Host Cell Invasion by Trypanosoma cruzi Is Potentiated by Activation of Bradykinin B₂ Receptors

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Abstract

The parasitic protozoan Trypanosoma cruzi employs multiple molecular strategies to invade a broad range of nonphagocytic cells. Here we demonstrate that the invasion of human primary umbilical vein endothelial cells (HUVECs) or Chinese hamster ovary (CHO) cells overexpressing the B₂ type of bradykinin receptor (CHO-B₂R) by tissue culture trypanmastigotes is subtly modulated by the combined activities of kininogens, kininogenases, and kinin-degrading peptides. The presence of captopril, an inhibitor of bradykinin degradation by kininase II, drastically potentiated parasitic invasion of HUVECs and CHO-B₂R, but not of mock-transfected CHO cells, whereas the B₂ antagonist HOE 140 or monoclonal antibody MBK3 to bradykinin blocked these effects. Invasion competence correlated with the parasites’ ability to liberate the short-lived kinins from cell-bound kininogen and to elicit vigorous intracellular free calcium ([Ca²⁺]ᵢ) transients through B₂R. Invasion was impaired by membrane-permeable cysteine proteinase inhibitors such as Z-(SBz)Cys-Phe-CHN₂ but not by the hydrophilic inhibitor 1-trans-epoxysuccinyl-l-leucyl-amido-(4-guanidino) butane or cystatin C, suggesting that kinin release is confined to secluded spaces formed by juxtaposition of host cell and parasite plasma membranes. Analysis of trypomastigote transfectants expressing various cysteine proteinase isoforms showed that invasion competence is linked to the kinin releasing activity of cruzipain, herein proposed as a factor of virulence in Chagas’ disease.

Key words: Trypanosoma cruzi • bradykinin • cruzipain • cysteine proteinases • kinin receptors

Introduction

Chagas’ disease, the chronic infection by the parasitic protozoan Trypanosoma cruzi, is a major cause of cardiomyopathy in rural Latin America. Transmitted by blood-sucking triatomine insects, the infective forms of T. cruzi (trypanmastigotes) rapidly enter the bloodstream, from where they disseminate the infection to multiple tissues. After invading macrophages, muscle, and other nucleated cells, the trypanmastigotes escape from endocytic vacuoles and migrate into the cytoplasm where they transform into round-shaped amastigotes, the replicating forms. Within 5–6 d, the host cells rupture, releasing large numbers of trypanmastigotes and amastigotes into interstitial spaces. Acute pathology and parasite tissue load subside with the onset of immunity, but the pathogen is not eradicated. After years of asymptomatic infection, 10–24% of the patients develop a severe chronic cardiomyopathy characterized by myocarditis, fibrosis, microcirculatory lesions, cardiomegaly, and conduction system abnormalities (1–3).

At the cellular level, T. cruzi trypanmastigotes invade nonphagocytic cells by a unique mechanism distinct from phagocytosis (4, 5). Penetration by tissue culture trypanmastigotes (TCTs)¹ is preceded by energy-dependent adhesive

¹Abbreviations used in this paper: ACE, angiotensin I–converting enzyme; BK, bradykinin; [Ca²⁺]ᵢ, intracellular free calcium; CHO, Chinese hamster ovary; DTT, dithiothreitol; H-kininogen, high molecular weight kininogen; HUVEC, human primary umbilical vein endothelial cell; TCT, tissue culture trypanmastigote.
interactions (6) involving the parasites’ surface glycoproteins (7, 8) and negatively charged host surface molecules (9). Depending on the host cell–parasite combination studied, invasion requires activation of the TGF-β signaling pathway (10) or stimulation of host cell receptors coupled to heterotrimeric G proteins (11, 12). Efforts to characterize the hitherto unknown Ca^{2+}–signaling agonist pointed to a crucial role of a cytosolic parasitic serine protease of 80 kD, oligopeptidase B (13). Although null mutants generated by targeted deletion of the oligopeptidase B gene were poorly infective (14), purified or recombinant oligopeptidase B alone failed to induce intracellular free calcium ([Ca^{2+}]_i) transients in the mammalian cells (13). Because addition of recombinant oligopeptidase B to null parasite extracts reconstituted [Ca^{2+}], signaling, it was suggested that the agonistic activity was generated by oligopeptidase B–mediated processing of a cytoplasmic T. cruzi precursor molecule (14).

