A Trypanosome-soluble Factor Induces IP₃ Formation, Intracellular Ca²⁺ Mobilization and Microfilament Rearrangement in Host Cells

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Abstract. Lysosomes are recruited to the invasion site during host cell entry by Trypanosoma cruzi, an unusual process suggestive of the triggering of signal transduction mechanisms. Previous studies showed that trypomastigotes, but not the noninfective epimastigotes, contain a proteolytically generated trypomastigote factor (PGTF) that induces intracellular free Ca²⁺ transients in several mammalian cell types. Using confocal time-lapse imaging of normal rat kidney (NRK) fibroblasts loaded with the Ca²⁺-sensitive dye fluo-3, we show that the initial intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) transient detected a few seconds after exposure to trypomastigote extracts is a result of Ca²⁺ release from intracellular stores. Removal of Ca²⁺ from the extracellular medium or inhibition of Ca²⁺ channels with NiCl₂ did not affect the response to PGTF, while depletion of intracellular stores with thapsigargin abolished it. [Ca²⁺]ᵢ transients induced by PGTF were shown to be coupled to the activity of phospholipase C (PLC), since the specific inhibitor U73122 completely blocked the response, while its inactive analogue U73343 had no effect. In addition, polyphosphoinositide hydrolysis and inositol 1,4,5-trisphosphate (IP₃) were detected upon cell stimulation with PGTF, suggesting the participation of IP₃-sensitive intracellular Ca²⁺ channels.

An immediate effect of the signaling induced by PGTF and live trypomastigotes was a rapid and transient reorganization of host cell microfilaments. The redistribution of F-actin appeared to be a direct consequence of increased [Ca²⁺]ᵢ, since thrombin and the Ca²⁺ ionophore ionomycin produced a similar effect, with a time course that corresponded to the kinetics of the elevation in [Ca²⁺]ᵢ. These observations support the hypothesis that PGTF-induced disassembly of the cortical actin cytoskeleton may play a role in T. cruzi invasion, by facilitating lysosome access to the invasion site. Taken together, our findings suggest that the proteolytically generated trypomastigote factor PGTF is a novel agonist that acts through the PLC/phosphoinositide signaling pathway of mammalian cells.

Intracellular pathogens show remarkable diversity in their mechanisms for invading mammalian cells. Recent evidence indicates that host cell invasion is in many cases preceded by the transduction of specific signals across the plasma membrane. Bacterial pathogens such as enteropathogenic Yersinia, Salmonella, and Escherichia coli induce responses in their host cells that are believed to facilitate invasion through distinct pathways (Bliska et al., 1993). Although the host cell entry mechanism of most intracellular pathogens resembles phagocytosis, in the sense that it involves F-actin-dependent plasma membrane extensions, exceptions to this rule have been found. One such exception is the intracellular protozoan parasite Trypanosoma cruzi, the causative agent of Chagas’ disease in man.

Invasion of nonphagocytic vertebrate cells by trypomastigotes, the infective stages of T. cruzi, proceeds by a unique mechanism that is independent of pseudopodia formation and actin polymerization (Schenkman et al., 1988; Tardieux et al., 1992). Disruption of the actin cytoskeleton with cytochalasin D enhances trypanosome entry, indicating that the mechanism of invasion is distinct from phagocytosis (Schenkman et al., 1991; Tardieux et al., 1992). In addition, an unusual process of recruitment and fusion of host lysosomes occurs directly beneath the invasion site, and this process is required for parasite internalization (Tardieux et al., 1992). These observations suggest that cortical actin rearrangements may be an early step in the T. cruzi invasion mechanism, to allow lysosomes easier access to the plasma membrane.

In searching for second messengers involved in the lysosome recruitment mechanism, it was found that T. cruzi trypomastigotes, but not the noninfective epimastigotes, induce repetitive, pertussis toxin-sensitive, transient eleva-
tions in the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) of normal rat kidney (NRK) cells (Tardieux et al., 1994). A soluble factor (PGTF) that induces Ca\(^{2+}\) signaling in several different mammalian cell types was subsequently extracted from trypomastigotes, and found to be dependent on the activity of a leupeptin-sensitive parasite alkaline peptidase (Burleigh and Andrews, 1995). Inhibition of the host cell [Ca\(^{2+}\)]\(_i\), transients, either by interfering with intracellular free Ca\(^{2+}\) levels or by inhibiting the trypanosome peptidase, decreased parasite entry, thereby suggesting a role for the PGTF-mediated signaling event in invasion (Tardieux et al., 1994; Burleigh and Andrews, 1995).

