Calcium Signaling Involves Na\(^+\)/H\(^+\) Exchanger and IP\(_3\) Receptor Activation in T. cruzi Epimastigotes

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1. Introduction

The intracellular calcium mobilization plays an important role in signaling systems that have been adapted to control diverse processes such as fertilization, proliferation, contraction, cellular metabolism, and secretion. In higher eukaryotes, the inositol phosphate pathway is a versatile signal system which transduces external signals through phospholipase C (PLC) activation and production of the second messengers inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG). Regarding calcium (Ca\(^{2+}\)) signaling, it is well established that IP\(_3\) produced by PLC activation diffuses into the cytosol and releases Ca\(^{2+}\) from intracellular stores, promoting intracellular calcium mobilization [1]. In the last years, it was demonstrated that protozoan parasites display an intracellular Ca\(^{2+}\) signaling with similarities to higher eukaryotes, including the participation of endoplasmic reticulum (ER) and mitochondria in Ca\(^{2+}\)-dependent signaling events [2].

In Trypanosoma cruzi, the etiologic agent that causes Chagas disease, the involvement of IP\(_3\)/Ca\(^{2+}\) pathway in parasite homeostasis, survival, and host cell invasion has been the focus of several studies (reviewed in [3]). Key components of this signaling pathway and enzymes associated with calcium signaling were reported in the parasite, such as PLC [4], phosphoinositide kinases [5,6], IP\(_3\) receptor [7], protein kinase C (PKC) [8–10], adenyl cyclase [11], and Ca\(^{2+}\)-dependent phosphodiesterase (PDE) [12], among others. The main Ca\(^{2+}\) stores in T. cruzi are lysosome-related organelles named acidocalcisomes, acidic vacuoles rich in phosphorus compounds and cations present in the three stages of
the parasite (epimastigotes, amastigotes, and trypomastigotes) [13]. Other intracellular calcium reservoirs are the ER and the mitochondrion. Each organelle has a pump responsible for Ca\(^{2+}\) intake: PMCA Ca\(^{2+}\)-ATPase in acidocalcisomes [14], SERCA Ca\(^{2+}\)-ATPase in endoplasmic reticulum (ER) [15], and a uniporter in mitochondrion [3]. As for calcium release, there are Ca\(^{2+}\)/H\(^+\) exchangers both in mitochondrion and acidocalcisomes [3], and an IP\(_3\) receptor, TcIP\(_3\)R, was first described in ER [7], although later its localization was demonstrated in acidocalcisomes [16].

In T. cruzi epimastigotes, various external signals trigger the mobilization of intracellular calcium; stimuli such as: insect’s intestine homogenate [17], nicotine [18], peptide derived from \(\alpha\)-D-globin [6], carbachol [19,20], and osmotic stress [21]. In previous works, we demonstrated the involvement of a Na\(^+\)/H\(^+\) exchanger (TcNHE1) in the early (PLC-independent) response of the biphasic calcium signal induced by extracellular stimuli, suggesting that IP\(_3\)R-dependent Ca\(^{2+}\) release could be responsible for the late response [19–21]. We also suggested that TcNHE1 activity could be regulated by PKC [21], enzymes identified as differentially expressed in the three stages of the parasite with important roles in parasite differentiation to the trypomastigote infective form [8]. On the other hand, adenyl cyclase is a key enzyme stimulated by calcium in T. cruzi [11], which catalyzes the formation of cyclic adenosine monophosphate (cAMP). The increase in cAMP levels activates protein kinase A (PKA) [22–24]. This kinase is a mediator important of many of the signal transduction pathways in eukaryote cells and is also implicated as a regulator of differentiation in T. cruzi [25].

In this work, we demonstrated that Ca\(^{2+}\) release triggered by high osmolarity treatment involves TcNHE1 activation. Our results suggest that Na\(^+\)/H\(^+\) exchanger is under regulation by protein kinases in T. cruzi epimastigotes. We also demonstrated that parasite IP\(_3\) receptor (IP\(_3\)R) present in intracellular membranes of epimastigotes is able to release calcium in response to exogenous IP\(_3\), Ryanodine (Ry) and Caffeine. This response is reverted in the presence of inhibitors of IP\(_3\)R and Ry receptors (RyR), suggesting the activity of a calcium channel with hybrid properties of IP\(_3\)/Ry receptors.

