Inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase by miconazole

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Lax, Antonio, Fernando Soler, and Francisco Fernandez-Belda. Inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase by miconazole. *Am J Physiol Cell Physiol* 283: C85–C92, 2002. First published February 20, 2002; 10.1152/ajpcell.00580.2001.—The inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase activity by miconazole was dependent on the concentration of ATP and membrane protein. Half-maximal inhibition was observed at 12 μM miconazole when the ATP concentration was 50 μM and the membrane protein was 0.05 mg/ml. When ATP was 1 mM, a low micromolar concentration of miconazole activated the enzyme, whereas higher concentrations inhibited it. A qualitatively similar response was observed when Ca^{2+} transport was measured. Likewise, the half-maximal inhibition value was higher when the membrane concentration was raised. Phosphorylation studies carried out after sample preequilibration in different experimental settings shed light on key partial reactions such as Ca^{2+} binding and ATP phosphorylation. The miconazole effect on Ca^{2+}-ATPase activity can be attributed to stabilization of the Ca^{2+}-free enzyme conformation giving rise to a decrease in the rate of the Ca^{2+} binding transition. The phosphoryl transfer reaction was not affected by miconazole.

calcium adenosine 5′-triphosphatase; sarcoplasmic reticulum membrane; imidazole antimycotics

MICONAZOLE (Mic) and other antifungal agents bearing an imidazole ring are known to have fungistatic or fungicidal action depending on the concentration used. The pharmacological effect is attributed to inhibition of cytochrome *P*-450 14-α-demethylase, thus avoiding lanosterol demethylation. This is a necessary step in the formation of ergosterol, a critical component of the fungal membrane. An alternative mechanism may be inhibition of the respiratory chain electron transport (39).

N_1-substituted imidazole drugs were initially described as potent inhibitors of different cytochrome *P*-450-dependent oxidative processes (3, 29, 34), even though other metabolic effects were later described. Of particular interest may be the effects on cellular Ca^{2+} homeostasis. Thus Mic and related compounds inhibit with high affinity the store-operated Ca^{2+} channel from rat thymocyte plasma membrane (1) and the Ca^{2+}-dependent K^+ channel from human erythrocyte (2). Therefore, the movement of Ca^{2+} and K^+ across the plasma membrane is impaired. It is also known that clotrimazole depletes intracellular Ca^{2+} stores, an effect that has been associated with the arrest of cell proliferation in normal and cancer cell lines (4).

Other studies have shown that imidazole antimycotics release Ca^{2+} from thapsigargin-sensitive Ca^{2+} pools in rat thymic lymphocytes (24). They also inhibit Ca^{2+} uptake after thapsigargin-mediated depletion of intracellular Ca^{2+} stores (24). Additionally, econazole, Mic, and SKF-96365 inhibit the Ca^{2+}-dependent ATPase activity in sarcoplasmic reticulum (SR) vesicles isolated from skeletal muscle (24). Inhibition of SR Ca^{2+}-ATPase and cardiac muscle contraction by clotrimazole has also been reported (35).

With this in mind, we analyzed the inhibition mechanism of this key intracellular transport system, i.e., SR Ca^{2+}-ATPase, by means of the imizadole-containing drug Mic.

Coupling between Ca^{2+} transport and ATP hydrolysis occurs through a cyclic sequence of phosphorylated and nonphosphorylated enzyme intermediates with or without bound Ca^{2+} (12, 15, 20). In a very basic reaction scheme (Fig. 1), the Ca^{2+}-free nonphosphorylated enzyme E interacts with cytoplasmic (external) Ca^{2+} to form $E{\cdot}Ca_2$. The Ca^{2+}-bound species interacts with ATP, leading to the steady-state accumulation of Ca^{2+}-bound phosphoenzyme ($EP{\cdot}Ca_2$) plus ADP. Subsequently, a conformational transition involving reorientation of the Ca^{2+} sites ensures Ca^{2+} dissociation into the luminal (internal) space, while phosphoenzyme (EP) hydrolysis produces the release of inorganic phosphate and recovery of E with externally oriented Ca^{2+} binding sites.

In the present study, we tested overall hydrolytic and transport activities under different experimental conditions. Furthermore, we focused on partial reactions related to ligand binding and conformational changes, such as Ca^{2+} binding and ATP binding/phosphorylation, that are critical to the ATP-dependent Ca^{2+} transport process.

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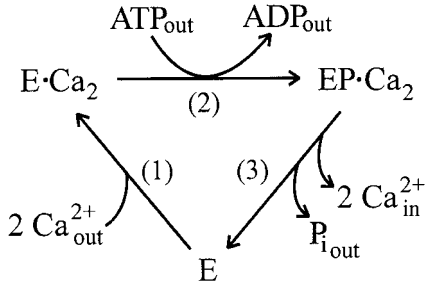

Fig. 1. Minimal reaction cycle of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase. 1, The Ca^{2+}-free enzyme E binds Ca^{2+} at the cytoplasmic side, giving $E \cdot Ca_2$. 2, The enzyme with external Ca^{2+} bond is phosphorylated by ATP, giving phosphoenzyme $(EP) \cdot Ca_2$ plus ADP. 3, The sequential breakdown of the ternary complex produces Ca^{2+} dissociation inside the SR (Ca^{2+}_{in}), as well as P_i release and recovery of the Ca^{2+}-free enzyme with external orientation.

MATERIALS AND METHODS

SR preparation. Fast-twitch skeletal muscle was obtained from the hind leg of female New Zealand rabbits (body wt 2–2.5 kg). Microsomal vesicles were prepared according to Eletr and Inesi (10). Isolated samples were aliquoted and stored at −80°C until use.

Protein concentration. The SR membrane concentration refers to milligrams of total protein per milliliter and was measured by the Lowry et al. (19) procedure. Bovine serum albumin was used as a standard.