Signal transduction events mediated by hormones and growth factors are often accompanied by hydrolysis of polyphosphoinositides, transient increases in [Ca\(^{2+}\)]\(_i\), and rapid actin cytoskeletal rearrangements (Chinkers et al., 1979; Kadowaki et al., 1986; Bretscher, 1991; Janney, 1994). Ca\(^{2+}\) signaling has also been shown to be associated with phagosome–lysosome fusion (Jaconi et al., 1990). The T. cruzi-induced Ca\(^{2+}\) signal was therefore proposed as playing a role in invasion through reorganization of host cell microfilaments and/or lysosome fusion (Tardieux et al., 1994). In this work we characterize the [Ca\(^{2+}\)]\(_i\) transients induced by the trypomastigote-soluble fraction containing the signaling factor PGTF (Burleigh and Andrews, 1995). We show that the initial [Ca\(^{2+}\)]\(_i\) elevation induced by PGTF in NRK cells is mediated by phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP\(_3\)) formation, and Ca\(^{2+}\) release from intracellular stores. The resulting increase in [Ca\(^{2+}\)]\(_i\) induces a transient reorganization of actin microfilaments, in a pattern consistent with a role in facilitating parasite invasion.

Materials and Methods

Materials

EGTA, leupeptin, thrombin, genistein, staurosporine, thapsigargin, and phospholipid were obtained from Sigma Immunochemical (St. Louis, MO), ionomycin from Calbiochem Novabiochem (La Jolla, CA), and H-7, phalloidin were obtained from Sigma Immunochemical (St. Louis, MO), EGTA, leupeptin, thrombin, genistein, staurosporine, thapsigargin, and Materials were washed in Hepes-buffered Ringer’s solution (Heuser, 1989) and re-suspended in the same solution before exposure to NRK cell monolayers.

Confocal Time-lapse Fluorescence Imaging

NRK cells were plated on glass coverslips at 2 × 10\(^6\) cells/cm\(^2\), cultured for 24 h and loaded with 5 μM fluo-3-AM (1% DMSO 0.2% pluronic F-127; Molecular Probes) for 45 min at 37°C. Coverslips were mounted in a perfusion chamber (Warner Instruments, New Haven, CT) and superfused with PBS\(^+\) or Ca\(^{2+}\) free PBS containing 10 mM EGTA at 37°C. Time-lapse images were collected with a confocal scanning laser microscope (Ex = 490 nm, Em = 510 nm) (MRC-500; Bio-Rad Labs., Richmond, CA) and recorded on an optical memory disc recorder (TQ-3031P; Parasonic, Secaucus, NJ) at 1.8 frames/s (scan time = 0.8 s, download time = 1.0 s) with no averaging. Kinetic plots were obtained from fluorescence values derived by averaging over a 5 × 5 pixel area in a series of images. 10 cells, showing a representative response profile for the whole population visualized (≈30 cells), were analyzed in each experiment.

Polyphosphoinositide Hydrolysis Analysis

NRK cells were plated at 1.5 × 10\(^6\) cells/cm\(^2\) in 12-well dishes and grown for 24 h to subconfluence. Wells were then rinsed twice with 10 ml Heps-buffered DMEM and incubated in 0.5 ml of this medium containing 2 μCi/ml [\(^{3}H\)]myo-inositol (105 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 20 h at 37°C. Cells were then treated with 20 mM LiCl for 1 min before the addition of agonist solution in the presence of 20 mM LiCl. After agonist treatment, cells were extracted with 750 μl of 20 mM formic acid for 30 min at 4°C. 1-ml packed/volume columns of anion-exchange resin AG1\(\times\)8, formate form, 100–200 mesh size (Bio Rad Labs., Hercules, CA) were used to bind inositol phosphates. Columns were sequentially washed with 2 ml of 2 M ammonium formate/0.1 M formic acid, 2 ml of water and 4 ml of 20 mM ammonium hydroxide. Cell extracts were then loaded and the column immediately washed with 3 ml of 40 mM ammonium hydroxide followed by 4 ml of 40 mM ammonium formate. Inositol phosphates were eluted with 4 ml of 2 M ammonium formate/0.1 M formic acid and the radioactivity determined in a scintillation counter (Paris and Pouyssegur, 1986).

