Inhibition of Ca^{2+} release from *Trypanosoma brucei* acidocalcisomes by 3,5-dibutyl-4-hydroxytoluene: role of the Na^+/H^+ exchanger

Anibal E. VERCESI*†, Mercedes T. GRIJALBA*² and Roberto DOCAMPO*¹

*Laboratory of Molecular Parasitology, Department of Pathobiology, College of Veterinary Medicine, University of Illinois, 2001 S. Lincoln Ave, Urbana, IL 61802, U.S.A., and †Departamento de Bioquímica, Universidade Estadual de Campinas, Campinas 13081, São Paulo, Brazil

Acidocalcisomes are acidic vacuoles present in trypanosomatids that contain a considerable fraction of intracellular Ca^{2+} . They possess a vacuolar-type H^+ -ATPase for H^+ uptake, a $\text{Ca}^{2+}/\text{H}^+$ countertransporting ATPase for Ca^{2+} uptake and a $\text{Ca}^{2+}/\text{nH}^+$ antiporter for Ca^{2+} release. *Trypanosoma brucei* procyclic trypomastigotes acidocalcisomes possess, in addition, an Na^+/H^+ antiporter that may participate in Ca^{2+} release from these organelles. In this work we show that the hydrophobic anti-oxidant 3,5-dibutyl-4-hydroxy toluene (BHT), at concentrations in the range 1–20 μM , inhibits Na^+ -induced Ca^{2+} release from the acidocalcisomes of digitonin-permeabilized procyclic trypomastigotes in a concentration-dependent manner. This effect supports the notion that Ca^{2+} release from this compartment is

regulated by the activity of the Na^+/H^+ antiporter. In the presence of BHT, Ca^{2+} release could still be obtained by nigericin-mediated alkalization of the acidocalcisomes, clearly demonstrating that the action of BHT is not at the level of the $\text{Ca}^{2+}/\text{nH}^+$ antiporter but on that of the Na^+/H^+ antiporter. In the same range of concentrations and depending on the pre-incubation time, BHT had an stimulatory or an inhibitory effect on the vacuolar H^+ -ATPase present in *T. brucei* acidocalcisomes. Since these effects of BHT were obtained at concentrations which are commonly used for its antioxidant properties, these results indicate that care should be exercised when attributing effects of BHT to only these properties.

INTRODUCTION

In previous communications we provided evidence that the *Trypanosoma brucei* procyclic and bloodstream trypomastigotes possess acidic vacuoles that accumulate Ca^{2+} via a vanadate-sensitive Ca^{2+} -ATPase [1–3]. The acidic pH of this compartment, which is termed the acidocalcisome, is generated and sustained by a bafilomycin A_1 -sensitive H^+ -ATPase and seems to be important for Ca^{2+} retention, since alkalization induced by nigericin, NH_4Cl or bafilomycin A_1 treatment was followed by Ca^{2+} release [1–3]. The stimulation of Ca^{2+} release by vacuole alkalization suggests that Ca^{2+} release is mediated by a $\text{Ca}^{2+}/\text{nH}^+$ antiporter. This hypothesis was further supported by the recent detection of an Na^+/H^+ antiporter in the acidocalcisomes of *T. brucei* procyclic trypomastigotes [2]. External Na^+ causes vacuolar alkalization, which leads to Ca^{2+} release from the organelles. Acidocalcisomes have also been found in other trypanosomatids such as *Trypanosoma cruzi* [4] and *Leishmania mexicana amazonensis* [5] and in *Toxoplasma gondii* [6].

In this paper we provide evidence that the Na^+/H^+ antiporter in the acidocalcisomes of *T. brucei* procyclic trypomastigotes can be inhibited by 3,5-dibutyl-4-hydroxytoluene (BHT). This inhibition prevents the Na^+ -dependent stimulation of Ca^{2+} release. These results support our previous proposition [2] that Ca^{2+} release from the acidocalcisomes of *T. brucei* procyclic trypomastigotes occurs via a $\text{Ca}^{2+}/\text{nH}^+$ antiporter and is promoted by the action of the Na^+/H^+ antiporter.

MATERIALS AND METHODS

Culture methods

T. brucei procyclic forms (ILTar 1 procyclics) were grown at 28 °C in medium SDM-79 [7] supplemented with haemin

(7.5 mg/l) and 10 % (v/v) heat-inactivated fetal calf serum. At 2–3 days after inoculation, cells were collected by centrifugation and washed twice in a buffer (buffer A) containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 5.5 mM glucose and 50 mM Hepes, pH 7.4, and resuspended in the same buffer. In some experiments NaCl in buffer A was replaced with a similar amount of choline chloride. The final concentration of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay [8] in the presence of 0.2 % deoxycholate.