Ca^{2+} in media. The free Ca^{2+} concentration was adjusted by adding appropriate volumes of $CaCl_2$ and/or ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) stock solutions, as described by Fabiato (11). The computer program used for calculation took into account the absolute stability constant for the Ca^{2+}-EGTA complex (32), the EGTA protonation equilibria (6), the presence of Ca^{2+} ligands, and the pH of the medium. "Absence of Ca^{2+}" or "Ca^{2+}-free medium" means that there was a sufficiently low level of free Ca^{2+} to prevent EP formation from ATP or the expression of Ca^{2+}-dependent ATPase activity.

Phospholipid vesicles. Liposomes were prepared by vortexing for 2 min at room temperature a mixture of 3 mg of egg yolk phosphatidylcholine (Avanti Polar Lipids) in 0.75 ml of medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, and 5 mM $MgCl_2$. The mixture was briefly sonicated to clarity and then centrifuged to remove unwanted material. Phospholipid was finally quantified by measuring the inorganic phosphate content (7).

Ca^{2+}-ATPase activity. The initial rate of inorganic phosphate release was measured by the colorimetric method of Lin and Morales (17). The experiments were performed at 25°C. A typical reaction medium contained 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 2 mM EGTA, 1.92 mM $CaCl_2$ (free Ca^{2+} was 10 μM), 0.05 mg SR protein/ml, 1 mM ATP, and a given Mic concentration when indicated. An ATP-regenerating system containing 2 mM phosphoenolpyruvate and 6 U/ml pyruvate kinase was included when the ATP concentration was lowered to 50 μM. The Ca^{2+} dependence was studied in a medium containing 1.5 μM A-23187 and 0.02 mg/ml membrane protein. EGTA concentration was 0.1 mM, and suitable $CaCl_2$ concentrations were added to yield the desired free Ca^{2+}. Experiments in the presence of phosphatidylcholine vesicles were carried out in the described 10 μM free Ca^{2+} medium containing 0.01 mg of SR protein/ml and a fixed Mic concentration of 30 μM. The precise composition of the reaction medium is described in the corresponding figure legends.

Ca^{2+} transport. The initial rate of Ca^{2+} transport was measured at 25°C with the aid of $^{45}Ca^{2+}$ as a radioactive tracer (23). The composition of the reaction medium was that described for Ca^{2+}-ATPase activity, although SR protein was 0.01 mg/ml and ~1,000 cpm $^{45}Ca^{2+}$/nmol Ca^{2+} was included. The transport process was stopped by filtering 1 ml of sample aliquots (0.01 mg) through HAWP Millipore filters (Milford, MA) (0.45-μm pore diameter). The filters were rinsed with 10 ml of ice-cold medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, and 1 mM $LaCl_3$ and then subjected to liquid scintillation counting. A blank assay, performed with a medium containing no ATP, was used to subtract nonspecific Ca^{2+} retained in the filter.

EP measurements. The accumulation of radioactive EP after addition of [γ-^{32}P]ATP was measured as described by de Meis (9). Samples were initially preincubated following different protocols.

Addition of Mic to $E \cdot Ca_2$. The initial Ca^{2+}-containing medium, i.e., 20 mM MOPS, pH 7.0, 80 mM KCl, 20 mM $MgCl_2$, 0.1 mM EGTA, either 0.103 mM $CaCl_2$ (10 μM free Ca^{2+}) or 1.1 mM Ca^{2+} (1 mM free Ca^{2+}), and 0.05 mg SR/ml, was preincubated in an ice-water bath with a given Mic concentration and allowed to stand for a variable period of time. In some experiments, 20 mM MOPS, pH 7.0, was substituted with 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.0.

Addition of Ca^{2+} to $E \cdot$Mic. SR vesicles (0.05 mg of SR/ml) equilibrated in a Ca^{2+}-free medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 20 mM $MgCl_2$, 0.1 mM EGTA, and a given Mic concentration were placed in an ice-water bath. After being mixed with 1.1 mM $CaCl_2$, the samples were allowed to stand for a certain period of time. In some experiments, 20 mM MES, pH 6.0, was used instead of 20 mM MOPS, pH 7.0.

Phosphorylation of preincubated samples was initiated at the ice-water temperature by adding a given [γ-^{32}P]ATP concentration, usually 50 μM. The reaction was stopped 1 s later by adding 2 ml of ice-cold quenching solution containing 250 mM perchloric acid and 4 mM sodium phosphate. Samples were 1:1 diluted by the denaturing acid solution. Quenched samples were filtered through 0.45-μm nitrocellulose filters (HAWP; Millipore). Filters were rinsed with 35 ml of ice-cold medium containing 125 mM perchloric acid and 2 mM sodium phosphate and then solubilized and counted by the liquid scintillation technique. Suitable blank assays were made by adding the quenching solution before [γ-^{32}P]ATP.

Data presentation. The experimental data points represent means ± SE of at least three independent determinations, each performed in duplicate.

RESULTS

Ca^{2+}-dependent ATP hydrolysis catalyzed by a preparation of skeletal SR vesicles is sensitive to the presence of Mic.

Dependence on ATP. The initial experiments were performed at neutral pH in the presence of 10 μM free Ca^{2+}, 50 μM ATP, 5 mM Mg^{2+}, and 5 mM oxalate. The membrane protein concentration was 0.05 mg/ml. Under these conditions, the hydrolysis rate was monotonically inhibited as the Mic concentration was raised. The $K_{0.5}$ value for inhibition was 12 μM (Fig. 2*A*). The ATP concentration was kept constant during the measurements with an ATP-regenerating system. When the free Ca^{2+} concentration was maintained at 10 μM but the ATP concentration was increased to 1 mM, a

