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The Effects of Phenothiazines and Other Calmodulin Antagonists on the Sarcoplasmic and Endoplasmic Reticulum Ca^{2+} Pumps

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ABSTRACT. The effects of a number of phenothiazines and other calmodulin antagonists on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) were investigated. The drugs used in this study were trifluoperazine, calmidazolium, fluphenazine, chlorpromazine, W-7, and calmodulin-binding peptide. Our results showed that calmidazolium and calmodulin-binding peptide were the most potent inhibitors of skeletal muscle SR Ca^{2+}-ATPase activity (isoform SERCA 1) (IC_{50} values of 0.5 and 7 μM, respectively), while W-7 was the least potent inhibitor (IC_{50}, 125 μM). All of the antagonists had little effect on the cerebellar ER Ca^{2+}-ATPase activity (isoform SERCA 2b), except for trifluoperazine, which had a biphasic effect, causing stimulation at low concentrations and inhibition at higher concentrations. Our results suggest that the effects of these calmodulin antagonists are independent of calmodulin and that they inhibit the Ca^{2+}-ATPase in an isoform-specific manner. It was found that these antagonists inhibit the skeletal muscle isoform of the Ca^{2+} pump by altering the Ca^{2+} affinity and the associated Ca^{2+}-binding steps, as well as possibly stabilising the E1 conformational state of the enzyme. BIOCHEM PHARMACOL **60**;12:1797–1806, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. Ca^{2+} pumps; Ca^{2+}-ATPase; SERCA; calmodulin antagonists; phenothiazine; Ca^{2+} homeostasis

Variation in the concentration of free Ca^{2+} in the cell, $[Ca^{2+}]_i$†, is one of the major signals involved in the control of many cellular processes [1, 2]. A number of proteins are important in controlling $[Ca^{2+}]_i$, including Ca^{2+} pumps located in the sarco- and endoplasmic reticulum. These intracellular Ca^{2+} pumps are often referred to as SERCA (Sarco(Endo)plasmic Reticulum Ca^{2+}-ATPase) and are essential for the maintenance of low Ca^{2+} concentration in the cytosol of the resting cell. SERCA Ca^{2+} pumps do this by translocating Ca^{2+} from the cytosol to the lumen of the SR or ER.

There are three distinct genes that code for SERCA Ca^{2+} pumps and these are expressed in a tissue-specific manner [3, 4]. The SERCA 1 gene product is expressed exclusively in fast-twitch skeletal muscle [3], the SERCA 2a isoform is expressed in cardiac and slow-twitch muscle, and the SERCA 2b isoform is the dominant isoform found in neuronal tissues [4–7]. SERCA 3 is expressed in non-muscle tissues such as the thymus, white blood cells, and the large intestines [4, 8]. All of the Ca^{2+} pumps have a large cytoplasmic region that contains the catalytic site (where ATP binds and phosphorylation occurs) and a transmembrane domain that forms a channel-like structure that allows Ca^{2+} translocation across the membrane [9–11]. Vesicles derived from the sarco/endoplasmic reticulum can accumulate Ca^{2+} by using the energy derived from ATP hydrolysis [12]. In addition, unlike the plasma membrane Ca^{2+} pumps, SERCA do not have a calmodulin-binding domain and consequently are not regulated by calmodulin [13, 14].

The mechanism by which the ATPase transports Ca^{2+} is usually discussed in terms of the model proposed by DeMeis and Vianna [15], involving the two main conformational states E1 and E2. In the E1 conformation, the two Ca^{2+}-binding sites of the ATPase are of high affinity and are exposed to the outer (cytoplasmic) side of the SR/ER membrane, whereas in the E2 conformation the two sites are of low affinity and are exposed to the inside of the SR/ER. Furthermore, in the E1 conformation the ATPase can be phosphorylated by MgATP, which drives the Ca^{2+} translocation process. It has been demonstrated that Ca^{2+} binding to the ATPase is both sequential and co-operative in nature [16]. This suggests that binding of the first Ca^{2+} ion is followed by a conformational change, $E1Ca \rightarrow E1'Ca$, which allows binding of the second Ca^{2+} ion, forming $E1'Ca_2$ [16].

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† *Abbreviations:* SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; FITC, fluorescein 5′-isothiocyanate; and $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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Calmodulin is a Ca^{2+}-binding protein that modulates a number of proteins regulating cellular function [17]. A class of antipsychotic drugs known as phenothiazines (which includes trifluoperazine, chlorpromazine, and fluphenazine) and which are commonly used to treat certain mental disorders were found to interact strongly with calmodulin and have thus been termed calmodulin antagonists. These and related compounds bind calmodulin in a Ca^{2+}-dependent manner and are also able to block the activation of enzymes by calmodulin [18]. They are therefore useful probes for the study of the relationship between calmodulin and calmodulin-modulated proteins [19]. More recently, these drugs have been shown to interact with several other proteins that do not contain calmodulin-binding sites, such as the D_2 dopamine receptor [20] and *N*-methyl-D aspartate (NMDA) receptors [21], and thus these compounds may affect cellular function by interfering with numerous biological processes.

There are believed to be between 2 and 5 Ca^{2+}-dependent phenothiazine-binding sites on calmodulin [19]. In addition, due to the hydrophobic and cationic nature of these calmodulin antagonists, it has been suggested that they may also interact with various calmodulin-binding proteins at their calmodulin-binding site. Alternatively, these drugs might also interact with and affect proteins at sites unrelated to calmodulin binding [20–22]. Here, we assess this latter possibility by investigating the effects of trifluoperazine and other calmodulin antagonists on SERCA Ca^{2+} pumps, which are not regulated by calmodulin and do not have a calmodulin-binding site [13, 14, 23].

MATERIALS AND METHODS

Materials

All calmodulin antagonists used were purchased from Calbiochem, FITC was from Molecular Probes, and all other reagents were obtained from Sigma.

Membrane Preparations and ATPase Activity Measurements

Pig cerebellar microsomes (essentially consisting of ER) were prepared as described [24]. SR was prepared from rabbit fast-twitch skeletal muscle as previously described by Michelangeli and Munkonge [25]. Ca^{2+}-ATPase was purified from the rabbit skeletal muscle in a fully uncoupled form and of greater than 95% purity as described [25]. SR Ca^{2+}-ATPase activities were determined using the coupled enzyme assay method, where Ca^{2+}-dependent ATP hydrolysis is linked to NADH oxidation (as described in [25]) in a buffer containing 40 mM HEPES/KOH (pH 7.2), 1 mM EGTA, 5 mM $MgSO_4$, 2 mM ATP, 0.42 mM phosphoenol pyruvate, 0.15 mM NADH, 7.5 IU pyruvate kinase, and 18 IU lactate dehydrogenase. SR vesicles or the purified Ca^{2+}-ATPase (15 μg) were incubated for 10 min at 37° in 2.5 mL of this buffer. ATPase activity was initiated by the addition of 90 μL of 25 mM $CaCl_2$ to give a free Ca^{2+} concentration of ~6 μM $CaCl_2$.

