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Heme-induced *Trypanosoma cruzi* proliferation is mediated by CaM kinase II

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ABSTRACT

Trypanosoma cruzi, the etiologic agent of Chagas disease, is transmitted through triatomine vectors during their blood-meal on vertebrate hosts. These hematophagous insects usually ingest approximately 10 mM of heme bound to hemoglobin in a single meal. Blood forms of the parasite are transformed into epimastigotes in the crop which initiates a few hours after parasite ingestion. In a previous work, we investigated the role of heme in parasite cell proliferation and showed that the addition of heme significantly increased parasite proliferation in a dose-dependent manner [1]. To investigate whether the heme effect is mediated by protein kinase signalling pathways, parasite proliferation was evaluated in the presence of several protein kinase (PK) inhibitors. We found that only KN-93, a classical inhibitor of calcium–calmodulin-dependent kinases (CaMKs), blocked heme-induced cell proliferation. KN-92, an inactive analogue of KN-93, was not able to block this effect. A *T. cruzi* CaMKII homologue is most likely the main enzyme involved in this process since parasite proliferation was also blocked when Myr-AIP, an inhibitory peptide for mammalian CaMKII, was included in the cell proliferation assay. Moreover, CaMK activity increased in parasite cells with the addition of heme as shown by immunological and biochemical assays. In conclusion, the present results are the first strong indications that CaMKII is involved in the heme-induced cell signalling pathway that mediates parasite proliferation.

Introduction

Heme (iron protoporphyrin IX) is a tetrapyrrole containing a central iron ion. It is an important molecule in the basic metabolism of all living organisms. It is involved in oxygen transport, respiration and drug detoxification. Heme regulates the transcriptional repressor Bach1, the transcription factor for Heme Oxygenase 1. Heme also regulates a Ras-mitogen-activated protein kinase (MAPK) pathway in mammalian systems [2]. It is well known that *Trypanosoma cruzi*, the etiologic agent of Chagas disease, requires heme for *its* metabolism [1,3,4]. Genome sequencing of this protozoan has shown that it lacks several genes encoding enzymes involved in heme biosynthesis [5]. Therefore, *T. cruzi* must acquire exogenous heme from its host. *A* majority of heme found within an organism is present in hemoglobin (Hb). However, in the midgut of hematophagous insects the free heme concentration greatly increases after Hb digestion. *T. cruzi* epimastigotes are the proliferative forms of this parasite and inhabit an environment (midgut) in the bug insect rich in heme generated during blood digestion.

Recently, we showed that epimastigotes acquire exogenous heme and the addition of heme increases parasite proliferation significantly in a dose-dependent manner [1]. Furthermore, we showed that neither Hb nor its peptides are able to promote the same increase as free heme. Therefore, we hypothesized that heme *is* involved in a signalling process that regulates proliferation.

Tremendous progress has been made over the last few decades in understanding key pathways that regulate cell growth and division. Common to all the regulatory transitions of the cell cycle is the ubiquitous second messenger, Ca^{2+} and its universally important intracellular receptor, calmodulin (CaM). Several recent studies have improved the understanding of how CaM regulates cell cycle transitions and proliferation rate [6,7]. The multifunctional Ca^{2+}/CaM kinases (CaMKs) are a family of serine/threonine protein kinases that include $CaMKK_{\alpha\beta}$, CaMKI, CaMKIV and CaMKII [8]. CaMKII is a major mediator of calcium signalling in the brain, the heart and other tissues [9]. Ogueta et al. [10,11] identified CaMKII in *T. cruzi* using monoclonal antibodies against rat brain CaMKII. In the present work, we show for the first time that CaMKII is

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activated in the presence of heme *in T. cruzi*. Furthermore, we demonstrate that such activation is involved in the proliferation of the epimastigote form of the parasite and therefore plays a role in Chagas disease transmission.