Other clues to understand the role of T. cruzi proteases in host cell invasion emerged from in vitro assays performed with synthetic inhibitors of cruzipain (15), the parasite’s major cysteine protease (16–18). Encoded by multiple polymorphic genes (19, 20), this cathepsin L–like protease is the most extensively characterized isoform expressed by replicating forms of the parasite (16–18, 21). Given the broad pH range of the activity profile and the high stability of cruzipain (17), the finding of antigen deposits of this molecule in foci of myocardial inflammation (22) suggested that this protease may contribute to pathology. Our findings that the substrate specificity of cruzipain resembles that of tissue kallikrein and that cruzipain releases the bradykinin (BK)–like vasoactive peptide lysylbradykinin (”kallidin”) from its large precursor forms, high (H–) and low (L–) molecular weight kininogens (23), suggested that T. cruzi may directly trigger the kinin system through the activity of this cysteine protease.

Here we demonstrate that the short-lived kinin peptides and their cognate G protein–coupled cellular receptors (24) are engaged in the signaling mechanisms leading to T. cruzi invasion. We also show that invasion of cells that overexpress the constitutive B_{1} subtype of BK receptor is critically modulated by the kinin-degrading activity of host kininase II, also known as the angiotensin I–converting enzyme (ACE). The finding that activation of the proinflammatory kinin cascade by trypanostigotes potentiates invasion may shed light on the molecular basis of Chagas’ disease pathophysiology.

Materials and Methods

Cells and Parasites. Chinese hamster ovary (CHO) cells transfected with the cDNA encoding the rat B_{2} type of BK receptor (B_{2-R}; CHO-B_{2-R}) or mock–transfected CHO cells (CHO-mock) were used (25). Subclone rB2CHO12/4 showed a maximum ^{3}H–BK binding activity of 1.3 pmol/mg of protein at passage 2. CHO cells were cultured in HAM’s F12, each supplemented with 10% (vol/vol) of FCS at 37°C in a humidified atmosphere containing 5% CO_{2}. Vero cells were cultured in DMEM with 10% FCS. Human primary umbilical vein endothelial cells (HUVECs) were obtained by treatment of umbilical veins with a 0.1% (wt/vol) collagenase IV solution (Sigma-Aldrich). Primary HUVECs were seeded in 25-cm^{2} flasks (Corning) coated with 2% porcine skin gelatin, and grown in M199 medium supplemented with 2 mM glutamine, 2.5 μg/ml amphotericin B, 100 μg/ml penicillin, 100 μg/ml gentamicin, 0.13% sodium bicarbonate, and 20% FCS. Cells were maintained at 37°C in a humidified 5% CO_{2} atmosphere until they reached confluency. After treatment with 0.02% trypsin/0.02% EDTA, HUVECs were seeded into 24-well plates with gelatin–coated glass coverslips and cultivated at 37°C for several days before being used in invasion assays.

T. cruzi epimastigotes (Dm28c clone) were cultivated at 28°C in LIT medium containing 10% FCS. TCTs were harvested from the supernatants of infected Vero cultures maintained in DMEM supplemented with 2% FCS (TCT–FCS). TCT transfectants overexpressing Dm28c genes encoding the major cruzipain (18) isoform (for simplicity, hereafter designated cruzipain–1) or cruzipain–2 (20, 23) were obtained by cloning full-length copies of each of these into the Smal–HindIII sites of pTEX plasmid (26). Log phase Dm28c epimastigotes were transfected by electroporation with a single pulse of 450 V, 500 μF in an electroporator (Bio-Rad Laboratories). The parasites were selected for growth in LIT medium containing 10% FCS and 200 μg/ml of genetin (Sigma–Aldrich) for six consecutive weeks and reselected at 800 μg/ml of genetin for four additional weeks. Metacyclogenesis was done by incubating stationary phase–transfected epimastigotes in Grace’s medium, pH 5.5, including 800 μg/ml of genetin for 7 d at 27°C. TCT transfectants were collected from Vero cell supernatants 3–4 d after infection with the metacyclics. Plasma contents were stable for at least 7 wk of culture in the absence of the selecting drug; TCT transfectants were tested in invasion assays after a 3-wk passage. The cysteine protease activity contained in cell lysates from transfected or wild-type parasites was measured as the rate of hydrolysis of Bz-CMK (20 μM) in Na_{2}HPO_{4}, 50 mM Na_{2}HPO_{4}, 200 mM NaCl, and 5 mM EDTA, pH 7.0, supplemented with 2.5 mM dithiothreitol (DTT), at 37°C. To prepare the parasite cell lysates, freshly released TCTs were washed twice in HBSS and re suspended in 300 μl of PBS, pH 7.2, containing 2 mM EDTA. Then, parasites were subjected to freeze and thaw cycles (two times), followed by the addition of Triton X-100 to 1%. Samples were kept on ice for 10 min and soluble material was recovered by centrifugation at 13,000 g. Protein concentration was determined by the DC–protein kit (Bio–Rad Laboratories). Peptidase activity was measured in lysates normalized to 2 μg/ml protein (final concentration). Enzyme stability tests were performed by mixing 2 μl of lysates (1 mg/ml) to 100 μl of 0.1 M glycine, pH 12, for 5 s. Assay buffer was added to 1 ml and the peptidase activity was measured as described above.