HPLC Fractionation of Inositol Phosphates

NRK cells were labeled as described above, except that the [\(^{3}H\)]myo-inositol concentration was 5 μCi/ml and 60-mm dishes were used to grow the cells. Cells were washed with 15 ml of PBS, 0.1% BSA for 15 min as described above and the reaction was terminated by removing the medium and adding boiling distilled water to the cells. Cell lysates were collected, boiled for 5 min, and centrifuged to remove particulate material. The soluble supernatant from two 60-mm dishes was pooled and the volume concentrated to 20 μl. Samples were injected into a SAX hydrophore anion-exchange analytical column (4.6 × 100 mm; Rainin Instrument Co., Inc., Woburn, MA). The flow rate was set at 1 ml/min and 0.8 ml fractions were collected. Inositol was eluted with water for 15 min. Inositol phosphate species were eluted in the order of inositol monophosphate (IP\(_1\)), inositol diphosphate (IP\(_2\)), IP\(_3\) by a 5-min linear gradient of 0–10% 1 M ammonium formate, pH 3.8, followed by 20 min isocratic elution with 10% 1 M ammonium formate. Elution then continued with a 5-min linear gradient of 10–100% 1 M ammonium formate followed by a 20-min isocratic elution with 1 M ammonium formate. The following standards were run in parallel to identify the eluted species: [\(^{3}H\)]myo-inositol (105 Ci/mmole; Amersham), [\(^{3}H\)]inositol-1-phosphate, and [\(^{3}H\)]inositol 1,4,5-trisphosphate (21 Ci/mmole; Dupont-New England Nuclear, Boston, MA).

F-actin Imaging

NRK cells were plated on glass coverslips at 2 × 10\(^6\) cells/cm\(^2\) and cultured for 24 h. Coverslips were washed with PBS\(^+\) and treated with agonist for the indicated periods of time. Fixation was performed with acetone at −20°C for 5 min. After fixation, coverslips were washed in TBS and incubated with 1 μg/ml FITC-labeled phalloidin (Sigma) in TBS containing 1% BSA for 1 h. Preparations were visualized using a fluorescence microscope (Zeiss Axiovert 135; Carl Zeiss, Inc., Thornwood, NY). Line-scan analysis of F-actin was performed on digital images recorded with a ULTRASONS Corp., Danbury, CT), and centrifuged at 700 g for 10 min at 4°C. The postnuclear supernatant was further centrifuged at 100,000 g for 30 min at 4°C and the resulting supernatant fraction was passed through a 1 ml Concanavalin-A–Sepharose column (Pharmacia Chemicals, Upsalla, Sweden). The bound fraction containing PGTF was collected and stored at −80°C (Burleigh and Andrews, 1995).

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videocamera (CCD-C72; Dage-MTI, Inc., Michigan City, IN), measuring the fluorescence brightness across a straight line drawn from the cell margin through the nucleus, using MetaMorph software (Universal Imaging Corp., Westchester, PA).

**Cell Invasion Assay**

Trypomastigotes were washed in Hepes-buffered Ringer's solution (Heuser, 1989) and resuspended in the same solution at 5 × 10⁷/ml. Coverslips with NRK cells plated at 2 × 10⁴ cells/cm² 24 h before, were pretreated with the indicated drugs for 15 or 30 min, washed and incubated with trypomastigotes for 20 min at 37°C. Three coverslips for each experiment were separately washed in cold PBS²⁺, fixed in 2% paraformaldehyde (PFA) for 20 min and incubated for 15 min with 500 mM NH₄Cl to quench remaining aldehyde groups of the fixative. The coverslips were then incubated with 10 μg/ml IgG from a rabbit immunized with *T. cruzi* trypomastigotes. After washing, rhodamine-conjugated anti-rabbit IgG antibodies (Boehringer Mannheim Corp., Indianapolis, IN) were added for 30 min in TBS containing 1% BSA, followed by washing and staining of host cell and parasite DNA with DAPI (Sigma) diluted to 10 μg/ml. DAPI-positive parasites with negative or faint immunolabeling were counted as intracellular, whereas parasites strongly labeled with anti-*T. cruzi* antibodies were considered extracellular and not counted.

**Results**

**Trypomastigote-induced [Ca²⁺]i Transients Are Mediated by Ca²⁺ Release from Intracellular Stores**

Trypomastigotes induce rapid and repetitive [Ca²⁺]i transients in host cells (Tardieux et al., 1994). This activity is associated with the soluble fraction of sonicated trypomastigotes and is mediated by a novel Ca²⁺-signaling factor (PGTF) whose generation requires a *T. cruzi* leupeptin-sensitive alkaline peptidase (Burleigh and Andrews, 1995).