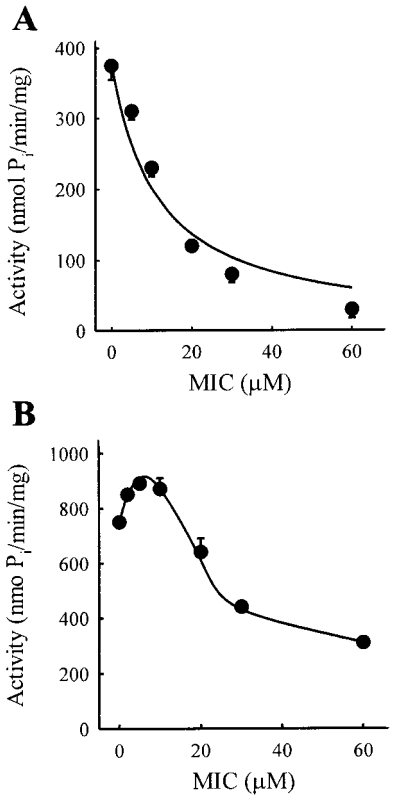

Fig. 2. Effect of ATP on Ca^{2+}-ATPase activity measured in the presence of miconazole (Mic). Hydrolytic activity was measured at 25°C in a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 2 mM EGTA, 1.92 mM $CaCl_2$, (10 μM free Ca^{2+}), 0.05 mg SR/ml, and a given Mic concentration. *A*: reaction was started by the addition of 50 μM ATP; 2 mM phosphoenolpyruvate and 6 U/ml pyruvate kinase were also present. *B*: reaction was started by adding 1 mM ATP in the absence of the ATP-regenerating system.

more complex dependence became evident (Fig. 2*B*). In this case, the rate of hydrolysis was activated when the Mic concentration was <10 μM and inhibited when the drug concentration was higher. It is clear that the Mic effect is dependent on the phosphorylating substrate concentration.

The effect of Mic on Ca^{2+} transport was also measured under the conditions described for the hydrolysis experiments. The buffered medium was at a neutral pH, free Ca^{2+} was 10 μM, ATP was 1 mM, and Mg^{2+} was 5 mM. The membrane protein concentration was 0.01 mg/ml, and the Ca^{2+}-chelating agent oxalate was also present. Here, again, the activation or inhibition by Mic was concentration dependent, i.e., a low Mic concentration produced activation, whereas higher concentrations exerted an inhibitory action (Fig. 3).

Dependence on membrane protein. The influence of the membrane protein concentration on the effect of Mic was also explored. This was assayed by measuring initial rates of ATP hydrolysis at a neutral pH in a medium containing 10 μM free Ca^{2+}, 1 mM ATP, 5 mM Mg^{2+}, and 5 mM oxalate. When the ATP hydrolysis rate was studied as a function of Mic concentration using 0.01, 0.05, or 0.15 mg SR/ml, a family of curves was generated. The degree of inhibition induced by a given Mic concentration fell as the protein concentration was raised and vice versa (Fig. 4*A*). In other words, the $K_{0.5}$ for inhibition increased progressively from 12 μM to 42 and 100 μM, respectively, as the membrane protein concentration was raised. We also selected conditions to observe hydrolytic activity inhibition in the standard 10 μM free Ca^{2+} medium using 0.01 mg protein/ml and 30 μM Mic. When the experiment was repeated in the presence of phosphatidylcholine vesicles, the enzyme activity was progressively protected as the phospholipid concentration was raised (Fig. 4*B*).

Dependence on Ca^{2+}. Initial rates of ATP hydrolysis were also measured in the presence of different free Ca^{2+} concentrations using SR vesicles leaky to Ca^{2+}. A plot of Ca^{2+}-ATPase activity vs. Ca^{2+} concentration, expressed as pCa, showed a bell-shaped dependence (Fig. 5). When the experiments were repeated in the presence of 30 μM Mic, half-maximal activation required a higher Ca^{2+} concentration and the maximal rate did not reach that observed in the absence of Mic.

A more detailed characterization was undertaken by analyzing single steps of the enzyme reaction cycle (Fig. 1). Data were obtained by preincubating samples under different conditions followed by a short phosphorylation with 50 μM [γ-^{32}P]ATP to evaluate the accumulation of EP. The assays were carried out at the ice-water temperature, and the phosphorylation was stopped after 1 s. Preliminary experiments indicated that the Mic effect was the same when the phosphorylation time was prolonged (data not shown). The SR protein concentration in these assays was 0.05 mg/ml.

In one case, SR vesicles at a neutral pH and in the absence of free Ca^{2+} were equilibrated at the ice-water temperature for 5 min with 20 μM Mic. Then, 1 mM free Ca^{2+} was added and preincubation was maintained at the same temperature for different periods of time. The time-dependent effect was evaluated by measuring the accumulation of EP after Ca^{2+} addition. The enzyme capacity to be phosphorylated by ATP in-

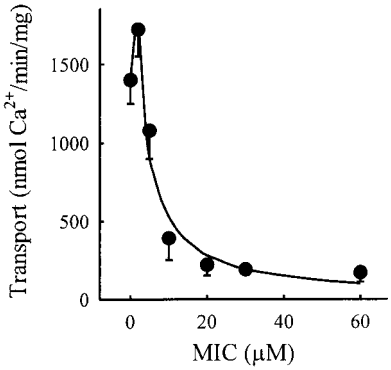

Fig. 3. ATP-dependent Ca^{2+} transport measured in the presence of Mic. SR vesicles (0.01 mg/ml) were equilibrated at 25°C in a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 2 mM EGTA, 1.92 mM $^{45}CaCl_2$ (~1,000 cpm/nmol), and a given Mic concentration. The transport process was initiated by the addition of 1 mM ATP and stopped by filtering 1-ml aliquots of reaction medium at different times. Filters were processed as described in MATERIALS AND METHODS.

