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Signal transduction induced in *Trypanosoma cruzi* metacyclic trypomastigotes during the invasion of mammalian cells

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Abstract

Penetration of *Trypanosoma cruzi* into mammalian cells depends on the activation of the parasite's protein tyrosine kinase and on the increase in cytosolic Ca^{2+} concentration. We used metacyclic trypomastigotes, the *T. cruzi* developmental forms that initiate infection in mammalian hosts, to investigate the association of these two events and to identify the various components of the parasite signal transduction pathway involved in host cell invasion. We have found that i) both the protein tyrosine kinase activation, as measured by phosphorylation of a 175-kDa protein (p175), and Ca^{2+} mobilization were induced in the metacyclic forms by the HeLa cell extract but not by the extract of *T. cruzi*-resistant K562 cells; ii) treatment of parasites with the tyrosine kinase inhibitor genistein blocked both p175 phosphorylation and the increase in cytosolic Ca^{2+} concentration; iii) the recombinant protein J18, which contains the full-length sequence of gp82, a metacyclic stage surface glycoprotein involved in target cell invasion, interfered with tyrosine kinase and Ca^{2+} responses, whereas the monoclonal antibody 3F6 directed at gp82 induced parasite p175 phosphorylation and Ca^{2+} mobilization; iv) treatment of metacyclic forms with phospholipase C inhibitor U73122 blocked Ca^{2+} signaling and impaired the ability of the parasites to enter HeLa cells, and v) drugs such as heparin, a competitive IP_3-receptor blocker, caffeine, which affects Ca^{2+} release from IP_3-sensitive stores, in addition to thapsigargin, which depletes intracellular Ca^{2+} compartments and lithium ion, reduced the parasite infectivity. Taken together, these data suggest that protein tyrosine kinase, phospholipase C and IP_3 are involved in the signaling cascade that is initiated on the parasite cell surface by gp82 and leads to Ca^{2+} mobilization required for target cell invasion.

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- Calcium response
- Cell invasion

Introduction

To enter mammalian cells, the protozoan parasite *Trypanosoma cruzi*, the etiological agent of Chagas' disease in man, requires the activation of signal transduction pathways both in the parasite and the host cell (1-7). In the process of cell invasion, the engagement of cell surface receptors is thought to trigger the signaling cascade that mobilizes various second messengers. Mobilization of one of such messengers, namely the calcium ion,

promotes in the host cell the rearrangement of microfilaments (8), the recruitment of lysosomes to the site of *T. cruzi* entry (9) and parasite internalization (10). The flow of information from the cell surface to the intracellular components, leading to an increase in cytosolic free Ca^{2+} concentration, possibly occurs in the following manner. Binding of the infective trypomastigote forms to the target cell induces in the latter the activation of pertussis toxin-sensitive heterotrimeric G protein (1). This, coupled to the activity of phospholipase C, generates inositol 1,4,5-triphosphate (IP_3) that participates in Ca^{2+} release from IP_3-sensitive intracellular stores (8).

As to the signaling events in *T. cruzi* during mammalian cell invasion, the available data are still fragmentary. Upon contact with target cells, Ca^{2+} mobilization is triggered in trypomastigotes. This Ca^{2+} response, which is required for parasite penetration, is not elicited in noninfective epimastigote forms (2,6). An early event in this cascade may include protein tyrosine kinase (PTK) activation, for treatment of trypomastigotes with PTK inhibitor genistein reduces cell invasion (3,7). Whether PTK activation is associated with the Ca^{2+} response remains to be established.

In metacyclic trypomastigotes, the insect-stage *T. cruzi* developmental forms that are infective to mammalian hosts, the interaction of the surface glycoprotein gp82 with an as yet to be defined host cell receptor appears to induce PTK activity (7). This molecule is also capable of inducing host cell Ca^{2+} mobilization (5) and has been implicated in the process of cell invasion (11). To advance our understanding on the metacyclic trypomastigote signal transduction pathway relevant for infection, experiments were performed i) to determine the possible association between PTK activation and Ca^{2+} response, ii) to examine the involvement of phospholipase C and IP_3 in the parasite entry into target cells, and iii) to confirm the role of gp82 as the cell surface receptor that mediates signaling to the parasite interior.

