

## Amiodarone Destabilizes Intracellular Ca<sup>2+</sup> Homeostasis and Biosynthesis of Sterols in *Leishmania mexicana*<sup>∇</sup>

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**Leishmaniasis represents a serious public health problem worldwide. The first line of treatment is based on glucantime and pentostan, which generate toxic effects in treated patients. We have recently shown that amiodarone, frequently used as an antiarrhythmic, possesses activity against *Trypanosoma cruzi* through the disruption of mitochondrial Ca<sup>2+</sup> homeostasis and the inhibition of parasite ergosterol biosynthesis, specifically at the level of oxidosqualene cyclase activity (G. Benaim, J. Sanders, Y. Garcia-Marchan, C. Colina, R. Lira, A. Caldera, G. Payares, C. Sanoja, J. Burgos, A. Leon-Rossell, J. Concepcion, A. Schijman, M. Levin, E. Oldfield, and J. Urbina, *J. Med. Chem.* 49:892–899, 2006). Here we show that at therapeutic concentrations, amiodarone has a profound effect on the viability of *Leishmania mexicana* promastigotes. Additionally, its effect on the viability of the parasite was greater against intracellular amastigotes than against promastigotes, and it did not affect the host cell. Using fluorimetric and confocal microscopy techniques, we also demonstrated that the mechanism of action of amiodarone was related to the disruption of intracellular Ca<sup>2+</sup> homeostasis through a direct action not only on the mitochondria but also on the acidocalcisomes. On the other hand, analysis of the free sterols in promastigotes incubated with amiodarone showed that this drug also affected the biosynthesis of 5-dehydroepisterol, which results in squalene accumulation, thus suggesting that amiodarone inhibits the squalene epoxidase activity of the parasite. Taken together, the results obtained in the present work point to a more general effect of amiodarone in trypanosomatids, opening potential therapeutic possibilities for this infectious disease.**

Chemotherapy is the most effective treatment against leishmaniasis, due to the lack of an effective vaccine (14, 20). The recommended first-line therapy for different forms of leishmaniasis includes pentavalent antimonials like glucantime and pentostan (8, 25). However, these drugs have secondary effects on the renal, cardiac, and hepatic systems (25). Resistance to pentavalent antimonial drugs is also an important factor that limits the treatments available in several countries (1, 13). Another disadvantage of antimonial therapy is the requirement for long-term parenteral administration in order to obtain optimal results (12, 21). At present, miltefosine appears to represent a major advance in the treatment of visceral and cutaneous leishmaniasis, despite its teratogenic characteristics, which limits its use during pregnancy (11).

Amiodarone (Fig. 1), an antiarrhythmic class III drug commonly used to treat several cardiomyopathies, has been the subject of recent studies as an antimycotic and parasitocidal agent, since this drug possesses excellent pharmacokinetic properties and a relative low cost. Thus, this drug has been shown to have potent effects against the yeast *Saccharomyces cerevisiae* as well as *Candida albicans* and other fungi (9). The

reported mechanism of action of amiodarone against *Saccharomyces cerevisiae* (19) and *Trypanosoma cruzi* (7) is, at least in part, the disruption of Ca<sup>2+</sup> homeostasis.

The crucial role of Ca<sup>2+</sup> in the regulation of many important processes for cellular viability has been demonstrated in different trypanosomatids (6, 23, 24). At the plasma membrane level, it has been reported that a Ca<sup>2+</sup> ATPase is stimulated by calmodulin in *T. cruzi* (3, 5), *Trypanosoma brucei* (4), and different species of *Leishmania* (2, 22). In all of these parasites, the regulation of cytoplasmic Ca<sup>2+</sup> is carried out by three major organelles: the endoplasmic reticulum; the unique mitochondrion that is present in these parasites; and the acidocalcisome, a special compartment that is devoted to the accumulation of polyphosphates and that is also probably involved in the regulation of Ca<sup>2+</sup> (17). The parasite mitochondrion possess a Ca<sup>2+</sup> electrophoretic uniporter in its internal membrane which utilizes the difference in the proton electrochemical potential between the intramitochondrial space and the cytoplasm as the driving force for the accumulation of this cation (2, 15). This mitochondrial Ca<sup>2+</sup> transport system is characterized by a low affinity for Ca<sup>2+</sup> but a high capacity for Ca<sup>2+</sup> accumulation. All these attributes are very similar to the mitochondrial Ca<sup>2+</sup> transport system in mammals (2, 15). The acidocalcisomes, however, are acidic organelles with high levels of pyrophosphates and polyphosphates which are capable of accumulating large amounts of Ca<sup>2+</sup> (16, 17). All these systems, which operate in a concerted fashion, contribute to the maintenance of intracellular Ca<sup>2+</sup> homeostasis, keeping

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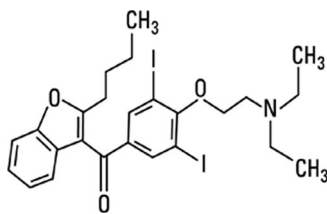


FIG. 1. Chemical structure of amiodarone.

the intracellular (cytoplasmic)  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) under 100 nM, well below the extracellular  $\text{Ca}^{2+}$  concentration (which is on the order of mM), thus allowing this cation to perform its essential function as an internal signaling messenger (6).

We have previously shown that amiodarone affects the viability of *T. cruzi* by disrupting mitochondrial  $\text{Ca}^{2+}$  homeostasis in the parasite (7). In the present study, we demonstrate that amiodarone also affects the viability of *Leishmania mexicana* by destabilizing  $\text{Ca}^{2+}$  homeostasis in both the mitochondrion and the acidocalcisome.

Similar to *T. cruzi*, however, *L. mexicana* contains ergosterol instead of cholesterol as the main sterol component on its membranes. This property constitutes a notable difference between trypanosomatids and humans and validates the use of the sterol biosynthetic pathway as a potential target for the development of new drugs (31). We have also demonstrated that amiodarone blocks *T. cruzi* ergosterol biosynthesis at the level of the oxidosqualene cyclase (7). In this context, in this work we also show that amiodarone inhibits the *L. mexicana* sterol pathway at the level of the squalene epoxidase, which is essential for the synthesis of 5-dehydroepisterol.