Microsomal Ca^{2+}-ATPase activity was measured according to the method of LeBel *et al.* [26] in a buffer containing 45 mM HEPES/KOH (pH 7.2), 6 mM $MgCl_2$, 2 mM NaN_3, 250 mM sucrose, and 12.5 μg/mL of A23187 with $CaCl_2$ and EGTA added to give a free Ca^{2+} concentration of approximately 1 μM. Microsomal membranes (75 μg/mL) were added to 1 mL of this buffer and incubated at 37°. The reaction was then initiated by the addition of 5.8 mM ATP. After 30 min, the reaction was stopped by the addition of 0.5 mL of ice-cold 6.5% (w/v) trichloroacetic acid. The concentration of phosphate liberated was determined by the formation of a blue phosphomolybdate complex. The samples were spun to bring down the protein precipitate. Five hundred millilitres of the resulting supernatant was added to 1.5 mL of copper acetate buffer (pH 4). After mixing, 0.25 mL of 5% ammonium molybdate was added followed by 0.25 mL of 2% *p*-methyl-aminophenol sulphate containing 5% sodium sulphite. The colour intensity was measured at 870 nm after 10 min and related to known phosphate standards. The Ca^{2+}-ATPase activity was assessed by measuring the absorbance difference of the samples at 870 nm in the absence and presence of 1 μM free Ca^{2+}. Ca^{2+} uptake was measured at 37° by monitoring changes in fluoresence of Fluo-3 as described by Michelangeli [27]. Briefly, either 200 μg of cerebellar microsomes or 15 μg of SR vesicles was incubated with calmodulin antagonists in 2 mL of buffer containing 40 mM Tris-phosphate, 100 mM KCl (pH 7.2), 10 mM phosphocreatine, and 10 μg/mL of creatine kinase, after which 125 nM Fluo-3 was added to the microsomal suspension and Ca^{2+} uptake initiated by the addition of 1.5 mM MgATP. Total Ca^{2+} accumulation was measured by the addition of the calcium ionophore A23187 (12.5 μg/mL). The initial rate of Ca^{2+} uptake was calculated by measuring the calcium accumulated during the first 200 sec. The calcium concentration was related to fluorescence using the equation

$$[Ca^{2+}] = K_d(F\text{-}Fmin)/(Fmax\text{-}F)$$

where K_d is the dissociation constant for Ca^{2+} binding to Fluo-3 (900 nM at 37°, pH 7.2, in 100 mM KCl) [24, 27, 28], F is the fluorescence intensity of sample at a given time, and Fmin and Fmax are the fluorescence intensities of the sample in 1 mM EGTA and 2 mM $CaCl_2$, respectively.

Measurements of Steps in the ATPase Mechanism by Fluorescence

Measurements of the E1–E2 equilibrium were determined by monitoring the changes in fluoresence of Ca^{2+}-ATPase labelled with FITC as described by Michelangeli *et al.* [29] and Froud and Lee [30]. ATPase was labelled with FITC to give a molar ratio of FITC:ATPase of approximately 0.5:1. Ca^{2+}-ATPase (1 mg) was incubated in 100 μL of 1 M KCl, 50 mM potassium phosphate, 0.25 M sucrose (pH 8) to

which was added 2.5 nmol FITC from a stock solution of FITC in dry dimethylformamide (5 mM). The reaction was left to stand for 1 hr at room temperature. Two hundred and fifty microlitres of 0.2 M sucrose, 50 mM Tris–HCl (pH 7.0) was added and the mixture left on ice until use. Labelled ATPase (10 μg) was added to 2.5 mL of 50 mM Tris, 50 mM maleate, 5 mM $MgSO_4$, 100 mM KCl, 0.1 mM EGTA (pH 6 or pH 7) at 25° and the fluorescence intensity monitored with a Perkin Elmer LS 50B spectrofluorimeter (exciting at 495 nm and detecting the emission at 525 nm). The change in FITC fluorescence was measured upon addition of 0.4 mM $CaCl_2$.

The tryptophan fluorescence of the purified Ca^{2+}-ATPase was recorded by using an excitation wavelength of 275 nm and measuring the emission at 330 nm in the presence and absence of calmodulin antagonists. Measurements were made in a buffer containing 20 mM HEPES/Tris, 100 mM KCl, 5 mM $MgSO_4$, and 100 μM Ca^{2+} at pH 7.2. The calcium dependence of fluorescence was observed by addition of EGTA to the ATPase in the buffer to give the required free Ca^{2+} concentration using the EGTA–Ca^{2+} constants given by Gould *et al.* [31].

Ca^{2+} Binding and Phosphorylation Measurements

Binding of $^{45}Ca^{2+}$ to the ATPase was measured by using the dual labelling technique of Michelangeli *et al.* [32]. ATPase (0.1 mg) was incubated at 25° in 1 mL of buffer containing 20 mM HEPES/Tris (pH 7.2), 100 mM KCl, 5 mM $MgSO_4$, 100 μM EGTA, 500 μM [^{3}H]glucose (0.2 Ci/mol), and $^{45}CaCl_2$ (3 Ci/mol) to give a free Ca^{2+} concentration of 6 μM. Samples were then rapidly filtered through Millipore HAWP filters (0.45 μm). Filters were left to dry, after which 8 mL of scintillant was added. The filters were then counted for both ^{3}H and $^{45}Ca^{2+}$. The amount of [^{3}H] glucose trapped on each filter was used to calculate the wetting volume for the filter, and the amount of Ca^{2+} trapped in this volume was subtracted from the total Ca^{2+} bound to the filter to give that bound to the ATPase.

Steady-state levels of phosphorylation of the ATPase by [^{32}P]γ-ATP were carried out at 25° in 20 mM HEPES/Tris (pH 7.2) containing 100 mM KCl, 5 mM $MgSO_4$, 100 μM $CaCl_2$, and 0.075 mg/mL of ATPase. The reaction was initiated by addition of 100 μM [^{32}P]γ-ATP (specific activity 10 Ci/mol) and quenched with 10% trichloroacetic acid, 0.2 M H_3PO_4 after 10 sec. The samples were then filtered through Whatman GF/C filters, washed, and counted.

RESULTS

Figure 1, A and B shows the inhibitory action of a number of calmodulin antagonists on skeletal muscle SR Ca^{2+}-ATPase activity. Comparative data indicate that calmidazolium (IC_{50}, 0.5 ± 0.1 μM) and the calmodulin-binding peptide (which corresponds to amino acid sequence 290–309 of calmodulin kinase II) (IC_{50}, 7.0 ± 0.6 μM) were the most potent inhibitors of SR Ca^{2+}-ATPase activity. Trifluoperazine, chlorpromazine, and fluphenazine had similar potencies (IC_{50} values of 45 ± 7 μM, 23 ± 5 μM, and 15 ± 4 μM, respectively), while W-7 was the least potent of the drugs tested (IC_{50}, 125 ± 20 μM). Since the IC_{50} values for these drugs with the purified SR Ca^{2+}-ATPase, where no other protein was present, were similar (IC_{50} values for calmidazolium, trifluoperazine, chlorpromazine, and flu-