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**Figure 1.** Extracellular Ca²⁺ is not required for the [Ca²⁺]i response of NRK fibroblasts to PGTF. NRK cells were loaded with fluo-3 and analyzed by confocal time-lapse fluorescence imaging after: (a) exposure to TSF in PBS²⁺; (b) exposure to TSF in Ca²⁺-free PBS containing 10 mM EGTA; (c) pretreatment for 15 min with 5 mM NiCl₂ followed by TSF; (d) pretreatment for 30 min with 0.5 μM thapsigargin, followed by TSF. Arrows indicate TSF addition. Fluorescence profiles of 10 representative single cells for each condition are shown.

To study the origin of the [Ca²⁺]i transients induced by the trypomastigote soluble fraction, we used fluo-3-AM-loaded NRK fibroblasts and confocal laser time-lapse imaging. The addition of 80 μl of trypomastigote-soluble fraction (TSF) to NRK cells in the presence of the same volume of PBS²⁺ induced a significant increase in [Ca²⁺]i after ~10 s in all cells visualized (Fig. 1 a). As described previously (Burleigh and Andrews, 1995), pretreatment of the soluble extract for 5 min with 50 μM leupeptin resulted in complete inhibition of the Ca²⁺ response (not shown).

[Ca²⁺]i transients in mammalian cells can be mediated by the opening of Ca²⁺ channels in the plasma membrane and/or by release of Ca²⁺ from intracellular stores. To examine the contribution of internal Ca²⁺ stores, external free Ca²⁺ was removed by perfusing cells for 10 min in PBS containing no Ca²⁺ and 10 mM EGTA. For these experiments, 20 μl of a fourfold concentrated TSF prepared in PBS²⁺ was used, resulting in an approximate final Ca²⁺ concentration of 0.2 mM. The presence of 50 times excess EGTA (10 mM) in the incubation buffer ensured chelation of all free Ca²⁺. As shown in Fig. 1 b, extracellular Ca²⁺ removal did not affect the [Ca²⁺]i transient induced

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**Figure 2.** Thapsigargin inhibits the [Ca²⁺]i response of NRK cells to live trypomastigotes. (a) untreated cells (Control), (b) cells pretreated with 0.5 μM thapsigargin for 30 min. Arrows indicate addition of live trypomastigotes at 10⁷/ml. Fluo-3 fluorescence imaging was as described in the legend of Fig. 1.

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**Figure 3.** Thapsigargin inhibits host cell invasion by *T. cruzi*. The number of intracellular parasites in NRK cells pretreated with Ringer's (Control) or 0.5 μM thapsigargin (15 min and 30 min) was determined after a 20-min infection period. The data represents the average of triplicate determinations ± SD.
Figure 4. PGTF induces polyphosphoinositide hydrolysis and IP₃ formation in NRK cells. (a) Total inositol phosphates released after 15-min stimulation with trypomastigote soluble fraction (TSF), 50 μM leupeptin-treated TSF (TSF + leupeptin) or ESF; (b) kinetics of inositol phosphate release after stimulation with TSF (●) or 0.5 U/ml thrombin (○). Each point represents the means of triplicate determinations ± SD; (c) HPLC fractionation of inositol phosphates released after 15-min stimulation with TSF (●) or 50 μM leupeptin-treated TSF (○). Peaks were identified through known standards run in parallel. IP, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate.

Figure 5. PLC inhibitor blocks the [Ca²⁺]ᵢ response of NRK fibroblasts to PGTF. NRK cells were pretreated for 3 min with: (a) 10 μM U73343 (inactive analogue of PLC inhibitor); (b) 10 μM U73122 (specific PLC inhibitor). Arrows indicate addition of TSF. Fluo-3 fluorescence imaging was as described in the legend of Fig. 1.

Depletion of Intracellular Ca²⁺ Stores Inhibits Invasion of NRK Cells by T. cruzi

NRK cells were exposed to 0.5 μM thapsigargin for 15 or 30 min, the drug was washed out and 5 × 10⁵ live trypomastigotes/ml were incubated with the cells for 20 min. After fixation and immunofluorescence staining, the number of trypomastigotes that invaded thapsigargin-treated cells during this period was found to be markedly lower than what was observed in untreated control cells. The strongest inhibition occurred in cells pretreated with thapsigargin.
Figure 6. *T. cruzi* induces microfilament rearrangement in NRK fibroblasts. Cells were stained with FITC-phalloidin after treatment for 30 s (a–d) or 15 min (e and f) with: (a) PBS2+; (b) TSF; (c) ESF; (d) 50 μM leupeptin-treated TSF (TSF + leupeptin); (e) PBS2+; (f) live trypomastigotes at 10^8/ml. Bar, 10 μm.

the drug for 30 min (Fig. 3). As mentioned above, a 30-min thapsigargin treatment results in complete inhibition of [Ca^{2+}]_i transients induced in NRK cells by live trypomastigotes (Fig. 2 b).

