Role in Host Cell Invasion of Trypanosoma cruzi-induced Cytosolic-free Ca\(^{2+}\) Transients

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Summary

Trypanosoma cruzi enters cells by a unique mechanism, distinct from phagocytosis. Invasion is facilitated by disruption of host cell microfilaments, and involves recruitment and fusion of host lysosomes at the site of parasite entry. These findings implied the existence of transmembrane signaling mechanisms triggered by the parasites in the host cells before invasion. Here we show that infective trypomastigotes or their isolated membranes, but not the noninfective epimastigotes, induce repetitive cytosolic-free Ca\(^{2+}\) transients in individual normal rat kidney fibroblasts, in a pertussis toxin-sensitive manner. Parasite entry is inhibited by buffering or depleting host cell cytosolic-free Ca\(^{2+}\), or by pretreatment with Ca\(^{2+}\) channel blockers or pertussis toxin. In contrast, invasion is enhanced by brief exposure of the host cells to cytochalasin D. These results indicate that a trypomastigote membrane factor triggers cytosolic-free Ca\(^{2+}\) transients in host cells through a G-protein-coupled pathway. This signaling event may promote invasion through modulation of the host cell actin cytoskeleton.

Materials and Methods

Materials. Trypsin, soybean trypsin inhibitor (SBTI), pertussis toxin (PTX), choler toxin (CTx), epidermal growth factor (EGF), A23187, cytochalasin D, verapamil, NiCl\(_2\), Hepes and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO), and MAFIA-AM and fluo-3 were obtained from Molecular Probes, Inc. (Eugene, OR).

Cells and Parasites. Normal rat kidney (NRK) cells were grown in 10 mM Hepes-buffered DMEM containing 5% FBS, at 37°C in a 5% CO\(_2\) atmosphere. Trypomastigotes from the T. cruzi Y strain were obtained from the supernatant of infected LLC-MK2 supernatants (1, 12). Epimastigotes from the Y strain were cultured in liver infusion tryptose medium containing 10% FBS at 28°C (13). T. brucei procyclics Tat 1.1 were grown in Cunningham’s medium (14) supplemented with 10% FBS and 25 mM Hepes, at 28°C. For some experiments, parasites were killed by a 3-min incubation at 56°C. Parasites were washed in Hepes-buffered Ringer’s solution (15) and resuspended in the same solution at 10\(^6\)/ml for time lapse confocal microscopy or invasion experiments.

Time Lapse Confocal Microscopy. NRK rat fibroblasts seeded on glass coverslips at a density of 2.5 \(\times 10^5\)/cm\(^2\) were loaded with 5 \(\mu\)M fluo-3/AM for 30 min at 37°C (16), transferred to a heated chamber on the stage of a Zeiss Axiovert microscope, perfused at 37°C with Hepes-buffered Ringer’s solution, and observed using a confocal imaging system (MRC-600; Bio-Rad Laboratories, Cambridge, MA), taking optical sections of approximately 2 \(\mu\)m in thick-
ness (17). Parasites were introduced into the chamber in Ringer's solution at a density of $10^9$/ml. Images were recorded at a rate of one frame per second on an optical memory disk (model TQ3031F; Panasonic, Secaucus, NJ) and analyzed subsequently using an image processor (Series 151; Itex, Woburn, MA) (17).

Cell Treatments. NRK cells were pretreated with 0.4 μg/ml PTx for 4 h at 37°C, before exposure to parasites or isolated membranes. Parasite membranes were prepared as described previously (18) and resuspended in Ringer's buffer at $10^8$ parasite equivalents/ml. Isolated membranes were treated with 150 μg/ml trypsin for 30 min.

