Ca^{2+} Transport by the Synaptosomal Plasma Membrane Ca^{2+}-ATPase and the Effect of Thioridazine†

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ABSTRACT: Thioridazine inhibits the activity of the synaptic plasma membrane Ca^{2+}-ATPase from pig brain and slightly decreases the rate of Ca^{2+} accumulation by synaptic plasma membrane vesicles in the absence of phosphate. However, in the presence of phosphate, thioridazine increases the rate of Ca^{2+} accumulation into synaptic plasma membrane vesicles. Phosphate anions diffuse through the membrane and form calcium phosphate crystals, reducing the free Ca^{2+} concentration inside the vesicles and the rate of Ca^{2+} leak. The higher levels of Ca^{2+} accumulation obtained in the presence of thioridazine could be explained by a reduction of the rate of slippage on the plasma membrane ATPase.

The nervous system contains a number of ion transporters that contribute to the maintenance of a low intracellular free Ca^{2+} concentration, including the plasma membrane Ca^{2+}-ATPase (PMCA)[1] that pumps Ca^{2+} out of the cell and an intracellular Ca^{2+}-ATPase (SERCA) that removes Ca^{2+} from the cytosol to internal stores. The synaptosomal plasma membrane Ca^{2+}-ATPase has been purified and well characterized (*1*−*3*). The activity of the protein is highly regulated, being stimulated by calmodulin, acidic phospholipids, controlled proteolysis, oligomerization, and protein kinases (*4*, *5*). A stoichiometric ratio of Ca^{2+} uptake to ATP hydrolyzed of 1:1 is obtained by measurements of initial rates (*2*), whereas a 2:1 ratio has been determined for the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (*6*). Under noninitial rate conditions, a level of Ca^{2+} uptake to ATP hydrolyzed of less than 2:1 is observed for the sacroplasmic reticulum Ca^{2+}-ATPase due to passive leak of transported Ca^{2+} out of the vesicle (*7*, *8*) and due to a slippage pathway that involves the release of bound Ca^{2+} from the phosphorylated intermediate to the cytoplasmic side of the membrane before its transport into the lumen, resulting in ATP hydrolysis uncoupled from Ca^{2+} transport (*9*−*11*). The amount of Ca^{2+} ions retained by SR vesicles can be increased by addition of phosphate or oxalate to the external medium (*12*). These anions diffuse into the lumen of the vesicles through specific transporters (*13*−*15*) and precipitate the Ca^{2+} ions in the lumen of the vesicles.

There have, as yet, been no reports on the potential importance of slippage and leak for Ca^{2+} transport by PMCA. de Meis (*16*) has shown that useful information about uptake and leak of Ca^{2+} by SERCA can be obtained from studies of the effects of drugs such as chlorpromazine. Here we report on studies of the effects of thioridazine and other antipsychotics on the functioning of the synaptosomal PMCA and show that thioridazine, while being an inhibitor of PMCA ATPase activity, can, under some conditions, produce a large increase in the rate of accumulation of Ca^{2+}, consistent with a decrease in the rate of slippage on PMCA.

MATERIALS AND METHODS

Materials. Thioridazine hydrochloride, fluphenazine hydrochloride, chlorpromazine hydrochloride, calmodulin from bovine brain, the phospholipids phosphatidylcholine (PC), type XI-E from egg yolk, and phosphatidylserine (PS) from bovine brain, thapsigargin, ammonium vanadate, and calmodulin−agarose were obtained from Sigma, and $^{45}Ca^{2+}$ was from American Radiolabeled Chemicals, Inc. All other reagents were of the highest purity available.

Preparation of Synaptic Plasma Membrane (SPM) Vesicles and Purified Plasma Membrane Ca^{2+}-ATPase. The fractions were prepared as described by Salvador and Mata (*1*). Briefly, the SPM vesicles were obtained by osmotic lysis of synaptosomes. The Ca^{2+}-ATPase was isolated from SPM vesicles solubilized in 0.6% (w/v) Triton X-100, using a calmodulin affinity column. The purified fraction was eluted in 20 mM Hepes/KOH, pH 7.4, 130 mM KCl, 1 mM $MgCl_2$, 15% glycerol, 0.06% Triton X-100, and 2 mM EDTA. All fractions were stored at −80 °C until use. The protein content was measured by the Bradford method (*17*).

