

## A Factor from *Trypanosoma cruzi* Induces Repetitive Cytosolic Free Ca<sup>2+</sup> Transients in Isolated Primary Canine Cardiac Myocytes

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An unusual 120-kDa alkaline peptidase contained in a trypomastigote soluble fraction (TSF) of *Trypanosoma cruzi* is associated with the induction of repetitive Ca<sup>2+</sup> transients and subsequent invasion by the parasite of a number of mammalian cell lines, including tissue culture L<sub>6</sub>E<sub>2</sub> myoblasts (B. A. Burleigh and N. W. Andrews, *J. Biol. Chem.* 270:5172–5180, 1995; S. N. J. Moreno, J. Silva, A. E. Vercesi, and R. Docampo, *J. Exp. Med.* 180:1535–1540, 1994; A. Rodríguez, M. G. Rioult, A. Ora, and N. W. Andrews, *J. Cell Biol.* 129:1263–1273, 1995; I. Tardieux, M. H. Nathanson, and N. W. Andrews, *J. Exp. Med.* 179:1017–1022, 1994). Using single cell spectrofluorometry and whole-cell patch clamping, we show that TSF produces rapid repetitive cytosolic Ca<sup>2+</sup> transients (each associated with cell contraction) in primary cardiac myocytes isolated from dogs. The response of myocytes to TSF was dose dependent in that increasing numbers of cells responded to increasing concentrations of TSF. The TSF-induced Ca<sup>2+</sup> transients could be obliterated when TSF was heated or treated with trypsin or the protease inhibitor leupeptin. Aprotinin, pepstatin A, and E-64 did not affect TSF activity. The TSF-induced Ca<sup>2+</sup> transients and trypomastigote cell invasion could not be inhibited by  $\alpha$  (prazosin)- or  $\beta$  (propranolol)-adrenergic blockers or L-type Ca<sup>2+</sup> channel blockers (verapamil, nisoldipine, or cadmium) or by removal of extracellular Ca<sup>2+</sup>. However, inhibition of pertussis toxin-sensitive G proteins and Ca<sup>2+</sup> release from the sarcoplasmic reticulum (with thapsigargin or ryanodine) prevented the TSF-induced Ca<sup>2+</sup> transients and cell invasion by trypomastigotes. These data suggest that cardiac myocyte pertussis toxin-sensitive G proteins are associated with the regulation of TSF-induced Ca<sup>2+</sup> transients and myocyte invasion by trypomastigotes but are independent of Ca<sup>2+</sup> entry into the cytosol via L-type Ca<sup>2+</sup> channels. The Ca<sup>2+</sup> transients are dependent on release of Ca<sup>2+</sup> from sarcoplasmic reticulum Ca<sup>2+</sup> stores, but this release is not dependent on extracellular Ca<sup>2+</sup> or on the classic model of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release in cardiac myocytes. Further, subthreshold depolarizations, together with cell contraction as demonstrated by whole-cell patch clamping, occurred with each Ca<sup>2+</sup> transient. However, the depolarizations were of magnitude insufficient to generate an action potential, providing further evidence for a lack of dependence on L-type Ca<sup>2+</sup> channels and other voltage-dependent channels (Na<sup>+</sup> and K<sup>+</sup> channels) in the generation of TSF-induced Ca<sup>2+</sup> transients. Our findings suggest that primary canine cardiac myocytes respond to TSF and parasite invasion in ways similar to those of the *in vitro* cell lines studied to date. Since cardiac myocytes are primary targets for *T. cruzi* in the vertebrate host, our study indicates that TSF may play a role in the pathogenesis of Chagas' disease in humans.

*Trypanosoma cruzi*, the protozoan parasite that causes Chagas' disease, invades nonphagocytic vertebrate cells by a unique mechanism independent of pseudopodium formation, which still occurs after actin filaments are disrupted with cytochalasin D (22, 23, 27). Trypomastigotes are in close contact with the host cell for up to 10 min prior to entry. During this association, lysosomes are recruited, fuse with one another and with the cell membrane directly adjacent to the invasion site, and appear to form the parasitophorous vacuole which the parasite enters (9, 27). These coordinated processes suggest the need for communication between the parasite and the host cell, eventually leading to lysosomal recruitment and parasite entry. Subsequently, infective trypomastigotes and their isolated membranes, but not noninfective epimastigotes, induce repetitive cytosolic free Ca<sup>2+</sup> transients in rat kidney fibroblasts *in vitro* (26). Induction of the Ca<sup>2+</sup> transients was shown to be pertussis toxin (PTx) sensitive and dependent on a phospholipase C/phosphoinositide signaling pathway (21, 26). A

120-kDa alkaline peptidase (produced by both epimastigotes and trypomastigotes) was involved in the generation of the repetitive cytosolic free Ca<sup>2+</sup> transients (which arose from intracellular stores) in a number of different mammalian cell lines, possibly by processing of precursors present only in infective trypomastigotes (5). Inhibition of the Ca<sup>2+</sup> transients was associated with interference with host cell invasion by the parasite (21, 26).

Although it has been demonstrated to occur in a wide range of mammalian cells *in vitro* (5), including tissue culture L<sub>6</sub>E<sub>2</sub> myoblasts (14), no studies to date have demonstrated the phenomenon for cells that form a natural target of *T. cruzi* in the host. In this work we show that rapid, repetitive cytosolic Ca<sup>2+</sup> transients were induced by a trypomastigote soluble fraction (TSF) in primary cardiac myocytes isolated from dogs. Further, the cardiac myocyte contracts with each Ca<sup>2+</sup> transient, but this event is not associated with the generation of an action potential. Our data suggest that the Ca<sup>2+</sup> transient is mediated by a PTx-sensitive G-protein-dependent release of Ca<sup>2+</sup> from sarcoplasmic reticulum (SR), independent of Ca<sup>2+</sup> entry via L-type Ca<sup>2+</sup> channels or from extracellular sources. We also

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show that inhibition of  $\text{Ca}^{2+}$  release from SR stores is associated with an inhibition of invasion of cardiac myocytes by *T. cruzi*.

#### MATERIALS AND METHODS

**Reagents.** Prazosin, propranolol, nisoldipine, cadmium, verapamil, EGTA [ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], leupeptin, aprotinin, PTx, thapsigargin, ryanodine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), trypsin, soybean trypsin inhibitor (SBTI), and pronase E were obtained from Sigma Chemical Co., St. Louis, Mo. Fura-2/AM was obtained from Molecular Probes, Inc., Eugene, Oreg. Bis-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester (MAPTAM) was obtained from Calbiochem Corp., San Diego, Calif. *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) was obtained from Boehringer Mannheim, Indianapolis, Ind.

***T. cruzi* strains and growth conditions.** Trypomastigotes from the *T. cruzi* Y strain were obtained from the supernatant of infected LLC-MK<sub>2</sub> cells (2). Epimastigotes from the Y strain were cultured in liver infusion tryptose medium (LIT) containing 10% fetal bovine serum (FBS) at 28°C and used as controls (5, 18).