Materials and methods

Reagents. Protease inhibitor cocktail (AEBSF, aprotinin, leupeptin, bestatin, pepstatin A and E-64), EDTA, EGTA, Triton X-100, Hepes, SDS, Tris base, glycine, acrilamide, and TEMED were purchased from SIGMA FINE CHEMICALS (St. Louis, MO, USA). Hemin (Heme-Cl) was obtained from PORPHYRIN PRODUCTS. BHI (brain–heart infusion medium) was obtained from DIFCO (Sparks, MD, USA). Fetal bovine serum (FBS) was purchased from CULTLAB (São Paulo, Brazil). SeeBlue® pre-stained standard (250, 98, 64, 50, 36, 30, 16, 6, and 4 kDa), anti-mouse and anti-rabbit secondary antibodies were obtained from INVITROGEN CORPORATION. Sodium orthovanadate (VO_4^{-3}), okadaic acid (OKA), phenylarsine oxide (PAO), LY 294002 (LY), bisindolylmaleimide I (BIS), H-9, H-89, roscovitine (R), 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN-93), (2-[*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) (KN-92), the myristoylated form of autocamtide-2-related inhibitory peptide (Myr-AIP), and bovine serum albumin (BSA) fraction V were purchased from CALBIOCHEM (L. Jolla, CA, USA). Sodium fluoride, Na^+/K^+ double tartrate and ammonium molybdate were purchased from REAGEN (RJ, Brazil). The polyclonal anti-phospho CaMKII and CaMK assay kit were obtained from UPSTATE BIOTECHNOLOGIES (Piscataway, NJ). Ethanol and methanol were purchased from MERK. All other reagents used were of analytical grade.

Parasites. Trypanosoma cruzi strain Dm 28c (CT-IOC-010) was provided from the Trypanosomatid Collection of the Oswaldo Cruz Institute, Fiocruz, Brazil. The protozoa were grown at 28 °C for *seven* days in BHI supplemented with 30 μM heme and 10% foetal calf serum (FCS). Cultures were kept in 100 mL bottles at an initial density of 20–30 $\times$ 10^6 cells/mL in 30 mL of medium. Growth was monitored by cell counting in a Neubauer chamber.

Effect of protein kinase inhibitors on parasite proliferation. On the seventh day of growth in BHI supplemented with 30 μM and 10% FBS, *T. cruzi* epimastigotes were washed twice in heme-free BHI and incubated with one of the following kinase inhibitors: LY294002 8 μM (LY), Bistyrphostin 0.05 μM (BIS), H-9 (5 μM), H-89 (0.24 μM), R (3.5 μM), or KN-93 (2 μM). The initial parasite concentration was 5 $\times$ 10^5 cells. Cell densities were determined by using a hemocytometer after five days [12]. To test the specificity of KN-93 effects, on the seventh day of growth in BHI supplemented with 30 μM *and* 10% FBS, *T. cruzi* epimastigotes were washed twice in heme-free BHI and incubated with different concentrations of KN-93 (0, 0.2, 2, 5, and 10 μM) or its ineffective analogue, KN-92 (2 μM). In some experiments, the parasites were incubated in the absence or in the presence of the Myr-AIP peptide (30 μM). In these experiments, the initial parasite concentration was 5 $\times$ 10^5 cells. The cells were incubated for five days and the cell density was determined using a hemocytometer.

Samples. The epimastigotes, at the exponential phase, were maintained in BHI supplemented with 10% FBS and 30 μM heme. The cells were washed in BHI supplemented with 10% FBS without heme. *T. cruzi* epimastigotes were incubated *either* in the absence or presence of 30 μM heme for 15 min. The cells were washed in a phosphate-buffered solution (PBS; 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2). After washing, the cells were lysed using a solution of 50 mM Hepes, 1 mM $MgCl_2$, 10 mM EDTA, and 1% Triton X-100 at a pH of 6.4, in the presence of a freshly added cocktail of protease inhibitors (1.04 mM AEBSF, 800 nM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, and 14 μM E-64) and phosphatase inhibitors (1 mM EDTA, 1 mM EGTA, 0.075 μM OKA, 2 mM VO_4^{-3} and 2 mM ammonium molybdate, and 0.18 mM PAO). After lysis, the cells were centrifuged at 10,000 rpm for 10 min at 4 °C, after which the supernatant was collected and kept for use.

