

Dual Role of Signaling Pathways Leading to Ca^{2+} and Cyclic AMP Elevation in Host Cell Invasion by *Trypanosoma cruzi*

ELISABET V. CALER,¹ RORY E. MORTY,¹ BARBARA A. BURLEIGH,² AND NORMA W. ANDREWS^{1*}

Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06520,¹ and the Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts 02155²

Received 6 July 2000/Returned for modification 19 August 2000/Accepted 4 September 2000

Cell invasion by the protozoan parasite *Trypanosoma cruzi* involves activation of host signaling pathways and the recruitment and fusion of lysosomes at the parasite entry site. A major signaling pathway regulating invasion of fibroblasts, epithelial cells, and myoblasts involves mobilization of Ca^{2+} from intracellular stores and requires the activity of a *T. cruzi* serine peptidase, oligopeptidase B (OPB). Deletion of the OPB gene results in a marked defect in trypomastigote virulence, consistent with a greatly reduced cell invasion capacity. Here we show that uptake by macrophages, on the other hand, is largely independent of OPB expression and sensitive to inhibition of by cytochalasin D. The residual invasion capacity of OPBnull trypomastigotes in fibroblasts still involves lysosome recruitment, although in a significantly delayed fashion. Transient elevations in intracellular Ca^{2+} concentrations were observed in host cells exposed to both wild-type and OPBnull trypomastigotes, but the signals triggered by the mutant parasites were less vigorous and delayed. The capacity of triggering elevation in host cell cyclic AMP (cAMP), however, was unaltered in OPBnull trypomastigotes. Modulation in cAMP levels preferentially affected the residual cell invasion capacity of OPBnull parasites, suggesting that this signaling pathway can play a dominant role in promoting cell invasion in the absence of the major OPB-dependent pathway.

Microbial pathogens have developed a remarkable variety of different strategies to disrupt or exploit mammalian cell processes in order to invade, survive, and propagate in their hosts. Signaling between pathogens and host cells has emerged as a key regulatory feature during mammalian cell invasion, as exemplified by enteric bacterial pathogens (11, 15). However, in contrast to bacteria, which often utilize host cell actin-driven uptake mechanisms, larger pathogens such as protozoa exhibit quite distinct and unusual infection strategies (1). *Trypanosoma cruzi*, the causative agent of Chagas' disease in humans, is a protozoan parasite capable of invading a large variety of cell types in its vertebrate host. Previous work from our laboratory revealed that invasion of many cell types by *T. cruzi* is independent of host actin polymerization and involves recruitment and fusion of host cell lysosomes at the site of parasite attachment (2, 25, 30, 32).

The directional movement and localized fusion of lysosomes at the *T. cruzi* attachment site suggested that a signal of parasite origin was locally transduced in host cells. This hypothesis was reinforced when trypomastigotes, the infective *T. cruzi* life cycle stages, were shown to activate phospholipase C and to trigger IP_3 -mediated Ca^{2+} release from host cell intracellular stores (24, 31). Characterization of this signaling pathway revealed that a parasite serine peptidase oligopeptidase B (OPB), is required for the generation of a soluble factor that triggers intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) transients in mammalian cells (4–6).

Deletion of the *T. cruzi* OPB gene severely impairs the ability of trypomastigotes to invade mammalian cells and to establish infections in mice, without affecting parasite growth

rates, differentiation, motility, or protein synthesis. The invasion defect of OPBnull trypomastigotes is associated with their inability to mobilize Ca^{2+} from thapsigargin-sensitive stores in mammalian host cells (6). Unlike wild-type (WT) parasites, the diminished invasion capacity of the OPBnull parasites, (about 25 to 30% of WT levels) was found to be refractory to pretreatment with thapsigargin, a drug that depletes intracellular Ca^{2+} stores (6). These data are consistent with the hypothesis that *T. cruzi* OPB functions in the generation of a Ca^{2+} signaling agonist for mammalian cells. This was directly demonstrated by reconstitution of the Ca^{2+} signaling activity in soluble extracts of OPBnull trypomastigotes with recombinant OPB (6). Interestingly, the residual level of host cell invasion by the OPBnull mutants was completely abolished when host cells were pretreated with the Ca^{2+} chelator MAPTA-AM, suggesting that the OPBnull trypomastigotes retain a requirement for host cell Ca^{2+} elevation for invasion (6).

In addition to Ca^{2+} signaling, *T. cruzi* trypomastigotes (but not the noninfective epimastigote forms) trigger elevation in host cell cyclic AMP (cAMP) levels. Furthermore, inhibition of host cell adenylyl cyclase inhibits parasite invasion, whereas stimulation of cAMP production enhances it (23). Modulation in cAMP levels was also found to affect Ca^{2+} -dependent exocytosis of lysosomes, similar to what has been reported for other Ca^{2+} -regulated secretory pathways (23). Taken together with the observation that both *T. cruzi* entry and lysosome exocytosis are enhanced by disruption of the host cell actin cytoskeleton (23), these findings point to important functional parallels between this parasite's unusual cell invasion mechanism and Ca^{2+} -regulated exocytosis (17, 23, 26).

The goal of the present study was to investigate the mechanisms underlying the residual capacity for cell invasion by the OPBnull trypomastigotes. Since deletion of the OPB gene abolishes the ability of *T. cruzi* to mobilize Ca^{2+} from host cell intracellular stores (6), it became important to determine if the cAMP signaling pathway was also affected by this mutation,

* Corresponding author. Mailing address: Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Ave., New Haven, CT 06536. Phone: (203) 737-2410. Fax: (203) 737-2630. E-mail: norma.andrews@yale.edu.

and if cAMP levels differentially affected the invasion capacity of OPBnull and WT parasites. We also investigated the role of lysosome recruitment in the residual infectivity of the OPBnull mutants, and compared the kinetics of [Ca²⁺]_i transient generation in host cells by live OPBnull and WT trypomastigotes by digital fluorescence microscopy analysis.

MATERIALS AND METHODS

Materials. Pertussis toxin (PTx), 3-isobutyl-1-methylxanthine (IBMX), isoproterenol hydrochloride (ISO) and MDL-12,330A were from Calbiochem; cytochalasin D, 4',6'-diamidino-2-phenylindole (DAPI), leupeptin, probenecid, and 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) were from Sigma. Fluo-3-AM and pluronic acid were from Molecular Probes. 2-[³H]adenine was from Amersham Life Science, and ZFA-FMK was from Enzyme Systems Products.

Mammalian cells and parasites. Normal rat kidney (NRK) fibroblasts and J774 cells were maintained in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum (DMEM-10% FBS) at 37°C in a humidified atmosphere containing 5% CO₂. OPBnull trypomastigotes were generated by targeted replacement of the two alleles of the OPB gene as described earlier (6). Y strain WT and OPBnull tissue culture trypomastigotes were maintained by weekly passages on LLCMK₂ cells as described previously (3). Epimastigotes were grown and maintained in liver infusion tryptose medium as described by Nogueira and Cohn (21).