#### MATERIALS AND METHODS

**Chemicals.** Amiodarone [(2-butyl-3-benzofuranyl)-[4-[2-(diethylamino)ethoxy] 3,5-diiodophenyl] methanone hydrochloride], EGTA, digitonin, fluorocarbonyl cyanide *P*-(trifluoromethoxy) phenylhydrazine (FCCP), bafilomycin A, nigericin, lectin from the coral tree (*Erythrina cristagalli*[r]), and L-polylysine were from Sigma (St. Louis, MO). Fura 2-acetoxymethyl ester (FURA 2-AM), rhodamine 123, rhod 2-AM, and acridine orange were from Molecular Probes (Eugene, OR).

**Culture of promastigotes and determination of cellular proliferation.** Promastigotes of *L. mexicana* were cultured in RPMI 1640 medium (Gibco) supplemented with 10% inactivated fetal bovine serum (Gibco) under continuous agitation at 29°C. The susceptibility of *L. mexicana* to amiodarone was evaluated by use of a parasite growth curve obtained in the absence or the presence of the drug and a Neubauer chamber. The drug was added 24 h after the cultures were established ( $10^6$  parasites/ml). At least three independent experiments were performed for each condition.

**Determination of percentage of infected macrophages.** J774G8 macrophages were maintained at 37°C and 5%  $\text{CO}_2$  in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum. For parasite infection, a proportion of 10 promastigotes to 1 macrophage was employed, and the infected cells were incubated for 24 h at 37°C in 5%  $\text{CO}_2$ . To determine the effect of amiodarone on intracellular amastigotes, growth curve studies were carried out in the presence or the absence of the drug. The percentage of infected macrophages was determined at 72 h posttreatment under a light microscope by the use of Giemsa stain. At least three independent experiments were performed for each condition.

**Determination of  $[\text{Ca}^{2+}]_i$ .** To measure  $[\text{Ca}^{2+}]_i$ , we used the fluorescent ratio-metric  $\text{Ca}^{2+}$  indicator Fura 2, whose excitation spectrum depends on the concentration of the cation, while its emission peak does not vary (30). Parasites were loaded with Fura 2-AM as described previously (7), with slight modifications. Briefly, parasites ( $4 \times 10^6$  promastigotes/1.5 ml of culture medium) were centrifuged at  $600 \times g$  for 2.5 min and were then washed twice with loading buffer (137 mM NaCl, 4 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8.5 mM  $\text{Na}_2\text{HPO}_4$ , 11 mM

glucose, 1 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgSO}_4$ , 20 mM HEPES-NaOH, pH 7.4) (32). The parasite pellet was resuspended in the same buffer, and a mixture of 6  $\mu\text{M}$  Fura 2-AM and 2.4 mM probenecid was applied. The parasites were then incubated at 29°C for 45 min under continuous agitation. Following two washes with this buffer, fluorescence measurements were performed in a Hitachi 2000 spectrofluorimeter at 29°C with continuous agitation by stirring in a cuvette; the spectrofluorimeter was coupled to a computer with an appropriate data acquisition system.  $[\text{Ca}^{2+}]_i$  was evaluated as described by Gryniewicz et al. (18) by applying the following equation:  $K_d \times [(R - R_{\min}) / (R_{\max} - R)] \times [F_{\min}(380) / F_{\max}(380)]$ , where  $K_d$  is the dissociation constant of Fura 2 (244 nM);  $R$  is the ratio of the fluorescence emission obtained after excitation at 340 nm/after excitation at 380 nm;  $R_{\max}$  and  $F_{\max}$  are the ratio of excitation fluorescence at 340 nm/excitation fluorescence at 380 nm and the fluorescence of Fura 2 at 380 nm, respectively, under saturated  $\text{Ca}^{2+}$  concentrations; and  $R_{\min}$  and  $F_{\min}$  are the ratio of excitation fluorescence at 340 nm/excitation fluorescence at 380 nm and the fluorescence of Fura 2 at 380 nm, respectively, in the absence of  $\text{Ca}^{2+}$ . Maximum and minimum values were obtained after the addition of 30  $\mu\text{M}$  digitonin, which allows the flow of  $\text{Ca}^{2+}$  to the interior of the cell. Then, 8 mM EGTA was added to chelate all the remaining  $\text{Ca}^{2+}$ .

**Determination of the mitochondrial membrane potential.** The mitochondrial membrane potential estimations were carried out with the fluorescent dye rhodamine 123, which presents maximum peaks in its excitation and emission spectra at 488 and 530 nm, respectively. Rhodamine 123, is a mitochondrion-specific cationic dye which allows visualization of the state of the electrochemical potential of this organelle since this dye is distributed between the internal and the external mitochondrial membrane according to the electrochemical potential. The experimental conditions used to load the parasites with this dye were essentially similar to the conditions described above for Fura 2-AM. Briefly, the parasites were loaded with 10  $\mu\text{g}/\text{ml}$  of rhodamine 123 for 30 min at 29°C. All measurements were performed in a Hitachi 2000 spectrofluorimeter at 29°C and under continuous agitation; the spectrofluorimeter was coupled to a computer with an appropriate data acquisition system.

**Determination of acidocalcisome alkalization level.** The accumulation of acridine orange in the acidocalcisomes was used as a probe for alkalization, as reported by Docampo et al. (16). Initially, promastigotes were washed twice with 130 mM KCl–1 mM  $\text{MgCl}_2$ –2 mM  $\text{KH}_2\text{PO}_4$ –20 mM Tris-HCl, pH 7.4. The promastigotes ( $10^9$  cells/ml) were then loaded with 2  $\mu\text{M}$  acridine orange for 5 min at 29°C and under constant agitation. Measurements were performed at an excitation  $\lambda$  of 488 nm and an emission  $\lambda$  of 530 nm in a Hitachi 2000 spectrofluorimeter under continuous agitation in a stirred cuvette at 29°C.

