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Altered Lipid Composition and Enzyme Activities of Plasma Membranes from *Trypanosoma (Schizotrypanum) Cruzi* Epimastigotes Grown in the Presence of Sterol Biosynthesis Inhibitors

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ABSTRACT. The accepted mechanism for the antiproliferative effects of sterol biosynthesis inhibitors (SBI) against the protozoan parasite *Trypanosoma (Schizotrypanum) cruzi*, the causative agent of Chagas' disease, is the depletion of specific parasite sterols that are essential growth factors and cannot be replaced by cholesterol, the main sterol present in the vertebrate host. However, the precise metabolic roles of these specific parasite steroles are unknown. We approached this problem by subjecting *T. cruzi* epimastigotes to two types of SBI, inhibitors of sterol C-14 demethylase and $\Delta^{24(25)}$ methyl transferase, and investigating the modification of lipid composition and enzyme activities in the plasma membranes of the parasite. We found in purified plasma membrane from SBI-treated cells that, together with the expected changes in the sterol composition, there was also an inversion of the phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio and a large increase in the content of saturated fatty acids esterified to phospholipids. The modification of the phospholipid headgroup composition correlated with a 70% reduction in the specific activity of the membrane-bound PC-PE-*N*-methyl transferase SBI-treated cells; it was shown that this inhibition was not due to a direct effect of the drug on the enzyme. Finally, the specific activity of the Mg^{2+}-dependent, vanadate-sensitive ATPase present in the membranes was also inhibited by *ca.* 50% in SBI-treated cells. The results suggest that one of the primary effects of the depletion of endogenous sterols induced by SBI in *T. cruzi* is a modification of the cellular phospholipid composition as a consequence of a reduced activity of PE-PC-*N*-methyl transferase and probably of the acyl Δ^9 and Δ^6 desaturases; this, in turn, could affect the activity of other enzymatic and transport proteins. BIOCHEM PHARMACOL 53;5:697–704, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. sterol biosynthesis inhibitors; ketoconazole; azasterol; phospholipids; phosphatidylcholine-phosphatidylethanolamine *N*-methyl transferase; *Trypanosoma cruzi*

SBI† are among the most powerful, broad spectrum antifungal agents known. They act by depleting essential and specific membrane components and/or by inducing the accumulation of toxic intermediate or side products of the biosynthetic pathway [1–3]. These compounds are also active against protozoan parasites such as *Trypanosoma (Schizotrypanum) cruzi*, the causative agent of Chagas' disease, which specifically require ergosterol and 24-ethyl-cholesta-5,7,22-trien-3β-ol for growth and cannot replace these components with cholesterol, the only sterol present in the vertebrate hosts or culture medium [4–9]. In fungi, the deleterious effects of an altered sterol composition on cell function have been related to the modulation of activity of several membrane-bound enzymes by ergosterol, the main endogenous sterol of these organisms [1, 2, 10–12] and to the alteration of the physical properties of the cellular membranes as a consequence of the accumulation of precursors or derivatives such as squalene, lanosterol, eburicol (24-methylenedihydrolanosterol), 14α-methyl-ergosta-8,24(24^1)-3β,6α-diol or obtusifolione [1–3, 13, 14]. However, in *T. cruzi*, SBI-induced growth inhibition and loss of cell viability have been correlated strictly with the complete depletion of the endogenous sterols and not with the accumulation of precursors or unnatural sterols [4–9, 15, 16]. In any case, nothing is known concerning the specific cellular functions of sterols in this and related organisms. As an initial approach to answering this question, we have

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† *Abbreviations:* SBI, sterol biosynthesis inhibitor(s); GLC/MS, gas–liquid chromatography coupled to mass spectrometry; MIC, minimal inhibitory concentration; 24(25)-SMT, $\Delta^{24(25)}$ sterol methyl transferase; PE-PC-*N*-methyl transferase, phosphatidylethanolamine-phosphatidylcholine-*N*-methyl transferase; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

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studied the alteration of lipid composition and enzyme activities in purified plasma membrane preparations obtained from *T. cruzi* epimastigotes grown in the absence or presence of two different types of SBI: the cytochrome P450-dependent C-14α demethylase inhibitor ketoconazole [1, 2, 4, 5, 7, 15] and two sterol 24-methyl-transferase (24(25)-SMT) inhibitors [8, 9, 17, 18].