Western blot analysis. Protein concentrations were determined by the method of Lowry et al. [13] with BSA as the standard. The proteins were then separated by 12% sodium dodecyl sulfate sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli [14] and transferred onto nitrocellulose membranes. Lysates from the parasite cells (80 μg) were used. The membranes were blocked in Tris-buffered saline (TBS) supplemented with 0.1% Tween (TT) plus 5% BSA for 1 h before incubation overnight in the primary phospho-CaMK II (1:2000) antibody. After removal of the primary antibody and washing five times in TT, the membranes were incubated in the secondary antibody conjugated to rabbit peroxidase for 1 h. Washed blots were then incubated with *a* chemiluminescence ECL kit (Amersham).

Enzyme assays. CaMKII activity was assayed as suggested by the manufacturer kit (Upstate Biotechnologies, Piscataway, NJ) of the kit using autocamtide-2 as the substrate and [γ^{32}P]-ATP (Amersham Biosciences, 6000 Ci/mmol). Reactions (a final volume of 50 μl) were incubated with the supernatant from *T. cruzi* epimastigote lysates (100 μg *of* protein) in the presence of 8 μg/mL CaM, 1 mM $CaCl_2$, 2 μM cAMP-dependent protein kinase peptide inhibitor, 2 μM protein kinase C peptide inhibitor, 100 μM autocamtide-2 and 100 μM [γ^{32}P]-ATP (500–1000 cpm/pmol) at 30 °C for 10 min. Aliquots were then removed, spotted onto phosphocellulose discs, washed three times in 0.75% phosphoric acid and once in acetone, dried and counted by liquid scintillation (Tri-Carb 2100 TR). Simultaneously, the protein contents in the aliquots were separated by 12% SDS–PAGE. After electrophoresis, the gels were stained with Coomassie blue, distained, dried and analyzed by autoradiography using a Storm 860 (Molecular Dynamics). Further conditions are described in Silva-Neto et al. [12].

Statistical analysis. Statistical analyses were conducted with GraphPad Prism 3 software (GraphPad Software, Inc., San Diego, CA). Data are presented as means ± standard deviation (SD). Data were analyzed by a one-way analysis of variance (ANOVA) and differences between groups were assessed by using the *Tukey* post-test. The level of significance was set at $p < 0.05$.

Results

The involvement of protein kinases in heme-induced cell proliferation

Heme is involved in *T. cruzi* cell proliferation in a dose-dependent manner. However, this effect is not observed when the cells are incubated in the presence of hemoglobin or globin-derived peptides [1]. Fig. 1A shows that the treatment of *T. cruzi* with 30 μM heme displays a significant increase on cell proliferation, approximately 30% in relation to the control group. To investigate whether the proliferative effect of heme is associated with a protein kinase (PK), we evaluated the effect of several PK inhibitors. The cells were maintained for 5 days in BHI medium supplemented with 30 μM heme, 10% FBS medium and each of the inhibitors. Among all of the inhibitors tested, only KN-93 (2 μM), a classical inhibitor of CaM kinases (CaMK), had a significant effect on cell proliferation mediated by heme (Fig. 1A). The other inhibitors did not inhibit heme-induced cell proliferation. None of inhibitors had an effect on cell proliferation in the absence of heme (data not shown).

CaMKs are usually maintained in an inactive state due to the presence of an autoinhibitory domain [15]. However, upon an