***T. cruzi* cell invasion assays and immunofluorescence.** Mammalian cells were plated at a density of 2.5 × 10⁴ cells/cm² in DMEM-10% FBS on 12-mm round coverslips placed in 6-cm plastic tissue culture dishes and grown for 48 h at 37°C in a humidified atmosphere containing 5% CO₂. Coverslips with attached cells were washed briefly with 2% FBS-DMEM and transferred to 3.5-cm dishes immediately prior to incubation for 1 h at 37°C with purified trypomastigotes at 5 × 10⁷ parasites/ml. For cell pretreatments, drugs were added at the following final concentrations: PTx, 0.4 μg/ml; cytochalasin D, 10 μM; 8-Br-cAMP, 1 mM; IBMX, 500 μM; isoproterenol hydrochloride, 10 μM. Infected cells were washed 3 times with cold phosphate-buffered saline (PBS), fixed in 2% (wt/vol) paraformaldehyde-PBS, and the number of intracellular parasites was determined by immunofluorescence as described previously (32). At least 200 host cells from randomly chosen microscopic fields were analyzed for each experimental point. For lysosome-parasite colocalization, infected cells were labeled with the LY1C6 monoclonal antibody against rat Lamp-1, as described previously (32).

Time-lapse fluorescence microscopy. Changes in the [Ca²⁺]_i of NRK cells exposed to WT or OPBnull mutant trypomastigotes were continuously measured at the single cell level, using time-lapse fluorescence video microscopy in an Axiovert 135 microscope (Carl Zeiss, Inc.). A total of 80 μl of trypomastigotes at a concentration of 10⁸ parasites/ml in PBS containing 10 μM leupeptin (to inhibit any OPB-dependent Ca²⁺-signaling activity released from damaged parasites) was added to NRK cells (preloaded for 1 h with 5 μM fluo-3-AM, 0.05% pluronic acid, and 0.5 mM probenecid) 30 s after the initiation of the time-lapse recording. Fluorescent images were collected with a digital camera (Orca II; Hamamatsu) through a 25× objective lens at time-lapse intervals of 2 s, using a computer-controlled shutter system (MetaMorph; Universal Imaging). Total duration of the recording was 600 s.

Determination of intracellular cAMP levels. Confluent monolayers of NRK cells in six-well dishes were prelabeled for 24 h with 2-[³H]adenine (23 Ci/mmol or 5 μCi/ml). After labeling, cells were preincubated in 500 μl of 1 mM IBMX for 10 min at 37°C, followed by the addition of the indicated drugs or parasites in 500 μl of Ringer's BSA buffer (14) and incubated for 30 min at 37°C. After drug or parasite treatment, cells were washed three times with Ringer's BSA, and reactions terminated by the addition of 1 ml of 5% trichloroacetic acid containing 1 mM ATP and 1 mM cAMP per well. Acid-soluble nucleotides were separated on ion-exchange columns as described previously (28). In the experiments with live *T. cruzi*, labeled cells were exposed to 5 × 10⁷ trypomastigotes or epimastigotes for 30 min at 37°C.

RESULTS

Host cell invasion by *T. cruzi* OPBnull trypomastigotes is refractory to pertussis toxin treatment of host cells. Our previous studies established that the *T. cruzi* serine hydrolase OPB is involved in the activation of a host cell signaling pathway that is a key event in the infection process (6). Consistent with the demonstration that the signaling pathway activated by OPB involves IP₃ generation and mobilization of Ca²⁺ from intracellular stores (24), the residual invasion capacity of the OPBnull mutants was found to be refractory to thapsigargin pretreatment of host cells (6). In order to extend this observation and to verify if the signaling pathway involved in intracellular

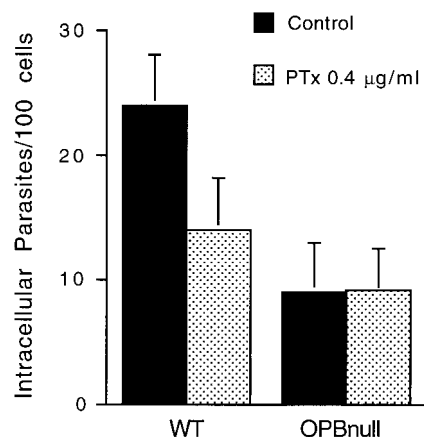


FIG. 1. PTx treatment of host cells does not affect the residual cell invasion capacity of OPBnull trypomastigotes. NRK fibroblasts were pretreated or not with 0.4 mg Ptx per ml overnight at 37°C. After treatment, the toxin was removed and the cells were exposed to WT or OPBnull trypomastigotes for 30 min. The data represent the average of triplicates ± the standard deviation (SD).

Ca²⁺ mobilization was effectively abolished in the OPBnull mutants, we investigated the effect of PTx treatment on the susceptibility of NRK cells to invasion by WT and OPBnull trypomastigotes. PTx catalyzes the ADP ribosylation of G_α and G_α_s, uncoupling these G-α subunits from their receptors and blocking signal transduction (16). Previous results showed, similarly to what is observed after thapsigargin treatment, that both mobilization of Ca²⁺ from intracellular stores and infection of NRK fibroblasts by *T. cruzi* trypomastigotes are inhibited when the host cells are pretreated with PTx (31). In Fig. 1, we show that host cell pretreatment with PTx has no effect on the invasion of OPBnull trypomastigotes, whereas it reduces the entry of WT parasites by ca. 40%. These results suggest that deletion of the OPB gene results in complete loss of the ability to signal host cells via the putative pertussis toxin-sensitive G-coupled receptor pathway, which generates IP₃ and mobilizes Ca²⁺ from intracellular stores (24, 31).

Host cell actin polymerization is not required for residual invasion by OPBnull trypomastigotes. We next investigated the role of the host cell actin cytoskeleton in the residual invasion capacity of the signaling-deficient OPBnull parasites. Earlier experiments established that disruption of the host cell actin cytoskeleton with cytochalasin D significantly facilitates invasion of fibroblasts and epithelial cells by WT *T. cruzi* (32). This effect was interpreted as a removal of the barrier posed by the cortical actin cytoskeleton to lysosome recruitment and fusion, required for cell entry by WT parasites in these cell types (23, 25, 32). Here we compared the effect of cytochalasin D treatment on the susceptibility to invasion by OPBnull and WT trypomastigotes in fibroblasts and in a phagocytic cell line. Inhibition of OPBnull invasion by cytochalasin D in fibroblasts would be an indication of an alternative uptake mechanism, dependent on host cell actin polymerization and not involving lysosome recruitment. The results obtained with the macrophage cell line would complement these findings, since in macrophages *T. cruzi* uptake would be expected to occur primarily by phagocytosis and thus require an intact actin cytoskeleton. Figure 2 shows that pretreatment of NRK cells with cytochalasin D enhanced invasion by OPBnull and WT (Fig. 2a) to a similar extent (66 and 55%, respectively), which is consistent with what was previously reported for WT *T. cruzi* invasion (32). On the other hand, cytochalasin D significantly inhibited