**Confocal microscopy determinations.** Promastigotes of *L. mexicana* were immobilized on coverslips on four- to eight-well plates coated with lectin from the coral tree (*Erythrina cristagalli*), which interacts with glycoproteins on the parasite surface membrane that contain oligosaccharides with galactosyl ( $\beta$ -1,4)-*N*-acetylglucosamine, as described by Rohloff et al. (28). This lectin was demonstrated to be able to immobilize *T. cruzi* (28) and also *L. mexicana* (this work) in confocal microscopy experiments while maintaining their viability. The lectin was dissolved in Dulbecco's phosphate-buffered saline (PBS) at a concentration of 0.5 mg/ml. The glass surfaces coated with poly-L-lysine were treated with the lectin solution for 30 min and air dried under a laminar-flow hood. Promastigotes in exponential phase were collected by centrifugation, washed three times with PBS, and resuspended in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM  $\text{MgSO}_4$ , 50 mM HEPES-NaOH, pH 7.4) without glucose. The cells were allowed to attach for 30 min at room temperature, and the unattached cells were removed by rinsing the coverslips three times with buffer A (28). Then, the fixed promastigotes were loaded with rhod 2-AM (10  $\mu\text{M}$ ) and rhodamine 123 (16  $\mu\text{g}/\text{ml}$ ) in Tyrode medium for 45 min at 29°C. Similarly, macrophages infected with promastigotes of *L. mexicana* (10 promastigotes to 1 macrophage) were loaded with rhod 2-AM (10  $\mu\text{M}$ ) and rhodamine 123 (16  $\mu\text{g}/\text{ml}$ ) in Tyrode medium for 45 min at 29°C and 5%  $\text{CO}_2$ . Under these conditions, the fluorescence of rhod 2-AM is mainly produced in intracellular  $\text{Ca}^{2+}$ -rich compartments due to its low affinity for  $\text{Ca}^{2+}$ , thus allowing monitoring of the changes in the mitochondrial  $\text{Ca}^{2+}$  concentration simultaneously with the changes in the electrochemical potential of this organelle with rhodamine 123, as described above.

**Free sterol content determinations.** Free sterol contents were determined by high-resolution gas-liquid chromatography coupled with mass spectrometry, as described previously (33). Briefly, for the extraction and the separation of neutral lipids, *L. mexicana* was cultured in the presence of amiodarone, as described above (see Table 2). Lipids were extracted with chloroform-methanol (2:1, vol/vol). The extract was dried and suspended in a minimum volume of chloroform. The chloroform suspension was applied to a silicic acid column (1.5 by 4 cm) and washed with 5 column volumes of chloroform to separate the neutral lipids from the other lipid fractions.

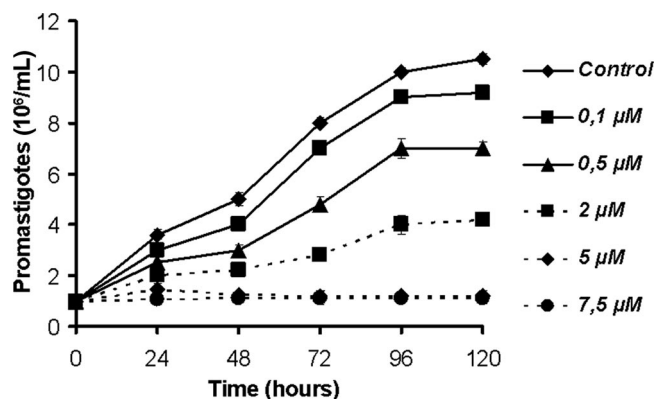


FIG. 2. Susceptibility of *L. mexicana* promastigotes to amiodarone. Populations of *L. mexicana* promastigotes were exposed to different concentrations of amiodarone. Experiments were performed at least in triplicate for each condition.

For the quantitative analysis of free sterols and structural assignment, the neutral lipids were then separated in a capillary high-resolution column (25 m by 0.20 mm [inner diameter]; Ultra-2; 5% phenyl-methyl-siloxane; film thickness, 0.33 μm) in a Hewlett-Packard HP-5890 series II gas chromatograph equipped with mass-sensitive detector HP-5971. The lipids were dissolved in chloroform and injected into the column at an initial temperature of 50°C (1 min), followed by a temperature increase to 280°C at a rate of 25°C/min and a further rise to 300°C at a rate of 1°C/min. The flow rate of the carrier gas (He) was kept constant at 0.6 ml/min. The injector temperature was 250°C; the detector was kept at 280°C.

## RESULTS

### Susceptibility of *L. mexicana* promastigotes to amiodarone.

*L. mexicana* promastigotes were exposed to different concentrations of amiodarone, and their viability was determined for 5 days. As can be seen in Fig. 2, amiodarone produced a dose-dependent inhibition of growth. Inhibition of 100% was obtained with a concentration of 5 μM amiodarone, and the 50% inhibitory concentration ( $EC_{50}$ ) was 900 nM (Fig. 2). This result indicates that amiodarone is able to affect the viability of these parasites with an even greater efficacy than it affects the viability of *Trypanosoma cruzi* epimastigotes, for which the drug concentration needed for 100% inhibition was 1 order of magnitude greater ( $EC_{50}$ , 9 μM) (7).

**Effect of amiodarone on *L. mexicana* intracellular amastigotes.** Macrophages were infected with promastigotes in order to evaluate the effects of different concentrations of amiodarone on the number of infected macrophages. Figure 3A shows that as the amiodarone concentration increased, a concomitant decrease in the proportion of infected macrophages was observed. This effect was more pronounced than the inhibition of the promastigotes that was observed, since the  $EC_{50}$  was 8 nM. This fact was also reflected in the MIC of 20 nM obtained under these conditions. Figure 3B also shows that amiodarone did not affect the viability of macrophages in culture, as expected.

