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# Pharmacological Differentiation between Intracellular Calcium Pump Isoforms

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## SUMMARY

We studied the $Ca^{2+}$-ATPase isoforms of sarco/endoplasmic reticulum (SERCA) derived from cerebellum, cardiac muscle, and skeletal muscle. The $Mg^{2+}$ dependence varied among the three enzyme preparations. The $Ca^{2+}$ transport in skeletal muscle vesicles, but not in cerebellar or cardiac vesicles, was activated by free $Mg^{2+}$ concentrations varying from 0.1 to 0.3 mM. Concentrations of $Mg^{2+}$ of >1 mM inhibited $Ca^{2+}$ transport in all three vesicle preparations but with more pronounced effect in cerebellar and cardiac vesicles. At 10–80 μM, trifluoperazine activated $Ca^{2+}$ uptake in cerebellar and cardiac vesicles but not in skeletal muscle vesicles. The activation was due to an increase in the coupling ratio between $Ca^{2+}$ transport and ATP hydrolysis and was observed only in the presence of ATP concentrations of >100 μM. The $Ca^{2+}$ transport in all three vesicle preparations was inhibited by trifluoperazine concentrations of >100 μM. The inhibition promoted by trifluoperazine was prevented by the addition of dimethylsulfoxide (10% v/v) to the medium. The $Ca^{2+}$ efflux from loaded vesicles was increased by arsenate and even more by trifluoperazine. In skeletal muscle vesicles, the efflux promoted by arsenate was several-fold faster than that promoted in vesicles derived from cerebellum or cardiac muscle. In skeletal muscle, the enhancement of $Ca^{2+}$ efflux promoted by both arsenate and trifluoperazine was antagonized by thapsigargin, $Ca^{2+}$, $Mg^{2+}$, and $K^+$. These agents partly antagonized the enhancement of $Ca^{2+}$ efflux promoted by trifluoperazine in cardiac vesicles but had little or no effect in the cerebellar vesicles. Finally, Mg·$P_i$ and Mg·ATP, the two substrates that phosphorylate the $Ca^{2+}$-ATPase, antagonized the effect of trifluoperazine in all of the preparations tested. The concentration of ATP needed was in the same range as that of the second $K_m$ value for ATP (50–300 μM) of the SERCA isoforms. The results indicate that the effect of the drugs on the cytosolic $Ca^{2+}$ homeostasis may vary depending on the target tissue.

Intracellular $Ca^{2+}$ plays an important role as a signaling agent in different types of cells (1, 2). The $Ca^{2+}$-ATPase of SERCA is essential for maintenance of a low cytosolic $Ca^{2+}$ concentration and is encoded by three distinct genes. The *SERCA 1* gene is expressed exclusively in fast skeletal muscle (3, 4). The *SERCA 2* gene gives rise to the SERCA 2a and SERCA 2b isoforms by alternative splicing (5–7). The SERCA 2a isoform is expressed in cardiac and slow skeletal muscle, whereas SERCA 2b is ubiquitous and is the dominant isoform found in the cerebellum (7–9). SERCA 3 is expressed in nonmuscle tissues such as platelets and lymphoid tissues (4, 10, 11).

All of the *SERCA* encodes a cytoplasmic region that contains the catalytic site and a transmembrane domain that forms a channel-like structure that allows $Ca^{2+}$ translocation across the membrane (12–15). Vesicles derived from the sarco/endoplasmic reticulum can accumulate $Ca^{2+}$ using the energy derived from ATP hydrolysis (16–18). The catalytic cycle can be reversed, and the enzyme releases $Ca^{2+}$ from the vesicles in a process that is coupled with the synthesis of ATP (18–22). The coupling between $Ca^{2+}$ efflux and ATP synthesis observed during the reversal of the $Ca^{2+}$ pump is lost when the substrates for the ATPase, ADP, and $P_i$ are excluded from the assay medium (23). Under these conditions, $Ca^{2+}$ leaks through the $Ca^{2+}$ pump without synthesis of ATP. Several drugs can uncouple reversal of the pump. They both inhibit ATP synthesis and induce an increase in the $Ca^{2+}$ efflux rate from the vesicles. The uncoupling drugs can be divided into two major groups: nonhydrophobic and hydrophobic drugs. Both classes of drugs seem to interact with the $E_2$ form of the enzyme, but they differ in their potency. Arsenate and heparin are nonhydrophobic drugs that impair ATP synthesis and increase $Ca^{2+}$ efflux at a rate similar to that measured during reversal of the pump (24–28). A variety of hydrophobic drugs, such as phenothiazines (29–31), local anesthetics (32), fatty acids (33), and ethanol (34), in-

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**ABBREVIATIONS:** SERCA, sarco/endoplasmic reticulum $Ca^{2+}$-ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

hibit ATP synthesis and promote $Ca^{2+}$ release through the pump at a much faster rate than that observed with the use of arsenate or heparin. Under this condition, the ATPase functions as a passive $Ca^{2+}$ channel. The channel function has been described only for the skeletal muscle isoform, and no data are available for the isoforms present in other tissues.

Several types of tissues, such as large intestine, spleen, thymus, and platelets, simultaneously express more than one SERCA isoform (4); it is not known why these cells express more than one isoform of $Ca^{2+}$-ATPase. We tested the effects of drugs that are known to interact with the $E_2$ conformation of skeletal muscle $Ca^{2+}$-ATPase as a tool to search for functional differences among the SERCA isoforms. It was found that the different $Ca^{2+}$-ATPase isoforms vary in $Mg^{2+}$ dependence and in the regulation of the channel function. The same drug can either activate or inhibit $Ca^{2+}$ transport depending on the SERCA isoform and on the conditions used. Extrapolation of these observations to the intact animal suggests that the same drug may have opposite effects on the homeostasis of cytosolic $Ca^{2+}$ in different tissues.

## Experimental Procedures

**Vesicle preparations.** The vesicles were prepared from the best animal source reported in the literature. Cerebella of adult rats were dissected, and vesicles from the endoplasmic reticulum were prepared according to Supattapone *et al.* (35). Canine cardiac sarcoplasmic vesicles were prepared according to Harigaya and Schwartz (17). The light fraction of skeletal muscle sarcoplasmic reticulum was prepared from rabbit fast skeletal muscle according to Eletr and Inesi (36). The vesicles were stored in liquid nitrogen until use. Protein concentration was estimated according to Lowry *et al.* (37).

The $Ca^{2+}$ uptake was not the result of contamination by membranes other than sarco/endoplasmic reticulum. In agreement with previous reports, thapsigargin (2 $\mu$M), a highly specific inhibitor of the $Ca^{2+}$ pump, inhibited a large fraction (70–85%) of the $Ca^{2+}$ uptake in cerebellum and abolished the $Ca^{2+}$ uptake in cardiac and skeletal muscle vesicles (38, 39).