**Parasite soluble factor preparation.** Trypomastigotes or epimastigotes were washed twice and resuspended in Dulbecco's phosphate-buffered saline (PBS) containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ( $\text{PBS}^{2+}$ ) at  $2 \times 10^8/\text{ml}$ . Parasites were killed by heating (56°C; 5 min) and then frozen at -80°C. Trypanosomes were thawed, sonicated on ice with a microtip for two bursts of 15 s at a setting of 2 (Model 250 sonicator; Branson Ultrasonics Corp., Danbury, Conn.), and centrifuged ( $700 \times g$  for 10 min at 4°C). The postnuclear supernatant was further centrifuged ( $100,000 \times g$  for 30 min at 4°C), and the resulting supernatant fraction was passed through a 1-ml concanavalin A-Sepharose column (Pharmacia Chemicals, Uppsala, Sweden). The unbound fraction containing the soluble fractions of trypomastigotes (TSF) and epimastigotes (epimastigote soluble fraction [ESF]) was collected and stored at -80°C (5).

**Parasite culture supernatant preparations.** Trypomastigotes and epimastigotes were each washed three times in PBS (at  $8,000 \times g$  and  $1,000 \times g$ , respectively) for 15 min at 4°C; resuspended in Eagle minimum essential medium (Life Technologies, Grand Island, N.Y.) containing 10% FBS and LIT containing 10% FBS, respectively, at  $2 \times 10^8/\text{ml}$ ; and incubated for 24 h at 37 and 28°C, respectively, and then organisms were removed (at  $10,000 \times g$  for 20 min at 4°C). Supernatants from trypomastigotes and epimastigotes were stored at -80°C.

**Canine cardiac myocytes.** Adult beagles were euthanized with barbiturate, and their hearts were removed and placed in cool Tyrode solution (pH 7.3) containing the following (millimolar concentrations are given):  $\text{MgCl}_2$ , 0.7;  $\text{NaH}_2\text{PO}_4$ , 0.9;  $\text{CaCl}_2$ , 2.0; NaCl, 124;  $\text{NaHCO}_3$ , 24; KCl, 4; and glucose, 5.5, as described previously (19). Briefly, the circumflex coronary artery or a branch of the left anterior descending coronary artery was cannulated and a portion of the left ventricle was excised. The cannulated segment was perfused at a flow rate of 18 ml/min with oxygenated solutions maintained at 37°C. The tissue initially was perfused with Tyrode solution. After 10 to 15 min, the perfusion was switched to a  $\text{Ca}^{2+}$ -free solution (pH 7.3) containing the following (millimolar concentrations are given): NaCl, 118; KCl, 4.8;  $\text{MgSO}_4$ , 1.2;  $\text{KH}_2\text{PO}_4$ , 1.2; glutamine, 0.68; glucose, 11;  $\text{NaHCO}_3$ , 25; pyruvate, 5; mannitol, 2; and taurine, 10. At approximately 3 to 5 min, collagenase (type II; 0.4 mg/ml; Worthington Biochemical Corp.) and BSA (0.5 mg/ml; Sigma Chemical Co.) were added and the perfusion was switched to the recirculating mode for 10 to 15 min. Digested tissue was sliced away from the subepicardial area, placed in 10 ml of enzyme solution, and swirled. The supernatant was collected, 10 ml of fresh  $\text{Ca}^{2+}$ -free solution with 0.4 mg of collagenase per ml and 0.5 mg of BSA per ml was added to the slurry, and the mixture was gently bubbled in a water bath maintained at 37°C. Supernatant was collected for six subsequent washes. The final pellet was washed in 10 ml of incubation buffer containing 2% BSA and the following (millimolar concentrations are given): NaCl, 118; KCl, 4.8;  $\text{MgSO}_4$ , 1.2;  $\text{KH}_2\text{PO}_4$ , 1.2; glutamine, 0.68; glucose, 11;  $\text{NaHCO}_3$ , 20; HEPES, 5; pyruvate, 5; taurine, 10; and  $\text{CaCl}_2$ , 0.5. After 30 min of settling, the pellet was washed a second time with incubation buffer now containing 1 mM  $\text{CaCl}_2$  and allowed to equilibrate at room temperature for 30 min. These cells were used for  $\text{Ca}^{2+}$  imaging experiments, patch clamp recording, and the cardiac myocyte infection assay. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

**Whole-cell patch clamp preparations.** Action potentials of single cardiac myocytes were recorded in the whole-cell patch configuration under current-clamp mode by using an Axopatch-1D amplifier (Axon Instruments, Burlingame, Calif.) interfaced with a personal computer (Dell System 320LX). Data acquisition and analysis were performed with a commercial program (PCLAMP, version 5.5.1; Axon Instruments). Seals were made in final incubation buffer containing 1 mM  $\text{CaCl}_2$ , and experiments were performed at 22 to 25°C. Pipettes were pulled from Gold Seal Accu-fill 90 Micropipets (Clay Adams, Becton Dickinson Co., Parsippany, N.J.) and had resistances of 1 to 3 M $\Omega$  when filled with internal solution. Series resistance from the pipette tip and cell capacitance were partially compensated, as was the liquid junctional potential between the pipette and the bath solutions (typically 5 to 10 mV). The residual series resistance ( $R_s$ ) was small

(<20% of the initial series resistance, where  $R_s$  was less than two times electrode resistance). Alterations in the reference potential were minimized by connecting the bath to the reference potential well (containing internal solution) with an agar bridge. Records were filtered at 2 kHz and sampled at a frequency of 10 kHz.

Action potentials were measured in incubation buffer containing 2.6 mM  $\text{CaCl}_2$  without BSA. The pipette solution (pH 7.3) contained the following (millimolar concentrations are given): potassium aspartate, 125; KCl, 20; ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1; ATP (sodium salt), 4;  $\text{MgCl}_2$ , 1; and HEPES, 5. Action potentials were generated by a suprathreshold stimulus command passed from the computer to the cell through the Axopatch-1D amplifier. Parasite fractions (1:10 or 1:5 dilutions as specified below), thapsigargin (1  $\mu\text{M}$ ), or verapamil (10 or 20  $\mu\text{M}$ ) was added directly to the chamber containing the cardiac myocytes during current clamp recording.