**PGTF Induces Polyphosphoinositide Hydrolysis and IP_3 Formation**

Inositol 1,4,5-trisphosphate, derived from PLC hydrolysis of polyphosphoinositides, is a known mediator of Ca^{2+} release from intracellular stores (Berridge, 1993). To determine if the TSF-containing PGTF could induce inositol phosphate hydrolysis in NRK fibroblasts, cells were labeled with [3H]myo-inositol for 20 h and inositol phosphates were extracted. LiCl was included to inhibit the recycling of inositol phosphates to inositol, and thus enhance the sensitivity of the assay (Majerus, 1992). Treatment of cells with the TSF for 15 min induced significant release of inositol phosphates (Fig. 4 a, TSF). On the other hand, when the TSF was treated with 50 μM leupeptin, a condition that inhibits PGTF generation (Burleigh and An-
no inositol phosphates were detected (Fig. 4 a, TSF + leupeptin). Furthermore, exposure of the cells to a soluble fraction of epimastigotes, the noninfective life cycle stages of T. cruzi that do not trigger a Ca\(^{2+}\) response (Tardieux et al., 1994; Burleigh and Andrews, 1995), also failed to induce inositol phosphate release (Fig. 4 a, ESF). Inositol phosphates were detectable as early as 1 min after the addition of the extract to cells, with a continuous release that lasted \(\sim\) 7.5 min. In a parallel assay, thrombin treatment resulted in a slightly faster response, with inositol phosphates being continuously released from the NRK cells during 5 min (Fig. 4 b). Fractionation of the inositol phosphate pool by HPLC confirmed that IP\(_3\) was generated in response to PGTF (Fig. 4 c). The IP\(_3\) peak detected after a 15-min treatment with TSF was greatly reduced when the extract was pretreated with leupeptin to block production of PGTF (TSF + leupeptin).

**Phospholipase C Mediates the PGTF-induced [Ca\(^{2+}\)], Transients**

Many receptor-mediated signaling events in mammalian cells are coupled to PLC activation, resulting in the formation of IP\(_3\) and diacylglycerol (Berridge, 1993). Our finding that PGTF induces IP\(_3\) generation in NRK cells implied the involvement of PLC. To obtain further evidence in this direction, we tested the effect of U73122, a specific PLC inhibitor, and its inactive analogue U73343 (Bleasdale et al., 1990). Incubation of fluo-3-loaded NRK cells for 3 min with 10 \(\mu\)M U73122 resulted in complete inhibition of the [Ca\(^{2+}\)], transients induced by the trypomastigote soluble extract, while U73343 had no effect (Fig. 5).

**PGTF and Live Trypomastigotes Induce Transient Microfilament Rearrangement**

Treatment of NRK cells with the TSF for 30 s induced marked alterations in the microfilament organization. After rapid acetone fixation and phalloidin staining, short F-actin fibers were visualized in the perinuclear area, associated with an overall reduction in stress fibers (Fig. 6, a and b). This effect was not observed when the trypomastigote extract was pretreated with leupeptin (to block the generation of PGTF) or when an epimastigote extract was used (Fig. 6, c and d). Live trypomastigotes induced a similar pattern of microfilament reorganization in some cells of the population (Fig. 6, e and f).

To further analyze the changes observed in the organization of microfilaments, images were recorded and the fluorescence intensity was measured by radial line scan analysis. When PBS\(^{2+}\) or leupeptin-pretreated TSF were added to the NRK cells, the F-actin distribution from the cell cortex to the nuclear area showed a characteristic high brightness pattern, corresponding to the overall stress fibers (Fig. 7, PBS, TSF + leupeptin). On the other hand, when either TSF, 0.5 U/ml thrombin or 5 \(\mu\)M ionomycin were added to the cells, an altered pattern was obtained, with reduced brightness in the cortex and nuclear areas (Fig. 7, TSF, Thrombin, Ionomycin).