Chemicals

ATP, arsenazo III, EGTA, nigericin, antimycin A, oligomycin, BHT and digitonin were purchased from Sigma. Bafilomycin A_1 was from Kamiya Biomedicals, Thousand Oaks, CA, U.S.A. Acridine Orange (AO) was obtained from Molecular Probes, Eugene, OR, U.S.A. All other reagents were of analytical grade.

Determination of Ca^{2+} movements

Variations in free Ca^{2+} concentrations were monitored by measuring the changes in the absorbance spectrum of arsenazo III [9] using an SLM Aminco DW2000 spectrophotometer at the wavelength pair 675/685 nm. No free-radical formation from arsenazo III occurred under the conditions used [10–12]. The concentration of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program, as described previously [1–3]. Each experiment was repeated at least three times with different cell preparations; the Figures show representative experiments.

Proton-pump activity

Acidification of digitonin-permeabilized cells was monitored by

Abbreviations used: AO, Acridine Orange; BHT, 3,5-dibutyl-4-hydroxytoluene.

¹ To whom correspondence should be addressed.

² Present address: Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil.

measuring the changes in the absorbance spectrum of AO [1,13], using an SLM Aminco DW2000 spectrophotometer at the wavelength pair 493/530 nm. Each experiment was repeated at least three times with different cell preparations; the Figures show representative experiments.

RESULTS AND DISCUSSION

The monitoring of Ca^{2+} release from acidocalcisomes of permeabilized *T. brucei* procyclic trypomastigotes in our previous studies was performed in reaction media containing antimycin A and vanadate to prevent re-uptake of the released Ca^{2+} by mitochondria and non-mitochondrial Ca^{2+} pools [1,2]. These cells possess high levels of endogenous respiratory substrates, and it is known that antimycin A can stimulate the production of oxygen radicals by mitochondria [14,15] which can be deleterious to the cell membranes [15]. In order to prevent possible membrane oxidation and Ca^{2+} leakage during our experiments we included low concentrations of the lipophilic antioxidant BHT [16] in the incubation media.

The experiment depicted in Figure 1 shows that there is less leakage of Ca^{2+} in the presence (trace a) than in the absence (trace b) of 20 μM BHT. This conforms to the above hypothesis for BHT membrane protection, but might also be due to specific inhibition of the process of Na^{+} -induced Ca^{2+} release previously described [2], as the activities of many membrane proteins have been found to be affected by a wide range of hydrophobic molecules, including BHT [17,18] and references cited therein). Although the osmotic support utilized in this experiment was 130 mM KCl, the cells were in buffer A containing 116 mM NaCl, and contaminant Na^{+} was therefore present (approximately 2.3 mM). In support of the hypothesis that the Ca^{2+} leakage observed (Figure 1, trace b) was caused by contaminant Na^{+} , when the cells were washed and resuspended in buffer A in which NaCl was replaced by choline chloride, no Ca^{2+} leakage occurred (Figure 1, broken line). To confirm that BHT was inhibiting the process of Na^{+} -induced acidocalcisome Ca^{2+} release, we studied this effect directly (Figure 2A). Traces a and b allow for comparison between the rates of Na^{+} -induced Ca^{2+} release in the presence (a) and absence (b) of BHT. A potent inhibition of Na^{+} -induced Ca^{2+} release by BHT was observed (trace a). Similar inhibition was also observed when BHT was added after Na^{+} -induced Ca^{2+} release was initiated (trace c) under the conditions of trace b. This effect could also be due to a stimulatory effect of BHT on Ca^{2+} uptake by acidocalcisomes or other compartments. However, BHT had no effect on ATP-dependent Ca^{2+} uptake by digitonin-permeabilized procyclics when used up to a concentration of 40 μM (results not shown). That the inhibition of Na^{+} -induced Ca^{2+} release by BHT was mediated by inhibition of acidocalcisome alkalization by Na^{+} , and not by inhibiting Ca^{2+} transport through the $\text{Ca}^{2+}/\text{nH}^{+}$ exchanger, was evidenced by the subsequent addition of nigericin, which alkalized the vacuole by catalysing a $\text{K}^{+}/\text{H}^{+}$ exchange (Figure 2A, trace c). This experiment shows that nigericin addition was followed by Ca^{2+} release, which is similar to what happened when nigericin was added at the beginning of the experiment in the absence of added NaCl and BHT (trace d). Figure 2(B) shows the inhibition by BHT of Ca^{2+} release when the permeabilized cells were incubated in 130 mM NaCl medium. In trace a, 20 μM BHT was present from the start of the experiment, whereas in trace b, BHT was added where shown.