**$Ca^{2+}$ uptake.** $Ca^{2+}$ uptake was measured in media containing 50 mM MOPS-Tris, pH 7.0, 100 mM KCl, 20 mM $P_i$, 2 mM phosphonolpyruvate, 30 units/ml pyruvate kinase, 5 $\mu$M FCCP, 0.02–0.05 mg of protein/ml, and various concentrations of [$^{45}$Ca]$CaCl_2$, $MgCl_2$, ATP, and other substrates as specified in the figure legends. The $Ca^{2+}$ concentration used in the experiments was saturating and ranged from 40 to 200 $\mu$M. The same results were obtained using a fixed high-$Ca^{2+}$ concentration. The reaction was stopped by filtration through Millipore filters (0.45 $\mu$m) (40). The filters were flushed six times with 5 ml of 3 mM $La(NO_3)_3$, and the remaining radioactivity was counted in a scintillation counter. The free $Mg^{2+}$ concentration was calculated using the association constants and computer program described by Fabiato and Fabiato (41). The $H^+$ ionophore FCCP was used to prevent $Ca^{2+}$ accumulation by possible mitochondrial contamination.

**ATPase activity.** ATP hydrolysis was determined by measuring the release of $P_i$ from [$\gamma$-$^{32}$P]ATP as described previously (42). $Mg^{2+}$-dependent ATPase activity was measured in the presence of 5 mM EGTA. The $Ca^{2+}$-stimulated ATPase activity was determined by subtracting the $Mg^{2+}$-dependent ATPase activity from the ATPase activity measured in the presence of both $Mg^{2+}$ and $Ca^{2+}$.

The $Ca^{2+}$-stimulated ATPase activity was inhibited by thapsigargin (2 $\mu$M) in all of the vesicle preparations tested. Other transport ATPases, such as $Na^+/K^+$-ATPase, $F_1$-ATPase, plant $H^+$-ATPase, and the calmodulin-dependent plasma membrane $Ca^{2+}$-ATPase, are not inhibited by thapsigargin, even at high concentrations of the drug (22, 43).

**$Ca^{2+}$ efflux.** For $Ca^{2+}$ efflux experiments, the vesicles were preloaded with $^{45}$Ca in a medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM $MgCl_2$, 20 mM $P_i$, 0.02–0.3 mM $CaCl_2$, 3 mM ATP, 5 $\mu$M FCCP, and 0.05–0.1 mg of protein/ml. After a 30–60-min incubation at 35°, the vesicles were centrifuged at 40,000 × *g* for 40 min, the supernatant was discarded, and the walls of the tubes were blotted to minimize the volume of residual loading medium. The pellets were kept on ice and resuspended in ice-cold 0.2 M sucrose. The $Ca^{2+}$-loaded vesicles were diluted within 2 min into efflux media to a final concentration of 0.02–0.05 mg of protein/ml. The efflux was arrested as described above for $Ca^{2+}$ uptake.

**Phosphorylation of phospholamban.** Assay medium and experimental procedures for phospholamban phosphorylation were as described by Tada *et al.* (44).

**Materials.** Arsenate, cAMP-dependent protein kinase, cAMP, ATP, FCCP, and trifluoperazine were obtained from Sigma Chemical (St. Louis, MO). Thapsigargin (LC Service, Woburn, MA) was dissolved in dimethylsulfoxide. After dilution, the final concentration of dimethylsulfoxide in the assay media was 0.5%. [$^{45}$Ca]$CaCl_2$ was purchased from Dupont (Wilmington, DE). All other reagents were of analytical grade.

## Results

**Effect of free $Mg^{2+}$.** The skeletal muscle (45, 46), cardiac muscle (47), and cerebellar (48) $Ca^{2+}$-ATPases have two $K_m$ values for ATP. The first is a high affinity $K_m$ value that reflects the binding of ATP to the catalytic site of the enzyme. The second $K_m$ reflects the binding of ATP with lower affinity to a regulatory site that leads to an increase in the rate of conversion between $E_2$ and $E_1$ forms of the enzyme (49). We now show that the $K_m$ and $V_{max}$ values varied among the different isoforms depending on the $Mg^{2+}$ and trifluoperazine concentration in the medium (Fig. 1 and Table 1).

The true substrate of the skeletal muscle $Ca^{2+}$-ATPase is the MgATP complex (50). Free $Mg^{2+}$ at concentrations of ≤2 mM activates both the rate of $Ca^{2+}$ transport (Fig. 2C) and the rate of ATP hydrolysis (Fig. 3C; Ref. 51). The activation was abolished, and a small inhibition was observed when the free $Mg^{2+}$ concentration in the medium was raised from 0.3 to 10.0 mM. We now show that the dual effect of $Mg^{2+}$ was not observed in cerebellar or cardiac vesicles (Figs. 2, A and B, and 3, A and B). In these preparations, increasing concentrations of free $Mg^{2+}$ failed to activate the transport and strongly inhibited both $Ca^{2+}$ uptake (Fig. 2, A and B) and ATPase activity (Fig. 3, A and B). The inhibition by free $Mg^{2+}$ became more pronounced when ATP concentration in the medium was decreased from 2 to 0.01 mM.

We were not able to measure both $K_m$ and $V_{max}$ values for ATP in the presence of $MgCl_2$ concentrations of <0.2 mM because of the difficulty of maintaining the concentration of free $Mg^{2+}$ constant over a wide range of ATP concentrations without varying significantly the concentration of free ATP. Thus, we were unable to establish which kinetic parameters of the enzyme were changed when the ATPase of skeletal muscle was activated by raising the free $Mg^{2+}$ concentration from 0.1 to 0.3 mM (Fig. 2C). In all of the SERCA isoforms, raising the free $Mg^{2+}$ concentration from 0.3 to 5.0 mM promoted a decrease in the first $V_{max}$ and an increase in the second $K_m$ value for ATP (Table 1). The major difference between SERCA 1 (skeletal muscle) and SERCA 2 (cardiac and muscle and cerebellum) isoforms in high $Mg^{2+}$ was a 2-fold decrease in the second $V_{max}$ for cerebellar and cardiac muscle $Ca^{2+}$-ATPases, which was not observed in skeletal

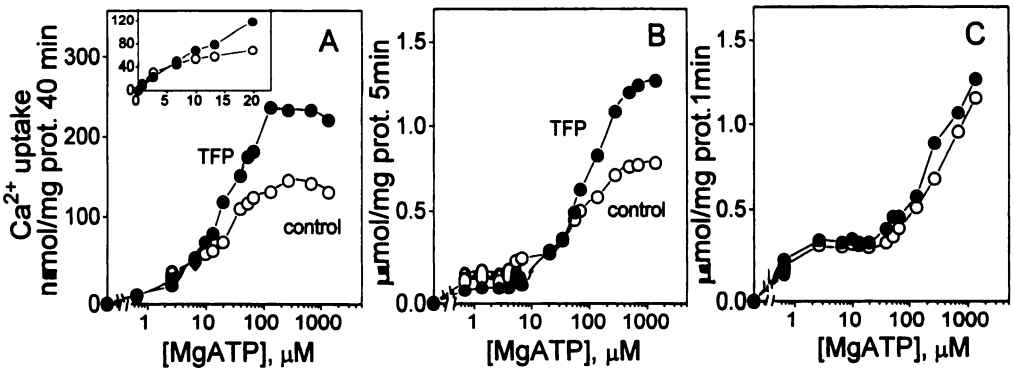


**Fig. 1.** ATP dependence of $Ca^{2+}$ uptake in the presence and absence of trifluoperazine (*TFP*). The $Ca^{2+}$ uptake of (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles was measured at 35° in media containing 50 mM MOPS-Tris, pH 7.0; 0.3 mM free $Mg^{2+}$; 20 mM $P_i$; (A) 40, (B) 60, or (C) 120 μM [$^{45}$Ca]$CaCl_2$; 100 mM KCl; 2 mM phosphenolpyruvate; 30 units/ml pyruvate kinase; 5 μM FCCP; and (A and C) 50 or (B) 20 μg of protein/ml in the presence of increasing concentrations of MgATP in the absence (○) or the presence of 40 μM trifluoperazine (●). *Inset*, data obtained with 0.7–19.8 μM MgATP on an expanded scale. Values are representative of three to six different experiments performed with three different vesicle preparations.