Figure 1. Subcellular changes in cytosolic Ca²⁺ in NRK cells exposed to distinct life cycle stages of T. cruzi. (a–d) Serial confocal microscopic images of NRK fibroblasts loaded with the Ca²⁺-sensitive dye fluo-3 and exposed to live, infective trypomastigotes. (a) Baseline image before exposure to T. cruzi. (b–d) Same field 200, 250, and 300 s after exposure to trypomastigotes. (e–h) Serial images of a different group of fibroblasts after exposure to live, noninfective epimastigotes. (e–g) Images before, 150, and 300 s after exposure to epimastigotes. (h) Same field after subsequent exposure to trypomastigote membranes. Fluorescence intensity in a–h are pseudocolored according to the color scale located below. Large increases in fluorescence intensity correspond to increasing [Ca²⁺], but subtle fluctuations in fluorescence intensity also occur because fluo-3 cannot be ratio-imaged (37). Scale bar (top left) is 25 μm. (i) Graphical representation of fluorescence intensity over time in rectangular regions within the two cells indicated in a–d (arrowheads). The increase in [Ca²⁺] occurs in each cell <100 s apart, in a repetitive and asynchronous fashion. (j) Graphical representation of fluorescence intensity over time in a rectangular region within the cell indicated in e–g (arrowhead). An increase in [Ca²⁺] occurred only after the cell was exposed to trypomastigote membranes.
at 37°C, followed by 150 μg/ml SBTI. Control membranes were treated only with SBTI. EGF was added in each experiment after about 4 min of image recording, at a final concentration of 5 nM.

**Invasion Assays.** The number of intracellular parasites was determined after a 20-min infection period, as detailed previously (1). Host cell pretreatments were performed at 37°C, as follows: 10 min with Ca²⁺-free medium, 1 h with 500 μM MAPT-AM, 5 min with 0.5 μM A23187, 15 min with 5 mM NiCl₂, 30 min with 100 μM verapamil, or 4 h with 0.4 μg/ml PTx or CTx. All drugs were removed before exposure of the cells to parasites in invasion assays. Where not stated, the free Ca²⁺ concentration in the medium was 1 mM. The Ca²⁺-free medium contained 5 mM EGTA. Ca²⁺ conditions in the medium were kept constant during host cell pretreatment and infection.

**Results and Discussion**

The effects of two distinct life cycle stages of *T. cruzi* on Ca²⁺ signaling in NRK fibroblasts were examined using confocal video microscopy. Shortly after being introduced to the chamber, the infective trypomastigote forms induced repetitive increases in cytosolic-free calcium ([Ca²⁺]ᵢ) in intracellular parasites were examined using confocal video microscopy. Shortly after being introduced to the chamber, the infective trypomastigote forms induced repetitive increases in cytosolic-free calcium ([Ca²⁺]ᵢ) in

![Graphical representation of fluorescence intensity over time in single NRK cells after: (a) no pretreatment and exposure to isolated trypomastigote membranes. Note repetitive increases in [Ca²⁺]ᵢ occurring 100 s apart. (b) Pretreatment with PTx followed by exposure to trypanosome membranes. No increase in [Ca²⁺]ᵢ is detected. Subsequent addition of EGF elicits a Ca²⁺ signal. (c) Pretreatment with PTx followed by a recovery period of 1 h, then exposure to trypanosome membranes. Unlike cells with no recovery period, a Ca²⁺ signal is detected in these cells. Subsequent addition of EGF again induces a Ca²⁺ signal.](image-url)

**Table 1. Ca²⁺ Signaling Induced in NRK Cells by Trypanosomes**

| Experiments                  | 1    | 2    | 3    | 4    | 5    | 6    |
|------------------------------|------|------|------|------|------|------|
| *T. cruzi* trypomastigote    | 20/85 (23%) * | 15/50 (30%) | 20/60 (33%) | 6/34 (17.6%) | 7/35 (20%) | 12/38 (32%) |
| *T. cruzi* epimastigote      | 0/45 (0%) | 0/60 (0%) | 0/52 (0%) | | | |
| *T. brucei* procyclic        | 0/55 (0%) | 0/45 (0%) | | | | |
| Trypomastigote membranes     | 40/46 (86%) | 20/45 (44%) | 11/22 (50%) | 35/65 (54%) | 19/45 (42%) | |
| Epimastigote membranes       | 0/45 (0%) | | | | | |
| Trypsin-treated              | | | | | | |
| trypomastigote membranes     | 1/55 (2%) | 0/43 (0%) | | | | |
| EGF                          | 45/52 (86%) | 52/55 (94%) | 48/48 (100%) | | | |
| PTx treated/trypomastigote   | 0/55 (0%) | 0/45 (0%) | | | | |
| PTx treated + recovery/trypomastigote membranes | 3/45 (7%) | 6/55 (11%) | 13/40 (32%) | | | |

Recorded images obtained as described in Materials and Methods were observed frame by frame and the number of cells showing at least one Ca²⁺ spike during a period of 400 s was determined.

