Clotrimazole Inhibits the Ca\(^{2+}\)-ATPase (SERCA) by Interfering with Ca\(^{2+}\) Binding and Favoring the E2 Conformation*

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Clotrimazole (CLT) is an antifungal imidazole derivative that is known to inhibit cytochrome P-450, ergosterol biosynthesis and proliferation of cells in culture, and to interfere with cellular Ca\(^{2+}\) homeostasis. We found that CLT inhibits the Ca\(^{2+}\)-ATPase of rabbit fast-twitch skeletal muscle (SERCA1), and we characterized in detail the effect of CLT on this calcium transport ATPase. We used biochemical methods for characterization of the ATPase and its partial reactions, and we also performed measurements of charge movements following adsorption of sarcoplasmic reticulum vesicles containing the ATPase onto a gold-supported biomimetic membrane. CLT inhibits Ca\(^{2+}\)-ATPase and Ca\(^{2+}\) transport with a K\(_{i}\) of 35 \(\mu M\). Ca\(^{2+}\) binding in the absence of ATP and phosphoenzyme formation by the utilization of ATP in the presence of Ca\(^{2+}\) are also inhibited within the same CLT concentration range. On the other hand, phosphoenzyme formation by utilization of P\(_{i}\) in the absence of Ca\(^{2+}\) is only minimally inhibited. It is concluded that CLT inhibits primarily Ca\(^{2+}\) binding and, consequently, the Ca\(^{2+}\)-dependent reactions of the SERCA cycle. It is suggested that CLT resides within the membrane-bound region of the transport ATPase, thereby interfering with binding and the conformational effects of the activating cation.

Clotrimazole (CLT) is an antifungal imidazole derivative widely used for the treatment of yeast infections. Like other drugs belonging to the same imidazole family (e.g. miconazole and ketoconazole), its antifungal effect is because of inhibition of cytochrome P-450, an enzyme involved in ergosterol biosynthesis by the yeast (1, 2). It has also been shown that CLT inhibits cell proliferation of several normal and cancer cells in vitro (3). Interference with cellular Ca\(^{2+}\) homeostasis contributes to this inhibition; in particular, the action of CLT involves depletion of intracellular Ca\(^{2+}\)-stores, also confirmed in Madin-Darby canine kidney cells, where CLT induces Ca\(^{2+}\) release from thapsigargin-sensitive Ca\(^{2+}\)-stores (4).

It is reported that CLT affects the Ca\(^{2+}\) pump activity of SR vesicles isolated from rabbit heart muscle, producing inhibition of the Ca\(^{2+}\)-dependent reactions of the ATPase (SERCA2) obtained from cardiac myocytes (5). With the experiments reported here, we found that, in fact, CLT inhibited the Ca\(^{2+}\)-ATPase. We investigated the effect of CLT on the Ca\(^{2+}\)-ATPase (SERCA1) associated with native membrane vesicles derived from rabbit fast-twitch skeletal muscle. Under optimal coupling conditions, the SERCA pump translocates two calcium ions per ATP molecule from the cytoplasm to the lumen of SR with a turnover rate of about 10 s\(^{-1}\), forming a 3 orders of magnitude Ca\(^{2+}\) gradient (6–9).

Altered SERCA function is relevant to pathogenic mechanisms of several diseases (10–12). Furthermore, SERCA function can be affected by several compounds of possible pharmacological interest, such as thapsigargin (TG) (13), cyclopiazonic acid (14), curcumin (15, 16), 2,5-di-tert-butyl-1,4-dihydroxybenzene (17), and 1,3-dibromo-2,4,6-tris(methylisothiourea)benzene (18).

In addition to biochemical characterization of the ATPase and its partial reactions, we performed direct measurements of charge translocation following adsorption of SR vesicles containing ATPase onto a biomimetic membrane supported by a gold electrode and activation by rapid mixing with a suitable substrate (19). This technique has been used successfully in studies of electrogenic transport by several membrane proteins, such as Na\(^{+}\),K\(^{+}\)-ATPase (20, 21), melibiose permease (22), and Na\(^{+}\)/proline antiporter (23), including the SERCA pump (24, 25).