Since alkalization of the acidocalcisomes induced by $\text{Na}^{+}/\text{H}^{+}$ exchange can be followed by monitoring changes in AO absorbance [2], we performed experiments to study the effect of BHT on the activity of the $\text{Na}^{+}/\text{H}^{+}$ exchanger. AO is a tertiary

Figure 1 Effect of BHT on endogenous Ca^{2+} release from permeabilized trypomastigotes

The cells (50 μl , final concentration in the cuvette: 0.3 mg of protein/ml) suspended in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 5.5 mM glucose and 50 mM Hepes buffer pH 7.4) were added to 2.5 ml of buffer B (130 mM KCl, 1 mM MgCl_2 , 2 mM potassium phosphate, 20 mM Tris/HCl, pH 7.4, 2 $\mu\text{g}/\text{ml}$ oligomycin, 1 μM antimycin A and 0.5 mM sodium α -vanadate), containing 0.5 mM K-ATP, and 40 μM arsenazo III. BHT (20 μM) was present in trace a. Digitonin (DIG, 28 μM), calcium ionophore A23187 (5 μM) and CaCl_2 (2.5 μM) were added where indicated. The broken line is for cells washed and resuspended in buffer A in which choline chloride replaced NaCl.

Figure 2 Inhibition by BHT of Na^{+} -induced Ca^{2+} release from permeabilized trypomastigotes

(A) The cells (0.3 mg of protein/ml) were added to buffer B (see Figure 1 legend) containing 0.5 mM K-ATP, 40 μM arsenazo III and 28 μM digitonin. Trace a: 40 mM NaCl and 20 μM BHT were added at the beginning of the experiment. Trace b: 40 mM NaCl was added at the beginning of the experiment. Trace c: the same conditions as in trace b, but 20 μM BHT and 1 μM nigericin (NIG) were added where indicated. Trace d: 1 μM nigericin was added at the beginning of the experiment. Calcium ionophore A23187 (5 μM) and CaCl_2 (2.5 μM) were added where indicated. (B) The cells (0.3 mg of protein/ml) were added to buffer B containing 0.5 mM K-ATP and 40 μM arsenazo III, in which 130 mM KCl was replaced by 130 mM NaCl. Trace a: 20 μM BHT was added at the beginning of the experiment. Trace b: 20 μM BHT was added where indicated. Broken line: conditions as in trace b without the addition of BHT. Digitonin (DIG; 28 μM) and CaCl_2 (2.5 μM) were added where indicated.

Figure 3 AO accumulation by permeabilized trypomastigotes

(A) The cells (0.3 mg of protein/ml) were added to buffer B (see Figure 1 legend) containing 28 μ M digitonin and 0.2 mM EGTA, in the absence of ATP. AO (2 μ M), bafilomycin A₁ (BAF, 2 μ M), NaCl (40 mM) and NH₄Cl (10 mM) were added where indicated. (B) Experimental conditions as in (A), but 0.5 mM ATP, 40 mM NaCl, 10 mM NH₄Cl and 20 μ M BHT (to trace indicated as BHT only) were added where indicated.

amine that becomes concentrated in acidic compartments undergoing changes in both absorbance and fluorescence properties [13].

Characterization of the Na⁺/H⁺ antiporter-containing acidic compartment was made by the addition of AO to permeabilized cells in the absence or presence of added ATP. In Figure 3(A), trace a shows that in the absence of ATP some AO is accumulated and retained during the time of observation. Addition of NH₄Cl (trace b) caused a fast release, while addition of bafilomycin A₁, a known H⁺-ATPase inhibitor [19], (trace c) caused a slower release of the dye. However, addition of NaCl did not change the AO absorbance, indicating that the acidic compartment that accumulated this amount of AO does not contain the Na⁺/H⁺ exchanger. However, when ATP was added after this first phase of AO accumulation, a further accumulation of AO occurred (Figure 3B). Interestingly, this extra AO accumulated after ATP addition could be totally released by the addition of 40 mM NaCl, indicating the existence of at least two types of acidic compartments in these cells: one that has and another that does not have the Na⁺/H⁺ antiporter. Addition of 20 μ M BHT before addition of NaCl prevented the Na⁺-induced alkalization of the vacuole. This experiment demonstrates that the latter acidic compartment is the one that contains Ca²⁺ and that the Na⁺/H⁺ antiporter activity is related to Ca²⁺ release from this compartment (acidocalcisome).