TABLE 1

**Effects of $Mg^{2+}$ and trifluoperazine on the kinetics of $Ca^{2+}$ transport in cerebellar, cardiac, and skeletal muscle vesicles**

Apparent $K_m$ and $V_{max}$ values for MgATP were obtained from the double-reciprocal (Lineweaver-Burk) plots. The conditions were the same as for Fig. 2 in the presence of 0.3 or 5.0 mM free $Mg^{2+}$, with increasing concentrations of ATP (0.66-1948 μM), and in the absence or the presence of 40 μM trifluoperazine. Values are mean ± standard error of three to six experiments with three different vesicle preparations. The $K_m$ and $V_{max}$ values are expressed in μM and nmol/mg/min, respectively.

| Addition | $Ca^{2+}$ uptake | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.3 mM free $Mg^{2+}$ | | | | 5.0 mM free $Mg^{2+}$ | | | |
| | $K_{m1}$ | $V_{max1}$ | $K_{m2}$ | $V_{max2}$ | $K_{m1}$ | $V_{max1}$ | $K_{m2}$ | $V_{max2}$ |
| Cerebellum | | | | | | | | |
| None | 4.9 ± 0.7 | 1.72 ± 0.23 | 47 ± 6 | 4.40 ± 0.23 | 3.3 ± 1.0 | 0.47 ± 0.6 | 146 ± 13 | 1.93 ± 0.18 |
| Trifluoperazine | N.D. | N.D. | 42 ± 6 | 7.08 ± 0.62 | 6.2 ± 1.7 | 0.57 ± 0.08 | 281 ± 45 | 4.18 ± 0.18 |
| Cardiac muscle | | | | | | | | |
| None | 4.0 ± 1.3 | 40 ± 8 | 192 ± 34 | 244 ± 16 | 20 ± 9 | 18 ± 7.6 | 616 ± 99 | 141 ± 36 |
| Trifluoperazine | 16 ± 5 | 44 ± 6 | 239 ± 36 | 343 ± 19 | 15 ± 10 | 17 ± 11.6 | 1159 ± 229 | 250 ± 23 |
| Fast-twitch muscle | | | | | | | | |
| None | 1.0 ± 0.13 | 280 ± 17 | 315 ± 20 | 926 ± 93 | 0.7 ± 0.005 | 126 ± 7 | 653 ± 14 | 812 ± 72 |
| Trifluoperazine | 1.05 ± 0.05 | 267 ± 17 | 290 ± 94 | 966 ± 40 | 1.85 ± 0.15 | 173 ± 15 | 483 ± 160 | 833 ± 8 |

N.D., not detected.

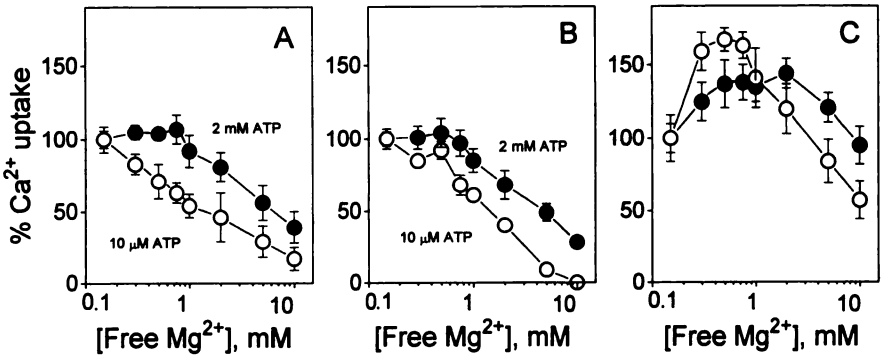


**Fig. 2.** $Mg^{2+}$ dependence of $Ca^{2+}$ uptake by (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles. The assay media and conditions were as described in the legend to Fig. 1 except that the media contained increasing concentrations of free $Mg^{2+}$, with 10 μM (○) or 2 mM ATP (●). The 100% $Ca^{2+}$ uptake was determined using 0.15 mM free $Mg^{2+}$. The values obtained at low and high ATP concentrations were (A) 52 ± 3.3 and 144 ± 13 nmol/mg of protein/40 min; (B) 130 ± 11 and 864± 60 nmol/mg of protein/10 and 5 min, respectively; and (C) 150 ± 24 and 681 ± 68 nmol/mg of protein/1 min. Values are mean ± standard error of three or four experiments with three different preparations. The absence of error bars indicates that the mean ± standard error is smaller than the symbol.

muscle $Ca^{2+}$-ATPase (Table 1). In Figs. 2 and 3, there is a decrease in the activity of all isoforms when the $Mg^{2+}$ concentration is raised from 0.3 to 5.0 mM.

**Effects of trifluoperazine.** Recently, it has been shown that the effect of trifluoperazine, a phenothiazine, varies depending on the vesicle preparation that was used. This drug inhibits $Ca^{2+}$ uptake in both platelet and skeletal muscle vesicles (28, 52, 53), whereas in brain vesicles, it stimulates $Ca^{2+}$ uptake (54). We show that both the cardiac and the cerebellar ATPases are activated by trifluoperazine (Fig. 4). Thus, unlike SERCA 1, both SERCA 2a (cardiac muscle) and SERCA 2b (cerebellum) are stimulated by low concentrations of trifluoperazine. The drug has a biphasic effect: it activates in the range of 10–80 μM and inhibits the $Ca^{2+}$

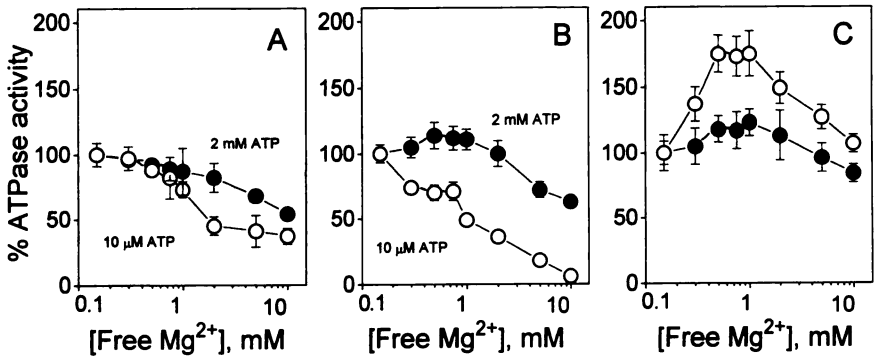


**Fig. 3.** $Mg^{2+}$ dependence of the $Ca^{2+}$-ATPase activity of (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles. ATPase activity was measured in a medium containing 50 mM MOPS-Tris, pH 7.0; 20 mM $P_i$; (A) 40, (B) 60, or (C) 120 μM $CaCl_2$; 100 mM KCl; 5 μM FCCP; increasing concentrations of free $Mg^{2+}$; and (A) 20, (B) 10, or (C) 5 μg of protein/ml with 10 μM (○) or 2 mM (●) [γ-$^{32}$P]ATP. The 100% $Ca^{2+}$-ATPase activity was determined using 0.15 mM free $Mg^{2+}$. Values obtained in low and high ATP concentration were (A) 18.2 ± 1.6 nmol/mg of protein/1 min and 1.11 ± 0.02 μmol/mg of protein/10 min; (B) 62.1 ± 5.4 nmol/mg of protein/30 sec and 3.71 ± 0.3 μmol/mg of protein/5 min; (C) 142 ± 9 nmol/mg of protein/30 sec and 7.26 ± 0.8 μmol/mg of protein/4 min. Values are mean ± standard error of three experiments with three different preparations. The absence of error bars indicates that the mean ± standard error is smaller than the symbol.