We found that CLT interferes specifically with Ca\(^{2+}\) binding to SERCA even in the absence of ATP. For this reason, the Ca\(^{2+}\)-dependent reactions of this enzyme are also inhibited.

MATERIALS AND METHODS

Chemicals—Calcium and magnesium chlorides and MOPS were obtained from Merck at analytical grade. ATP disodium salt (97%) and dithiothreitol (≥99%) were purchased from Fluka. Octadecanethiol (98%) from Aldrich was used without further purification. EGTA, calcium ionophore A23187, and 1-[o-chloro-o,a,a'-diphenylbenzyl]-imidazole (clotrimazole) were obtained from Sigma. The lipid solution contained diphytanoylphosphatidylcholine (Avanti Polar Lipids) and octadecylamine (≥99.0%, Fluka) (60:1) and was prepared at a concentration of 1.5% (w/v) in n-decane (Merck), as described by Bamburg et al. (26).

ATPase Preparation—Sarcoplasmic reticulum vesicles were obtained by extraction from the fast-twitch hind leg muscle of New Zealand White rabbit followed by homogenization and differential centrifugation, as described by Eletr and Inesi (27). The vesicles derived from longitudinal SR membrane contained only negligible amounts of the ryanodine receptor Ca\(^{2+}\) channel associated with junctional SR (light vesicles).

Free Ca\(^{2+}\) concentration was calculated with the computer program WinMAXC (28). Unless otherwise stated, 1 \(\mu M\) calcium ionophore A23187 was used to prevent Ca\(^{2+}\) accumulation by the SR vesicles (29).

ATPase Activity—Ca\(^{2+}\)-ATPase hydrolytic activity was determined following P\(_{i}\) production by a colorimetric method (30). The reaction mixtures contained 20 mM MOPS (pH 7.0), 80 mM KCl, 3 mM MgCl\(_2\), and...
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0.2 mM EGTA, 0.2 mM CaCl₂, 2 μg of SR protein/ml, and 3 μM A23187 ionophore and were preincubated with various concentrations of CLT for 30 min. The reaction was started by addition of 1 mM ATP. The incubation temperature was 37 °C.

igel: ([32P]Pi) was added to half of the samples to provide controls exhibiting no specific Ca²⁺ binding because it was demonstrated previously that TG prevents specific Ca²⁺ binding (13). The reaction temperature was 25 °C. The reaction mixture was filtrated with an 0.45-μm filter, and radioactivity was measured by a liquid scintillation counter.

Phosphorylation by [γ-32P]ATP—Enzyme phosphorylation by ATP was measured in a pH 7.0 ice-cold reaction mixture containing 20 mM MOPS, 80 mM KCl, 2 mM MgCl₂, 0.1 mg of SR protein/ml, and 3 μM A23187 ionophore after a preincubation with various concentrations of CLT for 30 min. Individual samples (1 ml) were started by the addition of 20 μl [γ-32P]ATP and quenched at 1 s with 1 M perchloric acid. After filtration and washing of the sample, radioactivity of the sample was measured with a liquid scintillation counter.

Phosphorylation by [32P]P₀—Enzyme phosphorylation with P₀ was measured at 25 °C in a pH 6.2 reaction mixture containing 50 mM MES, 10 mM MgCl₂, 2 mM EGTA, and 20% Me₃SO after a 30-min preincubation with various concentrations of CLT in the presence of 1 mM [32P]P₀, and 0.5 mg of SR protein/ml. The reaction was acid-quenched after a 10-min incubation by the addition of 1 M perchloric acid. The EP measurements were conducted using a filtration method.