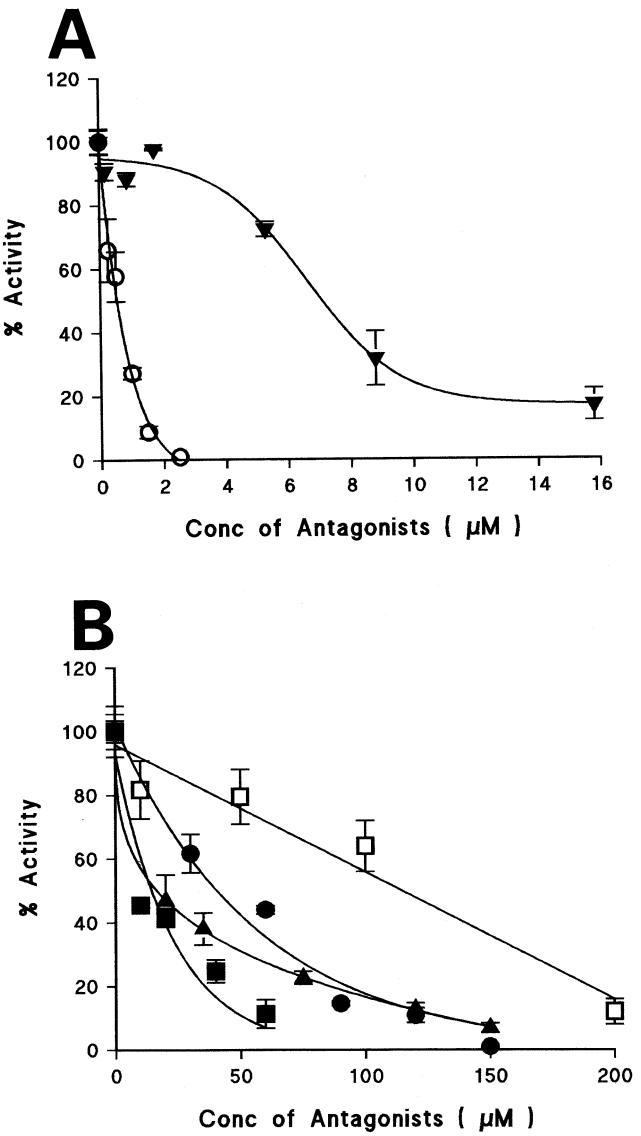

FIG. 1. The inhibition of the SR Ca^{2+}-ATPase by calmodulin antagonists. (A) shows the effects of different concentrations of calmidazolium (▼) and the calmodulin-binding domain peptide (○) on SR Ca^{2+}-ATPase activity at a constant free [Ca^{2+}] (approx. 6 μM). ATPase activity was measured at pH 7.2, and 37°. The data are presented as the means ± SD of between three and six determinations. (B) shows the effects of various concentrations of trifluoperazine (●), fluphenazine (■), chlorpromazine (▲), and W-7 (□) on SR Ca^{2+}-ATPase activity at constant free [Ca^{2+}] (6 μM). ATPase activity was measured at pH 7.2, and 37°. The data are presented as the means ± SD of between three and six determinations. One hundred percent activity corresponds to 11 IU/mg.

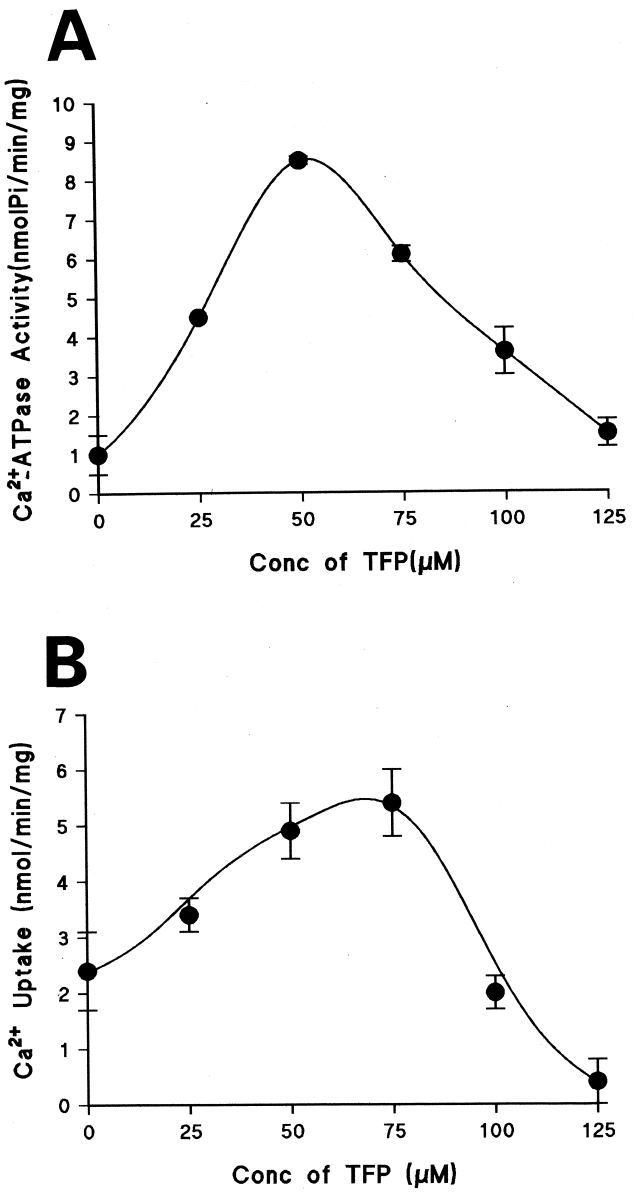

FIG. 2. The effects of trifluoperazine on cerebellar ER Ca^{2+}-ATPase activity and Ca^{2+} uptake. (A) shows the effects of trifluoperazine (TFP) on ER Ca^{2+}-ATPase activity from cerebellar microsomes. ER Ca^{2+}-ATPase activity was monitored at 37° (pH 7.2) in buffer containing 45 mM HEPES, 6 mM $MgCl_2$, 2 mM NaN_3, 250 mM sucrose, and 12.5 μg/mL of A23187, with $CaCl_2$ and EGTA added to give a free [Ca^{2+}] of approximately 1 μM. (B) shows the effects of trifluoperazine on uptake of Ca^{2+} by ER Ca^{2+}-ATPase. Three hundred micrograms of rat cerebellar microsomes was suspended in 2 mL of 40 mM Tris/phosphate buffer, 100 mM KCl, pH 7.2 at 37° in the presence of 10 mM phosphocreatine, 10 μg/mL of creatine kinase, and 1.25 μM Fluo-3. Ca^{2+} uptake was initiated by the addition of 1.5 mM MgATP. The data are presented as the means ± SD of between three and six determinants.

phenazine were 0.5 ± 0.1 μM, 55 ± 7 μM, 35 ± 7 μM, and 10 ± 3 μM, respectively), any effect observed with these antagonists is unlikely to be dependent upon their interaction with any other protein such as calmodulin.

Figure 2A shows the effect of trifluoperazine on Ca^{2+}-dependent ATP hydrolysis in cerebellar microsomes. At concentrations up to 50 μM, trifluoperazine increased the activity of the ER Ca^{2+}-ATPase approximately 9-fold. Concentrations above 50 μM decreased the activity of the Ca^{2+}-ATPase. Figure 2B shows the dose-dependent effect on ATP-dependent Ca^{2+} uptake in cerebellar microsomes. It was found that trifluoperazine had a similar effect on Ca^{2+} uptake. The slight difference in the concentration of trifluoperazine required to attain maximum activity by the two methods probably reflects the differences in experimental condition used, i.e. different ATP, Mg^{2+}, Ca^{2+}, K^+, etc.

