Clotrimazole, an Antimycotic Drug, Inhibits the Sarcoplasmic Reticulum Calcium Pump and Contractile Function in Heart Muscle*

(Received for publication, May 6, 1998, and in revised form, July 30, 1998)

Lenka Snajdrova, Ande Xu, and Njanoor Narayanan†

From the Department of Physiology, University of Western Ontario, London, Ontario N6A 5C1, Canada

Clotrimazole (CLT), an antimycotic drug, has been shown to inhibit proliferation of normal and cancer cell lines and its systemic use as a new tool in the treatment of proliferative disorders is presently under scrutiny (Benzaquen, L. R., Brugnara, C., Byers, H. R., Gattoni-Celli, S., and Halperin, J. A. (1995) Nature Med. 1, 594–540). The action of CLT is thought to involve depletion of intracellular Ca\(^{2+}\) stores but the underlying mechanism has not been defined. The present study utilized membrane vesicles of rabbit cardiac sarcoplasmic reticulum (SR) to determine the mechanism by which CLT depletes intracellular Ca\(^{2+}\) stores. The results revealed a strong, concentration-dependent inhibitory action of CLT on the ATP-energized Ca\(^{2+}\) uptake activity of SR (50% inhibition with \(~35 \mu M\) CLT). The inhibition was of rapid onset (manifested in <15 s), and was accompanied by a 7-fold decrease in the apparent affinity of the SR Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) and a minor decrement in the enzyme’s apparent affinity toward ATP. Exposure of SR to CLT in the absence or presence of Ca\(^{2+}\) resulted in irreversible inhibition of Ca\(^{2+}\) uptake demonstrating that the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free conformations of the Ca\(^{2+}\)-ATPase are CLT-sensitive. Introdution of CLT to the reaction medium subsequent to induction of enzyme turnover with Ca\(^{2+}\) and ATP resulted in instantaneous cessation of Ca\(^{2+}\) transport indicating that an intermediate enzyme species generated during turnover undergoes rapid inactivation by CLT. The inhibition of Ca\(^{2+}\) uptake by CLT was accompanied by inhibition of Ca\(^{2+}\)-stimulated ATP hydrolysis and Ca\(^{2+}\)-induced phosphoenzyme intermediate formation from ATP in the ATPase catalytic cycle. Phosphorylation of the Ca\(^{2+}\)-deprived enzyme with P\(_i\) in the reverse direction of catalytic cycle and Ca\(^{2+}\) release from Ca\(^{2+}\)-preloaded SR vesicles were unaffected by CLT. It is concluded that CLT depletes intracellular Ca\(^{2+}\) stores by inhibiting Ca\(^{2+}\) sequestration by the Ca\(^{2+}\)-ATPase. The mechanism of ATPase inhibition involves a drug-induced alteration in the Ca\(^{2+}\)-binding site(s) resulting in paralysis of the enzyme’s catalytic and ion transport cycle. CLT (50 \mu M) caused marked depression of contractile function in isolated perfused, electrically paced rabbit heart preparations. The contractile function recovered gradually following withdrawal of CLT from the perfusate indicating the existence of mechanisms in the intact cell to inactivate, metabolize, or clear CLT from its target site.

Clotrimazole (CLT)\(^1\) is an antimycotic imidazole derivative widely used for the treatment of yeast infections and its fungicidal action has been attributed to the inhibition of sterol 14a-demethylase, a microsomal cytochrome P-450-dependent enzyme (1, 2). Imidazole antifungics have also been shown to be potent inhibitors of many mammalian cytochrome P-450-mediated reactions (3–5). Recently, CLT has been shown to have a remarkable ability to inhibit the proliferation of normal and cancer cell lines \textit{in vitro} and \textit{in vivo} (6), and the potential of this drug as a new tool in the treatment of proliferative disorders is now being explored. Evidence from mechanistic studies indicates that the growth-blocking property of CLT may arise from its ability to interfere with cellular Ca\(^{2+}\)-homeostasis. Thus, it has been shown that CLT depletes intracellular Ca\(^{2+}\) stores in 3T3 cells (6), inhibits voltage- and ligand-stimulated Ca\(^{2+}\) influx mechanisms in GH3 cells, chromaffin cells, and thymocytes (7, 8), as well as Ca\(^{2+}\)-activated K\(^+\) channels in erythrocytes and thymocytes (9, 10). The effects of CLT on Ca\(^{2+}\) influx and Ca\(^{2+}\)-activated K\(^+\) channels may be secondary to the drug-induced depletion of intracellular Ca\(^{2+}\) stores (6). It has not been clarified whether the CLT-induced depletion of intracellular Ca\(^{2+}\) stores is due to activation of Ca\(^{2+}\) release from intracellular stores or due to inhibition of reuptake of Ca\(^{2+}\) back into intracellular stores.

In muscle cells, the intracellular membrane system of SR plays a central role in the storage and release of Ca\(^{2+}\) during the contraction-relaxation cycle. Upon myocyte excitation, Ca\(^{2+}\) is released from the SR through the ryanodine receptor-Ca\(^{2+}\) release channel to initiate muscle contraction (11–13). Subsequent muscle relaxation occurs upon sequestration of Ca\(^{2+}\) back into the SR lumen by an SR-associated Ca\(^{2+}\)-pumping ATPase (14–17). During the translocation of Ca\(^{2+}\) across the SR membrane, Ca\(^{2+}\)-ATPase serves as an energy transducer and a carrier for Ca\(^{2+}\) (15–17). The mechanism of Ca\(^{2+}\) transport by the SR Ca\(^{2+}\)-ATPase is recognized to involve cyclic transitions between two major conformational states, \(E_1\) and \(E_2\) (15–17). These two states differ in that the affinity for Ca\(^{2+}\) is high in the \(E_1\) conformation and low in the \(E_2\) conformation, and in that the Ca\(^{2+}\)-binding sites are exposed to the cytoplasmic side of SR in \(E_1\) but to the luminal side of SR in \(E_2\). The catalytic and ion transport cycle begins with the binding of 2 mol of Ca\(^{2+}\) ions followed by 1 mol of Mg\(^{2+}\)-ATP to the \(E_1\) form of the ATPase. An aspartic acid residue (Asp\(^{351}\)) in the active site is then phosphorylated by the terminal phosphate of ATP forming an acylphosphate. Phosphorylation of the enzyme results in a conformational change in the \(E_1\)PCa\(^{2+}\)–phosphoenzyme intermediate to the \(E_2\)PCa\(^{2+}\) form which has decreased Ca\(^{2+}\) affinity. The Ca\(^{2+}\)-binding sites are now everted so that they face the SR lumen to which Ca\(^{2+}\) is subsequently released.