Ca^{2+}-ATPase Activity. The Ca^{2+}-dependent ATPase activity was measured spectrophotometrically at 37 °C by using a coupled enzyme assay in the presence of 50 mM Hepes/KOH, pH 7.4, 100 mM KCl, 5 mM Na_3N, 2 mM $MgCl_2$, 3.16 μM free Ca^{2+}, 0.11 mM NADH, 0.42 mM phospho-

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[1] Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; PC, phosphatidylcholine; PMCA, plasma membrane Ca^{2+}-ATPase; PS, phosphatidylserine; SR, sarcoplasmic reticulum.

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enolpyruvate, 10 IU of pyruvate kinase, and 28 IU of lactate dehydrogenase. The SPM vesicles (40 μg of protein) were incubated in this medium for 2 min, and the reaction was started by addition of 1 mM ATP. The purified ATPase (2 μg), containing 0.06% Triton X-100, was mixed with lipid according to the method of Palacios et al. (*18*) and then diluted directly into the assay medium to give a final volume of 1 mL. The sample was then incubated for a further 2 min before starting the reaction by addition of 1 mM ATP.

Ca^{2+} Uptake. $^{45}Ca^{2+}$ transport was measured at 37 °C by the filtration technique (*19*). SPM vesicles (40 μg/mL) were incubated in an uptake medium containing 50 mM Hepes/KOH, pH 7.4, 100 mM KCl, 5 mM Na_3N, 2 mM $MgCl_2$, 100 μM $CaCl_2$, enough BAPTA to give 3.16 μM free Ca^{2+}, 0.12 μCi of $^{45}CaCl_2$ (approximately 20 400 cpm/nmol), and 0.1 μM thapsigargin (standard uptake medium). When required, 20 mM KH_2PO_4 and thioridazine were added to the medium in 1 mL total volume. After 4 min incubation at 37 °C, the uptake reaction was started by addition of 1 mM ATP and was stopped after 5 min or the time specified by filtration through Millipore filters (HAWP-045) and two washes with 5 mL of 20 mM Hepes and 1 mM $LaCl_3$. Filters were dried for 45 min at 80 °C, dissolved in scintillation fluid (Cytoscint, ICN), and counted. Three blanks without protein were filtered to determine the level of nonspecific calcium retained by the filters.

Data Fitting and Simulation. Data were fitted to the appropriate binding equations using the nonlinear least-squares routine in the SigmaPlot package (SPSS Inc, Chicago, IL). Simulations of uptake and release of Ca^{2+} were performed using the program FACSIMILE (MCPA Software Ltd, Sprotbrough, Doncaster, U.K.).

RESULTS

Effects of Thioridazine on ATPase Activity. PMCA was reconstituted by mixing lipids with the delipidated protein, eluted from the calmodulin−agarose column in 0.06% Triton-X100, followed by dilution into the assay buffer. This method produces unsealed vesicles in which all protein molecules have access to the substrates. Addition of thioridazine to PMCA reconstituted into bilayers of PC or PS, lead to inhibition of ATPase activity in either the presence or the absence of calmodulin with IC50 values of 77 μM (Figure 1); levels of inhibition were the same for periods of incubation with thioridazine between 2 and 60 min (data not shown). As shown by the broken line in Figure 1, the levels of inhibition do not fit well to a simple inhibition curve

$$v/v_{max} = K_1/(I + K_1) \quad (1)$$

where v_{max} and v are the rates of ATP hydrolysis in the absence and presence of thioridazine at a concentration I, respectively, and K_1 is the apparent dissociation constant for thioridazine. Greater inhibition of activity was observed at high concentrations of thioridazine than would have been expected from simple saturable inhibition, suggesting an additional contribution to inhibition, particularly important at higher concentrations of thioridazine. This additional inhibition was modeled as a nonsaturable component in which inhibition was linearly related to the concentration of thioridazine, so