TABLE 1. Effect of calmodulin antagonists on ATP-dependent Ca^{2+} uptake and percent activity of ER Ca^{2+}-ATPase

Calmodulin antagonists	ATP-dependent Ca^{2+} uptake (%)	Ca^{2+}-ATPase activity (% activity)
Calmidazolium		
5 μM	100 ± 3	88 ± 9
10 μM	103 ± 6	99 ± 2
20 μM	95 ± 7	91 ± 9
25 μM	95 ± 6	ND
40 μM	ND	85 ± 20
80 μM	ND	101 ± 5
Fluphenazine		
30 μM	103 ± 10	83 ± 16
60 μM	120 ± 17	93 ± 20
100 μM	97 ± 8	84 ± 12
Chlorpromazine		
10 μM	104 ± 12	93 ± 15
30 μM	115 ± 21	85 ± 20
60 μM	ND	108 ± 8
75 μM	94 ± 20	ND
100 μM	84 ± 12	65 ± 10
W-7		
10 μM	98 ± 7	83 ± 8
30 μM	96 ± 8	106 ± 9
50 μM	99 ± 6	ND
60 μM	ND	100 ± 10
80 μM	95 ± 8	94 ± 10

One hundred percent activity corresponds to typically 10–20 nmol/min/mg ATP hydrolysed, at 37° (pH 7.2) depending upon the membrane preparation used. Data represent means ± SD of five determinations. ND, not determined.

The microsomal fraction of cerebellum has been shown to be relatively abundant in ER Ca^{2+}-ATPase (isoform SERCA 2b) [4, 33, 34]. Table 1 shows the effects of the other calmodulin antagonists on ATP-dependent Ca^{2+} uptake and the activity of the ER Ca^{2+}-ATPase. It can be seen that in contrast to the SR Ca^{2+}-ATPase (which contains the SERCA 1 isoform), all calmodulin antagonists tested (over a range of concentrations) only minimally inhibited microsomal Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake. These findings suggest that these compounds inhibit Ca^{2+}-ATPases in an isoform-selective manner.

Figure 3 shows the typical bell-shaped dependence of ATPase activity on free Ca^{2+} concentration. This type of profile probably arises from the ATPase having two conformational states, E1 and E2 [15], with E1 having a high affinity for Ca^{2+} (<μM) and E2 a lower affinity (~mM)

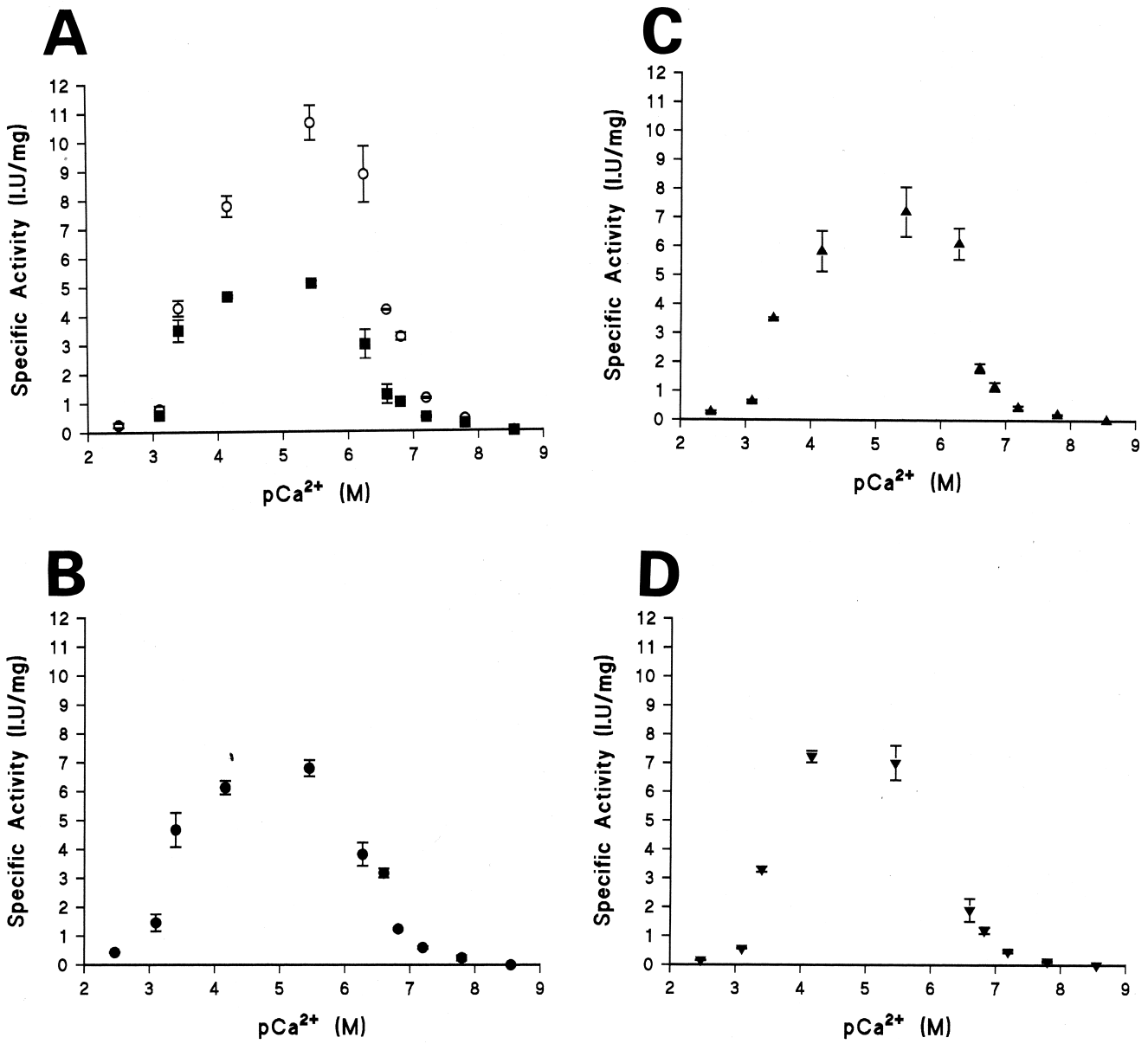

FIG. 3. Effects of calmodulin antagonists on SR Ca^{2+}-ATPase activity as a function of free [Ca^{2+}]. Calmodulin antagonists, at concentrations which caused between 30–50% inhibition, were preincubated for 10 min with SR in assay buffer (pH 7.2 and 37°). ATPase activity was measured in the presence of A23187 (10 μg/mL). The data are presented as the means ± SD of three determinations. (A) Control data (○) and fluphenazine, 10 μM (■); (B) trifluoperazine, 30 μM (●); (C) chlorpromazine, 20 μM (▲); (D) calmidazolium, 0.4 μM (▼).

[15]. We have previously suggested that the stimulatory phase of activity corresponds to Ca^{2+} binding to the E1 state, while the inhibitory phase corresponds, at least in part, to Ca^{2+} binding to the E2 state [33, 35]. It is clear that all the drugs reduced the V_{max} with respect to Ca^{2+}, since none of the drugs reached the maximal activity observed for ATPase alone. In addition, from the shape of these profiles, estimates of the K_m values for Ca^{2+} binding to the high-affinity (stimulatory) phase and the low-affinity (inhibitory) phase could be calculated and are given in Table 2. The estimated K_m values for the stimulatory phase for all of the antagonists except chlorpromazine were increased at least 2-fold, suggesting that these compounds may decrease the affinity of the Ca^{2+}-ATPase for Ca^{2+}. Table 2 also shows that the co-operativity of Ca^{2+} on the Ca^{2+}-ATPase was also affected by these antagonists. The estimated Hill coefficient in the absence of these antagonists was typically 1.6 similar to previously published values [33, 36]. However, the estimated Hill coefficient appeared to decrease in the presence of trifluoperazine, fluphenazine, and calmidazolium, becoming more non-co-operative. Table 2 also shows that the calmodulin antagonists affected the estimated K_m for the inhibitory phase, although to a lesser extent than the K_m for the stimulatory phase.

