loaded with quin 2 or BAPTA did not show any significant decrease in motility over a period of 4 h. To measure cytosolic Ca^{2+} concentration in the presence of buffering concentrations of the Ca^{2+} chelators, trypomastigotes were loaded with fura 2 AM as described above and then exposed to 50 μM of BAPTA AM for 1 h at 37°C as described above. Therefore, cytosolic Ca^{2+} was measured either in untreated cells or in cells exposed to 1 mM KCN, which is known to increase the $[Ca^{2+}]_i$ concentration of untreated parasites (21).

Invasion of Myoblasts Assay. Coverslips with attached L_6E_9 myoblasts were incubated with control and quin 2– or BAPTA-preloaded trypomastigotes for 4 h at 37°C in a CO_2 incubator. Four coverslips for each experimental point were separately washed in cold Hanks' solution, air-dried, fixed in absolute methanol, and stained with Giemsa. Parasite invasion was assessed by the percentage of myoblasts associated with parasites (i.e., parasites surface bound and internalized). A minimum of 200 cells were screened in each culture (22).

Determination of $[Ca^{2+}]_i$ by Spectrofluorometry. Experiments were performed in cuvettes placed in a thermostated (37°C) spectrofluorometer (model F-2000; Hitachi Instruments Inc., Danbury, CT) with 2.5 ml of incubation medium containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1 mM $CaCl_2$, and 50 mM Hepes, pH 7.0 (buffer B). *T. cruzi* trypomastigotes loaded as described before (18) were exposed to suspensions of L_6E_9 myoblasts or to myoblasts grown in coverslips. L_6E_9 myoblasts grown and isolated as described before (18, 19), were washed twice in buffer B and added directly to the trypomastigote suspension in the cuvette under continuous stirring. In other experiments the myoblasts were grown to confluence (about 10^6 cells/cm^2) on rectangular glass coverslips (2.2 × 0.9 cm) as indicated above, washed twice with buffer B, and added to the trypomastigotes suspensions or were loaded with fura 2 as described above, washed twice in buffer B, and exposed to unloaded trypomastigotes suspensions. The coverslips were mounted at an approximately 30° angle relative to the light beam in a special holder in a standard fluorimeter cuvette equipped with magnetic stirring. Excitation was at 340 and 380 nm and emission was at 510 nm. The fura 2 fluorescence response to $[Ca^{2+}]_i$ concentration was calibrated from the ratio of 340:380 nm fluorescence values after substraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. (23). Other experimental conditions and calibrations were as described before (18, 23, 24).

Results and Discussion

As shown by the pseudocolor ratio image in Fig. 1 *A*, when fura 2 was loaded into *T. cruzi* trypomastigotes by incubation with the acetoxymethylester derivative as described under Materials and Methods, a heterogeneous pattern of apparent $[Ca^{2+}]_i$ was observed in dried preparations, with discrete regions of higher Ca^{2+} concentration. In this regard, by using energy-dispersive x-ray elemental mapping, major accumulations of calcium were localized in organelles identifiable by scanning transmission electron microscopy throughout the body of *T. cruzi* epimastigotes with major concentrations in both the anterior and posterior regions (25). A $[Ca^{2+}]_i$ of about 20–30 nM was found in the cytosol of most of the trypomastigotes which compares favorably with the value that we detected previously using spectrofluorometric techniques (18).

Changes of $[Ca^{2+}]_i$ in the trypomastigotes during their contact with tissue culture myoblasts were studied at the single cell level. Trypomastigote suspensions were added to slides containing coverslips with confluent monolayers of L_6E_9 myoblasts and their interaction was followed by phase contrast and fluorescence microscopy after different times of incubation. It was observed that initial association of fura 2–loaded trypomastigotes to myoblasts increased the fluorescence of their cytoplasm when measured at 340 nm (Fig. 1 *B*). Ratio imaging is not shown in Fig. 1 *B* because of the high motility of the portions of the trypomastigotes bodies that were not associated to the myoblasts. After 1–2 h of contact of