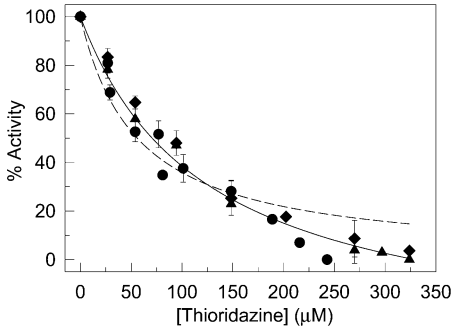


FIGURE 1: Effect of thioridazine on the Ca^{2+}-ATPase activity. ATPase activity was measured as indicated in the method section, using 2 μg of delipidated ATPase reconstituted with 10.6 μg of PC (▲) or PC plus 0.32 μg of calmodulin (◆) or PS (●) in 1 mL of the assay medium. Activities were measured at 37 °C in the presence of 1 mM ATP and thioridazine at the indicated concentrations. Data are means ± SD (bars) of four experiments from different preparations. The broken line shows the best fit to simple inhibitor binding (eq 1) giving a value for K_1 of 56 ± 7 μM. The solid line shows the best fit to a model with both saturable and nonsaturable inhibition (eq 2) described by dissociation constants K_1 and K_2 of 124 ± 17 μM and 330 ± 31 μM, respectively.

$$v/v_{max} = [K_1/(I + K_1)][1 - I/K_2] \quad (2)$$

where K_2 is the apparent dissociation constant for nonsaturable binding. The data fit well to eq 2 with values for K_1 and K_2 of 124 ± 17 μM and 330 ± 31 μM, respectively.

The nonsaturable component of inhibition could correspond to a detergent-like effect of thioridazine, which could result in significant depletion of the free concentration of thioridazine. Indeed, the inhibitory effect of thioridazine is observed to increase with decreasing concentration of protein, the inhibition of PMCA reconstituted with PS caused by 77 μM thioridazine increasing from 50% at 7 μg of PMCA to 82% at 1 μg of PMCA (data not shown).

The presence of 77 μM thioridazine had no significant effect on the Ca^{2+}-dependence of ATPase activity nor on the ATP-dependence of activity when measured at a constant free concentration of Mg^{2+} (data not shown). However, inhibition of ATPase activity by thioridazine was highly dependent on the free Mg^{2+} concentration (Figure 2) with inhibition by 77 μM thioridazine being about 50% at free Mg^{2+} concentrations between 0.42 and 1.5 mM but with lower levels of inhibition at higher concentrations of Mg^{2+}.

Effects of Thioridazine on Ca^{2+} Accumulation. Synaptic plasma membrane vesicles (SPM) containing the Ca^{2+}-ATPase were used to quantify Ca^{2+} accumulation. All assays were performed in the presence of 0.1 μM thapsigargin, a highly specific inhibitor of SERCA (*20*), to avoid any effects attributable to SERCA. The action of other ATPases was blocked by azide. Consequently, the Ca^{2+} ATPase activity and Ca^{2+} transport measured in SPM vesicles under these conditions are those due to PMCA.

As shown in Figure 3A, the ATPase activity of these vesicles was inhibited by thioridazine over the same concentration range observed in studies of purified PMCA (Figure 1). Figure 3B shows the effect of thioridazine on Ca^{2+} accumulation by SPM vesicles in the absence or presence of 20 mM phosphate. The level of Ca^{2+} accumulated by the vesicles is higher in the presence of phosphate than in its absence because of the formation of a calcium

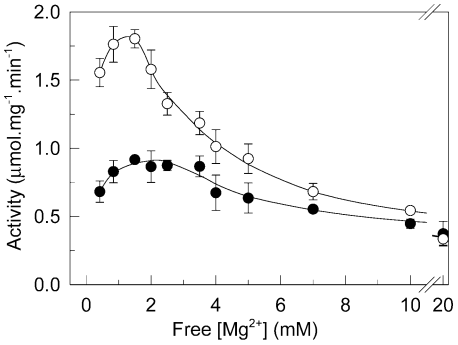


FIGURE 2: Effect of thioridazine on the Mg^{2+} dependence of ATPase activity. Two micrograms of ATPase was reconstituted as described in the Materials and Methods in 10.6 μg of PS and incubated in 1 mL of assay medium with the indicated concentrations of free Mg^{2+}. The ATPase activity was measured after addition of 1 mM ATP in the absence (empty symbols) or in the presence (filled symbols) of 77 μM of thioridazine. Data are means ± SD (bars) of four experiments from different preparations.

