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# Clomipramine and Related Structures as Inhibitors of the Skeletal Sarcoplasmic Reticulum $Ca^{2+}$ Pump

**Fernando Soler,[2] Fernando Plenge-Tellechea,[2,3] Isabel Fortea,[2] and Francisco Fernandez-Belda[2,4]**

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The $Ca^{2+}$-pumping activity of skeletal sarcoplasmic reticulum vesicles is half-maximally inhibited by 120 $\mu M$ clomipramine, 250 $\mu M$ desipramine, and 500 $\mu M$ imipramine or trimipramine. The inhibition is attributed to the dihydrodibenzazepine moiety, since 3-(dimethylamino)propionitrile, reproducing the aliphatic amine chain, has no inhibitory action. The inhibition is shown as a marked decrease of $Ca^{2+}$ binding at equilibrium in the absence of ATP and as a reduction of phosphorylation of the $Ca^{2+}$-free conformation by inorganic phosphate. Therefore, the drug effect is consistent with preferential interaction of tricyclic antidepressants with the $Ca^{2+}$-free conformation of the nonphosphorylated enzyme. An additional decrease in the apparent rate constant of enzyme dephosphorylation, i.e., in the release of phosphate from ATP during enzyme cycling was also noticed.

**KEY WORDS:** Clomipramine; tricyclic antidepressants; $Ca^{2+}$-pump; sarcoplasmic reticulum; skeletal muscle.

## INTRODUCTION

The $Ca^{2+}$-ATPase protein from sarcoplasmic reticulum (SR)[1] belongs to a family of intracellular membrane enzymes/transporters involved in the ATP-dependent sequestration of cytosolic $Ca^{2+}$. This $Ca^{2+}$ transport system is of paramount relevance since it is responsible for relaxation in fast-twitch skeletal muscle and cardiac myocytes (MacLennan *et al.*, 1985; Mintz and Guillain, 1997; Inesi *et al.*, 1998).

The functional activity of the SR $Ca^{2+}$ pump can be described by an operational model based on the coexistence of $Ca^{2+}$-dependent conformations of the enzyme, as well as phosphorylated and nonphosphorylated species (de Meis and Vianna, 1979). $Ca^{2+}$ binding to the nonphosphorylated enzyme at the cytosolic side is followed by the formation of $Ca^{2+}$-bound phosphoenzyme when incubated in the presence of ATP. A subsequent change in orientation and a decrease in binding affinity of the transport sites induces $Ca^{2+}$ release toward the reticulum lumen. Enzyme dephosphorylation and reorientation of the transport sites in the $Ca^{2+}$-free nonphosphorylated protein complete the reaction cycle.

The enzyme can be inhibited by a wide variety of hydrophobic molecules (Morris *et al.*, 1982; Ho *et al.*, 1983; Almeida *et al.*, 1986; Sokolove *et al.*, 1986; Michelangeli *et al.*, 1990; Martinez-Azorin *et al.*, 1992; Ishida and Honda, 1993; Seino *et al.*, 1994). In this regard, studies of drug effects on this system may contribute to a better understanding of the transport and catalytic coupling mechanism.

[1] Key to abbreviations: SR, sarcoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; EGTA, [ethylenebis(oxonitrilo)]tetraacetic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; A23187, calcimycin; EP, enzyme-phosphorylated intermediate.

[2] Departamento de Bioquimica y Biologia Molecular A, Facultad de Veterinaria, Universidad de Murcia en Espinardo. E-30071 Murcia, Spain.

[3] Permanent address: Centro de Estudios Biologicos, Universidad Autonoma de Ciudad Juarez, Avda. Lopez Mateos #20. C.P. 32310 Ciudad Juarez, Chihuahua, Mexico.

[4] Author to whom correspondence should be sent. email: fbelda@fcu.um.es.



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Clomipramine and other related compounds, bearing the dihydrodibenzazepine nucleus (Fig. 1), exert their therapeutic action by inhibiting the presynaptic uptake of amine neurotransmitters. Therefore, they have a pharmacological application for the treatment of endogenous depression (Montgomery, 1994). Moreover, they have been shown to interfere, at least *in vitro*, with enzyme activities involved in energy-coupling reactions, such as $Na^+/K^+$-ATPase from synaptic plasma membrane (Carfagna and Muhoberac, 1993) or $Ca^{2+}$-ATPase from erythrocyte membrane (Plenge-Tellechea *et al.*, 1999).

We have proved in this study that tricyclic antidepressants inhibit the $Ca^{2+}$-pump activity of isolated SR vesicles. The reported data have been analyzed taking into consideration key partial reactions of the catalytic and transport cycle. Our experimental approach sheds light on the inhibition mechanism of an energy-transduction system, the SR $Ca^{2+}$ pump, induced by the presence of antidepressant drugs.

## MATERIALS AND METHODS

### Microsomal Vesicles

SR membrane, mainly derived from longitudinal tubules of rabbit fast-twitch skeletal muscle, was obtained as described by Eletr and Inesi (1972). The membrane protein was evaluated by the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard. Samples were aliquoted and stored at −80 °C until use.

### $Ca^{2+}$ Concentration in the Reaction Medium

Free $Ca^{2+}$ was adjusted by proper additions of $CaCl_2$ and EGTA stock solutions as calculated by computation (Fabiato, 1988). The computer program considered the $Ca^{2+}$-EGTA absolute stability constant (Schwartzenbach *et al.*, 1957), the $H_4^+$-EGTA dissociation constants (Blinks *et al.*, 1982), and the presence of relevant electrolytes.

### Oxalate-Supported $Ca^{2+}$ Transport

Initial rates of $Ca^{2+}$ transport were measured at 25°C by radioactive tracer and sample filtration (Martonosi and Feretos, 1964). The standard reaction medium consisted of 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 1 mM EGTA, 0.967 mM [$^{45}$Ca]$CaCl_2$, 0.01 mg/ml of SR protein, 0.5 mM ATP, and a certain drug concentration when indicated. Free $Ca^{2+}$ was 10 μM and $^{45}$Ca-specific activity ~2,000 cpm/nmol. The reaction was started by adding ATP and maintained under stirring during the measurements. $Ca^{2+}$ uptake of 1-ml aliquots was stopped at sequential times by vacuum filtration. Filters containing the $Ca^{2+}$-loaded vesicles were rinsed with 2 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, and 1 mM $LaCl_3$ and the radioactivity retained was counted. The use of radioactive standards allowed us to express the transport data as nmol $Ca^{2+}$/mg of protein.