Figure 4 shows the dose-dependent inhibition by BHT of Na⁺-induced AO release from acidocalcisomes. A maximum inhibition was obtained with 25 μ M BHT and a significant inhibition could already be detected at 1.2 μ M.

Figure 4 Inhibition by BHT of Na⁺-induced AO release in permeabilized trypomastigotes

The cells (0.3 mg of protein/ml) were incubated in buffer B (see Figure 1 legend) containing 0.5 mM K-ATP (trace labelled ATP), 0.2 mM EGTA and 28 μ M digitonin. AO (2 μ M) was added where indicated (30 s after the cells). NaCl (40 mM), NH₄Cl (10 mM) and BHT (0, 1.2, 2.5, 5, 15 and 25 μ M) were added where indicated. The lower broken line represents one experiment in the absence of BHT, NaCl and NH₄Cl. The upper broken line was without the addition of ATP.

Figure 5 Effect of BHT on ATP-driven AO accumulation by permeabilized trypomastigotes

The cells (0.3 mg of protein/ml) were incubated in buffer B (Figure 1) containing 28 μ M digitonin, 0.2 mM EGTA and 2 μ M AO. BHT (0, 1.0, 2.5, 10 and 25 μ M) was added 30 s before ATP addition. ATP (0.5 mM) and NH₄Cl (10 mM) were added where indicated. The broken line represents one experiment where BHT was added 5 s before ATP addition.

When BHT was added from the beginning of these experiments we observed alterations in the extent of AO accumulation (results not shown). Since this effect could be due to an inhibition of H⁺ uptake by the acidocalcisomal H⁺-ATPase, we studied the effect of different concentrations of BHT on AO uptake. Figure 5 shows that when ATP was added 30 s after different concentrations of BHT were included in the medium, an inhibition of AO accumulation could already be detected using 1 μ M BHT, and complete inhibition was obtained using 25 μ M BHT. No further inhibition or AO release was detected at higher concentrations (at least up to 40 μ M) of BHT (results not shown). The

Figure 6 Comparison between the rates of BHT- and bafilomycin A_1 -induced AO release from permeabilized *T. brucei* procyclic trypomastigotes

The cells (0.3 mg of protein/ml) were incubated in buffer B (Figure 1) containing 28 μ M digitonin, 0.2 mM EGTA and 0.5 mM K-ATP. AO (2 μ M), BHT (20 μ M, trace b), bafilomycin A_1 (BAF; 2.0 μ M, trace a) or NH_4Cl (10 mM; trace c) were added where indicated. Control did not have any additional addition.

broken line shows that 20 μ M BHT caused an increase in the initial rate of AO accumulation when added 5 s before ATP. This is in agreement with other data [18] and references therein) showing that the effect of BHT on membrane-bound enzymes differs depending on the preincubation time. In this regard, the incubation of the cells with 2.5 μ M BHT during a prolonged period of time (3–35 min) caused a more effective inhibition in a time-dependent manner (results not shown).

We also performed experiments to compare the rates of vacuole alkalinization caused by bafilomycin A_1 and BHT (Figure 6). It can be observed that while bafilomycin A_1 addition caused a complete release of AO in less than 2 min (trace a), the AO release caused by the addition of BHT was very slow (trace b). This is in agreement with the results shown in Figure 3(A) and Figure 5, which indicate that bafilomycin A_1 could release AO from the compartment devoid of an Na^+/H^+ antiporter, whereas BHT could not.

Based on several studies regarding the effect of BHT and related compounds upon Ca^{2+} -ATPase activity [18], we can speculate that the effects of BHT on the H^+ -ATPase and the Na^+/H^+ antiporter probably are not related to alterations in the fluidity or viscosity of the bilayer, as expected with hydrophobic molecules. In the case of the Ca^{2+} -ATPase, the inhibitory effect of BHT is due to a reduction in the effective rates of the E2-E1 step for the MgATP-free and MgATP-bound forms [18]. BHT can either activate or inhibit the Ca^{2+} -ATPase, depending on its concentration. At low concentrations, the increase in the activity of Ca^{2+} -ATPase is strictly correlated with the presence of cholesterol and the chemical structure of the surrounding phospholipid [18]. In reconstituted systems, increased activity is always observed when phospholipids with shorter fatty acid chains are used [18].