* Values represent (number of cells with a Ca²⁺ spike)/(total number of cells observed).

1 30 min after toxin removal.

$ 60 min after toxin removal.
individually fibroblasts (Fig. 1, a−d, and i). [Ca²⁺] increases occurred within 200 s of exposure but were asynchronous, suggesting they were triggered by parasite attachment to individual cells. Trypomastigotes are 10–20 μm long, and at the density used (10⁹/ml), several could be visualized attached to some cells of the population. Invasion is a slower process, requiring at least 10 min of trypomastigote host cell interaction (11). [Ca²⁺] responses were also elicited by heat-killed (3 min at 55°C) trypomastigotes, or by membrane fractions isolated from trypomastigotes (Figs. 1, h and j, and 2 a). Treatment of the trypomastigote membranes with trypsin abolished their capacity to trigger [Ca²⁺] signals in NRK cells (Table 1). Epimastigotes, the T. cruzi life cycle stages which are not capable of invading vertebrate cells (13), did not induce [Ca²⁺] signaling (Fig. 1, e−g, and j), and neither did their isolated membranes (Table 1). Negative results were also obtained with procyclins of T. brucei, the exclusively extracellular African trypanosome (Table 1).

The induction of [Ca²⁺] transients exclusively by the infective trypomastigote forms suggested that Ca²⁺ signaling was necessary for invasion. Accordingly, the cells were treated in several ways to inhibit the [Ca²⁺] signals, and then exposed to parasites in invasion assays. Trypomastigote entry was significantly impaired by preloading the host cells with the membrane-permeant Ca²⁺ chelator MAPTA-AM, which acts as a buffer, clamping the [Ca²⁺] at resting levels (19) (Fig. 3). Depletion of host cell [Ca²⁺] before exposure to the parasites, by exposure of the fibroblasts to the Ca²⁺ ionophore A23187 (20) or to the intracellular Ca²⁺ chelator MAPTA-AM (21) in Ca²⁺-free medium, further inhibited trypanosome entry (Fig. 3). The decrease in infection rate induced by A23187 in the presence of Ca²⁺ was of marginal significance (p = 0.02). Since addition of the ionophore should have equilibrated the intracellular and extracellular Ca²⁺ concentrations, this suggests that, like parasite-induced transient Ca²⁺ increases, a sustained ionophore-induced Ca²⁺ increase is also permissive for T. cruzi invasion. Under each of the inhibitory conditions, however, parasite entry was still increased twofold by pretreatment of the host cells for 5 min with cytochalasin D (legend to Fig. 3). Removal of Ca²⁺ from the medium before and during infection also inhibited T. cruzi invasion (Fig. 3). This is not likely to reflect metabolic changes in the parasites, since the concentration of free Ca²⁺ in the cytoplasm of T. cruzi trypomastigotes is maintained at 10−20 nM, regardless of the presence or absence of external Ca²⁺ (22). Pretreatment of the host cells with the Ca²⁺ channel blockers NiCl₂ (23) or verapamil (24) also markedly reduced T. cruzi invasion (Fig. 3). NRK cells treated with NiCl₂ did not respond with Ca²⁺ signals when exposed to trypomastigote membranes (data not shown). These findings are consistent with a requirement for [Ca²⁺] increases in the host cells for T. cruzi entry, and suggest that Ca²⁺ influx is a necessary component of the parasite-induced signaling process. It is interesting to note that verapamil treatment has been reported to dramatically improve survival and myocardial disease in T. cruzi–infected mice (25).

It is noteworthy that the protocols used by us to buffer or deplete [Ca²⁺] do not interfere with particle ingestion by phagocytosis, in macrophages (26) or neutrophils (27, 28). In contrast, there is evidence that [Ca²⁺] transients are required for phagosome-lysosome fusion (28). Our finding that [Ca²⁺] transients are involved in T. cruzi entry into fibroblasts is therefore consistent with invasion being mediated by lysosomal fusion (1).