**Spectrofluorometric measurement of intracellular calcium in cardiac myocytes.** Cells were loaded with the fluorescent  $\text{Ca}^{2+}$  indicator fura-2/AM as described previously (30). Briefly, cells were incubated with 3  $\mu\text{M}$  fura-2/AM (30 min; 37°C) in a modified Krebs-Henseleit solution (pH 7.4) containing the following (millimolar concentrations are given): NaCl, 140; KCl, 5;  $\text{MgCl}_2$ , 2;  $\text{CaCl}_2$ , 1; glucose, 10; and HEPES, 10, and oxygenated with 100%  $\text{O}_2$  plus 5% FBS. Dye-loaded cells were washed at least three times (at  $37 \times g$  for 45 s) and resuspended in modified Krebs-Henseleit solution at a concentration of  $10^3/\text{ml}$ . Calcium transients were recorded by using a Nikon Diaphot inverted microscope modified for dual wavelength excitation (xenon lamp light source) at 340 and 380 nm and measurement of fluorescence emission at 510 nm, as described previously (6). With this system, a collimated light beam from a xenon lamp is passed through a computer-controlled electronic shutter system and focused onto a mirror. An oscillator deflects the beam alternately at 1,100 Hz through either a 340 or a 380 nm interference filter and into the epifluorescence port of the inverted microscope. A long-pass dichroic mirror deflects the light beam into a Nikon CF Fluor 40 $\times$  oil objective which focuses the excitation light onto an individual myocyte. Light returning through the objective is collected by a photomultiplier tube. A collimator device in front of the photomultiplier tube restricts fluorescent light to that from only the myocyte of interest, blocking out the background. Fluorescence signals were digitized and stored on a computer using DM3000 software for cation measurement or on videotape. Video images (4915 charge-coupled device camera; Cohu, Inc., San Diego, Calif.) were digitized (LG-3 frame grabber; Scion Corp., Walkersville, Md.) in a Macintosh Quadra 800 microcomputer (Apple Computer Corp., Cupertino, Calif.) for later evaluation with the National Institutes of Health Image Analysis software (version 1.57; Rasband, 1994). Video images were captured as described above with on-chip camera integration for eight videoframes (0.27 s). Cells were loaded into a chamber ( $5 \times 10^2$  cells in 500  $\mu\text{l}$  of modified Krebs-Henseleit solution) and allowed to equilibrate for 5 min in order for the cardiac myocytes to settle on the bottom of the chamber, and then healthy single cardiac myocytes were identified visually. Cells were imaged by measuring fluorescence at 340 and 380 nm every 0.3 s for 600 s. When the 340- and 380-nm fluorescence readings were stable, TSF or ESF (at dilutions of 1:10 unless otherwise specified) or trypomastigote supernatant or epimastigote supernatant (at dilutions of 1:20) was added directly to the chamber containing the cardiac myocytes. For some experiments, data are reported as ratios of the fluorescences emitted during excitation at 340 and 380 nm, which alter proportionally to cytosolic  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) (10). For cardiac myocyte treatments, cells were incubated (37°C) with receptor and/or channel antagonists as follows: 30 min with 10  $\mu\text{M}$  prazosin or 1  $\mu\text{M}$  propranolol; 10 min with  $\text{Ca}^{2+}$ -free medium (containing 5 mM EGTA with or without 0.5 mM MAPTAM); 60 min with 0.5 mM MAPTAM alone; 15 min with 5, 10, 50, or 100  $\mu\text{M}$  verapamil; 15 min with 10  $\mu\text{M}$  nisoldipine; 15 min with 200  $\mu\text{M}$  cadmium; 6 h with 0.4  $\mu\text{g}$  of PTx per ml; 30 min with 0.5  $\mu\text{M}$  thapsigargin; and 15 min with 2.0  $\mu\text{M}$  ryanodine. All drugs were removed (by two washes at  $37 \times g$  for 45 s) from the cardiac myocytes before exposure to TSF (with or without the treatments described above), ESF, or trypomastigotes.

In experiments to determine the number of cells induced to contract and produce a  $\text{Ca}^{2+}$  transient in response to various treatments, cardiac myocytes (after treatments and washing as described above) were pipetted into 96-well plates ( $10^2$  cells in 100  $\mu\text{l}$  of modified Krebs-Henseleit solution plus 5% FBS per well) and TSF was added directly to the medium. For these experiments, the number of single cardiac myocytes showing repetitive contractions and  $\text{Ca}^{2+}$  signals during a 600-s period per total number of single healthy myocytes examined is reported. For TSF inhibition experiments, TSF was incubated with the following inhibitor for 5 min at 37°C immediately prior to use in the assays: 100  $\mu\text{M}$  aprotinin, 250  $\mu\text{M}$  leupeptin, 250  $\mu\text{M}$  E-64, or 250  $\mu\text{M}$  pepstatin A; alternatively, it was incubated with 150  $\mu\text{g}$  of trypsin per ml for 30 min followed by 150  $\mu\text{g}$  of SBTI per ml or subjected to boiling for 3 min.

**Cardiac myocyte invasion assay.** Cells were pretreated with the indicated drugs used in spectrofluorometrically imaged myocytes as described above, washed (at  $37 \times g$  for 45 s), resuspended in modified Krebs-Henseleit solution plus 5% FBS, and incubated with trypomastigotes for 60 min at 37°C. Trypomastigotes were washed (at  $8,000 \times g$  for 15 min) and resuspended in modified Krebs-Henseleit solution plus 5% FBS before exposure to cardiac myocytes. The final concentration of trypomastigotes was calculated on the basis of the 40:1 ratio of parasites to cardiac myocytes (at least  $5 \times 10^2$  myocytes per assay). After