* This work was supported by Medical Research Council of Canada Grant MT 9553. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom all correspondence should be addressed: Dept. of Physiology, University of Western Ontario, London, Ontario N6A 5C1, Canada. Tel.: 519-661-3469; Fax: 519-661-3827; E-mail: nnarayan@physiology.uwo.ca.

\(^1\) The abbreviations used are: CLT, clotrimazole; SR, sarcoplasmic reticulum; Mg\(^{2+}\)SO\(_4\), dimethyl sulfoxide; NCD4, N-cyclohexyl-N’-(4-di-methylamino-1-napthyl) carbamidine; EP, phosphoenzyme; CPA, cyclopiazonic acid; TG, thapsigargin.
The Mg$^{2+}$-catalyzed hydrolysis of $E_{2}$P devoid of bound Ca$^{2+}$ ions results in the release of $P$ into the cytoplasm leaving $E_{2}$, which isomerizes to $E_{1}$ to complete the cycle. The Ca$^{2+}$-ATPase reaction mechanism is thought to be similar for different isoforms of this enzyme expressed in muscle and non-muscle tissues (18).

Given its robust Ca$^{2+}$ cycling properties, the SR membrane provides an excellent model system to investigate the mechanisms by which CLT depletes intracellular Ca$^{2+}$ stores. Here we present the results from studies using rabbit cardiac SR demonstrating a strong inhibitory action of CLT on the Ca$^{2+}$ ion transporting as well as energy transducing functions of the Ca$^{2+}$-ATPase. On the other hand, CLT was found to have no effect on Ca$^{2+}$ release from Ca$^{2+}$-preloaded SR vesicles. In isolated perfused beating rabbit hearts, infusion of CLT resulted in marked depression of contractile function.

**EXPERIMENTAL PROCEDURES**

**Materials**—4$^{55}$CaCl$\_2$ and [32P]Na$_2$PO$_4$ were purchased from New England Nuclear (Montreal, PQ, Canada), and [32P]ATP from Amersham (Oakville, ON, Canada). CLT was from Sigma. All other chemicals were from Sigma or BDH Chemicals (Toronto, ON, Canada).

**Preparation of SR Vesicles**—SR membrane vesicles were prepared from heart ventricles of New Zealand White rabbits (body weight 2.5–3 kg) as described previously (19). Following isolation, the SR vesicles were suspended in 10 mM Tris maleate (pH 6.8) containing 100 mM KCl and stored at −80 °C after quick-freezing in liquid N$_2$. Protein concentration was determined by the method of Lowry et al. (20) using bovine serum albumin as standard.

**Ca$^{2+}$ Transport and Ca$^{2+}$-ATPase Assays**—ATP-dependent, oxalate-facilitated Ca$^{2+}$ uptake by SR was determined using the Millipore filtration technique as described previously (21). The standard incubation medium for Ca$^{2+}$ uptake (total volume 250 μl) contained 50 mM Tris maleate (pH 6.8), 5 mM MgCl$_2$, 5 mM Na$_2$HPO$_4$, 106 mM Me$_2$SO, 4 mM [32P]Na$_2$PO$_4$, and SR (50 μg). The reaction mixture was initiated by the addition of 50 μM EGTA, 0.1 mM CaCl$_2$, and SR (50 μg of protein). The Ca$^{2+}$-dependence of phosphoenzyme formation was monitored in parallel assays in the absence of Ca$^{2+}$ and in the presence of EGTA. The reaction was initiated by the addition of ATP following preincubation of the rest of the assay mixture at 23 °C for 3 min, was allowed to proceed for 30 s and was quenched with 1 ml of 10% trichloroacetic acid containing 4 mM NaN$_3$. The acid-denatured protein was recovered by centrifugation, the pellets were washed with 0.2 M NaHPO$_4$, digested in 500 μl of 50 mM Tris-HCl (pH 7.5) containing 2% sodium dodecyl sulfate, and the 32P radioactivity was determined by liquid scintillation counting.

**Ca$^{2+}$ Release Assay**—[4$^{55}$Ca]$^{2+}$ release rates from SR vesicles passively loaded with [4$^{55}$Ca]Cl$_2$ were determined by Millipore filtration (25). Passive Ca$^{2+}$ loading was performed by incubating SR vesicles (1 mg of protein/ml) at 23 °C for 40 min in a medium containing 50 mM Tris maleate (pH 6.8), 100 mM KCl, 1 mM oxalate, 10% Me$_2$SO, and 40 μM TG. The SR vesicles were then resuspended in assay medium (50 mM Tris maleate, pH 6.8, containing 2 mM MgCl$_2$ and 1 mM EGTA) that was preincubated for 5 min at 37 °C. Subsequently aliquots of the incubation mixture were filtered through Millipore filters at 30-μs intervals for a period of 5 min. The filters were washed with 3 ml of ice-cold 10 mM Tris maleate buffer containing 120 mM KCl, 10 mM MgCl$_2$, and 10 μM ruthenium red, dried at 60 °C, and 45Ca$^{2+}$ radioactivity was determined by liquid scintillation counting.