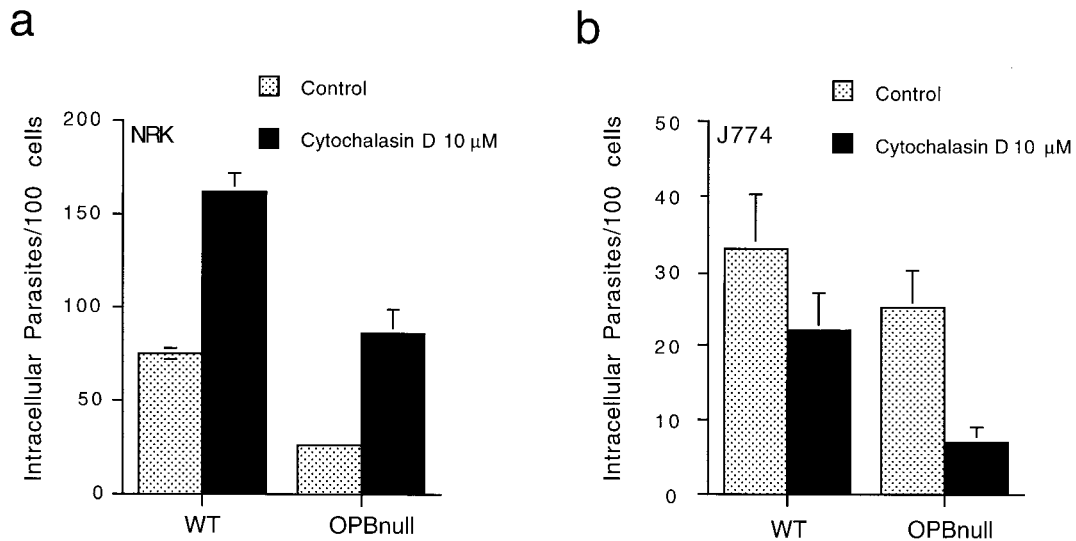


FIG. 2. Cytochalasin D enhances invasion of NRK cells and inhibits invasion of J774 macrophages by WT and OPBnull trypanomastigotes. Cells were pretreated with 10 μM cytochalasin D for 10 min at 37°C and then exposed to parasites for 1 h (a, NRK) or 30 min (b, J774). The data represent the average of triplicates ± the SD.

infection of J774 macrophages by OPBnull parasites (70%), whereas infection by WT trypanomastigotes was reduced to a lesser extent (33%) (Fig. 2b). These results suggest that the residual invasion mechanism of the signaling-deficient OPBnull trypanomastigotes may also involve lysosome recruitment, which is facilitated by disruption of the actin cytoskeleton.

OPBnull trypanomastigotes recruit host cell lysosomes with a delayed kinetics. Several lines of evidence indicate that *T. cruzi* requires lysosome recruitment to invade mammalian cells (23, 25, 32), and that a major component of this invasion mechanism is mediated by OPB-dependent signaling (6). The results discussed above show that the residual invasion capacity of signaling-defective OPBnull trypanomastigotes, although significantly less efficient than WT parasites, still has properties consistent with a requirement for lysosome mobilization. To directly investigate this issue, we determined the kinetics of association between trypanomastigotes and lysosomes in NRK fibroblasts following short-term infections.

Figure 3 shows representative immunofluorescence images obtained following 10-, 20-, and 30-min time courses of *T. cruzi* infection of NRK cells. Extracellular (attached) parasites were visualized by immunofluorescence staining using antibodies against *T. cruzi* added prior to cell permeabilization, and antibodies to the lysosomal glycoprotein Lamp-1 added after cell permeabilization were used to localize host cell lysosomes. This labeling procedure revealed that the residual invasion mechanism of OPBnull trypanomastigotes involves, similar to WT, an initial stage of lysosome clustering at the parasite attachment site, followed by fusion and formation of a parasite-containing intracellular vacuole which stains positive for lysosomal markers. However, whereas WT trypanomastigotes attached to host cells were frequently found associated with lysosomes in the earlier time points, lysosome recruitment by OPBnull parasites showed a delayed pattern (Fig. 3). Quantification of this process (Fig. 4a) showed that the highest percentage of extracellular parasites associated with lysosomes (partially internalized) was reached after 15 min with WT and only after 30 min with OPBnull parasites. The number of completely internalized parasites for the same time course experiment was also determined, revealing a larger number of

intracellular WT parasites in relation to OPBnull in all time points (Fig. 4b).

These results suggest that even though OPBnull trypanomastigotes appear to be able to induce recruitment and fusion of lysosomes at their invasion site, the process is clearly delayed in relation to WT parasites. This finding explains the significantly lower levels of invasion by OPBnull trypanomastigotes observed at a given time point.

[Ca²⁺]_i transients triggered in NRK fibroblasts by OPBnull trypanomastigotes are delayed. Previous results suggested that the alternative signaling mechanism leading to invasion of OPBnull parasites also involved [Ca²⁺]_i transients, because parasite entry is abolished when host cells are loaded with the Ca²⁺ chelator MAPTA-AM (6). However, OPBnull trypanomastigotes do not contain the OPB-dependent soluble agonist present in WT parasites, which triggers Ca²⁺ release from thapsigargin-sensitive intracellular stores (6). We therefore hypothesized that [Ca²⁺]_i transients, perhaps derived from an alternative source of Ca²⁺, might be generated during the interaction of live OPBnull trypanomastigotes with mammalian cells.

In order to initially characterize the pattern of Ca²⁺ signaling triggered by live trypanomastigotes, we performed time-lapse fluorescence imaging of NRK fibroblasts loaded with the Ca²⁺ sensitive dye fluo-3-AM. Figure 5 illustrates a time-lapse sequence in which a localized zone of [Ca²⁺]_i elevation was detected at the site of attachment of a live WT trypanomastigote. Figure 5a and e show phase-contrast images corresponding to the beginning and end, respectively, of the time lapse sequence. In panels b, c, and d are representative frames of the variation in fluo-3 fluorescence intensity detected in the host cell a few seconds after the parasite was stably attached. A localized elevation of [Ca²⁺]_i was observed at the site of trypanomastigote attachment, a pattern consistent with the stimulus originating from the parasite. Other cells in the vicinity without attached trypanomastigotes did not show any [Ca²⁺]_i transients during a 500-s observation period (not shown). This single cell response pattern is distinct from the overall [Ca²⁺]_i elevation observed in the majority of cells when WT trypanomastigote soluble extracts are added to fluo-3-loaded NRK fibroblasts