MATERIALS AND METHODS

Parasite

The EP stock [19] of *T. cruzi* was used throughout this study.

In Vitro *Studies*

The epimastigote form of the parasite (equivalent to that present in the Reduviid vector) was cultivated in liver infusion-tryptose medium supplemented with 5% newborn calf serum [19] at 28° and strong aeration (120 rpm). The cultures were initiated with a cell density of 2×10^6 epimastigotes/mL, and the drugs were added, alone or in combination, when the cultures reached a cell density of 1×10^7 epimastigotes/mL. Cell densities were measured by turbidimetry and with an electronic particle counter (model ZBI, Coulter Electronics Inc., Hialeah, FL).

Isolation and Purification of T. cruzi *Plasma Membranes*

A highly enriched plasma membrane fraction was obtained from washed epimastigotes by grinding with glass beads (Sigma) and differential centrifugation, using a previously described method [20].

Studies on Lipid Composition and Metabolism

For the analysis of the effects of the different drugs on the sterol and phospholipid composition of the plasma membranes from control and drug-treated cells, total lipids were extracted from the membranes and separated into neutral and polar lipid fractions by silicic acid column chromatography as described before [7, 8, 15, 16]. The neutral lipid fraction was first analyzed by TLC, on Merck 5721 silica gel plates with heptane:isopropyl ether:glacial acetic acid (60:40:4, by vol.) as developing solvent and by conventional GLC (isothermic separation in a 4-m glass column packed with 3% OV-1 on Chromosorb 100/200 mesh, with nitrogen as carrier gas at 24 mL/min and flame ionization detection in a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments, the neutral lipids were separated using a capillary high resolution column (30 m × 0.25 mm i.d. HP-5 MS column, 5% Phenyl-methylsilicone, 0.25 μm film thickness) in a computer-controlled Hewlett–Packard 5890 series II gas chromatograph equipped with an HP5971A mass sensitive detector. The lipids were injected in ethyl acetate and the column was kept at 50° for 1 min; then the temperature was increased to 250° at a rate of 10°/min and finally to 280° at a rate of 1°/min. The carrier gas (helium) flow was kept constant at 1.1 mL/min. Injector temperature was 250° and the detector was kept at 280°. The assignment of 5,7-diene structures was based on characteristic fragmentation patterns in mass spectrometry but also on chromatography in $AgNO_3$-impregnated Merck 5721 silica gel thin-layer plates and UV-VIS spectrophotometry, carried out in a Hewlett-Packard 8452A UV-VIS spectrophotometer under the control of a 33 MHz 486 computer.

The polar lipid fraction (containing mostly phospholipids) from the silicic acid column chromatography was further fractionated by TLC on Merck 5721 silica gel plates using chloroform:methanol:32.5% ammonia w/v (17:7:1, by vol.) as developing solvent [21]. The phospholipid spots were visualized using iodine, then scraped, and the total organic phosphorus was measured using the method of Ames and Dubin [22]. For the analysis of the fatty acids esterified to the membrane phospholipids, total phospholipids dissolved in 100 μL of chloroform were transmethylated by adding 200 μL of 2% H_2SO_4 in methanol and incubating at 60° for 1 hr. The reaction was stopped by adding 200 μL of distilled water. The methyl ester were extracted with petroleum ether, dried and analyzed quantitatively by GLC in a 2 m by 2 mm (i.d.) column packed with 10% SILAR GT on Chromosorb W (100–200 mesh) on a Varian 3700 gas chromatograph with a flame ionization detector [23]. The temperature program was 150° for 10 min, followed by a linear temperature increment of 3°/min up to 205° and then a constant 205° for an additional 25 min; nitrogen gas was used as carrier at 8 mL/min.