### $Ca^{2+}$ Binding at Equilibrium

Experiments on $Ca^{2+}$ binding in the absence of ATP were performed with the aid of $^{45}$Ca and $^3$H radioactive tracers (Champeil and Guillain, 1986). Unbound $Ca^{2+}$ was evaluated from the filter wet volume by using [$^3$H]glucose as a marker. The addition of either clomipramine or desipramine to the enzyme in a $Ca^{2+}$-saturating medium, *i.e.*, 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.105 mM [$^{45}$Ca]$CaCl_2$ (~5,000 cpm/nmol) (10 μM free $Ca^{2+}$), 1 mM [$^3$H]glucose (~10,000 cpm/nmol), and 0.4 mg/ml of SR protein, or in a nominally $Ca^{2+}$-free medium, *i.e.*, 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM glucose, and 0.4 mg/ml of SR protein, was followed by incubation at 25°C for at least 5 min. In the latter case, 0.02 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 3.15 mM [$^{45}$Ca]$CaCl_2$ (~5,000 cpm/nmol), and 1 mM [$^3$H]glucose (~10,000 cpm/nmol) was added to 0.6 ml of the enzyme suspension (final free $Ca^{2+}$ was 10 μM). The incubation was now prolonged for 1 min at 25°C. In any case, samples of 0.5 ml (0.2 mg of protein) were filtered under vacuum without any further washing to determine $^{45}$Ca and $^3$H associated with the filters. Specific $Ca^{2+}$ bound to the enzyme was calculated by subtracting unbound $Ca^{2+}$ retained by the filter.

### Assays of Radioactive Phosphoenzyme

Accumulated levels of EP were analyzed under different assay conditions. The drug concentration

effect on EP during the enzyme turnover was studied at 0°C by mixing equal volumes (0.5 ml) of leaky SR vesicles with the phosphorylating medium. When the enzyme (0.2 mg/ml of SR vesicles) was in the presence of 50 μM free $Ca^{2+}$, *i.e.*, 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 15 μM A23187, 0.1 mM EGTA, and 0.149 mM $CaCl_2$, the phosphorylation medium was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.149 mM $Ca^{2+}$, and 50 μM [γ-$^{32}$P]ATP (~20,000 cpm/nmol). Free $Ca^{2+}$ was unchanged after mixing. When the enzyme (0.2 mg/ml) was in a nominally $Ca^{2+}$-free medium, *i.e.*, 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 15 μM A23187, and 0.2 mM EGTA, the phosphorylating medium contained 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 50 μM [γ-$^{32}$P]ATP, and 0.298 mM $CaCl_2$ to give 50 μM free $Ca^{2+}$ after mixing. The drug tested was always present at the same concentration in both reaction media. Phosphorylation time was 5 s. The reaction was started under continuous vortexing and stopped by adding 1 ml of ice-cold 250 mM perchloric acid and 2 mM sodium phosphate. Acid-treated samples were placed in ice-water bath for 5 min before filtration through Millipore filters. The membrane protein retained by the filter was rinsed 5 times with 5 ml each of ice-cold 125 mM perchloric acid and 1 mM sodium phosphate. Radioactive $^{32}$P label was measured by liquid scintillation technique after solubilization of the filters. Data are expressed as nanomoles EP/milligram of protein.

The time course of EP formation was studied at 0°C by using $Ca^{2+}$-ATP as a substrate, *i.e.*, in the absence of $Mg^{2+}$ (Lacapere and Guillain, 1990). The enzyme suspension (0.2 mg) in 0.5 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $CaCl_2$, and 15 μM A23187 was manually mixed with 0.5 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $CaCl_2$, and 50 μM [γ-$^{32}$P]ATP (~20,000 cpm/nmol). The reaction was started under continuous vortexing and stopped in the second time scale by adding 1 ml of ice-cold quenching solution (250 mM perchloric acid and 2 mM sodium phosphate). Thereafter, samples were treated as described above. The drug tested was always present at the same concentration in both reaction media.

The ATP dependence was studied at 25°C by mixing 0.5 ml of enzyme suspension (0.2 mg/ml) containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 15 μM A23187, 0.1 mM EGTA, and 0.149 mM $CaCl_2$ (50 μM free $Ca^{2+}$) with 0.5 ml of phosphorylating medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.149 mM $CaCl_2$, and [γ-$^{32}$P]ATP in the range of 2 to 100 μM. Reaction media were supplemented with either 0.5 mM clomipramine or 1 mM desipramine when indicated. After 2 s, the reaction was stopped by 1 ml of ice-cold quenching solution and processed as described before.

Decay of radioactive EP during enzyme cycling was studied at 0°C after phosphorylation of leaky SR vesicles with radioactive ATP and the subsequent dilution with nonradioactive ATP. A volume of 0.2 ml composed of 20 mM Mops, pH 7.0, 80 mM Kl, 5 mM $MgCl_2$, 0.5 mg/ml of membrane protein, 37.5 μM A23187, 1 mM EGTA, and 0.967 mM $CaCl_2$ (10 μM free $Ca^{2+}$) was mixed at 0°C with 0.2 ml of medium containing 20 M Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.967 mM $CaCl_2$, and 50 μM [γ-$^{32}$P]ATP (~20,000 cpm/nmol). Five seconds later, the reaction medium was diluted with 5 ml of ice-cold medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.967 mM $CaCl_2$, and 25 μM ATP. The reaction was arrested at serial times by adding 2 ml of ice-cold stopping solution containing 460 mM perchloric acid and 3.7 mM sodium phosphate. Quenched samples (0.1 mg of protein) were filtered and processed as described before. The drug effect was studied by including clomipramine or desipramine in the reaction and dilution media.

EP level from [$^{32}$P]phosphate was measured at 25°C in a medium (0.2 ml) containing 50 mM Mes-Tris, pH 6.2, 10 mM $MgCl_2$, 2 mM EGTA, 0.5 mg/ml of SR protein, and 2 mM sodium [$^{32}$P]phosphate (~20,000 cpm/nmol). Clomipramine or desipramine was present when indicated. Equilibration was allowed to proceed for 15 min before addition of ice-cold quenching solution (5 ml) containing 125 mM perchloric acid and 2 mM sodium phosphate. Samples were maintained in ice for 5 min and then filtered and washed (five times) with the quenching medium as described for EP labeling from radioactive ATP. In some experiments, the [$^{32}$P]phosphate concentration was varied in the range of 0.2 to 2 mM.

