Tomatidine promotes the inhibition of 24-alkylated sterol biosynthesis and mitochondrial dysfunction in *Leishmania amazonensis* promastigotes

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(*Received 4 October 2011; revised 8 December 2011, 31 January and 23 February 2012; accepted 27 February 2012; first published online 1 May 2012*)

SUMMARY

Leishmaniasis is a set of clinically distinct infectious diseases caused by *Leishmania*, a genus of flagellated protozoan parasites, that affects ~12 million people worldwide, with ~2 million new infections annually. Plants are known to produce substances to defend themselves against pathogens and predators. In the genus *Lycopersicon*, which includes the tomato, *L. esculentum*, the main antimicrobial compound is the steroidal glycoalkaloid α-tomatine. The loss of the saccharide side-chain of tomatine yields the aglycone tomatidine. In the present study, we investigated the effects of tomatidine on the growth, mitochondrial membrane potential, sterol metabolism, and ultrastructure of *Leishmania amazonensis* promastigotes. Tomatidine (0·1 to 5 μM) inhibited parasite growth in a dose-dependent manner ($IC_{50} = 124 \pm 59$ nM). Transmission electron microscopy revealed lesions in the mitochondrial ultrastructure and the presence of large vacuoles and lipid storage bodies in the cytoplasm. These structural changes in the mitochondria were accompanied by an effective loss of mitochondrial membrane potential and a decrease in ATP levels. An analysis of the neutral lipid content revealed a large depletion of endogenous 24-alkylated sterols such as 24-methylene-cholesta-5, 7-dien-3β-ol (5-dehydroepisterol), with a concomitant accumulation of cholesta-8, 24-dien-3β-ol (zymosterol), which implied a perturbation in the cellular lipid content. These results are consistent with an inhibition of 24-sterol methyltransferase, an important enzyme responsible for the methylation of sterols at the 24 position, which is an essential step in the production of ergosterol and other 24-methyl sterols.

Key words: *Leishmania*, tomatidine, sterols, 24-sterol methyltransferase, mitochondria, trypanosomatids.

INTRODUCTION

Leishmaniasis is a complex set of neglected tropical diseases caused by more than 20 species of the *Leishmania* genus of protozoan parasites. These parasites are transmitted by infected female sandflies, *Phlebotomus* spp. (in the Old World) and *Lutzomyia* spp. (in the Americas) (Desjeux, 1996). The World Health Organization (WHO) estimates that ~12 million people are currently infected, with ~1–2 million new cases occurring every year (World Health Organization, 2011). There are 3 main clinical manifestations of leishmaniasis distributed worldwide: cutaneous, mucocutaneous, and visceral forms. In Brazil, *Leishmania amazonensis* causes a cutaneous form of the disease. In cases involving immune system failure, the disease can evolve into diffuse cutaneous leishmaniasis, which is severe and difficult to treat (Cuba-Cuba *et al*. 1985).

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The chemotherapeutic strategy currently employed for leishmaniasis uses pentavalent antimonial formulations as a first line of therapy; pentamidine, miltefosine, and amphotericin B are the second-line medications for resistant cases (Croft *et al*. 2006). However, these drugs are very toxic and can generate severe side-effects, and the number of resistant cases has increased significantly (Croft *et al*. 2005). Thus, there is an urgent need to develop new and safer drugs that are more accessible and less toxic.

Plants produce over 100 000 diverse, low-molecular-mass natural products known as secondary metabolites (Dixon, 2001). Tomatoes (*Lycopersicon esculentum*), a major food source for humans, accumulate a variety of secondary metabolites including phenolic compounds, phytoalexins, protease inhibitors, and glycoalkaloids. These molecules protect the plant against pathogens and predators. *Lycopersicon* species synthesize α-tomatine, a glycoalkaloid with 4 carbohydrate residues attached to the 3-OH group of the aglycone tomatidine (Fig. 1). Tomatine compounds are present in every part of the tomato plant. Immature green tomatoes contain up to 500 mg of tomatine per kg fresh fruit

doi:10.1017/S0031182012000522

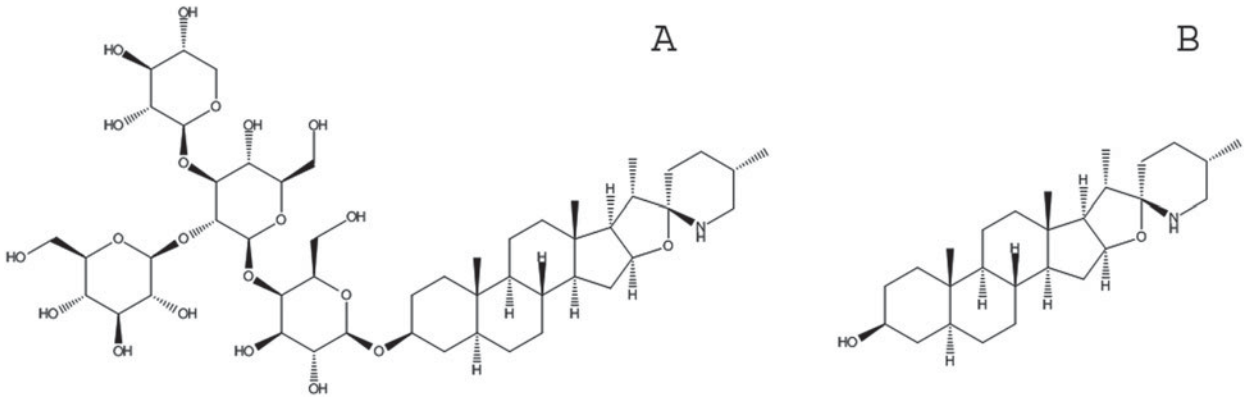

Fig. 1. Molecular structure of α-tomatine (A) and tomatidine (B).

weight. As the tomato ripens, the compound is degraded until the plant reaches maturity, at which time the levels in red tomatoes are reduced to ~5 mg of tomatine per kg fresh fruit weight. However, tomatidine is rarely found in the tomato fruit because tomatine degradation products are channeled into carotenoids and chlorophyll (Friedman, 2002). Tomatine forms strong, insoluble complexes with plant and animal sterols, which results in the disruption of cell membranes followed by the release of cytoplasmic contents and cell death. In contrast, the aglycone tomatidine does not interact with sterols (Blankemeyer *et al*. 1997; Friedman, 2002). It has also been reported that tomatine inhibits *Leishmania* trypanothione synthase *in vitro* (Saudagar and Dubey, 2011).

The aglycone tomatidine, produced by hydrolysis of the glycoalkaloid α-tomatine, potently inhibits ergosterol biosynthesis in fungi such as *Saccharomyces cerevisiae* (Simons *et al*. 2006). Structural analogues of tomatidine such as solanidine and solasodine, which are naturally present in potatoes and eggplant, respectively, have been described as growth inhibitors of the yeast-like alga *Prototheca wickerhamii*; these compounds demonstrated the ability to inhibit sterol biosynthesis *in vivo* and inhibit C24-sterol methyltransferase in a cell-free preparation (Mangla and Nes, 2000).