Enzymatic Assays

The activity of PE-PC-*N*-methyl transferase was assayed in freshly isolated membranes following the method of Kawasaki *et al.* [24]. Briefly, the purified membranes (final concentration 0.1 to 0.2 mg/mL) were incubated in a medium containing 125 mM Tris–HCl (pH 8.4), 1 mM L-cysteine and 50 μM *S*-adenosyl[*methyl*-^{14}C]methionine (55 mCi/mmol, Amersham) for varying amounts of time; then the reaction was stopped by adding 3 vol. of chloroform:methanol:concentrated HCl (2:1:0:2, by vol.). The organic phase was washed twice with equal volumes of 0.1 M KCl in 50% methanol and subsequently dried, and the phospholipids were separated by TLC as described above. The phospholipid spots were visualized using iodine, scraped, and counted using Aquasol (New England Nuclear) in a Beckman LS 5000 TD liquid scintillation spectrometer. Mg^{2+}-activated, vanadate-sensitive ATPase was assayed as described before [20].

Drugs

22,26-Azasterol (20-piperidin-2-yl-5α-pregnan-3β-20(*R*,*S*)-diol; [18, 25]) was synthesized and characterized as

described before [8, 9]. 24,25-(*R,S*)-Epiminolanosterol was synthesized according to Nes and collaborators [26, 27]. These drugs were dissolved in DMSO; the final DMSO concentration in the culture medium never exceeded 1% (v/v) and had no effect by itself on the proliferation of the epimastigotes. Ketoconazole (*cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) [11, 28] was provided by Ingrid Straziota, Janssen Pharmaceutica, Caracas, Venezuela. It was added to the culture or assay medium as an aqueous solution titrated to pH 2.4 with HCl and sterilized by filtration through membrane filters (mean pore size, 0.22 μm; Millipore Corp., Bedford, MA, U.S.A.).

RESULTS

Lipid Composition of Plasma Membranes from Control and SBI-Treated T. cruzi *Epimastigotes*

The plasma membranes isolated from control (untreated) and ketoconazole-treated epimastigotes (1 μM, 120 hr) were analyzed in terms of total protein, total phospholipid, and total sterol content. It was found that membranes from treated cells had a higher protein to phospholipid ratio than membranes from control cells (17 ± 3.8 vs 10 ± 3.4 nmol of phospholipid/mg protein, $P < 0.05$, N = 7), whereas the sterol to phospholipid molar ratio (0.30 ± 0.08) was indistinguishable in the two conditions. The detailed sterol and phospholipid composition of these membranes as well as those extracted from cells incubated for the same period of time in the presence of two 24(25)-SMT inhibitors, 22,26-azasterol and 24(*R,S*),25-epiminolanosterol, at their MIC [8, 9], is presented in Tables 1 and 2. It can be seen from Table 1 that the main membrane sterols found in control cells were 24-methyl-5,7,22-cholesta-trien-3β-ol (ergosterol) and its 24-ethyl analog plus smaller amounts of metabolic precursors such as episterol (ergosta-7,24(24^1)-dien-3β-ol), 5-dehydroepisterol (ergosta 5,7,24(24^1)-trien-3β-ol), and squalene. Cholesterol (taken passively from the growth medium; see Refs. 4, 5, 7, and 15) was also detected. In membranes from cells treated with 1 μM ketoconazole for 120 hr, it was found, as in previous studies with whole cells [4–9], that all endogenous sterols were replaced by 14α-methyl sterols such as 24-methylenedihydrolanosterol (eburicol), lanosterol, and 4,14-dimethyl-ergosta-8,24(24^1)-dien-3β-ol (obtusifoliol), while in the presence of the 24(25)-SMT inhibitors the native endogenous sterols were replaced by zymosterol (cholesta-8,24-dien-3β-ol) in the case of 22,26-azasterol or a mixture of zymosterol, cholesta-7,24-dien-3β-ol, and cholesta-5,7,24-trien-3β-ol in the case of 24(*R,S*),25-epiminolanosterol. A significant reduction in the content of cholesterol in the membranes of those cells was observed. Ergosta-8,24(24^1)-dien-3β-ol was also detected in significant amounts in cells treated with the sterol analogs, as these compounds are also strong inhibitors of sterol 24-methyl-reductase. For a detailed analysis of the effects of the 24(25)-SMT inhibitors on *T. cruzi* growth and sterol content, see Refs. 8 and 9. The effects of the SBI on the sterol composition of the cell membranes