Cell Invasion Assays. CHO-B_{2-R}, CHO-mock, or native HUVECs were plated on 13-mm round coverslips at a density of 2.5 × 10^{4} cells/cm^{2} in appropriate medium supplemented with 10% FCS and cultivated in 24-well plates for 48 h at 37°C in a 5% CO_{2} atmosphere. Before addition of TCTs, coverslips with attached cells were washed three times with HBSS and kept in serum–free medium containing 1 mg/ml BSA. The parasites added to the wells were freshly released from cultures of infected Vero cells cultivated in DMEM–FCS (2%). After removing cellular debris by low speed centrifugation (169 g), the parasite suspension was diluted three times in HBSS, spun down at 2,000 g, and gently resuspended in DMEM–BSA or M199–BSA (1 mg/ml each). Invasion assays with CHO cells or HUVECs were done in

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a total volume of 0.6 ml of DMEM-BSA or M199-BSA, respectively, at a parasite/host cell ratio of 2:1 for 3 h at 37°C, unless otherwise specified. When required, the medium was supplemented with 25 μM of captopril (CAP medium). The effects of receptor antagonists or antibodies were assayed by adding to CAP medium 0.1 μM HOE 140 (Aventis), 5–100 nM BK (Calbiochem), or 200 nM of mAbs. In some experiments, CAP medium was supplemented with purified H-kininogen (9 nM) 5 min before adding TCTs to HUVECs. Likewise, the effects of protease inhibitors were tested by adding 10 or 75 μM 1-trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane (E-64), 10 μM leupeptin (Sigma-Aldrich), 10 μM Z-(Sβ2)Cys-Phe-CHN₂(15), or 1 μM recombinant human cystatin C (from Dr. Magnus Abrahamsson, Lund University, Lund, Sweden) to the CAP medium. The effect of cruzipain-1 on invasion was evaluated by adding 10-fold dilutions of the activated protease to wells (0–10 nM final concentration) immediately after the addition of the parasites. The interaction was stopped by removing TCTs and washing cells three times with HBSS. Monolayers were fixed with Bouin and stained with Giemsa. Invasion was quantified by counting the number of intracellular parasites in a total of 100 cells per coverslip. Values represent means ± SD of at least three independent experiments, each done in triplicate under “blinded” conditions. Statistical analysis was done by one-way analysis of variance at a P = 0.05 significance level.