2. Results

2.1. Mobilization of Intracellular Calcium through Activity of a Na\(^+\)/H\(^+\) Exchanger Regulated by PKC and PKA

As previously described, TcNHE1 has consensus sites for phosphorylation and its activity in epimastigotes is affected by specific activators and inhibitors of PKC. In addition, the involvement of TcNHE1 in vacuolar alkalization, cytoplasmatic acidification, and intracellular calcium mobilization processes induced by hyperosmotic stress were demonstrated [21]. Here we determined the effect of TcNHE1 regulation by PKC on calcium signaling in epimastigotes.

Figure 1a shows that calcium release induced by mannitol (Control line, 100%) increased significantly when the parasites were pre-incubated with PKC-non physiologic activator PMA (+PMA, 117.3 ± 8.9%, \(n = 3\), \(p < 0.05\)). In addition, when epimastigotes were pre-treated with Chelerythrine (PKC inhibitor) or EIPA (Na\(^+\)/H\(^+\) exchanger inhibitor), the release of calcium induced by mannitol decreased significantly (+Che, 76.5 ± 6.4%; +EIPA, 38.9 ± 9.1%; \(n = 3\), \(p < 0.05\)) (Figure 1b).

Although there is no empirical evidence of TcNHE1 being substrate for PKA activity, the presence of consensus sites for phosphorylation by PKA opens this possibility. For this reason, we studied the effect of a semi-synthetic derivative of K525a (KT5720), inhibitor of PKA, on alkalization of acidocalcisomes in epimastigotes under hyperosmolarity conditions. Figure 2a shows that KT5720 significantly inhibited (+KT5720, 22 ± 7.2%, \(n = 3\), \(p < 0.01\)) the alkalization caused by 0.5 M mannitol (Control, 100%).
Figure 1. PKC effectors regulate Ca\(^{2+}\) mobilization induced by high osmolarity. Epimastigotes loaded with Fura 2 as described in Materials and Methods were resuspended in KRT buffer and stimulated (Control) or not (KRT) by addition of 0.5 M Mannitol at the time indicated by arrows. (a) Epimastigotes were pre-incubated during 10 min before the stimulation with 1 µM PMA (+PMA). (b) Epimastigotes were pre-incubated during 10 min before the stimulation with either 1 µM Chelerythrine (+Che) or 1 µM EIPA (+EIPA). A representative experiment is shown (n = 3, each performed in triplicate).

Forskolin is a non-physiological activator of mammalian Adenylate Cyclases (ACs). However, this agonist does not have effect over trypanosomatid ACs [26]. Therefore, we studied the effect of 8 Br-cAMP, an activator of PKA resistant to degradation by PDEs, on alkalinization of acidic vacuoles evoked by hyperosmotic stress. Under these conditions, the organellar alkalinization induced by mannitol (Control, 100%) was significantly increased (158 ± 7.8%, n = 3, p < 0.05) by activation of PKA (Figure 2b).

Finally, we analyzed the effect of 8 Br-cAMP and KT5720 on calcium signaling in epimastigotes. As showed in Figure 3, the addition of 8 Br-cAMP was also able to significantly increase calcium release induced by hyperosmotic stress, in a process dependent on EIPA-sensitive TcNHE1. These results suggest the possible regulation of TcNHE1 by PKA.

Taken together, our results suggest the participation of PKC and PKA in the regulation of TcNHE1 during the response of the parasite to high osmolarity.
Figure 2. PKA effectors affects acidocalcisome alkalinization induced by hyperosmotic stress. Epimastigotes were resuspended to a final density of 5 × 10^7 cells/mL in KRT buffer plus 10 μM of AO for 15 min prior to the addition of the different effectors. Cells were stimulated (Control) or not (KRT) by addition of 0.5 M Mannitol at the time indicated by arrows. (a) Epimastigotes were pre-incubated during 10 min before the stimulation with 25 nM KT5720 (+KT5720). (b) Epimastigotes were pre-incubated during 10 min before the stimulation with 1 mM 8 Bromo-cAMP (+8 Br cAMP). The fluorescence intensity (IF) was recorded at 493 nm in a spectrofluorometer. A representative experiment is shown (n = 3, each performed in triplicate).