**Effect of amiodarone on the  $[Ca^{2+}]_i$  of promastigote from *L. mexicana*.** The promastigotes were initially loaded with the  $Ca^{2+}$  indicator Fura 2. Figure 4 and Table 1 show that 5 μM amiodarone induced an increase in the  $[Ca^{2+}]_i$  in promastigote populations when an extracellular medium containing 2 mM  $Ca^{2+}$  was employed. In order to determine if the observed

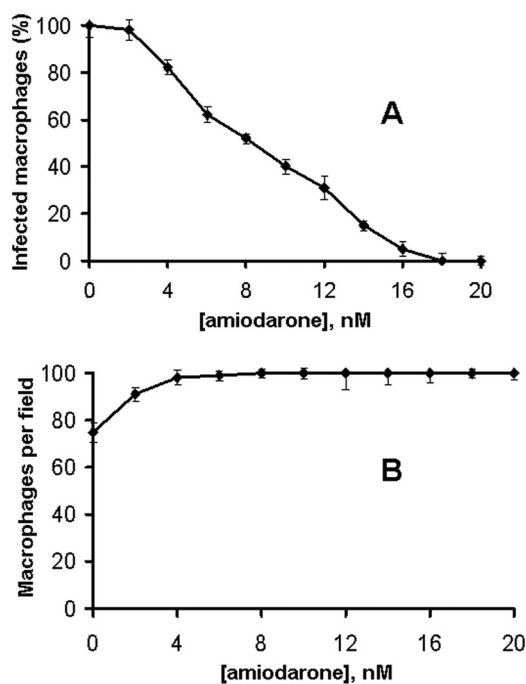


FIG. 3. Effect of amiodarone against intracellular amastigotes of *L. mexicana*. Macrophages infected with *L. mexicana* amastigotes were exposed to different concentrations of amiodarone. The percentage of infected macrophages (A) and the effect on noninfected macrophages (B) were determined at 72 h after the addition of the drug. Experiments were performed at least in triplicate for each condition.

increase in the  $[Ca^{2+}]_i$  was due to the entrance of this cation from the extracellular milieu through the opening of a  $Ca^{2+}$  channel in the plasma membrane or, instead, the cytoplasmic  $Ca^{2+}$  concentration was elevated as a result of the release of the cation from intracellular compartments, we performed the same experiment but in the absence of extracellular  $Ca^{2+}$  (and in the presence of EGTA). Since a similar response was obtained (Fig. 4B and Table 1), the results demonstrate that the increase in the parasite  $[Ca^{2+}]_i$  is produced by the  $Ca^{2+}$  released from intracellular organelles.

**Effect of amiodarone on the mitochondrial electrochemical potential of *L. mexicana*.** Promastigotes were loaded with rhodamine 123, a reagent that senses the electrochemical potential of the mitochondria. Figure 5A shows that amiodarone affected the mitochondrial electrochemical potential in the promastigotes. This result was corroborated when the parasites were exposed to FCCP, a classical protonophore uncoupler that dissipates the mitochondrial  $H^+$  gradient. Accordingly, FCCP generated the same effect as amiodarone (Fig. 5B). These results suggest that, similar to its effect in *T. cruzi*, amiodarone affects the parasite's mitochondria.

**Effect of amiodarone on acidocalcisomes from *L. mexicana*.** When the promastigotes were loaded with acridine orange (Fig. 6), it was observed that amiodarone generated a rapid alkalization of the parasite acidocalcisomes. This effect was similar to the effect generated by the vacuolar  $H^+$  ATPase inhibitor bafilomycin A (Fig. 6A). It was seen that only a partial alkalization was generated by amiodarone and/or bafilomycin A, since nigericin, an electroneutral  $K^+-H^+$  ex-

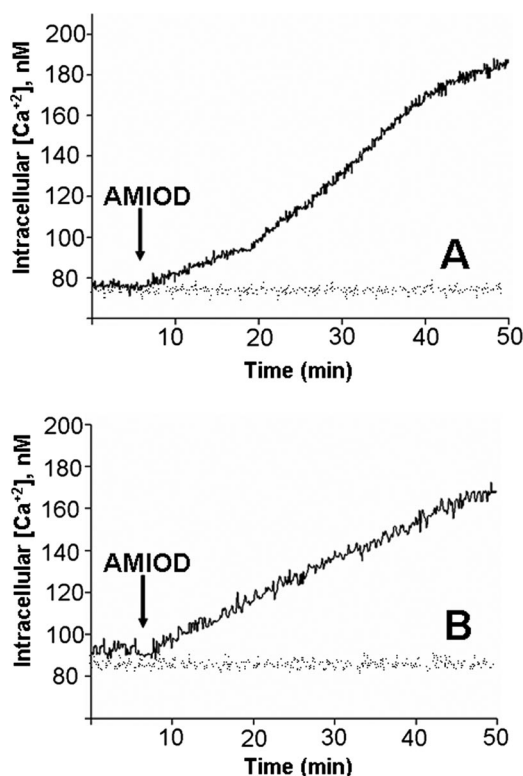


FIG. 4. Effect of amiodarone (AMIOD) on the  $[Ca^{2+}]_i$  of *L. mexicana* promastigotes. Populations of promastigotes ( $4 \times 10^6$  cells in 1.5 ml) of *L. mexicana* were loaded with Fura 2-AM ( $6 \mu M$ ) in a loading buffer (137 mM NaCl, 4 mM KCl, 1.5 mM  $KH_2PO_4$ , 8.5 mM  $Na_2HPO_4$ , 11 mM glucose, 1 mM  $CaCl_2$ , 0.8 mM  $MgSO_4$ , and 20 mM HEPES-NaOH, pH 7.4, containing 2.4 mM probenecid), and the  $[Ca^{2+}]_i$  was calculated as described in Materials and Methods. (A) Effect of  $5 \mu M$  amiodarone on the parasite  $[Ca^{2+}]_i$  in the presence of 2 mM  $Ca^{2+}$ ; (B) effect of  $5 \mu M$  amiodarone on promastigotes loaded with Fura 2 in the absence of external  $Ca^{2+}$ .

changer known to alkalinize the acidocalcisomes from these parasites (17), produced a second alkalinization response of a greater magnitude (Fig. 6A and 6B). These results suggest that the acidocalcisomes also participate in the increase in the parasite's  $[Ca^{2+}]_i$  produced by amiodarone.