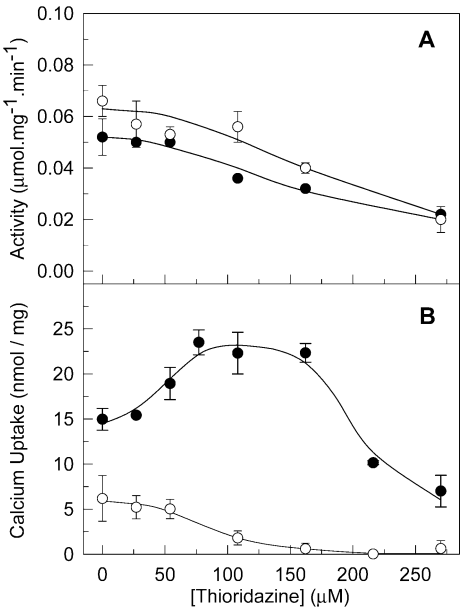


FIGURE 3: Effect of thioridazine on Ca^{2+}-ATPase activity and Ca^{2+} uptake by SPM vesicles. The ATPase activity (A) of 40 μg/mL of SPM was assayed in a reaction medium containing 0.1 μM thapsigargin without (empty symbols) or with (filled symbols) 20 mM KH_2PO_4. The reaction was started by addition of 1 mM ATP and thioridazine at the indicated concentrations. Ca^{2+} accumulation (B) was determined as described in the Materials and Methods section. SPM (40 μg/mL) was incubated for 4 min at 37 °C in the standard uptake medium (which also contained 0.1 μM thapsigargin) without (empty symbols) or with 20 mM KH_2PO_4 (filled symbols) and in the presence of the given concentrations of thioridazine. The reaction was started by addition of 1 mM ATP and was stopped 5 min later. Data are means ± SD (bars) of three experiments.

phosphate precipitate in the lumen of the vesicles, keeping a low free luminal Ca^{2+} concentration that prevents inhibition of Ca^{2+} transport and reduces the rate of leak of Ca^{2+} out of the vesicles. In the absence of phosphate ion, accumulation of Ca^{2+} is inhibited to some extent by thioridazine (Figure 3B) but, unexpectedly, in the presence of 20 mM phosphate Ca^{2+} accumulation is stimulated by thioridazine, a maximum activation of ca. 50−60% being observed over a thioridazine concentration range of 77−162 μM; at higher concentrations, thioridazine inhibited accumulation of Ca^{2+} in the presence of phosphate.

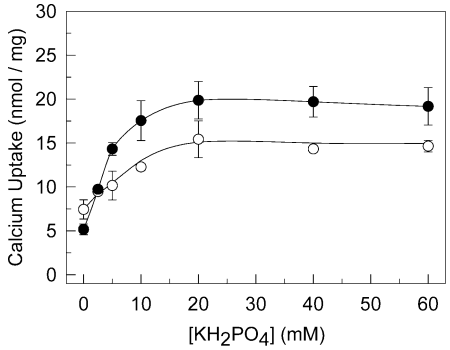


FIGURE 4: Phosphate concentration dependence of Ca^{2+} uptake. SPM vesicles (40 μg/mL) were incubated in the standard uptake medium in the absence (○) or presence (●) of 77 μM thioridazine. KH_2PO_4 was added into the medium at the indicated concentrations. Accumulation of Ca^{2+} was started by addition of 1 mM ATP and was stopped 5 min later as indicated in the Materials and Methods. Data are means ± SD (bars) of four experiments.

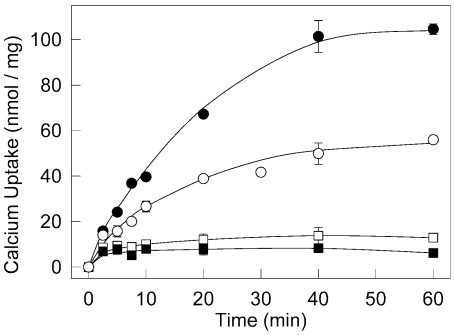


FIGURE 5: Time dependence of Ca^{2+} uptake by SPM vesicles. Ca^{2+} accumulation by SPM vesicles (40 μg/mL) was assayed as described in the Materials and Methods section, in the absence (□,■) or presence (○,●) of 20 mM KH_2PO_4 and without (empty symbols) or with (filled symbols) 77 μM thioridazine. The reaction was started by addition of 1 mM ATP. Data are means ± SD (bars) of four experiments.