TABLE 1. Free sterol composition of *Trypanosoma cruzi* plasma membranes isolated from epimastigotes grown in the absence or presence of different sterol biosynthesis inhibitors*

Compound	Control (no drug)	1 μM Ketoconazole	3 μM 22,26-Azasterol	10 μM 22,26-Azasterol	3 μM 24(R,S), 25-Epiminolanosterol
Exogenous					
Cholesterol	28	16.4	7.8	4.6	16.9
Endogenous, 14a-desmethyl					
Cholesta-8,24-dien-3β-ol (zymosterol)	<1	<1	47.5	76.9	16.0
Cholesta-5,7,24-trien-3β-ol	<1	<1	14.0	2.0	33.6
24-Methyl-5,7,22-Cholesta-trien-3β-ol (Ergosterol)	25	<1	10.1	2.9	<1
Cholesta-7,24-dien-3β-ol	<1	<1	5.2	1.6	13.7
Ergosta-8,24(24^1))-dien-3β-ol	<1	<1	10.6	10.1	<1
Ergosta-5,7-dien-3β-ol + ergosta-5,7,24(24^1)-trien-3β-ol	7.7	<1	<1	<1	<1
Ergosta-7,24(24^1)-dien-3β-ol	9.3	<1	<1	<1	<1
24-Ethyl-cholesta-5,7,22-trien-3β-ol	12	<1	<1	<1	<1
Endogenous, 14a-methyl					
Squalene	18	16.1	4.8	1.9	19.8
4,14-Dimethyl-ergosta-8,24(24^1)-dien-3β-ol (obtusifoliol)	<1	8.2	<1	<1	<1
Lanosterol	<1	17.2	<1	<1	<1
24-Methylenedihydrolanosterol (eburicol)	<1	42.1	<1	<1	<1

* Plasma membranes were isolated from epimastigotes grown in liver infusion-tryptose medium with and without the indicated drug concentrations for 120 hr, and sterols were extracted, and separated from polar lipids by silicic acid column chromatography as described in Materials and Methods. The sterols were analyzed by quantitative capillary GLC/MS using a Hewlett-Packard 598011 gas chromatograph with an HP5971 mass-sensitive detector. Results are expressed as weight percent and are a representative example of at least three independent experiments.

were concentration dependent, as illustrated for the case of 22,26-azasterol in Table 1. When we analyzed the phospholipid composition of the same membranes, we found (Table 2) that whereas in membranes from control cells PC and PE were the predominant species with a molar ratio of *ca.* 2:1, in cells treated with SBI, irrespective of the type of inhibitor used, the PC:PE ratio was inverted (to *ca.* 1:2). This was a consistent, statistically significant ($P < 0.05$) result. The effect was also concentration dependent with respect to the concentration of the SBI used, as shown in Table 2 for the case 22,26-azasterol. Concerning the other phospholipid species present (phosphatidylserine, phosphatidylinositol and sphingomyelin), no significant differences were detected between the membranes from control and treated cells. However, the total content of phospholipids in control and treated cells did not differ significantly (1.7 to 2.0% of the total dry weight). We also investigated the type of fatty acids esterified to the total phospholipid fraction and found (Table 3) that in membranes from cells treated with SBI there was a significant (2-fold, $P \leq 0.05$) increase in the ratio of saturated to unsaturated fatty acids when compared with the untreated controls. This effect was again independent of the type of SBI used.