Sterols are the major structural components of cell membranes and stabilize their structure by interacting with the fatty acyl moieties of membrane phospholipids to affect membrane fluidity. The sterol contents of microorganisms such as fungi and parasites of the Trypanosomatidae family differ from those of mammalian cells because of the predominant presence of ergosterol, episterol, and other 24-methyl sterols, which are completely absent in the host cells (de Souza and Rodrigues, 2009). During ergosterol biosynthesis, $\Delta^{24(25)}$-sterol methyltransferase incorporates a methyl group into the sterols at position 24. This enzyme is not expressed in mammalian cells; thus, it is an important target for the development of new therapeutics for diseases caused by these microorganisms. As previously reported, the main sterols in *Leishmania* are episterol and 5-dehydroepisterol, with negligible traces of ergosterol (Goad *et al*. 1984; Haughan *et al*. 1995; reviewed by Roberts *et al*. 2003; de Souza and Rodrigues, 2009).

In the present study, we evaluated the action of tomatidine on the growth, sterol metabolism and ultrastructure of *L. amazonensis* promastigotes. Tomatidine inhibited parasite growth at submicromolar concentrations and affected mitochondrial membrane potential ($\Delta\Psi_m$) and ultrastructure, potentially as a consequence of significant changes in sterol content and metabolism.

MATERIALS AND METHODS

Parasites

The MHOM/BR/75/Josefa strain of *L. amazonensis* was used; this strain was isolated from a patient with diffuse cutaneous leishmaniasis by C. A. Cuba-Cuba (Universidade de Brasília, Brazil). The strain was maintained by Balb/C footpad inoculation and, in the case of promastigotes, axenically cultured at 25 °C in Warren's medium (brain heart infusion plus hemin and folic acid; Warren, 1960) supplemented with 10% fetal bovine serum. Cell densities were determined daily on a haemocytometer using light microscopy.

Tomatidine

Tomatidine was acquired from Sigma Chemical Co. Stock solutions of the drug (2·5 mM) were prepared in dimethyl sulfoxide (DMSO). Thus, at the final tomatidine concentrations, the final DMSO concentrations in the assay media never exceeded 0·6% (vol/vol).

Growth curve

The cultures were initiated with a cell density of 1×10^6 cells/ml and drug was added 24 h later when the number of parasites in the cultures reached approximately $5-10 \times 10^6$ cells/ml. The cell density

https://doi.org/10.1017/S0031182012000522 Published online by Cambridge University Press

was determined daily on a haemocytometer using light microscopy. The same protocol of tomatidine treatment was used in all of the experiments in the present study. IC_{50} values were calculated using the Regression Wizard in Sigma Plot (version 10).

Nile red staining

After 48 h of growth in the presence of 5 μM tomatidine, *L. amazonensis* cells were harvested, washed twice with phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7·2, and 150 mM sodium chloride) and fixed in 4% freshly prepared formaldehyde in PBS for 20 min at room temperature. After washing twice in PBS, the cells were incubated in 10 μg/ml 9-diethylamino-5H-benzo[α]-phenoxazine-5-one (Nile red; acquired from Molecular Probes), a fluorescent dye with an affinity for phospholipids and neutral lipids (Greenspan *et al*. 1985), for 30 min at room temperature protected from light. The parasites were washed in PBS, pH 7·2. An aliquot of each cell suspension was collected and adhered to 0·1% poly-L-lysine-coated glass cover slips. Samples were mounted in 0·2 M *n*-propylgallate in glycerol:PBS (9:1) and yellow-gold fluorescence imagery of the neutral lipid inclusions was acquired using the appropriate filters (excitation, 485 nm; emission, 535 nm) in a Zeiss Axioplan epifluorescence microscope coupled to an Olympus X30 CCD camera. The images were further processed using Adobe Photoshop CS2 (Adobe Systems, Inc.).

Sterol analysis

To evaluate the effects of tomatidine on the sterol composition of the promastigotes, the total lipids from the control cells and the drug-treated cells (5×10^7 of each) were washed with PBS and extracted with a mixture of methanol:chloroform:water (2:1:0·8) in accordance with the method of Bligh and Dyer (1959). The samples were dried under a stream of dry N_2 and saponified with 25% ethanolic KOH for 1 h at 85 °C. Subsequently, samples were extracted with hexane and evaporated under a dry N_2 stream. For the acylation reaction, samples were re-suspended in a mixture containing 30 μl of acylating reagent and 30 μl of pyridine and incubated for 1 h at 65 °C. Gas chromatography with mass spectral (GC/MS) analysis was performed in a Shimadzu GCMS-QP2010 Plus system using a Restek® Rtx®-5MS (5% phenyl/95% dimethyl polysiloxane) column (30 m × 0·25 mm × 0·25 μm). The injector temperature was set at 250 °C. The column temperature was raised to 170–250 °C at a heating rate of 20 °C min^{-1} and then to 250–280 °C at a heating rate of 5 °C min^{-1} and held at 280 °C for 20 min. Helium was used as the carrier gas at a linear velocity of 41·9 cm s^{-1}. A sample volume of 1 μl was injected into the chromatograph. Electro-ionization (EI-70 eV) and a quadrupole mass analyser were utilized for scans from 50 to 700 amu. The interface temperature was set at 230 °C and the ion source was set at 200 °C. The components were identified by comparing their mass spectra with spectra in the NIST05 library stored in the computer controlling the mass spectrometer and the critical analysis of fragmentation patterns. Retention indices were also used to confirm the identity of the peaks in the chromatograms.

The effect of tomatidine on the uptake of ^{125}I-LDL

To evaluate the endocytosis of cholesterol, 1×10^7/ml cells were grown in the presence of 1% fetal calf serum, which favours the uptake of ^{125}I-labelled LDL (^{125}I-LDL). Control and drug-treated promastigotes were incubated with ^{125}I-LDL and observed after 48 h of incubation. The cells were then washed 3 times with PBS and the amount of radioactivity in each pellet was counted in a gamma counter. ^{125}I-LDL was obtained by iodination with ^{125}I-sodium iodide (280 μCi/mg protein; CNEN, São Paulo, Brazil) using Iodogen (100 μg/mg protein; Sigma-Aldrich Co, USA) following the manufacturer's instructions. To remove the free iodide, the reaction mixture was passed through 3 Sephadex G-50 spin columns (Sigma-Aldrich Co, USA).

Electron microscopy

Control cells and cells treated with tomatidine for 48 h (8×10^6) were fixed for 3 h at 4 °C in 2·5% glutaraldehyde (Sigma Chemical Co.) in 0·1 M cacodylate buffer (pH 7·2). After fixation, the cells were post-fixed for 30 min in a solution containing 1% OsO_4 and 1·25% potassium ferrocyanide in 0·1 M cacodylate buffer. The cells were then washed in the same buffer, dehydrated in acetone, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. The cells were observed in a Zeiss 900 electron microscope.