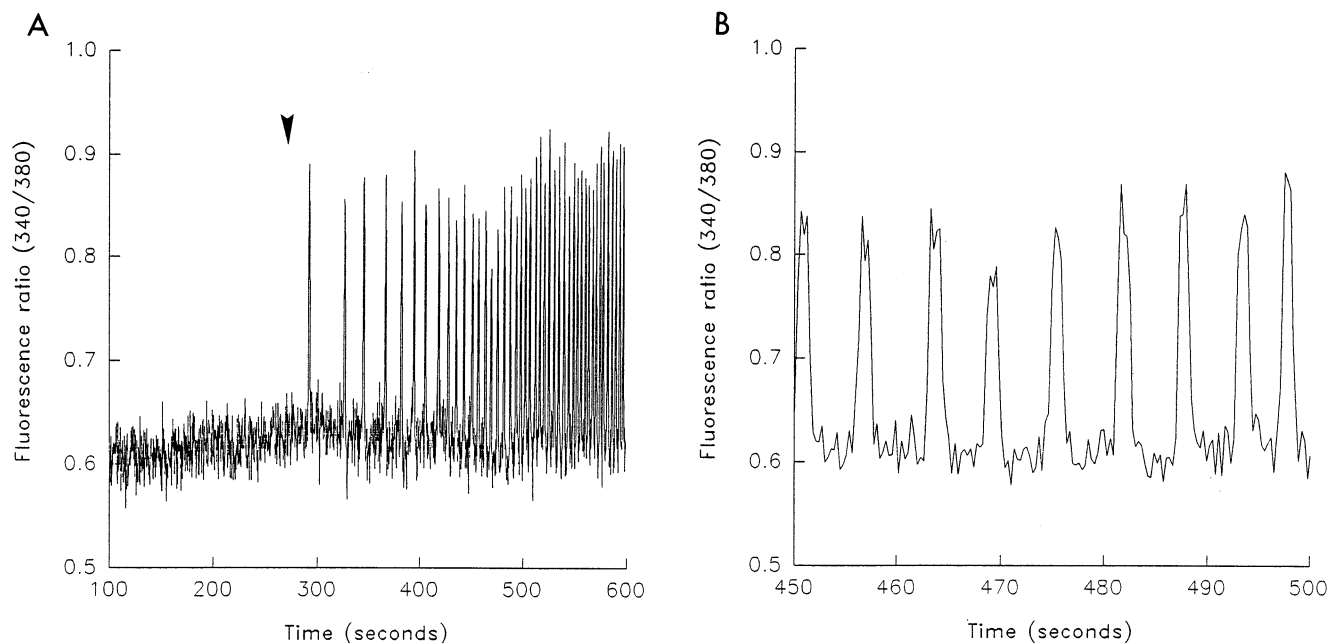


FIG. 1. (A) Representative tracing from a single cardiac myocyte of changes in the ratio of the fluorescences emitted during excitation at 340 and 380 nm [fluorescence ratio (340/380)] over time after the addition (arrowhead) of TSF (dilution, 1:10). (B) Same tracing as in panel A but with an expanded time axis (50 s) to show the morphology of individual  $\text{Ca}^{2+}$  transients.

exposure to parasites, myocytes were washed (at  $37 \times g$  for 45 s) twice in modified Krebs-Henseleit solution and once in distilled  $\text{H}_2\text{O}$  for a total  $\text{H}_2\text{O}$  contact time of 120 s to lyse cell-adhered but noninternalized parasites (1). Cells were then pipetted over an area (18 by 18 mm) on ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, Pa.), air dried, fixed (with acetone for 5 min), and air dried again. An automated slide stainer was used to perform immunoperoxidase staining as described previously (4). Briefly, endogenous peroxidases were inactivated by addition of a solution of 0.5%  $\text{H}_2\text{O}_2$  in methanol for 12 min at room temperature. The slides were then digested by incubation in a solution of pronase E at  $42^\circ\text{C}$  for 20 min. Nonspecific protein adhesion was blocked with 4% horse serum in 0.1 M PBS solution. Primary (obtained from experimentally *T. cruzi*-infected dogs) and secondary (rabbit anti-dog immunoglobulin G) antibodies were diluted in PBS solution containing 4% horse serum. The primary antibody was incubated at  $42^\circ\text{C}$  for 55 min; the biotinylated secondary antibody was incubated at  $42^\circ\text{C}$  for 22 min. The avidin-biotin peroxidase solution was incubated at  $42^\circ\text{C}$  for 35 min, and the chromogen, 3,3'-diaminobenzidine-4HCl, was incubated at  $42^\circ\text{C}$  for 5 min. The slides were counterstained, with Gills no. 2 hematoxylin, and then dehydrated, coverslipped, and examined by light microscopy. Each treatment experiment was performed in triplicate. Numbers of parasites in 100 infected cells per treatment were counted, and the data are presented as the means  $\pm$  standard errors of the means for triplicate experiments and expressed as percentages of control values.

## RESULTS

After isolation, the canine cardiac myocytes retained cross striations, were similar to each other in size (approximately 100 to 300  $\mu\text{m}$  long and 10  $\mu\text{m}$  wide), and were essentially rod shaped. Some cells could be seen to periodically contract spontaneously. These cells, as well as cells that contained few striations, had breaks in their cell wall with cytoplasm bulging out, or were rounded up and did not form a rod shape, were not imaged.

After the introduction of TSF (at a 1:10 dilution) into the chamber containing the cardiac myocytes, repetitive increases in cytosolic free  $\text{Ca}^{2+}$  (transient) levels were detected in individual myocytes (representative signal; Fig. 1A). Measurements made in 10 myocytes after the application of the TSF showed that the mean time from application to initiation of transients was 24.4 s (range, 18.0 to 30.0 s), the mean number of transients was 260 (range, 127 to 375 s), the mean number

of transients during 50-s periods after the onset of repetitive transients (frequency) was 15 (range, 1 to 27), and the mean duration for which cells produced repetitive transients was 866 s (range, 425 to 1,250 s). The mean duration of each transient was very uniform, irrespective of the frequency of transients, and was 2.4 s (range, 2.1 to 2.7 s) (Fig. 1B). All the  $\text{Ca}^{2+}$  transients arose from a common site in each cell and spread throughout the cell in a wave pattern typical of those described previously for cardiac myocytes (reviewed in reference 13). Each  $\text{Ca}^{2+}$  wave was associated with a shortening of the myocyte seen as a sudden visible contraction (Fig. 2). Hence, the number of  $\text{Ca}^{2+}$  transients produced by the cell was equal to the number of cell contractions.

When TSF was applied to cardiac myocytes, if the cells responded, their response tended to be uniform; the variation between assays tended to be in the number of cells responding and not in the degree to which a cell would respond to TSF. Using these findings (i.e., that the cells' response to TSF tended to be an all-or-none phenomenon and that each  $\text{Ca}^{2+}$  transient was associated with a cell contraction), we developed an assay to measure responses of cells to TSF after administration of various treatments (to the cells and to TSF) which depended on counting the number of cells that contracted after a particular treatment in relationship to the number of cells in an assay.

The response of myocytes to TSF was proportional to the amount of TSF added, with dilution resulting in fewer cells developing repetitive  $\text{Ca}^{2+}$  transients (Table 1). The number of transients per cell tended to decrease, although the size and duration of each transient tended to be similar to those of transients produced by higher concentrations of TSF (results not shown). The ESF, the life cycle stage of *T. cruzi* incapable of invading vertebrate cells, did not induce  $\text{Ca}^{2+}$  transients. Culture supernatants from trypomastigotes induced repetitive  $\text{Ca}^{2+}$  transients in myocytes, whereas supernatants from epimastigote cultures did not. Medium controls for trypomasti-