## Materials

Nitrocellulose filter units (HA type) with 0.45-μm pore size from Millipore were used for vacuum filtration. [γ-$^{32}$P]ATP, [$^{32}$P]phosphate, [$^{45}$Ca]$CaCl_2$, and [$^{3}$H]glucose were radioactive products of DuPont NEN. The calcium-standard solution (Titrisol) was

purchased from Merck. Clomipramine, desipramine, imipramine, and trimipramine were obtained from Sigma Chemical Co., as well as the Sigma-Fluor scintillation cocktail (ref. S-4023). 3-(Dimethylamino)-propionitrile was from Acros Chimica (former Janssen Chimica), Belgium. A23187 was from Roche Molecular Biochemicals (former Boehringer Mannheim). All other chemicals were of reagent grade. Clomipramine was dissolved in ethanol and trimipramine in dimethyl sulfoxide. The volume of organic solvent added was always lower than 1% of the total volume.

## RESULTS

Vesicular SR fragments exhibit linear rates of $Ca^{21}$ transport upon addition of ATP when equilibrated in the presence of oxalate as a lumenal precipitating agent. The steady state rate measured at 258C, in a pH 7.0 buffered medium, and under the presence of 0.5 mM ATP was ca. 1.5 mmol $Ca^{21}$/min and mg of protein. Figure 2A shows that the $Ca^{21}$-sequestering activity was inhibited by clomipramine in a dose-dependent manner. Half-maximal inhibition was achieved at a concentration ca. 120 mM. It is also shown that 3-(dimethylamino)propionitrile does not exert any inhibitory action when used up to a concentration of 2 mM (Fig. 2A, inset). It should be noted that the selected nitrile corresponds to the aliphatic chain present in the clomipramine structure. The inhibition induced by clomipramine is not dependent on the SR protein concentration (data not shown), ruling out any drug partitioning into the membrane. We also considered

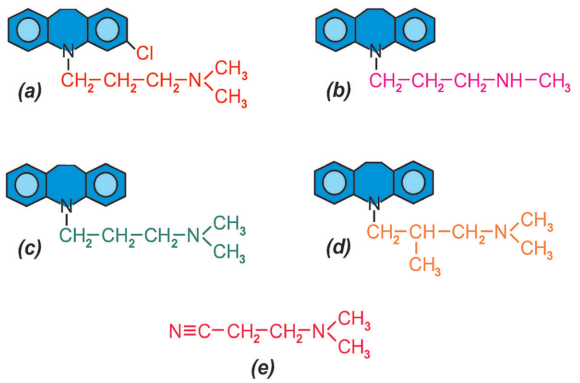
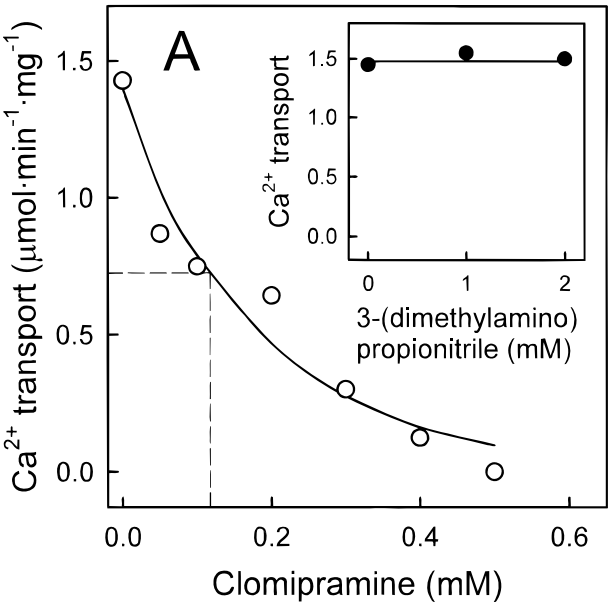



**Fig. 1.** Chemical structure of compounds under study. (a) Clomipramine; (b) desipramine; (c) imipramine; (d) trimipramine; and (e) 3-(dimethylamino)propionitrile



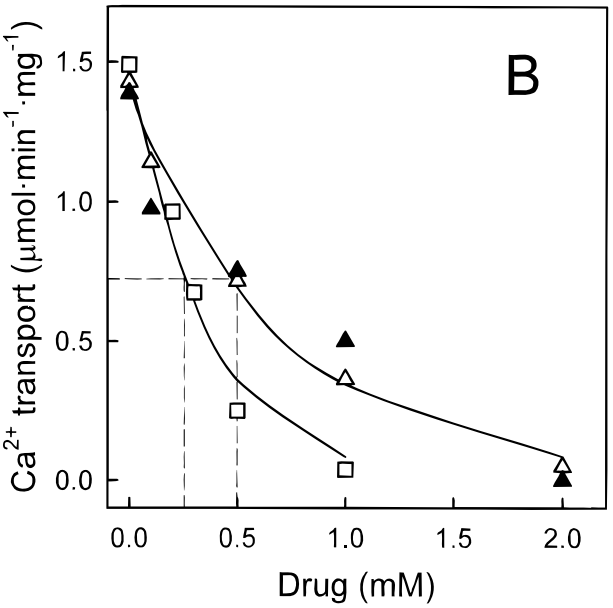




**Fig. 2.** Clomipramine (panel A) and related compounds (panel B) inhibit $Ca^{21}$ transport in SR vesicles, whereas 3-(dimethylamino)-propionitrile has no effect. $Ca^{21}$ transport was measured at 258C. The final reaction medium was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$ 5 mM $K^+$-oxalate, 1 mM EGTA, 0.967 mM [$^{45}$Ca]$CaCl_2$, 0.01 mg SR protein/ml, 0.5 mM ATP, and a certain concentration of clomipramine (V), desipramine (M), imipramine (n), or trimipramine (m). The reaction was stopped by filtration of 1 ml aliquots. Filters were rinsed with 2 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, and 1 mM $LaCl_3$. Radioactive $^{45}$Ca in the filters was measured by liquid scintillation counting. Inset data in panel A (v) were obtained in the presence of 3-(dimethylamino)propionitrile.

clomipramine-related structures to assess the effect on the specific ATP-dependent $Ca^{2+}$ transport of SR vesicles. Figure 2B shows that desipramine, imipramine, and trimipramine can interfere with the $Ca^{2+}$ pumping rate. The relative inhibition induced by a given concentration of drug was lower than in the case of clomipramine. In other words, these compounds displayed a lower potency of inhibition. Half-maximal inhibition for desipramine was 250 μM, whereas that for imipramine and trimipramine was 500 μM. The drug effect on $Ca^{2+}$ transport cannot be attributed to a counteracting $Ca^{2+}$-leak pathway since clomipramine and other related structures cannot induce release from $Ca^{2+}$-loaded vesicles (data not shown).