**Digital Imaging Fluorescence Microscopy.** The changes in [Ca²⁺], were determined using the fluorescent dye Fura 2-AM (Molecular Probes). Customized chambers used in these experiments were designed as follows: 30-mm plastic Petri dishes were drilled leaving 1-cm diameter holes in the center covered at the bottom by thin glass coverslips (0.7 mm) mounted with silicone glue. The plates were extensively washed and sterilized under a UV lamp for 20 min before use; coverslips for HUVECs contained 2% porcine gelatin. CHO cells and HUVECs were plated at 2 × 10⁵ cells per dish in the appropriate medium supplemented with 10 and 20% of FCS, respectively, and cultivated for 24 h at 37°C in 5% CO₂. The monolayers were washed three times with HBSS and incubated for 20 min at 37°C in a 5% CO₂ atmosphere using serum-free HAM’s F12 medium, pH 7.4, supplemented with 12.5 mM Hepes, 1 mg/ml BSA, 2.5 mM probenecid (Sigma-Aldrich), 25 μM captopril, and 6 μM Fura 2-AM. After rinsing three times to remove extracellular dye, the cells were maintained at 37°C for 15 min in the same medium without Fura 2-AM. Fura-loaded cells were analyzed in an Axiovert 100 microscope under an oil immersion 40× objective (ZEISS). Fluorescence images were collected by a digital CCD camera using a 510-nm filter. [Ca²⁺], was monitored at 36–37°C by alternating the excitation wavelengths between 334 and 380 nm using the Attofluor Ratio System (ZEISS). Raw fluorescence images were digitalized to a pixel assay, point density readings were taken for each image, and a visual display of the 340/380-nm ratio was produced. Before adding parasites or purified proteases, the variation of [Ca²⁺], of all cells in the field was monitored for 2 min; 20–30 cells which did not present spontaneous [Ca²⁺], transients were chosen as regions of interest. After initial monitoring, the cellular responsiveness to kinin receptor agonists was assessed by adding BK (5–50 nM) to CHO-B₁R or HUVECs preloaded with Fura 2-AM, using CAP medium. Vorgorous [Ca²⁺], transients were observed for nearly 100% of the monitored cells. Specificity of BK-induced transients was checked by adding 100 nM of HOE 140 to the monolayers before 50-nM agonist exposure. CHO-mock failed to induce significant [Ca²⁺], elevations up to 50 nM BK. When indicated, 100 nM of HOE 140 was applied to the cells before the application of enzyme or parasites. Assays with TCTs were carried out at a parasite/host cell ratio of 10:1. Responding cells were observed under light phase to confirm interaction with the parasites. The Attograph software was used to generate tracings representing [Ca²⁺], transients of individual cell responses, as well as average responses of 20–30 cells (n = 30). Purified cruzipain-1 was tested at 5 nM, after a 15-min activation of a stock solution (10-fold) with 2.5 mM DTT in PBS, pH 7.2. Controls included the addition of DTT-containing buffer alone or activated cruzipain-1 pretreated with 75 μM E-64 for 30 min. The specificity of HOE 140 was tested by pretreating HUVECs with 100 nM of the B₁R antagonist before stimulating the cells with 14 nM α-thrombin (Dr. Russolina Zingali, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil) or with 50 nM BK. In the last series of experiments, the [Ca²⁺], concentrations were determined by the two-point calibration in vivo method (27) after sequentially adding 20 μM ionomycin (Sigma-Aldrich) and 10 mM EGTA to the cultures.

**Purified Proteins and Abs.** Cruzipain (GP57/51) was isolated from crude aqueous extracts of Dm28c epimastigotes as described (28). Recombinant cruzain (cruzipain-1) lacking the COOH-terminal extension (18) was expressed in Escherichia coli (Dr. J.H. McKerror, University of California at San Francisco, San Francisco, CA). Recombinant cruzipain-2 devoid of the COOH terminus (20) was expressed in Saccharomyces cerevisiae (23) and partially purified by affinity chromatography on thiopropyl-Sepharose 6B (Amersham Pharmacia Biotech); SDS-PAGE revealed a major band of 29 kD. Purified H-kininogen was from Calbiochem. mAbs MBK3 (IgG1) recognizing the BK epitope in H-kininogen (10 nM) in 200 μl of a reaction buffer containing 50 mM Na₂HPO₄, pH 6.5, 200 μM NaCl, 5 mM EDTA, and 0.25 mM DTT. After incubation for 30 min at 37°C, the reaction was stopped by adding 75 μM E-64, 1 mg/ml BSA, and 25 μM captopril; samples were deproteinized with ice-cold TCA. After diluting the soluble fractions with the supplier’s buffer, the sam-
Trypomastigotes induce 