**Heart Perfusion and Measurement of Contractile Function**—Rabbits were anesthetized with sodium pentobarbital (35 mg/kg, intravenous), the hearts were excised and immediately cannulated for retrograde aortic perfusion of the coronary arteries with mammalian Ringer solution consisting of 154 mM NaCl, 5 mM KCl, 2.2 mM CaCl$_2$, 6 mM NaHCO$_3$, and 5.5 mM dextrose. The perfusion buffer was equilibrated with 95% O$_2$-5% CO$_2$, which maintained a pH of 7.4; the perfusion temperature was set at 37 ± 0.2 °C. The hearts were perfused at a constant flow rate of 25 ml/min using a peristaltic pump. After an initial 15–20 min of perfusion, when the spontaneous beating had stabilized, the hearts were paced electrically at a rate of 120 beats/min with a Grass SD9 stimulator via a platinum wire electrode inserted into the epicardium, at double threshold voltage and a duration of 5 ms. A latex balloon-tipped cannula filled with degassed H$_2$O was inserted into the lumen of the left ventricle for obtaining systolic left ventricular pressure (LVP) development. The cannula was connected via a pressure transducer (COBE, Bramalea, Canada) to a BioPac System Digital Monitor (model MP100) and a personal computer which allowed on-line monitoring of LVP and off-line calculation of developed pressure, rate of pressure development (+dP/dt), and rate of relaxation (−dP/dt).

**Data Presentation**—Unless specified otherwise, the experimental values represent the average of at least three independent experiments using separate SR preparations performed in duplicate. The data are presented as mean ± S.E.

---

Under the standard Ca$^{2+}$ uptake assay conditions employed in this study, 0.1 μM TG was found to produce complete inhibition of Ca$^{2+}$ sequestration by the SR.
Inhibition of Ca\(^{2+}\)-ATPase by Clotrimazole

**RESULTS**

Effect of CLT on the Time Course of ATP-dependent Ca\(^{2+}\) Uptake by SR—The ATP-dependent, oxalate-facilitated Ca\(^{2+}\) uptake by SR vesicles is a useful, commonly used parameter to measure the Ca\(^{2+}\)-pump (Ca\(^{2+}\)-ATPase) function of SR in vitro (15). Fig. 1 shows the time course of ATP-dependent Ca\(^{2+}\) uptake by cardiac SR vesicles measured in the absence of CLT and in the presence of three selected concentrations of CLT. The rates of Ca\(^{2+}\) uptake by SR were strongly inhibited in the presence of CLT; the degree of inhibition increased with increasing concentration of CLT.

CLT and SR Protein Concentration Dependence on the Inhibitory Action of CLT on Ca\(^{2+}\) Uptake—In the experiments shown in Fig. 2A, the ATP-dependent Ca\(^{2+}\) uptake was determined in the absence of CLT and in the presence of varying concentrations of CLT (16–120 μM); the amount of SR in the assay medium was kept constant at 30 μg of protein/ml (i.e. 7.5 μg of protein/250 μl of assay medium, see “Experimental Procedures”). It is seen that CLT inhibited Ca\(^{2+}\) uptake strongly and in a concentration-dependent manner. Under these conditions, 50% inhibition of Ca\(^{2+}\) uptake was observed at ~35 μM CLT; nearly complete inhibition of Ca\(^{2+}\) uptake occurred at CLT concentrations of 80–120 μM.

The results presented in Fig. 2B demonstrate the influence of SR protein concentration in the assay medium on the inhibitory action of CLT on Ca\(^{2+}\) uptake by SR. In these experiments, the SR protein concentration in the assay medium was varied from 0.02 to 0.32 mg/ml and Ca\(^{2+}\) uptake was measured in the absence of CLT and in the presence of two selected concentrations of CLT (32 and 64 μM). It can be seen that a given concentration of CLT, increasing the concentration of SR in the reaction mixture leads to progressively less inhibition of Ca\(^{2+}\) uptake. This relationship is more pronounced at the low (32 μM) CLT concentration. Thus, the CLT concentration dependence of Ca\(^{2+}\) uptake inhibition is an apparent function of the stoichiometric relationship of the inhibitor and Ca\(^{2+}\)-ATPase (see “Discussion”).

Effect of CLT on Ca\(^{2+}\)-ATPase Activity—Since CLT inhibited ATP-dependent Ca\(^{2+}\) uptake by SR, the effect of CLT on Ca\(^{2+}\)-ATPase activity (ATP hydrolysis) of SR was investigated. Incubation of SR vesicles with varying concentrations of CLT (16–120 μM) under the assay conditions identical to that used for Ca\(^{2+}\) uptake led to concentration-dependent inhibition of Ca\(^{2+}\)-stimulated ATPase activity (Fig. 3A). This inhibition of ATPase activity occurred at the same concentration range of CLT required for inhibition of Ca\(^{2+}\) uptake (cf. Fig. 2A). Therefore the observed reduction of Ca\(^{2+}\) uptake is a consequence of a primary inhibition of ATPase activity by CLT. The Ca\(^{2+}\)-stimulated ATPase activity of SR measured in the absence of CLT in these experiments (Fig. 3A), however, exceeded the Ca\(^{2+}\) uptake activity determined under identical assay conditions (Fig. 2A). This excess ATP hydrolysis is apparently due to uncoupling of ATP hydrolysis and Ca\(^{2+}\) transport prevailing in the isolated SR vesicles and/or contribution of other ATPase(s) unrelated to the Ca\(^{2+}\)-pumping ATPase. In order to examine the specific effect of CLT on the SR Ca\(^{2+}\)-pumping ATPase, additional experiments were performed where TG (0.1 μM)-sensitive Ca\(^{2+}\)-ATPase activity of SR was determined in the absence of CLT and in the presence of varying concentrations of CLT. Also, the assays were performed in the absence of oxalate

---

3 Densitometric scanning of cardiac SR proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the band corresponding to the Ca\(^{2+}\)-ATPase accounted for about 10% of the total protein. Analysis of the number of Ca\(^{2+}\)-ATPase catalytic units/protein weight unit based on steady state levels of EP (Fig. 7) gave a value of ~4%. The lower value based on EP measurements is to be expected since only one-half of the total enzyme molecules seem to undergo phosphorylation (Ref. 40 and reports cited therein).
and in the presence of a Ca\(^{2+}\) ionophore (A23187, 3 \(\mu M\)) in the assay medium which permitted linear rates of ATP hydrolysis. The results showed concentration-dependent inhibition of TG-sensitive ATPase activity by CLT (Fig. 3B). The concentration of CLT required for half-maximal inhibition of TG-sensitive Ca\(^{2+}\)-ATPase activity (~36 \(\mu M\), Fig. 3B) and Ca\(^{2+}\)-stimulated ATPase activity (~30 \(\mu M\), Fig. 3A) was found to be essentially similar. The TG-sensitive ATPase activity measured in the absence of CLT and in the presence of ionophore (Fig. 3B) was about 4-fold higher than the Ca\(^{2+}\)-stimulated ATPase activity measured in the absence of ionophore and in the presence of oxalate (Fig. 3A). Such difference in enzyme activity is likely due to stimulation of the SR Ca\(^{2+}\)-ATPase by the ionophore (26, 27). 