It has been shown that inhibitors such as nonylphenol, thapsigargin, and mastoparan affect the E1–E2 equilibrium

TABLE 2. Effect of different calmodulin antagonists on K_m for both the high-affinity stimulatory phase and the low-affinity inhibitory phase. The table also shows Hill coefficient values in the presence and absence of compounds

Calmodulin antagonists	Stimulatory phase K_m	Inhibitory phase K_m	Hill coefficient (n_{Hill})
Control	0.25 μM	0.32 mM	1.6
Calmidazolium (0.5 μM)	0.56 μM	0.40 mM	1.1
Fluphenazine (20 μM)	0.50 μM	0.50 mM	0.9
Chlorpromazine (25 μM)	0.32 μM	0.40 mM	1.4
Trifluoperazine (50 μM)	0.50 μM	0.32 mM	1.0

K_m values and Hill coefficient were calculated from data presented in Fig. 3.

of the ATPase [29, 36, 37]. This step can be conveniently monitored from changes in the fluorescence of FITC-labelled Ca^{2+}-ATPase [29, 30]. It has been shown that addition of Ca^{2+} results in a decrease in fluorescence intensity that can be attributed to the E2 → E1 conformational step [29, 36, 37]. Figure 4A shows that the fluorescence change observed at pH 6, upon the addition of $CaCl_2$ was 6% for the control. The fluorescence response to Ca^{2+} was smaller in the presence of the calmodulin antagonists, indicating that the E2 → E1 step had shifted towards E1 for all antagonists. Figure 4A shows that this step was more greatly affected by calmidazolium, although other antagonists also shifted this equilibrium, but required greater concentrations to do so. Figure 4B also shows that these drugs reduced the FITC-ATPase fluorescence upon addition of Ca^{2+} in a similar manner at pH 7.0. The In addition, in the presence of fluphenazine (10 μM), the fluorescence change became slower compared to control (Fig. 4C).

Calcium binding and dissociation can be studied through changes in the tryptophan fluorescence of the ATPase. On addition of Ca^{2+} to the purified ATPase, there is an increase in tryptophan fluorescence which has been attributed to the E1Ca–E1′Ca transition, with the E2, E1, and E1Ca forms having relatively low tryptophan fluorescence intensities and E1′Ca and E1′Ca_2 having higher fluorescence intensities [29, 38]. Addition of EGTA results in Ca^{2+} dissociation and a subsequent decrease in tryptophan fluorescence. Figure 5A shows that the tryptophan fluorescence of the purified ATPase increased by 8.5% upon addition of Ca^{2+}. Figure 5A also shows that calmidazolium and trifluoperazine reduced this increase in tryptophan fluorescence upon addition of Ca^{2+}, again suggesting that calmidazolium is more effective than trifluoperazine in affecting these steps. The other calmodulin antagonists tested appeared to dramatically quench the absolute tryptophan fluorescence, such that no meaningful data could be obtained with regard to the fluorescence change upon addition of Ca^{2+}. Figure 5B shows how the tryptophan fluorescence changed with free [Ca^{2+}]. In the absence of calmodulin antagonists, the apparent K_d for Ca^{2+} binding to the ATPase under these experimental conditions was ~2 μM. In the presence of 20 μM trifluoperazine or 5 μM calmidazolium, the change in fluorescence was reduced and the apparent K_d increased to 25 and 20 μM, respectively.

Table 3 shows the effect of trifluoperazine (35 μM) on the phosphorylation of the ATPase by [^{32}P]γ-ATP in the presence of Ca^{2+}. It was observed that trifluoperazine decreased ATP-dependent phosphorylation. In addition, 35 μM trifluoperazine reduced the amount of $^{45}Ca^{2+}$ bound to the ATPase nearly 2-fold at a free Ca^{2+} concentration of 6 μM.

DISCUSSION

Genetic and immunological analyses have shown that there are several distinct Ca^{2+}-ATPase isoforms in non-muscle tissues [4]. These isoforms have similar kinetic properties to each other and to the muscle isoforms of the Ca^{2+}-ATPase [33, 39]. However, they may have different sensitivities to inhibitors, and some of the mechanistic steps may have different rates [33, 35, 40]. The SR/ER Ca^{2+}-transport ATPase (SERCA) is a 100-kDa protein that differs structurally and immunologically from the Ca^{2+}-transport ATPase of the plasma membrane [13, 14]. The SERCA family of Ca^{2+} pumps, in contrast to the plasma membrane Ca^{2+} pump, does not bind calmodulin and calmodulin does not stimulate its activity [23, 41]. The present data show that the effects of compounds referred to as calmodulin antagonists are independent of calmodulin, as they inhibited both the ATPase activity of SR vesicles and purified Ca^{2+} ATPase from skeletal muscle in a similar manner. Our results thus confirm that calmodulin antagonists do not exclusively exert their inhibitory effect by acting on either calmodulin or calmodulin-dependent enzymes via a calmodulin-binding domain. From our results, it would appear that of the aromatic calmodulin antagonists, calmidazolium is by far the most potent inhibitor of the Ca^{2+}-ATPase (IC_{50}, 0.5 μM), thus making it the most potent inhibitor of the Ca^{2+}-ATPase thus far known, apart from thapsigargin. Its structure is also far bulkier and more complex (containing 4 aromatic rings) compared to trifluoperazine, fluphen-