An overall effect on $Ca^{2+}$ transport can be more deeply analyzed by considering key partial events of the enzyme reaction cycle. With this aim, we started by measuring equilibrium $Ca^{2+}$ binding to the nonphosphorylated enzyme. Microsomal vesicles suspended in a medium containing 10 μM free $Ca^{2+}$ were incubated at 25°C with a certain concentration of either clomipramine or desipramine. Five minutes later, the enzyme suspension was filtered under vacuum. ATP was absent to prevent any ATP hydrolysis and, therefore, the formation of phosphorylated species. When the enzyme high-affinity transport sites, located at the cytosolic side, were previously saturated by $Ca^{2+}$ the subsequent addition of clomipramine or desipramine did not perturb the $Ca^{2+}$-bound species (Fig. 3A). We also studied whether or not the $Ca^{2+}$-free form of the enzyme was affected by antidepressants. The experimental protocol consisted of a preliminary equilibration, at 25°C for at least 5 min, of SR vesicles in the presence of EGTA (*i.e.*, in the absence of $Ca^{2+}$) with either clomipramine or desipramine, followed by the addition of sufficient $Ca^{2+}$ (10 μM free $Ca^{2+}$) to saturate the high-affinity sites. Direct measurements of $^{45}Ca^{2+}$ binding to the nonphosphorylated enzyme preincubated with drug indicate that clomipramine or desipramine can interfere with the equilibrium $Ca^{2+}$ binding at the cytosolic side (Fig. 3B). Although data points were collected 1 min after the $Ca^{2+}$ addition, no further effect was noted when the incubation time was prolonged.

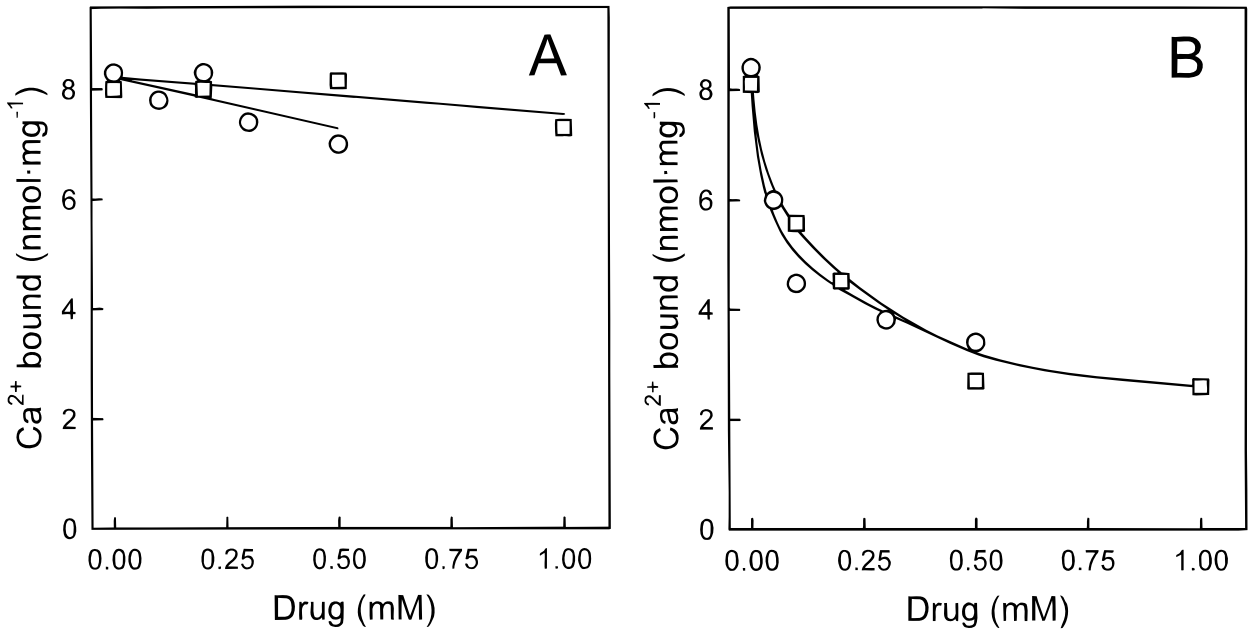



**Fig. 3.** Clomipramine and desipramine display higher interaction affinity for the $Ca^{2+}$ free than for the $Ca^{2+}$-bound conformation of the enzyme. (A) The enzyme suspension containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.105 mM [$^{45}$Ca]$CaCl_2$, 1 mM [$^3$H]glucose, and 0.4 mg SR protein/ml was preincubated at 25°C for 5 min with various concentrations of either clomipramine (○), or desipramine (□). (B) The enzyme suspension containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM glucose, and 0.4 mg SR protein/ml was mixed at 25°C and maintained for at least 5 min with various concentrations of either clomipramine (○) or desipramine (□). $Ca^{2+}$ binding was elicited by adding 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 3.15 mM [$^{45}$Ca]$CaCl_2$, and 1 mM [$^3$H]glucose (final free $Ca^{2+}$ was 10 μM). In any case, aliquots of 0.5 ml were filtered under vacuum. Double radioactive counting was carried out without any rinsing of the filters. Clomipramine was raised up to 0.5 mM because of solubility problems.

Partial reactions involved in the formation and decomposition of phosphorylated species were also considered. The addition of ATP to the $Ca^{2+}$-bound enzyme is followed by a rapid phosphoryl transfer and the subsequent accumulation of EP. The initial set of experiments was carried out at 0°C, in order to slow down the enzyme turnover; the radioactive EP was measured 2 s after mixing the enzyme suspension with the phosphorylation medium. In one case, $Ca^{2+}$-saturated SR vesicles were first exposed to clomipramine and then mixed with radioactive ATP. In the other, SR vesicles in a nominally $Ca^{2+}$-free medium were preequilibrated with clomipramine before the simultaneous addition of $Ca^{2+}$ and ATP. The accumulated level of EP was affected by the presence of drug, although the degree of inhibition was dependent on the starting enzyme conformation. A preliminary incubation of SR vesicles plus $Ca^{2+}$ before the addition of clomipramine protected to a large extent against inhibition (Fig. 4A). However, the inhibition was clear when clomipramine was added to the vesicles in the absence of $Ca^{2+}$. Experiments in the presence of desipramine were qualitatively similar (Fig. 4B). A slight inhibition was observed when the drug was added to the microsomal membrane in the presence of $Ca^{2+}$, whereas a clear inhibition was noted when desipramine was added to the vesicles in the absence of $Ca^{2+}$.