Effects of tomatidine on the mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) is an important parameter of mitochondrial function that is used as an indicator of cell health. The $\Delta\Psi_m$ of control cells and cells treated with tomatidine for 48 h were measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; obtained from Molecular Probes/Invitrogen). This lipophilic cationic probe accumulates in the mitochondrial matrix according to the membrane potential. In healthy cells with a high $\Delta\Psi_m$, JC-1 spontaneously forms complexes known as

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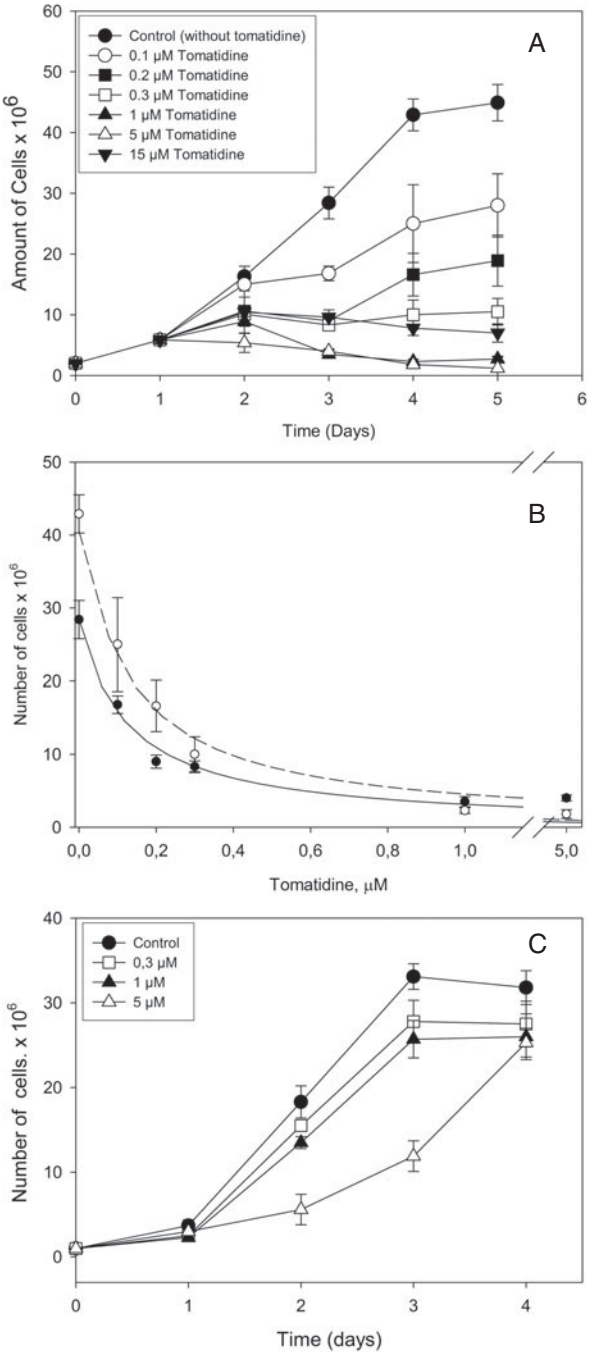

Fig. 2. (A) Growth curve of *Leishmania amazonensis* in the presence of tomatidine. Promastigotes (1×10^6/ml) were grown in Warren's medium supplemented with 10% fetal bovine serum at 25 °C. After 24 h of growth, tomatidine was added at the indicated concentrations. No tomatidine was added to the control sample. Each point represents the mean ± S.E. of 3 independent experiments. (B) Tomatidine sensitivity. The points for days 3 (closed circles) and 4 (open circles) (48 and 72 h in the presence of tomatidine, respectively) were plotted against tomatidine concentration and fitted with the inhibition equation $n = N/(1 + [\text{tomatidine}]/K_{50})$, where N is the number of cells in the absence of tomatidine, K_{50} is the tomatidine concentration yielding 50% inhibition, and n is the cell concentration at any inhibitor concentration. (C) Cell viability after tomatidine treatment. The cells were incubated as in (A) with different tomatidine concentrations, and after 48 h in contact with the drug, they were harvested, washed twice with 2 ml of fresh growth medium, re-suspended to 1×10^6 cells/ml and allowed to grow until day 4 in the absence of tomatidine. Each point represents the mean ± S.E. of 3 independent experiments.

J-aggregates, showing intense red fluorescence (emission at 590 nm). In apoptotic or unhealthy cells with a low $\Delta\Psi_m$, JC-1 remains in its monomeric cytosolic form and shows only green fluorescence (emission at 530 nm) (Cossarizza *et al.* 1993; Mukherjee *et al.* 2002; Mehta and Shaha, 2004). The cells (1×10^7 cells/ml) were washed twice with PBS and re-suspended in a reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES/K^+ pH 7·2, 2 mM KH_2PO_4, 1 mM $MgCl_2$, and 500 μM EGTA. JC-1 (10 μg/ml) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 2 μM; obtained from Sigma Chemical Co.) were added at the specific times indicated in the chart. FCCP is an effective protonophore that causes dissipation of the mitochondrial membrane potential (Johnson *et al.* 1981). Each set of curves was repeated 3 times and the figure is representative of these experiments.

The effects of tomatidine on cytosolic ATP content

The ATP contents after tomatidine treatment for 1 and 48 h were determined by the luciferin/luciferase method, as previously described (Yang *et al.* 2002). Cells ($2{\cdot}5 \times 10^6$) were harvested and washed twice with a buffer containing 116 mM NaCl, 5·4 mM KCl, 0·8 mM $MgSO_4$, 50 mM HEPES/KOH pH 7·2, and 5·5 mM glucose. The cell suspension was then incubated in boiling water for 5 min and cooled and aliquots of 10 μl were taken for the determination of ATP using the luciferase ATP assay kit (Promega). The amount of ATP in each sample was calculated from a standard curve prepared with ATP and expressed as a percentage of the ATP in the control cells.

Statistical analysis

The data were expressed as means ± S.E. (standard error) from at least 3 independent experiments. The statistical significance was calculated using a paired *t*-test. P values less than 0·05 were considered statistically significant.

RESULTS

The effect of tomatidine on the growth of Leishmania amazonensis *promastigotes*

To determine whether tomatidine promoted changes in cell proliferation, we constructed growth curves

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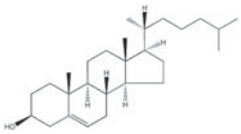
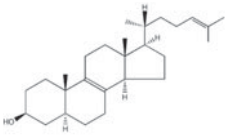
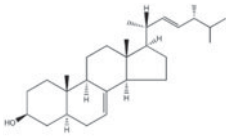
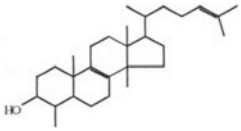
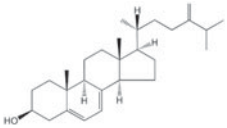
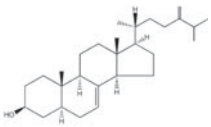
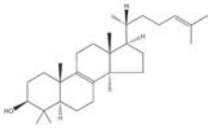
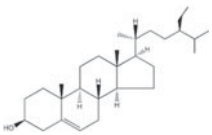
Table 1. Effects of tomatidine on the free sterol contents of *Leishmania amazonensis* promastigotes

(Total lipids were extracted from the control cells and cells treated with tomatidine for 48 h. The extracted lipids were saponified and analysed by gas chromatography and mass spectrometry. For each condition, lipids corresponding to $1{\cdot}7 \times 10^6$ cells were injected. The compositions are expressed as mass percentages. ND, not detected. The total areas under the peaks (representing 100%) are in millions of arbitrary units; the areas are 128, 143 and 195 for the parasites treated with 0, 0·3 and 1 μM tomatidine, respectively.)