Measurement of Charge Movements—Charge movements were measured by adsorbing the SR vesicles containing the Ca²⁺-ATPase onto a biomimetic membrane supported by a gold electrode (the so-called solid-supported membrane (SSM)). The SSM consisted of an octadecanethiol monolayer covalently bound to a gold surface via the sulfur atom with a phospholipid monolayer on top of it (31). After adsorption, usually carried out at an applied potential of +0.1 V, the protein was activated by a rapid injection of a solution containing the appropriate substrate, e.g. ATP or Ca²⁺ ions. If at least one electrogenic step is involved in the relaxation process, a current transient (pre-steady state) can be recorded along the external circuit. The acquisition of transients obtained under different experimental conditions, together with their subsequent elaboration, can provide important kinetic information about protein function and/or its modulation by drugs, peptides, and small soluble proteins. In particular, from a current transient, three main data can be extracted: the peak current, the translocated charge, and the decay time constants. The charge is obtained by numerical integration of the current transient, whereas the decay time constants are provided by a multieponential fitting of the transient (19, 20, 24, 25). The time constants are strictly correlated with the rate constants of the reaction steps involved in the activation of the pump (32). The preparation of the gold electrodes and the whole experimental setup as well as the solution exchange technique are described in detail by Tadini-Buoninsegni et al. (25).

For the activation experiments, the composition of the solutions was the following: (a) for Ca²⁺ concentration jumps, the non-activating solution contained 150 mM choline chloride, 25 mM MOPS (pH 7.0), 0.25 mM EGTA, 1 mM MgCl₂, 0.2 mM dithiothreitol, and the activating solution had the same composition plus the required CaCl₂ amount added from a 5, 50 or 500 mM stock solution; (b) for ATP concentration jumps, the non-activating solution contained 150 mM choline chloride,
On and Off Ca\textsuperscript{2+} Concentration Jumps in the Presence of CLT in Pre-steady State—A Ca\textsuperscript{2+} concentration jump, performed with the SSM-based technique, yielded a current transient that could be ascribed to Ca\textsuperscript{2+} binding to the ATPase. An ON signal was obtained following the injection of the Ca\textsuperscript{2+}-containing solution (Fig. 3A, step 1), and an OFF signal was obtained upon calcium release from the binding site following injection of a solution containing EGTA and no calcium (Fig. 3A, step 2) (25). We then performed these experiments in the presence of increasing concentrations of CLT to assess the effect of the drug on Ca\textsuperscript{2+} binding in the absence of ATP. In agreement with direct measurements of Ca\textsuperscript{2+} binding (Fig. 2), we found that charge movements attributed to Ca\textsuperscript{2+} binding were also reduced by CLT.

Fig. 4 compares a saturating calcium concentration jump in the absence and in the presence of 5 \textmu M CLT, showing a clear reduction of the signal in the presence of the drug. Moreover, a titration with increasing Ca\textsuperscript{2+} concentrations (Fig. 5) shows that the signal is reduced to about one-third of that recorded in the absence of the inhibitor (25). On the other hand, despite the reduction in maximal binding, the \(K_d\) and \(n\) values (0.53 and 1.7 \textmu M, respectively) obtained from fitting to the Hill function are very close to those obtained in the absence of drug, either by measurements of charge movements (25) or by direct measurements with a radioactive tracer (33). This indicates that non-saturating CLT reduces the amount of Ca\textsuperscript{2+} bound but does not affect the binding characteristics (i.e. affinity) of the enzyme molecules that are not totally inhibited.

We also found that titrations with increasing CLT concentrations in the presence of 10 or 100 \textmu M Ca\textsuperscript{2+} concentrations (Fig. 6) yield the same inhibition curve. These results are in agreement with the experiments on enzyme phosphorylation with ATP (Fig. 2) and ATPase hydrolytic activity (Fig. 1) and imply that increasing the Ca\textsuperscript{2+} concentration above the saturating level does not protect the enzyme from CLT. Therefore, the CLT inhibition is not competitive with respect to Ca\textsuperscript{2+}. On the other hand, we found that the effective CLT concentration is lower when the inhibitor is added before (\(K_I = 7\) \textmu M, as in Fig. 6) rather than after Ca\textsuperscript{2+} (\(K_I = 35\) \textmu M, as in Figs. 1, 2, and 7). A similarly greater resistance of the Ca\textsuperscript{2+}-bound conformation of the enzyme (Ca\textsubscript{2}E1) as compared with the Ca\textsuperscript{2+}-free conformation (E2) was found previously with respect to TG (13) and miconazole (34).