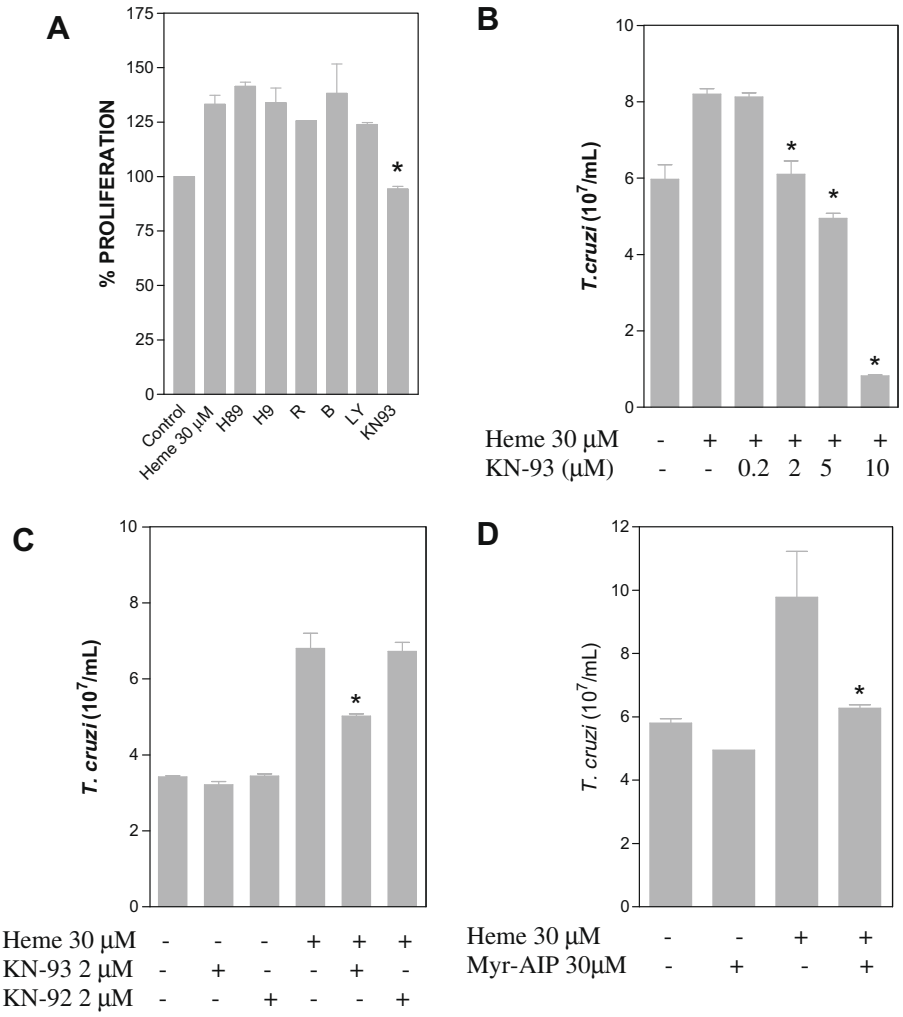

Fig. 1. Effect of different PK inhibitors on *T. cruzi* epimastigote proliferation. *T. cruzi* epimastigotes were maintained for five days in BHI supplemented with 10% FBS in the absence (control) or presence of 30 μM heme, as well as in the presence of different protein kinase (PK) inhibitors. (A) *T. cruzi* epimastigotes were incubated with the following protein kinase inhibitors: H-89 (H89), H-9 (H9), roscovitine (R), bisindolylmaleimide I (B), LY294002 (LY), and KN-93 (KN93). Cell proliferation (represented as a percent) was calculated based on absolute values of the treated parasites related to the control group. (B) *T. cruzi* epimastigotes were incubated with different concentrations of KN-93 (2 μM, a classical inhibitor of CaMKs). (C) Cells were incubated in the presence of KN-92 (2 μM, an inactive analogue of KN-93). (D) Cells were incubated in the presence of Myr-AIP (30 μM, a myristoylated CaMKII inhibitory peptide). All data are presented as means ± SD. $^{*}P < 0.01$ in relation to the group treated with 30 μM heme as determined by Tukey test. Results are representative of three independent experiments.