Sterol		Retention time (min)	m/z (%)	Base peak	Control %	Tomatidine 0·3 μM (%)	Tomatidine 1 μM (%)
Cholest-5-en-3β-ol (exogenous cholesterol)		20·6	458 (50)	129	8·0	20·6	17·5
Cholesta-8,24-dien-3β-ol (zymosterol)		22·1	456 (100)	456	<1	53·2	58·4
Cholesta-7,22-dien-3β-ol		22·8	456 (19)	343	ND	7·5	3·1
4,14-Dimethyl-zymosterol		23·7	484 (58)	379	ND	4·5	5·5
14-Desmethyl ergosta-5,7,24(24^1)-trien-3β-ol (5-dehydroepisterol)		23·9	468 (28)	363	62·5	ND	ND
Ergosta-7,24(24^1)-dien-3β-ol (episterol)		24·3	470 (8)	343	13·1	ND	ND
Lanosta-8,24-dien-3β-ol (lanosterol)		25·1	498 (38)	393	0·2	3·6	3·7
Stigmast-5-en-3β-ol (β-sitostertol)		25·35	486 (43)	129	17·6	6·6	7·4
Unidentified steroid		25·85	484 (13)	386	9·8	3·5	3

for cells in the presence and absence of the drug (Fig. 2A). A significant reduction in the growth rate was observed during the treatment, which was dose-dependent (Fig. 2B) up to the concentration of 5 μM. The IC_{50} values obtained after 48 and 72 h of treatment were 117 ± 30 and 124 ± 59 nM, respectively. A decrease in swimming mobility was also observed together with the reduction in the growth. In the presence of DMSO alone, the solvent used to prepare the tomatidine solutions, there was a small decrease in cell proliferation, less than 10% at 0·6% DMSO, the final concentration in a 15 μM tomatidine treatment (data not shown), which was not considered to be significant.

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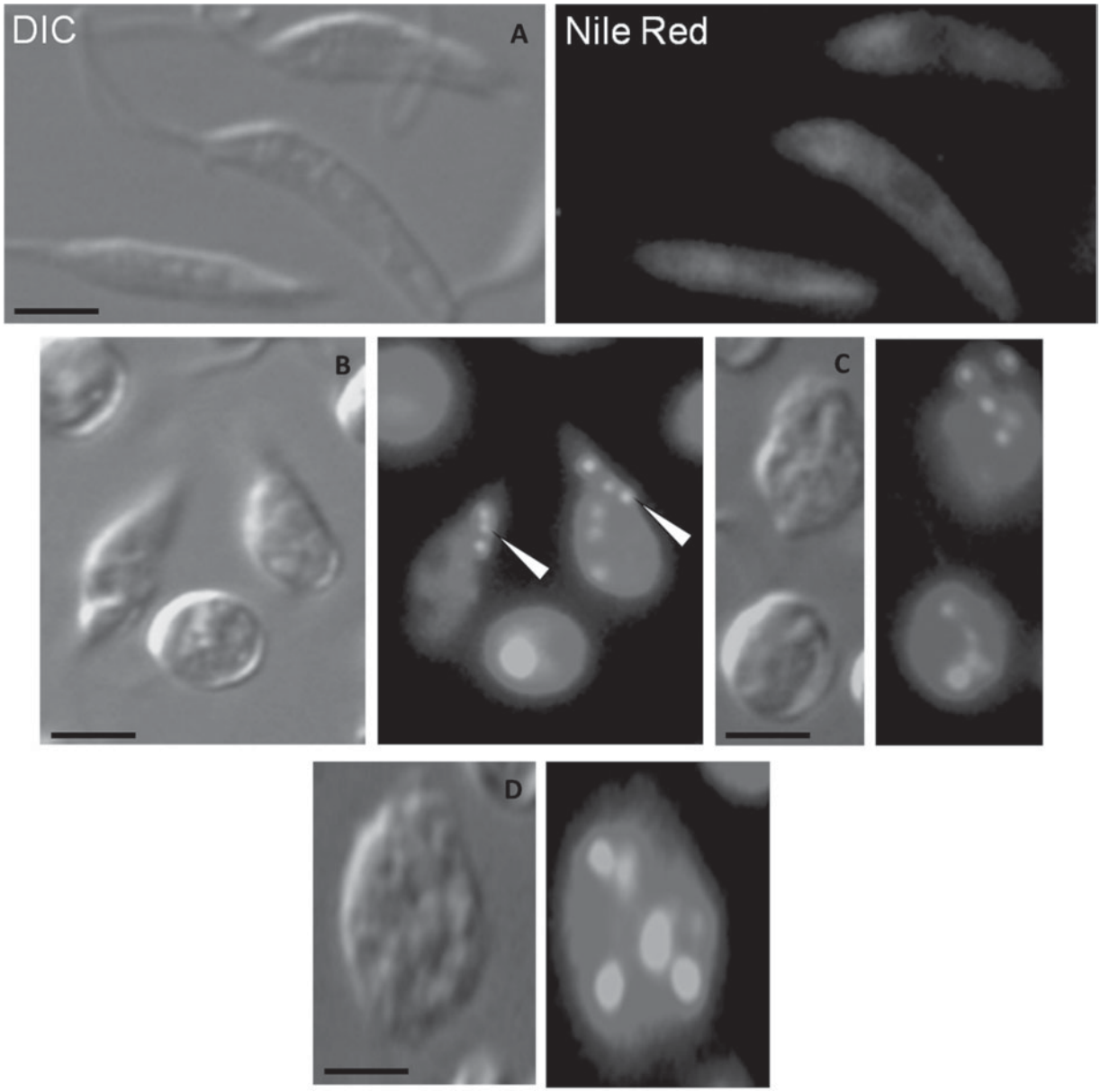

Fig. 3. Fluorescence microscopy of *Leishmania amazonensis* labelled with Nile red. The parasites were grown in the absence (A) or presence of 5 μM tomatidine (B–D) for 72 h. The cells were harvested, fixed and allowed to equilibrate with the fluorescent lipophilic dye Nile red, as described in the Materials and Methods section. The panels on the left show differential interferential contrast images (DIC) and those on the right show fluorescent images. The arrows point to the vacuoles that have Nile red accumulation. Scale bars: (A) 3·5 μm; (B) 3·0 μm; (C) 3·0 μm; (D) 1·7 μm.

After being exposed to various tomatidine concentrations, *L. amazonensis* promastigotes promptly recovered their growth capacity after the removal of the inhibitor (Fig. 2C) after which the cells needed 4 days to reach the growth level of the control cells.