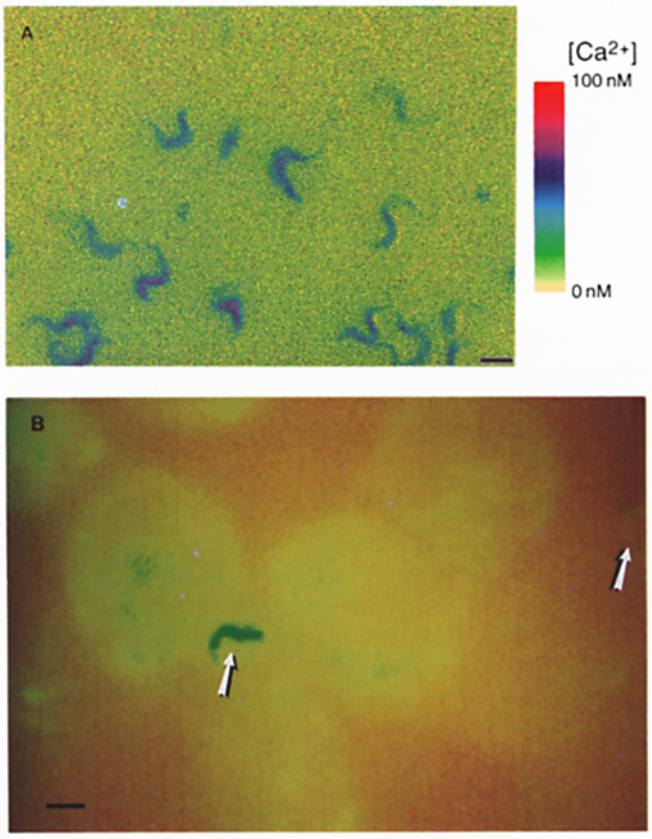

Figure 1. Digitized fluorescence images of fura 2–loaded trypomastigotes. (*A*) Digitized images ($R_{340/380}$) of fura 2–loaded trypomastigotes in suspension. Cells (15 μl of trypomastigotes suspension) were placed on a slide and observed with a microscope. Fluorescence images were collected as described under Materials and Methods. Since the trypomastigotes were highly motile, the images were taken immediately after the preparation was dried. (*B*) Digitized fluorescence image (340 nm) or fura 2–loaded trypomastigotes in contact with fura 2–loaded myoblasts. A confluent myoblast monolayer of fura 2–loaded myoblasts in a coverslip was placed on a slide and observed with a microscope as described under Materials and Methods. The image shows a highly fluorescent trypomastigote associated to a myoblast, and a free trypomastigote (*arrows*). Bars, 2 μm.

fura 2–loaded trypomastigotes with myoblasts, it was possible to identify the intracellular parasites in each of the slides examined by fluorescence microscopy, since they were highly fluorescent (Fig. 2 *A*), in contrast to extracellular, nonassociated parasites (Fig. 1, *A* and *B*). To investigate if changes in $[Ca^{2+}]_i$ also occurred in the host cells as has been reported before (10–13), both trypomastigotes and myoblasts were loaded with fura 2. In some cells, only the parasites were highly fluorescent (Fig. 2 *A*). Since parasite motility markedly decreases intracellularly (26), ratio imaging was possible as shown by the pseudocolor ratio image of Fig. 2 *B*. The cytosolic Ca^{2+} concentration of trypomastigotes was about 100 nM, which was three to five times higher than in the extracellular, nonassociated parasites (Fig. 1 *A*). A $[Ca^{2+}]_i$ of about 20–30 nM was found in most of the myoblasts. Because calibrations were performed against bulk solutions, this value was substantially lower than would be determined from in-cell calibrations (13, 23). Some myoblasts showed a higher $[Ca^{2+}]_i$ around the intracellular parasites, and there was an apparent spreading of the elevated $[Ca^{2+}]_i$ into the remainder of the cell (Fig. 2 *D*). Control incubations containing fura 2–loaded trypomastigotes and unloaded myoblasts showed only highly fluorescent trypomastigotes inside the myoblasts. No changes in the regions of the myoblasts surrounding the parasites were detected (data not shown). Controls with only fura 2–loaded myoblasts showed only patches of fluorescence