Transients in CHO-B2R, 

Mock or CHO-B2R loaded with Fura 2-AM in Ham's F12 medium supplemented with 1 mg/ml of BSA, 12.5 mM Hepes, and 25 μM captopril. The changes of [Ca^{2+}]_i were determined on CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addition of TCTs to CHO-B2R, such as primary HUVECs. The addi...
have increased their susceptibility to infection. Consistent with this prediction, the invasion index for CHO-B2R in the presence of CAP medium was nearly 4.5-fold higher than that for CHO-mock (Fig. 3 A), whereas captopril had no effect on the invasion of CHO-mock (not shown). Similarly to the inhibitory effects of HOE 140 on TCT-induced \([Ca^{2+}]_i\) transients, addition of the B2R antagonist decreased the invasion of CHO-B2R in CAP medium, whereas it had no effect on the invasion of CHO-mock (Fig. 3 A). Similar effects were noted when we attempted to downregulate B2R by applying high concentrations of the agonist, i.e., 0.1 \(\mu M\) BK, to these cultures. Using primary cultures of HUVECs, we noted that parasite invasion was enhanced threefold in the presence of 25 \(\mu M\) captopril, whereas absence of captopril or inclusion of HOE 140 in CAP medium significantly attenuated invasion (Fig. 3 B). Although HUVECs were less sensitive to invasion than CHO-B2R, TCT invasion of HUVECs in the presence of CAP medium was likewise reduced by the addition of high concentrations (0.1 \(\mu M\)) BK (Fig. 3 B). In conclusion, TCT invasion of target cells that overexpress B2R is markedly enhanced in the presence of captopril, suggesting that the short-lived kinin agonist is protected from degradation by the kininase II inhibitor.

**Processing of Cell-bound Kininogens Is Required for TCT Invasion.** The fact that TCT invasion is potentiating by kininase II inhibitors and reduced in cultures exposed to specific B2R antagonist or to high levels of exogenous BK pointed to the possibility that a low amount of plasma kininogen either remains associated with the cell surfaces of target cells and/or is displayed by the parasites, in either way serving as a source for precursors of kinase-signaling peptides. Consistent with this concept, the addition of an exogenous supply of H-kininogen (9 nM) to the serum-free CAP medium resulted in a greater than threefold increase in parasite invasion (Fig. 4 A). To test the hypothesis that HUVEC susceptibility to TCT invasion is influenced by the levels of kinin precursors and to further substantiate our notion that kinins are indeed involved in the signaling mechanism, we tested the effects of an mAB (MBK3) to BK that cross-reacts with the kinin segment of human and bovine kininogens (27). Our data show that

**Figure 2.** TCTs induce cytosolic \([Ca^{2+}]_i\), transients in HUVECs through the B2R. The changes in \([Ca^{2+}]_i\) were determined by adding TCTs (parasite/host cell ratio of 10:1) to monolayers of HUVECs preloaded with Fura 2-AM, loaded, plated on gelatin-coated glass coverslips, maintained in M199 medium with 12.5 mM Hepes, 1 mg/ml BSA, and 25 \(\mu M\) captopril. (A) TCTs added to HUVECs, average responses; (D) individual cell response; (B) TCTs added to HUVECs that were previously treated with 100 nM HOE 140, average cell responses; (E) individual cell response; (C) epimastigotes (EPI) added to HUVECs, average responses; (F) epimastigotes added to HUVECs, individual cell responses. (G–I) Internal controls showing the sensitivity of HUVECs to BK and selectivity of the B2R antagonist HOE 140. Tracings represent average cell responses. (G) Cytosolic \([Ca^{2+}]_i\) changes induced by 14 nM \(\alpha\)-thrombin; (H) effect of 100 nM HOE 140 on thrombin-induced responses; (I) responses induced by 5 nM BK; (K) effect of 100 nM HOE 140 on responses induced by BK.

**Figure 3.** T. cruzi invasion is enhanced in mammalian cells overexpressing B2R. (A) Invasion assays were performed with CHO-B2R or CHO-mock in HAM’s F12 medium containing 1 mg/ml of BSA in the presence or absence of 25 \(\mu M\) captopril. Dm28c TCTs were added at a ratio of 2:1 (parasite vs. CHO) and the interaction proceeded for 3 h at 37°C in the presence or absence of 100 nM HOE 140 or BK, as indicated. (B) TCTs were added to HUVECs (ratio of 2:1) in M199 medium containing 1 mg/ml BSA. The interaction proceeded for 3 h in the presence or absence of 25 \(\mu M\) captopril. HOE 140 or BK, 100 nM each, was added to the CAP medium immediately before addition of the parasites. Values represent the number of intracellular parasites per 100 cells as means ± SD of three independent experiments. Significant differences \((P < 0.05)\) between paired bars are marked by asterisks \((* *, **, ***)).
preincubation of HUVECs with 300 nM MBK3 in CAP medium significantly reduced the potentiating effects of captopril, whereas the addition of unrelated Ab (IgG1) failed to interfere with TCT invasion, and HKL6, an mAb directed against an epitope of kininogen domain D6, had only marginal inhibitory activity (Fig. 4 B). The results suggest that MBK3 compromises parasite invasion of HUVECs, either by blocking the processing of cell-bound kininogens and/or by “neutralizing” the liberated effector kinin peptide.