increase in intracellular calcium the binding of the Ca^{2+}/CaM complex to the kinase disrupts the interaction of the autoinhibitory domain. KN-93 *also* binds to the Ca^{2+}/CaM binding site and inhibits the kinase activity [16]. In some cells, such as fibroblasts and HeLa cells, KN-93 causes an anti-proliferative effect [17]. In the case of *T. cruzi*, we show that KN-93 does not cause an anti-proliferative effect. However, this inhibitor does abolish heme-induced cell proliferation (Fig. 1A). These results indicate that heme participates in cellular signalling during cell proliferation. In order to test the specificity of the effects of KN-93, different concentrations of this drug was added to the medium at different concentrations and the results show a significant decrease *in* cell proliferation when the drug concentration increased (Fig. 1B). *A* drastic anti-proliferative effect of 10 μM KN-93 was seen. This anti-proliferative effect is most likely due to a toxic effect at this concentration. The specificity of KN-93 was further confirmed using KN-92, an inactive analogue of KN-93, in a similar assay. KN-92 did not effect epimastigote proliferation (Fig. 1C). These results indicate that the inhibition of cell proliferation mediated by heme is dependent on CaMK.

The CaMK signalling cascade contains three members: CaMK kinase (CaMKK; α and β), CaMKI and CaMKIV [8]. CaMKK is the upstream member of the cascade and activates CaMKI and CaMKIV through phosphorylation of their "activation loop" containing a Ser/Thr. Phosphorylation of the Ser/Thr in CaMKI and CaMKIV strongly augments *its* kinase activity [18]. Both CaMKI and CaMKIV can be inhibited by KN-93. In contrast with the human kinome, CaMKs are poorly represented in trypanosomatids with only 13 genes predicted to encode active enzymes in *T. cruzi* [19].

Another member of the CaMK family, CaMKII, is not a target of CaMKK. Unlike CaMKI and CaMKIV, CaMKII has an additional association domain located carboxyl terminal to its regulatory domain. CaMKII has been described as being markedly activated by autophosphorylation at Thr^{286} and markedly inhibited in the presence of autocamtide-2 related inhibitory peptide (AIP) or its myristoylated form (Myr-AIP). AIP functions by binding to the substrate-binding site for autophosphorylation on CaMKII. Myr-AIP (KKALRRQEAVDAL) is a peptide derived from the CaMKII substrate autocamtide-2, with an alanine substituted for threonine at the 9-position. This amino acid substitution results in a highly specific and potent inhibitor of CaMKII. Unlike the KN series of inhibitors, the binding of Ca^{2+}/CaM is not affected by AIP or Myr-AIP [20]. Thus, Myr-AIP was added to the parasite cultures to test *the* ability *of* this peptide to block heme-mediated proliferation (Fig. 1D).

According to the results, Myr-AIP is able to block the proliferation at the same rate as KN-93.

Autophosphorylation of CaMKII in the presence of heme

To assess the activation of CaMKII, we determined the level of phospho-CaMKII present in the parasite lysates by immunoblotting with an anti-phospho-CaMKII antibody. The antibody used was designed to recognise the autophosphorylation site *at* Thr^{286} and to determine the level of enzyme activation. As shown in Fig. 2A, the level of phospho-CaMKII increased with heme treatment. Fig. 2B demonstrates the same level of protein in all of the treatment samples. Densitometry confirmed that enzyme activation occurs in a short time (about 15 min). The level of phospho-CaMKII is twice as high as the control after 30 min of treatment (Fig. 2C). These results confirm the involvement of heme and CaMKII in *T. cruzi* proliferation.

CaMKII enzymatic activity is increased by heme

In order to directly examine CaMKII enzymatic activity, we performed an *in vitro* kinase assay. *T. cruzi* was incubated in the presence or absence of heme and/or Myr-AIP. In all conditions, the parasites were lysed after incubation and submitted to an *in vitro* phosphorylation assay in the presence of [γ^{32}P]-ATP and autocamtide as the substrate. Samples were fractionated in a denaturing polyacrylamide gel and analyzed by autoradiography. The profile of phosphorylated proteins from the parasite homogenates can be seen in Fig. 3. In addition, a difference between the untreated cells and the cells treated with heme can be seen when comparing the phosphorylated protein profiles. Parasite extracts obtained from the cells previously incubated in the presence of Myr-AIP and then exposed to heme showed a significant decrease in the total level of phosphorylation (Fig. 3, lane 4). These data demonstrate a close correlation between heme stimulation and CaMKII activation. Simultaneously, aliquots were counted by liquid scintillation. As shown, cells treated with heme for 30 min had an increase in kinase activity *of* up to 35%, while the cells treated with both Myr-AIP and heme had a decrease in enzymatic activity compared to the control (Fig. 4). Altogether, these results strongly support the participation of CaMKII in hememediated *T. cruzi* proliferation.