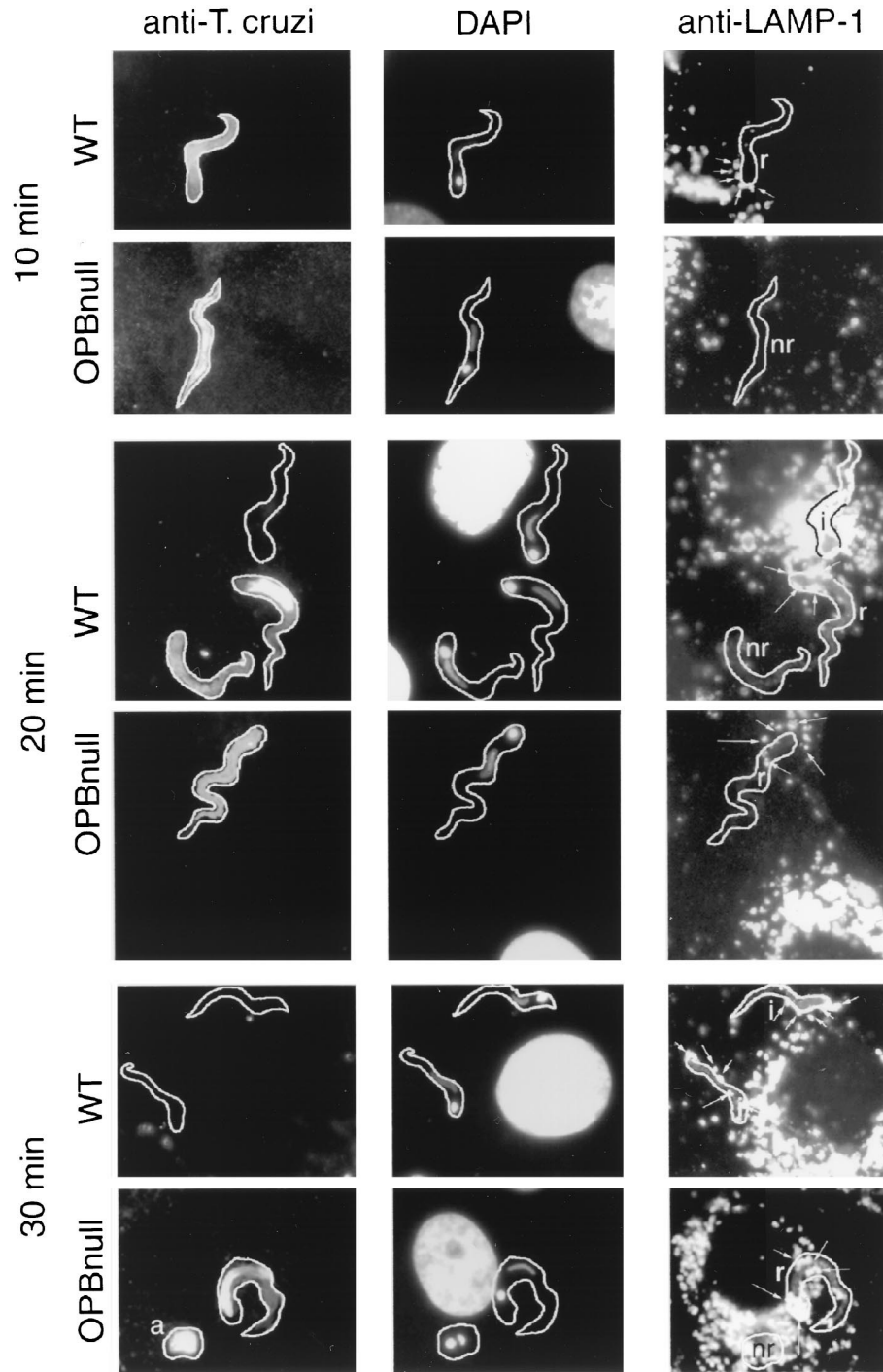


FIG. 3. Kinetics of lysosome recruitment by WT and OPBnull trypanosomes in NRK fibroblasts. NRK cells were exposed to 10⁸ WT or OPBnull trypanosomes per ml for 10, 20, or 30 min at 37°C. Immunofluorescence was performed using (i) anti-*T. cruzi* antibodies prior to cell permeabilization to detect extracellular parasites (left column), (ii) DAPI to detect NRK and parasite nuclei (center column) or (iii) anti-Lamp-1 monoclonal antibody after cell permeabilization to detect host cell lysosomes (right column). Lysosomes recruited to the sites of parasite attachment are indicated by arrows. i, Intracellular parasites; r, extracellular parasites recruiting lysosomes; nr, extracellular parasites not recruiting lysosomes.

(6). We thus proceeded to image fluo-3-AM-loaded NRK cells exposed to WT and OPBnull trypanosomes at the single cell level and to register the time point after contact with the parasites in which individual cells responded with an [Ca²⁺]_i elevation.

Time-lapse imaging experiments showed that live OPBnull trypanosomes are still able to induce a transient [Ca²⁺]_i increase in NRK fibroblasts. We analyzed a total of 28 independent time-lapse experiments in order to compare the kinetics of the signaling triggered by WT and OPBnull trypano-

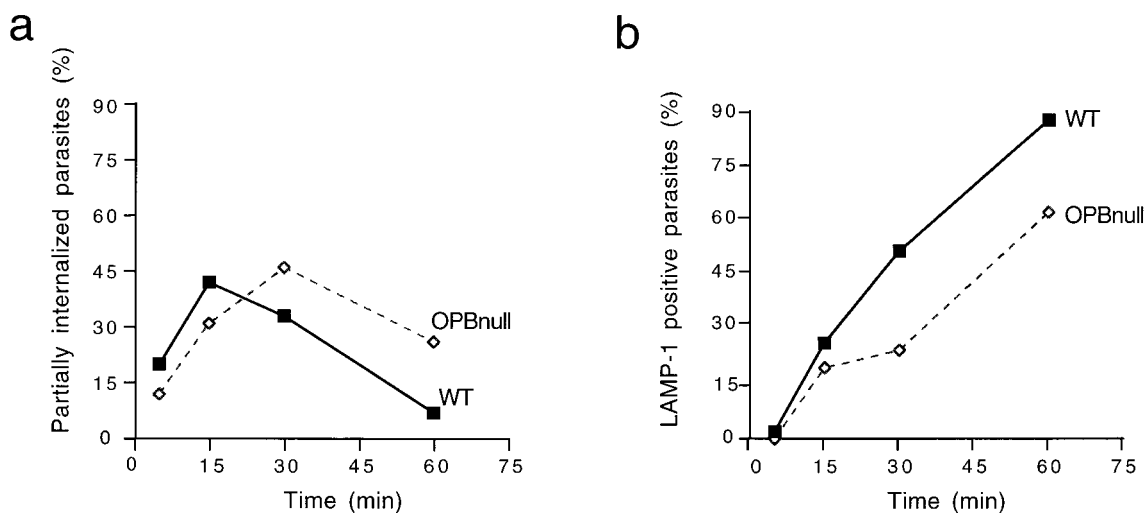


FIG. 4. OPBnull trypanosomes recruit lysosomes and invade cells in a delayed pattern. NRK cells were exposed to WT or OPBnull trypanosomes for the indicated periods of time. Parasites attached to the cells and partially internalized were stained with an anti-*T. cruzi* antibody; recruitment of lysosomes was visualized by staining with an anti-Lamp-1 monoclonal antibody after cell permeabilization, and the total number of parasites was determined by nuclear staining with DAPI. (a) Percent parasites recruiting lysosomes = (number of anti-*T. cruzi*-Lamp-1 double-positive parasites/total parasites) \times 100. (b) Percent internalized parasites = (Lamp-1-positive parasites/total parasites) \times 100.

mastigotes. Table 1 shows that the number of NRK fibroblasts responding with a transient $[Ca^{2+}]_i$ increase to OPBnull trypanosomes is 39% less than the number of cells responding to WT trypanosomes during a 10-min period. Figure 6 shows the response kinetics of individual cells after exposure to OPBnull or WT trypanosomes. A total of 47% of the NRK cells exposed to WT parasites responded with a $[Ca^{2+}]_i$ elevation within 200 s, whereas only 17% of the cells responded to OPBnull trypanosomes in the same time interval (Table 1). In addition, the fraction of experiments in which only one or no cells responded was 32% (nine experiments) for OPBnull trypanosomes, versus 10.5% (three experiments) for WT trypanosomes (Table 1, Fig. 6). The *P* value obtained in a Student's unpaired *t* test of the mean response time of cells exposed to WT or OPB null parasites was 0.0074, thus confirming that the difference between the two groups is highly significant. These findings are consistent with the results of the lysosome recruitment time course experiments (Fig. 3 and 4), and suggest that OPB null trypanosomes trigger less-vigorous Ca^{2+} signaling in NRK cells, which leads to less efficient lysosome recruitment and invasion compared to WT parasites.