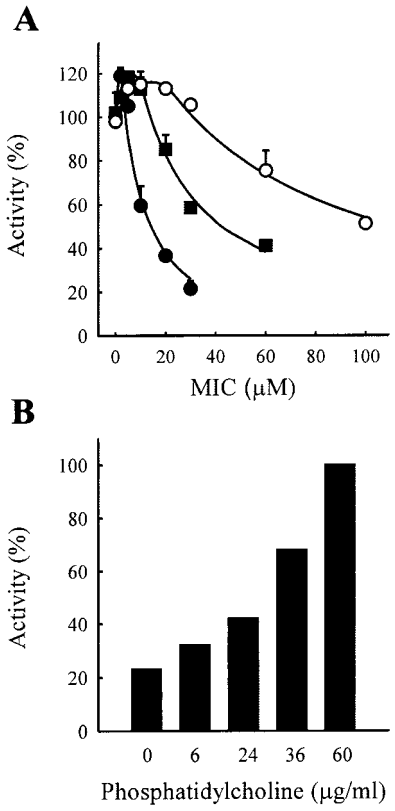

Fig. 4. Effect of membrane protein and exogenous lipid on Ca^{2+}-ATPase activity measured in the presence of Mic. *A*: reaction was carried out at 25°C in a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 2 mM EGTA, 1.92 mM $CaCl_2$ (10 μM free Ca^{2+}), and a given Mic concentration. The reaction was started by adding 1 mM ATP, and the SR protein concentration was 0.01 (●), 0.05 (■), or 0.15 mg/ml (○). *B*: experiments were performed in the presence of 0.01 mg protein/ml and 30 μM Mic by using the described 10 μM free Ca^{2+} medium. A final concentration of phospholipid vesicles was also present when indicated.

creased slowly in the minute time scale when 1 mM free Ca^{2+} was added to a medium containing SR vesicles in the presence of Mic (Fig. 6). The hyperbolic increase showed a value of ~2.5 nmol EP/mg protein 30 min after Ca^{2+} addition. It is also shown that EP reached maximal values of ~3.2 nmol/mg protein from the initial time point when preincubation was performed in the absence of Mic.

Alternatively, SR vesicles resuspended in a Ca^{2+}-containing medium were set in an ice-water bath, and then 20 μM Mic was added. Incubation after Mic addition was prolonged for different periods of time and was finished by a 1-s phosphorylation step, with [γ-^{32}P]ATP added first and then acid solution. When the vesicles were initially resuspended in the presence of 10 μM free Ca^{2+}, the enzyme very rapidly lost the capacity to be phosphorylated by ATP (Fig. 7*A*). Preincubation with 20 μM Mic for 10 s was sufficient to drastically decrease the phosphorylating capacity of the enzyme. Control experiments performed without Mic in the preincubation medium provided the maximal level of phosphorylation.

Interestingly, when the vesicles were resuspended in a 1 mM free Ca^{2+} medium, the subsequent addition of

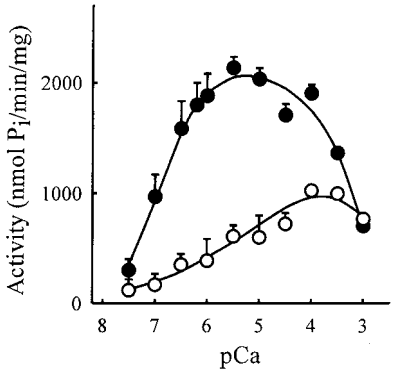

Fig. 5. Effect of Mic on Ca^{2+}-ATPase activity measured as a function of Ca^{2+} concentration. The reaction medium consisted of 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.02 mg SR/ml, 1.5 μM A-23187, and a given $CaCl_2$ concentration to yield a certain Ca^{2+} concentration (pCa). The reaction was initiated at 25°C by adding 1 mM ATP (●). The reaction medium was supplemented with 30 μM Mic when indicated (○). pCa is the negative logarithm of free Ca^{2+} expressed as molar concentration.

20 μM Mic produced only a small decrease in the accumulated EP. The EP level in the absence of Mic preincubation was 3.2 nmol/mg protein (Fig. 7*B*), and this value decreased to 2.6 nmol/mg protein after preincubation with 20 μM Mic.

Dependence on enzyme conformation and pH. The effect of Mic concentration on EP was measured at the ice-water temperature by performing both the preincubation of samples and phosphorylation by ATP under different conditions. When the experiments were performed at pH 7.0 and Mic was preincubated for 1 min with SR vesicles already in the presence of 1 mM free Ca^{2+}, the effect of Mic concentration on EP was moderate (Fig. 8). As a reference, 57% of the maximal EP

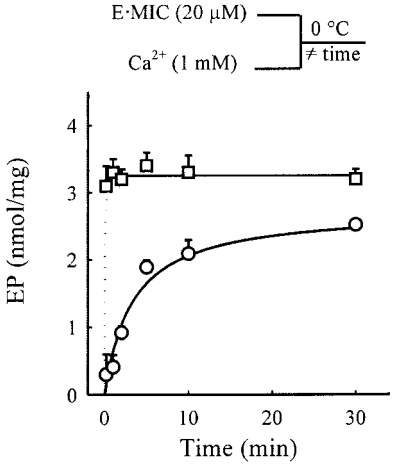

Fig. 6. Time-dependent EP accumulation after addition of Ca^{2+} to SR vesicles in the presence of Mic. SR vesicles (0.05 mg/ml) in a Ca^{2+}-free medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 20 mM $MgCl_2$, and 0.1 mM EGTA were mixed at the ice-water temperature for 5 min with 20 μM Mic. The addition of 1.1 mM Ca^{2+} (1 mM free Ca^{2+}) marked the preincubation *time 0*. Phosphorylation of samples after different preincubation times was initiated by adding 50 μM [γ-^{32}P]ATP and stopped after 1 s by acid quenching. The experimental data show EP levels when the preincubation was performed in the absence (□) or presence (○) of 20 μM Mic.