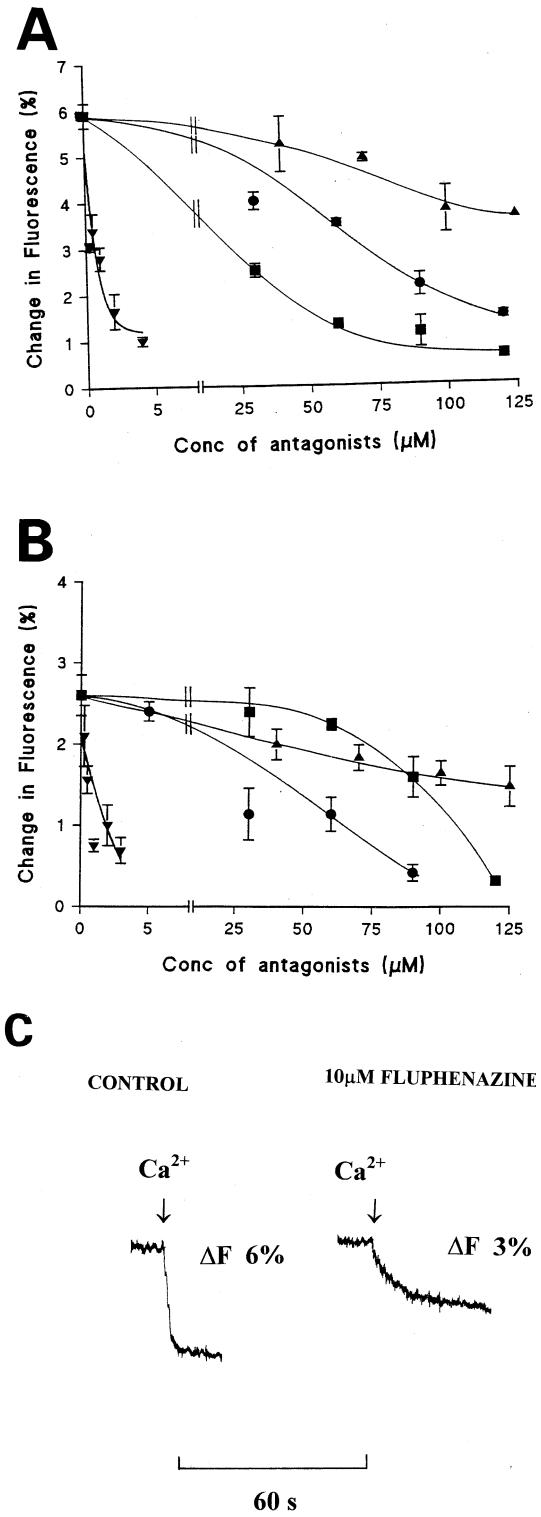

FIG. 4. The effects of calmodulin antagonists on the fluorescence intensity of FITC-labelled Ca^{2+}-ATPase upon the addition of Ca^{2+}. Measurements were made in a buffer containing 2.5 mL of 50 mM Tris, 50 mM maleate, 5 mM $MgSO_4$, 100 mM KCl, 0.1 mM EGTA at pH 6 (A) or pH 7 (B) and 25°. Fluorescence change was estimated upon the addition of 0.4 mM Ca^{2+}. Fluphenazine (■); trifluoperazine (●); chlorpromazine (▲); calmidazolium (▼). (C) shows the rate of decrease upon addition of 0.4 mM Ca^{2+} to the FITC-labelled Ca^{2+}-ATPase at pH 6, in the presence and absence of 10 μM fluphenazine. Each data point is the mean ± SD of three determinations.

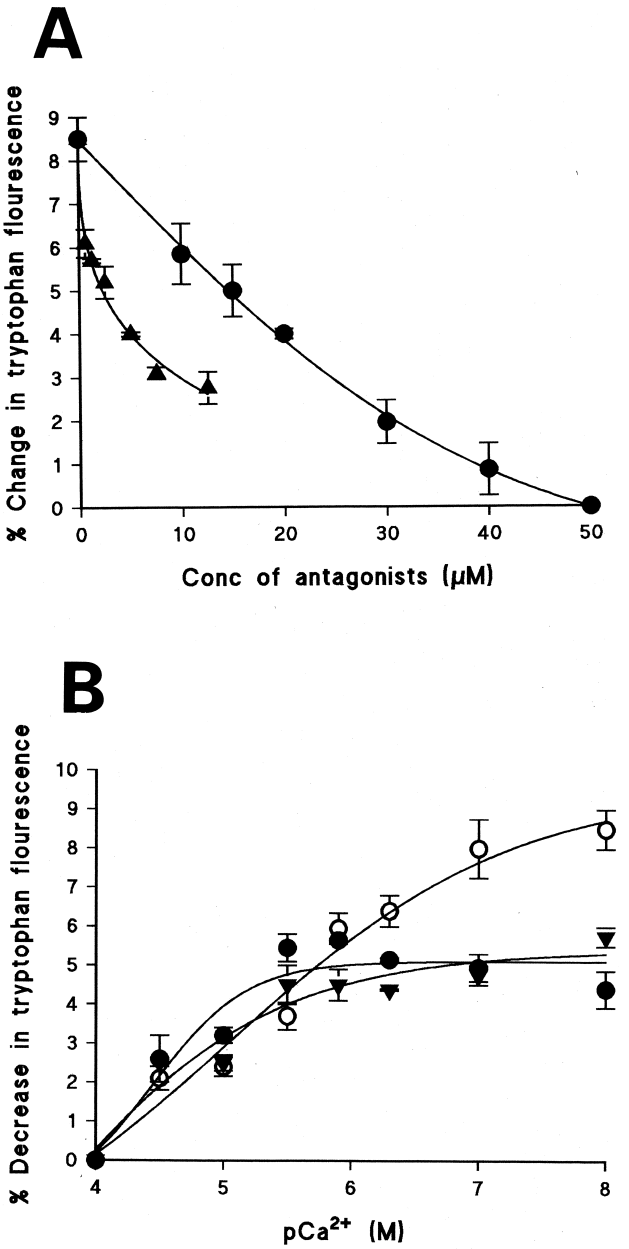

FIG. 5. The effects of trifluoperazine and calmidozolium on the change in tryptophan fluorescence induced by Ca^{2+}. (A) The effect of varying concentrations of trifluoperazine (●) and calmidazolium (▼) on the % change in tryptophan fluorescence intensity of the purified ATPase (0.5 μM). The ATPase was preincubated in the presence of trifluoperazine at pH 7.2 in 20 mM HEPES/Tris, 100 mM KCl, 5 mM $MgSO_4$. The change in fluorescence was observed by addition of EGTA and Ca^{2+} to the ATPase in the buffer to give the required free Ca^{2+} concentration. (B) Percent change in tryptophan fluorescence intensity of the purified ATPase (0.2 μM) as a function of free [Ca^{2+}], in the absence (○) and presence of 20 μM trifluoperazine (●) and 0.5 μM calmidazolium (▼). Measured at pH 7.2 in 20 mM HEPES/Tris, 100 mM KCl, 5 mM $MgSO_4$, and 100 μM Ca^{2+}. The calcium dependence of fluorescence was observed by addition of EGTA to the ATPase in the buffer to give the required free Ca^{2+} concentrations (i.e. 10 nM to 100 μM) using the binding constants given in [29]. Each data point represents the mean ± SD of 3 determinations.

TABLE 3. Effect of trifluoperazine on the phosphorylation of the SR Ca^{2+}-ATPase by [^{32}P]ATP and $^{45}Ca^{2+}$ binding at 6 μM free [Ca^{2+}].

	[^{32}P]ATP-dependent phosphorylation (nmol/mg)	$^{45}Ca^{2+}$ bound (nmol/mg)
Control	5.3 ± 0.2	24 ± 1
Trifluoperazine (35 μM)	3.2 ± 0.1	15 ± 1

The values are the means ± SD of 3 determinations.

azine, and chlorpromazine, which are very similar in structure to each other. W-7, on the other hand, has the least bulky structure, consisting as it does of two fixed aromatic rings, and is also the least potent. It would therefore appear that there is a correlation between the potency of inhibition of these antagonists and their hydrophobicity and size. This property has also been suggested to be important in making calmidazolium the most potent antagonist of calmodulin [42].

The calmodulin-binding peptide was also a very potent inhibitor of the SR ATPase (IC_{50}, 7 μM). This 20-mer peptide has a high proportion of basic amino acids (30%) concentrated within the first half of the peptide. It is therefore likely that this peptide acts by inhibiting the Ca^{2+}-ATPase in a similar fashion to other known Ca^{2+}-ATPase peptide inhibitors, i.e. mastoparan [36] and melittin [43], which are also highly basic in character.

Trifluoperazine and other calmodulin antagonists were found to inhibit SR Ca^{2+}-ATPase (SERCA 1) activity, while trifluoperazine showed a biphasic response with cerebellar ER Ca^{2+}-ATPase, causing a stimulation at low concentrations while inhibiting at higher concentrations. Since the ER Ca^{2+}-ATPase consists mainly of the SERCA 2b isoform [4], this finding suggests that these drugs affect the SERCA Ca^{2+} pumps in an isoform-specific manner. Such an isoform-specific affect of trifluoperazine has also recently been suggested by Engelender and DeMeis [40]. In this and other related studies, it was suggested that trifluoperazine affected ATPase phosphorylation by Pi, with the pump uncoupling to promote Ca^{2+} efflux [44–46]. In addition, the activation promoted by trifluoperazine in cerebellum and cardiac vesicles could be related to the ATP regulatory binding site of the enzyme, since at low ATP concentration this stimulation does not occur [40]. Thus, it is clear that other steps apart from the E1–E2 could also be affected by the calmodulin antagonists. We have previously shown that the Ca^{2+}-ATPase activity of our cerebellar microsomes comes exclusively from SERCA rather than plasma membrane Ca^{2+}-ATPase, since little or no affect on activity was observed by the addition of 3 μM vanadate [35], which would completely inhibit the plasma membrane Ca^{2+}-ATPase [47]. Thus, the effects of these calmodulin antagonists on cerebellar microsomes cannot be due to the presence of any contaminating plasma membrane Ca^{2+}-ATPase in our microsomal preparations.