Lipid composition

To evaluate the effects of tomatidine on the sterol content of *L. amazonensis* promastigotes, sterols from control and drug-treated cells were extracted and quantitatively analysed by gas chromatography/mass spectrometry (GC/MS). Table 1 shows that cells exposed to tomatidine for 48 h displayed a large decrease in the levels of endogenous 24-alkylated sterols, mainly 24-methylene-cholesta-5,7-dien-3β-ol (5-dehydroepisterol) but also episterol and β-sitosterol, and an increase in the levels of endogenous 24-dealkylated sterols, mainly cholesta-8,24-dien-3β-ol (zymosterol), cholesta-7,22-dien-3β-ol, 4,14-dimethyl-zymosterol, and lanosterol. In addition, the drug-treated cells showed a higher percentage of exogenous cholesterol than the control cells and a decrease in the level of an unidentified sterol. The sterol composition of control cells is almost identical to those described in the literature for *L. amazonensis* (Rodrigues *et al*. 2002; Lorente *et al*. 2004). On the

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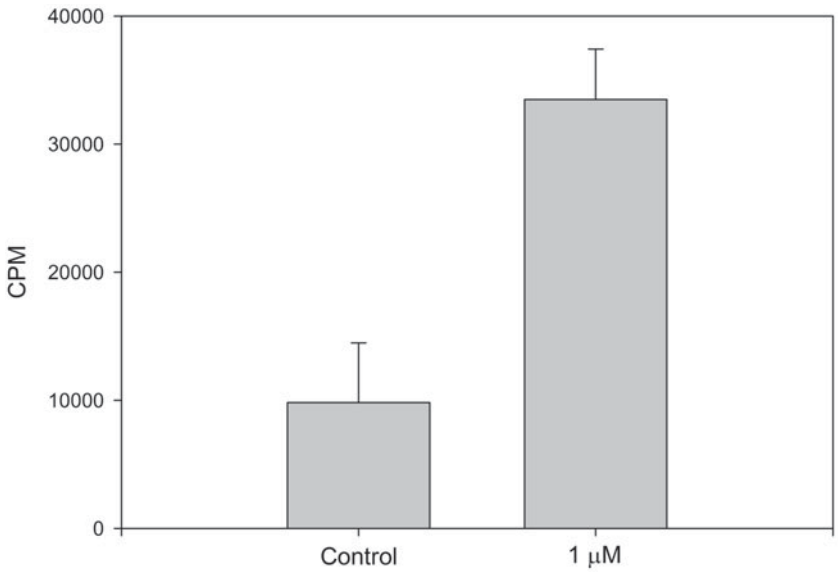

Fig. 4. ^{125}I-LDL uptake by *Leishmania amazonensis* is increased in cells treated with tomatidine. Control and drug-treated cells were incubated with ^{125}I-LDL at 24 h after growth initiation; after 48 h, the cells were washed 3 times with PBS. The amount of radioactivity in each pellet was counted in a gamma counter. CPM, counts per minute. The points represent the means ± S.E., $n = 3$.

other hand, the sterols that accumulate after the tomatidine treatment are fully compatible with the inhibition of the SMT (Rodrigues *et al*. 2002).

Neutral lipids in vesicles

The increase of external cholesterol suggested the presence of lipid accumulation in the tomatidine-treated parasites. To confirm this observation, the accumulation of the fluorescent dye Nile red was studied. This dye is an excellent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy. It has intense fluorescence in organic solvents and hydrophobic lipids; however, the fluorescence is fully quenched in water. Therefore, Nile red acts as a fluorescent hydrophobic probe, particularly when the cells are examined for yellow-gold fluorescence (Greenspan *et al*. 1985). After loading with Nile red, the control cells showed a poor, diffuse orange fluorescence, whereas the tomatidine-treated cells displayed intense yellow fluorescence concentrated in rounded organelles (Fig. 3).

The effect of tomatidine on the uptake of ^{125}I-LDL by L. amazonensis

To verify possible differences in exogenous lipid uptake, control and tomatidine-treated promastigotes were cultivated with ^{125}I-LDL in medium with 1% fetal calf serum, which favours ^{125}I-LDL uptake. Fig. 4 demonstrates that after 48 h of treatment, 1 μM tomatidine was sufficient to significantly increase the uptake of ^{125}I-LDL. This increased uptake was probably responsible for the accumulation of the exogenous cholesterol observed in the sterol analysis (Table 1).

Ultrastructural alterations of L. amazonensis *promastigotes induced by tomatidine*

Transmission electron microscopy was used to reveal the ultrastructural lesions in the organelles and the structures of the drug-treated promastigotes. Figure 5A shows a control promastigote with normal ultrastructure of the mitochondrion (M), nucleus (N), plasma membrane and Golgi complex (arrow). One of the most evident alterations in the tomatidine-treated promastigotes was observed in the mitochondria, which had intense swelling (Fig. 5B) with a loss of matrix contents (Fig. 5C). Changes in the mitochondrial membrane were also observed at lower concentrations of tomatidine (0·3 μM) (Fig. 5B, arrowhead). At this concentration, we observed the presence of small vesicles inside the flagellar pocket (Fig. 5C) and large vacuoles similar to multivesicular bodies (Fig. 5D, arrowhead).

At higher tomatidine concentrations (5 and 15 μM), several alterations were observed. Figure 6A shows an autophagosome (A) near a mitochondrial profile, which has many cristae (arrow). Fig. 6B shows the presence of several lipid storage bodies, which were sometimes wrapped in an endoplasmic reticulum-like structure (arrowhead). Figure 6C and D show the presence of several vesicles containing part of the cytoplasm and myelin-like figures inside the flagellar pocket (star). After treatment with 15 μM tomatidine, the mitochondria were swollen with a loss of the mitochondrial matrices (Fig. 6E–F). We also