**E-64–sensitive Proteases Mediate Kinin Release by Living Trypomastigotes.** Given the indications that the major cysteine proteinase isofrom, cruzipain-1, releases kinins from H-kininogen (23), and that an accessible BK epitope is required for parasite invasion (Fig. 4 B), we sought to examine if living trypomastigotes are able to release kinins from purified H-kininogen. To this end, washed TCTs were resuspended in CAP medium and 10 nM of purified H-kininogen was added to the suspension. ELISA measurements of the kinin concentrations in culture supernatants revealed the presence of this peptide within 30 min (Fig. 5 A). The processing reaction, which evolved in the absence of reducing agents, converted a small fraction (~10%) of available H-kininogens into immunoreactive kinins over a limited time period. Significantly, the kinin-releasing activity was completely abolished by pretreating the parasites with an irreversible inhibitor of cysteine proteinases, E-64. These data suggest that TCTs engage cysteine proteinases in releasing kinins from soluble H-kininogens. As trypomastigotes are poorly endocytic (30), the membrane-impermeable E-64 inhibitor either binds to secreted cysteine proteases or acts on activated enzymes as they reach the parasites’ flagellar pocket (17).

**Trypomastigotes Release Different Cruzipain Isoforms.** As pointed out in a previous study (23), the Met-Lys and Arg-Ser flanking site bonds of BK are cleaved at different efficiencies by two distinct recombinant cruzipain isoforms, namely cruzain (cruzipain-1) and cruzipain-2. Assays performed with each of these genetically engineered proteinases (both truncated on the COOH-terminal extension) confirmed that they are able to release immunoreactive kinins from purified H-kininogen, albeit with different efficiencies (Fig. 5 B). Consistent with the demonstration of kinin–releasing activity by living trypomastigotes, analysis of the supernatants from parasite suspensions revealed the presence of peptidases that were activated by DTT and inhibited by E-64 (Fig. 6 A). Immunoprecipitation using isoform-specific Abs mAb212 (anti–cruzipain-1) and rab222 (anti–cruzipain-2) followed by fluorogenic substrate assay suggested that both isoforms were present in the supernatants (Fig. 6 B). Hence, TCTs released cysteine protease isoforms that may be involved in parasite-induced kinin release.

**The Kinin Pathway of Parasite Invasion Is Linked to the Expression of Cruzipain-1.** As both recombinant cruzipain-1 and cruzipain-2 act as kininogenases in vitro, we investigated if the engagement of B2R by the TCT may be linked to differential expression of these isoforms. To this end, we used the episomal pTEX plasmid to generate TCT transfectants containing full-length copies of the gene encoding for the major cruzipain-1 isoform (TCT-cz1) or for the...
Fig. 6. Differential infectivity of TCTs overexpressing cruzipain isoforms. (A) Analysis of the cysteine protease activity present in supernatants from wild-type Dm28c TCTs. Parasite suspensions (2 × 10^7 cells/ml) were incubated at 37°C for 2 h in DMEM without FCS. The filtered supernatants were tested for cysteine activity at 37°C using 20 μM e-NH2-Cap-Leu-(SBz)Cys-MCA in 50 mM Na2HPO4, pH 6.5, 200 mM NaCl, 5 mM EDTA, and 5 mM DTT. The graph depicts enzyme activity found in trypomastigote supernatants (Supnt) activated with DTT (■) or pretreated with 30 μM E-64 (○). (B) Protein A–agarose beads were loaded with anti-cruzipain-1 or anti-cruzipain-2 and the immunobeads were added to supernatants obtained from wild-type TCT suspensions. After 2 h of incubation at room temperature, the immune complexes associated with the beads were washed with PBS and the initial rates of hydrolysis by enzymes associated with the solid phase were monitored in the presence or absence of 30 μM E-64, as specified above. Results (initial rates) are represented by the mean of three independent experiments. Statistical significance (P < 0.05) is indicated by asterisks.