Material and Methods

T. cruzi, mammalian cells and cell invasion assay

T. cruzi strain CL (12) was used throughout this study. Parasites were maintained by cyclic passage in mice and in axenic cultures. Liver infusion tryptose medium (13) and Grace's medium (Gibco, Gaithersburg, MD, USA) were used, respectively, to grow parasites and to obtain cultures enriched in metacyclic forms. Metacyclic trypomastigotes were purified by passage through a DEAE-cellulose column, as described (14). HeLa cells, the human carcinoma-derived epithelial cells, and human leukemic K562 cells (15) were grown at 37°C in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), streptomycin (100 µg/ml) and penicillin (100 U/ml) in a humidified 5% CO_2 atmosphere. Mammalian cell invasion assays were carried out as previously described (16) by seeding 10^6 metacyclic forms onto each well of 24-well plates containing 13-mm diameter round glass coverslips coated with 2 x 10^5 HeLa cells. After 1-h incubation, the triplicate coverslips were washed in phosphate-buffered saline (PBS) and stained with Giemsa.

Detection of tyrosine phosphorylated *T. cruzi* proteins

In standard experiments, 5 x 10^7 parasites were incubated for 20 min at 37°C in the absence or presence of a mammalian cell extract, equivalent to 160 µg/ml protein, in a total volume of 200 µl. After washing with PBS, the parasites were disrupted at 4°C in a lysis solution containing phosphatase and protease inhibitors (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton

X-100, 1 mM $NaVO_4$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 25 µg/ml leupeptin, and 25 µg/ml antipain). Lysates were dissolved in loading buffer and subjected to electrophoresis in a 10% SDS-polyacrylamide gel, under reducing conditions, and the proteins were transferred to a nitrocellulose membrane. Following blockage with PBS containing 5% defatted milk, the nitrocellulose membrane was incubated with anti-phosphotyrosine antibodies (mouse monoclonal antibody from Sigma Chemical Co., St. Louis, MO, USA) diluted in PBS-milk for 1 h at room temperature. After several washes in PBS containing 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated anti-mouse IgG. The final reaction was developed by chemiluminescence using the ELC Western blotting detection reagent and Hyperfilm-MP from Amersham (Buckingham, UK).

Preparation of mammalian cell extract

The cell extract used in phosphorylation and Ca^{2+} signaling experiments was prepared as follows: after several washes in PBS, HeLa or K562 cells were sonicated on ice with an ultrasonic processor XL (2 pulses of 30 s each) in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 25 µg/ml leupeptin, and 25 µg/ml antipain). After ascertaining cell disruption under a phase contrast microscope, the sonicated preparation was centrifuged at 12,000 *g* for 5 min and the supernantant was collected and immediately used for experiments or stored at -80°C until use.

Determination of intracellular Ca^{2+} concentration

To measure *T. cruzi* cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, parasites (7 x 10^7 cells/ml) were washed in Tyrode solution (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.53 mM $MgCl_2$, 1.36 mM $CaCl_2$, and 5.5 mM glucose) and incubated with fura-2/AM (Sigma) for 3 h at room temperature, and non-incorporated fura-2 was washed out. Fluorescence was read with a fluorophotometer SPEX AR-CM system (Spex Industries, Edison, NJ, USA) with dual wavelength excitation (340 and 380 nm) and emission at 505 nm. The increase in parasite $[Ca^{2+}]_i$ after the addition of a mammalian cell extract to 2.5 ml of parasite suspension was calculated as described by Grynkiewicz et al. (17). For each preparation we determined R_{max} and R_{min}, which correspond to the fluorescence ratio at 340 and 380 nm in the presence of saturating Ca^{2+} after treatment with 50 µM digitonin, and in the absence of Ca^{2+}, upon addition of 10 mM ethylene glycol-bis(ß-aminoethyl ether) tetraacetic acid (EGTA), respectively. Some experiments were carried out with parasites in buffer A, pH 7.2, without Ca^{2+} and containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 50 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES).