In summary, BHT is an effective inhibitor of both the Na^+/H^+ exchanger and the H^+ -ATPase of the acidocalcisomes of *T. brucei* procyclic trypomastigotes at concentrations which are commonly used for its antioxidant properties [16]. This indicates that care should be exercised when attributing effects of BHT only to its antioxidant properties.

The true nature of acidocalcisomes is a matter of speculation. It is possible that acidocalcisomes correspond to what has been described before in *Trypanosoma cyclops* [20], *Trypanosoma*

rhodesiense [21], *T. cruzi* [22] and *Leishmania major* [23] as polyphosphate [20] or electron-dense [21–23] vacuoles. These vacuoles were detected by energy-dispersive X-ray microanalysis [20–23]. When initially observed in *T. cyclops* [20] they were termed polyphosphate bodies, on the basis of a high phosphorus content and an appearance similar to algal polyphosphate bodies, although the nature of the phosphate compound present was not chemically analysed. They had an appreciable calcium content and, possibly, some zinc [20]. Although in *T. rhodesiense* an acid phosphatase activity was detected cytochemically in vacuoles of similar size to the calcium-containing electron-dense vacuoles, the non-enzymic binding of heavy metals by the matrix of electron-dense vacuoles precluded an assignment of enzyme activity to them [21]. *T. cruzi* epimastigotes were also shown to possess electron-dense vacuoles containing large amounts of magnesium, potassium, calcium, phosphorus and zinc [22]. Electron-probe X-ray microanalysis of *L. major* promastigotes demonstrated that their electron-dense vacuoles were also rich in phosphorus, magnesium, calcium, sodium and zinc [23]. Further work on the correspondence of acidocalcisomes and electron-dense vacuoles in different trypanosomatids is underway in our laboratory.

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REFERENCES

- Vercesi, A. E., Moreno, S. and Docampo, R. (1994) *Biochem. J.* **304**, 227–233
- Vercesi, A. E. and Docampo, R. (1996) *Biochem. J.* **315**, 265–270
- Scott, D. A., Moreno, S. N. J. and Docampo, R. (1995) *Biochem. J.* **310**, 789–794
- Docampo, R., Scott, D. A., Vercesi, A. E. and Moreno, S. N. J. (1995) *Biochem. J.* **310**, 1005–1012
- Lu, H.-G., Zhong, L., Chang, K. P. and Docampo, R. (1996) *J. Biol. Chem.* **272**, 9464–9473
- Moreno, S. N. J. and Zhong, L. (1996) *Biochem. J.* **313**, 655–659
- Brun, R. and Schönenberg, M. (1979) *Acta Trop.* **36**, 289–292
- Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Scarpa, A. (1979) *Methods Enzymol.* **56**, 301–338
- Docampo, R., Moreno, S. and Mason, R. P. (1983) *J. Biol. Chem.* **258**, 14920–14925
- Moreno, S., Docampo, R. and Mason, R. P. (1984) *J. Biol. Chem.* **259**, 14609–14616
- Moreno, S., Docampo, R. and Vercesi, A. E. (1992) *J. Biol. Chem.* **267**, 6020–6026
- Palmgren, M. G. (1991) *Anal. Biochem.* **192**, 316–321
- Boveris, A., Oshino, N. and Chance, B. (1972) *Biochem. J.* **128**, 617–630
- Kowaltowski, A. J., Castilho, R. F. and Vercesi, A. E. (1995) *Am. J. Physiol. (Cell Physiol.)* **269**, C141–C147
- Kagan, V. E., Serbinova, E. A. and Packer, L. (1990) *Arch. Biochem. Biophys.* **280**, 33–39
- Sokolove, P. M., Albuquerque, E. X., Kauffman, F. C., Spande, T. F. and Daly, J. W. (1986) *FEBS Lett.* **203**, 121–126
- Michelangeli, F., Orlowski, S., Champeil, P., East, J. M. and Lee, A. G. (1990) *Biochemistry* **29**, 3091–3101
- Bowman, E. J., Siebers, A. and Altendorf, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7972–7976
- Vickerman, K. and Tetley, L. (1977) *Ann. Soc. Belge Med. Trop.* **57**, 441–455
- Williamson, J. and McLaren, D. J. (1981) *J. Protozool.* **28**, 460–467
- Dvorak, J. A., Engel, J. C., Leapman, R. D., Swyt, C. R. and Pella, P. A. (1988) *Mol. Biochem. Parasitol.* **31**, 19–26
- LeFurgey, A., Ingram, P. and Blum, J. J. (1990) *Mol. Biochem. Parasitol.* **40**, 77–86