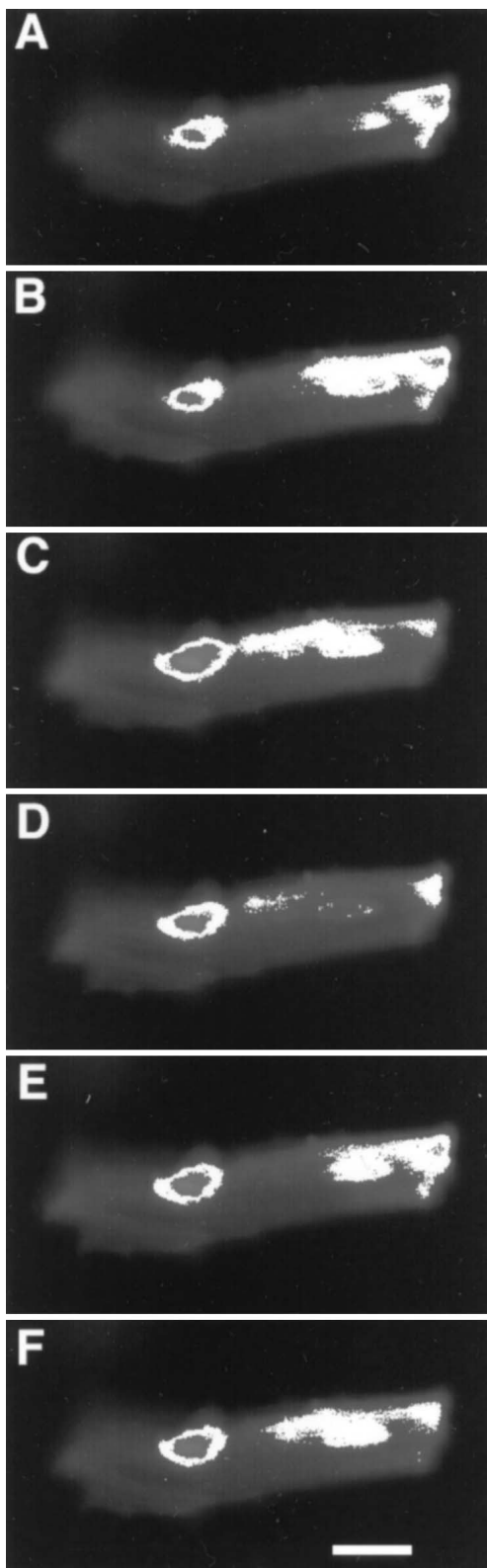


FIG. 2. Serial microscope images of canine cardiac myocytes loaded with the  $\text{Ca}^{2+}$ -sensitive dye fura-2/AM and exposed to TSF. The frames were taken 0.27 s apart. Fluorescence intensity is pseudocolored on a grey scale, with white being the maximum intensity and black being minimum intensity. Large increases in fluorescence intensity correspond to increasing  $[\text{Ca}^{2+}]_i$ . Note the development of a wave starting in the right of the cell (A) and progressing towards the nucleus (B and C); dissipation of the wave is shown in frame D. Cell lengths (as measured

gote supernatant and epimastigote supernatant did not produce  $\text{Ca}^{2+}$  transients in myocytes (results not shown). Treatment of TSF with trypsin (followed by SBTI) or heating ( $100^\circ\text{C}$  for 3 min) prior to application abolished its capacity to trigger the repetitive  $\text{Ca}^{2+}$  transients (Table 1). Treatment of TSF with SBTI alone did not inhibit the response of the cells (results not shown).

We next pretreated TSF (the trypomastigote soluble concanavalin A-unbound fraction) with four specific subset protease inhibitors prior to application to the cardiac myocytes to determine if proteolytic activity was associated with the induction of  $\text{Ca}^{2+}$  transients. Leupeptin was 100% effective in preventing the TSF-induced  $\text{Ca}^{2+}$  transients, but aprotinin, pepstatin A, and E-64 did not affect the number of cells responding to TSF (Table 1).

To determine if  $\alpha$ - and/or  $\beta$ -receptors played a role in induction of the  $\text{Ca}^{2+}$  transients and contraction of the myocytes, the cells were pretreated with  $\alpha$ -adrenergic (prazosin) and  $\beta$ -adrenergic (propranolol) receptor-blocking agents prior to the application of TSF. None of these agents reduced the response to TSF (Table 2). We next attempted to determine if second-messenger activation was associated with the generation of TSF-induced  $\text{Ca}^{2+}$  transients. Myocytes treated with PTx ( $0.4 \mu\text{g/ml}$  for 6 h) to block PTx-sensitive G proteins prior to the application of TSF were completely inhibited from responding to TSF. PTx-treated myocytes still retained normal morphology and basal  $\text{Ca}^{2+}$  levels (dead myocytes ball up and show an approximately 10-fold increase in basal  $\text{Ca}^{2+}$  levels) and showed functional integrity during patch clamping experiments (11). As  $\text{Ca}^{2+}$  transients could be mediated by the opening of  $\text{Ca}^{2+}$  channels in the plasma membrane, we also determined if blocking  $\text{Ca}^{2+}$  channels or removing the availability of  $\text{Ca}^{2+}$  from the extracellular environment would alter the generation of TSF-induced  $\text{Ca}^{2+}$  transients. Myocytes pretreated with cadmium ( $200 \mu\text{M}$ ), nisoldipine ( $10 \mu\text{M}$ ), or verapamil ( $100 \mu\text{M}$ ) showed no reduction in the number of cells responding to TSF (Table 2). As expected, lower concentrations of verapamil ( $5$ ,  $10$ , and  $50 \mu\text{M}$ ) also did not affect the myocytes' response to TSF (results not shown). Myocytes preincubated in a  $\text{Ca}^{2+}$ -free solution did respond to TSF. The size and rapidity to peak development of the  $\text{Ca}^{2+}$  transient produced in response to TSF suggested that the most likely source of the  $\text{Ca}^{2+}$  was intracellular stores. To investigate the role of intracellular  $\text{Ca}^{2+}$  stores, myocytes were pretreated with thapsigargin ( $0.5 \mu\text{M}$  for 30 min) or ryanodine ( $2.0 \mu\text{M}$  for 15 min). Under both conditions, there was a complete inhibition of the  $\text{Ca}^{2+}$  transients, even in response to high concentrations (dilution of 1:1) of TSF.

In patch clamp experiments, myocytes did not develop action potentials in association with TSF-induced  $\text{Ca}^{2+}$  transients and cell contractions (Fig. 3A). However, after the TSF application (but not after application of heat-treated TSF or control solutions) cells did develop subthreshold depolarizations (Fig. 3B). When TSF was washed out of the cell patch chamber, the TSF-induced cell contractions and subthreshold depolarizations disappeared immediately (Fig. 3C). The contractions and subthreshold depolarizations induced by TSF in myocytes were unaffected by verapamil ( $10$  or  $20 \mu\text{M}$ ) treatment (Fig. 3D) but were completely eliminated when myocytes were treated with thapsigargin (at  $0.5 \mu\text{M}$ ) (Fig. 3E).

from end to end) are (in micrometers) 120.9 (A), 122.5 (B), 119.3 (C), and 118.7 (D). (E and F) A second  $\text{Ca}^{2+}$  wave begins from the same site as that in frames A to D, progressing towards the nucleus. Cell lengths are 124.1 and 117.8  $\mu\text{m}$ , respectively. Bar = 20  $\mu\text{m}$ .