Cruzipain-2 gene (TCT-cz2). Consistent with the higher stability of recombinant cruzipain-2 at alkaline pH (Lima, A.P.C.A., unpublished observations), we found that a significant proportion of the overall cysteine protease activity present in TCT-cz2 lysates (30%) was attributed to enzymes that resist inactivation at pH 12 (Fig. 6 C), whereas only a minor fraction (8%) of alkaline-resistant cysteine peptidases were present in TCT-cz1 (not shown) or wild-type cells (Fig. 6 C). Hence, the profile of isoforms expressed by TCT-cz2, although still dominated by the alkaline-sensitive major cruzipain-1 isoform, is skewed towards increased production of the cruzipain-2–like isoforms. Remarkably, invasion assays performed with target cells that do not overexpress B2R, i.e., CHO-mock or Vero cells, revealed that TCT-cz1 was consistently less infective (expressed for CHO-mock; Fig. 6 D, gray bar at left) compared with TCT-cz2 (Fig. 6 D, gray bar at right) or wild-type parasites (Fig. 3 A). TCT-cz1 efficiently invaded CHO-B2R cells, and this effect was reduced almost to the reference level of CHO-mock by the addition of HOE 140 (Fig. 6 D, black bars at right), indicating that the B2R pathway is used by the cz1 transfectants. By contrast, overexpression of cruzipain-2 did not significantly enhance invasion of CHO-B2R cells compared with CHO-mock (Fig. 6 D, right). Furthermore, HOE 140 did not reduce TCT-cz2 infectivity for CHO-B2R (Fig. 6 D, right), suggesting that these parasites signal through kinin-independent pathways. Collectively, these data suggest that T. cruzi’s ability to engage the kinin signaling pathway of invasion is preferentially linked to a dominant expression of the major cysteine protease isoform (cruzipain-1).

Purified Cruzipain Triggers [Ca2+]i Transients in Cells Exposing B2R. Given the indications that cruzipain-1 is found in the supernatants from wild-type TCTs and that living trypomastigotes release kinins through E-64–sensitive protease(s), we tested the ability of purified cruzipain-1 to induce [Ca2+]i transients. Indeed, we found that the parasite protease triggered robust [Ca2+]i transients in CHO-B2R loaded with Fura-2 (Fig. 7 B). This response was almost nullified by adding HOE 140 at 50 nM (Fig. 7 D) to the CAP medium, whereas [des Arg9-Leu8-BK]-benzamidine, an antagonist of the B2 subtype, failed to interfere (data not shown). Importantly, the catalytic activity of cruzipain-1 was essentially required for the B2R stimulation because pretreatment with E-64 abrogated the [Ca2+]i response (Fig. 7 C). These data suggest that cruzipain-1 generates a kinin–like signaling factor by processing kininogen displayed by CHO cells. Unlike the strong [Ca2+]i transients in CHO-B2R, cruzipain-1 induced only a minor [Ca2+]i response in CHO-mock (Fig. 7 A). This low response was not inhibited by HOE 140 or by [des Arg9-Leu8-BK] (data not shown), suggesting that the minor [Ca2+]i transients in
Figure 7. Cruzipain induces [Ca^{2+}]_i transients in CHO cells through the B2R. Activated cruzipain-1 or E-64-treated cruzipain-1 were added to monolayers of CHO cells in Ham’s F12 medium supplemented with 1 mg/ml of BSA, 25 μM captopril. The protease was diluted 10-fold to 5 nM. (A) CHO-mock; (B) CHO-B2R exposed to cruzipain; (C) CHO-B2R exposed to E-64–treated cruzipain; (D) CHO-B2R pretreated with 100 nM HOE 140 for 120 s and then exposed to cruzipain-1 (indicated by arrows). Addition of the proteinase buffer alone did not elicit any [Ca^{2+}]_i transients in CHO cells. The y-axis represents the ratio of fluorescent absorbances at 334 and 380 nm. The tracings represent average cell responses.

Discussion

In this study, we demonstrate that trypomastigote invasion of host cells expressing BK B2Rs is drastically increased due to signaling by kinin peptides. The presence of captopril in the culture medium was a crucial prerequisite for the increased invasiveness of target cells expressing B2R, most likely because BK was protected from rapid degradation by ACE/kininase II (31). The effects induced by captopril suggest that T. cruzi tissue tropism may be influenced by variable levels and activities of kinin–degrading peptidases expressed in different host tissues. Indeed, highly vascularized tissues such as kidney parenchyma and lungs abundantly expressing ACE (32) are virtually spared from T. cruzi parasitism, whereas other tissues undergo massive infection and destruction during acute infection (33). Thus, variable expression levels of ACE may modulate the severity of lesions and fibrosis induced by this pathogen in the cardiovascular system of chagasic patients.