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Figure 3. PKA effectors regulate Ca^{2+} mobilization induced by high osmolarity. Epimastigotes loaded with Fura 2 as described in Materials and Methods were resuspended in KRT buffer and stimulated by the addition of 0.5 M Mannitol at the time indicated by arrows. Epimastigotes were pre-incubated during 10 min before the stimulation with either KRT buffer (Control) or 1 mM 8 Bromo-cAMP (+8 Br cAMP) or 25 nM KT5720 (+KT5720) or 1 μM EIPA (+EIPA). A representative experiment is shown (n = 3, each performed in triplicate).
2.2. Calcium Release through Channels Belonging to the IP3/Ry Receptor Superfamily

In the last few years, it was demonstrated that TcIP3R is involved in parasite growth, metacyclogenesis, infectivity and virulence, probably through regulation of mitochondrial metabolism [27,28]. However, the effect of agonists on the activity of the endogenous protein was not yet analyzed.

Here we proposed to study the effect of IP3 and Ry on the mobilization in vivo of intracellular calcium in epimastigotes of T. cruzi. To demonstrate the release of Ca2+ from intracellular stores mediated by IP3, a suspension of the microsomal fraction of membranes (105,000 g) was loaded with Ca2+ in the presence of sodium azide (NaN3) to inhibit the action of mitochondrial ATPases. Figure 4 shows that the addition of exogenous IP3 was able to provoke the release of Ca2+ from this preparation. In addition, inhibition of the signal was observed when 2-Aminoethyl diphenylborinate (2-APB) and heparin, both inhibitors of IP3 receptors, were used. As control, resuspended parasites were incubated in buffer pH 7.2 (Figure 4). The results indicate the presence of a receptor in the microsomal membranes of epimastigotes, able to release Ca2+ in response to IP3. Since the microsomal fraction contains acidocalcisomes [19,21] and TcIP3R was described in these organelles [16], the results suggest that this receptor is responsible for the Ca2+ release observed.

![Figure 4](image_url)

**Figure 4.** Effect of IP3 on the calcium signal in membranes of T. cruzi epimastigotes. The membrane fractions (105,000 g) were obtained as described in Materials and Methods, pre-incubated in presence of 3mM NaN3, 5 μM CaCl2, and 2 mM ATP-Tris and loaded with Fura-2. The arrow indicates the addition of the effectors: KRT buffer (Control) or 20 μM IP3-Na+ (IP3) or 100 μM 2-APB + 20 μM IP3-Na+ (IP3 + 2-APB) or 0.5 mg/mL Heparin + 20 μM IP3-Na+ (IP3 + 2-APB). The data correspond to a representative experiment (n = 5, each performed in duplicate).

Receptors that release calcium in response to Ry treatment have been reported in vertebrates and invertebrates, and even in some unicellular organisms [29]. To know if the IP3R present in the membrane fraction was also able to recognize Ry and therefore increase calcium levels in the medium, tests were carried out by adding both activators (exogenous Ry and caffeine) and inhibitors (Ruthenium red and heparin) of RyRs. Figure 5a shows that the addition of exogenous Ry induced the release of Ca2+ as in the case of IP3, whereas when the fractions were pre-treated with both inhibitors, a decrease of the calcium signal was observed. In addition, the addition of caffeine increased the Ca2+ release, while caffeine + Ry combined were able to increase the calcium signal more than Ry and caffeine alone (Figure 5b). This indicates the presence of a calcium channel with hybrid properties of IP3/Ry receptors.
Figure 5. Effect of Ryanodine (Ry) on the calcium signal in membranes of *T. cruzi* epimastigotes. The membrane fractions (105,000 g) were obtained as described in Materials and Methods, pre-incubated in the presence of 3mM NaN₃, 5 µM CaCl₂, and 2 mM ATP-Tris and loaded with Fura-2. (a) The arrow indicates the addition of the effectors: KRT buffer (Control) or 1 µM Ryanodine (Ry) or 50 µM Ruthenium Red + 1 µM Ryanodine (Ry + RR) or 0.5 mg/mL Heparin + 1 µM Ryanodine (Ry + Heparin). (b) The arrow indicates the addition of the effectors: KRT buffer (Control) or 1 µM Ryanodine (Ry) or 10 µM Caffeine (Caffeine) or 1 µM Ryanodine + 10 µM Caffeine (Ry + Caffeine). The data correspond to a representative experiment (n = 5, each performed in duplicate).