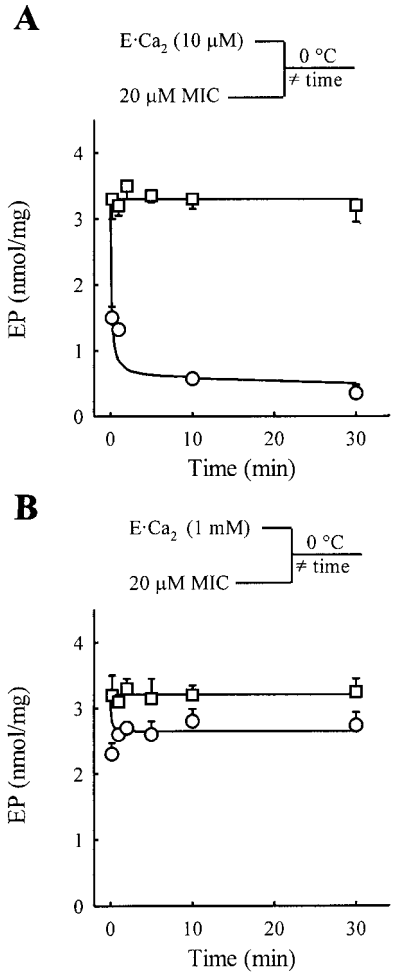

Fig. 7. Time-dependent EP accumulation after addition of Mic to SR vesicles in the presence of Ca^{2+}. *A*: SR vesicles (0.05 mg/ml) in a 10-μM free Ca^{2+} medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 20 mM $MgCl_2$, 0.1 mM EGTA, and 0.103 mM $CaCl_2$ were preincubated at the ice-water temperature for a given period of time with 20 μM Mic (○). *B*: SR vesicles (0.05 mg/ml) in a 1 mM free Ca^{2+} medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 20 mM $MgCl_2$, 0.1 mM EGTA, and 1.1 mM $CaCl_2$ were preincubated for a given period of time with 20 μM Mic (○). EP was evaluated by 1-s phosphorylation at the ice-water temperature using 50 μM [γ-^{32}P]ATP. Control experiments without a Mic preincubation were performed and data are shown in *A* and *B* (□).

level was accumulated when 30 μM Mic was included in the preincubation medium. When the vesicles in the absence of Ca^{2+} were exposed to Mic and then preincubated for 1 min with 1 mM free Ca^{2+}, the phosphorylating capacity of the enzyme was clearly lower. Thus 7 μM Mic produced a 50% inhibition of EP accumulation. Note that the same Mic concentration induced only a 12% inhibition when added to the vesicles in the presence of Ca^{2+}. The inhibitory pattern at pH 6.0 was qualitatively similar, i.e., the inhibition was higher when Mic was added to the vesicles before Ca^{2+}, although some differences were observed (Fig. 8*B*). Namely, preincubation for 1 min of SR vesicles in the presence of 1 mM free Ca^{2+} with 4 μM Mic inhibited EP accumulation by 3%, but, when 4 μM Mic was initially mixed with the vesicles in the absence of Ca^{2+} and the subsequent preincubation with 1 mM free Ca^{2+} lasted 1 min, EP accumulation was inhibited by 50%.

Dependence on phosphorylation. The phosphorylation partial reaction was studied by measuring EP as a function of ATP concentration. In these experiments, SR vesicles were equilibrated at the ice-water temperature in the presence of 1 mM free Ca^{2+} before a given Mic concentration was added. The preincubation was

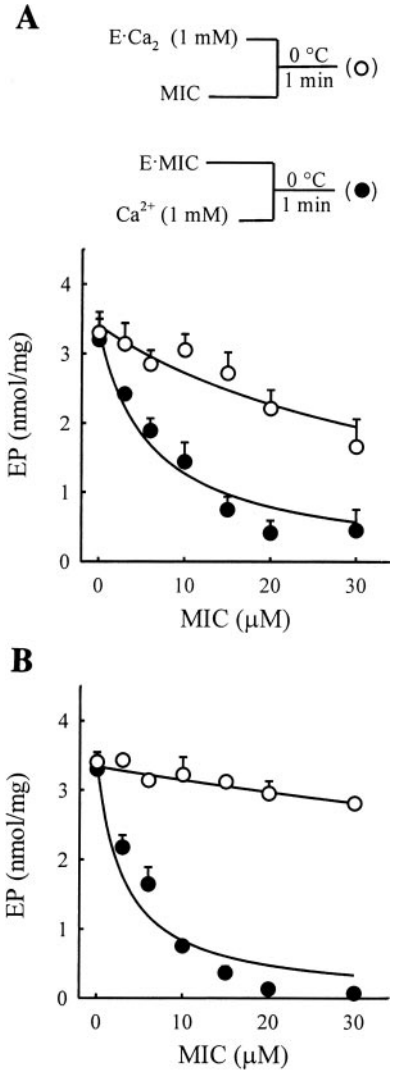

Fig. 8. Mic concentration effect on EP when samples were preincubated in the presence of Ca^{2+}. *A*: experiments were performed at pH 7.0. The initial medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1.1 mM $CaCl_2$ (1 mM free Ca^{2+}), and 0.05 mg/ml SR protein was preincubated at the ice-water temperature for 1 min with a given Mic concentration (○). Vesicles in a Ca^{2+}-free medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.05 mg/ml SR protein, and a given Mic concentration were preincubated at the ice-water temperature for 1 min with 1.1 mM $CaCl_2$ (1 mM free Ca^{2+}) (●). *B*: experiments were performed at pH 6.0. SR vesicles in a 1-mM free Ca^{2+} medium were preincubated at the ice-water temperature for 1 min with a given Mic concentration (○). In the absence of Ca^{2+}, SR vesicles were mixed with a given Mic concentration and then preincubated with 1 mM free Ca^{2+} (●). In all cases, samples after preincubation were phosphorylated for 1 s with 50 μM [γ-^{32}P]ATP.

maintained at the ice-water temperature for 1 min, and the subsequent 1-s phosphorylation step was performed using different [γ-^{32}P]ATP concentrations. Control data obtained with no previous Mic preincubation step showed that EP increased linearly when the radioactive ATP increased from 1 to 100 μM (Fig. 9*A*). The presence of 10 μM Mic during preincubation did not alter the dependence of EP on ATP concentration. When the Mic concentration was raised to 20 μM, EP accumulation was lower, although the dependence on ATP concentration was still linear. When the EP level is plotted on a relative scale as a function of the ATP concentration on a logarithmic scale, it is clear that Mic did not affect either the maximal level of phosphorylation or the apparent affinity for ATP (Fig. 9*B*).