OPBnull trypanosomes retain the capacity to elevate host cell cAMP levels, and their invasion capacity is preferentially affected by cAMP modulation. Previous studies demonstrated

that WT trypanosomes stimulate cAMP production in NRK cells and that host cell invasion can be modulated by agents that alter intracellular cAMP levels (23). We investigated if deletion of the OPB gene, in addition to abolishing the parasite's capacity to mobilize Ca^{2+} from intracellular stores, also affected its ability to elevate cAMP. Figure 7a shows that WT and OPBnull trypanosomes induce similar levels of intracellular cAMP elevation after an exposure period of 30 min. The cAMP values measured under these conditions are clearly in the linear range of detection, since treatment with isoproterenol, an agonist that elevates cAMP through stimulation of the β -adrenergic receptor, induces a significantly higher response (Fig. 7a). Also similarly to what is observed with WT parasites, exposure of NRK cells to OPBnull epimastigotes does not result in elevated cAMP (Fig. 7a). These results indicate that the OPB-dependent signaling pathway that controls Ca^{2+} mobilization from intracellular stores is independent from the cAMP-stimulatory pathway. Both pathways, however, are detected only in the infective trypanosome forms and are therefore likely to play important roles in the cell invasion process.

To obtain insight into the role of the cAMP signaling pathway in *T. cruzi* invasion, we investigated the effect of agents that modulate host cell cAMP levels on susceptibility to infection by WT or OPBnull mutant trypanosomes. Pretreatment

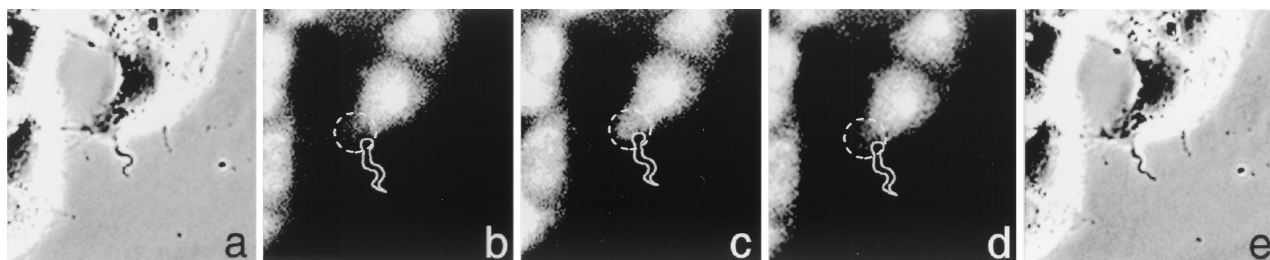


FIG. 5. Localized Ca^{2+} signaling response generated by *T. cruzi* trypanosomes in NRK cells. (a) Phase-contrast image of a stably attached trypanosome at the beginning of the time lapse recording. (b, c, and d) Fluorescence images of fluo-3-loaded NRK cells at 39, 41, and 43 s, respectively, after initiation of the time-lapse recording, showing a localized transient $[Ca^{2+}]_i$ elevation at the site of parasite attachment. (e) Phase-contrast image obtained after finalization of the time-lapse recording. Dashed circles indicate the region of the cell where a localized $[Ca^{2+}]_i$ elevation occurred as a consequence of parasite attachment.

TABLE 1. [Ca²⁺]_i transients generated by OPBnull and WT *T. cruzi* trypomastigotes in NRK fibroblasts

Parasite strain	No. of movies	Total RC ^a	No. of movies with RC of ≤1 (%)	t _r <200 s (%) ^b
WT	14	38	3 (10.5)	18 (47)
OPBnull	14	23	9 (32)	4 (17)

^a RC, total number of cells responding to trypomastigotes with a [Ca²⁺]_i transient. Movies with an RC of ≤1 represent the number of individual movies in which only one or no cell responded.

^b t_r, time elapsed from addition of trypomastigotes to the moment when the first [Ca²⁺]_i transient was detected. A t_r of <200 s represents the number of cells that responded in the initial 200 s after exposure to the parasites.

of NRK cells with the adenylyl cyclase inhibitor MDL-12,330A reduces invasion by both WT and OPBnull trypomastigotes (Fig. 7b), whereas exposure to isoproterenol or 8-Br-cAMP (a membrane-permeant analogue of cAMP) stimulates infection by both parasite types (Fig. 7c). Interestingly, whereas the stimulatory effect of isoproterenol and 8-Br-cAMP on susceptibility to WT trypomastigotes invasion is small (20 to 25% increase), a significantly stronger effect is observed for invasion by OPBnull trypomastigotes (69% increase), with the numbers of intracellular parasites reaching levels similar to those normally obtained with WT parasites. These results suggest that in the absence of the major OPB-dependent signaling pathway, cAMP stimulation plays an important role in the lysosome-mediated residual invasion phenotype observed in the OPBnull parasites.

DISCUSSION

Recent studies have provided extensive evidence that activation of signaling cascades in host cells plays a central role in the *T. cruzi* cell invasion mechanism (5, 20, 34). Mobilization of Ca²⁺ from intracellular stores has been specifically implicated, since cell loading with Ca²⁺ chelators and Ca²⁺ store depletion by thapsigargin effectively inhibit trypomastigote entry (7, 24, 31). An investigation of the *T. cruzi* Ca²⁺ signaling capacity revealed that the infective trypomastigotes forms contain a soluble factor capable of triggering intracellular free Ca²⁺ transients in several mammalian cell types and that production

of this factor requires the activity of a parasite serine peptidase, OPB (4, 5, 6, 24, 31). Deletion of the *T. cruzi* OPB gene by targeted replacement resulted in a significant attenuation of the parasite's virulence for mice and in a 60 to 70% reduction in their ability to invade mammalian cells. Since OPBnull trypomastigotes do not contain the OPB-dependent soluble Ca²⁺ signaling factor, the retention of a residual invasion capacity in the mutant parasites suggested the existence of an alternative mechanism for host cell entry (6).

The major pathway utilized by *T. cruzi* to invade several epithelial and fibroblast cell lines was shown to involve lysosome recruitment and fusion at the parasite attachment site (23, 25, 32). Experimental conditions that affect lysosome distribution or that affect fusion of lysosomes with the plasma membrane significantly interfere with trypomastigote invasion (23, 25, 26). Several lines of evidence support the idea that Ca²⁺-regulated lysosome exocytosis may be a ubiquitous process that is subverted by *T. cruzi* as a mechanism for cell invasion. Regulated exocytosis of granules with lysosomal characteristics has been more frequently described in cells of the hematopoietic lineage, such as platelets, mast cells, neutrophils, and cytotoxic lymphocytes (22). Recent evidence indicates, however, that intracellular free Ca²⁺ elevations can trigger exocytosis of conventional lysosomes in many cell types and that the process may be regulated by a transmembrane protein with Ca²⁺-binding properties, synaptotagmin VII (19, 26). Taken together, the available evidence suggests that activation of host cell signaling pathways and Ca²⁺ elevation at the site of parasite attachment results in localized lysosome recruitment, docking, and fusion, events that contribute to formation of the *T. cruzi*-containing intracellular vacuole.

