Thapsigargin Causes Ca\(^{2+}\) Release and Collapse of the Membrane Potential of *Trypanosoma brucei* Mitochondria in Situ and of Isolated Rat Liver Mitochondria*

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Thapsigargin, previously reported to release Ca\(^{2+}\) from non-mitochondrial stores of different cell types, as well as nigericin, were found, when used at high concentrations, to release Ca\(^{2+}\) and collapse the membrane potential of *Trypanosoma brucei* bloodstream and procyclic trypomastigotes mitochondria in situ. At similarly high concentrations (>10 \(\mu\)M), thapsigargin was also found to release Ca\(^{2+}\) and collapse the membrane potential of isolated rat liver mitochondria. These results indicate that care should be taken when attributing the effects of thapsigargin in intact cells to the specific inhibition of the sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPase family of calcium pumps. In addition, we have found no evidence for an increase in intracellular Ca\(^{2+}\) by release of the ion from intracellular stores by nigericin, measuring changes in cytosolic Ca\(^{2+}\) by dual wavelength spectrofluorometry in fura-2-loaded *T. brucei* bloodstream trypomastigotes or measuring Ca\(^{2+}\) transport in digitonin-permeabilized cells.

The use of digitonin to permeabilize the plasma membrane of different trypanosomatids (1–8) has allowed the identification of two intracellular Ca\(^{2+}\) pools. Ca\(^{2+}\) uptake by the first pool is inhibited by antimycin A, FCCP, and ruthenium red and stimulated by respiratory substrates, phosphate, and acetate. This pool has a high capacity and low affinity for Ca\(^{2+}\) and is able to buffer external Ca\(^{2+}\) at concentrations in the range of 0.6–0.7 \(\mu\)M (1–8). These are characteristics typical of the endoplasmic reticulum (10). Recently, the presence of a third Ca\(^{2+}\) pool sensitive to changes in intracellular pH has been suggested in *Trypanosoma brucei* bloodstream trypomastigotes (11) on the basis of the changes observed in the fluorescence of fura-2-loaded cells when nigericin was included in the incubation medium. In contrast, the sesquiterpene lactone and tumor promoter thapsigargin was shown to apparently release Ca\(^{2+}\) from a non-mitochondrial pool insensitive to nigericin (12). Nigericin has also been shown to increase cytosolic Ca\(^{2+}\) in *Leishmania donovani* promastigotes (13). Since we have been unable to detect any increase in intracellular Ca\(^{2+}\) by thapsigargin in *Trypanosoma cruzi* amastigotes and epimastigotes using fura-2-loaded cells (7), we re-examined the effect of nigericin and thapsigargin on Ca\(^{2+}\) homeostasis in *T. brucei* using fura-2-loaded and digitonin-permeabilized cells.

We report here that neither thapsigargin nor nigericin, when used at low concentrations, are able to increase the intracellular Ca\(^{2+}\) concentration of either *T. brucei* bloodstream or procyclic trypomastigotes by the release of Ca\(^{2+}\) from intracellular stores. In addition, we report that high concentrations of thapsigargin and nigericin are able to release Ca\(^{2+}\) from *T. brucei* and rat liver mitochondria, probably as a result of the collapse of their membrane potential.

**MATERIALS AND METHODS**

**Culture Methods—** *T. brucei* procyclic forms (ILTar 1 procyclics) were grown at 28 °C in medium SDM-79 (14) supplemented with hemin (7.5 mg/liter) and 10% heat-inactivated fetal calf serum. Two to three days after inoculation, cells were collected by centrifugation and washed twice in Dulbecco's phosphate-buffered saline. *T. brucei* bloodstream forms (monomorphic strain 427 from clone MTat 1.4, otherwise known as variant 117) were isolated from infected mice or rats as described previously (15) and kept in separation buffer containing 44 mM NaCl, 55 mM glucose, 57 mM Na\(_2\)HPO\(_4\), and 3 mM NaH\(_2\)PO\(_4\), pH 8.0, until use, and after loading with fura-2. The final concentration of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay (16) in the presence of 0.2% deoxycholate. Rat liver mitochondria were isolated as described before (17).

**Chemicals—** ATP, calcium ionophore A23187, sodium orthovanadate, FCCP, succinate, arsenazo III, EGTA, Triton X-114, nigericin, and digitonin were purchased from Sigma. Thapsigargin was purchased from Sigma (T9035, more than 99% pure, as indicated by the manufacturer) or from LC Services. Identical results were obtained when thapsigargin from different sources was used. fura-2/AM was from Molecular Probes, Inc., Eugene, OR. All other reagents were analytical grade.

**Spectrofluorometric Determinations—** fura-2 determinations were performed as described before (18). Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program as described before (6).