TABLE 1. Ca<sup>2+</sup> signaling induced in canine cardiac myocytes after treatment with various substances

Treatment (dilution) <sup>a</sup>	Result of expt <sup>b</sup> :						Mean
	1	2	3	4	5	6	
Control <sup>c</sup>	0/82 (0)	0/114 (0)	0/79 (0)	0/92 (0)	0/95 (0)		0/92 (0)
TSF (1:20)	8/81 (10)	10/92 (11)	18/119 (15)	7/86 (8)	15/98 (15)	9/89 (10)	11/112 (10)
TSF (1:10)	24/80 (30)	35/106 (33)	32/101 (32)	18/78 (23)	20/91 (22)	26/96 (27)	26/92 (28)
TSF (1:5)	40/98 (41)	47/110 (43)	37/90 (41)	42/98 (43)	48/107 (45)	38/95 (40)	42/100 (42)
ESF (1:10)	1/97 (1)	0/92 (0)	0/107 (0)				0/99 (0)
TS (1:20) <sup>d</sup>	15/94 (16)	21/96 (22)	19/103 (13)	12/89 (13)			17/96 (18)
ES (1:20) <sup>e</sup>	1/99 (1)	0/89 (0)	0/109 (0)	0/105 (0)	0/86 (0)		0/98 (0)
Heat-Tx TSF <sup>f</sup>	0/79 (0)	0/100 (0)	1/102 (1)	0/92 (0)			0/93 (0)
Tryp-Tx TSF <sup>g</sup>	5/105 (5)	3/99 (3)	0/88 (0)	2/109 (2)			3/100 (3)
Leupeptin <sup>h,i</sup>	0/96 (0)	0/118 (0)	0/106 (0)	0/104 (0)	0/89 (0)		0/103 (0)
Pepstatin A <sup>h,i</sup>	22/76 (29)	35/107 (33)	30/101 (30)	24/96 (25)	23/113 (20)		27/99 (27)
E-64 <sup>h,i</sup>	25/104 (24)	34/118 (29)	35/106 (33)	41/112 (37)	31/102 (30)	27/98 (28)	32/107 (30)
Aprotinin <sup>i,j</sup>	39/113 (35)	24/94 (26)	31/103 (30)	29/89 (33)	37/109 (34)		32/102 (31)

<sup>a</sup> Where not indicated, TSF was used at a dilution of 1:10.

<sup>b</sup> Data represent the numbers of myocytes showing repetitive contractions and Ca<sup>2+</sup> signals during a 600-s period/total numbers of single healthy-appearing myocytes counted in each preparation, with percentages in parentheses.

<sup>c</sup> Modified Krebs-Henseleit solution plus 5% FBS.

<sup>d</sup> TS, trypomastigote culture supernatant.

<sup>e</sup> ES, epimastigote culture supernatant.

<sup>f</sup> Heat-Tx, heat treated (100°C for 3 min).

<sup>g</sup> Tryp-Tx, trypsin treated (150 µg/ml for 30 min followed by 150 µg of SBTI per ml).

<sup>h</sup> Concentration, 250 µM.

<sup>i</sup> Treatment performed at 37°C for 5 min.

<sup>j</sup> Concentration, 100 µM.

Using an immunofluorescence assay to determine parasitism of cardiac myocytes after incubation with trypomastigotes, we were able to determine if those treatments which inhibit the induction of repetitive Ca<sup>2+</sup> transients in response to TSF were also associated with an inhibition of trypomastigote invasion of cells. There was a significant ( $P < 0.001$ ) reduction in parasitism when cells were pretreated with PTx (Fig. 4). Pretreatment of cardiac myocytes with the Ca<sup>2+</sup>-channel-blocking agents verapamil, cadmium, and nisoldipine did not significantly ( $P > 0.05$ ) reduce parasite invasion below control levels. Trypomastigote invasion was slightly reduced, but not to significant ( $P > 0.05$ ) levels, when Ca<sup>2+</sup> was removed from the medium prior to and during exposure to trypomastigotes. Trypomastigote invasion was significantly ( $P < 0.01$ ) reduced from control levels by pretreating cells with the membrane-permeant Ca<sup>2+</sup> chelator MAPTAM, which acts to clamp Ca<sup>2+</sup> at

resting levels (12). Further inhibition of parasitism ( $P < 0.001$ ) occurred when the cells were pretreated with MAPTAM in a Ca<sup>2+</sup>-free medium (containing 5 mM EGTA). Also, there was a markedly significant ( $P < 0.001$ ) reduction in parasitism when cells were pretreated with thapsigargin and ryanodine (Fig. 4).

## DISCUSSION

In this study, we demonstrate that a soluble fraction from *T. cruzi* trypomastigotes, but not from epimastigotes, mediates repetitive Ca<sup>2+</sup> transients in primary canine cardiac myocytes. Live trypomastigotes, trypomastigote membrane preparations, and a soluble fraction from trypomastigotes have been reported to produce repetitive Ca<sup>2+</sup> transients in rat kidney fibroblasts in vitro (26) and single Ca<sup>2+</sup> transients in a wide

TABLE 2. Ca<sup>2+</sup> signaling induced in canine cardiac myocytes after treatment with various substances

Treatment	Result of expt <sup>a</sup> :					Mean
	1	2	3	4	5	
Control <sup>b</sup>	0/121 (0)	0/113 (0)	0/109 (0)	0/102 (0)	0/105 (0)	0/110 (0)
TSF <sup>c</sup>	31/99 (31)	38/118 (31)	26/103 (25)	41/111 (37)	30/112 (27)	33/109 (30)
Prazosin (10 µM; 30 min)	35/109 (32)	27/107 (25)	29/92 (32)	31/102 (30)		31/103 (30)
Propranolol (1 µM; 30 min)	40/114 (35)	31/108 (29)	38/104 (37)	32/97 (33)		35/106 (33)
PTx (0.4 µg/ml; 6 h)	0/76 (0)	0/82 (0)	0/88 (0)	0/71 (0)		0/79 (0)
Verapamil (100 µM; 15 min)	21/84 (25)	36/91 (40)	37/103 (36)	31/94 (33)	29/102 (28)	31/95 (33)
Cadmium (200 µM; 15 min)	25/81 (31)	40/115 (35)	33/95 (35)	35/98 (36)	29/107 (27)	33/99 (33)
Nisoldipine (10 µM; 15 min)	28/105 (27)	29/101 (29)	22/89 (25)	31/95 (33)	26/100 (26)	27/98 (28)
Ca <sup>2+</sup> -free medium <sup>d</sup>	29/116 (25)	30/96 (31)	22/107 (21)	26/103 (25)		27/105 (26)
Thapsigargin (0.5 µM; 30 min)	0/102 (0)	0/111 (0)	0/98 (0)	0/103 (0)		0/99 (0)
Ryanodine (2.0 µM; 15 min)	0/87 (0)	0/118 (0)	0/92 (0)	0/97 (0)		0/79 (0)

<sup>a</sup> Data represent the numbers of single myocytes in individual experiments showing repetitive contractions and Ca<sup>2+</sup> signals during a 600-s period/total numbers of single healthy-appearing myocytes counted in each preparation, with percentages in parentheses.

<sup>b</sup> Modified Krebs-Henseleit solution plus 5% FBS.

<sup>c</sup> Dilution, 1:10.

<sup>d</sup> Plus 5 mM EGTA.