TABLE 3. Fatty acid composition of the phospholipid fraction of *Trypanosoma cruzi* plasma membranes isolated from epimastigotes grown in the absence or presence of different sterol biosynthesis inhibitors*

Fatty acid	Control (no drug)	1 μM Ketoconazole	3 μM 24(R,S),25-Epiminolanosterol
16:0	9.7 ± 0.7	12.6 ± 0.4	11.8 ± 0.9
16:1	2.8 ± 0.3	1.4 ± 0.3	ND†
18:0	11.2 ± 0.4	22.4 ± 1.7	22.1 ± 1.9
18:1	36.4 ± 1.8	33.4 ± 3.6	31.8 ± 2.9
18:2	39.1 ± 2.9	30.2 ± 3.6	34.3 ± 3.6
18:3	0.8 ± 0.1	ND	ND
SFA‡	20.9 ± 2.7§	35.0 ± 2.3‖	33.9 ± 2.5‖
UFA¶	79.1 ± 3.3§	65.0 ± 4.7‖	66.1 ± 4.9‖
SFA/UFA	0.26 ± 0.04§	0.57 ± 0.08¶	0.51 ± 0.07‖

* Plasma membranes were isolated from epimastigotes grown in liver infusion-typtose medium with and without the indicated drug concentrations for 120 hr and polar lipids were separated by silicic acid column chromatography and TLC as described in Materials and Methods; the fatty acids esterified to this fraction were transmethylated and quantitatively analyzed by GLC as described in Materials and Methods. The results (means ± SEM, from at lease three independent experiments) are expressed as mol%.

† ND = not detected.

‡ Saturated fatty acids.

¶ Unsaturated fatty acids

§ Significant ($P < 0.05$) differences from the treated groups.

‖ Significant ($P < 0.05$) differences from the control groups but not among the treated groups.

Membrane-Bound Enzyme Activities

As the phospholipid headgroup changes in the SBI-treated cells were restricted to the PE/PC pair, we studied the activity of the enzyme involved in this metabolic transformation, PE-PC-*N*-methyl transferase [24, 29]. Figure 1 shows that the activity of this enzyme, assayed by measuring the incorporation of radioactivity from S-adenosyl[*methyl*-^{14}C]methionine into total phospholipids, was reduced by 70% in membranes obtained from cells treated with 1 μM ketoconazole for 120 hr when compared with membranes from control cells. It can also be seen that the incorporation of ketoconazole into the assay medium (1 μM) did not affect the activity of the enzyme from control cells. Table 4 shows that in membranes of epimastigotes treated with 1 μM ketoconazole or 10 μM 22,26-azasterol for 120 hr the radioactivity specifically incorporated into PC was reduced significantly when compared with that present in membranes obtained from control cells ($P < 0.05$), whereas the radioactivity measured in the PE pool (which contained *N*-methyl-PE and *N,N*-dimethyl-PE as well as PE) was not significantly different between the two types of cells.

We also investigated the activity of another essential membrane enzyme/transporter: the Mg^{2+}-activated, vanadate-sensitive ATPase previously reported in this and related organisms [30–31]. It was found that the activity of the enzyme in membranes obtained from ketoconazole-treated epimastigotes (1 μM, 120 hr) was reduced significantly when compared with control cells (5.2 ± 0.87 vs. 8.3 ± 1.7 nmol/min · mg protein, $P < 0.05$, N = 4). The enzyme activity was inhibited 75% in the presence of 50 μM orthovanadate under our assay conditions; no effects of ke-