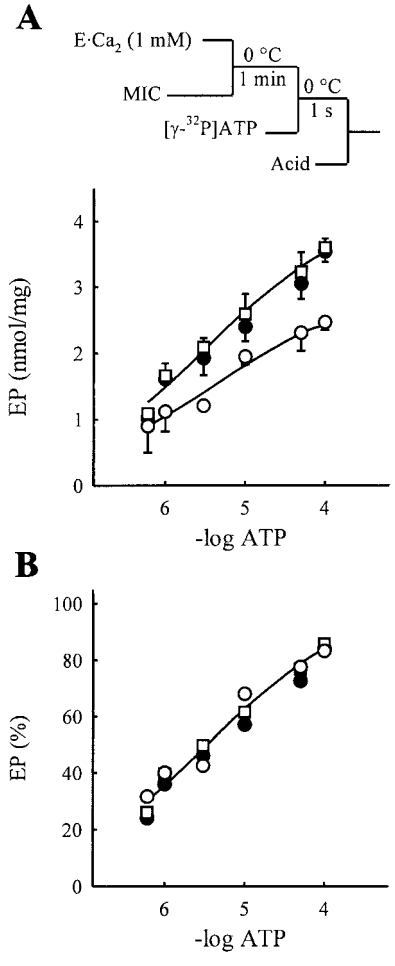

Fig. 9. Effect of Mic on ATP phosphorylation rate. *A*: SR vesicles (0.05 mg/ml) in a medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 20 mM $MgCl_2$, 0.1 mM EGTA, and 1.1 mM $CaCl_2$ (1 mM free Ca^{2+}) were phosphorylated at the ice-water temperature for 1 s with a given concentration of [γ-^{32}P]ATP (□). In some experiments, preincubation for 1 min before phosphorylation at the ice-water temperature of SR vesicles in the 1 mM free Ca^{2+} medium with 10 μM (●) or 20 μM Mic (○) was performed. *B*: EP levels in a relative scale were plotted as a function of the ATP concentration. Symbols are as defined in *A*.

DISCUSSION

A common feature of many different compounds affecting the Ca^{2+}-ATPase activity of isolated SR vesicles is their hydrophobic character (8, 22, 26, 27, 33, 36). Some of these compounds, including high-affinity inhibitors (30, 37), exhibit only inhibitory action, whereas others (5, 25, 38) are known to exert activating effect. Mic can be included in a group (22, 26, 18) that exerts activation when used at a low concentration and inhibition when used at higher concentrations (Figs. 2*B* and 4*A*). The dual effect of Mic on Ca^{2+}-ATPase activity can be explained by binding/interaction to two different sites of the enzyme.

Mic can also activate or inhibit the rate of Ca^{2+} transport, depending on the concentration used, as observed during ATP hydrolysis (Fig. 3). This confirms that the Mic effect is on the Ca^{2+}-ATPase protein. However, the dependence of transport and hydrolysis on Mic concentration is not exactly the same (cf. Figs. 3 and 4*A*).

The hydrophobic nature of drugs usually promotes their incorporation into the membrane, and when this happens, activation or inhibition is dependent on both drug and membrane concentration. The Mic effect also shares this feature because the degree of inhibition is dependent on membrane protein concentration (Fig. 4*A*). One milligram of SR protein contains ~4 nmol of active enzyme, as deduced from the maximal phosphorylation level. Therefore, a membrane protein concentration of 0.05 mg/ml is equivalent to 0.2 μM Ca^{2+}-ATPase. If we consider that the $K_{0.5}$ for inhibition is 12 μM (Fig. 2*A*), a Mic-to-ATPase ratio of 60 can be calculated. This means that 50% inhibition of the enzyme activity requires the participation of 60 drug molecules. Therefore, Mic is not a high-affinity inhibitor of the enzyme (30), and the protecting effect when the membrane concentration is raised (Fig. 4*A*) can be attributed to drug partitioning. Incorporation of Mic into the lipidic phase was confirmed when phosphatidylcholine vesicles were included in the reaction medium (Fig. 4*B*). Full protection against inhibition was observed using 10 times the phospholipid concentration present in the sample of SR membrane.

A brief ATP phosphorylation at low temperature is a valuable tool for distinguishing between $E{\cdot}Ca_2$ and E accumulation, because only the Ca^{2+}-bound enzyme can be phosphorylated by ATP (see Fig. 1). Using this approach, the partial reaction $E + 2Ca^{2+} \rightarrow E{\cdot}Ca_2$ (*transition 1* in Fig. 1) was studied. The Ca^{2+}-free to Ca^{2+}-bound transition in the absence of Mic takes place in the millisecond time scale (16). However, the transition from the Ca^{2+}-free to the Ca^{2+}-bound state in the presence of Mic is slow and requires the addition of mM Ca^{2+} (Fig. 6). This suggests that the enzyme is retained in the Ca^{2+}-free conformation when Mic is added to SR vesicles in the absence of Ca^{2+}.

Furthermore, the Ca^{2+}-bound to Ca^{2+}-free transition can be rapidly elicited by Mic when the vesicles are initially in the presence of low μM Ca^{2+} (Fig. 7*A*). This confirms the stabilization of the Ca^{2+}-free enzyme by

Mic. Nonetheless, the Ca^{2+}-bound conformation can be stabilized in the presence of Mic when SR vesicles are previously equilibrated in the presence of millimolar Ca^{2+} (Fig. 7*B*). The final equilibrium of Ca^{2+} and Mic binding to the enzyme is independent of the order of addition, although the slow Ca^{2+} binding transition in the presence of Mic is responsible for the distinct behavior of the Ca^{2+}-bound and Ca^{2+}-free enzymatic forms that we observe. Stabilization of the Ca^{2+}-free enzyme is the main effect of clotrimazole (35) and other high-affinity inhibitors of the enzyme (14, 31, 37).

