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Evaluation of the Presence of a Thapsigargin-Sensitive Calcium Store in Trypanosomatids Using *Trypanosoma evansi* as a Model

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ABSTRACT: Ca^{2+} plays an important role in the regulation of several important activities in different trypanosomatids. These parasites possess a Ca^{2+} transport system in the endoplasmic reticulum (ER) involved in Ca^{2+} homeostasis, which has been reported to be insensitive to thapsigargin, a classical inhibitor of the sarcoplasmic–ER Ca^{2+} adenosine triphosphatase (ATPase) (SERCA) in most eukaryotic cells. However, currently there is a controversy regarding the existence of a thapsigargin-sensitive ER Ca^{2+} store in these parasites. Therefore, we decided to explore the effect of this inhibitor using different methodological approaches. First, we selected *Trypanosoma evansi* as a parasite model to warrant the homogeneity of the population because this parasite has only a single life cycle, i.e., bloodstream-form trypomastigotes. Second, we compared the thapsigargin effect on Ca^{2+} homeostasis by spectrophotometrical Ca^{2+} measurements using 3 different approaches: whole-cell populations, cells that have been permeabilized by treatment with digitonin, and intact single cells. Our results demonstrate that a low concentration of thapsigargin induces Ca^{2+} release from intracellular Ca^{2+} stores in this parasite, which can be observed independently of the method used. Furthermore, the addition of thapsigargin before or after nigericin did not abolish its effect, showing that thapsigargin acts specifically on the ER. In conclusion, our results indicate the presence of a nonmitochondrial thapsigargin-sensitive Ca^{2+} store in *T. evansi*.

Calcium plays an important role in the regulation of multiple cellular activities in different trypanosomatids (Docampo and Moreno, 1996; Stodjl and Clarke, 1996; Ruiz et al., 1998). The presence of different Ca^{2+} transport systems in these parasites warrants an efficient regulation of the intracellular calcium concentration ($[Ca^{2+}]_i$), which can reach approximately 4 orders of magnitude with respect to the extracellular milieu (Benaim, 1996). These mechanisms are associated with intracellular specialized organelles, as well as in the plasma membrane (Ruben and Hutchinson, 1991; Docampo, 1993; Benaim, 1996; Docampo and Moreno, 2001). As in other eukaryotic cells, a Ca^{2+} transport system has been localized at the endoplasmic reticulum (ER), which is involved in the intracellular Ca^{2+} homeostasis. This system is characterized by adenosine triphosphate (ATP) stimulation and sodium orthovanadate inhibition (Vercesi et al., 1991; Moreno, Docampo et al., 1992). Thapsigargin is a potent and classical inhibitor of the sarcoplasmic–ER Ca^{2+} adenosine triphosphatase (ATPase) (SERCA) (Thastrup et al., 1990), causing the release of Ca^{2+} from the inositol-1,4,5-trisphosphate–sensitive store in different cells. However, there is a controversy regarding the effect of thapsigargin on the ER Ca^{2+} store in trypanosomatids. Thus, although some groups have detected the presence of a nonmitochondrial Ca^{2+} store sensitive to thapsigargin in *Trypanosoma brucei* at low concentration (1 μM) (Ruben and Akins, 1992), other groups failed to detect any effect of thapsigargin, even at higher concentration (8 μM), in both *T. brucei* (Vercesi et al., 1993) and *Trypanosoma cruzi* (Moreno, Vercesi et al., 1992), using whole cells or cells that have been exposed to treatment with digitonin. In contrast, other studies in trypanosomatids aimed at correlating diverse cellular events with $[Ca^{2+}]_i$ such as cell cycle control or invasion of mammalian cells and have shown that these processes are sensitive to low concentration of thapsigargin (1 μM) (Stojdl and Clarke, 1996; Yoshida et al., 2000; Neira et al., 2002).

Although species or cellular stage differences may account for this discrepancy, another plausible explanation might be related to the different methodological approaches used in these studies. Therefore, we decided to explore the effect of thapsigargin on the Ca^{2+} homeostasis in *Trypanosoma evansi* by comparing spectrophotometric Ca^{2+} measurements in cell populations (whole or digitonin-treated cells) and intact single parasites. The latter approach, recently reported by our group, allows for the measurement of free cytosolic calcium concentration with higher time resolution (Mendoza et al., 2001, 2002). Furthermore, the use of *T. evansi* as a model is advantageous because it is a parasite in which the only cellular stage is the bloodstream form (trypomastigote), thus avoiding the possible problems related to heterogeneity of the populations used for studies of Ca^{2+} homeostasis in other trypanosomatids. Finally, there are only few reports with respect to the way in which *T. evansi* regulates its intracellular calcium concentration (Mendoza et al., 2001, 2002) and there is no report on the effect of thapsigargin in this parasite.