3. Discussion

The mechanisms involved in the Ca²⁺ release from intracellular reservoirs in lower eukaryotic cells remained poorly understood for decades. An important biochemical event in the calcium signal is the activity of Na⁺/H⁺ exchangers, which have been implicated in the regulation of pH homeostasis, in the control of cell volume and in the adaptation to high salinity, helping to avoid the harmful effects of Na⁺ in key biochemical processes [30].

In *T. cruzi* epimastigotes, it was demonstrated the involvement of a Na⁺/H⁺ exchanger sensitive to EIPA, TcNHE1, in the early response of the biphasic calcium signaling induced by extracellular stimuli [18,21]. The functionality of Na⁺/H⁺ exchangers plays a central role in the ionic homeostasis, and therefore these exchangers are subject to the action of different factors for its regulation. It is well known that, in different cell types, Na⁺/H⁺ exchangers are regulated via phosphorylation by PKC, thus mediating the response to many of the signals involved in the control of proliferation cellular, differentiation, volume, and osmolarity changes [30,31]. In *T. cruzi*, the analysis of the sequence of TcNHE1 revealed conserved sites of phosphorylation by protein kinase C ([ST]–x–[RK]) located in the C-terminal region of the protein [21]. Here we showed that calcium release induced by hyperosmotic stress increased significantly when the parasites were pre-incubated with phorbol ester (PMA), a non-physiological activator of PKC. In addition, when epimastigotes were pre-treated with chelerythrin or EIPA, the release of calcium induced by mannitol decreased significantly. These results suggest that the role of PKC in the regulation of Na⁺/H⁺ exchanger in epimastigotes is similar to what occurs in other eukaryotic cells [32].

On the other hand, the analysis of the TcNHE1 sequence [21] revealed consensus sites for cAMP-dependent PKA. Among other signaling pathways, the adenyl cyclase that gen-
erates cAMP, PKA, and several PDEs were informed as active enzymes in *T. cruzi* [26,33]. cAMP regulates fundamental pathways in many types of organisms; it is involved in the control of growth, differentiation, and in the processes of osmoregulation in trypanosomatids [34], with the activation of PKA as a possible effector mechanism [25]. Our results suggest the possible involvement of PKA in the regulation of TcNHE1 under conditions of hyperosmotic stress. In epimastigotes, the inhibitor of PKA, KT5720, induced a decrease in organellar alkalization induced by high osmolarity. Moreover, the presence of 8 Br-cAMP, a non-hydrolysable agonist of PKA, significantly increased the organellar alkalization and the calcium release induced by hyperosmotic stress. Nevertheless, we do not discard the involvement of other effectors and signaling pathways not analyzed here. For instance, a cAMP Response Protein (CARP) identified in *T. cruzi* could act as PKA-independent cAMP effector [35], as proposed for *T. brucei* [26].