Purification of recombinant protein J18

The recombinant protein J18 (18) containing the entire sequence of the metacyclic trypomastigote surface molecule gp82 fused to glutathione S-transferase (GST) was purified from IPTG-induced *Escherichia coli* suspensions according to the procedure detailed in Ref. 19, followed by electroelution as described (20).

Heparin loading by reverse permeabilization

The method of Kobayashi et al. (21) was used, with slight modifications. Metacyclic trypomastigotes were incubated in the following sequence of solutions kept at 2°C: I) 20 min in 10 mM EGTA, 120 mM KCl, 5 mM ATP, 2 mM $MgCl_2$, 20 mM N-tris

(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 6.8, at 2°C; II) 90 min in 120 mM KCl, 5 mM ATP, 2 mM $MgCl_2$, 20 mM TES, pH 6.8, at 2°C; III) 30 min in 120 mM KCl, 5 mM ATP, 10 mM $MgCl_2$, 20 mM TES, pH 6.8, at 2°C; IV) 30 min in 140 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 5.6 mM glucose, 2 mM MOPS, pH 7.1, at 22°C. To load heparin, 1 mg/ml heparin was added to the solutions used in steps I-IV. The parasites were warmed to 22-25°C and $CaCl_2$ was added at 15-min intervals to give the following concentrations: 0.001, 0.01, 0.1 and 1.6 mM. After loading with heparin, parasite viability was ascertained by the ethidium bromide test (22). Control parasites were treated in the same manner except that heparin was not added.

Treatment of parasites with different drugs

Metacyclic trypomastigotes were treated with 1 µM thapsigargin at 37°C for 30 min. Control parasites were incubated under the same conditions in the presence of 0.1% DMSO, equivalent to the DMSO concentration in the thapsigargin-treated samples. Caffeine treatment was performed by incubating the parasites at 37°C for 30 min with 10 mM of the drug. In both cases, the parasites were washed in buffer A before use. To inhibit phospholipase C, the parasites were treated for 4 min at 37°C in the presence of 10 µM U73122, or were incubated with 100 µM neomycin for 4 h at 37°C, washed in PBS and then used for the invasion assay. Parasites used as controls were incubated under the conditions described above in the absence of U73122 or neomycin.

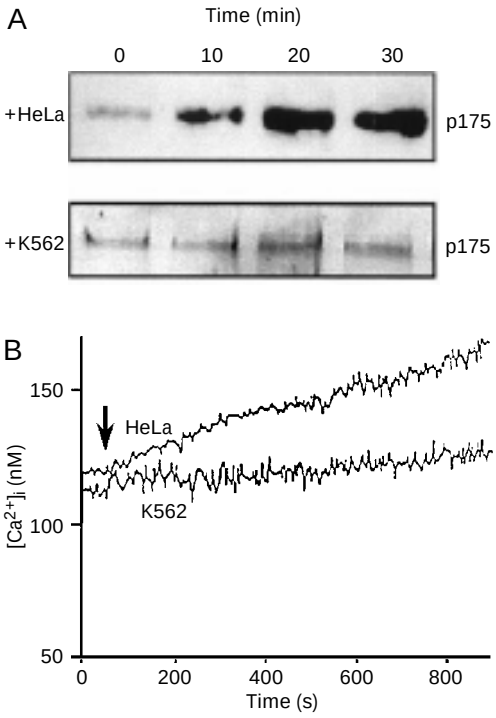

Figure 1 - Phosphorylation of p175 and Ca^{2+} mobilization induced in *T. cruzi* metacyclic trypomastigotes. *A*, Parasites were incubated at 37°C for the indicated periods of time in the presence of HeLa cell or *T. cruzi*-resistant K562 cell extract, equivalent to 160 µg/ml protein. After washing in PBS, the parasites were lysed with detergent and the total lysates were subjected to SDS-PAGE and analyzed by immunoblotting using anti-phosphotyrosine antibodies. *B*, Sonicated extract of HeLa or K562 cells, containing an equivalent of 25 µg protein, was added to fura-2-loaded parasites at the indicated time (arrow) and $[Ca^{2+}]_i$ was measured. Results representative of three experiments are presented.