In this study, first we measured Ca^{2+} transport by intracellular organelles in situ, using arsenazo III in digitonin-treated cells of *T. evansi* as described previously (Benaim et al., 1990). Figure 1 shows the effect of a low concentration of thapsigargin (1 μM) on Ca^{2+} transport in a permeabilized cell of *T. evansi* cells in the presence of oligomycin (a F_1–F_0 mitochondrial ATPase inhibitor) and antimycin A (a respiratory chain inhibitor). As observed (Fig. 1), thapsigargin produced a Ca^{2+} release under this condition. We included antimycin A in the assay because little is known about mitochondrial function and the possible effect of this drug in *T. evansi* mitochondrion. In addition, this drug has been used by other authors in the experiments used to evaluate the thapsigargin effect in *T. brucei* procyclic trypomastigotes (Vercesi et al., 1993) and in *T. cruzi* (Docampo et al., 1993). However, identical results were obtained when antimycin A was omitted from the medium (results not shown). These results are in agreement with the fact that *T. evansi* belongs to *T. brucei* clade trypanosome (Stevens and Gibson, 1999) and only has bloodstream forms that possessed an undeveloped mitochondrion depending only on glycolysis to generate ATP. In these trypanosomes, Ca^{2+} uptake by mitochondria depends on the electrochemical proton gradient membrane generated by an oligomycyn-sensitive ATPase. Therefore, the addition of oligomycin prevents Ca^{2+} uptake by the mitochondria in these assays. Indeed, we have previously shown that oligomicin produced a release of Ca^{2+} from the mitochondrial pool in *T. evansi* (Mendoza et al., 2002). It can be seen that in the absence of ATP, Ca^{2+} uptake is low (Fig. 1Aa, Ba) and thapsigargin induces a low Ca^{2+} release (Fig. 1Aa) when compared with the effect produced in the presence of ATP (Fig. 1Ab). Basically, most of the Ca^{2+} uptake is due to an ATP-dependent Ca^{2+} sequestration. These results suggest the presence of a nonmitochondrial Ca^{2+} store that depends on a thapsigargin-sensitive Ca^{2+} ATPase. These results contrast with those reported in other trypanosomatids, where thapsigargin (1–8 μM) did not have any effect on intracellular Ca^{2+} release in digitonin-treated cells of *T. cruzi*, *T. brucei* (Moreno, Vercesi et al., 1992; Vercesi et al., 1993), or *Herpetomonas* sp. (Sodré et al., 2000). Figure 1Ab shows that the addition of the Ca^{2+} ionophore A23187 produces a large Ca^{2+} release, probably from other compartments, such as acidocalcisomes, in which Ca^{2+} uptake is also an ATP-dependent process (Docampo and Moreno, 2001). In addition, as seen in Figure 1Ac, when digitonized cells were pretreated with vanadate, a significant decrease in thapsigargin-induced Ca^{2+} release is observed, in comparison with the effect of thapsigargin alone (Fig. 1Ab). This inhibition suggests that a ''P''-type Ca^{2+} ATPase, probably a SERCA Ca^{2+} pump, is responsible for this effect as reported previously in other trypanosomatids (Vercesi et al.,