The phosphorylation reaction can be studied in the absence of rapid kinetic technology by decreasing temperature, increasing $Ca^{2+}$, and removing $Mg^{2+}$ (Shigekawa *et al.*, 1983; Lacapere and Guillain, 1990). The assay was conducted at 0°C and the reaction was started by mixing equal volumes of the enzyme suspension containing 5 mM $Ca^{2+}$ with a phosphorylation medium containing 50 μM [γ-$^{32}$P]ATP and 5 mM $Ca^{2+}$. At this $Ca^{2+}$ concentration and in the absence

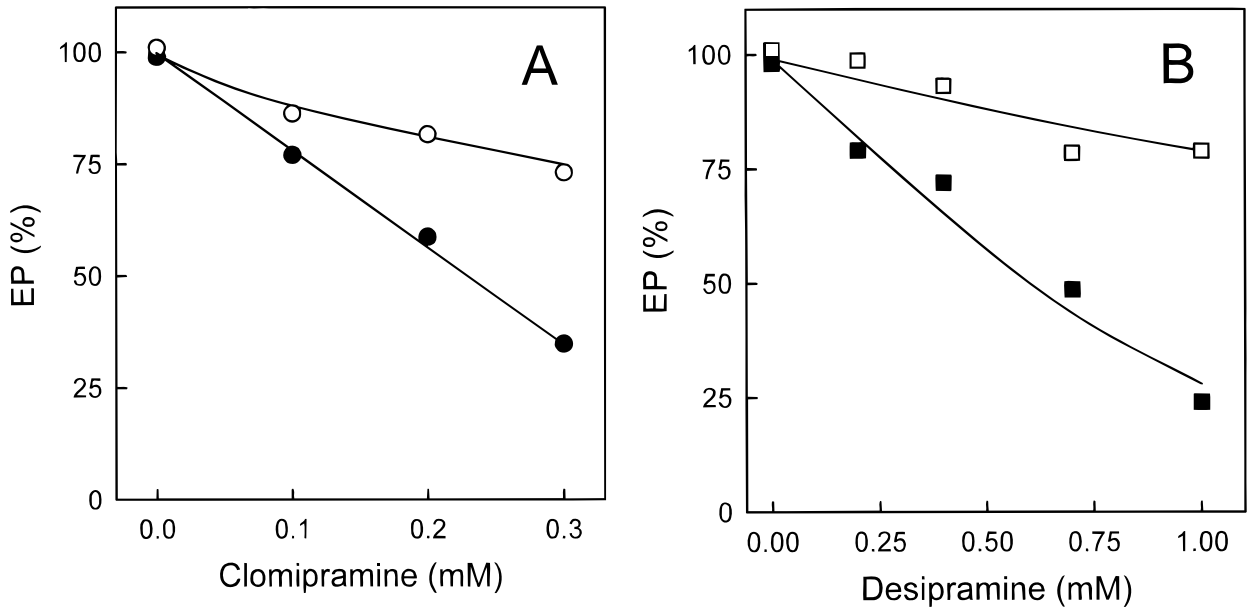



**Fig. 4.** The clomipramine or desipramine effect on EP is larger when the drug is added to the $Ca^{2+}$-free enzyme before phosphorylation. (A) SR vesicles (0.2 mg/ml) in a $Ca^{2+}$-saturating medium (20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 15 μM A23187, 0.1 mM EGTA, and 0.149 mM $CaCl_2$) were first incubated at 25°C for 5 min with a certain clomipramine concentration (abscissa axis). Phosphorylation at 0°C was started by mixing 0.5 ml of the enzyme suspension with 0.5 ml of medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 50 μM [γ-$^{32}$P]ATP, 0.1 mM EGTA, 0.149 mM $CaCl_2$ and the clomipramine concentration present in the enzyme suspension (○). Alternatively, SR vesicles (0.2 mg/ml) in the absence of $Ca^{2+}$ (20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 15 μM A23187, and 0.2 mM EGTA) were incubated with a certain clomipramine concentration for 5 min at 25°C. Phosphorylation at 0°C was initiated by mixing 0.5 ml of enzyme suspension (in the $Ca^{2+}$-free medium containing clomipramine) with 0.5 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 50 μM [γ-$^{32}$P]ATP, 0.298 mM $CaCl_2$ and the same clomipramine concentration (●). (B) SR vesicles (0.2 mg/ml) in a $Ca^{2+}$-saturating medium was incubated for 5 min at 25°C with a certain desipramine concentration (abscissa axis). Phosphorylation at 0°C was started by mixing the enzyme suspension containing desipramine with the phosphorylating medium supplemented with the same desipramine concentration (□). EP was also formed after incubation of SR vesicles in the absence of $Ca^{2+}$ with desipramine followed by the addition of phosphorylating medium containing $Ca^{2+}$ and desipramine at the same concentration (■). The reaction media composition was as described in (A) but including desipramine instead of clomipramine. Phosphorylation time in all experiments was 5 s and radioactive EP was evaluated as detailed in the experimental section.

of $Mg^{2+}$ the phosphorylating substrate is $Ca^{2+}$-ATP. The slow time course of EP formation measured under the above-mentioned conditions displayed an initial rate of 1.4 nmol/s and mg of protein and a maximal phosphorylation level of 3.5 nmol/mg. The apparent rate constant was ca. 0.5 $s^{-1}$ (Fig. 5). The experiments were repeated in the presence of either 0.3 mM clomipramine or 1 mM desipramine by including the corresponding drug in both reaction media. This avoided any change in drug concentration after mixing. Figure 5 indicates that none of the kinetic parameters were modified by clomipramine or desipramine, ruling out any functional effect on the phosphorylation reaction by ATP.

The steady-state accumulation of EP was also measured at 25°C and in the presence of 50 μM $Ca^{2+}$ and 5 mM $Mg^{2+}$. In this case, the phosphorylation was maintained for 2 s and the [γ-$^{32}$P]ATP concentration was varied between 1 and 50 μM. The total EP in the absence of drug increased as a function of the ATP concentration, reaching an asymptotic level (ca. 3.5 nmol/mg) at the higher substrate concentrations (Fig. 6). A similar dependence was observed in the presence of either 0.5 mM clomipramine or 1 mM desipramine, although the clomipramine effect was more pronounced. Desipramine decreased the asymptotic level at ca. 3 nmol/mg and clomipramine at ca. 1.5 nmol/mg.

$Ca^{2+}$ translocation from the cytosolic to the lumenal side is functionally linked to EP breakdown, leaving the $Ca^{2+}$-free nonphosphorylated enzyme ready to start a new reaction cycle. We studied the dephosphorylation rate at 0°C by first phosphorylating leaky vesicles with 25 μM [γ-$^{32}$P]ATP for 5 s and then diluting 12.5-fold with cold ATP at the same concentration.