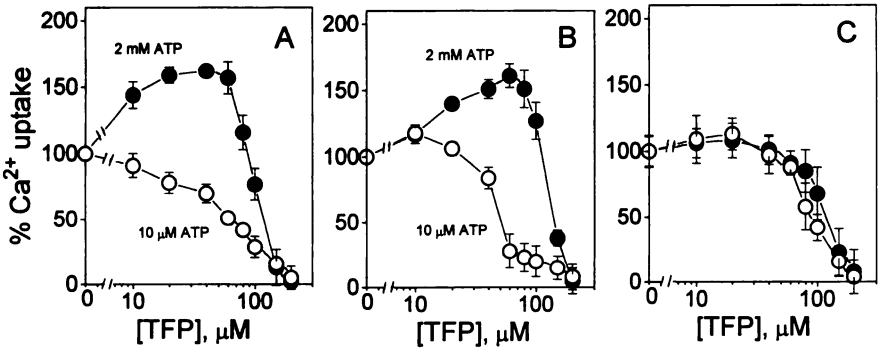


**Fig. 4.** Effects of trifluoperazine (*TFP*) on $Ca^{2+}$ uptake of (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles. Medium composition and conditions were as described in the legend to Fig. 1 except that the media contained increasing concentrations of trifluoperazine, 0.3 mM free $Mg^{2+}$, and either 10 μM (○) or 2 mM ATP (●). The 100% $Ca^{2+}$ uptake was determined in the absence of trifluoperazine. The values obtained in low and high ATP concentration were (A) 34 ± 2 and 110 ± 4 nmol/mg of protein/40 min; (B) 198 ± 10 and 926 ± 33 nmol/mg of protein/10 and 5 min, respectively; and (C) 259 ± 27 and 823 ± 91 nmol/mg of protein/1 min. Values are mean ± standard error of three experiments with three different preparations. The absence of error bars indicates that the mean ± standard error is smaller than the symbol.

transport when its concentration is raised above 100 μM (Fig. 4). The stimulation of $Ca^{2+}$ uptake by trifluoperazine in cerebellar and cardiac muscle vesicles was abolished by thapsigargin (2 μM) (data not shown). An interesting new finding was that the activation promoted by trifluoperazine in cerebellar and cardiac vesicles seems to be related to the regulatory ATP binding site of the enzyme. When the ATP concentration is decreased to a level sufficient to saturate only the catalytic site, trifluoperazine no longer activates the ATPase, and like in skeletal muscle vesicles, it inhibits only $Ca^{2+}$ uptake (Fig. 4, A and B). In skeletal muscle vesicles, the effect of trifluoperazine is the same for both high and low ATP concentrations (Fig. 4C). The effect of trifluoperazine does not depend on the $Mg^{2+}$ concentration in the medium. In the presence of either 0.3 or 5.0 mM free $Mg^{2+}$, trifluoperazine (40 μM) enhanced the second $V_{max}$ for $Ca^{2+}$ uptake in cerebellar and cardiac muscle vesicles but had no effect on skeletal muscle vesicles (Fig. 1 and Table 1). In addition, it abolished the biphasic MgATP dependence in cerebellar vesicles, so that the first $K_m$ was no longer detected, and only one $K_m$ for MgATP (low affinity) could be measured (Fig. 1A, *inset*).

The stimulatory effect of trifluoperazine was not related to alterations in the interaction of SERCA with regulatory proteins, such as phospholamban. This was tested by inactivating the phospholamban present in cardiac vesicles through phosphorylation using protein kinase A. Phosphorylation of phospholamban did not alter the trifluoperazine effect on $Ca^{2+}$ uptake (data not shown), nor did trifluoperazine alter the endogenous phosphorylation level of phospholamban (55). Ruthenium red (100 μM) had no effect on the stimulation of $Ca^{2+}$ uptake promoted by trifluoperazine (data not shown).

**Coupling between $Ca^{2+}$ transport and ATP hydrolysis.** In skeletal muscle vesicles, two $Ca^{2+}$ ions are transported for each ATP molecule hydrolyzed (56). This coupling ratio is observed only when a high oxalate concentration is used as a $Ca^{2+}$-precipitating agent (56) or during the initial two or three catalytic cycles of the enzyme in transient kinetic experiments (57). When $P_i$ is used as a $Ca^{2+}$-precipitating agent and at incubation intervals of >2 sec, the rate of $Ca^{2+}$ uptake by the vesicles represents a balance between the rates of $Ca^{2+}$ influx and $Ca^{2+}$ efflux, and the coupling ratio is <2 (58). Under these conditions, the coupling between $Ca^{2+}$ transported and ATP hydrolyzed in cerebellar vesicles was lower than that in vesicles from muscle. In three experiments, the coupling ratios between $Ca^{2+}$ uptake and ATP hydrolysis for vesicles from cerebellum, cardiac muscle, and skeletal muscle were 0.03, 0.40, and 0.37, respectively. Essentially, the same values were found with the use of 2 or

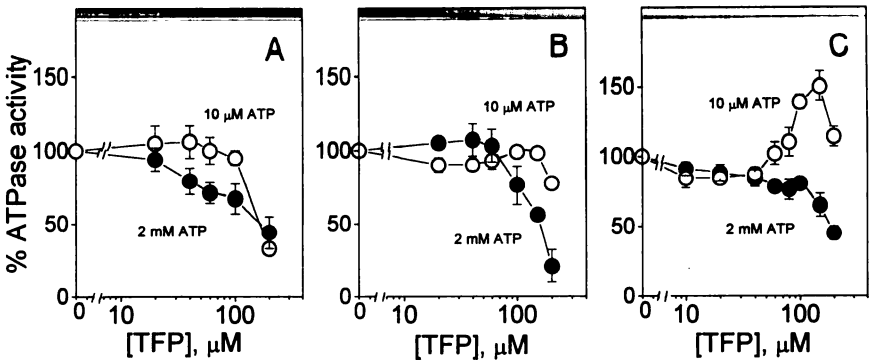


**Fig. 5.** Effects of trifluoperazine (*TFP*) on the $Ca^{2+}$-ATPase activity of (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles. ATPase activity was measured in a medium containing 50 mM MOPS-Tris, pH 7.0; 20 mM $P_i$; (A) 40, (B) 60, or (C) 120 μM $CaCl_2$; 0.3 mM free $Mg^{2+}$; 100 mM KCl; 5 μM FCCP; increasing concentrations of trifluoperazine; and (A) 50, (B) 10, or (C) 5 μg of protein/ml with 10 μM (○) or 2 mM [$\gamma$-$^{32}$P]ATP (●). The 100% $Ca^{2+}$-ATPase activity was determined in the absence of trifluoperazine. Values obtained in low and high ATP concentration were (A) 39 ± 1 nmol/mg of protein/2 min and 1.10 ± 0.09 μmol/mg of protein/10 min; (B) 105 ± 5 nmol/mg of protein/45 sec and 2.82 ± 0.03 μmol/mg of protein/6 min; and (C) 318 ± 22 nmol/mg of protein/30 sec and 9.88 ± 0.53 μmol/mg of protein/4 min. Values are mean ± standard error of three or four experiments with three different preparations. The absence of error bars indicates that the mean ± standard error is smaller than the symbol.