Regarding the late phase of the biphasic behavior of the Ca\(^{2+}\) signal induced by extracellular stimuli, in previous studies, it was hypothesized that the activity of an IP\(_3\)R (presumably located in ER or acidic vacuoles) could be responsible for the second increment in the Ca\(^{2+}\) signal [19–21]. At the same time, Hashimoto et al. reported the cloning of TcIP\(_3\)R, whose expression is crucial for epimastigote growth and metacyclogenesis [7]. The receptor functionally (IP\(_3\)-mediated Ca\(^{2+}\) release) in DT40-3KO cells expressing the receptor was also demonstrated [7,27]. Complementing these findings, in this work, we demonstrate for the first time the activity of the endogenous TcIP\(_3\)R, present in epimastigote microsomal membranes, able to release Ca\(^{2+}\) in response to exogenous IP\(_3\) and sensitive to IP\(_3\)R inhibitors. Interestingly, this calcium channel activity was also stimulated by Ry and caffeine, known as RyR agonists [36], and the response was reverted in the presence of RyR inhibitors. Since IP\(_3\)Rs and RyRs have different agonists and inhibitor profiles, these results suggest the activity of a Ca\(^{2+}\) channel with hybrid properties of IP\(_3\)/Ry receptors in *T. cruzi*. The hypothesis that primitive organisms have a single, ancestral calcium channel with hybrid properties of both receptors was also proposed based on similar results in *Toxoplasma gondii* [37]. Supporting this supposition, there are significant differences in the pore-forming transmembrane domain of TcIP\(_3\)R (among other IP\(_3\)Rs/RyRs sequences from pathogenic parasites) compared to human IP\(_3\)Rs and RyRs, which exist as two separate types of Ca\(^{2+}\) channels. In fact, those differences make the pathogen-specific channels an attractive target for anti-parasitic drugs [38], in the same way as that suggested for TcPI3K [5]. Moreover, the disruption of intracellular Ca\(^{2+}\) homeostasis was strongly suggested as therapeutic strategy against Chagas disease [39].

Taking together our results and previous studies, we infer that, in the triatomine rectum, *T. cruzi* epimastigotes experience hyperosmotic stress produced by presence of NaCl from triatomine urine. Under these conditions, parasite differentiation to infective trypomastigotes (metacyclogenesis) takes place. Thus, hyperosmotic conditions in the culture medium partially mimic the environment that epimastigotes must face in the rectum of triatomine bugs. Morphologically, we showed that high osmolarity caused either by NaCl or Mannitol, maintained in culture medium, led parasites to adopt an intermediate form between epimastigote and trypomastigote, which is characterized by possessing nucleus-adjacent kinetoplasts and increased flagellar length [40,41]. Biochemically, this stimulus has effects on the cell membrane at the level of activation of several signaling enzymes and the consequent formation of second messengers.

In brief, we could postulate the mechanism described in Figure 6 to explain the Ca\(^{2+}\)-related signaling pathways involved in the response of *T. cruzi* epimastigotes to high osmolarity.
The proposed steps, although not necessarily sequential, can be summarized as:

i. Hyperosmotic stress produced by urine presence activates TcNHE1 in acidocalcisomes, either by a cytosolic Na⁺ gradient or as part of the mechanism to regulate the cytoplasmatic pH [21]. The TcNHE1 activity could also be positively regulated by cAMP produced by adenyl cyclase [34].

ii. The neutralization of the pH gradient between the organellar lumen and the cytosol (alkalinization) produced by TcNHE1 activity is necessary for the release of Ca²⁺ from acidocalcisomes [19,42], through a Ca²⁺/H⁺ exchanger present in these acidic vacuoles [3].

iii. The released Ca²⁺ acts as cofactor of lipid kinases whose activity is also stimulated by hyperosmotic stress [6,41]. Thus, the sequential activity of phosphoinositide kinases [5] leads to increment in the levels of phosphatidylinositol-4,5-bisphosphate (PIP₂). Ca²⁺ also activates PLC, which uses PIP₂ as a substrate to form DAG and IP₃ [4].

iv. The released IP₃ binds to TcIP₃R [7] present in acidocalcisomes [16], producing a second release of Ca²⁺ (this study, [20]). Subsequently, DAG and Ca²⁺ activate PKC [9].
which triggers other signaling pathways [8]. Ca\textsuperscript{2+} also produces activation of other mechanisms that lead to cellular responses [28,33].

v. In addition to being a substrate of PLC, the incremented levels of PIP\textsubscript{2} could also act as a cofactor for phospholipase D (PLD) which would in turn increment the levels of phosphatidic acid (PA) (Santander et al., unpublished). Consistently with unpublished results and observations from our group, non-classic PLDs homologous were recently described in T. cruzi [43]. PIP\textsubscript{2} can also be a substrate of TcP13K, an enzyme that produces phosphatidylinositol-3,4,5-trisphosphate (PIP\textsubscript{3}) which could trigger the PKB/Akt signaling pathway [5].