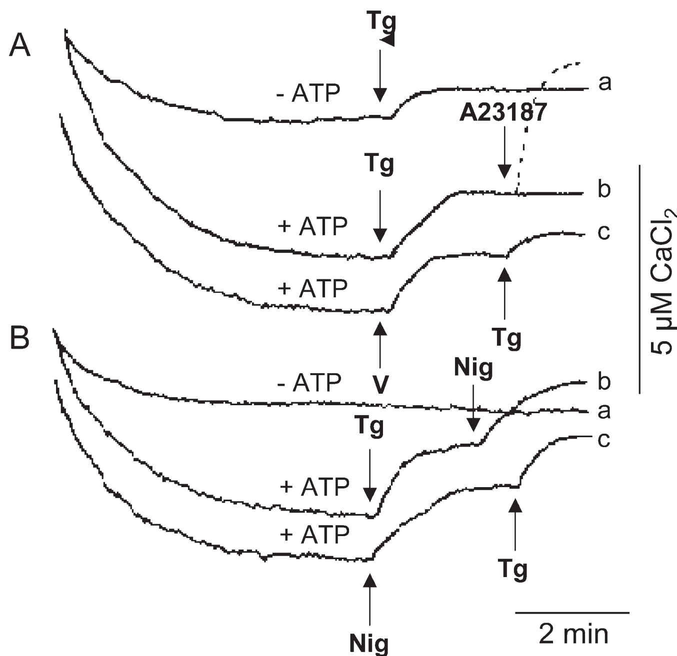

FIGURE 1. Effect of thapsigargin on Ca^{2+} uptake by digitonin-permeabilized cells of *Trypanosoma evansi*. The reaction medium contains 125 mM sucrose, 65 mM KCl, 10 mM Tris–HCl, 2.5 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.4, 40 μM Arsenazo III, 30 μM digitonin, 5 μM $CaCl_2$, 2 μg/ml oligomycin, and 2 μg/ml antimycin A in the presence or absence of 1 mM ATP, at a final density of 10^6 cells/ml. Calcium movements were followed by measuring the changes in the absorbance spectrum of the metallochromic indicator using an Aminco DW-2a dual wavelength (675–685 nm) spectrophotometer at 30 C. The arrows indicate the additions of thapsigargin (1 μM) (Tg), calcium ionophore A23187 (10 μM), sodium vanadate (400 μM) (V), and nigericin (4 μM) (Nig). Traces shown are representative of at least 3 independent experiments conducted on separate preparations.

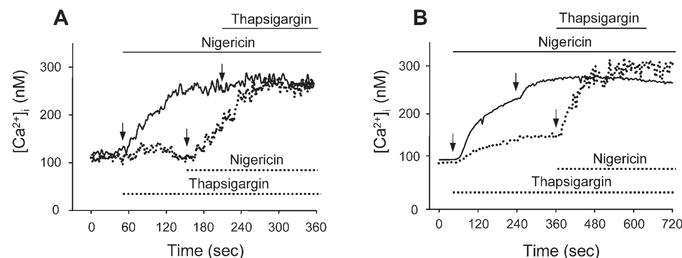

FIGURE 2. Effect of the thapsigargin in $[Ca^{2+}]_i$ in single (**A**) and population (**B**) cells loaded with Fura-2 (8 μM) of *Trypanosoma evansi* incubated in the absence of extracellular Ca^{2+} (100 μM ethyleneglycoltetraacetic acid). The medium contains 145 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 11 mM glucose, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid–NaOH (pH 7.3). In cell populations, the experiments were carried out in a Hitachi F-2000 spectrofluorometer. In single cells, a fluorescence-imaging apparatus (Ion Optix Co., Milton, Massachusetts) was used to obtain fluorescent recordings of Fura-2–loaded parasites, and the solutions were delivered to the parasite using a DAD-12 perfusion system (Adam & List Associates, Westbury, New York). The arrows indicate the additions of thapsigargin (2 μM) and nigericin (8 μM) in the medium. In the dot trace thapsigargin was applied before nigericin, and in the solid trace thapsigargin was applied after nigericin in both panels (A and B). All the experiments were carried out at 30 C. Traces shown are representative of at least 3 independent experiments conducted on separate preparations.

1991; Moreno, Docampo et al., 1992). Second, to evaluate the effect of thapsigargin on $[Ca^{2+}]_i$ in whole *T. evansi* cells loaded with Fura-2 as Ca^{2+} indicator, we followed $[Ca^{2+}]_i$ in single cells or cell populations based on the methods described by Mendoza et al. (2001) and Moreno and Zhong (1996), respectively. Our experiments were performed in ethyleneglycoltetraacetic acid–containing buffers; therefore, the increase in $[Ca^{2+}]_i$ reflected its release from intracellular stores. As shown in Figure 2 (single cell [A] and cell populations [B]), addition of thapsigargin (2 μM) produces a small increase in $[Ca^{2+}]_i$ of about 35 and 50 nM, respectively. Our results are consistent with those reported in *T. brucei* by Ruben and Akins (1992), showing the existence of a thapsigargin-sensitive Ca^{2+} pool in whole-cell populations. These authors reported that the addition of 1 μM thapsigargin elevated $[Ca^{2+}]_i$ by approximately 50–75 nM, thus indicating that a thapsigargin-sensitive store has a low calcium capacity, which is characteristic of the ER. Our experiments (Fig. 2A) using $[Ca^{2+}]_i$ measurements in single cells strongly support their conclusion. In contrast, other researchers have not detected a thapsigargin-induced Ca^{2+} release from intracellular stores at low concentrations (1–2 μM) using the whole-cell population approach either in *T. brucei* (Verccesi et al., 1993) or in *T. cruzi* (Moreno, Vercesi et al., 1992; Docampo et al., 1993). The latter authors (Docampo et al., 1993) reported an increase in $[Ca^{2+}]_i$ in the absence of extracellular calcium only when they used high concentrations of thapsigargin (20 μM). Our results demonstrate that independent of the different methodological approaches used, thapsigargin at low concentration produces a clear effect on Ca^{2+} homeostasis in *T. evansi*.