TABLE 2. Phospholipid composition of *Trypanosoma cruzi* plasma membranes isolated from epimastigotes grown in the absence of presence of different sterol biosynthesis inhibitors*

Phosholipid species	Control (no drug)	1 μM Ketoconazole	3 μM 22,26-azasterol	10 μM 22,26-azasterol	3 μM 24(R,S), 25-Epiminolanosterol
Phosphatidylcholine (PC)	46.7 ± 6.7†	26.4 ± 4.4‡	30.4 ± 4.9	27.7 ± 4.6‡	30.3 ± 3.9‡
Phosphatidylethanolamine (PE)	26.4 ± 4.8†	46.9 ± 5.8‡	39.6 ± 5.5	48.8 ± 6.5‡	36.5 ± 4.5‡
Phosphatidylinositol (PI)	16.2 ± 3.1	15.4 ± 2.8	16.5 ± 2.5	17.5 ± 2.3	18.2 ± 2.2
Phosphatidylserine (PS)	6.3 ± 1.6	5.1 ± 1.7	7.3 ± 1.7	3.0 ± 1.1	5.0 ± 1.5
Sphingomyelin (SPM)	4.4 ± 1.1	6.2 ± 1.8	6.2 ± 1.7	3.0 ± 1.3	10 ± 1.9

* Plasma membranes were isolated from epimastigotes grown in liver infusion-tryptose medium with and without the indicated drug concentrations for 120 hr, and polar lipids were separated by silicic acid column chromatrography and TLC as described in Materials and Methods. The different phospholipid species present were quantitated measuring organic phosphorus. The results (means ± SEM of at least three indepent experiments) are expressed as mol %.

† Significant ($P < 0.05$) differences from the treated groups.

‡ Significant ($P < 0.05$) differences from the control groups but not among the treated groups.

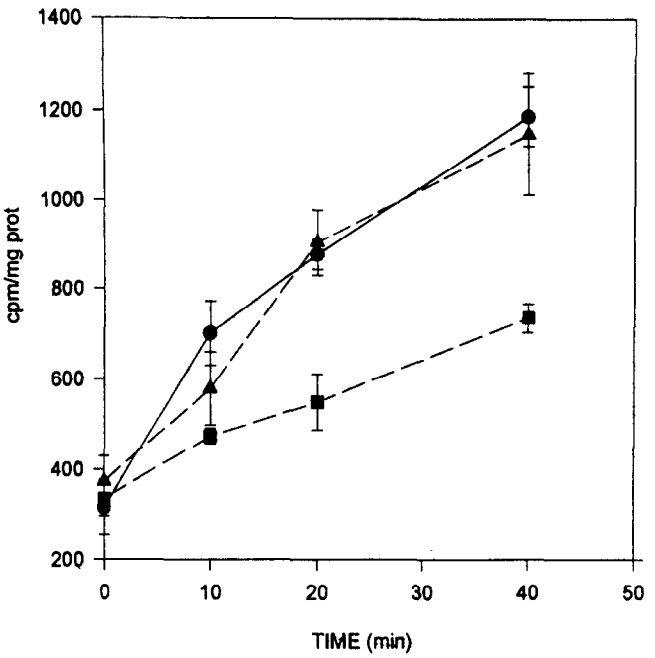

FIG. 1. Activity of PE-PC-*N*-methyl transferase in membranes from control and ketoconazole-treated *T. cruzi* epimastigotes. The enzyme activity was measured as described in Materials and Methods in a medium containing 125 mM Tris–HCl (pH 8.4), 1 mM L-cysteine, 50 μM S-adenosyl[*methyl*-^{14}C]methionine (55 mCi/mmol, Amersham) and 0.2 mg/mL of membrane protein. The radioactivity incorporated in the total phospholipid fraction is plotted as a function of incubation time for (●) membranes from control cells, (■) membranes from cells treated with 1 μM ketoconazole for 120 hr, and (▲) membranes from control cells plus 1 μM ketoconazole in the assay medium. Bars indicate SEM from four independent experiments.

toconazole when directly added to the assay medium were detected, up to a 10 μM concentration of the drug.