In this study, we have undertaken further experiments to analyse the mechanism of inhibition of the SR Ca^{2+}-ATPase by these calmodulin antagonists. The mechanism of the Ca^{2+}-ATPase is usually described in terms of the E1–E2 model originally proposed by De Meis and Vianna [15]. This model postulates two major conformational states of the enzyme, E1 and E2. These two states differ in their affinity for Ca^{2+}, with the E1 state having high-affinity (cytoplasmic-facing) Ca^{2+}-binding sites and the phosphorylated form of E2 having low-affinity (luminal-facing) Ca^{2+}-binding sites. Following binding of two Ca^{2+} and ATP to E1, phosphorylation of the ATPase can take place to form the $E1PCa_2$ intermediate. After a conformational change to $E2PCa_2$, loss of the Ca^{2+} ions into lumen of the SR allows dephosphorylation of the ATPase and a return to the E1 state, to repeat the cycle. Results from the Ca^{2+} dependency of ATPase activity indicate that the calmodulin antagonists not only reduced the maximum activity of Ca^{2+}-ATPase, but also increased the K_m for Ca^{2+} binding to the high-affinity (stimulatory) site. This suggests that the affinity of Ca^{2+} binding to the E1 form of ATPase is reduced by ~2-fold in the presence of these compounds. Calcium-binding experiments showed that the level of $^{45}Ca^{2+}$ bound to the SR ATPase at a free Ca^{2+} concentration of 6 μM was reduced by a factor of nearly two in the presence of trifluoperazine, demonstrating that this antagonist affects Ca^{2+} binding.

Using FITC-labelled Ca^{2+}-ATPase, the calmodulin antagonists shifted the E2–E1 equilibrium of the enzyme towards E1, in contrast to thapsigargin, which stabilises the E2 form of the enzyme [37]. However, we have recently shown that the wasp venom peptide mastoparan, which is also a potent inhibitor of the SR Ca^{2+}-ATPase, acts by stabilising the E1 form of the pump [36].

The effects of calmodulin antagonists on the tryptophan fluorescence changes induced by Ca^{2+} were also reduced up to half, suggesting that these antagonists affect the normal sequence of conformational changes that occur on Ca^{2+} binding. The tryptophan fluorescence change as a function of Ca^{2+} also suggests that calmodulin antagonists decrease the affinity of ATPase for Ca^{2+}, consistent with the activity versus [Ca^{2+}] data and $^{45}Ca^{2+}$-binding data. In the absence of calmodulin antagonists, the activity of the ATPase as a function of Ca^{2+} was also co-operative, with a Hill coefficient of 1.6. This co-operativity was reduced or abolished in the presence of these compounds, also suggesting that the Ca^{2+}-binding events had been altered in such a way that Ca^{2+} binding no longer followed a two-step process (i.e. binding of the first Ca^{2+} induced a conformational change which allowed binding of the second Ca^{2+}). One interpretation of our data could be that in the presence of calmodulin antagonists Ca^{2+}-binding sites are altered in such a way that the two Ca^{2+} now bind independently of one another. Therefore, the binding of the second Ca^{2+} would not be dependent on a conformational change produced by binding of the first Ca^{2+}. This finding is similar to our previous study, which showed that mastopa-

ran shifts the E2 → E1 equilibrium towards E1 and this also decreased the affinity of ATPase for Ca^{2+} and abolished the co-operativity of Ca^{2+} binding [36].

In summary this study has shown that the SERCA family of Ca^{2+} pumps can be affected by calmodulin antagonists in a manner which does not require the presence of calmodulin or a calmodulin binding domain. It must therefore be stated that the mode of action of these drugs when used in patients, may not necessarily be due to their effects on calmodulin-modulated processes. Also the inhibition induced by these calmodulin antagonists is isoform specific; inhibiting the SERCA 1 more than the SERCA 2b. The mechanism by which these antagonists inhibit the SERCA 1 Ca^{2+} pump, appears, at least in part, to be by altering the Ca^{2+} affinity and the associated Ca^{2+} binding steps, in addition to possibly stabilizing the enzyme in an E1 conformational state.