The low levels of cell invasion observed when host cells are exposed to OPBnull trypomastigotes are not affected by depletion of intracellular Ca²⁺ stores with thapsigargin, while invasion by WT parasites is greatly reduced (6). Here we show that host cell pretreatment with PTx, a condition previously shown to inhibit infection by WT *T. cruzi* (31), also does not affect invasion by OPBnull trypomastigotes. These findings reinforce the hypothesis that OPBnull trypomastigotes lost the capacity to activate the G protein-dependent host cell signaling pathway that leads to IP₃ formation and Ca²⁺ mobilization from intracellular stores and that has been directly linked to lysosome

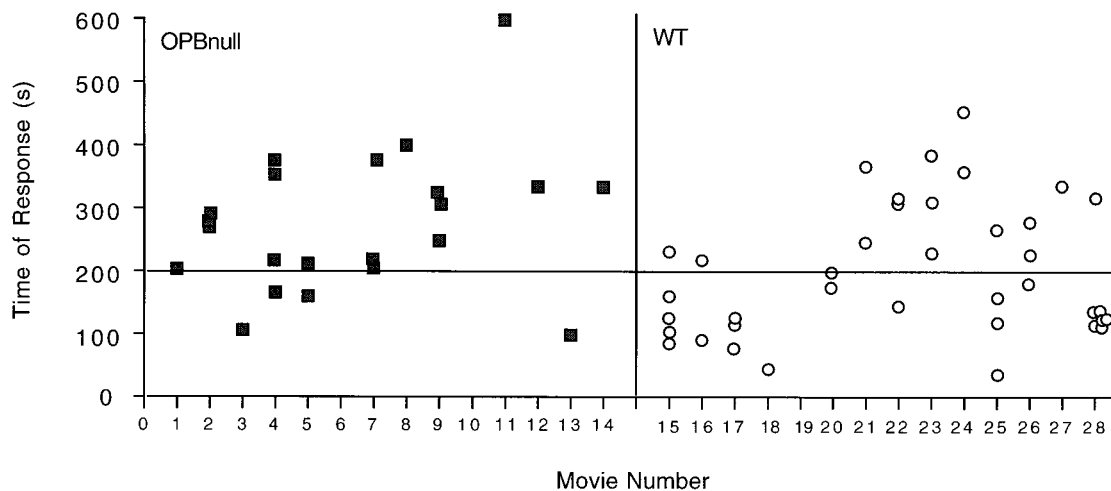


FIG. 6. OPBnull trypomastigotes trigger delayed Ca²⁺ signaling in NRK cells. WT (■) or OPBnull (□) trypomastigotes were added to NRK cells preloaded with the Ca²⁺-sensitive dye fluo-3. Time-lapse images were acquired, and the time frame in which each individual [Ca²⁺]_i elevation occurred was determined. The plot shows the total number of responsive cells in each individual movie for a recording period of 600 s.

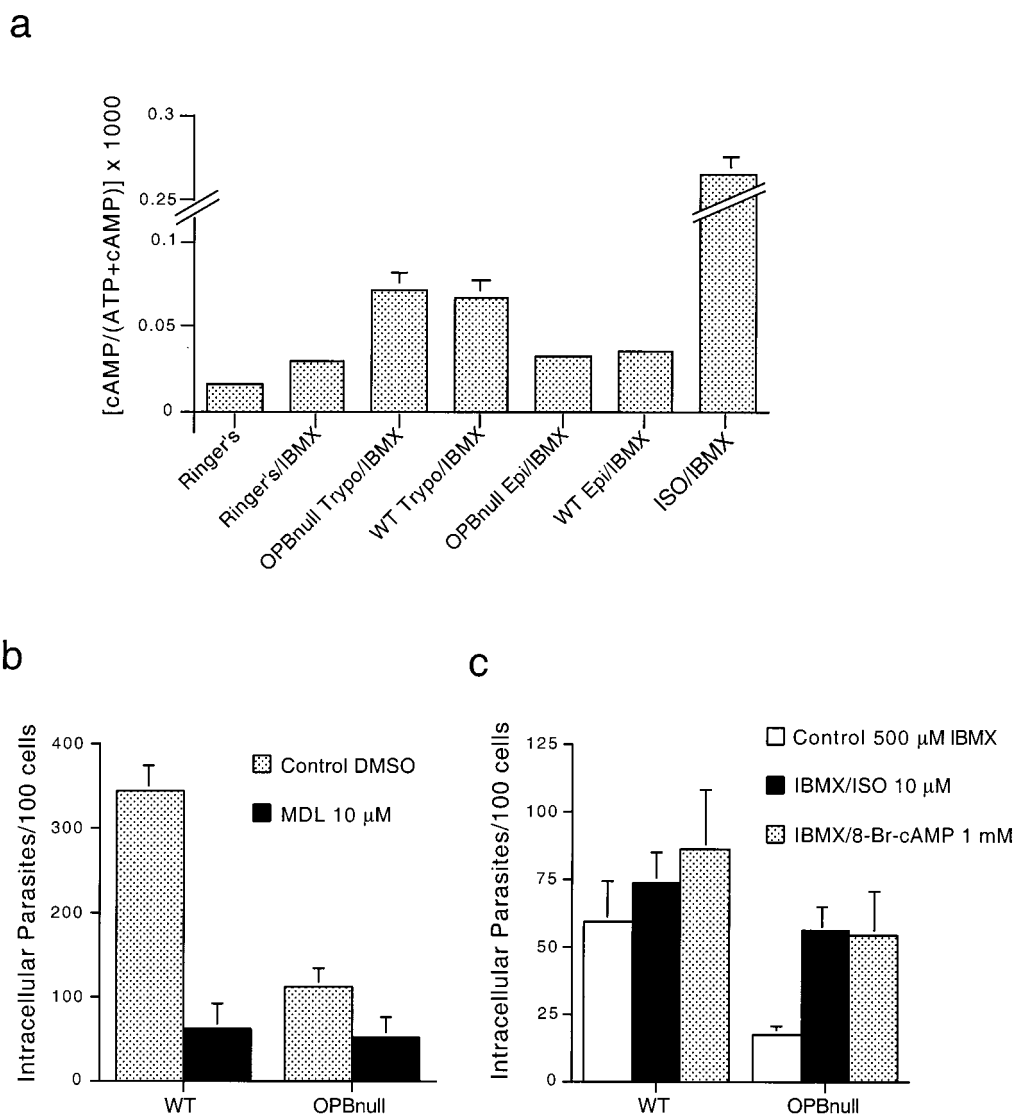


FIG. 7. Residual cell invasion by OPBnull trypanosomes is modulated by cAMP. (a) Intracellular levels of cAMP were measured in IBMX-pretreated NRK cells after exposure to WT and OPBnull trypanosomes and epimastigotes or exposure to isoproterenol. Infection of NRK cells by OPBnull trypanosomes was quantitated after pretreatment of the host cells with different cAMP modulators: b, 10 μ M MDL-12,330A for 30 min at 37°C; and c, 10 μ M isoproterenol or 1 mM 8-Br-cAMP for 30 min at 37°C. The data represent the average of triplicate determinations \pm the SD.

recruitment (5, 6). Such observations raised the possibility that the residual invasion capacity of OPBnull mutants might be dependent on a completely different mechanism, not involving lysosome recruitment.