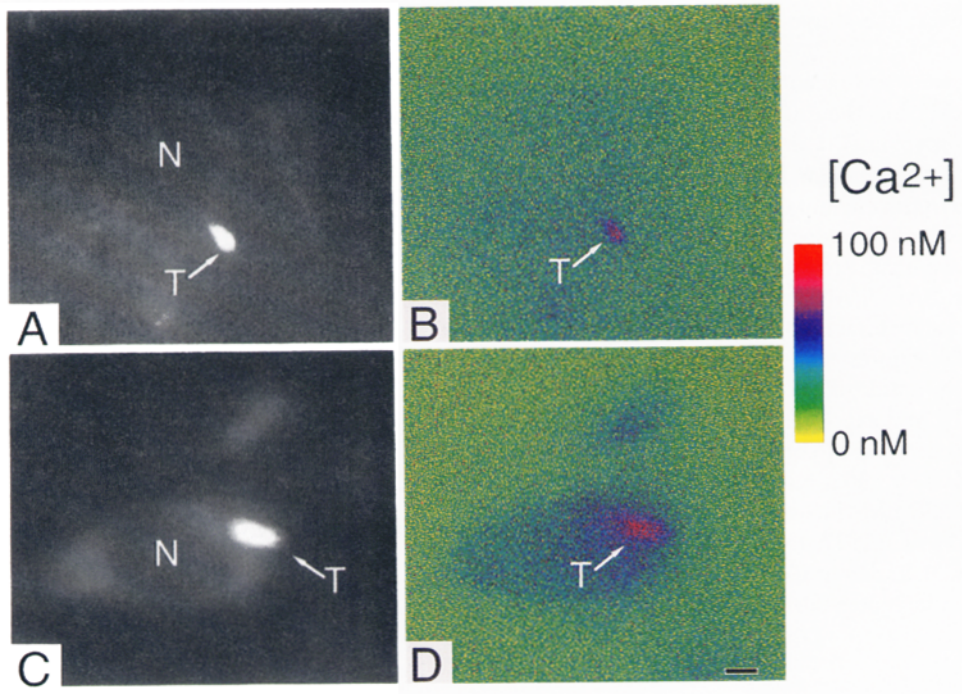

Figure 2. Digitized images of fura 2–loaded trypomastigotes after internalization into fura 2–loaded L_6E_9 myoblasts. Experimental details are as under Materials and Methods. (*A* and *C*) Total fluorescence. (*B* and *D*) Digitized $R_{340/380}$ images. (*A* and *B*) Images of a preparation incubated for 1 h in the presence of trypomastigotes. (*C* and *D*) Images of a preparation incubated for 2 h in the presence of trypomastigotes showing a myoblast infected with a trypomastigote that is intensively fluorescent. There is also an increase in fluorescence in the region of the myoblast surrounding the trypomastigote. Bar, 2 μm.