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References

1. Berridge MJ, Inositol triphosphate and calcium signalling. *Nature* **361:** 315–325, 1993.
2. Clapham DE, Calcium signalling. *Cell* **80:** 259–268, 1995.
3. MacLennan DH, Brandl CJ, Korczak B and Green NM, Amino-acid sequence of a $Ca^{2+}Mg^{2+}$-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementry DNA sequence. *Nature* **316:** 696–700, 1985.
4. Wu KD, Lee WS, Wey J, Bungard D and Lytton J, Localization and quantification of endoplasmic reticulum Ca^{2+}-ATPase isoform transcripts. *Am J Physiol* **269:** C775–C784, 1995.
5. Lytton J, Zarain-Herzberg A, Periasamy M and MacLennan DH, Molecular cloning of the mammalian smooth muscle sarco(endo)plasmic reticulum Ca^{2+}-ATPase. *J Biol Chem* **264:** 7059–7065, 1989.
6. Brandl CJ, DeLeon S, Martin DR and MacLennan DH, Adult forms of the Ca^{2+}-ATPase of sarcoplasmic reticulum: Expression in developing skeletal muscle. *J Biol Chem* **262:** 3768–3774, 1987.
7. Miller KK, Verma A, Snyder SH and Ross CA, Localization of an endoplasmic reticulum calcium ATPase mRNA in rat brain by *in situ* hybridization. *Neuroscience* **43:** 1–9, 1991.
8. Wuytack F, Papp B, Verboomen H, Raeymaekers L, Dode L, Bobe R, Enouf J, Bokkala S, Authi KS and Casteels R, A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. *J Biol Chem* **269:** 1410–1416, 1994.
9. Andersen JP and Vilsen B, Primary ion pumps. *Curr Opin Cell Biol* **2:** 722–730, 1990.
10. Inesi G and Kirtley MR, Structural features of cation transport ATPases. *J Bioenerg Biomembr* **24:** 271–283, 1992.
11. Vilsen B, Structure–function relationship in the Ca^{2+}-ATPase of sarcoplasmic reticulum studied by use of the substrate analogue CrATP and site-directed mutagenesis: Comparison with the Na^{+},K^{+}-ATPase. *Acta Physiol Scand* **154:** 1–146, 1995.
12. Trotta EE and DeMeis L, Adenosine 5′-triphosphate–orthophosphate exchange catalyzed by the Ca^{2+}-transport ATPase of brain. Activation by a small transmembrane Ca^{2+} gradient. *J Biol Chem* **25:** 7821–7825, 1978.
13. Eggermont JA, Vrolix M, Raeymaekers L, Wuytack F and Casteels R, Ca^{2+}-transport ATPases of vascular smooth muscle. *Circ Res* **6:** 266–278, 1988.
14. Verbist J, Wuytack F, Raeymaekers L, Van Leuven F, Cassiman J and Casteels R, A monoclonal antibody to the calmodulin-binding ($Ca^{2+}Mg^{2+}$)-dependent ATPase from pig stomach smooth muscle inhibits plasmalemmal ($Ca^{2+}Mg^{2+}$)-dependent ATPase activity. *Biochem J* **240:** 633–640, 1986.
15. DeMeis L and Vianna A, Energy interconversion by the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum. *Annu Rev Biochem* **48:** 275–292, 1979.
16. Inesi G, Kurzmack M, Coan C and Lewis DE, Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles. *J Biol Chem* **255:** 3025–3031, 1980.
17. Cheung WY, Calmodulin plays a pivotal role in cellular regulation. *Science* **207:** 19–27, 1980.
18. Levin RM and Weiss B, Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phophodiesterase. *Mol Pharmacol* **13:** 690–697, 1977.
19. Marshak DR, Lukas TJ and Watterson DM, Drug–protein interactions: Binding of chlorpromazine to calmodulin, calmodulin fragments, and related calcium binding proteins. *Biochemistry* **24:** 144–150, 1985.
20. Wilson JM, Sanyal S and Van Tol HH, Dopamine D_2 and D_4 receptor ligands: Relation to antipsychotic action. *Eur J Pharmacol* **351:** 273–286, 1998.
21. Lidsky TI, Yablonsky-Alter E, Zuck LG and Banerjee SP, Antipsychotic drug effects on glutamatergic activity. *Brain Res* **764:** 46–52, 1997.
22. Gietzen K, Sadorf I and Bader H, A model for the regulation of the calmodulin-dependent enzymes erythrocyte Ca^{2+}-transport ATPase and brain phosphodiesterase by activators and inhibitors. *Biochem J* **207:** 541–548, 1982.
23. Wibo M, Morel N and Godfraind T, Differentiation of Ca^{2+}-pumps linked to plasma membrane and endoplasmic reticulum in the microsomal fraction from intestinal smooth muscle. *Biochim Biophys Acta* **649:** 651–660, 1981.
24. Mezna M and Michelangeli F, Alkali metal ion dependence of inositol 1,4,5-triphosphate-induced calcium release from rat cerebellar microsomes. *J Biol Chem* **270:** 28097–28102, 1995.
25. Michelangeli F and Munkonge F, Methods of reconstitution of the purified sarcoplasmic reticulum (Ca^{2+}-Ma^{2+})-ATPase using bile salt detergents to form membranes of defined lipid to protein ratios or sealed vesicles. *Anal Biochem* **194:** 231–236, 1991.
26. LeBel D, Poirier GG and Beaudoin AR, A convenient method for the ATPase assay. *Anal Biochem* **85:** 86–89, 1978.
27. Michelangeli F, Measuring calcium uptake and inositol 1,4,5-triphosphate-induced calcium release in cerebellar microsomes using fluo-3. *J Fluorescence* **1:** 203–206, 1991.
28. Sayers LG and Michelangeli F, The effects of tetrahexyl ammonium cations (THA^{+}) on inositol 1,4,5-triphosphate-induced calcium release from porcine cerebellar microsomes: THA^{+} can induce calcium release selectively from the $InsP_3$-sensitive calcium stores. *Biochim Biophys Acta* **1152:** 177–183, 1993.
29. 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.
30. Froud RJ and Lee AG, Conformational transitions in the Ca^{2+} + Mg^{2+}-activated ATPase and the binding of Ca^{2+} ions. *Biochem J* **237:** 197–206, 1986.
31. Gould GW, McWhirter J, East JM and Lee AG, A model for the uptake and release of Ca^{2+} by sarcoplasmic reticulum. *Biochem J* **245:** 739–749, 1987.

32. Michelangeli F, Orlowski S, Champeil P, Grimes EA, East JM and Lee AG, Effects of phospholipids on binding of calcium to (Ca^{2+}-Mg^{2+})-ATPase. *Biochemistry* **29:** 8307–8312, 1990.
33. Michelangeli F, Di Virgilio F, Villa A, Podini P, Meldolesi J and Pozzan T, Identification, kinetic properties and intercellular localization of the (Ca^{2+}-Mg^{2+})-ATPase from the intracellular stores of chicken cerebellum. *Biochem J* **275:** 555–561, 1991.
34. Michelangeli F, The effects of amino acid-reactive reagents on the functioning of the inositol 1,4,5 triphosphate-sensitive calcium channel from rat cerebellum. *Cell Signal* **5:** 33–39, 1993.
35. Brown GR, Benyon SL, Kirk CJ, Wictome M, East JM, Lee AG and Michelangeli F, Characterisation of a novel Ca^{2+}-pump inhibitor (bis-phenol) and its effects on intracellular Ca^{2+} mobilization. *Biochim Biophys Acta* **1195:** 252–258, 1994.
36. Longland CL, Mezna M and Michelangeli F, The mechanism of inhibition of the Ca^{2+}-ATPase by mastoparan. Mastoparan abolishes cooperative Ca^{2+} binding. *J Biol Chem* **274:** 14799–14805, 1999.
37. Wictome M, Michelangeli F, Lee AG and East JM, The inhibitors thapsigargin and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone favour the E2 form of the Ca^{2+}, Mg^{2+}-ATPase. *FEBS Lett* **304:** 109–113, 1992.
38. Fernandez-Belda F, Kurmack M and Inesi G, A comparative study of calcium transients by isotopic tracer, metallochromic indicator, and intrinsic fluorescence in sarcoplasmic reticulum ATPase. *J Biol Chem* **259:** 9687–9698, 1984.
39. Lytton J, Westlin M, Burk SE, Shull GE and MacLennan DH, Functional comparison between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J Biol Chem* **267:** 14483–14489, 1992.
40. Engelender S and De Meis L, Pharmacological differentiation between intracellular calcium pump isoforms. *Mol Pharmacol* **50:** 1243–1252, 1996.
41. Raeymaekers L, Wuytack F, Eggermont J, De Schutter G and Casteels R, Isolation of a plasma-membrane fraction from gastric smooth muscle. Comparison of the calcium uptake with that in endoplasmic reticulum. *Biochem J* **210:** 315–322, 1983.
42. Gietzen K, Wuthrich A and Bader H, R24571: A new powerful inhibitor of red blood cell Ca^{2+}-transport ATPase and of calmodulin-regulated functions. *Biochem Biophys Res Commun* **101:** 418–425, 1981.
43. Baker KJ, East JM and Lee AG, Mechanism of inhibition of Ca^{2+}-ATPase by myotoxin a. *Biochem J* **307:** 571–579, 1995.
44. De Meis L, Fast efflux of Ca^{2+} mediated by the sarcoplasmic reticulum Ca^{2+}-ATPase. *J Biol Chem* **266:** 5736–5742, 1991.
45. Wolosker H and De Meis L, Ligand-gated channel of the sarcoplasmic reticulum Ca^{2+} transport ATPase. *Biosci Rep* **15:** 365–376, 1995.
46. Rocha JB, Wolosker H, Souza DO and De Meis L, Alterations of Ca^{2+} fluxes in brain microsomes by K^+ and Na^+: Modulation by sulphated polysaccharides and trifluoperazine. *J Neurochem* **66:** 772–778, 1996.
47. Carafoli E and Zurini M, The plasma membrane Ca^{2+}-pumping ATPase of plasma membranes. Purification, reconstruction and properties. *Biochim Biophys Acta* **683:** 279–301, 1982.