One possibility was that this alternative invasion mechanism might resemble phagocytosis, requiring host cell actin polymerization. Our data, however, show that invasion of NRK cells by OPBnull trypanosomes is enhanced by host cell pretreatment with cytochalasin D, similarly to what is observed with WT parasites. However, in J774 macrophages cytochalasin D has a more pronounced inhibitory effect on infection by OPBnull than WT parasites suggesting that in phagocytic cells the main route of internalization of the mutant parasites involves actin polymerization. Taken together with the results obtained with PTx treatment, the effect of cytochalasin D indicates that the invasion pathway utilized by OPBnull trypanosomes is independent of OPB-mediated signaling but still involves a mechanism that is facilitated by depolymerization of host cell

actin microfilaments. The apparent more important role of phagocytosis in the uptake of OPBnull parasites by macrophages suggests that the actin-independent pathway utilized for invasion may be less vigorous in these mutants, resulting in a larger fraction of the parasites being engulfed instead of driving their own internalization.

We thus proceeded to directly verify if lysosome recruitment was involved in the cell entry process of OPBnull trypanosomes. Time course experiments revealed that lysosomes are recruited to the sites of both WT and OPBnull trypanosome attachment and that lysosomal markers are similarly gradually incorporated into the nascent parasitophorous vacuoles. However, a marked difference was observed in the kinetics of the process: lysosome recruitment and host cell internalization were significantly slower with OPBnull trypanosomes. Taken together with the observation that in macrophages OPBnull trypanosomes appear to be taken up predominantly by an actin polymerization-dependent phagocytic mechanism, these

results suggest that the invasion capacity of the mutants, although similar to WT with respect to lysosome recruitment, is much less vigorous.

To further understand the basis for the delayed lysosome recruitment phenotype, we investigated the capacity of OPB-null trypomastigotes to trigger intracellular Ca²⁺ transients in NRK cells. As discussed above, previous studies demonstrated that extracts of OPBnull trypomastigotes are deficient in the OPB-dependent factor that mobilizes Ca²⁺ from host cell intracellular stores (6). Since the process of lysosome recruitment appears to be directly linked to intracellular Ca²⁺ elevations, the delayed lysosome recruitment phenotype of OPBnull parasites was predicted to also require Ca²⁺, perhaps mobilized from an alternative source. Direct observations of the interaction between live trypomastigotes and NRK cells loaded with Ca²⁺-sensitive dye confirmed this prediction: OPBnull trypomastigotes are still capable of triggering [Ca²⁺]_i transients, although the process is clearly delayed in relation to what is observed with WT parasites. The more potent response induced by WT trypomastigotes allowed us to visualize events in which the [Ca²⁺]_i transient generated was clearly localized at the parasite attachment site. The responses triggered by OPBnull trypomastigotes, although still detectable at the single cell level, were significantly less intense, precluding detection of a localized response. Although the lack of effect of thapsigargin treatment strongly suggests that OPBnull trypomastigotes are not mobilizing Ca²⁺ from host cell intracellular stores, the alternative source of Ca²⁺ utilized is currently unknown. One interesting possibility is that the "stretch"-induced Ca²⁺ channels that have been detected on the plasma membrane of many cell types (18) might be involved. The vigorous motility exhibited by *T. cruzi* trypomastigotes is not affected by deletion of the OPB gene, so it is conceivable that active motility after host cell attachment could result in the opening of such channels and in Ca²⁺ influx from the extracellular medium. Our observations are consistent with a scenario in which the delayed Ca²⁺ transients triggered by OPBnull trypomastigotes would be the result of Ca²⁺ influx through plasma membrane channels, whereas the more intense response observed with WT parasites would also include Ca²⁺ mobilization from intracellular stores, through the PTx-sensitive, IP₃-mediated signaling pathway that is dependent on the OPB-generated Ca²⁺ agonist.

Previous work in our laboratory showed that host cell cAMP levels modulate both Ca²⁺-dependent lysosome exocytosis and lysosome-mediated *T. cruzi* invasion, again highlighting the interesting similarities between these two processes (23). Although the exact mechanism by which cAMP regulates lysosome exocytosis is not known, it is possible that cAMP-dependent protein kinase A mediates phosphorylation of vesicle membrane components involved in docking and fusion events (10) or that the effects observed are due to phosphorylation-dependent disassembly of cortical cytoskeleton components (8, 12, 27) or modulation of microtubule-dependent vesicular transport (9, 13, 33). We showed previously that membrane-permeant analogs of cAMP enhance *T. cruzi* entry into NRK fibroblasts and that the infective trypomastigotes are able to stimulate cAMP production in host cells (23, 32). Here we found that OPBnull trypomastigotes retain the capacity of elevating host cell cAMP and that the residual invasion capacity of these parasites can be restored close to WT levels by host cell treatment with drugs that stimulate cAMP production. These results indicate that *T. cruzi* is able to trigger at least two independent signaling pathways that facilitate lysosomal recruitment and fusion, a process required for successful invasion of fibroblasts by both WT and OPBnull parasites. The

finding that cAMP levels have a more marked influence on the invasion of OPBnull trypomastigotes suggests that cAMP is modulating the invasion process independently of the major IP₃-dependent signaling pathway that mobilizes Ca²⁺ from intracellular stores. Although cAMP can affect a number of different cellular mechanisms, it is noteworthy that elevation in cAMP levels induces dispersion of lysosomes from the perinuclear area to the cell periphery, the site where *T. cruzi* invasion occurs (14, 29). It is thus conceivable that when the number of lysosomes in the proximity of the plasma membrane is increased, Ca²⁺ influx through plasma membrane channels may provide a sufficient stimulus for lysosome exocytosis and for parasite entry to occur.

ACKNOWLEDGMENTS

This work was supported by an NIH grant and a Burroughs-Wellcome Molecular Parasitology Scholar Award to N.W.A. and by a fellowship from the South African Foundation for Research Development to R.M.

We are very grateful to C. Berlot (Physiology Department, Yale University) for help with cAMP determinations and to H. Tan for excellent technical assistance.