**Evaluation of effect of amiodarone on *L. mexicana* promastigotes and amastigotes by confocal microscopy.** The  $[Ca^{2+}]_i$  and the membrane electrochemical potentials of promastigotes (Fig. 7A) and of intracellular amastigotes from an infected macrophage (Fig. 7B) were also evaluated by confocal micros-

TABLE 1. Effect of amiodarone on free cytoplasmic  $Ca^{2+}$  concentration of *L. mexicana* promastigotes

$Ca^{2+}$ concn in extracellular medium	Free cytoplasmic $Ca^{2+}$ concn (nM) <sup>a</sup>	
	Control	Amiodarone <sup>b</sup>
2 mM	$98 \pm 14$ ( $n = 10$ )	$185 \pm 11$ ( $n = 12$ )
0 mM	$94 \pm 18$ ( $n = 8$ )	$172 \pm 11$ ( $n = 9$ )

<sup>a</sup> Free cytoplasmic  $Ca^{2+}$  concentrations were determined with Fura 2, as described in Materials and Methods.

<sup>b</sup> Amiodarone concentration,  $5 \mu M$ .

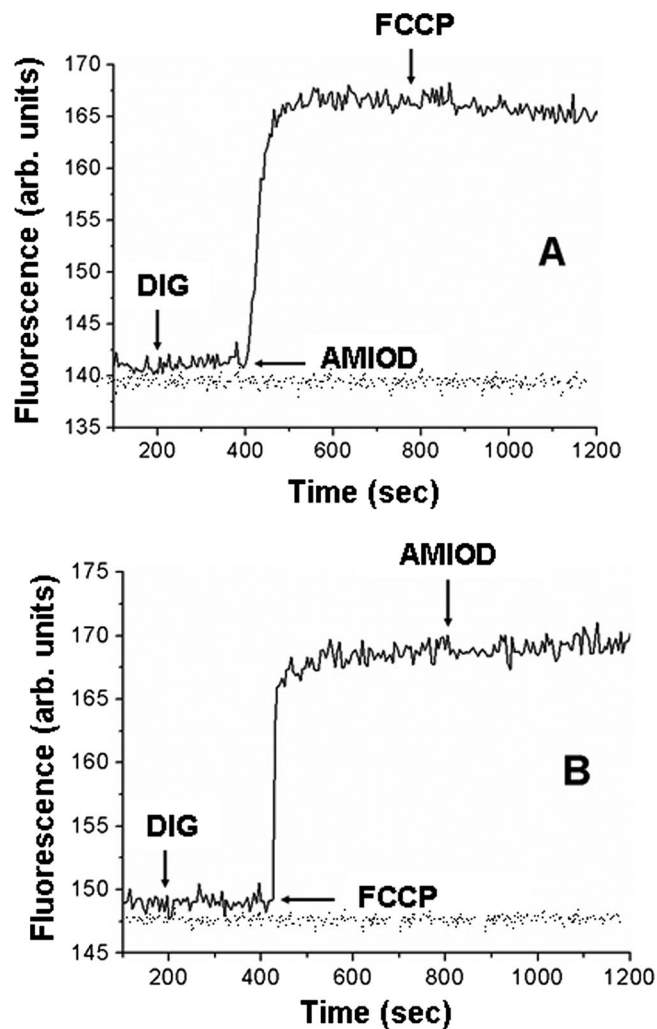


FIG. 5. Action of amiodarone (AMIOD) on the mitochondrial electrochemical potential of *L. mexicana* promastigotes. Parasites ( $4 \times 10^6$  cells in 1.5 ml) were incubated in the presence of rhodamine 123 ( $10 \mu g/ml$ ) for 30 min at room temperature in the same loading buffer (137 mM NaCl, 4 mM KCl, 1.5 mM  $KH_2PO_4$ , 8.5 mM  $Na_2HPO_4$ , 11 mM glucose, 1 mM  $CaCl_2$ , 0.8 mM  $MgSO_4$ , 20 mM HEPES-NaOH, pH 7.4). The cells were permeabilized with  $1 \mu M$  digitonin (DIG). (A) Effect of amiodarone ( $5 \mu M$ ), followed by the addition of FCCP ( $1 \mu M$ ), on the mitochondrial electrochemical potential; (B) effect of FCCP ( $1 \mu M$ ), followed by the addition of amiodarone ( $5 \mu M$ ), on the mitochondrial electrochemical potential.

copy in order to identify the intracellular locus of action involved in the effect of amiodarone in these parasites.

The rhod 2 fluorescence level allowed the measurement of the amount of  $Ca^{2+}$  released from the organelles. The use of this indicator is based on its relatively low affinity for calcium, so that the fluorescence observed is visible, in principle, only in compartments with relatively high calcium concentrations. An increase in the fluorescence is obtained only when calcium is released from intracellular compartments and reaches a concentration high enough to be detected by the indicator, which is also present in the cytoplasm. On the other hand, rhodamine 123 measures energized mitochondria (Fig. 7A and B, top panels). The bottom panel of Fig. 7A shows that amiodarone