At a submaximally effective concentration of CLT (32 \(\mu M\)), inhibition of TG-sensitive Ca\(^{2+}\)-ATPase activity was observed at varying Ca\(^{2+}\) concentrations (Fig. 3B, inset). The inhibitory effect of CLT on Ca\(^{2+}\)-ATPase activity was associated with decrements in maximal velocity \(V_{\text{max}}\) (nmol of P/mg of protein/3 min): control, 2491 ± 62; +32 \(\mu M\) CLT, 1573 ± 31) as well as the enzyme’s apparent affinity for Ca\(^{2+}\) \(K_{0.5}\) for Ca\(^{2+}\) (\(\mu M\)): control, 0.8 ± 0.06; +32 \(\mu M\) CLT, 2.8 ± 0.10.

Effect of CLT on Ca\(^{2+}\)-uptake by SR at Varying Concentrations of Ca\(^{2+}\) and ATP—The results presented in Fig. 4 show the effect of two selected concentrations of CLT (32 or 64 \(\mu M\)) on Ca\(^{2+}\) uptake by SR at a wide range of Ca\(^{2+}\) concentrations (9 nm- 71 \(\mu M\)). CLT inhibited Ca\(^{2+}\) uptake at all Ca\(^{2+}\) concentrations tested. With a submaximally effective concentration of CLT (32 \(\mu M\)), the inhibitory effect could not be overcome with increasing Ca\(^{2+}\) concentration. The kinetic parameters derived from the data shown in Fig. 4 are summarized in Table I. It can be seen that the inhibitory action of CLT is associated with decrements in the apparent affinity of the Ca\(^{2+}\)-ATPase for Ca\(^{2+}\), Hill coefficient \(n_{\text{Hill}}\) for Ca\(^{2+}\), as well as \(V_{\text{max}}\) of Ca\(^{2+}\) transport.

When Ca\(^{2+}\)-uptake assays were performed in the presence of various ATP concentrations, CLT inhibition was found to be independent of ATP concentration (Fig. 5). Approximately 60% decrease in the \(V_{\text{max}}\) of Ca\(^{2+}\) uptake was observed in the presence of 32 \(\mu M\) CLT \(V_{\text{max}}\) (nmol of Ca\(^{2+}\)/mg of protein/min): control, 120; +32 \(\mu M\) CLT, 50. This inhibitory action of CLT also appeared to involve a modest decrease in the apparent affinity of the Ca\(^{2+}\)-ATPase for ATP \(K_{0.5}\) for ATP (\(\mu M\)): control, 0.11; +32 \(\mu M\) CLT, 0.20.

Blockade of Ca\(^{2+}\) Uptake by Addition of CLT during Ca\(^{2+}\)-ATPase Turnover Cycle—To investigate the effect of CLT on Ca\(^{2+}\)-ATPase during its turnover, CLT was added to the Ca\(^{2+}\) uptake assay medium 90 s after initiating Ca\(^{2+}\)-ATPase turnover by the addition of ATP. The time course of Ca\(^{2+}\) uptake was monitored prior to, and following the addition of CLT, for several minutes. It was found that addition of CLT (32 or 64 \(\mu M\)) during the turnover cycle of Ca\(^{2+}\)-ATPase resulted in apparently instantaneous inhibition of further Ca\(^{2+}\) uptake by SR vesicles (Fig. 6). No subsequent increase in Ca\(^{2+}\) content of SR vesicles was observed even after prolonged incubation.

Effect of CLT on Ca\(^{2+}\)-dependent Phosphoenzymatic Formation—One of the well characterized intermediate steps in Ca\(^{2+}\)-ATPase reaction pathway is the formation of a phosphoencezyme intermediate (EP) upon the sequential binding of Ca\(^{2+}\) followed by ATP to the ATPase on the cytoplasmic side of the SR (see Introduction). Since CLT inhibited the overall reaction of Ca\(^{2+}\)-activated ATP hydrolysis by the SR Ca\(^{2+}\)-ATPase (Fig. 3), the effect of CLT on Ca\(^{2+}\)-dependent enzyme phosphorylation with ATP was examined. The results showed that in SR vesicles incubated with Ca\(^{2+}\) and ATP, EP formation was strongly inhibited by CLT (Fig. 7, panel A).
Table I

Effects of CLT on the kinetic parameters of Ca\textsuperscript{2+} uptake by SR

The kinetic parameters were derived from the data shown in Fig. 4 using the procedure described under "Experimental Procedures."

| Parameter                  | Control          | 32 \mu M CLT | 64 \mu M CLT |
|----------------------------|------------------|--------------|--------------|
| \(V_{\text{max}}\), nmol of Ca\textsuperscript{2+}-mg of protein \(^{-1}\)min\(^{-1}\) | 208 ± 5.7       | 112 ± 6.6    | 53 ± 7.7     |
| \(K_{0.5}\) for Ca\textsuperscript{2+} (\mu M) | 1.16 ± 0.16     | 7.4 ± 1.6    | 6.2 ± 2.1    |
| Hill coefficient (\(n_H\)) | 1.6 ± 0.28       | 0.78 ± 0.07  | 0.7 ± 0.1    |

FIG. 5. Effect of CLT on ATP-dependent Ca\textsuperscript{2+} uptake by SR at varying ATP concentrations. SR vesicles were preincubated for 3 min at 37 °C in the standard assay medium (see "Experimental Procedures") in the absence of CLT (●) or in the presence of 32 \mu M CLT (■). The Ca\textsuperscript{2+} uptake reaction was initiated by the addition of ATP and was allowed to proceed for 1 min. In panel A, Ca\textsuperscript{2+} uptake activity is plotted against ATP concentration; panel B shows the double reciprocal plot of the data for the ATP concentration range 0.1–5 mM. The data represent mean ± S.E. of three experiments using separate SR preparations.