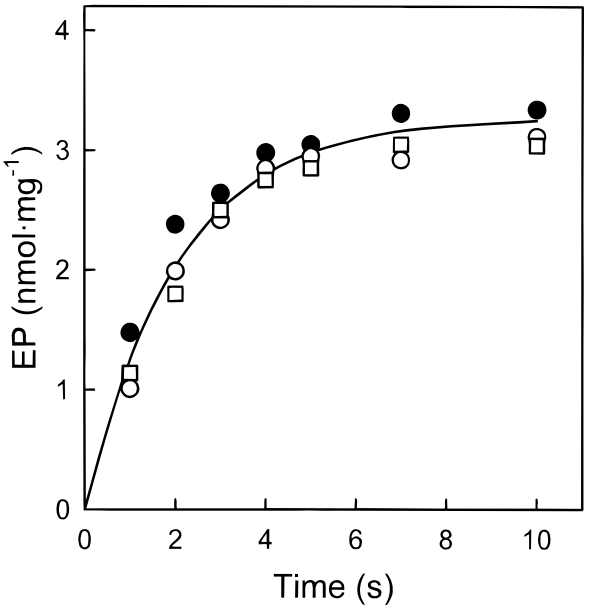



**Fig. 5.** Clomipramine and desipramine do not affect the phosphorylation partial reaction by ATP. Experiments at 0°C were performed by mixing the enzyme suspension in the presence of 5 mM $Ca^{2+}$ with the phosphorylating medium containing 5 mM $Ca^{2+}$. Clomipramine or desipramine was present at the same concentration in both reaction media. The drug under study was added to the enzyme already in the presence of $Ca^{2+}$. The final reaction medium was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $CaCl_2$, 25 μM [γ-$^{32}$P]ATP, 0.1 mg SR/ml, 7.5 μM A23187, and either 0.3 mM clomipramine or 1 mM desipramine. The reaction (1 ml) was quenched by 250 mM perchloric acid and 2 mM sodium phosphate (1 ml). Samples were placed for 5 min in ice-water bath before filtration. Filters were rinsed five times with 5 ml each of ice-cold 125 mM perchloric acid and 1 mM sodium phosphate. EP was evaluated after radioactive counting of the filters. Experiments in the absence of drug (●), in the presence of 0.3 mM clomipramine (○), or in the presence of 1 mM desipramine (□).

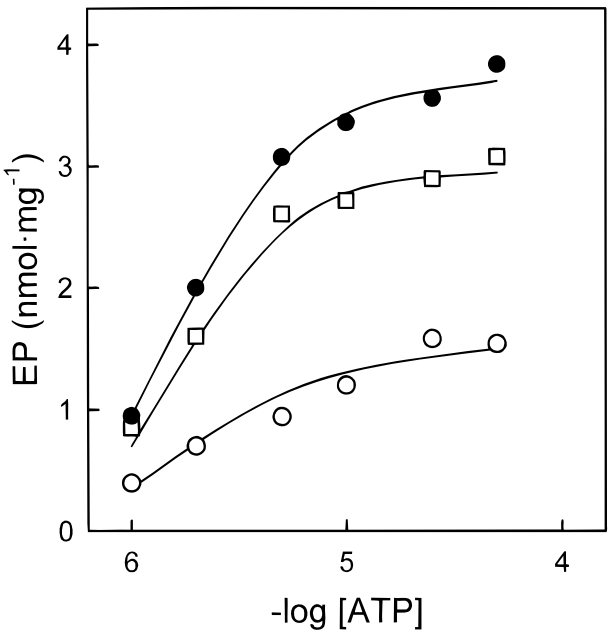



**Fig. 6.** Clomipramine and desipramine decrease the EP level under standard turnover conditions. The final reaction medium was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.149 mM $CaCl_2$, 0.1 mg SR/ml, 7.5 μM A23187, and either 0.5 mM clomipramine or 1 mM desipramine. [γ-$^{32}$P]ATP was varied in the range of 1 to 50 μM. The drug was always added to the medium once the enzyme was in the presence of $Ca^{2+}$. The final drug concentration was present in both reaction media. The phosphorylation was maintained for 2 s at 25°C and stopped by acid quenching. Samples were processed as described in the Materials and Methods section. Data points in the absence of drug (●), in the presence of 0.5 mM clomipramine (○), or in the presence of 1 mM desipramine (□). The abscissa axis correspond to M concentrations of ATP expressed as negative log values.

This allows the maintainence of steady-state conditions during the assay. The kinetics of radioactive EP decay was followed at various times after acid quenching of the samples. The results of Fig. 7 indicate that the rate of EP decomposition fits a two-exponential process with approximately 60 and 40% amplitudes and apparent rate constants ca. 1 and 0.11 $s^{-1}$, respectively. Clomipramine (0.5 mM) or desipramine (1 mM) affected the rate of dephosphorylation giving single apparent rate constants of 0.17 or 0.13 $s^{-1}$, respectively.

The dephosphorylation step was also studied as an independent event taking advantage of the partial reactions reversibility. The experimental approach consisted of equilibration of SR vesicles in a nominally $Ca^{2+}$-free medium with 2 mM [$^{32}$P]phosphate and 10 mM $Mg^{2+}$. The assay was carried out at 25°C, using pH of 6.2 and absence of $K^+$, in order to favor the accumulation of the $Ca^{2+}$-free phosphorylated species. The presence of either clomipramine or desipramine was accompanied by a concentration-dependent decrease in the level of $Ca^{2+}$-free EP. Half-maximal inhibition was induced by 90 μM clomipramine or 280 μM desipramine (Fig. 8). The inhibitory effect was also observed when the $Ca^{2+}$-free EP was measured as a function of the [$^{32}$P]phosphate concentration (Fig. 8, inset). This plot shows the dependence of EP on the phosphate concentration when 0.5 mM desipramine was present.