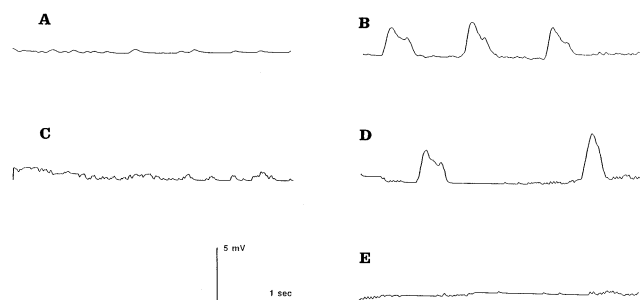


FIG. 3. Representative current clamp recordings from isolated canine cardiac myocytes before TSF application (A), after TSF (dilution, 1:10) application (B), after washing out of TSF (C), after verapamil (50  $\mu$ M) treatment (D), and after thapsigargin (0.5  $\mu$ M) treatment (E). Note the development of subthreshold depolarizations after TSF was applied (B) and their disappearance after it was washed out of the preparation (C). Verapamil treatment failed to affect TSF-induced subthreshold depolarizations (D), but thapsigargin treatment completely eliminated TSF-induced subthreshold depolarizations (E).

range of other cells in vitro (5). In these studies, although the latency period from application of TSF to the onset of the transient was similar, the duration of each transient and number of transients varied markedly from our results. In cardiac myocytes, each transient lasted less than 3 s, after which the  $[Ca^{2+}]_i$  returned to baseline levels before the first transient was quickly followed by another one. Some myocytes exhibited repetitive  $Ca^{2+}$  transients for as long as 20 min, producing over 850 transients during that time. This difference is most likely due to a difference in cell type. In cardiac myocytes, the rapid mobilization of  $Ca^{2+}$  from SR stores results in a massive increase in cytosolic free  $Ca^{2+}$  levels necessary for actin-myosin activation and contraction of the cell, seen visibly as a shortening of the cell length. After  $Ca^{2+}$  release, there is an almost equally rapid removal of the  $Ca^{2+}$  from the cytosol back into the SR stores, where it is available to generate the next  $Ca^{2+}$  transient. Given that a cardiac myocyte may need to produce a  $Ca^{2+}$  transient and contract as often as 200 times a minute,

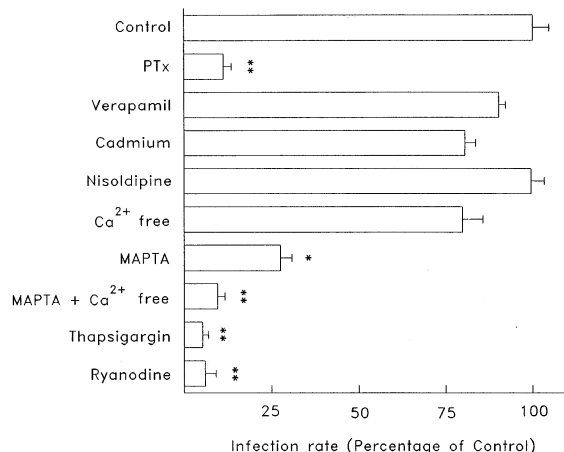


FIG. 4. Rates of infection (presented as percentages of control levels) of cardiac myocytes by *T. cruzi* trypomastigotes after myocytes were treated with modified Krebs-Henseleit solution plus 5% FBS (Control),  $Ca^{2+}$ -free medium (plus 5 mM EGTA; 10 min), verapamil (100  $\mu$ M; 15 min), cadmium (200  $\mu$ M; 15 min), nisoldipine (10  $\mu$ M; 15 min), MAPTAM (0.5 mM; 60 min) with and without  $Ca^{2+}$ -free medium (plus 5 mM EGTA), thapsigargin (0.5  $\mu$ M; 30 min), ryanodine (2.0  $\mu$ M; 15 min), and PTx (0.4  $\mu$ g/ml; 6 h). Data are presented as the means  $\pm$  standard errors of the means for triplicate experiments. \*\*,  $P < 0.001$ ; \*,  $P < 0.01$ .

there are extremely efficient mechanisms (SR  $Ca^{2+}$  pumps and the ability of the SR to store large amounts of  $Ca^{2+}$ ) for returning the  $[Ca^{2+}]_i$  to baseline levels (3).

As in other studies, the response of myocytes to TSF was proportional to the concentration of TSF applied to the cells (5). However, in cardiac myocytes, the number of cells responding to TSF varied in proportion to concentration, with the responses of each cell being relatively uniform. In NRK cells, the size of the  $Ca^{2+}$  transient in individual cells varied proportionally to TSF concentration (5). This suggests that individual myocytes have a threshold to the effects of TSF, which might be due to the alterations in myocytes within any one preparation of cells induced by the Langendorff perfusion technique compared with the relative homogeneity of the cell populations in in vitro cell preparations.

The fact that heat-treated TSF and trypsin-treated TSF failed to elicit  $Ca^{2+}$  transients in myocytes suggested that the effects of TSF were not caused by changes in electrolyte concentrations ( $Na^+$ ,  $K^+$ , or  $Ca^{2+}$ ) and that the active fraction was most likely a protein. Further, pretreatment of TSF with the specific protease inhibitor leupeptin resulted in a complete loss of activity, whereas E-64, aprotinin, and pepstatin A had no effect (5). These findings argue that TSF-induced  $Ca^{2+}$  transients are coupled to proteolytic activity. Recent studies have indicated that the only detectable protease in TSF is an unusual alkaline peptidase of 120 kDa (5) that is expressed in all life cycle stages of *T. cruzi*. It has been proposed that the peptidase cleaves a trypomastigote-specific substrate, a processing event required for the activation of a factor responsible for inducing  $Ca^{2+}$  transients in host cells (5). Our data showing that supernatants from trypomastigote (but not epimastigote) cultures also induced  $Ca^{2+}$  transients suggest that trypomastigotes secrete a factor with TSF-like activity into the surrounding medium. The trypomastigote substrate for the 120-kDa peptidase has not yet been identified, but it displays properties not dissimilar to those of precursors of mammalian hormones or growth factors (5, 21). Further evidence for binding of an agonist on host cell membranes rather than penetration of the cell to affect subcellular transduction pathways directly is shown by the termination of  $Ca^{2+}$  transients, cell contraction, and subthreshold depolarizations after washing out of TSF from the cell patch chamber (Fig. 3).

A feature of cardiac manifestations of Chagas' disease in mice and dogs is  $\beta$ -adrenergic hyporesponsiveness, possibly due to a decrease in  $\beta$ -adrenergic receptor density and affinity or to an uncoupling of the  $\beta$ -adrenergic receptor from the  $G_s$  protein (15, 16). Cardiac  $\alpha$ -adrenergic activation is a major pathway in the activation of phospholipase C-mediated intracellular  $Ca^{2+}$  transients (29). The finding that  $\alpha$ - and  $\beta$ -adrenergic receptor blockers (prazosin and propranolol, respectively) did not depress TSF-induced  $Ca^{2+}$  transients suggests that the  $Ca^{2+}$  transients and contraction of the myocytes are not dependent on intact  $\alpha$ - and  $\beta$ -adrenergic receptor activity. Our data do not allow us to speculate on which of the many known cell surface receptors may be associated with TSF activity, if in fact this activity is associated with a known receptor.