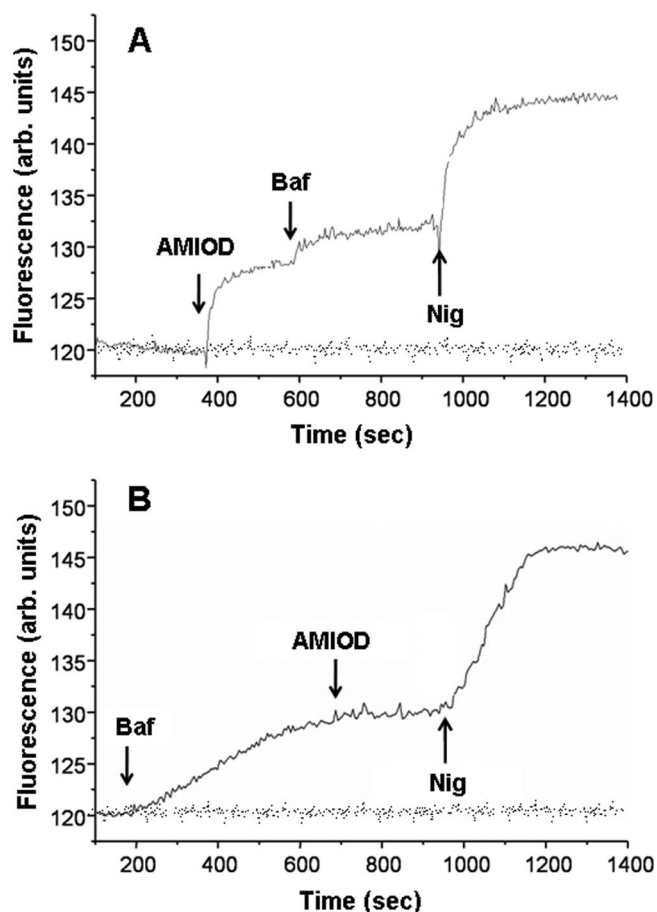


FIG. 6. Effect of amiodarone (AMIOD) on acidocalcisomes from *L. mexicana* promastigotes. Parasites ( $10^9$  cells/ml) were loaded with acridine orange ( $2 \mu\text{M}$ ) in a buffer containing 130 mM KCl, 1 mM  $\text{MgCl}_2$ , 2 mM  $\text{KH}_2\text{PO}_4$ , and 20 mM Tris-HCl, pH 7.4, as described in Materials and Methods. (A) Effect of amiodarone ( $5 \mu\text{M}$ ), followed by the addition of bafilomycin A (Baf;  $5 \mu\text{M}$ ) and then nigericin (Nig;  $2 \mu\text{M}$ ), on the acid level in acidocalcisomes; (B) effect of bafilomycin A ( $5 \mu\text{M}$ ), followed by the addition of amiodarone ( $5 \mu\text{M}$ ) and then nigericin ( $2 \mu\text{M}$ ), on the acid level in acidocalcisomes. arb., arbitrary.

generated the release of  $\text{Ca}^{2+}$  from the mitochondrion toward the parasite cytoplasm (red). It can be also observed that rhodamine 123 was released from the parasite mitochondrion as a product of the collapse of the electrochemical potential of the mitochondrion (green). The merged image (yellow) indicates that the mitochondrion is at least one of the organelles involved in the release of  $\text{Ca}^{2+}$  caused by amiodarone. In fact, acidocalcisomes were hardly seen under these experimental conditions because of their much smaller size. A similar effect was observed with intracellular amastigotes of *L. mexicana* that were loaded under similar conditions and treated with 20 nM amiodarone (Fig. 7B, arrows). Thus, the cytoplasmic  $\text{Ca}^{2+}$  concentration is elevated at the expense of the collapse of the electrochemical potential of the mitochondrion and the concomitant release of  $\text{Ca}^{2+}$ . Figure 7 also shows that amiodarone does not seem to affect the  $\text{Ca}^{2+}$  homeostasis in the host cell, since the cytoplasm remains black, which suggests that  $[\text{Ca}^{2+}]_i$  was not apparently altered (Fig. 7B).

**Effect of amiodarone on synthesis of free sterols in *L. mexicana* promastigotes.** After quantification of the neutral lipid fraction in a gas chromatograph equipped with a mass-sensitive detector, it was possible to determine that control promastigotes contained 67% 5-dehydroepisterol, similar to the values reported by others (27). On the other hand, we also demonstrated in this experiment that these parasites contain 19.2% squalene at the basal level. Interestingly, when promastigotes were exposed to 900 nM amiodarone, which corresponded to the  $\text{EC}_{50}$  of this drug, a drastic reduction (Table 2) in the amount of the main sterol, 5-dehydroepisterol, was seen and the accumulation of a significant amount of squalene (88.46%) was seen. These results strongly suggest that amiodarone affects the squalene epoxidase activity, which is essential for overall sterol biosynthesis (Table 2 and Fig. 8).

## DISCUSSION

The results obtained in this work clearly demonstrate that amiodarone generates a dose-dependent effect on *L. mexicana* promastigote populations. It had an  $\text{EC}_{50}$  (900 nM) that was 1 order of magnitude lower than that which we found for *T. cruzi* epimastigotes ( $\text{EC}_{50}$ ,  $9 \mu\text{M}$ ) (7) and that was also significantly less than the  $\text{EC}_{50}$ s of other drugs with inhibitory actions on *L. mexicana*, such as glibenclamide, an inhibitor of  $\text{K}^+$ ATP[ $\text{r}$ ] channels ( $\text{EC}_{50}$ ,  $54.3 \mu\text{M}$ ), as reported by our group (29). To evaluate the effect of this drug on the clinically relevant stage of the disease caused by *L. mexicana*, we performed experiments with macrophages infected with *L. mexicana* amastigotes. It was possible to observe that amiodarone was 2 orders of magnitude more potent against parasites at this stage ( $\text{EC}_{50}$ , 8 nM) than it was against free promastigotes. Interestingly, the effect of amiodarone on *L. mexicana* amastigotes was more than 2 orders of magnitude less than it was against cells infected with *T. cruzi* amastigotes ( $\text{EC}_{50}$ s, 8 nM and  $2.7 \mu\text{M}$ , respectively). It was also interesting to observe that the drug did not affect the viability of the host cells.