The effect of phosphate concentration on Ca^{2+} accumulation was studied in the presence and in the absence of thioridazine (Figure 4). The level of accumulation of Ca^{2+} increases with increasing phosphate concentration up to about 20 mM when the effect of phosphate saturates in both the absence and presence of thioridazine. The small inhibitory effect of thioridazine seen in the absence of phosphate is reversed at concentrations of phosphate above ca. 4 mM (Figure 4). The time dependence of Ca^{2+} accumulation is shown in Figure 5, with or without phosphate, which shows that at all times thioridazine increases the level of accumulation of Ca^{2+} in the presence of phosphate but not in the absence of phosphate.

Passive Leak of Ca^{2+} from SPM Vesicles. The observation that levels of accumulation of Ca^{2+} by SPM increase in the presence of phosphate suggests that the vesicles could be leaky to Ca^{2+}. This possibility was tested by actively loading the vesicles with Ca^{2+} in the presence of ATP and then studying passive Ca^{2+} leak as a function of time after inhibiting PMCA with vanadate (Figure 6A) or by removing the external Ca^{2+} with EGTA (Figure 6B). As shown, inhibition of PMCA leads to rapid release of accumulated Ca^{2+}, faster in the absence of phosphate than in its presence and faster when the ATPase is inhibited by removal of external Ca^{2+} than when it is inhibited by vanadate (Figure 6). The passive leak of Ca^{2+} fits to a single-exponential

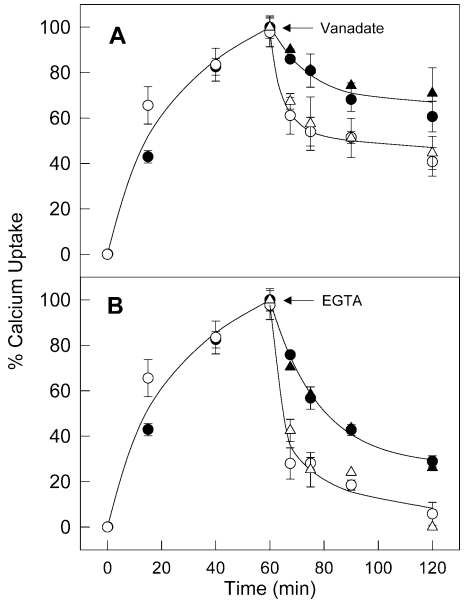
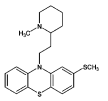
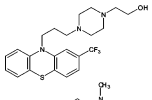
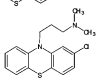


FIGURE 6: Passive leak of Ca^{2+} from actively loaded vesicles. SPM vesicles (40 μg/mL) were incubated in the standard uptake medium in the absence (empty symbols) or presence (filled symbols) of 20 mM KH_2PO_4 and without (○,●) or with (△,▲) 77 μM thioridazine. The reaction was started by addition of 1 mM ATP, and 60 min later, 100 μM vanadate (A) or 2 mM EGTA (B) was added. Accumulation of Ca^{2+} was measured at the indicated times. Data are means ± SD (bars) of four experiments. The 100% of Ca^{2+} uptake corresponded to 8.48 ± 0.75 and 52.55 ± 2.57 nmol/mg in the absence or presence of phosphate, respectively, and 7.77 ± 1.87 and 104.61 ± 2.32 nmol/mg with 77 μM thioridazine in the absence or presence of phosphate, respectively. Data are means ± SD (bars) of six experiments.

process under all conditions. When the PMCA is blocked with vanadate, the rate of passive leak decreases from 0.15% ± 0.04% Ca^{2+} uptake/min in the absence of phosphate to 0.045% ± 0.006% Ca^{2+} uptake/min in the presence of phosphate, the rates not being affected significantly by the presence of 77 μM thioridazine. Similarly, when PMCA is blocked by addition of EGTA, the rate of leak decreases from 0.21% ± 0.08% Ca^{2+} uptake/min in the absence of phosphate to 0.055% ± 0.004% Ca^{2+} uptake/min in the presence of phosphate, the rates again being unaffected by the presence of 77 μM thioridazine.