DISCUSSION

The antiproliferative effects of SBI against fungal organisms are currently understood as a combination of effects resulting from the depletion of the normal endogenous sterols plus the accumulation of cytotoxic sterol precursors and derivatives [1–3, 11, 13]. This last hypothesis has been advanced particularly for the allylamines, inhibitors of squalene epoxidase, where the cytotoxic effects have been associated with the accumulation of squalene [3, 13] (although there are other interpretations; see Ref. 1) and for many azoles, inhibitors of cytochrome P450-dependent sterol C-14α demethylase, for which the onset of growth arrest correlates with the accumulation of 4-methyl sterols such as obtusifoliol and 24-methylene-dihydrolanosterol (eburicol) as well as oxysterols such as 14α-methyl-ergosta-8,24(24^1)-dien-3β,6α-diol in *Saccharomyces cerivisiae* and *Candida albicans* and obtusifolione in *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Aspergillus fumigatus* [1, 2, 32, 33]. 4-Methyl sterols (particularly 4β-methyl) and oxysterols

TABLE 4. Activity of PE-PC-*N*-methyl transferase in *Trypanosoma cruzi* plasma membranes isolated from epimastigotes grown in the absence or presence of different sterol biosynthesis inhibitors*

Membranes	PE-PC-*N*-methyl transferase activity (pmol/min · mg protein)	
	Phospholip fraction	
	PC	PE
Control cells (no drug)	8.5 ± 0.7†	11.4 ± 2.5
Treated cells		
1 μM Ketoconazole	5.0 ± 0.7	8.6 ± 1.7
Control cells (no drug)	10.6 ± 1.8†	7.8 ± 1.5
Treated cells		
10 μM 22,26-Azasterol	6.3 ± 1.5	8.8 ± 1.6

* Plasma membranes were isolated from epimastigotes grown in the absence or presence of the indicated SBI concentration for 120 hr, and the activity of PE-PC-*N*-methyl transferase was measured as described in Materials and Methods. No significant effects were observed when ketoconazole (1 μM) or 22,26-azasterol (10 μM) was included in the assay medium (see Fig. 1). Values are means ± SEM of four independent experiments.

† Significant ($P < 0.05$) difference from the respective treated group.

are known to disrupt the packing of phospholipid molecules in bilayers, altering the basic permeability barrier properties of the membranes as well as lipid-protein interactions [10, 34–42]. However, detailed studies on the effects of SBI on growth and sterol content in *T. cruzi* have shown that growth arrest and loss of cell viability are associated with the depletion of the endogenous parasite sterols and not with the accumulation of any particular precursors or derivatives in the parasite membranes [6–9, 15]. Thus, although *T. cruzi* epimastigotes accumulate large amounts of 4-methyl sterols such as lanosterol and eburicol in the presence of azole derivatives [4–8, 15], growth inhibition is only observed when endogenous 4,14-desmethyl sterols drop below a critical level [7, 15]. This has been further shown by the simultaneous incubation of these cells with suboptimal concentrations of ketoconazole and mevinolin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase [43, 44], which leads to complete growth arrest despite the fact that the levels of 4,14-methyl sterols are lower in the cells treated with both drugs than in those treated with the azole alone [7]. This interpretation has received further support from recent studies using inhibitors of sterol 24-methyl transferase, which induced complete growth inhibition for both proliferative stages (epimastigotes and intracellular amastigotes) despite the fact that they induce the accumulation of only cholesta-type sterols such as zymosterol (cholesta-8,24-dien-3β-ol), cholesta-7,24-dien-3β-ol, and cholesta-5,7,24-trien-3β-ol [8, 9], which have all the molecular properties required to induce the "fluid-ordered" state of the phospholipid molecules ("bulk" role) in cellular membranes [10, 35, 45]. Thus, *T. cruzi* seems to have very strict requirements concerning the structural features required to perform the "metabolic" functions of sterols.