**Determination of Ca\(^{2+}\) Movements—** Variations in free Ca\(^{2+}\) concen-
trations were followed by measuring the changes in the absorbance spectrum of arsenazo III (18), using the SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm or with a calcium-selective electrode (2). No free radical formation from arsenazo III occurred under the conditions used (19, 20). The calibrations were performed by the sequential addition of known concentrations of EGTA. The initial Ca²⁺ concentration in the solution was obtained by atomic absorption spectrophotometry and the Ca²⁺ concentration after each EGTA addition was calculated by employing an iterative computer program as described before (6, 8).

**Estimation of Mitochondrial Membrane Potential**—These measurements were made using safranine O (5) or a TPP⁺-selective electrode in combination with a calomel reference electrode (2) as described in the references.

**RESULTS**

It has been reported (11, 12) that nigericin and thapsigargin are able to increase intracellular Ca²⁺ in *T. brucei* bloodstream trypanomastigotes by releasing it from intracellular stores. We therefore investigated the effect of these compounds on fura-2-loaded *T. brucei* bloodstream trypanomastigotes in the presence of 1 mM EGTA to bring extracellular Ca²⁺ to a very low concentration ([Ca²⁺] = 0.075 nM). Under these conditions (Fig. 1, dashed line), there was no change in [Ca²⁺], during the incubation time. Addition of nigericin (1 μM) or thapsigargin (1 μM), in any order, did not result in any significant change in fluorescence (not shown). When a very high concentration (2.75 μM) of nigericin was tested, without (not shown) or after addition of 8 μM thapsigargin (Fig. 1, trace a), no changes were detected. When 8 μM thapsigargin was used either before (Fig. 1, TG) or after nigericin (not shown), no changes were observed during the incubation period.

Fura-2-loaded *T. brucei* bloodstream trypanomastigotes suspended in buffer containing [Ca²⁺] = 1 mM had a fluorescence intensity corresponding to a cytosolic Ca²⁺ concentration in the range of 20–30 nM, as we have described previously (6). When high concentrations of nigericin (2.75 μM) (Fig. 1, trace b) were used in the presence of extracellular [Ca²⁺] > 1 mM, a significant transient increase in fluorescence was observed in agreement with the results reported previously (11, 12). No effects were detected when thapsigargin (8 μM) was added in the presence of high extracellular calcium (not shown). Taken together, these results indicate that the increase in cytosolic Ca²⁺ induced by addition of nigericin in the presence of high external Ca²⁺ is probably not due to Ca²⁺ release from intracellular stores.

To further demonstrate that neither nigericin nor thapsigargin was able to increase [Ca²⁺], through Ca²⁺ release from mitochondrial stores of *T. brucei* bloodstream trypanomastigotes, we used the digitonin-permeabilization technique described previously (6). Fig. 2 shows the oligomycin-insensitive (non-mitochondrial, Ref. 6) Ca²⁺ uptake by digitonin-permeabilized "*T. brucei* bloodstream trypanomastigotes. A fast decrease in Ca²⁺ concentration started immediately after addition of digitonin and lowered the ambient free Ca²⁺ concentration to at least 0.05–0.1 μM (Fig. 2, trace a). The subsequent addition of nigericin (2.75 μM) or thapsigargin (8 μM) in any order (Fig. 2 and not shown) did not cause any change in the ambient Ca²⁺ concentration. In contrast, addition of the calcium ionophore A23187 released the Ca²⁺ taken up, as well as the endogenous Ca²⁺. Vanadate (Fig. 2, trace b) totally inhibited Ca²⁺ uptake and slowly released the accumulated Ca²⁺. Further addition of thapsigargin did not change this slow rate of Ca²⁺ release caused by vanadate, whereas addition of calcium ionophore rapidly released the Ca²⁺ taken up and the endogenous Ca²⁺.

Nigericin is known to uncouple oxidative phosphorylation in rat liver mitochondria when used at high concentrations (10 μM) (21–23). Previous works on the effects of nigericin on intracellular Ca²⁺ homeostasis in *L. donovani* (13) and *T. brucei* (11, 12) reported the use of 4 μM (13) and 1–2.75 μM (11, 12) nigericin. When we examined the effect of similar concentrations of nigericin on the mitochondrial Ca²⁺ uptake (in the presence of vanadate, Ref. 6) by digitonin-permeabilized *T. brucei* bloodstream trypanomastigotes, we observed that addition of the drug after a steady state was attained led to the Ca²⁺ release (not shown). Even thapsigargin (8 μM) was able to release Ca²⁺ from these mitochondria in situ (not shown).