It has been postulated that thapsigargin could have unspecific effects in trypanosomatids and other cells. Thus, thapsigargin at high concentrations (4–20 μM) was able to release Ca^{2+} from mitochondria in *T. cruzi* and *T. brucei*, probably by collapsing the mitochondrial membrane potential and not by the classical SERCA pump inhibition (Docampo et al., 1993; Vercesi et al., 1993). Therefore, we decided to determine if the Ca^{2+} mobilization induced by low concentrations of thapsigargin (1–2 μM) was indeed from the ER or from other sources, including the mitochondria and the acidocalcisomes. For this purpose, we evaluated the effect of thapsigargin in combination with nigericin (a K^+–H^+ exchanger), which is known to produce Ca^{2+} release from intracellular stores (acidocalcisomes and mitochondria) as a consequence of the collapse of the proton gradient present in the membrane of these organelles (Ruben et al., 1991; Vercesi et al., 1993; Docampo et al., 1995; Mendoza et al., 2002). Interestingly, Figures 1B, 2A, B (whole and digitonin-treated cells, respectively) show that thapsigargin, at low concentrations, produces Ca^{2+} release before and after (Figs. 1Bb, Bc, 2A, B) nigericin treatment. The effects of thapsigargin and nigericin on intracellular calcium concentration and Ca^{2+} transport were additive. Even more, regardless of the order in which the treatment was given, any of these drugs elevated $[Ca^{2+}]_i$ independently. These data support the notion that these drugs act on different intracellular Ca^{2+} stores in *T. evansi*, i.e., one small thapsigargin-sensitive store that resides within the ER and another larger nigericin-sensitive store that resides in organelles whose calcium accumulation is dependent on the proton gradient present in their membrane. These results, together with those described above, where thapsigargin produces a Ca^{2+} release even in the presence of oligomycin, rule out the participation of a nonmitochondrial component in the effect of thapsigargin at low concentrations in *T. evansi* (Fig. 1). These results are in agreement with those reported for *T. brucei*, where a thapsigargin-sensitive Ca^{2+} pool apparently resides in the ER (Ruben and Akins, 1992).

In agreement with our findings, the presence of a SERCA-type Ca^{2+} ATPase in these parasites has been supported by cloning and sequencing in *T. brucei* (Nolan et al., 1994), *T. cruzi* (Furuya et al., 2001), and *Leishmania mexicana amazonensis* (Lu et al., 1997). In *T. brucei*, this Ca^{2+} ATPase activity in vitro possesses a high affinity for Ca^{2+}, which is sensitive to vanadate and to low concentrations of thapsigargin (Nolan et al., 1994). However, in *T. cruzi*, this pump was insensitive to low concentrations (0.3–1 μM) of thapsigargin (Furuya et al., 2001). In the same study, the authors pointed out differences between the sequences of the M3 and S3 fragments in these trypanosomatids, which are responsible for the sensitivity of SERCA pumps to thapsigargin (Furuya et al., 2001). They concluded that differences in the transmembrane sequences of the thapsigargin-binding site could account for the observed differences in thapsigargin sensitivity of these pumps in these trypanosomatids (Norregaard et al., 1994). In this regard, it is interesting

to note that *T. evansi* belongs to the *T. brucei* clade of trypanosomes (Stevens and Gibson, 1999). Thus, it is also possible that the effect of thapsigargin observed in *T. evansi* is a consequence of a closer phylogenetic relationship between this parasite and *T. brucei*, when compared with other trypanosomatids.

The thapsigargin-sensitive Ca^{2+} store in *T. evansi* is presumed to reside in the ER based on (1) the low capacity of this store, (2) intracellular localization because it occurs in the absence of extracellular calcium, (3) ATP dependency and sensitivity to vanadate, (4) specific Ca^{2+} release by low concentration of thapsigargin, and (5) the fact that nigericin and thapsigargin effects were additive and have different sizes. In conclusion, the results obtained in this work indicate that the ER is the intracellular Ca^{2+} store targeted by low concentrations of thapsigargin for Ca^{2+} release in *T. evansi*, as reported for *T. brucei* (Ruben and Akins, 1992). These results also suggest that the Ca^{2+} pump present in this organelle has strong similarities with the SERCA pump that has been described in higher eukaryotic cells.

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