Similar to the effect on epimastigotes and amastigotes from *T. cruzi* (7), amiodarone increased the  $[\text{Ca}^{2+}]_i$  in the promastigotes and amastigotes of *L. mexicana* at least in part by the same mechanism that it does in *T. cruzi* epimastigotes and amastigotes, since we could demonstrate that this drug induces the release of  $\text{Ca}^{2+}$  from the unique mitochondrion present in these parasites. This was demonstrated in several ways. First, we showed that amiodarone can simulate the effect of the protonophore FCCP on the release of rhodamine 123 from the mitochondrial internal membrane, which indicates the collapse of the electrochemical potential. This was first performed with cell populations (Fig. 5). Second, this was demonstrated with individual cells by the use of confocal microscopy with isolated promastigotes and amastigotes present in infected macrophages. This mechanism of action of amiodarone in *L. mexicana* (and similar to that in *T. cruzi*) is different from that in fungi reported by Gupta et al. (19) and Courchesne and Ozturk (10), since those authors observed that even though in these organisms the drug also acted by disrupting  $\text{Ca}^{2+}$  homeostasis, the increase in the  $[\text{Ca}^{2+}]_i$  was mediated by an MD-1 caffeine-sensitive  $\text{Ca}^{2+}$  channel in the plasma membrane.

Remarkably, in this study we also demonstrated that amio-

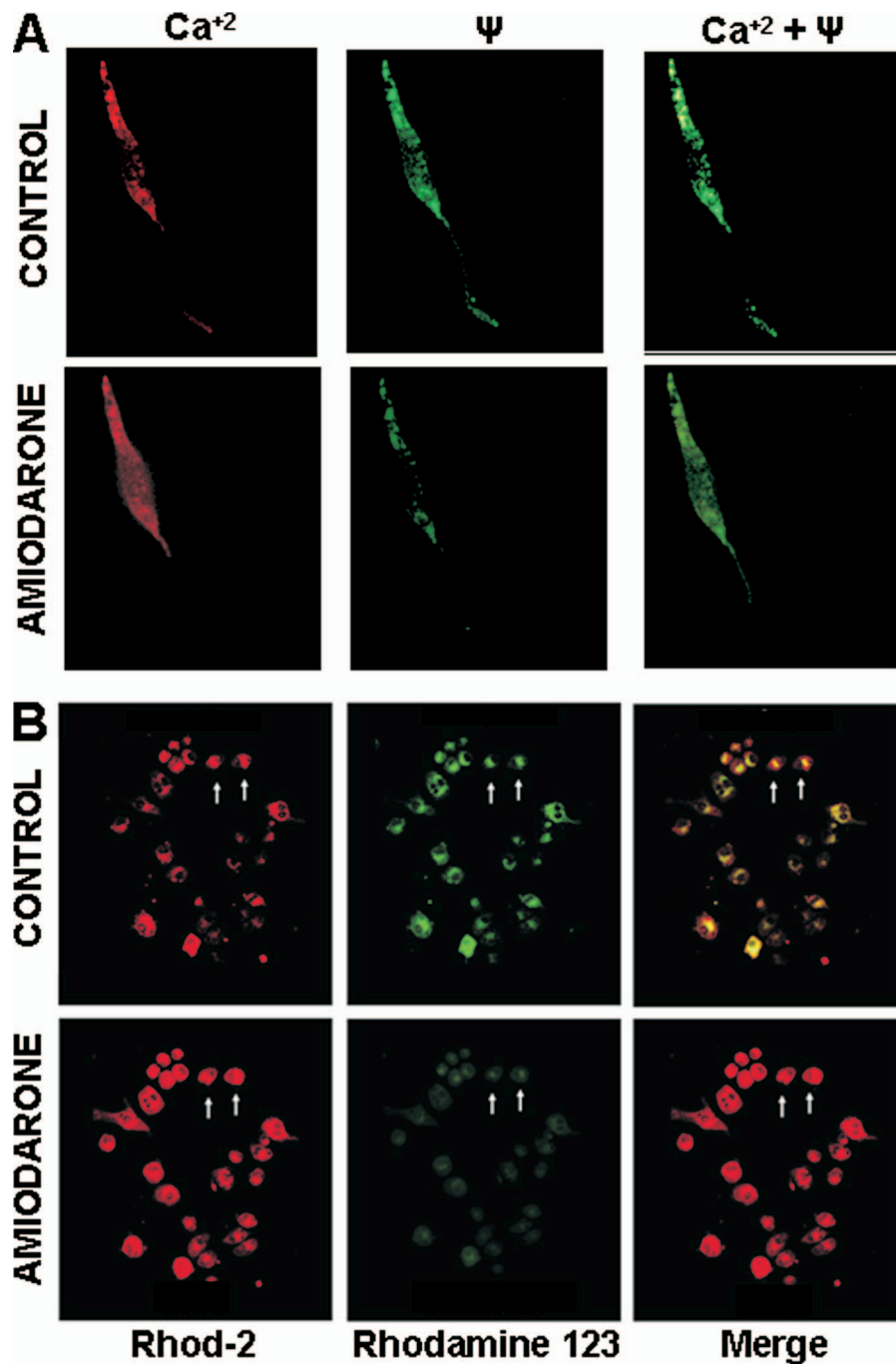


FIG. 7. Determination of the intracellular site of action of amiodarone in promastigotes and amastigote-infected macrophages from *L. mexicana* by confocal microscopy. (A) *L. mexicana* promastigotes were loaded with rhod 2 (red) and rhodamine 123 (green), as described in Materials and Methods. The top row shows control cells not exposed to amiodarone, while the bottom row shows cells treated with 5  $\mu$ M amiodarone for 20 min. (B) Amastigotes inside an infected macrophage loaded with rhod 2 (red) and rhodamine 123 (green), as described in Materials and Methods. The top row shows a control cell not exposed to amiodarone, while the bottom row shows the same cell treated with 20 nM amiodarone after 20 min.  $\Psi$ , electrochemical potential.

darone is able to affect another important organelle characteristic of all trypanosomatids, namely, the acidocalcisomes. Our results clearly demonstrated that, besides the mitochondrion, these organelles are also targeted by amiodarone, since the drug induced a rapid alkalinization very similar to that induced by bafilomycin A, a specific inhibitor of the vacuolar H<sup>+</sup>

ATPase responsible for acidification and subsequent Ca<sup>2+</sup> accumulation characteristic of these organelles (16, 17). We did not test for this possibility in our previous study with *T. cruzi* (7). Taken together, these results strongly support the idea that both mitochondria and acidocalcisomes are involved in the increment of the [Ca<sup>2+</sup>]<sub>i</sub> induced by amiodarone in *L. mexi-*