FIG. 6. Cessation of Ca\textsuperscript{2+} uptake by SR upon addition of CLT during Ca\textsuperscript{2+}-ATPase turnover cycle. Ca\textsuperscript{2+} uptake was initiated by the addition of ATP (at zero time in the figure) to the standard incubation medium (see "Experimental Procedures") preincubated for 3 min at 37 °C. At the time (1.5 min) indicated by the arrow, CLT (final concentration 32 (■) or 64 (▲) \mu M) or vehicle solution (●, 4% Me\textsubscript{2}SO) was added to the reaction mixture. The time course of Ca\textsuperscript{2+} uptake was monitored prior to, and following the addition of CLT/vehicle solution for several minutes as indicated. The data represent mean ± S.E. of three experiments using separate SR preparations.

characteristic functional difference between Ca\textsuperscript{2+} bound and Ca\textsuperscript{2+}-free conformations of the Ca\textsuperscript{2+}-ATPase is that the former undergoes phosphorylation with ATP but not P\textsubscript{i} whereas the latter undergoes phosphorylation with P\textsubscript{i} but not ATP (15). Since we observed an inhibitory action of CLT on Ca\textsuperscript{2+}-dependent enzyme phosphorylation with ATP (Fig. 7, panel A) and Ca\textsuperscript{2+}-stimulated ATP hydrolysis (Fig. 3), the effect of CLT on phosphorylation of the Ca\textsuperscript{2+}-free ATPase with P\textsubscript{i} was also investigated. Interestingly, it was observed that CLT did not exert any inhibitory effect on enzyme phosphorylation with P\textsubscript{i} even at a high concentration of 100 \mu M (Fig. 7, panel B).

Irreversible Nature of the Inhibitory Action of CLT on the SR Ca\textsuperscript{2+}-ATPase—In order to assess whether the inhibitory effect of CLT on the SR Ca\textsuperscript{2+}-ATPase was reversible, the following experiment was performed. SR vesicles were incubated with CLT (80 \mu M) for 3 min at 37 °C in the absence or presence of Ca\textsuperscript{2+}. Subsequently, the SR vesicles were recovered by centrifugation, washed extensively with 10 mM Tris maleate buffer containing 100 mM KCl (pH 6.8), and the time course of Ca\textsuperscript{2+} uptake was determined under standard assay conditions. SR vesicles subjected to the same experimental protocol but without CLT in the incubation medium served as control for this experiment. The results from this experiment showed that exposure of the SR to CLT (in the absence or presence of Ca\textsuperscript{2+}) resulted in an apparently irreversible inhibition of Ca\textsuperscript{2+}-ATPase function (Fig. 8).

Effect of CLT on Ca\textsuperscript{2+} Release—The possibility that CLT may also influence Ca\textsuperscript{2+} release from the SR was investigated by determining the effect of CLT on unidirectional Ca\textsuperscript{2+} release from passively Ca\textsuperscript{2+}-loaded SR vesicles. As shown in Fig. 9, CLT (10 or 80 \mu M) did not influence the rate of Ca\textsuperscript{2+} release from the SR. Therefore the inhibitory effect of CLT on Ca\textsuperscript{2+} uptake observed in this study can be attributed entirely to inhibition of the SR Ca\textsuperscript{2+}-ATPase.

Effect of CLT on Cardiac Contractile Function—In view of the strong inhibitory action of CLT on Ca\textsuperscript{2+} uptake by cardiac SR observed in vitro, it was of considerable interest to examine the effect of this drug on cardiac contractile function. In isolated, electrically paced rabbit heart preparations perfused at a constant perfusate flow rate, CLT (50 \mu M) produced marked depression of contractile function as evidenced by decrements in developed LVP as well as maximum rates of pressure development and relaxation (Fig. 10 and Table II). These effects were discernible within 1 min after initiating perfusion with CLT. Interestingly, reperfusion with normal buffer following CLT, resulted in gradual recovery of contractile function.

**DISCUSSION**

The results presented here demonstrate that micromolar concentrations of CLT strongly inhibits Ca\textsuperscript{2+} transport in isolated cardiac SR vesicles. The inhibition of Ca\textsuperscript{2+} transport is a consequence of primary inhibition of the SR Ca\textsuperscript{2+}-ATPase by...
CLT has provided insights into the mechanism of action of this drug. CLT inhibits the SR Ca\textsuperscript{2+}-ATPase rapidly, and in an apparently irreversible fashion since even after extraction of the inhibitor from the incubation medium, Ca\textsuperscript{2+}-ATPase did not regain its ion transporting activity (Fig. 8). The inhibitory action of CLT was accompanied by a striking decrease in the affinity of the Ca\textsuperscript{2+}-ATPase for Ca\textsuperscript{2+} (Table I) as well as diminished cooperativity of Ca\textsuperscript{2+} binding to the enzyme (as judged from lower value for the Hill coefficient). These findings suggest that the inhibitory action of CLT is associated with a major alteration in the functional properties of the Ca\textsuperscript{2+}-binding sites located in the transmembrane region of the ATPase (16, 17). Since the inhibitory effect of CLT could not be overcome by increasing the concentration of free Ca\textsuperscript{2+}, CLT inhibition is non-competitive with respect to Ca\textsuperscript{2+}. Thus, the structural perturbation in the Ca\textsuperscript{2+}-ATPase produced by CLT not only affects the enzyme’s affinity for Ca\textsuperscript{2+} but also causes a decrease in the maximal rate of Ca\textsuperscript{2+} transport. The CLT-induced changes in kinetic characteristics, together with the irreversible nature of CLT inhibition demonstrates that CLT is not a Ca\textsuperscript{2+}-chelating agent decreasing the concentration of free Ca\textsuperscript{2+} available in the medium, but rather it appears to produce structural perturbations in the Ca\textsuperscript{2+}-ATPase, which impact adversely on enzyme function. It must be noted that half-maximal inhibition of Ca\textsuperscript{2+} transport required a large stoichiometric excess of CLT over Ca\textsuperscript{2+}-ATPase in the assay medium (Fig. 2B). On the other hand, introduction of CLT during Ca\textsuperscript{2+}-ATPase turnover cycle resulted in instantaneous cessation of Ca\textsuperscript{2+} transport (Fig. 6), and extensive washing of the SR membranes after exposure to CLT did not result in recovery of Ca\textsuperscript{2+}-ATPase function (Fig. 8). From these observations it is not clear whether CLT-induced structural perturbations in the Ca\textsuperscript{2+}-ATPase stem from direct interaction of the drug with the Ca\textsuperscript{2+}-ATPase or indirectly due to partitioning of the drug in the membrane vesicles. It is possible that several molecules of CLT bind to hydrophobic “cavities” in the Ca\textsuperscript{2+}-ATPase, and to accommodate the intruding CLT, the ATPase undergoes structural rearrangement that results in loss of its enzymatic and ion transport functions. Such a mechanism has been suggested for the inhibitory action of anesthetics on Ca\textsuperscript{2+}-ATPases (28), and direct anesthetic binding to the SR Ca\textsuperscript{2+}-ATPase has been demonstrated recently (29). It is noteworthy that CLT inhibited the energy transducing and ion transporting functions of the Ca\textsuperscript{2+}-ATPase at the same concentration range. Thus, impaired coupling of catalytic and ion transport events does not contribute to the inhibitory action of CLT on the SR Ca\textsuperscript{2+} pump.