Results

Both p175 phosphorylation and Ca^{2+} mobilization are induced in metacyclic trypomastigotes by HeLa cells but not by the K562 cell extract

We observed that the activation of *T. cruzi* PTK, as measured by the specific increase in the phosphorylation levels of a trypomastigote 175-kDa protein (p175), is induced by exposure of parasites to cultured HeLa cells or to the sonicated extract. As the HeLa cell extract can be stored frozen without losing its phosphorylation-inducing activity, for practical reasons we have used the sonicated preparations in our study. The first assay to determine the association between PTK activation and Ca^{2+} response was carried out by i) incubating metacyclic trypomastigotes with HeLa cell or the K562 cell extract for varying periods of time, and then processing for immunoblot analysis using anti-phosphotyrosine antibodies, and ii) adding HeLa cell or K562 cell extract to fura-2-loaded parasites and measuring the increase in $[Ca^{2+}]_i$. As shown in Figure 1, the HeLa cell extract triggered an increase in the parasite cytosolic Ca^{2+} concentration in a manner compatible with the increase in the levels of p175 phosphorylation. By contrast, the extract of K562 cells, which are resistant to *T.*

cruzi infection, did not show any PTK- or Ca^{2+} signal-inducing activity. The results were also negative when a two times more concentrated K562 cell extract was used.

Treatment of metacyclic trypomastigotes with genistein inhibits p175 phosphorylation and Ca^{2+} response

The next assay to determine the association between PTK activation and Ca^{2+} response was performed by i) incubating metacyclic trypomastigotes, untreated or treated with 250 µM genistein for 30 min at 37ºC, in the absence or in the presence of HeLa cell extract for 20 min at 37ºC, and then processing for anti-phosphotyrosine immunoblotting, and ii) adding the HeLa cell extract to fura-2-loaded parasites, untreated or treated with genistein, and measuring $[Ca^{2+}]_i$. Figure 2 shows that genistein, an inhibitor of protein tyrosine kinase (23) capable of inhibiting metacyclic trypomastigote entry into HeLa cells by ~75% (7), blocked both the p175 tyrosine phosphorylation and Ca^{2+} mobilization, suggesting that the two events are associated.

p175 phosphorylation and Ca^{2+} response in metacyclic forms are mediated by gp82

To confirm the assumption that the metacyclic stage surface glycoprotein gp82, which binds to HeLa cells in a receptor-dependent manner (11), plays a role in signal transduction, the following assay was performed. The PTK activation and Ca^{2+} response were determined upon incubation of parasites with HeLa cell extract in the absence or in the presence of J18, a recombinant protein containing the full-length gp82 sequence which binds to HeLa cells in the same manner as the native gp82 (19). In the PTK assay, the concentration of J18 in the reaction mixture was 250 µg/ml. For $[Ca^{2+}]_i$ measurement, 50 µl of HeLa cell extract containing 250 µg/ml of J18 was added to fura-2-loaded parasites. As shown in Figure 3, J18 inhibited both p175 phosphorylation- and Ca^{2+} signaling-inducing activity of the HeLa cell extract. This result raises the possibility of isolating the signaling molecules from the HeLa cell extract using a gp82 affinity column. When metacyclic trypomastigotes were exposed to monoclonal antibody 3F6 directed at gp82,