**Increase in [Ca\(^{2+}\)], Is Sufficient to Induce the Centripetal F-actin Rearrangement**

The F-actin reorganization induced by PGTF in NRK cells could be detected 15 s after the addition of the trypomastigote soluble extract, reaching its maximum effect in 30 s.
Figure 8. The PGTF-induced F-actin rearrangement is transient. NRK cells were stained with FITC-phalloidin after treatment for 30 s or 2 min with: (a and b) PBS; (c and d) TSF; (e and f) 0.5 U/ml thrombin; (g and h) 5 μM ionomycin. Bar, 10 μm.
Neither treatment interfered with the F-actin rearrangement induced by thrombin or thrombin (Paris and Pouyssegur, 1986) or 5 μM ionomycin (Hallam et al., 1988) (Fig. 8, c–h). Leupeptin did not inhibit the F-actin rearrangement induced by thrombin or ionomycin (not shown). Recovery of the actin rearrangement induced by thrombin was also observed by 2 min (Fig. 8, c and i), while the effect of ionomycin was longer lasting (Fig. 8, g and h). The kinetics of the F-actin reorganization induced by these agonists coincided with the elevation in $[\text{Ca}^{2+}]_i$ (Fig. 9). Since ionomycin induces direct $\text{Ca}^{2+}$ influx through the plasma membrane without the involvement of other second messengers, we conclude that the centripetal F-actin reorganization observed in NRK cells in response to PGTF is probably a direct consequence of an increase in $[\text{Ca}^{2+}]_i$.

**Effects of Tyrosine Kinase and Protein Kinase C Inhibition**

Inhibition of the trypomastigote-induced $\text{Ca}^{2+}$ signaling pathway by pertussis toxin (Tardieux et al., 1994) suggested the involvement of a trimeric G protein-linked receptor. To investigate the alternative possibility, the involvement of a tyrosine kinase type of receptor, NRK cells were pretreated for 15 min with 30 μM genistein (Akiyama et al., 1987), before addition of the TSF. As shown in Fig. 10 a, treatment with this tyrosine kinase inhibitor did not affect the $[\text{Ca}^{2+}]_i$ response. In addition, the F-actin reorganization induced by PGTF (Fig. 10 g) and parasite invasion (Fig. 10 j) were also not affected, suggesting that genistein-sensitive protein kinases are not involved in these processes.

NRK cells were also pretreated with the protein kinase C (PKC) inhibitors H-7 (100 μM for 15 min; Kawamoto and Hidaka, 1984) and staurosporine (0.1 μM for 15 min, Tamaoki et al., 1986). Neither treatment interfered with the $[\text{Ca}^{2+}]_i$ response (Fig. 10, b and c), nor with the centripetal F-actin reorganization induced by PGTF (Fig. 10, h and i). However, the inhibitors themselves, particularly staurosporine, induced overall changes in the microfilament pattern of NRK cells (Fig. 10, e and f). The efficiency of invasion by *T. cruzi* was not affected by pretreatment of the host cells with H-7, and it was enhanced by staurosporine (Fig. 10, k and l). The latter result was probably a consequence of the staurosporine-induced overall microfilament depolymerization, similarly to what was observed earlier in cytochalasin D–treated cells (Tardieux et al., 1992).

**Discussion**

In this work we characterize signal transduction events occurring in NRK fibroblasts upon exposure to PGTF, a proteolytically generated factor from the protozoan parasite *T. cruzi* that induces $[\text{Ca}^{2+}]_i$ transients in several mammalian cell types (Burleigh and Andrews, 1995).

We investigated the origin of the $[\text{Ca}^{2+}]_i$ transients induced by PGTF-containing soluble extracts of trypanomastigotes. As controls, we used similar extracts prepared from epimastigotes that do not contain PGTF, or trypanomastigote fractions treated with leupeptin to inhibit PGTF generation. We conclude that the initial $[\text{Ca}^{2+}]_i$ elevation induced by PGTF in NRK cells is mediated by $\text{Ca}^{2+}$ release from intracellular stores, as the signal is not dependent on extracellular $\text{Ca}^{2+}$ and is completely abolished by thapsigargin. We also show that emptying the intracellular $\text{Ca}^{2+}$ stores with thapsigargin significantly inhibits NRK cell entry by *T. cruzi*, confirming previous findings implicating the PGTF-induced $[\text{Ca}^{2+}]_i$ transients in invasion (Tardieux et al., 1994).