The inhibition of Ca\textsuperscript{2+} transport by CLT was found to be independent of ATP concentration. Interestingly, while the Ca\textsuperscript{2+}-dependent phosphoenzyme formation with ATP was strongly inhibited by CLT, the Ca\textsuperscript{2+}-independent phosphoenzyme formation with Pi in the reverse direction of the catalytic cycle was unaffected by CLT. These findings suggest that CLT does not induce major structural alteration at the catalytic site located in the extramembranous region of the Ca\textsuperscript{2+}-ATPase (16, 17). This situation is similar to that encountered upon derivatization of SR Ca\textsuperscript{2+}-ATPase with the fluorescent carbodiimide NCD4 which also results in inhibition of Ca\textsuperscript{2+}-dependent ATP utilization but not Ca\textsuperscript{2+}-independent phosphoenzyme formation with Pi (30). Evidence has been obtained suggesting that NCD4 induces a perturbation within or near the transmembrane domain of the ATPase (30). It is noteworthy that CLT inhibited the energy transducing and ion transporting functions of the Ca\textsuperscript{2+}-ATPase at the same concentration range. Thus, impaired coupling of catalytic and ion transport events does not contribute to the inhibitory action of CLT on the SR Ca\textsuperscript{2+} pump.

The inhibition of Ca\textsuperscript{2+} transport by CLT was found to be independent of ATP concentration. Interestingly, while the Ca\textsuperscript{2+}-dependent phosphoenzyme formation with ATP was strongly inhibited by CLT, the Ca\textsuperscript{2+}-independent phosphoenzyme formation with Pi in the reverse direction of the catalytic cycle was unaffected by CLT. These findings suggest that CLT does not induce major structural alteration at the catalytic site located in the extramembranous region of the Ca\textsuperscript{2+}-ATPase (16, 17). This situation is similar to that encountered upon derivatization of SR Ca\textsuperscript{2+}-ATPase with the fluorescent carbodiimide NCD4 which also results in inhibition of Ca\textsuperscript{2+}-dependent ATP utilization but not Ca\textsuperscript{2+}-independent phosphoenzyme formation with Pi (30). Evidence has been obtained suggesting that NCD4 induces a perturbation within or near the transmembrane domain of the ATPase (30). It is noteworthy that CLT inhibited the energy transducing and ion transporting functions of the Ca\textsuperscript{2+}-ATPase at the same concentration range. Thus, impaired coupling of catalytic and ion transport events does not contribute to the inhibitory action of CLT on the SR Ca\textsuperscript{2+} pump.

**Fig. 8.** Irreversible nature of the inhibitory action of CLT on the SR Ca\textsuperscript{2+}-ATPase. SR vesicles (250 μg of protein) were incubated for 3 min at 37 °C in buffer A containing Ca\textsuperscript{2+} (composition of buffer A: 50 mM Tris maleate (pH 6.8), 5 mM MgCl\textsubscript{2}, 120 mM KCl, 5 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.1 mM EGTA, and 0.1 mM CaCl\textsubscript{2} (free Ca\textsuperscript{2+}, 9.7 μM); total volume 5 ml) or buffer B lacking Ca\textsuperscript{2+} (composition of buffer B: same as buffer A except that Ca\textsuperscript{2+} was omitted and the concentration of EGTA was increased to 0.25 mM) in the absence of CLT (control) or in the presence of 80 μM CLT. Subsequently, the SR vesicles were recovered by centrifugation, washed with 10 mM Tris maleate buffer containing 100 mM KCl (pH 6.8), and the time course of Ca\textsuperscript{2+} uptake was determined under standard assay conditions (see “Experimental Procedures”). The data represent mean ± S.E. of three experiments using separate SR preparations. ○ and ●, control SR incubated in the absence and presence of Ca\textsuperscript{2+}, respectively. □ and ■, SR incubated with CLT in the absence and presence of Ca\textsuperscript{2+}, respectively.

**Fig. 9.** Effect of CLT on Ca\textsuperscript{2+} release from Ca\textsuperscript{2+}-loaded SR vesicles. SR vesicles passively loaded with \textsuperscript{45}CaCl\textsubscript{2} were transferred to a Ca\textsuperscript{2+} release medium to initiate Ca\textsuperscript{2+} release. Ca\textsuperscript{2+} release was monitored in the absence of CLT (○) and in the presence of 10 (●) or 80 (▲) μM CLT in the Ca\textsuperscript{2+} release assay medium (see “Experimental Procedures”). The figure shows the Ca\textsuperscript{2+} content of SR vesicles (expressed as % of initial Ca\textsuperscript{2+} load) at various time intervals following incubation in the Ca\textsuperscript{2+}-release medium. The initial Ca\textsuperscript{2+} load of SR vesicles (prior to initiating Ca\textsuperscript{2+} release) was 572 nmol of Ca\textsuperscript{2+}/mg of protein. Results from a single experiment are shown here. Similar results were obtained in two additional experiments.