We have demonstrated previously (6) that *T. brucei* bloodstream trypanomastigotes mitochondria in situ only take up Ca²⁺ in the presence of ATP but not in the presence of respiratory substrates (6). In order to better analyze the effects of nigericin and thapsigargin on mitochondria, we studied the effect of these drugs on *T. brucei* procylic trypanomastigotes mitochondria in situ. These mitochondria are able to take up Ca²⁺ in the presence of respiratory substrates and in the absence of ATP (6), and therefore there is no interference with Ca²⁺ uptake by other cellular pools. Fig. 3 shows Ca²⁺ uptake by digitonin-permeabilized *T. brucei* procylic trypanomastigotes in the presence of succinate. When the cells were added to the reaction medium, a decrease in Ca²⁺ concentration started after a period of about 30 s and continued until the ambient free Ca²⁺ concentration was lowered to about 0.7–0.8 μM, in agreement with a previous report (6). The subsequent addition of antimycin A was followed by the release of all the Ca²⁺ taken up. If nigericin was added instead of antimycin, a higher Ca²⁺ release than that observed with antimycin A was detected, thus indicating, as expected (21–23), a clear effect on these mitochondria in situ. Fig. 4, trace...
Fig. 3. Ca\textsuperscript{2+} uptake by digitonin-permeabilized T. brucei procyclic trypomastigotes. The reaction medium contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 2.5 mM potassium phosphate, 1 mM MgCl\textsubscript{2}, 2 mM succinate, 40 \(\mu\)M arsenazo III, 45 \(\mu\)M digitonin, and 8.0 \(\mu\)M Ca\textsuperscript{2+}. Cells (7.03 mg of protein/ml) were added to the reaction medium, and after a steady state was attained, 1 \(\mu\)M antimycin A (AA) or 2.75 \(\mu\)M nigericin (NIG) was added. Other experimental conditions are as described in the legend to Fig. 2.

A. shows that thapsigargin (12.5 \(\mu\)M) also released Ca\textsuperscript{2+} from the mitochondria in situ of digitonin-permeabilized procyclic trypomastigotes. In this experiment vanadate was included to inhibit Ca\textsuperscript{2+} uptake by the non-mitochondrial pool (6). Addition of FCCP after thapsigargin did not release any additional Ca\textsuperscript{2+}, but addition of the calcium ionophore A23187 caused the release of endogenous Ca\textsuperscript{2+} (Fig. 4, trace a). The Ca\textsuperscript{2+} release caused by thapsigargin (Fig. 4, trace a) was similar to that caused by antimycin A (AA, Fig. 4, trace b). When FCCP was added to the incubation medium (Fig. 4, trace B) Ca\textsuperscript{2+} uptake was completely inhibited. Addition of thapsigargin under these conditions did not release any endogenous Ca\textsuperscript{2+}, whereas the calcium ionophore A23187 released a considerable amount of endogenous Ca\textsuperscript{2+}, as it has been reported previously (6).

Since Ca\textsuperscript{2+} release from these mitochondria in situ could be due to the collapse of their membrane potential, we investigated the effect of nigericin and thapsigargin on the mitochondrial membrane potential of digitonin-permeabilized T. brucei using the safranine O method (5, 7, 8).

Fig. 5 shows the determination of the mitochondrial membrane potential of digitonin-permeabilized T. brucei procyclic trypomastigotes. Addition of safranine was followed by an increase in absorbance at the wavelength pair 511–533 compatible with the stacking of the dye to the energized mitochondrial inner membrane. Addition of thapsigargin (12.5 \(\mu\)M) caused a rapid and extensive decrease in the membrane potential which was completed by the further addition of antimycin A.

Fig. 6 shows that addition of different concentrations of nigericin to T. brucei procyclic (Fig. 6A) or bloodstream (Fig. 6B) trypomastigotes mitochondria in situ caused a dose-dependent collapse of their membrane potential similarly to that caused by FCCP, valinomycin, or, in the case of bloodstream trypomastigotes mitochondria (6), to oligomycin (Fig. 6B, dashed line).

Thapsigargin has been shown to cause the release of Ca\textsuperscript{2+} from the endoplasmic reticulum in several different cell types (including platelets, lymphocytes, neutrophils, macrophages, hepatocytes, adrenal chromaffin, and parotid acinar cells) (24).
without affecting their mitochondrial activity (25). It has been indicated that this is because thapsigargin has a remarkable specificity for the sarcoplasmic and endoplasmic reticulum Ca$^{2+}$-ATPase family of calcium pumps (28). Since our results indicated that high concentrations of thapsigargin were able to release Ca$^{2+}$ from mitochondria of isolated rat liver mitochondria, we further investigated the characteristics and presence of this effect in isolated rat liver mitochondria to verify if this effect of thapsigargin was specific for *T. brucei* mitochondria.