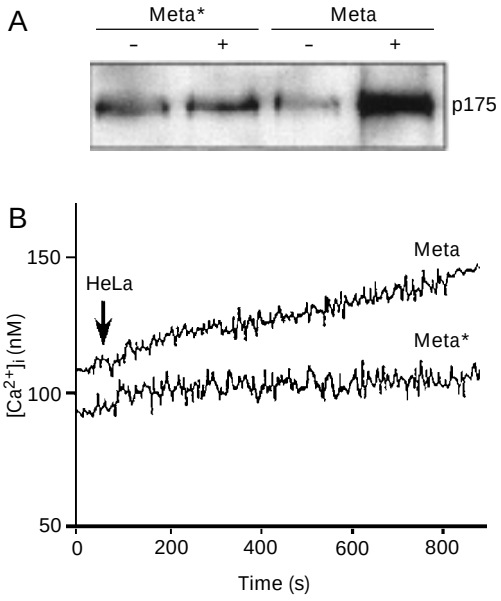

Figure 2 - Inhibition of *T. cruzi* metacyclic trypomastigote p175 phosphorylation and Ca^{2+} signaling by genistein. *A*, Metacyclic forms, untreated (Meta) or treated with 250 µM of protein tyrosine kinase inhibitor genistein (Meta*), were incubated in the absence (-) or in the presence (+) of HeLa cell extract at 37ºC for 20 min, and then processed for anti-phosphotyrosine immunoblotting. *B*, HeLa cell extract was added at the indicated time (arrow) to fura-2-loaded parasites, untreated (Meta) or treated with 250 µM genistein (Meta*), and $[Ca^{2+}]_i$ was measured. Results representative of three experiments are shown.

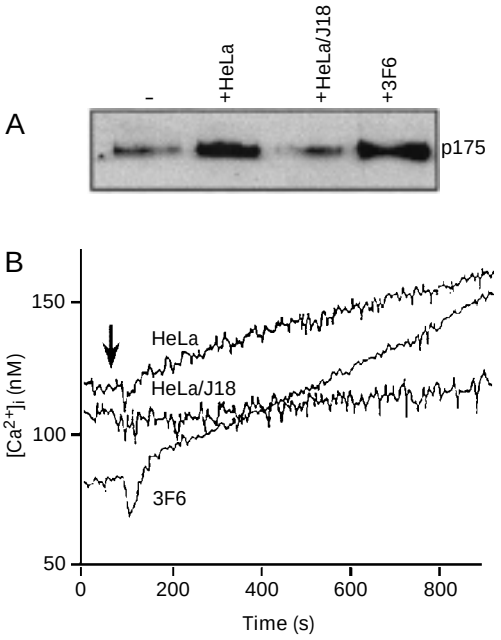

Figure 3 - Involvement of the *T. cruzi* metacyclic trypomastigote surface molecule gp82 with p175 tyrosine phosphorylation and Ca^{2+} response. *A*, Parasites were incubated in the absence (-) or in the presence of HeLa cell extract, unmixed (+HeLa) or mixed with recombinant protein J18 which contains the full-length gp82 sequence (+HeLa/J18), or were incubated with a monoclonal antibody directed at gp82 (+3F6). After 20 min at 37ºC, the parasites were processed for anti-phosphotyrosine immunoblotting. *B*, HeLa cell extract, mixed or not with J18, or monoclonal antibody 3F6 was added at the indicated time (arrow) to fura-2-loaded metacyclic trypomastigotes and $[Ca^{2+}]_i$ was measured.

at 10 µg/ml, both PTK activation and Ca^{2+} mobilization were triggered (Figure 3), supporting the idea of the involvement of gp82 in the signaling cascade.

Metacyclic trypomastigote Ca^{2+} required for host cell invasion is released from intracellular stores possibly in an IP_3-mediated manner

In a series of experiments, we determined whether target cell invasion by metacyclic forms was dependent on Ca^{2+} released from the parasite intracellular compartments, more specifically from IP_3-susceptible stores. First, the metacyclic forms either untreated or treated with thapsigargin, a sesquiterpene lactone that depletes intracellular Ca^{2+} stores in many mammalian cell types by specific inhibition of endoplasmic reticulum Ca^{2+}-ATPase (24), were incubated with HeLa cells for 1 h at 37°C in DMEM containing 2% FCS. Following washes in PBS, HeLa cells were stained with Giemsa and the number of intracellular parasites was counted in a total of 500 cells. As shown in Figure 4A, thapsigargin-treated parasites entered HeLa cells in significantly lower numbers as compared to untreated controls. Consistent with this observation, Ca^{2+} mobilization was barely detectable in thapsigargin-treated parasites upon stimulation with monoclonal antibody 3F6, in contrast to untreated controls (Figure 5).