ATP Concentration Jumps in the Presence of CLT—An ATP concentration jump in the presence of Ca\textsuperscript{2+}, performed on a SSM with SR vesicles containing Ca\textsuperscript{2+}-ATPase adsorbed onto it, produces a current transient because of the electrogenicity of Ca\textsuperscript{2+} release into the lumen of the SR and, to some extent, to Ca\textsuperscript{2+} re-binding to the protein after the hydrolytic cleavage of P-E\textsubscript{2} intermediate (Fig. 3B, steps 2 and 4). It was previously determined that the formation and hydrolytic cleavage of the phosphorylated intermediate are not electrogenic (35).

Fig. 7 shows the effect of 35 \textmu M CLT on a 100 \textmu M ATP concentration jump in the presence of saturating Ca\textsuperscript{2+} (100 \textmu M). It is clear that the current transient because of Ca\textsuperscript{2+} translocation is affected by CLT. In fact, starting from no CLT and increasing CLT concentration up to 35 \textmu M, peak currents are progressively decreased. Furthermore, integration of the current transients yields values for moved charges that exhibit a behavior very similar to that of peak currents with respect to the effect of CLT (Fig. 7, inset).

It is noteworthy that exponential fitting of the current transients yields two time constants for current decay. The two constants, \(\tau_1\) and \(\tau_2\), have average values of 27 ± 2 and 42 ± 3 ms, respectively and do not appear to be significantly affected by CLT (not shown), irrespective of the effect of CLT on peak currents and moved charge.
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![Image 1](https://example.com/image1.png)

**FIGURE 5.** Titration of SERCA with calcium in the presence of 5 μM CLT obtained by Ca²⁺ concentration jumps experiments. Values are normalized with respect to the bound charge for a saturating Ca²⁺ concentration jump (10 μM) in the absence of CLT. The solid line represents the fitting to a Hill function \( Q = Q_{\text{norm, max}} \cdot C^n / (K_C^n + C^n) \), which yields \( K_C = 0.53 \pm 0.03 \) μM and \( n = 1.7 \pm 0.2 \). The composition of the solution is indicated under “Materials and Methods.”

![Image 2](https://example.com/image2.png)

**FIGURE 6.** Dependence of the normalized charge following Ca²⁺ concentration jumps on CLT concentration. Free calcium concentration was 10 μM (filled circle and solid line) or 100 μM (open circle and dashed line). The composition of the solution is indicated under “Materials and Methods.”

**DISCUSSION**

CLT is an antifungal agent, the effect of which is attributed to inhibition of cytochrome P-450 and ergosterol biosynthesis in yeast [1, 2]. It is also shown that CLT inhibits cell proliferation in vitro [3], interferes with calcium homeostasis in culture cells [3, 4], and inhibits the calcium pump (SERCA2) obtained with the microsomal fraction of heart muscle [5]. We have characterized in detail the effect of CLT on the Ca²⁺-ATPase activity associated with microsomal vesicles obtained from skeletal muscle. This enzyme preparation is highly purified and well suited to characterization of the catalytic cycle and its partial reactions [6–9]. In addition, several SERCA inhibitors have been described [13–18], thereby yielding useful grounds for comparison of inhibitory mechanisms. Finally, the simultaneous use of biochemical and biophysical methods in the characterization of the CLT inhibitory mechanism has given us the opportunity to demonstrate clearly the functional relevance of charge transfer measurements and their correspondence to partial reactions of the ATPase cycle.