Effects of Other Inhibitors on Accumulation of Ca^{2+}. To test the structural specificity of the effects of thioridazine, Ca^{2+} transport was assayed with other inhibitors of PMCA. Table 1 shows that vanadate and tetrahydrobenzoquinone (BHQ) reduced ATPase activity and also reduced Ca^{2+} transport. However, the phenotiazines fluphenazine and chlorpromazine at concentrations that reduced ATPase activity increased Ca^{2+} accumulation in the presence of phosphate ion, almost doubling the levels of accumulation.

Table 1: Effects of Inhibitors of Ca^{2+}-ATPase Activity on Ca^{2+} Uptake

	Activity (μmol.mg^{-1}.min^{-1})[a]	Ca^{2+} uptake (nmol / mg of protein / 15 min)[b]
Control	1.75 ± 0.12 (100%)	24.2 ± 3.7 (100%)
 Thioridazine (77 μM)	0.91 (49.7%)	43.04 ± 6.46 (178.93 %)
 Fluphenazine (65 μM)	0.92 ± 0.09 (56%)	42.59 ± 5.11 (173.95 %)
 Chlorpromazine (100 μM)	0.95 ± 0.07 (51.6%)	36.76 ± 5.42 (152.16 %)
Vanadate (50 μM)	0.07 (4.1%)	4.01 ± 1.44 (18.45 %)
BHQ (1.5 mM)	0.93 (51.7%)	14.01 ± 2.11 (57.27%)

[a] ATPase activity was assayed using 2 μg of purified PMCA reconstituted in PS as described in Materials and Methods. [b] Ca^{2+}-uptake was assayed in 40 μg of SPM vesicles in the presence of 20 mM phosphate.

DISCUSSION

Thioridazine is a phenothiazine derivative with a selective dopaminergic-blocking action on the limbic dopamine receptors, which acts clinically at 100−800 mg daily doses as an antipsychotic (*21*, *22*). The aim of this work has been to study its action on the synaptosomal PMCA because PMCA plays an important role in lowering Ca^{2+} levels involved in neurotransmitter release and thioridazine is used for treatment of processes involved in the alteration of the synaptic transmission. Thioridazine inhibited the ATPase activity of the synaptic plasma membrane Ca^{2+}-ATPase from pig brain, the level of inhibition being unaffected by the presence of calmodulin or by the phospholipids, phosphatidylcholine or phosphatidylserine, used to reconstitute PMCA (Figure 1). The concentration of free Mg^{2+} in the medium had a large effect on the level of inhibition by thioridazine, only very low levels of inhibition being detected at high free concentrations of Mg^{2+} (Figure 2). Takara and Alonso (*23*) also reported a protective effect of Mg^{2+} on the inhibition of the Ca^{2+}-ATPase activity of the SERCA by haloperidol, a drug that is chemically different from phenothiazines but that shares a neuroleptic effect. The results suggest a competition between thioridazine and Mg^{2+} for binding to a site on PMCA at which binding of Mg^{2+} leads to stimulation of activity.

Thioridazine inhibited both ATPase activity and accumulation of Ca^{2+} by SPM vesicles in the absence of phosphate ion (Figures 3 and 5). However, in the presence of phosphate ion, addition of low concentrations of thioridazine leads to an increase in the rate of accumulation of Ca^{2+} (Figure 5) although inhibition was observed at concentrations of thioridazine above ca. 150 μM (Figure 3). Engelender and de Meis (*24*) also observed that accumulation of Ca^{2+} by cerebellar endoplasmic reticulum vesicles in the presence of phosphate was stimulated by trifluoperazine, even though trifluoperazine inhibited ATPase activity. They also showed that the presence of trifluoperazine increased the rate of passive leak of Ca^{2+} from actively loaded vesicles.

The level of accumulation of Ca^{2+} by SPM vesicles will be a balance between the rate of transport of Ca^{2+} into the lumen of the vesicles and the rates of any leak pathways out of the vesicles. As shown in Figure 6, accumulated Ca^{2+} leaks out of SPM vesicles when PMCA activity is blocked by addition of vanadate or EGTA. The rate of leak is faster in the absence of phosphate than in its presence, consistent with a passive leak process, the presence of phosphate reducing the lumenal free concentration of Ca^{2+} and so reducing the rate of passive leak. Leak of Ca^{2+} is unlikely

to be due to reversal of the Ca^{2+}-ATPase with movement of Ca^{2+} out of the lumen of the vesicles linked to the synthesis of ATP from ADP and phosphorylated ATPase, since ADP concentrations were low in these experiments and since in Figure 6A the ATPase was inhibited by vanadate and the external Ca^{2+} concentration was micromolar, conditions under which reversal of the Ca^{2+}-ATPase is inhibited (*24*). Passive leak of Ca^{2+} from SR vesicles has been suggested to be a simple passive process involving binding to the Ca^{2+}-ATPase (*9*, *24*, *25*).