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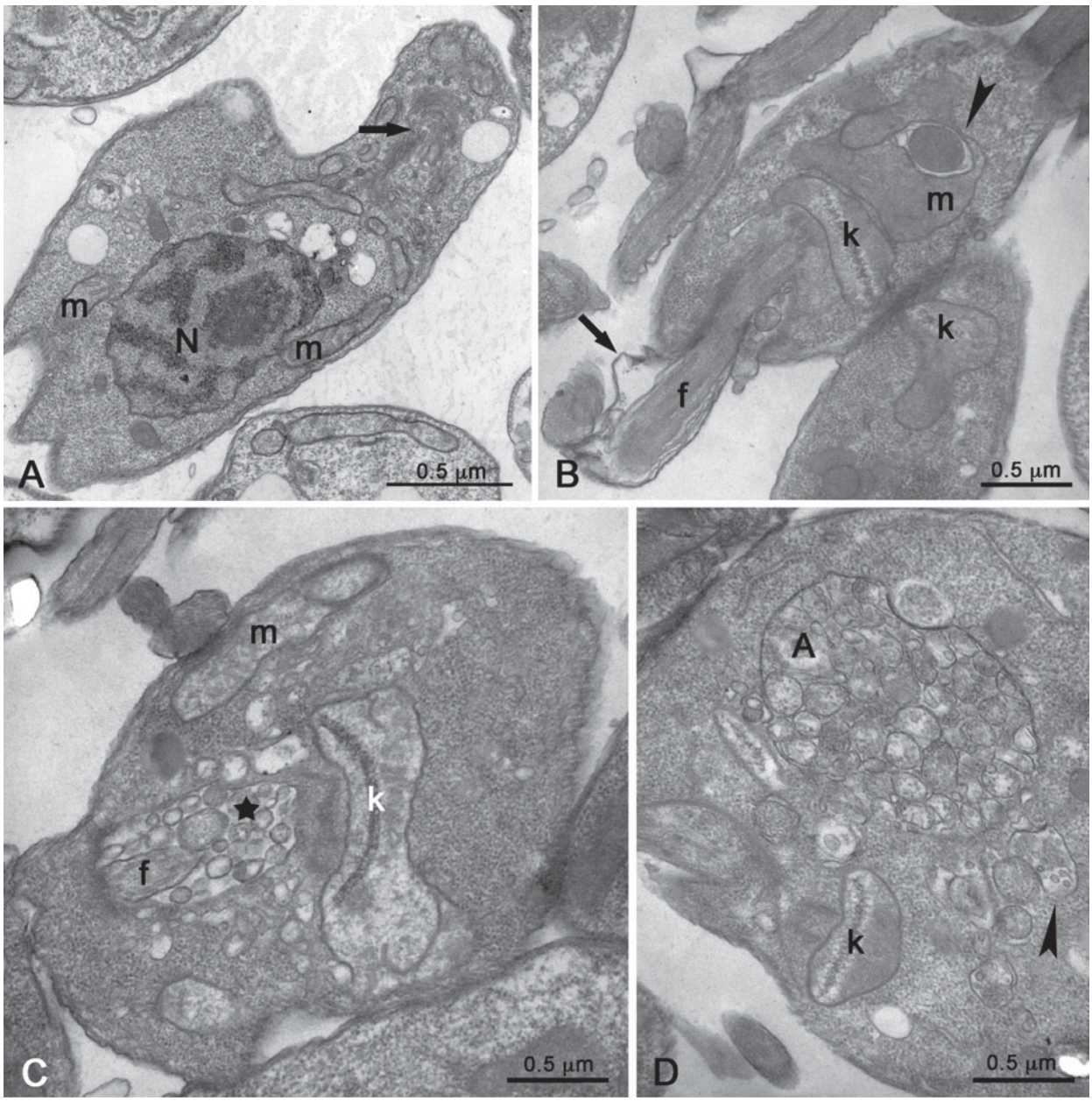

Fig. 5. (A–D) Ultrathin sections of *Leishmania amazonensis* promastigotes treated with vehicle (A; the control) and 0·3 μM tomatidine (B–D). (A) The control parasites show normal morphology in the nucleus, mitochondria, Golgi complex (arrow), and plasma membrane. (B–D) Promastigotes treated with 0·3 μM tomatidine for 48 h, as described in Fig. 2, display several morphological alterations: mitochondrial swelling (B–C), the presence of small vesicles inside the flagellar pocket (C, star), large autophagosomes containing several vesicles (A), and structures similar to multivesicular bodies (D, arrowhead). A, autophagosome; f, flagellum; k, kinetoplast; m, mitochondrion.

observed myelin-like figures (Fig. 4E, arrowhead) and autophagosomes (Fig. 6E, star) in the cytoplasm.

The effects of tomatidine on the mitochondrial membrane potential ($\Delta\Psi_m$)

According to the transmission electron microscopy analysis, the main organelle affected by tomatidine treatment was the mitochondrion, which showed membrane alterations, intense swelling and the loss of matrix contents (Figs 5B–C and 6E–F). To determine whether or not these morphological changes caused physiological changes in the mitochondria, we measured the $\Delta\Psi_m$ of the control and drug-treated promastigotes. Figure 7 shows that cells treated either with 0·3 or 1 μM tomatidine for 48 h had a reduced $\Delta\Psi_m$, as indicated by a low fluorescence intensity ratio (590/530 nm). This result was confirmed by the absence of fluorescence changes after the addition of the protonophore carbonyl cyanide *p*- trifluoromethoxyphenylhydrazone (FCCP; 2 μM), in contrast to the signal drop observed in control cells with FCCP, as a consequence of the complete abolition of $\Delta\Psi_m$.

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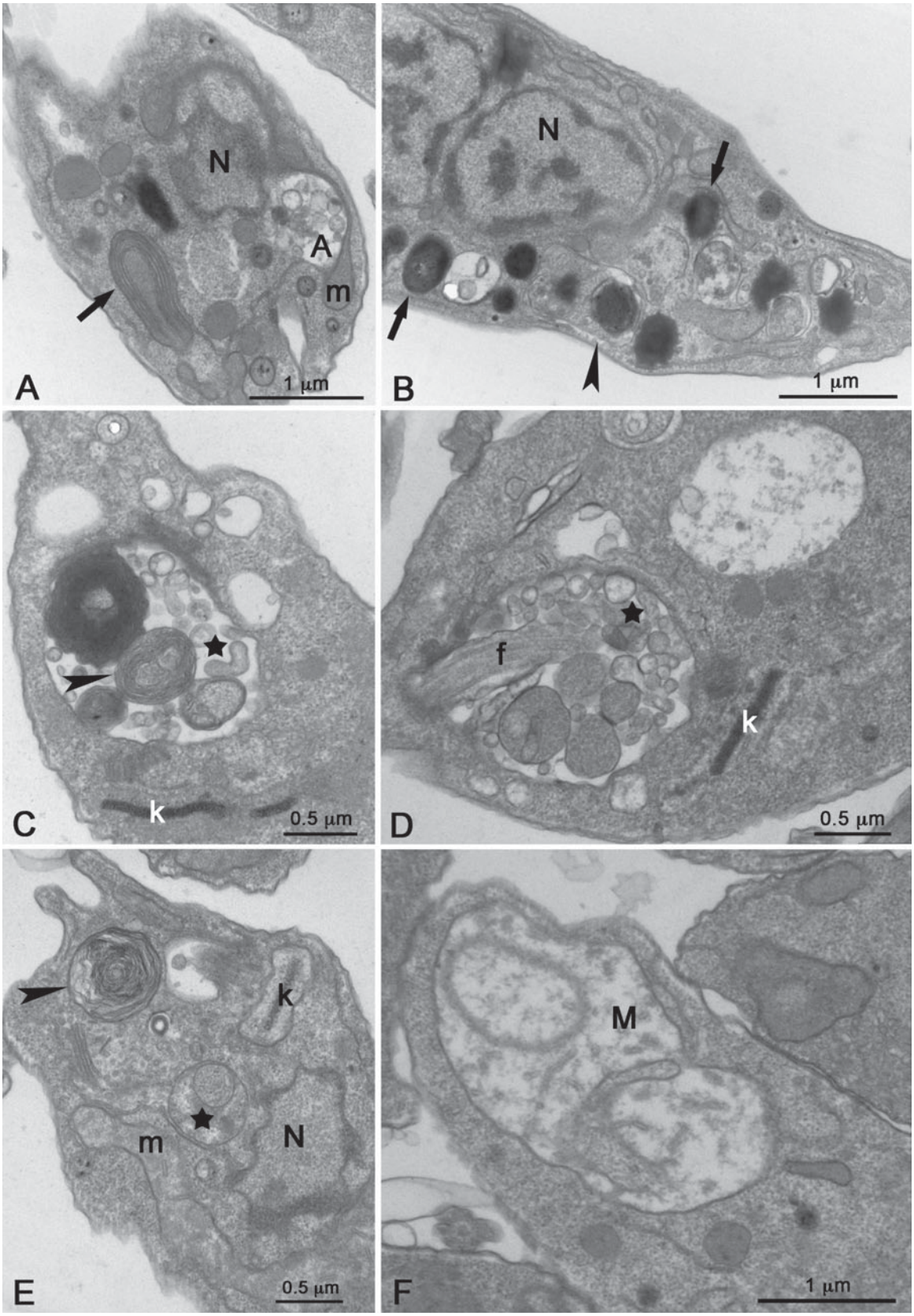