A role for heterotrimeric G proteins in signal transduction is well established (29). Treatment of intact cells with PTx results in ADP-ribosylation of members of a restricted class of G protein α-subunits, uncoupling them from their receptors and thus blocking signal transduction (30, 31). The PTx-sensitive G-protein subtypes Go₁ and Go₅ are involved in the regulation of Ca²⁺ influx channels, and in the activation of phospholipase C, which generates IP₃-mediated Ca²⁺ release from internal stores (29, 31, 32). Incubation of NRK fibroblasts with PTx for 4 h inhibited their [Ca²⁺] response to T. cruzi (Fig. 2 b). However, PTx-treated cells still responded normally to EGF, which induces [Ca²⁺] transients by interaction with a receptor tyrosine kinase, in a pathway independent of trimeric G proteins (33) (Fig. 2 b). The inhibitory effect of PTx on the [Ca²⁺] signal triggered by trypomastigote membranes was partially reversed after recovery periods of 30 min to 1 h (Table 1, Fig. 2 c). Taken together, these findings indicate that a PTx-sensitive host cell G protein is involved in the regulation of the Ca²⁺ signals elicited by T. cruzi.

Invasion of NRK fibroblasts by T. cruzi trypomastigotes was inhibited when the cells were pretreated with PTx (Fig. 3). The partial inhibition is probably due to recovery of the fibroblast population from PTx, since the toxin was removed before the 20-min infection period (see Table 1). CTx, which

**Figure 3.** Effect of inhibitors of Ca²⁺ signaling on the invasion of NRK cells by T. cruzi trypomastigotes. Effect of buffering (MAPTA) or depleting (MAPTA/Ca²⁺-free and A23187/Ca²⁺-free) host cell [Ca²⁺], of blocking plasma membrane Ca²⁺ channels (NiCl₂ and verapamil), or of pretreatment with PTx or CTx before infection. Each condition, except A23187 in the presence of Ca²⁺ and CTx, significantly inhibits invasion. (⁎) p < 0.005; (⁎⁎) p < 0.0001. Values shown are the mean ± SD of triplicate samples from representative experiments. In matched experiments, cytochalasin D (2 μM) was added during the last 5 min of the host cell pretreatment. The ratios (number of intracellular parasites with cytochalasin pretreatment)/(number of intracellular parasites without cytochalasin pretreatment) were (control) 2.0; (MAPTA) 2.7; (Ca²⁺ free) 2.0; (MAPTA in Ca²⁺ free) 3.2; and (A23187 in Ca²⁺ free) 3.2; and (PTx) 3.3.**

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ADP-ribosylates the α-subunit of Gs but not of Gi trimeric G proteins (34), affected neither invasion (Fig. 3) nor the [Ca^{2+}]_i transients induced by trypomastigote membranes (data not shown). Thus, *T. cruzi* invasion depends on Ca^{2+} signals elicited by interaction between the parasite membrane and a host cell surface component coupled to a pathway including a PTx-sensitive G protein. It is interesting to note that exposure of the PTx-treated fibroblasts to cytochalasin D for 5 min enhanced invasion to levels similar to those observed in control cells (legend to Fig. 3), suggesting that the action of the toxin can in large part be bypassed by disassembly of the actin cytoskeleton.

Ca^{2+} signaling also appears to be important for cell entry by *Salmonella typhimurium*. Increases in [Ca^{2+}]_i were detected in populations of Henle-407 cells exposed to this bacterium, and Ca^{2+} channel blockers had an inhibitory effect in invasion (35). Although observations at the single cell level still have to be performed to confirm these findings, it is possible that [Ca^{2+}]_i transients are involved in regulating the extensive cytoskeletal rearrangements known to accompany *Salmonella* invasion. The *Salmonella* mechanism for cell invasion is clearly distinct from the one used by *T. cruzi*, since it is dependent on host cell membrane ruffling and inhibited by cytochalasins (36). Further characterization of the [Ca^{2+}]_i fluxes occurring in these two systems is needed to clarify the possible involvement of distinct signaling pathways.

In view of our previous findings (1) cytosolic-free Ca^{2+} transients may be required for at least two steps of the *T. cruzi* invasion process: (a) local rearrangement of the cortical actin cytoskeleton allowing lysosome access to the plasma membrane, and (b) lysosome fusion at the site of trypanosome entry. Characterization of the trypomastigote ligand and the host cell receptor mediating the [Ca^{2+}]_i response may reveal a specific signal transduction pathway that couples cytoskeletal organization and membrane traffic.

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