REFERENCES

- Sibley, L. D., and N. W. Andrews. 2000. Cell invasion by un-palatable parasites. *Traffic* **1**:100-106.
- Andrews, N. W. 1995. Lysosome recruitment during host cell invasion by *Trypanosoma cruzi*. *Trends Cell Biol.* **5**:133-137.
- Andrews, N. W., K. S. Hong, E. S. Robbins, and V. Nussenzweig. 1987. Stage-specific surface antigens expressed during the morphogenesis of vertebrate forms of *Trypanosoma cruzi*. *Exp. Parasitol.* **64**:474-484.
- Burleigh, B. A., and N. W. Andrews. 1995. A 120-kDa alkaline peptidase from *Trypanosoma cruzi* is involved in the generation of a novel Ca²⁺-signaling factor for mammalian cells. *J. Biol. Chem.* **270**:5172-5180.
- Burleigh, B. A., E. V. Caler, P. Webster, and N. W. Andrews. 1997. A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca²⁺ signaling in mammalian cells. *J. Cell Biol.* **136**:609-620.
- Caler, E. V., S. Vaena de Avalos, P. A. Haynes, N. W. Andrews, and B. A. Burleigh. 1998. Oligopeptidase B-dependent signaling mediates host cell invasion by *Trypanosoma cruzi*. *EMBO J.* **17**:4975-4986.
- Dorta, M. L., A. T. Ferreira, M. E. M. Oshiro, and N. Yoshida. 1995. Ca²⁺ signal induced by *Trypanosoma cruzi* metacyclic trypomastigote surface molecules implicated in mammalian cell invasion. *Mol. Biochem. Parasitol.* **73**:285-289.
- Downey, G. P., E. L. Elson, B. D. Schwab, S. C. Erzurum, S. K. Young, and G. S. Worthen. 1991. Biophysical properties and microfilament assembly in neutrophils: modulation by cyclic AMP. *J. Cell Biol.* **114**:1179-1190.
- Eppl, H. J., K. M. Kreusel, C. Hanski, J. D. Schulzke, E. O. Riecken, and M. Fromm. 1997. Differential stimulation of intestinal mucin secretion by cholera toxin and carbachol. *Pflügers Arch.* **433**:638-647.
- Fushimi, K., S. Sasaki, and F. Marumo. 1997. Phosphorylation of serine 256 is required for cAMP-dependent regulatory exocytosis of the aquaporin-2 water channel. *J. Biol. Chem.* **272**:14800-4.
- Galán, J. E., and J. B. Bliska. 1996. Cross-talk between bacterial pathogens and their host cells. *Annu. Rev. Cell. Dev. Biol.* **12**:221-255.
- Goldman, J. E., and B. Abramson. 1990. Cyclic AMP-induced shape changes of astrocytes are accompanied by rapid depolymerization of actin. *Brain Res.* **528**:189-96.
- Hayakawa, T., R. Bruck, O. C. Ng, and J. L. Boyer. 1990. DBcAMP stimulates vesicle transport and HRP excretion in isolated perfused rat liver. *Am. J. Physiol.* **259**:G727-G735.
- Heuser, J. E. 1989. Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J. Cell Biol.* **108**:855-864.
- Ireton, K., and P. Cossart. 1998. Interaction of invasive bacteria with host signaling pathways. *Curr. Opin. Cell Biol.* **10**:276-283.
- Ismail, T. P., and J. Shine. 1992. G-protein coupled receptors. *Curr. Opin. Cell Biol.* **4**:195-202.
- Kima, P. E., B. Burleigh, and N. W. Andrews. Surface targeted lysosomal membrane glycoprotein (Lamp-1) enhances lysosome exocytosis and cell invasion by *Trypanosoma cruzi*. *Cell. Microbiol.*, in press.
- Lee, J., A. Ishihara, G. Oxford, B. Johnson, and K. Jacobson. 1999. Regulation of cell movement is mediated by stretch-activated calcium channels. *Nature* **400**:382-386.
- Martinez, I., S. Chakrabarti, T. Hellevik, J. Morehead, K. Fowler, and N. W. Andrews. 2000. Synaptotagmin VII regulates Ca²⁺-dependent exocytosis of lysosomes in fibroblasts. *J. Cell Biol.* **148**:1141-1149.

20. **Ming, M., M. E. Ewen, and M. E. A. Pereira.** 1995. Trypanosome invasion of mammalian cells requires activation of the TGF β signaling pathway. *Cell* **82**:287–296.
21. **Nogueira, N., and Z. Cohn.** 1976. *Trypanosoma cruzi*: mechanism of entry and intracellular fate in mammalian cells. *J. Exp. Med.* **143**:1402–1420.
22. **Page, L. J., A. J. Darmon, R. Uellner, and G. M. Griffiths.** 1998. L is for lytic granules: lysosomes that kill. *Biochim. Biophys. Acta* **1401**:146–156.
23. **Rodriguez, A., I. Martinez, A. Chung, C. H. Berlot, and N. W. Andrews.** 1999. cAMP regulates Ca²⁺-dependent exocytosis of lysosomes and lysosome-mediated cell invasion by trypanosomes. *J. Biol. Chem.* **274**:16754–16759.
24. **Rodriguez, A., M. G. Rioult, A. Ora, and N. W. Andrews.** 1995. A trypanosome-soluble factor induces IP3 formation, intracellular Ca²⁺ mobilization and microfilament rearrangement in host cells. *J. Cell Biol.* **129**:1263–1273.
25. **Rodriguez, A., E. Samoff, M. G. Rioult, A. Chung, and N. W. Andrews.** 1996. Host cell invasion by trypanosomes requires lysosomes and microtubule/kinesin-mediated transport. *J. Cell Biol.* **134**:349–362.
26. **Rodriguez, A., P. Webster, J. Ortego, and N. W. Andrews.** 1997. Lysosomes behave as Ca²⁺-regulated exocytic vesicles in fibroblasts and epithelial cells. *J. Cell Biol.* **137**:93–104.
27. **Rovere, P., L. Inverardi, J. R. Bender, and R. Pardi.** 1996. Feedback modulation of ligand-engaged alpha L/beta 2 leukocyte integrin (LFA-1) by cyclic AMP-dependent protein kinase. *J. Immunol.* **156**:2273–2279.
28. **Salomon, Y., C. Londos, and M. Rodbell.** 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548.
29. **Schenkman, S., N. W. Andrews, V. Nussenzweig, and E. S. Robbins.** 1988. *Trypanosoma cruzi* invade a mammalian epithelial cell in a polarized manner. *Cell* **55**:157–165.
30. **Schenkman, S., C. Diaz, and V. Nussenzweig.** 1991. Attachment of *Trypanosoma cruzi* trypomastigotes to receptors at restricted cell surface domains. *Exp. Parasitol.* **72**:76–86.
31. **Tardieux, I., M. H. Nathanson, and N. W. Andrews.** 1994. Role in host cell invasion of *Trypanosoma cruzi*-induced cytosolic free Ca²⁺ transients. *J. Exp. Med.* **179**:1017–1022.
32. **Tardieux, I., P. Webster, J. Ravesloot, W. Boron, J. A. Lunn, J. E. Heuser, and N. W. Andrews.** 1992. Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells. *Cell* **71**:1117–1130.
33. **Tousson, A., C. M. Fuller, and D. J. Benos.** 1996. Apical recruitment of CFTR in T-84 cells is dependent on cAMP and microtubules but not Ca²⁺ or microfilaments. *J. Cell Sci.* **109**:1325–1334.
34. **Yoshida, N., S. Favoreto, Jr., A. T. Ferreira, and P. M. Manque.** 2000. Signal transduction induced in *Trypanosoma cruzi* metacyclic trypomastigotes during the invasion of mammalian cells. *Braz. J. Med. Biol. Res.* **33**:269–278.

Editor: W. A. Petri, Jr.