The Mic effect on Ca^{2+} binding was exploited by performing 1-min preincubations as described in Fig. 8. This demonstrated that the phosphorylating capacity of the enzyme was lower when Mic was added to the enzyme in the absence of Ca^{2+}, i.e., when Mic was added before millimolar concentrations of Ca^{2+}. In contrast, the EP level was higher when Mic was added to the enzyme in the presence of Ca^{2+}, i.e., when millimolar concentrations of Ca^{2+} were added before Mic. This is consistent with a decrease in the rate of the Ca^{2+} binding transition induced by Mic.

The existence of a pH-dependent equilibrium between enzymatic forms, which affects Ca^{2+} binding, has been proposed (13, 28). Acidic pH favors the accumulation of E, whereas alkaline pH favors the accumulation of $E{\cdot}Ca_2$. According to this idea, when Mic is added to the enzyme in the absence of Ca^{2+}, the decrease in EP would be higher at pH 6 than at pH 7 because of the greater stabilization of the Ca^{2+}-free enzyme at acidic pH (Fig. 8). When Mic was added to the enzyme in the presence of mM Ca^{2+}, the inhibition was lower at pH 6 than at pH 7. This can be attributed to a slower enzyme turnover at acidic pH. The decrease in EP is due to the accumulation of Ca^{2+}-free species after ATP addition.

The partial reaction $E{\cdot}Ca_2 + ATP \rightarrow EP{\cdot}Ca_2 + ADP$ (*transition 2* in Fig. 1) was studied by preincubating SR vesicles in the presence of mM Ca^{2+} to saturate the transport sites and then phosphorylating with different ATP concentrations. When the data were plotted on a relative scale, it was clear that Mic did not affect either the maximal rate of phosphorylation or enzyme affinity for ATP (Fig. 9*B*). This indicates that Mic does not affect the ATP phosphorylation reaction. However, we noted that the inhibition of Ca^{2+}-ATPase activity by Mic was lower in the presence of 1 mM ATP than in the presence of 50 μM (Fig. 2). The protective role on the overall catalytic cycle induced by mM ATP can be explained by the activating effect exerted by the substrate, leading to the steady-state accumulation of Ca^{2+}-bound against Ca^{2+}-free enzymatic species.

A dose of 200 mg of ketoconazole, taken once daily, is used as treatment or prophylaxis against fungal infection (21). The oral dose translates into a peak plasma concentration of up to 7 μg/ml, i.e., 13.2 μM (21). Our in vitro inhibition data with Mic show $K_{0.5}$ values in a similar concentration range. Therefore, the inhibition of SR Ca^{2+}-ATPase by Mic and an alteration of the cytoplasmic free Ca^{2+} may be involved in the antifungal activity and/or the adverse effects associated with the use of this drug.

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REFERENCES

1. **Alvarez J, Montero M, and Garcia-Sancho J.** Cytochrome P450 may regulate plasma membrane Ca^{2+} permeability according to the filling state of the intracellular Ca^{2+} stores. *FASEB J* 6: 786–792, 1992.
2. **Alvarez J, Montero M, and Garcia-Sancho J.** High affinity inhibition of Ca^{2+}-dependent K^+ channels by cytochrome P-450 inhibitors. *J Biol Chem* 267: 11789–11793, 1992.
3. **Ballard SA, Lodola A, and Tarbit MH.** A comparative study of 1-substituted imidazole and 1,2,4-triazole antifungal compounds as inhibitors of testosterone hydroxylations catalysed by mouse hepatic microsomal cytochrome P-450. *Biochem Pharmacol* 37: 4643–4651, 1988.
4. **Benzaquen LR, Brugnara C, Byers HR, Gatton-Celli S, and Halperin JA.** Clotrimazole inhibits cell proliferation in vitro and in vivo. *Nat Med* 1: 534–540, 1995.
5. **Bigelow DJ and Thomas DD.** Rotational dynamics of lipid and the Ca-ATPase in sarcoplasmic reticulum. The molecular basis of activation by diethyl ether. *J Biol Chem* 262: 13449–13456, 1987.
6. **Blinks J, Wier W, Hess P, and Prendergast F.** Measurement of Ca^{2+} concentrations in living cells. *Prog Biophys Mol Biol* 40: 1–114, 1982.
7. **Bottcher CJF, van Gent CM, and Pries C.** A rapid and sensitive submicro phosphorus determination. *Anal Chim Acta* 24: 203–204, 1961.
8. **Chadwick C and Thomas EW.** Inhibition of (Ca^{2+}+Mg^{2+})-ATPase by carbodiimides. A structure-activity study. *Biochim Biophys Acta* 827: 419–423, 1985.
9. **De Meis L.** Approaches to studying the mechanisms of ATP synthesis in sarcoplasmic reticulum. *Methods Enzymol* 157: 190–206, 1988.
10. **Eletr S and Inesi G.** Phospholipid orientation in sarcoplasmic membranes: spin-label ESR and proton MNR studies. *Biochim Biophys Acta* 282: 174–179, 1972.
11. **Fabiato A.** Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol* 157: 378–417, 1988.
12. **Fernandez-Belda F, Fortea MI, and Soler F.** Testing the versatility of the sarcoplasmic reticulum Ca^{2+}-ATPase reaction cycle when p-nitrophenyl phosphate is the substrate. *J Biol Chem* 276: 7998–8004, 2001.
13. **Forge V, Mintz E, and Guillain F.** Ca^{2+} binding to sarcoplasmic reticulum ATPase revisited. *J Biol Chem* 268: 10953–10960, 1993.
14. **Fortea MI, Soler F, and Fernandez-Belda F.** Unravelling the interaction of thapsigargin with the conformational states of Ca^{2+}-ATPase from skeletal sarcoplasmic reticulum. *J Biol Chem* 276: 37266–37272, 2001.
15. **Inesi G.** Mechanism of calcium transport. *Annu Rev Physiol* 47: 573–601, 1985.
16. **Inesi G.** Sequential mechanism of calcium binding and translocation in sarcoplasmic reticulum adenosine triphosphatase. *J Biol Chem* 262: 16338–16342, 1987.
17. **Lin T and Morales M.** Application of a one-step procedure for measuring inorganic phosphate in the presence of proteins: the actomyosin ATPase system. *Anal Biochem* 77: 10–17, 1977.
18. **Logan-Smith MJ, Lockyer PJ, East JM, and Lee AG.** Curcumin, a molecule that inhibits the Ca^{2+}-ATPase of sarcoplasmic reticulum but increases the rate of accumulation of Ca^{2+}. *J Biol Chem* 276: 46905–46911, 2001.
19. **Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ.** Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951.