TABLE 2. Effect of amiodarone on the biosynthesis of free sterols in *L. mexicana* promastigotes

Sterol	Retention time (min)	Mass % after treatment with:	
		Control	Amiodarone
Exogenous cholesterol	24.9	13.8	11.54
Endogenous 14-desmethyl ergosta-5,7,24(24 <sup>1</sup> )-trien-3 $\beta$ -ol (5-dehydroepisterol)	28.9	67.0	ND <sup>a</sup>
Ergosta-7,24(24 <sup>1</sup> )-dien-3 $\beta$ -ol (episterol)	29.2	12.2	ND
Cholesta-8,24-dien-3 $\beta$ -ol (zymosterol)	25.9	ND	ND
Endogenous 14-methyl lanosterol	30.8	3.0	ND
Squalene epoxide	22.6	4.0	ND
Squalene	20.5	19.2	88.46

<sup>a</sup> ND, not detected.

*cana*, and this could be an argument for the potent effect of amiodarone on the viability of *L. mexicana*.

In this study, we also demonstrated that amiodarone inhibits the free sterol biosynthesis pathway in *L. mexicana* promastigotes. Indeed, we were able to show the accumulation of large

amounts of squalene after amiodarone treatment, which suggests the inhibition of squalene epoxidase (Fig. 8), which is essential for the biosynthesis of sterols (31). Accordingly, the levels of 5-dehydroepisterol, the most abundant sterol present in this parasite (27), fell dramatically upon addition of the drug. This result is, in principle, different from that reported in our previous work with *T. cruzi* (7), in which we found that amiodarone inhibits oxidosqualene cyclase. However, it is perfectly conceivable that amiodarone simultaneously inhibits squalene epoxidase and oxidosqualene cyclase in *L. mexicana*, since the accumulation of squalene observed would prevent the later accumulation of squalene epoxide. Moreover, these parasites being different, it is possible that squalene epoxidase behaves differently in *L. mexicana* compared to *T. cruzi*. Moreover, these two enzymes are contiguous in the sterol biosynthetic pathway in both parasites. For this reason, both substrates, squalene and squalene epoxide, have very similar structures and sizes and differ only by the presence of an epoxide moiety in *T. cruzi*. Thus, we consider it plausible that amiodarone could inhibit both enzymes. However, by the experimental approach that we have used, it can only be suggested that the first enzyme is inhibited.

In any case, the inhibition of the sterol synthesis pathway

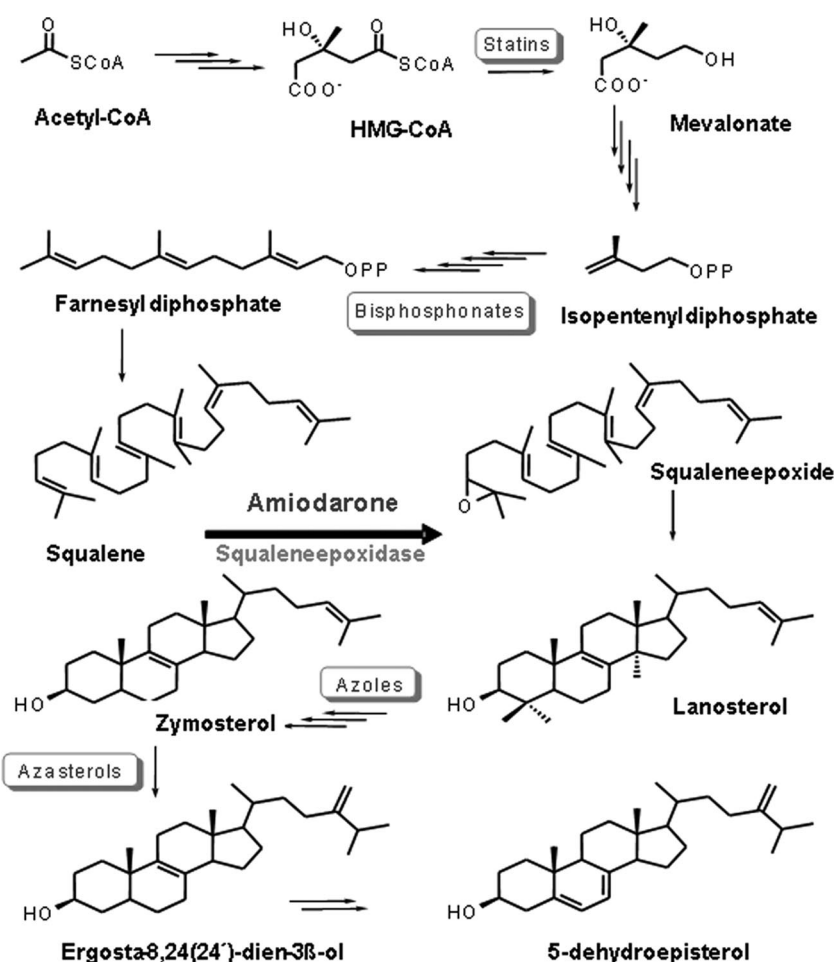


FIG. 8. Part of the sterol biosynthetic pathway in *Leishmania mexicana* showing the possible site of action of amiodarone. Acetyl-CoA, acetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

could be a second argument that explains the effect of amiodarone on the viability of *L. mexicana*.

The results obtained in the present work point to a more general effect of amiodarone against trypanosomatids, which was indeed expected, since the mechanisms of action reported in *T. cruzi* imply that this family of parasites has targets that are common, such as the mitochondria, acidocalcisomes, and sterol biosynthesis. The possible therapeutic use of amiodarone for the treatment of leishmaniasis was addressed in a recently published case report (26). In that report, the authors postulated that a human patient with leishmaniasis had been cured by the use of amiodarone. These results also open the possibility that amiodarone may be used in combination with other drugs, such as miltefosine and/or inhibitors of sterol biosynthesis, and this possibility needs to be investigated.

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