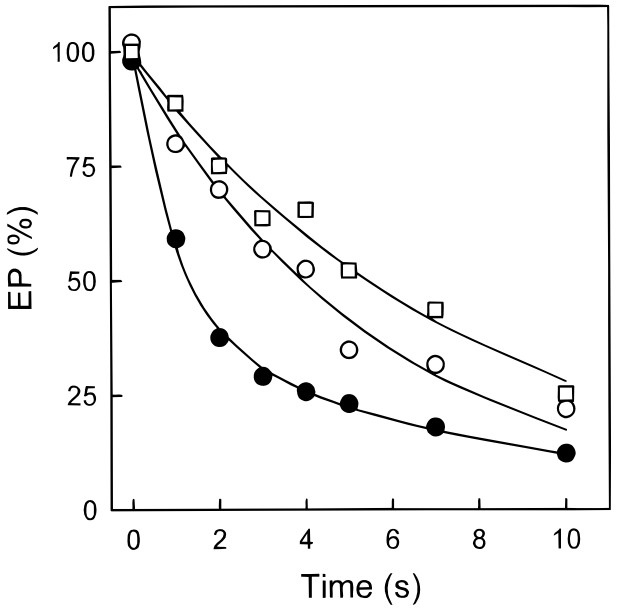



**Fig. 7.** Clomipramine and desipramine decrease the rate of the enzyme dephosphorylation. Labeling of radioactive EP under turnover conditions was obtained at 0°C by mixing 0.2 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mg/ml SR protein, 37.5 μM A23187, 1 mM EGTA, and 0.967 mM $CaCl_2$ with 0.2 ml of medium 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.967 mM $CaCl_2$, and 50 μM [$\gamma^{32}$P]ATP. Following a 5-s incubation, the mixture was diluted with 5 ml of ice-cold medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.967 mM $CaCl_2$, and 25 μM ATP. Samples were quenched at different times by 2 ml of acid solution (460 mM perchloric acid and 3.7 mM sodium phosphate). [$^{32}$P]EP was determined by scintillation counting after sample filtration and rinsing of the filters. Experiments in the absence of drug (●), in the presence of 0.5 mM clomipramine (○), or in the presence of 1 mM desipramine (□).

## DISCUSSION

The present data show that tricyclic antidepressants inhibit the ATP-dependent $Ca^{2+}$ transport of the skeletal SR membrane and our goal was to analyze

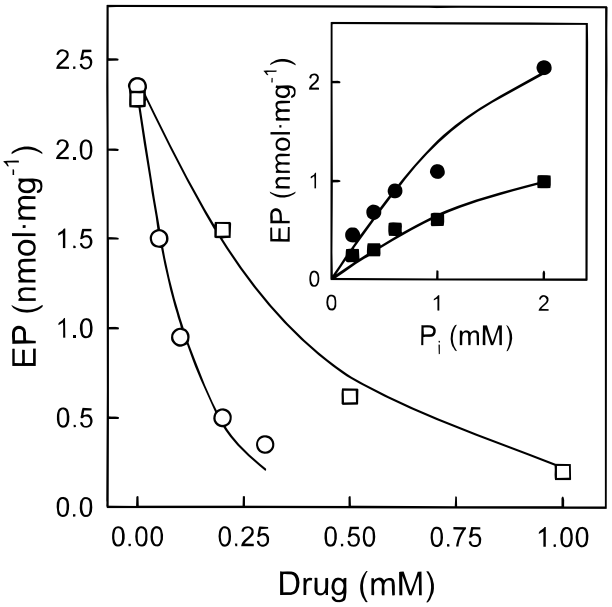



**Fig. 8.** Clomipramine and desipramine decrease the EP formed from inorganic phosphate. Radioactive EP in the absence of $Ca^{2+}$ was formed in a medium 50 mM Mes-Tris, pH 6.2, 10 mM $MgCl_2$, 2 mM EGTA. 0.5 mg/ml SR, and 2 mM sodium [$^{32}$P]phosphate. Following 15-min equilibration at 25°C, samples of 0.2 ml were quenched with 5 ml of ice-cold perchloric acid (125 mM) and sodium phosphate (2 mM), and then filtered and rinsed. Filters were collected for determination of $^{32}$P. Data points in the presence of clomipramine (○), or desipramine (□). Inset: Phosphorylation media and conditions as described for the main panel, but including different concentrations of [$^{32}$P]phosphate. The EP dependence on the phosphate concentration was studied in the absence (●) or in the presence of 0.5 mM desipramine (■).

the mechanism of inhibition. The use of drugs with closely related structures may also help to uncover structural features accounting for the inhibitory phenomenon.

Hydrophobic molecules bearing nucleophilic groups have been extensively reported as inhibitors of membrane enzymes and, in particular, of $Ca^{2+}$-ATPase from SR (Morris *et al.*, 1982; Ho *et al.*, 1983; Almeida *et al.*, 1986; Sokolove *et al.*, 1986; Michelangeli *et al.*, 1990; Martinez-Azorin *et al.*, 1992; Ishida and Honda, 1993; Seino *et al.*, 1994). The drugs considered in this study are secondary or tertiary amines linked to the dihydrodibenzazepine nucleus through the nitrogen atom. The inhibitory effect can be clearly attributed to the tricyclic moiety. The conclusion was reached by using 3-(dimethylamino)propionitrile. This compound, reproducing the aliphatic chain present in clomipramine or imipramine, does not affect the $Ca^{2+}$-pumping activity (Fig. 2A, inset). A more careful examination indicates that the presence of a chlorine substitution in the tricyclic structure, as occurs in clomipramine, produces the maximal potency of inhibition (Fig. 2A). The removal of CI from clomipramine to give imipramine is associated with a decrease in the inhibition potency (Fig. 2B). Moreover, the addition of a methyl group to the aliphatic amine moiety, such as in trimipramine, does not alter the inhibitory profile (Fig. 2B). However, the exchange of a tertiary for a secondary amine, as occurs when imipramine is converted into desipramine, increases the inhibition potency (Fig. 2B). Therefore, the presence of a nucleophilic group in the tricyclic moiety and a less-branched structure in the aliphatic chain seems to be critical for the inhibition potency.

By using $^{45}Ca^{2+}$ and equilibrium conditions, we observed that up to 0.5 or 1 mM tricyclics has no effect on $Ca^{2+}$ binding when added to the enzyme in the presence of 10 μM free $Ca^{2+}$ (Fig. 3A). There was a slight effect at the higher drug concentrations that was no longer observed when the $Ca^{2+}$ added was raised (data not shown). By contrast, inhibition was observed when 10 μM free $Ca^{2+}$ was added to the enzyme in the presence of tricyclics (Fig. 3B). Drug concentration was raised up to the solubility limit that was approximately coincident with the respective maximal effect on $Ca^{2+}$ transport, *i.e.*, 0.5 mM for clomipramine or 1 mM for desipramine. Therefore, the effect of tricyclics on $Ca^{2+}$ binding is dependent on the conformational state of the nonphosphorylated enzyme, being the drug affinity higher for the $Ca^{2+}$-free conformation. This may suggest that the dihydrodibenzazepine nucleus of the drugs interfere with the proper packing of the enzyme transmembrane domain where the $Ca^{2+}$-binding sites are located.

The influence of the enzyme conformational state on inhibition was also proved under turnover conditions at 0°C by measuring the EP level after ATP phosphorylation. The inhibition of EP by clomipramine (Fig. 4A) or desipramine (Fig. 4B) was always higher when the enzyme was initially in the $Ca^{2+}$-free form. It means that the observed effect at 0°C reflects the temperature-sensitive and $Ca^{2+}$-dependent interconversion between the two nonphosphorylated conformations of the enzyme.