20. **MacLennan DH, Rice WJ, and Green NM.** The mechanism of Ca^{2+} transport by sarco(endo)plasmic reticulum Ca^{2+}-ATPases. *J Biol Chem* 272: 28815–28818, 1997.
21. **Martindale.** *The Extra Pharmacopoeia* (28th ed.) London: Pharmaceutical Press, 1982, p. 426–429.
22. **Martinez-Azorin F, Teruel JA, Fernandez-Belda F, and Gomez-Fernandez JC.** Effect of diethylstilbestrol and related compounds on the Ca^{2+}-transporting ATPase of sarcoplasmic reticulum. *J Biol Chem* 267: 11923–11929, 1992.
23. **Martonosi A and Feretos R.** The uptake of Ca^{2+} by sarcoplasmic reticulum fragments. *J Biol Chem* 239: 648–658, 1964.
24. **Mason MJ, Mayer B, and Hymel LJ.** Inhibition of Ca^{2+} transport pathways in thymic lymphocytes by econazole, miconazole, and SKF-96365. *Am J Physiol Cell Physiol* 264: C654–C662, 1993.
25. **Melgunov VI, Jindal S, and Belikova MP.** Short-chain alkanols and the functional efficiency of the Ca pump in the sarcoplasmic reticulum of rabbit skeletal muscles. *FEBS Lett* 227: 157–160, 1988.
26. **Michelangeli F, Orlowski S, Champeil P, East JM, and Lee AG.** Mechanism of inhibition of the (Ca^{2+}-Mg^{2+})-ATPase by nonylphenol. *Biochemistry* 29: 3091–3101, 1990.
27. **Morris SJ, Silbergeld EK, Brown RR, and Haynes DH.** Erythrosin B (USFD&C Red 3) inhibits calcium transport and ATPase activity of muscle sarcoplasmic reticulum. *Biochem Biophys Res Commun* 104: 1306–1311, 1982.
28. **Pick U and Karlish SJD.** Regulation of the conformational transition in the Ca-ATPase from sarcoplasmic reticulum by pH, temperature and calcium ions. *J Biol Chem* 257: 6120–6126, 1982.
29. **Rodrigues AD, Gibson GG, Ioannides C, and Parke DV.** Interactions of imidazole antifungal agents with purified cytochrome P-450 proteins. *Biochem Pharmacol* 36: 4277–4281, 1987.
30. **Sagara Y and Inesi G.** Inhibition of the sarcoplasmic reticulum Ca^{2+} transport ATPase by thapsigargin at subnanomolar concentrations. *J Biol Chem* 266: 13503–13506, 1991.
31. **Sagara Y, Wade JB, and Inesi G.** A conformational mechanism for formation of a dead-end complex by the sarcoplasmic reticulum ATPase with thapsigargin. *J Biol Chem* 267: 1286–1292, 1992.
32. **Schwartzenbach G, Senn H, and Anderegg G.** Komplexone. XXIX. Ein grosser Chelateffekt besonderer. *Helv Chim Acta* 40: 1886–1900, 1957.
33. **Seino A, Furukawa KI, Miura T, Yaginuma T, Momose K, and Ohizumi Y.** 3′,3′′,5′,5′′-Tetraiodophenolsulfonephtalein is a selective inhibitor of Ca^{2+}-pumping ATPase in intracellular Ca^{2+} stores. *J Biol Chem* 269: 17550–17555, 1994.
34. **Sheets JJ, Mason JI, Wise CA, and Estabrook RW.** Inhibition of rat liver microsomal cytochrome P-450 steroid hydroxylase reactions by imidazole antimycotic agents. *Biochem Pharmacol* 35: 487–491, 1986.
35. **Snajdrova L, Xu A, and Narayanan N.** Clotrimazole, an antimycotic drug, inhibits the sarcoplasmic reticulum calcium pump and contractile function in heart muscle. *J Biol Chem* 273: 28032–28039, 1998.
36. **Sokolove PM, Albuquerque EX, Kauffman FC, Spande TF, and Daly JW.** Phenolic antioxidants: potent inhibitors of the (Ca^{2+}+Mg^{2+})-ATPase of sarcoplasmic reticulum. *FEBS Lett* 203: 121–126, 1986.
37. **Soler F, Plenge-Tellechea F, Fortea I, and Fernandez-Belda F.** Cyclopiazonic acid effect on Ca^{2+}-dependent conformational states of the sarcoplasmic reticulum ATPase. Implication for the enzyme turnover. *Biochemistry* 37: 4266–4274, 1998.
38. **Starling AP, Hughes G, East JM, and Lee AG.** Mechanism of stimulation of the calcium adenosinetriphosphatase by jasmone. *Biochemistry* 33: 3023–3031, 1994.
39. **Wispelwey B and Neu HC.** Antifungal agents. In: *Human Pharmacology: Molecular to Clinical* (3rd. ed.), edited by Brody TM, Larner J, and Minneman KP. St. Louis, MO: Mosby-Year Book, 1998, p. 735–744.