Treatment of cells with PTx results in ADP-ribosylation of some G-protein  $\alpha$ -subunits, uncoupling them from their receptors and resulting in blockade of signal transduction (25). When myocytes were pretreated with PTx, not only did they retain their functional and morphologic integrity (11), but TSF-induced  $Ca^{2+}$  transients were inhibited and there was a significant decrease in the rate of infection by trypomastigotes, confirming similar findings with NRK cells (21, 26). Our findings suggest that cardiac myocyte G proteins are involved in the regulation of trypomastigote invasion and TSF-induced

Ca<sup>2+</sup> transients. Indeed, TSF-induced Ca<sup>2+</sup> transients are dependent on an intact phospholipase C pathway and also result in an elevation of the inositol 1,4,5-triphosphate level (21). Further, TSF-induced elevations of Ca<sup>2+</sup> levels in NRK cells directly resulted in a redistribution of F-actin, supporting the hypothesis that TSF-induced disassembly of the cortical actin cytoskeleton may play a role in *T. cruzi* invasion (21). Although these findings have yet to be confirmed with primary cardiac myocytes, the results presented in this paper strongly suggest that myocytes respond to TSF and parasite invasion in ways similar to those of the in vitro cell lines studied to date.

The generally accepted model to explain excitation-contraction coupling in cardiac myocytes has been termed Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (8). The model depends on Ca<sup>2+</sup> influx into the cell via Ca<sup>2+</sup> channels as Ca<sup>2+</sup> current (and to a lesser degree via Ca<sup>2+</sup>-Na<sup>+</sup> exchange), leading to release of sufficient Ca<sup>2+</sup> (10 to 65 times more than the amount that enters via the Ca<sup>2+</sup> current) from the SR to activate contraction (3, 8). Treating myocytes with specific Ca<sup>2+</sup> channel blockers (cadmium, nisoldipine, and verapamil) did not reduce myocyte response to TSF (Table 2) or decrease *T. cruzi* invasion of myocytes (Fig. 4), suggesting that L-type Ca<sup>2+</sup> channel function is not necessary for the generation of TSF-induced Ca<sup>2+</sup> transients or for trypomastigote invasion of myocytes. Further, extracellular Ca<sup>2+</sup> is not required for either process to occur, as cells treated with TSF in a Ca<sup>2+</sup>-free solution showed neither a reduction in myocyte infection rate nor a significant loss of response to TSF. The lack of dependency on extracellular Ca<sup>2+</sup> influx would also discount the involvement of a cytosolic factor present in several cell lines (Ca<sup>2+</sup> influx factor) in the generation of the Ca<sup>2+</sup> transients (20).

Further evidence for a lack of requirement of L-type Ca<sup>2+</sup> channels and other voltage-dependent channels (Na<sup>+</sup> and K<sup>+</sup> channels) in the generation of TSF-induced Ca<sup>2+</sup> transients was shown by the inability of TSF to produce an action potential in association with repetitive cell contractions and Ca<sup>2+</sup> transients (Fig. 3). However, TSF (but not inactive TSF) did induce subthreshold depolarizations in myocytes, but not until approximately 3 to 7 min after TSF application. The subthreshold depolarizations were of magnitude insufficient to reach the action potential threshold and therefore could not induce an action potential and associated activation of voltage-dependent currents. Further, the subthreshold depolarizations were not dependent on L-type Ca<sup>2+</sup> channels, as verapamil treatment had no effect on their generation, but were dependent on SR Ca<sup>2+</sup> release, as they were obliterated by thapsigargin treatment. The ionic mechanism for the subthreshold depolarizations may be similar to that proposed for the generation of delayed afterdepolarizations (32). Increases in [Ca<sup>2+</sup>]<sub>i</sub> during the TSF-induced Ca<sup>2+</sup> transient would be expected to cause Na<sup>+</sup> influx via the transient outward current, I<sub>ti</sub>, secondary to activation of either nonspecific cation channels (7) or the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (17). Because we did not block the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger with Ni<sup>2+</sup>, we could not discount Na<sup>+</sup> entry through this channel. However, pretreatment of NRK cells with Ni<sup>2+</sup> did not prevent TSF-induced Ca<sup>2+</sup> transients (21).

Clamping the [Ca<sup>2+</sup>]<sub>i</sub> of cardiac myocytes at resting levels with MAPTAM and depleting cells of Ca<sup>2+</sup> by pretreating them with MAPTAM in a Ca<sup>2+</sup>-free solution before exposure to trypomastigotes significantly reduced the trypomastigote infection rate (Fig. 4). Similar results have been reported for NRK cells in vitro (26) and for rat heart myoblasts with bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid as a Ca<sup>2+</sup>-chelating agent (24), suggesting that Ca<sup>2+</sup> transients were necessary for invasion. As our findings suggested that the source of the Ca<sup>2+</sup> to produce the transient was not extracellular, we

examined the role of intracellular stores of Ca<sup>2+</sup> in the Ca<sup>2+</sup> transient. Pretreatment of myocytes with thapsigargin, which blocks ATPase for the SR Ca<sup>2+</sup> pump and therefore depletes SR Ca<sup>2+</sup> stores (28), and ryanodine, which blocks SR Ca<sup>2+</sup> release (31), resulted not only in a complete inhibition of TSF-induced Ca<sup>2+</sup> transients but also in a marked inhibition of the rate of infection of myocytes. These results confirm previous findings suggesting that TSF-induced Ca<sup>2+</sup> transients were mediated by release of Ca<sup>2+</sup> from SR stores and that TSF-induced Ca<sup>2+</sup> transients are a requirement for invasion (21, 26).

In summary, our results suggest that TSF-induced Ca<sup>2+</sup> transients in cardiac myocytes do not rely on  $\alpha$ - or  $\beta$ -adrenergic receptor activation or the influx of Ca<sup>2+</sup> into the cell through L-type Ca<sup>2+</sup> channels, are G-protein dependent, and are the result of SR Ca<sup>2+</sup> release. The results suggest that primary cardiac myocytes respond to TSF and parasite invasion in ways similar to those of the in vitro cell lines studied to date (5, 21, 26). Because Ca<sup>2+</sup> transients can be induced by supernatants from trypomastigote cultures but not by those from epimastigote cultures, we propose that while in close contact with a host cell, the trypomastigote releases a factor (cleaved from the parasite by protease activity) which passes from the parasite to bind with an as yet unknown cell surface membrane receptor. Receptor activation leads to a rise in the level of intracellular Ca<sup>2+</sup> (released from SR Ca<sup>2+</sup> stores), a requirement for lysosomal movement to the cell membrane adjacent to the parasite, and internalization of the parasite. Such communication is essential for the parasite to reach an intracellular site necessary for multiplication and escape of host immune processes. The fact that such processes occur in cardiac myocytes (primary targets for *T. cruzi* in the vertebrate host) exemplifies the importance that TSF may have in the pathogenesis of Chagas' disease in humans.

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