CLT. Furthermore, CLT was found not to influence Ca\textsuperscript{2+} release from Ca\textsuperscript{2+}-preloaded SR vesicles. Taken together, these findings suggest strongly that the ability of CLT to deplete intracellular Ca\textsuperscript{2+} stores observed in other cell types (6) likely stems from a “thapsigargin-like” inhibitory action of this drug on the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase.

Analysis of the characteristics of Ca\textsuperscript{2+}-ATPase inhibition by
Inhibition of Ca\textsuperscript{2+}-ATPase by Clotrimazole

Rabbit Heart Paced at 120 BPM

![Graph showing contractile function](image)

**FIG. 10. Effect of CLT on cardiac contractile function.** Contractile function was assessed in isolated perfused, electrically paced (120 beats/min) rabbit heart as described under “Experimental Procedures.” Shown are contractions recorded during perfusion with normal buffer (control), 2 min post-perfusion with buffer containing 50 \(\mu\)M CLT, and 10 min post-perfusion with normal buffer (washout) after CLT. Stock solution of CLT was prepared in Me\textsubscript{2}SO and then diluted with perfusion buffer to yield a CLT concentration of 50 \(\mu\)M. The final concentration of Me\textsubscript{2}SO in the perfusate was 5%. The normal buffer (control) contained an equivalent concentration of Me\textsubscript{2}SO vehicle. The effects of CLT on contractile function parameters are summarized in Table II. LVP, left ventricular pressure.

### Table II

| Parameter                  | Perfusion condition                                                                 |
|----------------------------|-------------------------------------------------------------------------------------|
|                            | Perfusion with normal buffer | 2 min post-perfusion with 50 \(\mu\)M CLT | 10 min post-perfusion with normal buffer after CLT |
|----------------------------|-------------------------------|---------------------------------|-----------------------------------------------|
| LVP (mm Hg)                 | 91                            | 35                              | 89                              |
| \(+dP/dt\) (mm Hg/s)        | 446                           | 219                             | 620                             |
| \(-dP/dt\) (mm Hg/s)        | 229                           | 188                             | 368                             |

**Effects of CLT on cardiac contractile function**

Contractile function was assessed in isolated perfused, electrically paced (120 beats/min) rabbit heart as described under “Experimental Procedures.” Segments of 20 consecutive contractions such as those depicted in Fig. 10 were analyzed to obtain the average value shown for each parameter. Similar finding were obtained in two additional isolated heart preparations. LVP, left ventricular pressure (systolic); \(+dP/dt\), maximum rate of pressure development; \(-dP/dt\), maximum rate of relaxation.

Conditions of this enzyme by Ca\textsuperscript{2+} (31). These observations are consistent with the view that CLT-induced structural perturbation involving the Ca\textsuperscript{2+}-binding sites of the ATPase contributes to the inhibition of ATP hydrolysis and Ca\textsuperscript{2+} transport.

Comparison of the observed characteristics of Ca\textsuperscript{2+}-ATPase inhibition by CLT with those reported for other inhibitors of the SR Ca\textsuperscript{2+}-ATPase such as TG and CPA, reveals certain similarities as well as striking differences. (i) Both TG and CPA react with and stabilize the Ca\textsuperscript{2+}-deprived, \(E_2\) form of the enzyme (32–36). Our finding that exposure of the Ca\textsuperscript{2+}-ATPase to CLT in the absence or presence of Ca\textsuperscript{2+} results in irreversible inhibition of the enzyme (Fig. 8) suggests that both the Ca\textsuperscript{2+}-deprived \(E_1\) state and the Ca\textsuperscript{2+}-liganded \(E_2\) state of the ATPase are CLT-sensitive. (ii) The protective effect of Ca\textsuperscript{2+} against TG and CPA inhibition of the Ca\textsuperscript{2+}-ATPase is lost upon induction of enzyme turnover with ATP, and an intermediate species generated during turnover undergoes rapid inactivation by CLT. In any case, the inhibitory action of CLT is manifested even when the catalytic and ion transport sites of the enzyme are occupied. (iii) Ca\textsuperscript{2+}-dependent enzyme phosphorylation with ATP is inhibited by TG (33), CPA (36), and CLT (Fig. 7, panel A). On the other hand, phosphorylation of the Ca\textsuperscript{2+}-deprived enzyme with P\textsubscript{i} is inhibited by TG (33) but not CLT (Fig. 7, panel B). This difference suggests that structural perturbations introduced by TG, but not CLT, affect the nucleotide-binding site in the catalytic domain of the ATPase. Consistent with this view, CLT was found to cause only a slight decrease in the enzyme’s affinity toward ATP (Fig. 5) when compared with the much greater decrements in ATP binding affinity observed upon exposure of the ATPase to TG (37). (iv) The Ca\textsuperscript{2+}-binding affinity of the ATPase is markedly decreased upon interaction of the enzyme with TG (32, 33, 38), CPA (34), and CLT (Table I). Thus, a structural perturbation affecting the properties of the Ca\textsuperscript{2+}-binding sites appears to be a common feature underlying the mechanism of action of all three drugs. A recent study which examined the TG sensitivity of chimeric Ca\textsuperscript{2+}-ATPase/Na\textsuperscript{+},K\textsuperscript{+}-ATPase molecules has suggested the transmembrane segments M3 and/or M4 as the potential TG target site in the Ca\textsuperscript{2+}-ATPase (39). Location of the CLT-binding sites will shed more light on the role played by the region of the Ca\textsuperscript{2+}-ATPase protein that is recognized by this drug.

CLT was found to cause marked depression of contractile function in the isolated perfused beating heart (Fig. 10 and Table II). The inhibition of contractile function was not due to compromised coronary flow because the perfusate flow rate was held constant during perfusion. Furthermore, we observed that under these conditions, CLT (50 \(\mu\)M) produced a modest decrease (−20%) in coronary perfusion pressure, suggesting a vasodilator (not constrictor) effect (results not shown). Interestingly, the contractile function recovered gradually following withdrawal of CLT from the perfusion medium. This finding contrasts with the apparently irreversible effect of CLT on the SR Ca\textsuperscript{2+}-ATPase observed *in vitro* and suggests the existence of mechanisms in the intact cell to inactivate, metabolize, or clear the drug from its target site. This observation and the effects and mechanisms of action of CLT on SR function described here might be of relevance in the design and evaluation of CLT and/or its derivatives for their therapeutic applications *in vivo*. The possibility that the CLT-induced impairment in cardiac contractile function is due to mechanisms other than the inhibitory action of CLT on the SR Ca\textsuperscript{2+} pump observed *in vitro* cannot be discounted, however. The potential direct or indirect effects of CLT at the level of myofilaments and on ion transport systems other than the SR Ca\textsuperscript{2+}-ATPase (e.g., plasma membrane Ca\textsuperscript{2+}-ATPase, Na\textsuperscript{+},K\textsuperscript{+}-ATPase, Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger) remain to be investigated.