Because the presence of captopril or HOE 140 did not interfere with parasite invasion of CHO-mock (this study), Vero cells (not shown), or L6E9 myoblasts (Docampo, R., personal communication), parasites likely invade these cell lines by alternative mechanisms such as the TGF-β–dependent transducing pathway (7) or oligopeptidase B–mediated production of [Ca^{2+}]_i signaling agonists (13, 14). Whereas the latter pathway is thought to involve proteolytic processing of a cytoplasmic precursor protein of the parasite (14), the kinin–mediated signal transduction route described herein likely depends on the processing of a host-derived precursor, namely kininogen(s). Preliminary analysis of a limited panel of well-established T. cruzi strains and clones indicates that invasion through the kinin-transducing pathway is not ubiquitous (Scharfstein, J., unpublished observations), indicating that clones from this highly diverse parasite species (34–37) may vary with respect to the ability to release proinflammatory kinins.
One of the conundrums of this study is the source of kininogens involved in the invasion process. Kininogens are secretory proteins displaying high affinity binding sites for heparan sulfate proteoglycans exposed on most cell surfaces (38), and thus we speculate that serum-derived kininogens attach to HUVECs or CHO cells during cultivation in vitro. At first sight, the finding that purified cruzipain is able to release kinins from cell-bound kininogens seems paradoxical because papain-like enzymes are rather sensitive to inhibition by the cystatin-like domains of kininogens (39). However, it is well known that the cystatin inhibitory sites in domain D3 of kininogen’s heavy chain overlaps with the cell binding site that docks it to endothelial cells (40), and therefore may not be available for cruzipain inhibition. Our finding that FITC-labeled cruzipain failed to bind to HUVECs (data not shown) is consistent with this interpretation. Further, our demonstration that activated cruzipain stimulates vigorous Ca\textsuperscript{2+} transients in CHO-B\textsubscript{2}R, and that HOE 140 and E-64 block this effect, points to proteolytic release of kinin agonists, either directly by secreted forms of the protease or indirectly by the activation of a kallikrein-type kininogenase. The finding that membrane-permeable cysteine protease inhibitor Z-(SBz)Cys-CHN\textsubscript{2}, but not hydrophilic inhibitor E-64, conveyed partial albeit significant protection of HUVECs, is consistent with such a “dual signaling” model.

Previous studies have revealed that [Ca\textsuperscript{2+}], transients induced by G protein–coupled receptor agonists stimulate the recruitment and fusion of lysosomes to the plasma membrane at sites where trypomastigotes are attached to host cells (4, 5). Considering that adhesive interactions between TCTs and host cells favor their reciprocal activation (41), it is conceivable that kinin signaling is most effective when the processing reaction is restricted to sites formed by juxtaposition of host and parasite cell membranes. Hence, signals emanating from host contacts with a few trypomastigotes, e.g., in CHO-mock or in HOE 140–treated CHO-B\textsubscript{2}R, may promote the delivery of host lysosomal cargos to secluded intercellular spaces, and this incipient activation process may generate a reduced environment in these segregated sites thereby sustaining activation of cruzipain-type kininogenases. The finding that membrane-permeable cysteine protease inhibitor Z-(SBz)Cys-CHN\textsubscript{2}, but not hydrophilic inhibitor E-64, conveyed partial albeit significant protection of HUVECs, is consistent with such a “dual signaling” model.

As B\textsubscript{2}R is constitutively expressed by endothelial cells, smooth muscle, fibroblasts, epithelial cells, and neuronal cells (24), and the B\textsubscript{2} subtype of kinin receptors is upregulated during inflammation (42), increased cellular invasion may be just one example of a physiopathological response...
that kinins and their metabolites may induce in tissues exposed to *T. cruzi*. For example, triggering of kinin receptors from vascular endothelial cells may facilitate the transmigration of the trypanastigotes across capillaries, modulate the expression of vascular adhesion molecules, and/or promote plasma leakage into interstitial spaces, and thus contribute to the microvascular changes observed in patients with chronic cardiomyopathy (43). Unraveling the delicate interplay between the parasite cysteine proteinases and the multiple components of the kinin cascade system may provide fresh insights into the molecular pathogenesis of Chagas’ disease.

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