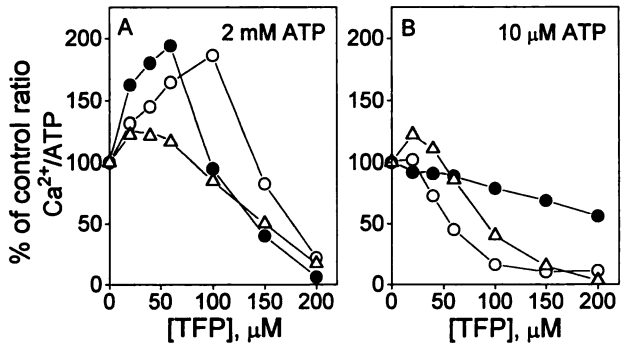


**Fig. 6.** Effects of trifluoperazine (*TFP*) on coupling ratio ($Ca^{2+}$/ATP) between $Ca^{2+}$ uptake and ATPase activity. $Ca^{2+}$ uptake and ATPase activity were measured as described in legends to Figs. 4 and 5 in the presence of increasing concentrations of trifluoperazine and either (A) 2 mM or (B) 10 μM ATP, using (●) cerebellar, (○) cardiac, or (△) skeletal muscle vesicles. The 100% coupling ratio of $Ca^{2+}$ to ATP was determined in the absence of trifluoperazine and corresponded to (A) 0.03, 0.40, and 0.37 or (B) 0.06, 0.18, and 0.67 for cerebellar, cardiac, and skeletal muscle vesicles, respectively. Values are mean ± standard error of three to five experiments with three different preparations.

0.01 mM ATP. These coupling ratios were calculated using the experimental values shown in Figs. 4 and 5.

The effect of trifluoperazine on $Ca^{2+}$-ATPase activity varied greatly depending on the isoform and ATP concentrations used (Fig. 5). In cerebellar and cardiac vesicles, the drug had no effect on the $Ca^{2+}$-ATPase when measured with 2 mM ATP and in the same concentration range of trifluoperazine that stimulated $Ca^{2+}$ uptake (Fig. 5, A and B). In skeletal muscle vesicles, trifluoperazine has the same effect on the ATPase activity (Fig. 5C) in the presence of 2 mM ATP as it has on $Ca^{2+}$ uptake (Fig. 4C). When ATP concentration was decreased to 10 μM, inhibition of the ATPase activity of cerebellar and cardiac vesicles was observed only when the trifluoperazine concentration was >100 μM (Fig. 5, A and B). In contrast, with 10 μM ATP, the ATPase activity of skeletal muscle vesicles was stimulated by trifluoperazine (Fig. 5C). As a result of the different patterns of activation and inhibition, the coupling ratio varied depending on both trifluoperazine and ATP concentrations used. The activation of $Ca^{2+}$ uptake by trifluoperazine observed with 2 mM ATP in SERCA 2 isoforms was associated with an increase in the coupling ratio between $Ca^{2+}$ transport and ATP hydrolysis (Fig. 6A). In all three vesicle preparations, the inhibition of $Ca^{2+}$ uptake promoted by high trifluoperazine concentrations (>100 μM) was associated with a decrease in the coupling ratio (Fig. 6).

**Effect of dimethylsulfoxide.** In skeletal muscle, inhibition of the $Ca^{2+}$-ATPase by trifluoperazine was antagonized by the addition of 10–20% (v/v) dimethylsulfoxide to the medium (52). The mechanism of this antagonism was discussed in detail previously (59, 60). We show that the effects of trifluoperazine in cardiac and cerebellar vesicles are also

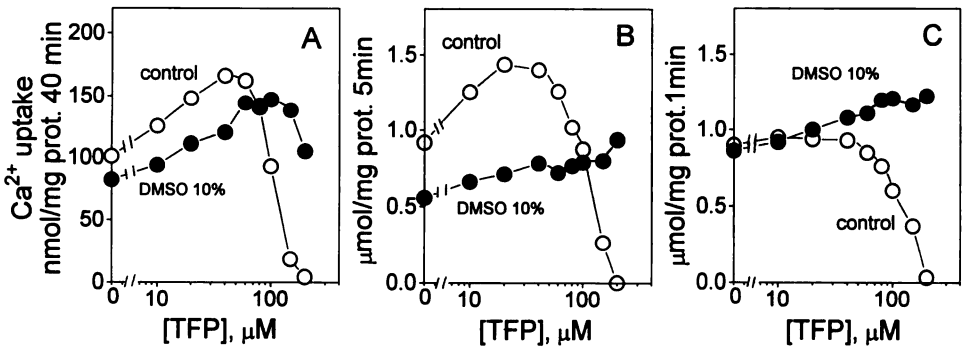


**Fig. 7.** Effect of trifluoperazine (*TFP*) on $Ca^{2+}$ uptake in (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles in the presence and absence of dimethylsulfoxide. The medium composition and conditions were as described in the legend of Fig. 1, except that the media contained increasing concentrations of trifluoperazine, 2 mM free $Mg^{2+}$, and 2 mM ATP in the absence (○) or the presence (●) of 10% (v/v) dimethylsulfoxide. Values are representative of three different experiments performed with three different vesicle preparations.

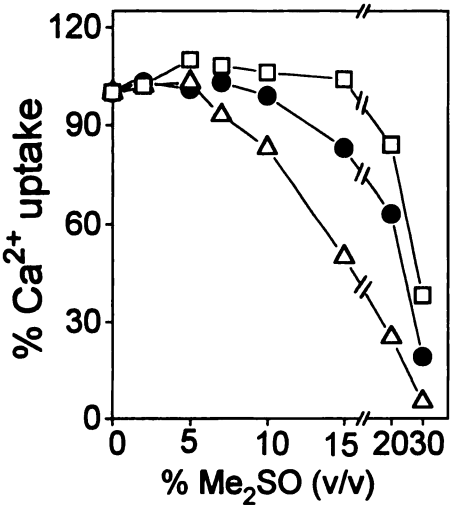


**Fig. 8.** Effect of dimethylsulfoxide ($Me_2SO$) on $Ca^{2+}$ uptake of SERCA isoforms. $Ca^{2+}$ uptake was measured in a medium containing 50 mM MOPS-Tris, pH 7.0; 0.3 mM free $Mg^{2+}$; 20 mM $P_i$; (●) 40, (△) 60, or (□) 120 μM [$^{45}$Ca]$CaCl_2$; 100 mM KCl; 2 mM ATP; 2 mM phosphenolpyruvate; 30 units/ml pyruvate kinase; 5 μM FCCP; and 50 (●, □) or 20 (△) μg of protein/ml in the presence of increasing concentrations of dimethylsulfoxide. The 100% $Ca^{2+}$ uptake was determined in the absence of dimethylsulfoxide and corresponded to 141 ± 15 nmol/mg of protein/40 min for cerebellar vesicles (●), 916 ± 74 nmol/mg of protein/5 min for cardiac vesicles (△), and 804 ± 91 nmol/mg of protein/1 min (□) for skeletal muscle vesicles. Values are representative of three different experiments performed with three different vesicle preparations.