Next, we tested the effect of caffeine, which inhibits IP_3-induced Ca^{2+} release in different cell types (25,26). Caffeine-treated parasites and untreated controls were seeded onto HeLa cells and incubated as described above. The infectivity of caffeine-treated parasites was significantly decreased (Figure 4B) and this fact was correlated with a greatly reduced Ca^{2+} response (Figure 5). In another assay, we used parasites subjected to reversible permeabilization to incorporate heparin, a blocker of IP_3-activated Ca^{2+} release in smooth muscle cells (21,27), as described in the Methods section. After incubation with HeLa cells for 1 h at 37°C in DMEM-2% FCS, the number of intracellular parasites was counted in 500 Giemsa-stained cells. As compared to controls, the heparin-loaded parasites displayed a reduced cell invasion rate (Figure 4C). We also determined whether the invasive capacity of Li^+-treated parasites was reduced. Lithium is assumed to block the recycling of IP_3, but treatment with Li^+ may eventually result in loss of the IP_3 signal. For instance, exposure of agonist-stimulated cerebral cortex slices or adrenal glomerulosa to Li^+ for longer than 5 min has been reported to suppress IP_3 generation (28,29). Metacyclic trypomastigotes treated with 10 mM LiCl for 45 min at

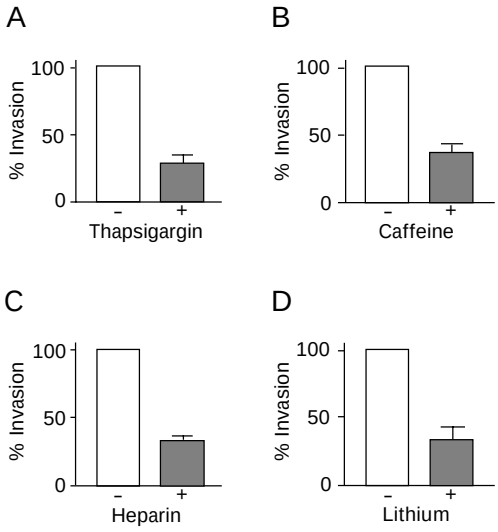

Figure 4 - Effect of treatment of *T. cruzi* metacyclic trypomastigotes with thapsigargin or drugs that affect the IP_3 signal on HeLa cell invasion. Parasites were: *A*, incubated at 37°C for 30 min in the absence or in the presence of 1 µM thapsigargin; *B*, treated or not for 30 min at 37°C with 10 mM caffeine; *C*, loaded or not with 1 mg/ml heparin as described in the Methods section, or *D*, treated or not with 10 mM Li^+ at room temperature for 45 min, and then seeded onto HeLa cells. After 1-h incubation at 37°C, the number of intracellular parasites was counted in a total of 500 Giemsa-stained cells and the percentage of invasion determined. The values are means ± SD of three independent assays.

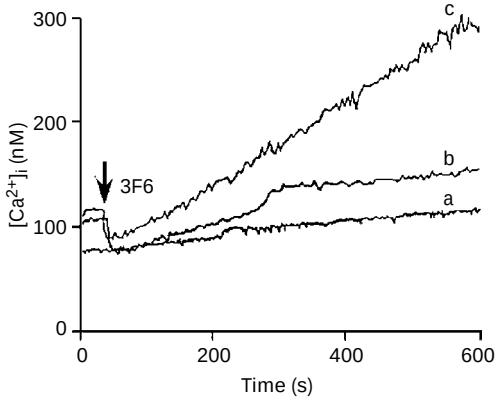

Figure 5 - Inhibitory effect of thapsigargin and caffeine on *T. cruzi* metacyclic trypomastigote Ca^{2+} mobilization. Monoclonal antibody 3F6, directed at the metacyclic stage surface molecule gp82, was added at the indicated time (arrow) to fura-2-loaded parasites, either untreated (c) or pretreated with thapsigargin (a) or caffeine (b) at 37°C for 30 min, and $[Ca^{2+}]_i$ was measured.