Importantly, as shown in Figure 6, the presence of thioridazine does not affect the rate of passive leak of Ca^{2+} out of SPM vesicles, so the explanation for the effect of thioridazine on the level of accumulation of Ca^{2+} seen in the presence of phosphate (Figures 3 and 5) cannot be a slowing of the rate of passive leak. An alternative possibility is that thioridazine affects the rate of slippage on PMCA, slippage being the process in which the phosphorylated intermediate of the Ca^{2+}-ATPase releases its bound Ca^{2+} on the cytoplasmic side of the membrane rather than into the lumen. An effect on the rate of slippage has been proposed to be the explanation for the effect of curcumin on Ca^{2+} accumulation by SR, where curcumin increased the rate of accumulation of Ca^{2+} despite being an inhibitor of ATPase activity (*26*). The observation of fast passive leak of Ca^{2+} from SPM vesicles (Figure 6) would explain why an effect of thioridazine is only seen in the presence of phosphate; in the absence of phosphate, passive leak of Ca^{2+} would be fast enough to mask any effect of thioridazine on slippage.

To show that slippage on the Ca^{2+}-ATPase is sufficient to explain the observed results, we have carried out simulations of a simple uptake scheme for SPM, including slippage and leak, according to the highly simplified scheme shown in Figure 7. The scheme is based on that used previously to describe accumulation of Ca^{2+} by the Ca^{2+}-ATPase of sarcoplasmic reticulum (*11*, *27*). As with the SR Ca^{2+}-ATPase, it is assumed that the Ca^{2+}-ATPase can exist in one of two conformations, E1 or E2. Following binding of a single Ca^{2+} ion, the ATPase can bind ATP and then phosphorylate to give E2PCa. E2PCa can release Ca^{2+} into the lumen of the vesicle, resulting in transport of Ca^{2+}, or it can release the bound Ca^{2+} ion on the outside of the vesicle in the process of slippage, resulting in the futile hydrolysis of ATP. Dephosphorylation of E2P results in regeneration of E2, which can recycle to E1. Simple passive leak of Ca^{2+} out of the vesicles can also occur. The rate constants used in the simulations were largely those used previously for simulations of the SR Ca^{2+}-ATPase but with a slower rate of phosphorylation to account for the slower rate of accumulation of Ca^{2+} by SPM than by SR vesicles (Table 2). A parameter having an important effect on the simulations is the internal volume of the vesicles, which is not known. Volumes of SR vesicles have been estimated to be a few microliters per milligram of protein (*28*); in the simulations, we have assumed an internal volume of 1.5 μL/mg protein.

Passive leak of Ca^{2+} from Ca^{2+}-loaded vesicles was simulated in the absence and presence of phosphate (Figure 7B) according to the simple scheme $Ca_{in} \rightarrow Ca_{out}$ and compared to the experimental data (Figure 6). Rate constants of 2×10^{-7} and 5×10^{-8} s^{-1} for passive leak in the absence and presence of phosphate, respectively, give profiles for the loss of Ca^{2+} that fit to single-exponential processes with