Figs. 7 shows that high concentrations of thapsigargin (>10 μM) caused a dose-dependent Ca$^{2+}$ release from isolated rat liver mitochondria without a significant inhibition of Ca$^{2+}$ uptake. The effect of thapsigargin on the membrane potential of isolated rat liver mitochondria is shown in Fig. 8. Collapse of the mitochondrial membrane potential was evident with similar concentrations of thapsigargin than those that caused Ca$^{2+}$ release. A control with the highest amount of the solvent of thapsigargin used (dimethyl sulfoxide) is shown in Figs. 7, trace f, and 8, pointed line, to indicate that the effect observed was not due to the solvent.

**DISCUSSION**

Nigericin and thapsigargin, when used at a relatively low concentration (1.0 μM), were unable to increase [Ca$^{2+}$], of *T. brucei* bloodstream trypanomastigotes (Fig. 1). Even when used at high concentrations, neither nigericin nor thapsigargin (Fig. 2) was able to release Ca$^{2+}$ from non-mitochondrial stores of digitonin-permeabilized *T. brucei* bloodstream trypanomastigotes. When used at a high concentration (2.75 μM), nigericin was able to increase [Ca$^{2+}$], (Fig. 1, traces a and b), and this increase was higher in the presence of a high extracellular Ca$^{2+}$ concentration (Fig. 1, trace b), in agreement with a previous report (11). However, at this high concentration, nigericin caused Ca$^{2+}$ release from the mitochondria in situ of permeabilized cells (Fig. 3) and collapsed their mitochondrial membrane potential (Fig. 6), in agreement with its known uncoupling effect on oxidative phosphorylation of rat liver mitochondria (21–23). On the other hand thapsigargin, when used at high concentrations (>8 μM), was unable to increase [Ca$^{2+}$], of *T. brucei* bloodstream trypanomastigotes (Fig. 1) but was able to stimulate Ca$^{2+}$ release from *T. brucei* bloodstream and procyclic (Fig. 4) trypanomastigotes mitochondria in situ and to collapse their mitochondrial membrane potential (Fig. 5).

Our results differ from the results previously reported on the effect of nigericin (11) and thapsigargin (12) on *T. brucei* bloodstream trypanomastigotes. Differences due to the different strain or differentiation stage of the *T. brucei* bloodstream trypanomastigotes used in those reports cannot be ruled out. In this regard, it should be noted that we have used similar (1 μM, 12) or higher concentrations (8 μM, Figs. 1 and 2) of thapsigargin from the same sources in our experiments with different results (see Fig. 1 and Figs 1 and 2 of Ref. 12). On the other hand, the possible uncoupling effect of nigericin at the very high concentrations used in previous studies in trypanosomatids (4 μM (Ref. 13); 1–2.75 μM (Refs. 11 and 12)) could have led, through ATP depletion, to an increase in [Ca$^{2+}$].

Taken together our results do not support the hypothesis of the presence of a nigericin-sensitive non-mitochondrial Ca$^{2+}$ pool in *T. brucei* long slender bloodstream trypanomastigotes (11). In addition, we were unable to find a non-mitochondrial thapsigargin-sensitive Ca$^{2+}$ pool in *T. brucei* bloodstream and procyclic trypanomastigotes as well as in other trypanosomatids that we have examined thus far, including *T. cruzi* epimastigotes and amastigotes (7) and *L. donovani* promastigotes. A number of previous studies (24, 27) have indicated that thapsigargin causes influx of calcium from the extracellular medium across the plasma membrane into the cytoplasm. Most investigators have proposed that this thapsigargin-induced calcium influx results from a thapsigargin-induced depletion of intracellular stored calcium, which by some unknown mechanism then causes increased calcium influx (27). Our results favor this hypothesis, since in trypanosomes there is a lack of thapsigargin-induced depletion of intracellular stored calcium and concomitantly no increase in [Ca$^{2+}$], through Ca$^{2+}$ influx.

Another important consequence of the present work concerns the use of thapsigargin as a tool for the study of Ca$^{2+}$ fluxes in intact cells. Thapsigargin was able to cause Ca$^{2+}$ release from mitochondria in situ of *T. brucei* as well as from isolated rat liver mitochondria. This indicates that care should

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1 A. E. Vercesi and R. Docampo, unpublished results.
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be taken when attributing the effects of thapsigargin in intact cells to the specific inhibition of the sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPase family of calcium pumps. In this regard the use of thapsigargin in intact cells at concentrations of 10 \(10^{-27}\) or \(10^{-50}\) pM (28) has been reported recently.

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