room temperature before addition to HeLa cells were internalized in significantly lower numbers than the untreated controls (Figure 4D). The viability of the parasites was not affected by treatment with thapsigargin, caffeine, lithium or heparin loading. They retained their normal morphology and motility and were fully viable, as evaluated by Trypan blue staining and the ethidium bromide test.

Phospholipase C inhibitor blocks Ca^{2+} response and reduces parasite infectivity

IP_3 is generated by phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-biphosphate (30). As our results indicated the participation of IP_3 in signal transduction required for cell invasion by metacyclic forms, we reasoned that inhibition of the parasite phospholipase C would affect its ability to enter host cells. To test that hypothesis, the parasites were treated with phospholipase C inhibitor U73122 (31) and then assayed for HeLa cell invasion. As shown in Figure 6A, U73122-treated parasites entered HeLa cells in significantly lower numbers than untreated controls. In addition to U73122, the effect of neomycin, another phospholipase C inhibitor (32), was examined. Although to a lesser extent than U73122, neomycin also inhibited the parasite infectivity (Figure 6B). Consistent with the inhibition of IP_3-mediated Ca^{2+} release by the drug, no significant Ca^{2+} response was detected in U73122-treated parasites (Figure 6C). However, we have noted a higher basal level of cytosolic Ca^{2+} concentration in U73122-treated parasites, suggesting also the possibility of a nonspecific effect of the drug in releasing Ca^{2+} from intracellular stores (33).

The inhibitory effect of different drugs is reversible

To test whether the effect of the various drugs was reversible, metacyclic trypomastigotes were treated with the drug, washed in PBS and then left to rest for 1 h at room temperature before seeding onto HeLa cells. Unlike the parasites used for the cell invasion assay immediately after drug treatment, the metacyclic forms incubated for 1 h in the absence of thapsigargin, caffeine, Li^+ or U73122 entered HeLa cells in numbers comparable to those of untreated controls, indicating the reversible effect of these compounds. The reversibility of heparin action was not determined, but its reversible antagonism of IP_3-activated Ca^{2+} release has been demonstrated in other cell types (27).

Discussion

In the present study we analyzed the signal transduction events in *T. cruzi* metacyclic trypomastigotes required for mammalian cell invasion. Previous studies have shown the requirement of protein tyrosine kinase activation, that results in p175 phosphorylation, and the increase in cytosolic Ca^{2+} concentration (5-7). Our results indicate that Ca^{2+} mobilization in metacyclic