Fig. 6. Effects of 5 μM (A–C) and 15 μM (D–F) tomatidine on the ultrastructure of *Leishmania amazonensis* promastigotes. (A) The presence of an autophagosome (A) in contact with a mitochondrion. The arrow points to a mitochondrion containing many cristae. (B) The presence of several lipid-storage bodies (arrows), which sometimes appear surrounded by endoplasmic reticulum profiles (arrowhead). (C–D) The presence of many vesicles and a myelin-like figure (arrowhead) inside the flagellar pocket (star). (E–F) Alterations in the mitochondria: an intense swelling with a loss of matrix contents, the presence of a myelin-like figure (arrowhead), and an autophagosome in the cytoplasm (star). f, flagellum; M, mitochondrion; k, kinetoplast; N, nucleus.

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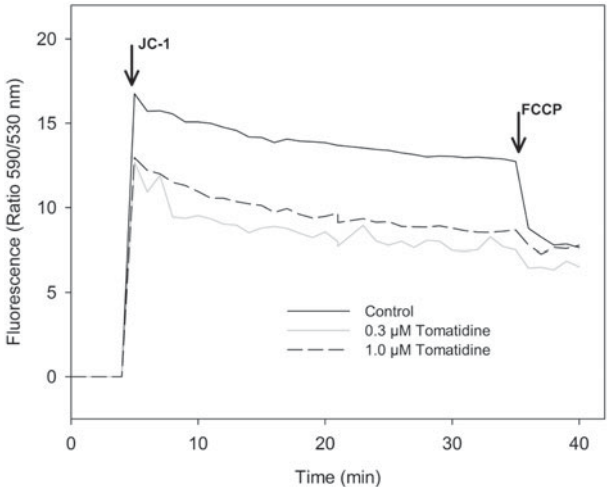

Fig. 7. Effects of tomatidine on the mitochondrial membrane potential ($\Delta\Psi_m$). The $\Delta\Psi_m$ of the control cells and cells treated with tomatidine for 48 h were measured by the addition of JC-1, as described in the Materials and Methods section. The reduced $\Delta\Psi_m$ in treated cells is characterized by a reduction in the fluorescence intensity ratio (590/530 nm). JC-1 (10 μg/ml) and FCCP (2 μM) were added at the times indicated in the figure. Each experiment was repeated at least 3 times; a representative figure of these experiments is shown.

Measurement of intracellular ATP levels after treatment with tomatidine

In view of the effect of tomatidine on the $\Delta\Psi_m$ and considering that 70% of the total intracellular ATP in *Leishmania* cells is supplied by oxidative phosphorylation in the mitochondria, with only ~30% provided by glycolysis (Sen *et al.* 2006), we also analysed their intracellular ATP contents. As shown in Fig. 8A, a significant decrease in ATP was observed after 48 h of treatment with 1 μM tomatidine. In contrast, no significant change was observed in the ATP content after a short treatment period (1 h) (Fig. 8B).

DISCUSSION

In this work, we demonstrated that tomatidine inhibits the growth of *L. amazonensis* promastigotes while promoting changes in their sterol content, ultrastructure, and $\Delta\Psi_m$. Our anti-proliferation studies revealed that the effect was dose dependent at lower concentrations of tomatidine (between 0·1 and 5 μM), with an IC_{50} of approximately 0·120 μM. The dose of 15 μM was slightly less effective than 5 μM. This finding may be a consequence of the over-expression of enzymes involved in the sterol biosynthesis pathway, which was previously observed in *Trypanosoma cruzi* after treatment with sterol biosynthesis inhibitors (SBIs) (Hankins *et al.* 2005).

The parasites treated with 1 or 5 μM tomatidine had important structural and metabolic alterations that compromised growth. However, when the drug was removed from the media, the cells recovered their normal growth rate, even at a concentration of 5 μM tomatidine, for which the recovery took 3 days. These results suggest that the effects of tomatidine are fully reversible.

Exposure to tomatidine for 48 h triggered a large reduction in the level of endogenous 24-alkylated sterols in *L. amazonensis*, including 5-dehydroepisterol, β-sitosterol and its precursor episterol, with a concomitant accumulation of 24-desalkyl sterols, mainly zymosterol, cholesta-7, 22-dien-3β-ol, lanosterol, and 4,14-dimethyl-zymosterol; the last 2 are precursors of zymosterol biosynthesis (Rodrigues *et al.* 2002). Previous studies in *L. amazonensis* and *L. donovani* promastigotes treated with 22,26-azasterol, an inhibitor of $\Delta^{24(25)}$-sterol methyltransferase, showed significant reductions in C_{28}-sterols and an accumulation of C_{27}-sterols, mainly endogenous ergosta-5,7,24 (24^1)-trien-3β-ol and cholesta-7,24-dien-3β-ol, and exogenous cholesterol derived from the culture medium (Haughan *et al.* 1995; Rodrigues *et al.* 2002). Therefore, our results are consistent with an inhibition of the enzyme $\Delta^{24(25)}$-sterol methyltransferase, which is responsible for the incorporation of methyl groups at position 24 of sterol intermediates, an essential step for the production of ergosterol and other 24-methyl sterols in protozoan parasites (Urbina *et al.* 1996; Nes, 2000; de Souza and Rodrigues, 2009). This inhibitory effect of tomatidine was also proposed for the C24-sterol methyltransferase of *S. cerevisiae* (Simons *et al.* 2006). Moreover, Mangla and Nes (2000) reported that the tomatidine analogues solanidine and solasodine had direct effects on a version of this enzyme found in *P. wickerhamii*. The accumulation of zymosterol, which is a substrate for the 24-sterol methyltransferase in *Leishmania* (Nes, 2000; Rodrigues *et al.* 2002), indicates that demethylases were not inhibited by tomatidine. The 24-methyltransferase enzyme is a potential chemotherapeutic target because it is absent in mammalian cells. The untreated promastigote cells had a relatively high percentage of sitosterol that also decreased in the presence of tomatidine. Because our results point to a strong inhibition of C24 methyltransferase activity, the ethylation at C24 may suggest the existence of a second enzyme with C24-sterol ethyltransferase activity that is less sensitive to this inhibition. More studies are needed to confirm this hypothesis.