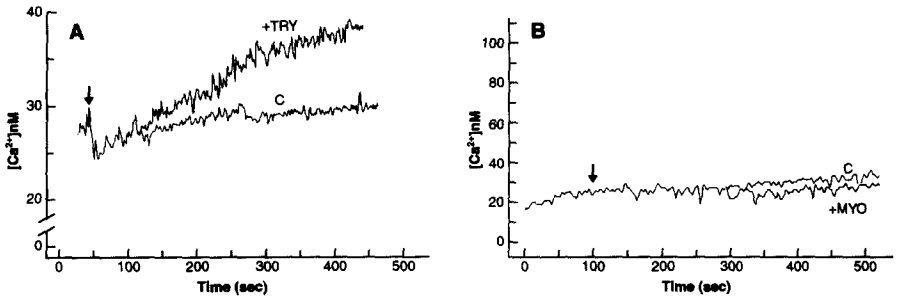

Figure 3. $[Ca^{2+}]_i$ concentration during *T. cruzi*–myoblast interaction. (*A*) Effect of trypomastigotes addition on the $[Ca^{2+}]_i$ of confluent fura 2–loaded myoblasts. Additions were where indicated by the arrow (*+TRY*, addition of 5×10^7 trypomastigotes/ml; *c*, control with the same volume of buffer added). (*B*) Effect of myoblasts addition on the $[Ca^{2+}]_i$ of fura 2–loaded trypomastigotes (5×10^7 cells/ml). Additions were where indicated by the arrow (*+MYO*, addition of 10^6 myoblasts/ml; *c*, control with the same volume of buffer added). These experiments are representative of at least three different experiments with different cell preparations.

in some cells apparently associated with parasites (data not shown).

T. cruzi trypomastigotes have been shown to induce repetitive cytosolic-free Ca^{2+} transients in individual normal rat kidney cells loaded with fluo-3 within 200 s of exposure, suggesting that they were triggered by parasite attachment to individual cells (10). We confirmed those results by spectrofluorometry using fura 2–loaded myoblasts challenged with trypomastigotes. An increase in $[Ca^{2+}]_i$ was detected 100–200 s after the exposure of myoblasts to a suspension of trypomastigotes (Fig. 3 *A*). However, when fura 2–loaded trypomastigotes were exposed to suspensions of myoblasts (Fig. 3 *B*) or to myoblasts grown on coverslips in equivalent concentrations (data not shown), no increase in the trypomastigotes $[Ca^{2+}]_i$ was detected for at least 30 min. These results indicate that Ca^{2+} mobilization initially occurs in the host cells upon attachment of trypomastigotes, as suggested before (10), and that the trypomastigotes increase in $[Ca^{2+}]_i$ occurs only after their association to the host cells. Since invasion of host cells by *T. cruzi* trypomastigotes does not occur in all cells of the population and is asynchronous, this increase in $[Ca^{2+}]_i$ is only detectable at the single cell level (Fig. 1 *B* and Fig. 2).

When *T. cruzi* trypomastigotes were preloaded with the $[Ca^{2+}]_i$ chelator quin 2-AM (50 μM) and then used to infect confluent monolayers of L_6E_9 myoblasts, a decrease in the association of the parasites with the myoblasts was observed (Table 1). To investigate if the impairment in the ability of trypomastigotes to infect the myoblasts was associated with a lowering of cytosolic Ca^{2+} level, we incubated trypomastigotes with another $[Ca^{2+}]_i$ chelator, BAPTA AM, which has a similar molecular structure and a comparable affinity for Ca^{2+} as does quin 2, but has weak fluorescence emission (20). We could then use fura 2 as an indicator for cytosolic Ca^{2+} in cells preloaded with BAPTA and exposed to KCN. Under these conditions, Ca^{2+} concentration in control cells was 20–30 nM. After exposure to KCN for 30 min, the cytosolic Ca^{2+} concentration was 100–120 nM. No significant changes were observed in the $[Ca^{2+}]_i$ of BAPTA-loaded trypomastigotes after exposure to KCN under similar conditions. Similar to pretreatment with quin 2, pretreatment with BAPTA partially impaired the ability of trypomastigotes to associate with myoblasts (Table 1).

In conclusion, an increase in the trypomastigotes $[Ca^{2+}]_i$ appears to follow parasite association to the host cells and is apparently required for invasion. The evidence in favor of this hypothesis is the following: first, there is an increase in $[Ca^{2+}]_i$ in trypomastigotes upon association with the myoblasts (Fig. 1 *B*); second, it is possible to differentiate between intracellular and nonassociated parasites because intracellular parasites are highly fluorescent (Fig. 2 *A*); and third, pretreatment of the parasites with the Ca^{2+} chelators quin 2 or BAPTA buffered $[Ca^{2+}]_i$ and decreased myoblasts invasion by ~40 and 63%, respectively (Table 1). Finally, other authors (27) have recently observed that pretreatment with the Ca^{2+} ionophore ionomycin, which elevated $[Ca^{2+}]_i$ in trypomastigotes, significantly enhanced the infective capacity of the parasites.