The level of accumulated EP at 0°C was not affected by clomipramine or desipramine when measured in the presence of 5 mM $Ca^{2+}$, 50 μM [γ-$^{32}$P]ATP, and in the absence of $Mg^{2+}$ (Fig. 5). In these experiments, the nonphosphorylated enzyme was initially in the $Ca^{2+}$-bound conformation and the catalytic turnover was expected to be slow (Lacapere and Guillain, 1990). Our data indicate that the ATP phosphorylation step is not the target of the antidepressant drug. Nonetheless, the EP level was clearly inhibited when the phosphorylation conditions were those of the $Ca^{2+}$ transport experiments, *i.e.*, 25°C, 50 μM $Ca^{2+}$ and 5 mM $Mg^{2+}$. The inhibition induced by 0.5 mM clomipramine was higher than that induced by 1 mM desipramine. This behavior was reproduced at different ATP concentrations (Fig. 6). This is consistent with a higher inhibition potency of clomipramine with respect to desipramine, as already shown in the $Ca^{2+}$ transport measurements. The EP dependence profile on ATP concentration indicates that tricyclics do not modify the ATP binding affinity to the enzyme. In these experiments, clomipramine or desipramine was added to the enzyme in the $Ca^{2+}$-bound conformation; therefore, the inhibition of the EP accumulation was elicited as a consequence of the enzyme cycling.

Phosphorylation of the $Ca^{2+}$-free enzyme by inorganic phosphate was also inhibited by clomipramine or desipramine (Fig. 8). These equilibrium measurements, performed at pH 6.2, revealed that drug concentrations giving half-maximal effect were lower than the corresponding half-maximal values obtained from the $Ca^{2+}$ transport (Fig. 2A, B) or $Ca^{2+}$ binding experiments (Fig. 3B) at pH 7.0. The selected conditions, including pH, can explain the observed differences. Thus, $Ca^{2+}$ transport was measured under steady-state conditions. It means that different enzyme species are simultaneously present, whereas the drug effect takes place on a single enzyme conformation (the $Ca^{2+}$-free

nonphosphorylated form). However, the phosphorylation experiments by inorganic phosphate (Fig. 8) correspond to equilibrium conditions with a single enzymic form. In this case, all the enzyme is in the $Ca^{2+}$-free nonphosphorylated conformation, being the target of the drug. The $Ca^{2+}$ binding experiments (Fig. 3B) correspond to a third situation, *i.e.*, equilibrium conditions but coexistence of two ($Ca^{2+}$-bound and $Ca^{2+}$-free) nonphosphorylated species. In this case, drug and $Ca^{2+}$ interact with a different enzyme conformation. This highlights that an observed effect on a partial reaction may be difficult to extrapolate to the overall $Ca^{2+}$-pump performance, since the evaluation of a partial reaction may require different assay conditions. This includes the presence or absence of $Ca^{2+}$ ionophore, high or low temperature, different pH or substrate concentrations, equilibrium or steady state measurements, etc. In any case, our data indicate that tricyclic antidepressants interact with the $Ca^{2+}$-free conformation of the $Ca^{2+}$-ATPase protein as occurs with other specific inhibitors of the enzyme including the high-affinity inhibitors thapsigargin (Sagara *et al.*, 1992) and cyclopiazonic acid (Soler *et al.*, 1998). The inhibition data reported here are of experimental interest but they are not directly related to the pharmacological effect of tricyclic antidepressants.

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## REFERENCES

Almeida, L. M., Vaz, W. L., Stümpel, J., and Madeira, V. M. C. (1986). *Biochemistry* **25**, 4832–4839.
Blinks, J., Wier, W., Hess, P., and Prendergast, F. (1982). *Progr. Biophys. Mol. Biol.* **40**, 1–114.
Carfagna, M. A., and Muhoberac, B. B. (1993). *Mol. Pharmacol.* **44**, 129–141.
Champeil, P., and Guillain, F. (1986). *Biochemistry* **25**, 7623–7633.
de Meis, L., and Vianna, A. (1979). *Annu. Rev. Biochem.* **48**, 275–292.
Eletr, S., and Inesi, G. (1972). *Biochim. Biophys. Acta* **282**, 174–179.
Fabiato, A. (1988). *Methods Enzymol.* **157**, 378–417.
Ho, M.-M, Scales, D. J., and Inesi, G. (1983). *Biochim. Biophys. Acta* **730**, 64–70.
Inesi, G., Wade, R., and Rogers, T. (1998). *Ann. N.Y. Acad. Sci.* **853**, 195–206.
Ishida, Y., and Honda, H. (1993). *J. Biol. Chem.* **268**, 4021–4024.
Lacapere, J. J., and Guillain, F. (1990). *J. Biol. Chem.* **265**, 8583–8589.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.
MacLennan, D. H., Brandi, C. J., Korczak, B., and Green N. M. (1985). *Nature* **316**, 696–700.
Martinez-Azorin, F., Teruel, J. A., Fernandez-Belda, F., and Gomez-Fernandez, J. C. (1992). *J. Biol. Chem.* **267**, 11923–11929.
Martonosi, A., and Feretos, R. (1964). *J. Biol. Chem.* **239**, 648–658.
Michelangeli, F., Orlowski, S., Champeil, P., East, J. M., and Lee, A. G. (1990). *Biochemistry* **29**, 3091–3101.
Mintz, E., and Guillain, F. (1997). *Biochim. Biophys. Acta* **1318**, 52–70.
Montgomery, S. A. (1994). *Annu. Rev. Med.* **45**, 447–457.
Morris, S. J., Silbergeld, E. K., Brown, R. R., and Haynes, D. H. (1982). *Biochem. Biophys. Res. Commun.* **104**, 1306–1311.
Plenge-Tellechea, F., Soler, F., and Fernandez-Belda, F. (1999). *Arch. Biochem. Biophys.* **370**, 119–125.
Sagara, Y., Fernandez-Belda, F., de Meis, L., and Inesi, G. (1992). *J. Biol. Chem.* **267**, 12606–12613.
Schwartzenbach, G., Senn, H., and Anderegg, G. (1957). *Helv. Chim. Acta* **40**, 1886–1900.
Seino, A., Furukawa, K.-I., Miura, T., Yaginuma, T., Momose, K., and Ohizumi, Y. (1994). *J. Biol. Chem.* **269**, 17550–17555.
Shigekawa, M., Wakabayashi, S., and Nakamura, H. (1983). *J. Biol. Chem.* **258**, 8698–8707.
Sokolove, P. M., Albuquerque, E. X., Kauffman, F. C., Spande, T. F., and Daly, J. W. (1986). *FEBS Lett.* **203**, 121–126.
Soler, F., Plenge-Tellechea, F., Fortea, I., and Fernandez-Belda, F. (1998). *Biochemistry* **37**, 4266–4274.