As shown in Fig. 4, tomatidine-treated cells endocytosed much more LDL than the control cells. It is possible that the parasites attempt to counterbalance altered sterol biosynthesis with higher cholesterol uptake from the medium. In *L. donovani* (Haughan *et al.* 1995) and *L. amazonensis* promastigotes (Rodrigues *et al.* 2002), the inhibition of 24-methyltransferase with azasterols also caused an

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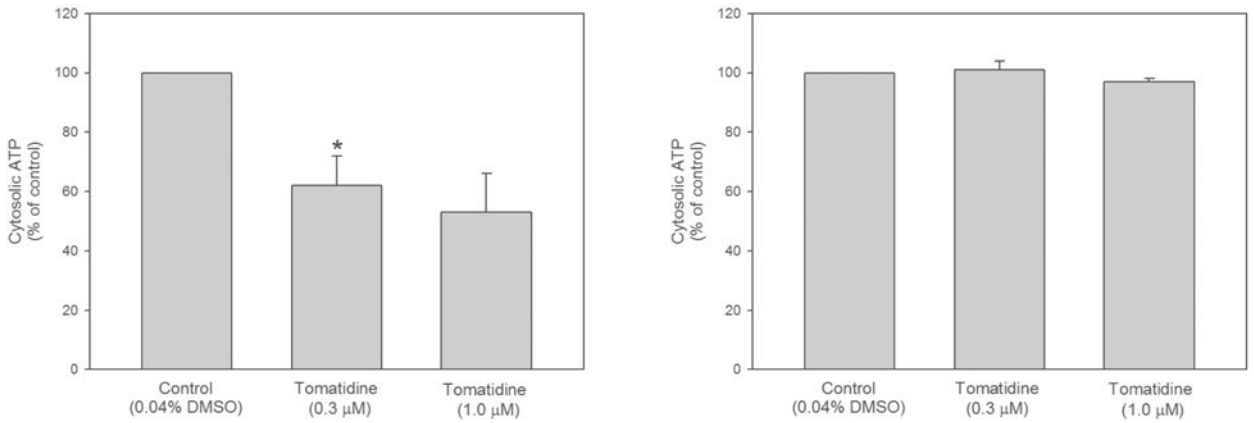

Fig. 8. Effects of tomatidine on cellular ATP levels. (A) Cellular ATP levels were determined after 48 h of treatment with tomatidine. A significant reduction in intracellular ATP content was detected after 48 h; however, after 1 h of treatment (B), the ATP levels of treated promastigotes remained the same as that of the control cells. The data are expressed as the mean percentages ± S.E., $n = 3$. Asterisks (*) indicate a significant difference ($P < 0{\cdot}05$) compared with the control.

increase in the amount of exogenous cholesterol uptake.

Sterols are important structural elements in cellular membranes and ultrastructural studies on *T. cruzi* and *L. amazonensis* treated with SBIs have provided evidence that membrane disorganization is a dominant factor that stimulates growth inhibition and cell lysis (Lazardi *et al.* 1990, 1991; Vannier-Santos *et al.* 1995; Vivas *et al.* 1996; Rodrigues *et al.* 2002, 2005, 2007; Roberts *et al.* 2003; Granthon *et al.* 2007; Rodrigues and de Souza, 2008; de Souza and Rodrigues, 2009). Transmission electron microscopy revealed significant ultrastructural alterations in *L. amazonensis* promastigotes treated with tomatidine for 48 h, namely, mitochondrial swelling, intense vacuolization and enlargement of the flagellar pocket. These results are similar to those observed when *L. amazonensis* promastigotes, *T. cruzi* epimastigotes and *Crithidia deanei* (an endosymbiont-bearing trypanosomatid) were treated with $\Delta^{24(25)}$-sterol methyltransferase inhibitors (Vivas *et al.* 1996, 1997; Rodrigues *et al.* 2002, 2007; Palmié-Peixoto *et al.* 2006). Altering the amounts and/or structures of free sterols leads to defects in endocytic pathways in mammalian cells and yeast (Pichler and Riezman, 2004). In cells treated with SBIs, the appearance of autophagic structures is probably due to the recycling of abnormal lipids and membranes during the processing of damaged organelles (Rodrigues *et al.* 2002; Santa-Rita, *et al.* 2005).

Our experiments using Nile red confirmed the presence of neutral lipid-rich vacuoles. Nile red emits strong fluorescence when in a lipid-rich environment. This dye has an emission that is environmentally sensitive; therefore, intracellular fat vacuoles filled with neutral lipids such as sterols, lipoproteins and triglycerides fluoresce yellow-gold with Nile red staining, whereas polar lipids such as phospholipids fluoresce red. More studies are needed to establish whether these vacuoles contain exogenous or endogenous lipids. The parasites treated with tomatidine had a notable increase in the uptake of LDL (a cholesterol-rich lipoprotein) from the external medium and GC-MS showed an increased relative amount of cholesterol in the neutral lipid fraction.

In trypanosomatids, the mitochondrion is one of the main organelles affected by SBI treatment (Lazardi *et al.* 1990, 1991; Vivas *et al.* 1996; Rodrigues *et al.* 2002, 2007; Palmie-Peixoto *et al.* 2006; de Souza and Rodrigues, 2009). The presence of large amounts of sterols in the mitochondrial membranes of *T. cruzi* epimastigotes may explain the severity of this effect (Rodrigues *et al.* 2001). Ergosterol and/or other 24-alkylated sterols are essential for mitochondrial function. Tomatidine affects mitochondrial ultrastructure and physiology, as observed by transmission electron microscopy, and leads to the loss of $\Delta\Psi_m$ with a concomitant decrease in ATP levels. The loss of the energy-transducing properties of the mitochondrial inner membrane is probably related to alterations in its lipid composition. These results are in agreement with a prior study (Rodrigues *et al.* 2007) that demonstrated the effects of SBIs on the $\Delta\Psi_m$ of *L. amazonensis* promastigotes treated with various inhibitors of $\Delta^{24(25)}$-sterol methyltransferase. The maintenance of the $\Delta\Psi_m$ is essential for the survival of the cell and the study of this mitochondrial functional parameter has become a focus in the investigation of apoptosis regulation, and many researchers have demonstrated the major functional impact of mitochondrial dynamics on apoptosis (reviewed by Shaha, 2006). The decrease in intracellular ATP after several hours of tomatidine treatment could contribute to cell death (Sen *et al.* 2007).

Tomatidine treatment resulted in decreased swimming mobility in *L. amazonensis* promastigotes. It may be related to the decrease in ATP or/and to the

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morphological alterations since the treated cells lost their elongated shape and acquired a round shape. In conclusion, our results indicate that tomatidine interferes with the growth, ultrastructure, sterol content and mitochondrial function of *L. amazonensis* promastigotes and should be considered as a potential new drug for the treatment of leishmaniasis.

ACKNOWLEDGMENTS

We would like to thank Mileane Busch for technical assistance. This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and the Programa de Núcleos de Excelência (PRONEX).

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