Although previous reports have indicated an increase in cytosolic Ca^{2+} in the host cells after *T. cruzi* invasion, none has reported an increase in $[Ca^{2+}]_i$ in the trypomastigotes. Morris et al. (11), found an increase in $[Ca^{2+}]_i$ of fura 2–loaded umbilical vein endothelial cells (from 55 ± 18 nM to 110 ± 20 nM) 4 d after infection, well after transformation of trypomastigotes into amastigotes, when amastigotes were actively duplicating in the host cells. On the other hand, Low et al. (13) found an increase in $[Ca^{2+}]_i$ in fura 2–loaded BSC-1 cells 1 d after infection (the earliest time point examined) and a subsequent decrease up to about 5 d of infection to a minimum of about 7 nM. Osuna et al. (12) found a threefold increase in $[Ca^{2+}]_i$ of aequorin-loaded HeLa cells after 1-h interaction with trypomastigotes. Treatment of the host

Table 1. *Effect of Quin 2 and BAPTA Preloading on the Interaction between Trypomastigotes and Myoblasts*

Chelator	Percent myoblasts associated with *T. cruzi* (mean ± SEM) (% R)*
None	55.3 ± 6.8
Quin 2	33.5 ± 8.3 (40)
BAPTA	20.2 ± 3.6 (63)

* R, Percent reduction compared to control (no chelator treated).

cells with EDTA, used as Ca^{2+} chelator, or verapamil, used as a calcium channel blocker, before and during their interaction with trypomastigotes, reduced the parasitization percentages by *T. cruzi* to about 72 and 55%, respectively (12). Recently, Tardieux et al. (10), using fluo 3–loaded NRK fibroblasts, found that trypomastigotes induce an increase in $[Ca^{2+}]_i$ in the host cells within 200 s of interaction. Parasite entry was inhibited by buffering or depleting host cell cytosolic Ca^{2+} with MAPTA, or calcium ionophore A-23187 in a Ca^{2+}-free medium containing 5 mM EGTA, respectively, or by pretreatment of the host cells with Ca^{2+} channel blockers, such as verapamil. We confirmed this early increase in $[Ca^{2+}]_i$ in myoblasts loaded with fura 2 upon contact with trypomastigotes (Fig. 3 *A*). In addition, we found that myoblasts showed a higher $[Ca^{2+}]_i$ around intracellular trypomastigotes (Fig. 2 *D*). Although we cannot rule out that this increase may represent remains of an early host cell response, the apparent spreading of the elevated $[Ca^{2+}]_i$ from the region surrounding the nonmotile trypomastigotes (and therefore intracellular; 26) into the remainder of the cell, suggests that it occurs after internalization. This would agree with other reports indicating increases in $[Ca^{2+}]_i$ in the host cells several minutes or hours after interaction with trypomastigotes (11–13) and would indicate the existence of multiple Ca^{2+} transients in host cells during infection. In summary, it appears to be well established that Ca^{2+} is an important signal for both trypomastigotes and host cells during their interaction.

We thank Professor Gregorio Weber, from the Department of Biochemistry, University of Illinois, for the use of the digital fluorescence microscopy imaging system and for his encouragement, and Drs. Norma Andrews and Felipe Kierszenbaum for communicating their results before publication.

This work was supported by a grant from the National Institutes of Health (AI-23259) to R. Docampo and S. N. J. Moreno. J. Silva was supported by a grant from the National Institutes of Health (GM-11223 to G. Weber) and is a John Simon Guggenheim Fellow. A. E. Vercesi is a Biotechnology Career Fellow of the Rockefeller Foundation.

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Received for publication 8 February 1994 and in revised form 3 June 1994.

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