As outlined in Fig. 3B, the ATPase (SERCA) cycle requires initial activation through Ca²⁺ binding from the exterior of the vesicles (step 1) followed by utilization of ATP to form a phosphorylated intermediate (step 2). The bound Ca²⁺ is then dissociated into the lumen of the vesicles (Fig. 3B, step 2) followed by hydrolytic cleavage of the phospho-enzymic (step 3). Using direct binding assays by a radioactive tracer as well as measurements of charge movements by the SSM-based technique, we found that the initial Ca²⁺ binding, before utilization of ATP, is inhibited by CLT (Figs. 2, 4, and 6). It is of interest that plots of charge as a function of Ca²⁺ concentration (Fig. 5) yield affinity constants and cooperative behavior identical to that of equilibrium binding isotherms obtained in the absence of CLT by direct measurements with isotopic tracer (33). The correspondence of the two types of measurements is highly satisfactory and demonstrates the specificity of charge movement with regard to Ca²⁺ binding and its inhibition by CLT.

Considering the absolute Ca²⁺ requirement for enzyme activation, it is expected that steady state ATPase activity will be inhibited in parallel with inhibition of Ca²⁺ binding by CLT. In fact, we found that steady state ATPase activity and Ca²⁺ transport were inhibited by the same CLT concentrations producing inhibition of Ca²⁺ binding. This was demonstrated by measuring P_i by a colorimetric method after adding ATP to the enzyme in the presence of Ca²⁺ (Fig. 1) as well as by measuring charge translocation following an ATP concentration jump by the SSM-based technique (Fig. 7). Here again, identical results were obtained by the biochemical and biophysical methods. An interesting observation is that, whereas CLT clearly decreases the amount of moved charge (Fig. 7), the decay kinetics of the remaining current transient is not affected by CLT (not shown). Therefore, although an increasing number of enzyme molecules are inactivated by CLT, the remaining enzyme molecules do not bind CLT and continue to function normally. This suggests a specific inhibitory site for CLT within the ATPase molecule whereby the ATPase molecules are inhibited independent of each other. Furthermore, comparative experiments performed by addition of CLT before or after Ca²⁺ suggest that the Ca²⁺-free conformation of the enzyme is more sensitive to CLT and is stabilized by the bound inhibitor.

We also found that phospho-enzymic formation by utilization of ATP in the presence of Ca²⁺ is inhibited by CLT within the same concentration range as Ca²⁺ binding (Fig. 2) and ATPase hydrolytic activity (Fig.
with a pattern consistent with noncompetitive inhibition. However, phosphoenzyme formation by utilization of P_i in the reverse direction of cycle is only minimally affected by CLT (Fig. 2), as also observed by Snajdrova et al. (5). This reverse reaction is not dependent on activation by Ca^{2+} and in fact requires removal of bound Ca^{2+} (36). Therefore, it is apparent that the ATPase catalytic mechanism is not primarily affected by CLT but only secondarily, through interference with activation by Ca^{2+} binding. Such a specific interference with a partial reaction (i.e. Ca^{2+} binding) is of interest when compared with the global inhibition of the ATPase cycle produced by TG. So far, 1,3-dibromo-2,4,6-tris(methylsulfothionium)benzene is the only other ATPase inhibitor producing specific inhibition of a single partial reaction, i.e. the E1-P → E2-P transition (37).

It should be noted that CLT shows a rather high affinity for SERCA with a K_i of 35 μM in the presence of Ca^{2+} and K_i of 7 μM in the absence of Ca^{2+}. Considering the hydrophobic character of the CLT molecule and the location of cation-binding sites in the membrane-bound region of transport ATPases, it is apparent that CLT interferes at this location with the binding of the activating cation and with the conformational change involved in cation binding. In addition to the significance of the CLT effect with regard to the catalytic and possibly pharmacological mechanism, we consider that stabilization of the Ca^{2+}-free, E2 conformation of the enzyme, leaving the catalytic site in a functional state as revealed by the P_i reaction, may be an extremely useful tool in structural and crystallization studies of cation transport ATPases. In fact, ATPase crystal structures of the highest resolution are presently obtained by incorporation of two (rather than one) inhibitors, permitting detection of ordered water molecules and advanced electrostatic calculations yielding estimates of the proton occupancy of acidic residues (38). At the same time, the availability of several inhibitors supports organic synthesis of highly specific and potent compounds, guided by the available crystal structure of the ATPase (39, 40).

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