Discussion

Recently, we established that heme is an important proliferative factor for *T. cruzi* epimastigotes. These epimastigotes uptake heme from the environment and store it in reservosomes [1]. Here, we demonstrated that heme increases *T. cruzi* proliferation through the activation of CaMKII. *Therefore,* since the proliferative form of this parasite lives in the gut of blood sucking bugs where the heme concentration is high (free or bound to proteins and peptides), we suggest that heme may influence the metabolism of the protozoan.

Recent studies show that heme promotes proliferation of endothelial progenitor cells through the AKT pathway [21]. Our results show that parasite proliferation, which is exclusively dependent on heme, is totally abolished by CaMK inhibition. These results are also corroborated by previous findings. *A* selective CaMK inhibitor has been demonstrated to have an anti-proliferative effect on a variety of mammalian cells [6]. In our case, proliferation of *T. cruzi* treated with KN-93 differs dramatically from the untreated group. These data show that *KN-93* acts as a fundamental regulator of parasite proliferation in the presence of heme. The specificity of CaMK action was confirmed using KN-92, an inactive form of the inhibitor.

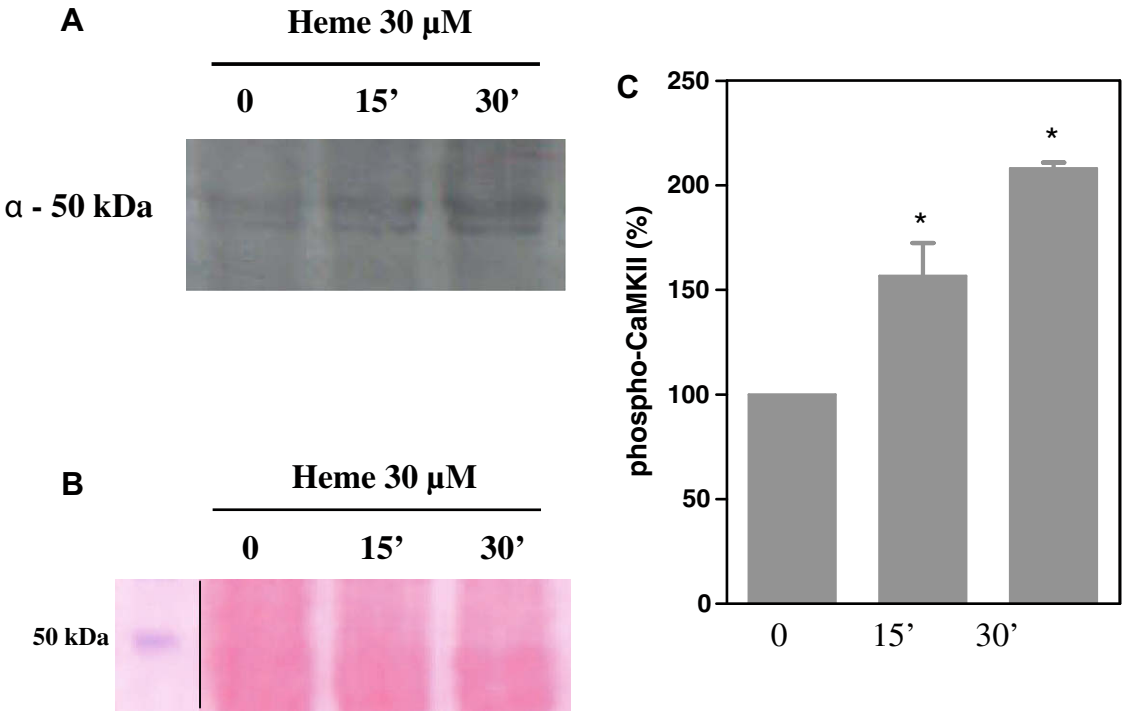

Fig. 2. Induction of CaMKII by hemin in *T. cruzi* epimastigotes. (A) *T. cruzi* epimastigotes were incubated in the absence (control) or presence of 30 µM heme for 15 or 30 min. *T. cruzi* were lysed and the supernatant was submitted to 12% SDS–PAGE, transferred onto nitrocellulose and immunoblotted with a