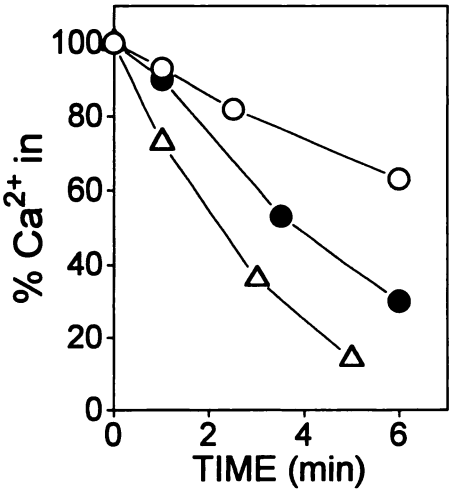


**Fig. 9.** Time course of $Ca^{2+}$ efflux from (○) cerebellar, (●) cardiac, and (△) skeletal muscle vesicles. Microsomal vesicles were loaded with [$^{45}$Ca]$CaCl_2$ in the presence of 20 mM $P_i$ as described in Experimental Procedures. The loaded vesicles were pelleted and resuspended to a final concentration of 50 (○ and △) or 20 (●) μg of protein/ml in medium containing 50 mM MOPS-Tris, pH 7.0, and 5 mM EGTA at 35°. The figure shows the $Ca^{2+}$ remaining in the vesicles after different incubation intervals. The $Ca^{2+}$ load varied from 58 to 111 nmol of $Ca^{2+}$/mg of protein for cerebellar vesicles, 0.8 to 1.6 μmol of $Ca^{2+}$/mg of protein for cardiac muscle vesicles, and 1.7 to 2.4 μmol of $Ca^{2+}$/mg of protein for skeletal muscle vesicles. Values are representative of three different experiments performed with three different vesicle preparations.

antagonized by dimethylsulfoxide (Fig. 7). Notice that in the absence of the drug, the organic solvent promoted a decrease of $Ca^{2+}$ transport in both cardiac and cerebellar vesicles (Fig. 7). In skeletal muscle, an inhibition of transport was observed only when the concentration of dimethylsulfoxide was raised above 15% (Fig. 8). Fig. 8 shows that the cerebellar and cardiac muscle vesicles are inhibited by lower concentrations of organic solvent than the skeletal muscle vesicles.

**$Ca^{2+}$ efflux.** In a previous report, we showed that $Ca^{2+}$ leaks through the skeletal muscle $Ca^{2+}$-ATPase when vesicles previously loaded with $Ca^{2+}$ are incubated in media containing none of the ligands of the ATPase (23, 29–31, 61). This efflux is not coupled to the synthesis of ATP and is referred to as passive $Ca^{2+}$ efflux. The rate of passive $Ca^{2+}$ efflux is decreased when natural ligands of the pump such as $Ca^{2+}$, $Mg^{2+}$, and $K^+$ are added to the medium (23). Thapsigargin, a highly specific inhibitor of SERCA, also decreases the passive $Ca^{2+}$ efflux of skeletal muscle vesicles, indicating that this efflux occurs through the $Ca^{2+}$-ATPase (30). The rate of passive $Ca^{2+}$ efflux was found to vary in the different preparations, being slower in cerebellar vesicles than in cardiac or skeletal muscle vesicles (Fig. 9 and Table 2). In these experiments, the vesicles were loaded with $Ca^{2+}$ using $P_i$ as a $Ca^{2+}$-precipitating agent, so that the concentration of free $Ca^{2+}$ inside different vesicle preparations was determined by the solubility product of calcium phosphate regardless of the total amount of calcium within the vesicles (58). Thus, the differences in $Ca^{2+}$ efflux rate are probably related to differences in the number of $Ca^{2+}$-ATPase units present in each vesicle preparation and not to different $Ca^{2+}$ concentrations in the vesicle lumen.

Thapsigargin and the cations $Ca^{2+}$, $Mg^{2+}$, and $K^+$ were able to inhibit the passive efflux of $Ca^{2+}$ from vesicles derived from muscle tissues (Fig. 10B and Table 2), but these agents were less effective in cardiac muscle than in skeletal muscle vesicles (Fig. 10C and Table 2). For cerebellar vesicles, however, thapsigargin and physiological cations had a negligible effect on the rate of passive $Ca^{2+}$ efflux (Fig. 10A and Table 2).

In earlier reports (24–26), it has been shown that arsenate increases the rate of $Ca^{2+}$ efflux from skeletal muscle vesicles. Engelender *et al.* (28) observed that arsenate had a negligible effect on vesicles derived from blood platelets that

TABLE 2

**Effect of cations and thapsigargin on the $Ca^{2+}$ efflux rate from cerebellar, cardiac, and skeletal muscle vesicles**

Vesicles were loaded with [$^{45}$Ca] $CaCl_2$ using 3 mM ATP and diluted in a medium containing 50 mM MOPS-Tris, pH 7.0, and 5 mM EGTA with no addition or with 200 μM free $Ca^{2+}$, 10 mM $MgCl_2$, 100 mM KCl, or 2 μM thapsigargin, in either the absence or the presence of 50 μM trifluoperazine at 35°c. Values are mean ± standard error of three experiments with three different preparations. Total $Ca^{2+}$ inside the vesicles after loading varied from 60 to 110 nmol/mg of protein for cerebellar vesicles, 0.95 to 1.55 μmol/mg of protein for cardiac vesicles, and 1.93 to 2.40 μmol/mg of protein for skeletal muscle vesicles.

| Addition | Calcium efflux rate | | |
|---|---|---|---|
| | Cerebellum | Cardiac muscle | Fast-twitch muscle |
| | *nmol/mg/min* | | |
| Without trifluoperazine | 5.66 ± 0.25 | 269 ± 16 | 504 ± 38 |
| $CaCl_2$ (200 μM) | 5.23 ± 0.47 | 182 ± 17 | 234 ± 17 |
| $MgCl_2$ (10 mM) | 3.92 ± 0.24 | 168 ± 9 | 246 ± 26 |
| KCl (100 mM) | 5.83 ± 0.52 | 178 ± 13 | 347 ± 35 |
| Thapsigargin (2 μM) | 5.29 ± 0.16 | 202 ± 21 | 183 ± 11 |
| With trifluoperazine (50 μM) | 15.0 ± 1.34 | 455 ± 33 | 1853 ± 74 |
| $CaCl_2$ (200 μM) | 12.21 ± 0.95 | 235 ± 17 | 160 ± 10 |
| $MgCl_2$ (10 mM) | 8.29 ± 0.31 | 316 ± 29 | 189 ± 14 |
| KCl (100 mM) | 11.59 ± 1.24 | 324 ± 31 | 344 ± 27 |
| Thapsigargin (2 μM) | 14.05 ± 1.57 | 374 ± 23 | 107 ± 6 |