**Acknowledgments**—We are grateful to Dr. Lian Jiang for drawing our attention to the work of Benzaquen *et al.* (Ref. 6) on the biological actions of CLT. We thank Lily Jiang for secretarial assistance and Bruce Arppe for preparing photographs of illustrations.
REFERENCES

1. Vanden Bossche, H., Willemsens, G., Cools, W., Marichal, P., and Lauwers, W. (1983) Biochem. Soc. Trans. 11, 665–667
2. Yoshida, Y., and Aoyama, Y. (1987) Biochem. Pharmacol. 36, 229–235
3. Ayub, M., and Levell, J. (1988) J. Steroid Biochem. 31, 65–73
4. Ballard, S. A., Lodola, A., and Tarbit, M. H. (1988) Biochem. Pharmacol. 37, 4643–4651
5. Sheets, J. J., Mason, J. I., Wise, C. A., and Estabrook, R. W. (1986) Biochem. Pharmacol. 35, 487–491
6. Benzaquen, L. R., Brugnara, C., Byers, H. R., Gattoni-Celli, S., and Halperin, J. A. (1995) Nature Med. 1, 534–540
7. Villalobos, C., Fonteriz, R., Lopez, M., Garcia, A. G., and Garcia-Sancho, J. (1992) FASEB J. 6, 2742–2747
8. Alvarez, J., Montero, M., and Garcia-Sancho, J. (1990) Biochem. J. 274, 183–197
9. Alvarez, J., Montero, M., and Garcia-Sancho, J. (1990) J. Biol. Chem. 267, 11789–11793
10. Brugnara, C., Armstrong, C. C., Sakamoto, M., Rifai, N., Alper, S. L., and Platt, O. (1995) J. Pharmacol. Exp. Ther. 273, 266–272
11. Fleischer, S., and Inui, M. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 333–364
12. Coronado, R., Marrissette, J., Sukhareva, M., and Vaughan, D. M. (1994) Am. J. Physiol. 266, C1485–C1504
13. Franzini-Armstrong, C., and Protasi F. (1997) Physiol. Rev. 77, 699–729
14. Feher, J. J., and Fabiato, A. (1990) in Calcium and the Heart (Langer, G. A., ed) pp. 199–268, Raven Press Ltd., New York
15. Inesi, G., Submilla, C., and Kirtley, M. E. (1990) Physiol. Rev. 70, 749–760
16. Lytton, J., and MacLennan, D. H. (1992) in The Heart and Cardiovascular System (Fozzard, H. A., Haber, E., Jennings, R. B., Katz, A. M., and Morgan, H. E., ed) 2nd Ed., pp. 1203–1221, Raven Press, New York
17. Guillain, F., and Mintz, E. (1997) Biochim. Biophys. Acta 1318, 52–70
18. Lytton, J., Westlin, M., Burk, S. E., Shull, G. E., and MacLennan D. H. (1992) J. Biol. Chem. 267, 14483–14489
19. Sulakke, P. V., and Narayanan, N. (1978) Biochem. J. 261, 171–180
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21. Narayanan, N. (1981) Biochim. Biophys. Acta 678, 442–459
22. Fabiato, A. (1988) Methods Enzymol. 157, 378–417
23. Narayanan, N., Newland, M., and Neuderf, D. (1983) Biochim. Biophys. Acta 735, 53–66
24. Narayanan, N., Su, N., and Bedard, P. (1991) Biochim. Biophys. Acta 1070, 83–91
25. Hawkins, C., Xu, A., and Narayanan, N. (1994) Biochim. Biophys. Acta 1191, 231–243
26. Sulakke, S. J., and Sulakke P. V. (1979) Gen. Pharmacol. 10, 103–113
27. Narayanan, N., Jones, D. L., Xu, A., and Yu, J. C. (1996) Am. J. Physiol. 271, C1032–C1040
28. Lopez, M. M., and Kosk-Kosicka, D. (1995) J. Biol. Chem. 270, 28239–28245
29. Kosk-Kosicka, D., Fomitcheva, I., Lopez, M. M., and Eckenhoff, R. G. (1997) FEBS Lett. 402, 189–192
30. Sumbilla, C., Cantilina, T., Collins, J. H., Malak, H., Lakowicz, J. R., and Inesi, G. (1991) J. Biol. Chem. 266, 12882–12889
31. Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan D. H. (1989) Nature 339, 476–478
32. Sagara, Y., Wade, J. B., and Inesi, G. (1992) J. Biol. Chem. 267, 1286–1292
33. Sagara, Y., Fernandez-Belda, F., de Meis, L., and Inesi, G. (1992) J. Biol. Chem. 267, 12606–12613
34. Seidler, N. W., Jona, I., Vegh, M., and Martonosi, A. (1989) J. Biol. Chem. 264, 17816–17823
35. Karon, B. S., Mahaney, J. E., and Thomas, D. D. (1994) Biochemistry 33, 13928–13937
36. Plenge-Tellechea, F., Soler, F., and Fernandez-Belda, F. (1997) J. Biol. Chem. 272, 2794–2800
37. Desesús, F., Girardet, J. L., and Dupont, Y. (1993) FEBS Lett. 332, 229–232
38. Wictome, M., Michelangelis, F., Lee, A. G., and East, J. M. (1992) FEBS Lett. 304, 109–113
39. Norregaard, A., Vileen, B., and Andersen, J. P. (1993) FEBS Lett. 336, 248–254
40. Nakamura, S., Suzuki, H., and Kanazawa, T. (1997) J. Biol. Chem. 272, 6232–6237