polyclonal phospho-CaMKII antibody. (B) Equal protein loading was checked by Ponceau S staining of the transferred nitrocellulose membrane prior to immunoblot analysis. (C) Quantification of the level of phospho-CaMKII was determined by densitometry. The bands were analyzed using Adobe Photoshop 5.0. All data are presented as means ± standard error of the mean (SEM). $^{*}P < 0.05$ in relation to the control group as determined by Tukey test. Results are representative of three independent experiments. The thin line in the nitrocellulose membrane represents the place where lanes were spliced.

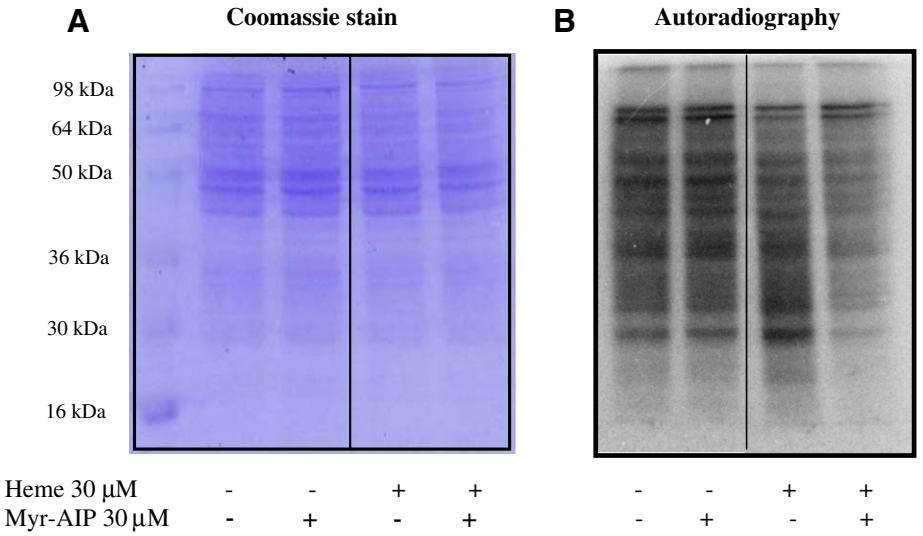

Fig. 3. Effect of Myr-AIP on the total level of phosphorylation by CaMKII. *T. cruzi* epimastigotes were incubated in the presence or absence of Myr-AIP (30 μM) for 20 min, followed by *a* incubation in the absence or presence of 30 μM heme for 60 min. The *T. cruzi* epimastigotes were then lysed and the supernatant was assayed for CaMKII activity, as suggested by the manufacturer (Upstate Biotechnologies, Piscataway, NJ) using autocamtide as the substrate. Aliquots were submitted to 12% SDS–PAGE, stained with Coomassie blue and analyzed by autoradiography. (A) Coomassie stain of the loading control. Lane 1: untreated *T. cruzi* epimastigotes (without heme and Myr-AIP; control); lane 2: Parasites incubated with Myr-AIP (30 μM) for 20 min; lane 3: Parasites incubated with heme (30 μM) for 60 min; lane 4: Parasites incubated with Myr-AIP (30 μM) for 20 min and then incubated with heme (30 μM) for 60 min. (B) Autoradiography. The thin line represents the place where the lanes were spliced.