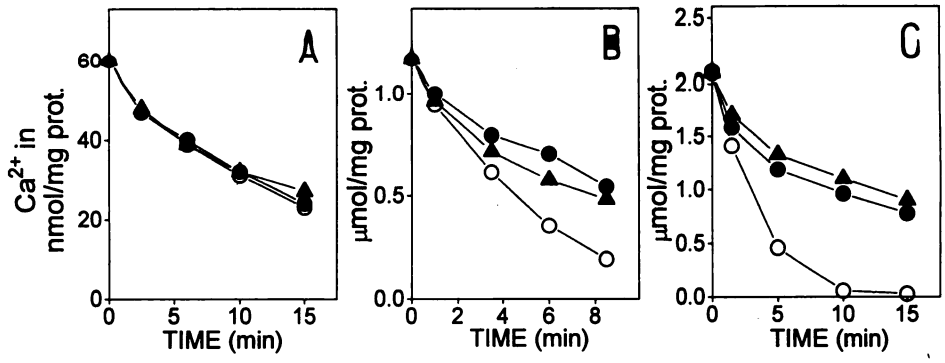


**Fig. 10.** Effect of $Ca^{2+}$ and thapsigargin on $Ca^{2+}$ efflux from (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles. Microsomal vesicles were loaded with [$^{45}$Ca]$CaCl_2$ in the presence of 20 mM $P_i$ and diluted to a final concentration of 50 (A and C) or 20 (B) µg of protein/ml in medium containing 50 mM MOPS-Tris, pH 7.0, and 5 mM EGTA, with no other additions (○), or containing concentrations of (●) 200 µM free $Ca^{2+}$ or (▲) 2 µM thapsigargin. The figure shows the $Ca^{2+}$ remaining in the vesicles after different incubation intervals. Values are representative of three different experiments performed with three different vesicle preparations.

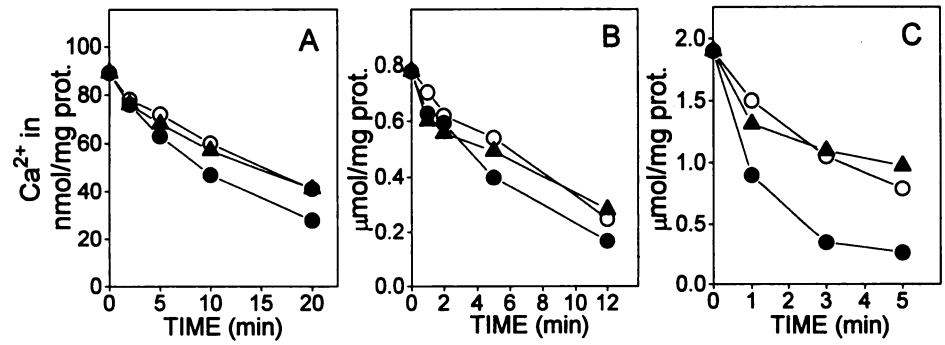


**Fig. 11.** Arsenate-induced $Ca^{2+}$ efflux and blockage by thapsigargin. The (A) cerebellar, (B) cardiac, or (C) skeletal muscle vesicles were loaded with [$^{45}$Ca]$CaCl_2$ in the presence of 20 mM $P_i$ and diluted to a final concentration of 50 (A and C) or 20 (B) µg of protein/ml in medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM $MgCl_2$, 5 mM EGTA, and either (○) no other additions, (●) 10 mM arsenate, or (▲) 2 µM thapsigargin plus 10 mM arsenate. The figure shows the $Ca^{2+}$ remaining in the vesicles after different incubation intervals. Values are representative of three or four different experiments performed with three different vesicle preparations.

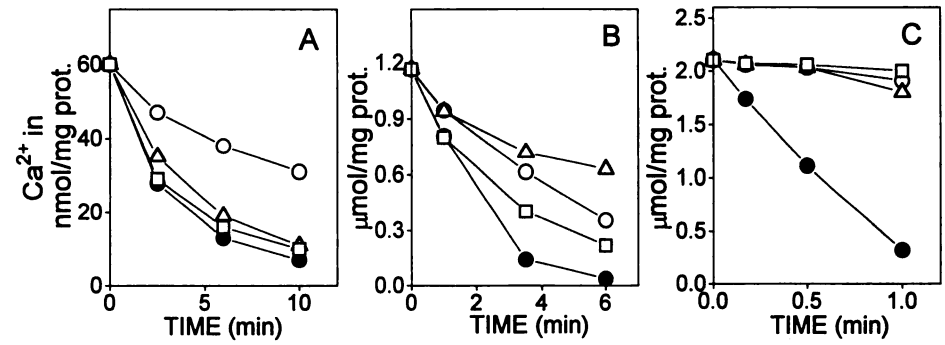


**Fig. 12.** Effect of $Ca^{2+}$ and thapsigargin on trifluoperazine-induced $Ca^{2+}$ efflux. The (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles were loaded with [$^{45}$Ca]$CaCl_2$ in the presence of 20 mM $P_i$ and diluted to a final concentration of 50 (A and C) or 20 (B) µg of protein/ml in medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM EGTA, and (○) either no other additions, (●) 50 µM trifluoperazine, (△) 50 µM trifluoperazine plus 200 µM free $Ca^{2+}$ concentration, or (□) 50 µM trifluoperazine plus 2 µM thapsigargin. The figure shows the $Ca^{2+}$ remaining in the vesicles after different incubation intervals. Values are representative of three different experiments performed with three different vesicle preparations.

were enriched with SERCA 2b and SERCA 3 isoforms. We show that the effect of arsenate on skeletal muscle was completely antagonized by thapsigargin and that arsenate promoted only a slight increase in $Ca^{2+}$ efflux in cerebellar and cardiac muscle vesicles (Fig. 11). Unlike arsenate, trifluoperazine was found to increase $Ca^{2+}$ efflux from all three vesicle preparations (Fig. 12). Similar to the efflux in the presence of EGTA alone (Fig. 10), the enhancement of the $Ca^{2+}$ efflux promoted in skeletal muscle by trifluoperazine was antagonized by thapsigargin, $Ca^{2+}$, $Mg^{2+}$, and $K^+$ (Fig. 12C and Table 2). Thapsigargin and cations were less effective in antagonizing the effect of trifluoperazine in cardiac muscle vesicles (Fig. 12B and Table 2) and least effective in cerebellar vesicles (Figs. 12A and Table 2).

The effect of trifluoperazine in cardiac and cerebellar vesicles was antagonized by Mg·$P_i$ (Fig. 13) and by Mg·ATP (Fig. 14), but similar to Fig. 12, the antagonism was more pronounced in skeletal muscle vesicles than in cardiac or cerebellar vesicles. Notice in Fig. 14 that the concentrations of Mg·ATP that antagonized the efflux of $Ca^{2+}$ promoted by

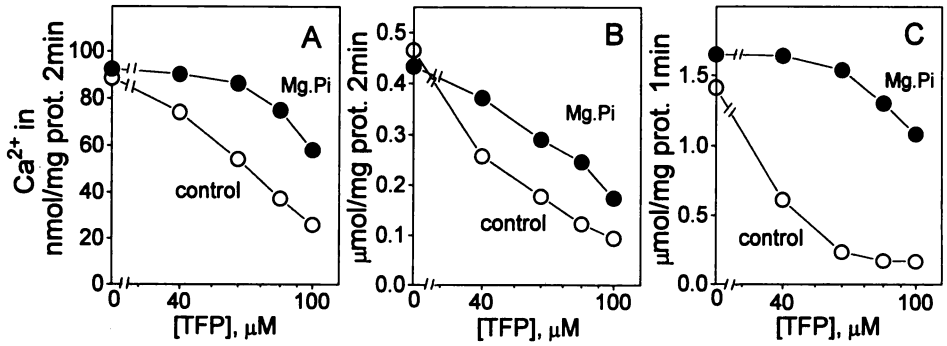


**Fig. 13.** Effect of Mg·$P_i$ on trifluoperazine (*TFP*)-induced $Ca^{2+}$ efflux. The (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles were loaded with [$^{45}$Ca]$CaCl_2$ in the presence of 20 mM $P_i$ and diluted to a final concentration of 50 (A and C) or 20 (B) µg of protein/ml in medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM EGTA, and increasing concentrations of trifluoperazine in either the absence (○) or the presence (●) of 4 mM $MgCl_2$ plus 4 mM $P_i$. Values are representative of three different experiments performed with three different vesicle preparations.