The marked changes of the phospholipid headgroup composition and fatty acid content in SBI-treated cells

(Tables 2 and 3) and the fact that these changes were independent of the type of SBI used or sterol precursors accumulated in the cell membranes indicated that the metabolic machinery responsible for the synthesis of these membrane components is affected by the depletion of the endogenous 4-desmethyl sterols. The significant reduction of the activity of the PE-PC-*N*-methyl transferase in the membranes from treated cells (Fig. 1 and Table 4), despite the increased levels of its phospholipid substrate (PE) in these membranes (Table 2), provided a molecular explanation for the headgroup composition changes. These results are also completely consistent with those obtained by Bloch's group[24, 29] in the yeast sterol auxotroph GL7, which showed that PC biosynthesis in yeast membranes from cells grown in the presence of ergosterol is much higher than in membranes from cells grown in cholesterol, through an increase in the specific activity of PE-PC-*N*-methyl transferase. These facts suggest that one of the specific cellular functions associated with ergosterol and analogs in *T. cruzi* is the regulation of the synthesis of the main phospholipid species of the membranes, PC, by modulation of the enzyme involved in the last step of its biosynthetic pathway.

The reduction in the content of 18:1 and 18:2 fatty acids and the concomitant increase in the saturated (16:0 and 18:0) components in the phospholipid fraction of membranes from SBI-treated cells indicate that probably the Δ^9 and Δ^6 desaturases were also affected. Similar results were obtained in *Candida albicans*, which in the presence of azole derivatives such as clotrimazole, econazole, miconazole, and ketoconazole suffers a marked reduction of the content of 18:1 fatty acids, suggesting an effect on the Δ^9 desaturase [1, 2, 12, 46]. However, another possible explanation for the increase in the phospholipid saturated fatty acid content in SBI-treated epimastigotes could be an attempt by the cell to compensate for the decreased lipid bilayer stability due to the large increase in PE content. It is well known that PEs with polyunsaturated fatty acid chains tend to promote nonbilayer (H_{II}) phases at physiological temperatures and that this tendency is reduced sharply when the chains are saturated [47–49]. This explanation has also been advanced to account for the large increase in saturated fatty acids observed in *T. cruzi* epimastigotes exposed to the antiplatelet compound ajoene, which inhibits specifically *de novo* PC synthesis, possibly through a direct effect on PE-PC-*N*-methyl transferase [23].

Finally, the decreased activity of the Mg^{2+}-activated ATPase in membranes from ketoconazole-treated cells is due most probably to the altered lipid (both sterols and phospholipids) composition of these membranes. Its reduced activity can lead to drastic alteration in the ionic content and osmotic balance of the cells as this enzyme is thought to be responsible for the maintenance of a protonmotive force through the plasma membranes of kinetoplastid parasite, involved in ion and non-electrolyte transport [30–31]. In fact, one characteristic phenomenon associated with the depletion of endogenous sterols in *T. cruzi* epimastigotes is rounding of the cells and a dramatic swelling of the single giant mitochondrion present in them, which suggests an altered osmotic balance of the cells [50, 51]. In fungi it has also been shown that azole antifungals alter the activity of ATPases from both mitochondria and plasma membranes [1, 2, 34].

In conclusion, this study has shown that one of the biochemical effects of the depletion of endogenous sterols induced by SBI in *T. cruzi* epimastigotes is a marked alteration of the phospholipid headgroup composition of the membranes, which is associated with a reduced activity of the key enzyme PE-PC-*N*-methyl transferase. This altered phospholipid composition, together with the alteration of the fatty acid content of the membranes and the accumulation of sterol precursors should modify the physical properties of the lipid matrix [20], which, in turn, could affect the activity of other membrane-bound proteins.

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