vi. DAG is also a substrate for DAGK, producing PA. DAGK activity is highly incremented in intermediate forms during epimastigote differentiation under hyperosmotic stress conditions. In agreement, dephosphorylation of PA by the action of phosphatidate phosphatases (PAPs) is inhibited [41].

vii. Incremented PA could act as part of the dynamic complex that binds microtubules to plasma membrane [41]. That binding would be required to maintain the parasite flagellum in an extended stage in intermediate forms [40], as part of the rearrangement that the subpellicular array of trypanosomes undergoes in morphological transitions [44]. In addition, PA could act as precursor of CDP-DAG, required to complete the PI cycle [45].

In summary, the hyperosmotic stress triggers signals that lead epimastigotes to biochemical and morphological changes indicative of early stages of differentiation. Thus, calcium-related enzymatic activities stimulated by the high osmolarity are responsible, at least in part, for structural and biochemical changes that occur during the differentiation to intermediate stage, while other stimuli would also be required to complete the parasite metacyclogenesis. Nevertheless, the study of the early events that induces this process could shed light about developing better strategies in order to avoid the parasite differentiation to the human-infective form.

4. Materials and Methods

Organisms and growth conditions. Trypanosoma cruzi Tulahuen strain was used. Epimastigotes were grown at 28 °C in modified Warren medium supplemented with 10% fetal bovine serum (FBS), as described previously [46]. Cells in logarithmic growth phase were harvested by centrifugation at 2000 g for 10 min, and washed twice with 25 mM Tris–HCl, pH 7.35, 1.2 mM MgSO\textsubscript{4}, 2.6 mM CaCl\textsubscript{2}, 4.8 mM KCl, 120 mM NaCl, and 100 mM glucose [Krebs-Ringer-Tris (KRT) buffer].

Treatment in cellulo with enzyme effectors. For measuring TcNHE1 inhibition, cells were harvested as described above and incubated with 1 µM 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) for 10 min prior to the addition of effectors [21]. To measure the effects of PKC and PKA activators/inhibitors on TcNHE1 activity, epimastigotes were incubated with 1 µM phorbol-12-myristate-13-acetate ester (PMA), 1 µM Chelerythrine, 25 nM KT5720, 1 mM 8 Bromo-cAMP, or the carrier 0.1% DMSO during 60 min before measurement of alkalinization or calcium signaling described below.

Measurement of alkalinization in acidic vesicles. The alkalinization of vesicles in intact epimastigotes was assayed by measuring changes in fluorescence of Acridine Orange (AO) as previously described [21]. Briefly, parasites harvested in the logarithmic phase of growth were suspended in 1.5 mL of KRT buffer (5 × 10\textsuperscript{7} cells/mL) and loaded in thermostated cuvettes at 28 °C. Subsequently, cells were incubated with 10 µM of AO for 15 min prior to the addition of the different effectors. Alkalinization was measured by integrating the total fluorescence signal obtained for each treatment and then comparing the areas under the curves. The area under the curve for control was defined 100% and percentages corresponding to the areas under the curves for effectors were calculated. The experiments were carried out in a Fluoromax-Spex 3 spectrofluorometer (Horiba Jovin Yvon\textsuperscript{TM}, Edison Township, NJ, USA) at excitation and emission wavelengths of 493 and 530 nm, respectively.
Measurement of intracellular $[\text{Ca}^{2+}]$ with Fura 2-AM. Intracellular $[\text{Ca}^{2+}]$ was determined as described previously [21]. Briefly, epimastigotes were harvested, washed once in KRT buffer and resuspended at $2 \times 10^8$ cells/mL. Cells were subsequently incubated with 4 $\mu$M Fura 2-AM in the dark for 60 min at 28 °C in a water bath with mild agitation, washed twice with ice-cold KRT buffer, incubated for 20 min at 28 °C with agitation, and kept in ice in the dark until use. For fluorescence measurement, an aliquot of Fura 2-loaded epimastigote suspension was diluted into 1.5 mL KRT buffer (final concentration $5 \times 10^7$ cells/mL), and placed in polystyrene cuvettes. For determinations of intracellular $[\text{Ca}^{2+}]$, $5 \times 10^7$ cells/mL were loaded in thermostatically controlled cuvettes at 28 °C, to which the corresponding effectors were added. The experiments were carried out in a Fluoromax-Spez 3 spectrofluorometer (Horiba Jovin Yvon™, Edison Township, NJ, USA). The fluorescence was recorded at an excitation and emission wavelength of 340/380 nm and 500 nm, respectively. Cell autofluorescence was recorded in parallel with cells without the fluorophore. Calcium release in response to effectors was determined by integrating the total fluorescence signal obtained. This value shows the percentage of calcium released, relative to the area under the transient curve for control (defined 100%). The results are expressed as fluorescence changes of the 340/380 ratio of Fura-2/Ac.