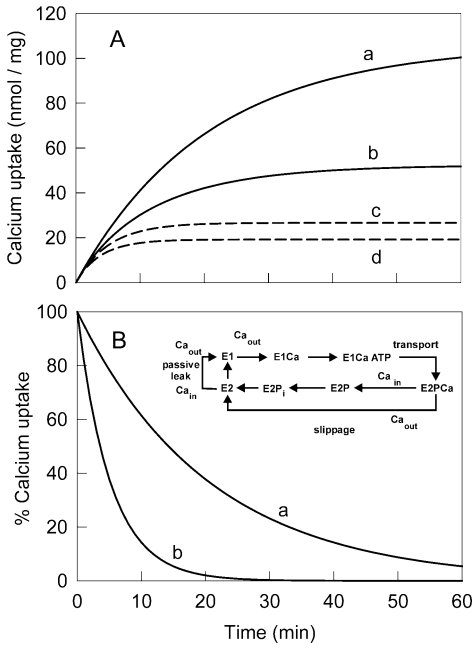


FIGURE 7: Simulations of Ca^{2+} accumulation and passive efflux. Simulations were performed using the parameters in Table 2 with a concentration of ATPase of 0.012 μM, initial Ca^{2+} and ATP concentrations of 120 μM and 0.8 mM, respectively, and a lumenal volume of 1.5 μL/mg of protein. Panel A shows the effects of passive leak and slippage on accumulation of Ca^{2+} with the rate constants for passive leak and slippage given in Table 2: (a) presence of 20 mM phosphate and 77 μM thioridazine; (b) 20 mM phosphate and no thioridazine; (c) 77 μM thioridazine and no phosphate; (d) no thioridazine and no phosphate. Panel B is a simulation of passive leak from Ca^{2+}-loaded vesicles: (a) in the presence of 20 mM phosphate; (b) in the absence of phosphate. The insert shows the reaction scheme used in the simulations.

Table 2: Rate Constants Used in the Simulations Shown in Figure 7

step	rate constant	
	forward	reverse
$E2 \rightarrow E1$	13 s^{-1}	47 s^{-1}
$E1 + Ca_{out} \rightarrow E1Ca$	1×10^8 M^{-1} s^{-1}	100 s^{-1}
$E1Ca + ATP \rightarrow E1CaATP$	1×10^8 M^{-1} s^{-1}	1×10^3 s^{-1}
$E1CaATP \rightarrow E2PCa$	0.3 s^{-1}	0
$E2PCa \rightarrow E2P + Ca_{in}$	6×10^3 s^{-1}	1.8×10^5 M^{-1} s^{-1}
$E2P \rightarrow E2$	100 s^{-1}	0
$E2PCa \rightarrow E2P + Ca_{out}$		
no thioridazine	100 s^{-1}	0
+ 77 μM thioridazine	0	0
$Ca_{in} \rightarrow Ca_{out}$		
no phosphate	2×10^{-7} s^{-1}	0
+ 20 mM phosphate	5×10^{-8} s^{-1}	0

rates of 0.2 and 0.05 min^{-1}, in agreement with the experimentally determined values. Accumulation of Ca^{2+} was simulated assuming that the presence of thioridazine had no effect on the rate of passive leak, in agreement with the experimental data shown in Figure 6, and also making the simplifying assumption that the presence of phosphate had no effect on the rate of slippage. Assuming a rate constant for slippage of 100 s^{-1} in the absence of thioridazine with no slippage in the presence of 77 μM thioridazine gives a time dependence for accumulation of Ca^{2+} in the presence of phosphate in good agreement with the experimental data (compare Figures 7A and 5). The simulations show that, in the absence of phosphate, levels of accumulation of Ca^{2+} are little affected by the presence of thioridazine, in contrast

to the large effect of thioridazine seen in the presence of phosphate (Figure 7A), confirming that passive leak of Ca^{2+} in the absence of phosphate is fast enough to mask the effects of thioridazine on the rate of slippage on the Ca^{2+}-ATPase. The simulations shown in Figure 7A predict a small increase in the level of accumulation of Ca^{2+} on addition of thioridazine, compared to the experimentally observed decrease (Figure 5), but this is probably due to the neglect of the inhibitory effect of thioridazine on ATPase activity in the simulations; although an inhibitory effect could have been included in the simulations, this was felt not to be justified given the highly simplified nature of the simulations, designed purely to show the relative effects of leak and slippage in the absence and presence of phosphate.

Activation of Ca^{2+} accumulation in the presence of phosphate was also observed with other phenothiazines that inhibited ATPase activity (Table 1) but not with other inhibitors of Ca^{2+}-ATPase such as vanadate or BHQ, indicating that the mechanism of action must be structurally specific for these drugs.

In summary, this study showed that thioridazine and other phenothiazines inhibited PMCA activity and Ca^{2+} accumulation by SPM vesicles in the absence of phosphate. However, in the presence of phosphate, these drugs activated Ca^{2+} transport. These effects can be explained by a reduction in the rate of slippage on PMCA. This slippage is masked by fast passive leak of Ca^{2+} out of the vesicles when Ca^{2+} accumulation is measured in the absence of phosphate.

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