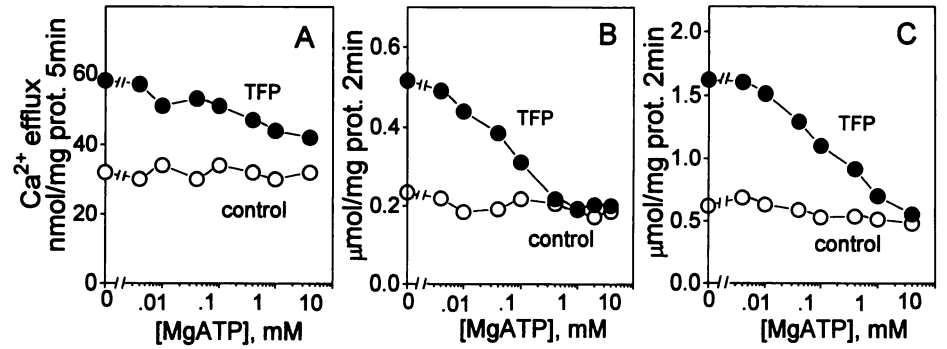


**Fig. 14.** The effect of MgATP on $Ca^{2+}$ efflux. The (A) cerebellar, (B) cardiac, and (C) skeletal muscle vesicles were loaded with [$^{45}$Ca]$CaCl_2$ in the presence of 20 mM $P_i$ using ATP and diluted to a final concentration of 50 (A and C) or 20 (B) µg of protein/ml in medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM EGTA, and increasing concentrations of MgATP in either the absence (○) or the presence (●) of 50 µM trifluoperazine (*TFP*). Values are representative of three different experiments performed with three different vesicle preparations.

trifluoperazine were in the same range as those found for the second $K_m$ value (50–500 µM) (Table 1).

## Discussion

The present data show that the SERCA 2b and SERCA 2a isoforms can be differentiated from SERCA 1 through modulation of $Ca^{2+}$ uptake and ATPase activity by $Mg^{2+}$ (Figs. 2 and 3), stimulation of $Ca^{2+}$ uptake by trifluoperazine (Figs. 1 and 4), sensitivity to dimethylsulfoxide (Fig. 8), and the effects of the uncoupling agents arsenate and trifluoperazine on the rate of passive $Ca^{2+}$ efflux (Figs. 11–14).

The SERCA 2b isoform is concentrated most densely in the cerebellum, especially in Purkinje cells (9). SERCA 3 is also expressed in these cells, but it represents only 10% of the total SERCA expressed in the cerebellum (4). Trifluoperazine was found to stimulate $Ca^{2+}$ transport in vesicles derived from either the cerebellum (Fig. 4) or the forebrain, which lacks SERCA 3 (data not shown). Therefore, the effects described in this report cannot be attributed to the low amount of SERCA 3 present in cerebellar Purkinje cells.

Using microsomes derived from COS cells, Lytton *et al.* (62) showed that SERCA 2b has a lower turnover rate for both $Ca^{2+}$ transport and ATP hydrolysis than other SERCA isoforms. In agreement with this observation, we found that the rate of transport in cerebellar vesicles, which are enriched with the SERCA 2b isoform, was slower than that measured in cardiac (SERCA 2a) or skeletal (SERCA 1) muscle vesicles. As far as we know, the coupling ratio between $Ca^{2+}$ transport and ATP hydrolysis for SERCA 2b has not been determined. At present, we do not know the reason for the low coupling coefficient of the cerebellar isoform observed in Fig. 6. It may be related to the earlier finding that the $Ca^{2+}$-transport ATPase of brain is able to form and support smaller $Ca^{2+}$ gradients than the SERCA 1 ATPase of skeletal muscle (18). We do not discard, however, the possibility that portions of cerebellar vesicles are leaky or that the $Ca^{2+}$ pumps are orientated in a random manner in the membrane, a condition that may lead to a decrease in the coupling ratio.

In earlier reports, it was shown that trifluoperazine, arsenate, $P_i$, and $Mg^{2+}$ interact with the $E_2$ conformation of SERCA 1 isoform (25, 26, 29). Thus, the different effects observed in the present study with trifluoperazine, arsenate, $P_i$, and $Mg^{2+}$ indicate that the $E_2$ conformations of the SERCA 1 and SERCA 2 isoforms are also different.

The binding of ATP to the regulatory site of the enzyme (Fig. 1) seems to play a role in regulating the coupling between transport and catalytic activity in the different $Ca^{2+}$-ATPase isoforms. This conclusion is derived from the following findings: (i) the stimulation of $Ca^{2+}$ uptake promoted by trifluoperazine in cerebellar and cardiac vesicles was observed only when 2 mM ATP was used to load the vesicles (Fig. 4, A and B), and it was the result of an increase in the coupling ratio between $Ca^{2+}$ uptake and ATPase activity (Fig. 6); and (ii) MgATP in the concentration range that binds to the regulatory site was able to antagonize the efflux of $Ca^{2+}$ promoted by trifluoperazine in all three vesicle prepa-

rations (Fig. 14). The increase in passive $Ca^{2+}$ efflux promoted by trifluoperazine was not the result of a nonspecific leakage of $Ca^{2+}$ through the membrane because several ligands of the pump were able to antagonize the effect of the drug. In addition, trifluoperazine increased passive $Ca^{2+}$ efflux in a concentration range in which $Ca^{2+}$ uptake either was not inhibited or was stimulated by the drug (Figs. 12 and 13). These data indicate that similar to skeletal muscle isoform, SERCA 2 isoforms can mediate $Ca^{2+}$ efflux as a $Ca^{2+}$ channel.

Modulation of channel function of the ATPase by cations and thapsigargin distinguished the cerebellar $Ca^{2+}$-ATPase isoform (SERCA 2b) from that found in cardiac muscle (SERCA 2a) (Figs. 10 and 12). These differences may be related to the presence of different carboxyl-terminal regions in the SERCA 2b and SERCA 2a isoforms. These two isoforms are completely identical, except the last four amino acids of SERCA 2a are replaced by a tail of 49 amino acids in SERCA 2b. This stretch of amino acids is hydrophobic and might constitute an 11th transmembrane domain for SERCA 2b (5, 6, 63). The putative 11th transmembrane domain of SERCA 2b may render it less sensitive to cations and thapsigargin than the SERCA 2a isoforms.

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