The observation that thapsigargin inhibits the $[\text{Ca}^{2+}]_i$ transients induced in NRK cells by both soluble extracts and intact trypanomastigotes indicates that these responses have a similar origin. One difference, however, is observed: the response to the soluble trypanomastigote fraction occurs synchronously in the majority of the target cells, while intact parasites induce asynchronous, repetitive $[\text{Ca}^{2+}]_i$ oscillations in only a few cells of the population. While the reason for this difference is not yet clear, it is

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**Figure 9.** Correlation between the NRK cell $[\text{Ca}^{2+}]_i$ response and the percentage of cells showing F-actin rearrangement over time. The fluorescence intensity profile for $[\text{Ca}^{2+}]_i$, shown corresponds to the average of measurements performed in 10 individual fluo-3-loaded NRK cells. The percentage of cells with F-actin reorganization was determined by counting altered cells in a total of 500, after exposure to TSF, thrombin (0.5 U/ml) or ionomycin (5 μM), and staining with FITC-phalloidin. Agonists were added at time = 0 s.
Figure 10. Protein kinase inhibitors do not block Ca\(^{2+}\) signaling, F-actin rearrangement and host cell invasion by T. cruzi. NRK fibroblasts were pretreated with genistein (30 μM, 15 min), staurosporine (0.1 μM, 15 min), or H-7 (100 μM, 15 min). (a–c) single cell [Ca\(^{2+}\)]\(_i\) responses of fluo-3-loaded NRK fibroblasts to TSF. Arrow indicates TSF addition; (d–f) FITC-phalloidin staining of cells treated for 30 s with PBS\(^{2+}\); (g–i) FITC-phalloidin staining of cells treated for 30 s with TSF. (j–l) invasion of T. cruzi trypomastigotes into NRK cells. The data represents the average of triplicates ± SD. Bar, 10 μm.
conceivable that live parasites release small amounts of PGTF intermittently, as they associate with individual host cells. Low concentrations of PGTF could be the factor responsible for the slower Ca^{2+} oscillations observed in cells stimulated by live parasites. The frequency of Ca^{2+} oscillations induced through the inositol phospholipid signaling pathway of mammalian cells often depends on the concentration of the extracellular agonist (Woods et al., 1986).

The role of IP_3 in releasing Ca^{2+} from intracellular stores through IP_3-sensitive channels is well documented (Berridge, 1993). We have detected a specific increase in polyphosphoinositide hydrolysis with the generation of IP_3 after addition of PGTF to NRK cells. The kinetics of inositol phosphate release induced by the TSF in the presence of LiCl was similar to that induced by hormones and growth factors in other cell types (Paris and Pouyssegur, 1986; Rosenbach and Greenlee, 1991). Taken together with the inhibitory effect of a specific PLC inhibitor, our results strongly suggest that IP_3 is involved in the intracellular Ca^{2+} mobilization induced by PGTF in NRK cells.

Signaling mediated by PLC activation and IP_3 formation is usually triggered by the stimulation of specific plasma membrane receptors, often coupled to intracellular messengers through GTP-binding regulatory proteins (G proteins). The early finding that pertussis toxin inhibits the T. cruzi-induced [Ca^{2+}], transients (Tardieux et al., 1994) suggests the involvement of a trimeric G protein containing a PTx-sensitive G_N subunit, perhaps associated with a seven-transmembrane class of receptor. However, we cannot at this point exclude the possibility that PGTF may be a nonspecific agonist similar to the cationic amphiphilic neupeptides and veno peptides that directly activate G proteins in the absence of a specific receptor (Mousli et al., 1990).

The observed recruitment and fusion of host cell lysosomes at the invasion site, and the enhancement in parasite entry after disruption of microfilaments with cytochalasin D (Tardieux et al., 1992) suggested that actin reorganization might be an early step in the T. cruzi invasion process. Ca^{2+} regulation of the actin cytoskeleton through actin-binding proteins is extensively documented (Vanderkhove, 1990; Watts and Howard, 1992; Stossel, 1993; Weeds and Maciver, 1993). There is also evidence that reorganization of the cortical actin cytoskeleton is necessary to allow vesicles to reach exocytotic sites in stimulated secretory cells (Burgouyne and Cheek, 1987; Linstedt and Kelly, 1987; Aunis and Bader, 1988; De Camilli et al., 1990). Therefore, we were interested in verifying if the [Ca^{2+}] transients induced by T. cruzi trypomastigotes had an effect on the organization of host cell microfilaments. We found that addition of the PGTF-containing TSF to NRK cells resulted in a rapid and transient reorganization of the cortical actin cytoskeleton. Live trypomastigotes induced similar changes, but in an asynchronous fashion that allowed detection of F-actin reorganization in only a few cells of the population at a given time. This observation correlates well with the asynchronous pattern of Ca^{2+} signaling induced in NRK cells by live trypomastigotes (Tardieux et al., 1994; Fig. 2).