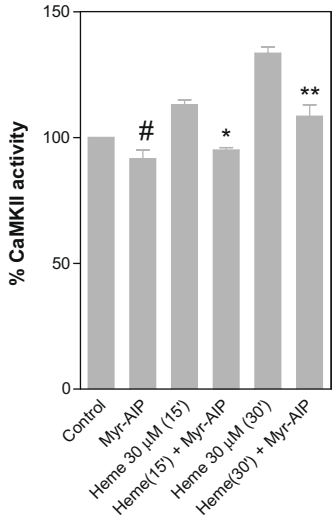

Fig. 4. CaMKII activity in *T. cruzi* epimastigotes. *T. cruzi* epimastigotes were incubated with the Myr-AIP (30 μM) inhibitor for 20 min followed by a incubation in the absence or presence of heme (30 μM) for 15 or 30 min. Cells were lysed and the supernatant was assayed for CaMKII activity using autocamtide as the substrate, as suggested by the manufacturer (Upstate Biotechnologies, Piscataway, NJ). The percent values of CaMKII activity were calculated based on the absolute values of the treated parasites in relation to the control group (without both heme and Myr-AIP). All data are presented as means ± SD, $^{\#}P > 0.05$ in relation to the control group, while $^{*}P < 0.05$ in relation to the 30 μM heme group treated for 15 min and $^{**}P < 0.01$ in relation to the group treated with 30 μM heme for 30 min. Statistical significance was determined by Tukey test. The results are representative of two independent experiments.

Multifunctional CaMKs, including CaMKI, CaMKII and CaMKIV, have been associated with cell cycle transitions [6]. In HeLa cells, KN-93 causes a G_1 arrest [17]. *In* normal fibroblasts, KN-93 treatment also arrested cells in G_1 with a low level of cyclin-dependent kinase-4 (cdk4) activity, an important regulator of the cell cycle [22]. The most intensely investigated member of the multifunctional CaMKs is CaMKII. Unlike many other protein kinases, CaMKII does not contain phosphorylated residues analogous to the activation domains of CaMKI and CaMKIV. Thus, CaMKII is not phosphorylated by CaMKK [18]. In order to test the possibility of heme involvement in CaMKK and CaMKIV phosphorylation, we examined the phosphorylation levels of these kinases using specific antibodies (data not show) and no increase was observed.

In HeLa cells the Proto-oncogene *c-fos* mRNA is induced by heme. This induction is MAPK-Independent and CaMKII-dependent. The presence of a specific CaMKII inhibitor (Myr-AIP) reduced the induction of *c-fos* mRNA. Interestingly, the level of phospho-CaMKII rapidly increases with hemin treatment. However, this increase in phospho-CaMKII does not occur in the presence of Myr-AIP [23]. These data indicate a relationship between heme and CaMKII activation.

In the present work, we show that the addition of Myr-AIP to cell cultures *of T. cruzi* blocked the proliferation of this parasite in the presence of heme. As found in HeLa cells, the addition of heme *to* the medium showed a significant increase in the activity of CaMKII, reaching more than *a* 30% increase in 30 min. Supporting our data, is the fact that the level of phospho-CaMKII (indicating its level of activation) in heme-treated cells also increased more than double compared to the untreated control.

Parasites *in the* invertebrate host's midgut are continuously adjusting their own signalling pathways to ongoing events during blood digestion. In the malaria parasite cycle, the activation of CaMK was previously demonstrated during cell maturation in the mosquito midgut [12]. However, the key modulators of such activation were not identified. Heme-mediated signalling through CaMK probably relies on a set of signalling molecules yet to be identified. Phospho-proteomic analysis of epimastigotes has shown that CaMK phosphorylation sites are the most abundant motifs (13.9%) found in sequenced phospho-peptides. These data emphasize the role of *this* protein kinase family in parasite biology [24]. The recent availability of both the *T. cruzi* genome and its protein kinase complement (the kinome) will likely shed light on such *a* question in the future [19]. Despite the importance of the heme molecule in the biology of *T. cruzi,* there are few studies of heme metabolism in the literature. Here, we have shown for the first time that heme is responsible for parasite proliferation and suggest the specific mechanism by which heme regulates parasite proliferation occurs through activation of CaMKII.

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