Preparation of membranes. Washed epimastigotes were resuspended in five volumes of 50 mM HEPES (pH 7.4) containing 0.25 M sucrose, 5 mM KCl, 1 mM EDTA, 1mM PMSF, 1 $\mu$g/mL aprotinin 4 $\mu$g/mL leupeptin. The suspension was frozen at −180 °C in liquid nitrogen and thawed; this freeze/thaw cycle was performed three times. Membrane fractions were prepared as described previously [5] with some modifications. Briefly, the homogenate was centrifuged at 100 g and 100 g for 15 min to remove cell debris and flagellar membranes, respectively. The supernatant was centrifuged at 105,000 $\times$ g for 45 min and the obtained pellet was washed, resuspended with 50 mM HEPES (pH 7.4) and used as a source of membranes. The protein content of membrane samples was determined by the Bradford method [47], with bovine serum albumin as the standard.

Determination of the incorporation and release of $\text{Ca}^{2+}$ from microsomal fractions. The determinations of free $\text{Ca}^{2+}$ in the incubation medium were determined by the use of the fluorescent indicator Fura-2 acid in a Fluoromax-Spez 3 spectrofluorometer (Horiba Jovin Yvon™, NJ, USA). For the determinations of $\text{Ca}^{2+}$ incorporation and release, a microsomal membrane fraction (105,000 g) obtained as described above was used. For this, the fractions (124 $\mu$g/mL of proteins) were resuspended in Tris-HCl 4 mM, 100 mM KCl, 2 mM MgCl$_2$, 250 mM sucrose, pH 7.2 (Buffer C) and loaded in thermostatically controlled cuvettes at 28 °C. Subsequently, 3 mM sodium azide plus 5 $\mu$M CaCl$_2$ and 5 $\mu$M Fura-2/Ac were added. After incubation for 10 min, ATP-dependent $\text{Ca}^{2+}$ uptake was induced by the addition of 2 mM ATP-Tris and monitored by the decrease in fluorescence of Fura-2/Ac (ex = 340 and 380 nm, em = 500 nm). The results are expressed as fluorescence changes of the 340/380 ratio of Fura-2/Ac. Calcium release in response to effectors was determined by integrating the total fluorescence signal obtained; this value reflects the total amount of calcium released. The transient curve of control was considered the base for comparison as described [19]. An effector was considered to enhance calcium release when the curve of the treatment was significantly different to the mean of control curve plus five standard deviations.

Treatment in vitro with enzyme effectors. To analyze inhibition or activation on IP$_3$R/RyR, 100 $\mu$M 2-Aminoethoxydiphenyl-borate (2-APB), 0.5 mg/mL Heparin, 50 $\mu$M Ruthenium Red, 10 $\mu$M Caffeine, 1 $\mu$M Ryanodine, or 20 $\mu$M IP$_3$-Na$^+$ were used [36,48]. Effectors or carriers were added to microsomal membrane preparations just before measuring $\text{Ca}^{2+}$ incorporation and release as described above.

Statistical and Graphical Analysis. The results are representative of three or more independent experiments, unless otherwise stated. Graphical and statistical analysis of the data was performed using OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA) and CorelDRAW X4 (Corel Corporation, Ottawa, Ontario, Canada) softwares. Graphical
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