An increase in [Ca^{2+}] appears to be sufficient for the rapid centripetal F-actin reorganization induced by T. cruzi in NRK cells. This interpretation is based on two sets of data. First, a similarly altered microfilament pattern was detected after exposure of the NRK cells to the Ca^{2+} ionophore ionomycin. Second, there was a good correlation between the kinetics of the Ca^{2+} signal and that of the F-actin rearrangement. Therefore, the [Ca^{2+}] rise triggered by PGTF may be directly responsible for changes in the organization of actin, possibly by activating Ca^{2+}-dependent microfilament-severing proteins such as gelsoxin (Watts and Howard, 1992; Weeds and Maciver, 1993). However, we must also consider a direct role for PLC-mediated polyphosphoinositide hydrolysis in disassembly of the cortical cytoskeleton, since it has been proposed that release of phosphoinositide-binding actin regulatory proteins can lead to capping, severing, and sequestration of actin monomers (Goldschmidt-Clemont et al., 1991; Stosseg, 1993; Janmey 1994).

Centripetal F-actin reorganization such as the one induced in NRK cells by T. cruzi has been reported in other cell types, and shown to accompany cellular processes such as locomotion, pseudopod extension, and secretion (Heath, 1983; Norman et al., 1994). In microglial cells, mobilization of Ca^{2+} from intracellular stores by bacterial lipopolysaccharide is associated with reorganization of actin filaments into a diffuse perinuclear pattern (Bader et al., 1994). Such rearrangement appears to involve a reduction in F-actin content in the cortical region and an increase in the cell interior (Norman et al., 1994).

It remains to be determined if host cell invasion by T. cruzi involves a local disassembly of the cortical actin cytoskeleton, and if these changes can be correlated with a localized cytosolic Ca^{2+} wave. An area of elevated [Ca^{2+}], was recently detected in L6E9 myoblasts around cell-associated T. cruzi trypomastigotes (Moreno et al., 1994). Lysosome recruitment and fusion may be regulated by a localized increase in [Ca^{2+}], similarly to what occurs during azurophil granule fusion in neutrophils (Jaconi et al., 1990), neurotransmitter release in synaptic terminals (Smith et al., 1988) and perforin release from cytotoxic T lymphocytes (Poenie et al., 1987). It is interesting to note that neutrophil azurophil granules and the lytic granules of cytotoxic lymphocytes are considered to have a common biogenesis with lysosomes, based on the presence of hydrolytic enzymes and of lysosomal membrane glycoproteins (Jaconi et al., 1990; Griffiths and Isaaz, 1992).

NRK cell pretreatment with genistein, a tyrosine kinase inhibitor, and of H-7 and staurosporine, inhibitors of PKC and other kinases, had no detectable effect on the [Ca^{2+}], transients or on the centripetal F-actin reorganization induced by PGTF. However, staurosporine significantly enhanced parasite invasion. We believe this effect is related to the overall microfilament disassembly that occurred in NRK cells after exposure to staurosporine. Disruption of the actin cytoskeleton with cytochalasin D causes a similar degree of enhancement in T. cruzi invasion (Tardieux et al., 1992). The disruptive effect of staurosporine on the general organization of the actin cytoskeleton has been often overlooked, and may explain previous observations attributed to other protein kinase dependent processes (e.g., Roubey et al., 1991; Zheleznyak and Brown, 1992; Parton et al., 1994). The lack of inhibition by staurosporine and H-7 in our studies also indicate that PKC activity is probably not required for T. cruzi invasion of NRK cells. Fur-
therefore, the observation that genistein does not affect cell entry by T. cruzi in these cells provides additional evidence that the process is distinct from phagocytosis, which has been shown to be inhibited by genistein (Greenberg et al., 1993).

The signal transduction events we detected in NRK fibroblasts after exposure to the proteolytically-generated trypanastigote factor PGTF are very similar to those following cell stimulation by several hormones and growth factors. A few seconds after PGTF stimulation different cell types respond with an elevation in [Ca\(^{2+}\)], that reaches concentrations between 300 and 700 nM (Burleigh and Andrews, 1995). We demonstrate here that there is a rapid, PLC-dependent polyphosphoinositide hydrolysis with production of IP\(_3\), Ca\(^{2+}\) release from intracellular stores and a dramatic F-actin reorganization in NRK cells. Inhibition of various steps in this signal transduction cascade results in block of the T. cruzi invasion process, strongly suggesting a role for PGTF in mediating the parasite-host cell interaction.

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