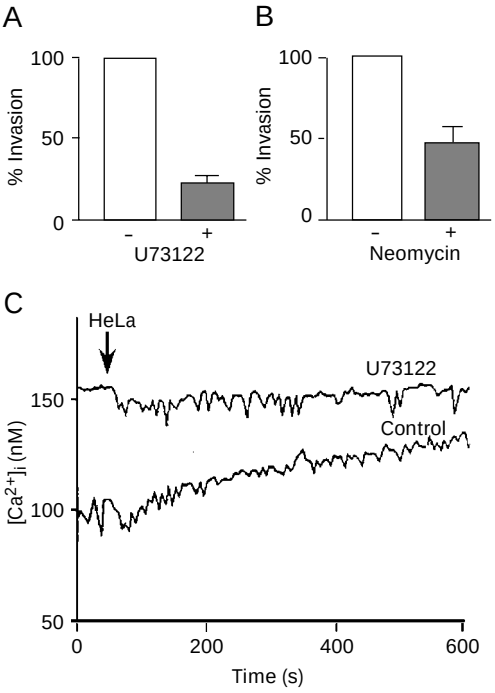

Figure 6 - Inhibition of HeLa cell invasion and Ca^{2+} response by treatment of *T. cruzi* metacyclic trypomastigotes with phospholipase C inhibitor. Parasites were treated or not with: *A*, 10 µM U73122 for 4 min at 37ºC, and *B*, 100 µM neomycin at 37ºC for 4 h and then washed. Following incubation of parasites with HeLa cells at 37ºC for 1 h, percent invasion was determined by counting the number of intracellular parasites in a total of 500 Giemsa-stained cells. *C*, HeLa cell extract was added to fura-2-loaded parasites, either untreated (control) or treated with 10 µM U73122 for 4 min at 37ºC, and $[Ca^{2+}]_i$ was measured. The values are means ± SD of three assays.

forms is dependent on PTK activation, so that the inhibition of the enzyme activity with the specific inhibitor genistein also inhibits the Ca^{2+} response (Figure 2).

The signaling cascade in metacyclic trypomastigotes is presumably initiated by recognition of the surface glycoprotein gp82 by its target cell receptor. Thus, if this recognition is prevented, for instance by the presence of gp82 recombinant protein, PTK activation and Ca^{2+} response cannot be induced (Figure 3). On the other hand, binding of the monoclonal antibody 3F6 to gp82, possibly by mimicking the parasite ligand-host cell receptor interaction, triggers p175 phosphorylation and Ca^{2+} mobilization (Figure 3). The next event in the metacyclic stage signal transduction pathway, following engagement of gp82, is probably PTK activation. How gp82, which is anchored to the plasma membrane via the glycosylphosphatidylinositol (GPI) moiety (34), associates with tyrosine kinase is not known. In mammalian cells, at least two possibilities of association of GPI-anchored surface molecules with tyrosine kinases have been reported. The neuron survival factor neurturin, for instance, was shown to signal through multicomplex receptors that consist of receptor tyrosine kinase and a member of a GPI-linked family of receptors that determine ligand specificity (35). Protein tyrosine kinases have been found in complexes immunoprecipitated from T cells with antibodies directed at GPI-linked proteins (36,37). Interaction of GPI-anchored proteins and kinases could also be mediated by transmembrane linker proteins (38).

Activation of phospholipase C, that leads to IP_3 generation and Ca^{2+} mobilization, is possibly the next step in the metacyclic trypomastigote-signaling pathway. We have found that treatment of parasites with the phospholipase C inhibitor U73122 blocked the Ca^{2+} response and the ability to invade HeLa cells (Figure 6). Whether different phospholipase C isozymes are present in *T. cruzi*, and in this case which form is activated during target cell invasion, is one of the many questions that remain to be investigated. Also to be clarified is the mode of association of protein PTK or p175 with phospholipase C.

The increase in cytosolic Ca^{2+} necessary for metacyclic trypomastigote entry into host cells may originate from thapsigargin-sensitive intracellular Ca^{2+} stores in an IP_3-mediated manner. First, treatment of parasites with thapsigargin, which depletes intracellular Ca^{2+} stores by inhibiting endoplasmic reticulum Ca^{2+}-ATPase (24), significantly reduced both the Ca^{2+} response (Figure 5) and the ability to enter HeLa cells (Figure 4). Second, drugs that interfere with IP_3-mediated Ca^{2+} release, such as heparin, caffeine and lithium, reduced the parasite infectivity (Figure 4). Although our data suggest that IP_3-mediated Ca^{2+} release occurs in metacyclic trypomastigotes, this event has not been detected in studies with other *T. cruzi* developmental forms in spite of the presence of inositol phosphates (39,40). The basis for such discrepancies has yet to be determined.

Although a number of questions still remain to be answered, taken together, our results suggest that interaction of the metacyclic trypomastigote surface molecule gp82 with its host cell receptor triggers a signaling cascade leading to sequential activation of protein tyrosine kinase and phospholipase C, with consequent generation of IP_3 that promotes Ca^{2+} mobilization necessary for parasite internalization.

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