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THE EFFECT OF TRIFLUOROPERAZINE ON THE SARCOPLASMIC RETICULUM MEMBRANE

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The inhibitory effect of trifluoroperazine (25–200 μM) on the sarcoplasmic reticulum calcium pump was studied in sarcoplasmic reticulum vesicles isolated from skeletal muscle. It was found that the lowest effective concentrations of trifluoroperazine (10 μM) displaces the Ca^{2+} dependence of sarcoplasmic reticulum ATPase to higher Ca^{2+} concentrations. Higher trifluoroperazine concentrations (100 μM) inhibit the enzyme even at saturating Ca^{2+}. If trifluoroperazine is added to vesicles filled with calcium in the presence of ATP, inhibition of the catalytic cycle is accompanied by rapid release of accumulated calcium. ATPase inhibition and calcium release are produced by identical concentrations of trifluoroperazine and, most likely, by the same enzyme perturbation. These effects are related to partition of trifluoroperazine into the sarcoplasmic reticulum membrane, and consequent alteration of the enzyme assembly within the membrane structure, and of the bilayer surface properties. The effect of trifluoroperazine was also studied on dissociated ('chemically skinned') cardiac cells undergoing phasic contractile activity which is totally dependent on calcium uptake and release by sarcoplasmic reticulum, and is not influenced by inhibitors of slow calcium channels. It was found that trifluoroperazine interferes with calcium transport by sarcoplasmic reticulum in situ, as well as with the role of sarcoplasmic reticulum in contractile activation.

Introduction

An inhibitory effect of chlorpromazine on the calcium pump of sarcoplasmic reticulum was initially reported by Balzer et al. [1,2]. Another phenothiazine, trifluoroperazine, has been recently used in attempts to uncover a role of calmodulin in functional regulation of sarcoplasmic reticulum. It was reported that trifluoroperazine inhibits the calmodulin dependent phosphorylation of a minor protein component (approx. 60 kDa) of the sarcoplasmic reticulum membrane, and thereby reduces the activity of the calcium pump [3]. It was also found that trifluoroperazine interacts directly with a glycoprotein (approx. 53 kDa) component of the sarcoplasmic reticulum membrane, and reduces the activity of the calcium pump through this interaction [4]. We describe here a series of experiments which were performed in order to characterize the effects of trifluoroperazine on sarcoplasmic reticulum vesicles, and test their relevance to contractile activation in myocytes.

Experimental methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as previously described [8]. Dissociated myocytes were obtained from rat hearts by perfusion with collagenase [5], and suspended in 50 mM Mops (pH 7.4), 118 mM NaCl, 4.7 mM KCl, 12 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, and 5 mM glucose. Protein was

determined by the Folin method. Total calcium was measured, and free Ca^{2+} (in the presence of EGTA) estimated as previously described [7], taking into account pH and other ligands [9].

Calcium uptake by sarcoplasmic reticulum vesicles was measured by the filtration method [14] or by double wavelength spectrophotometry in the presence of metallochromic indicators [16]. Steady-state ATPase activity was assessed by determination of P_i production by the molybdovanadate method [12,13]. ^{32}P-labeled phosphoenzyme and $^{32}P_i$ production in the initial phase of the reaction following addition of [γ-^{32}P]ATP was determined as in Ref. 11.

Calcium uptake by sarcoplasmic reticulum in dissociated cardiac myocytes was measured by filtration methods [5]. The contractile behavior of the myocytes was observed by light microscopy and recorded on videotapes which were later replayed for examination of large populations of cells. Each droplet of myocyte suspension observed under the microscope contained $\gg 10^3$ single cells. The contractile behavior of at least 30 cells selected randomly on the video screen was then tabulated per each experimental variable [5].

Electron microscopic observation were carried out on suspensions of sarcoplasmic reticulum vesicles negatively stained with 1% uranyl acetate.

All experiments were carried out at 25°C.

Results

It is known that addition of ATP or other suitable substrates to sarcoplasmic reticulum vesicles is followed by rapid uptake of calcium by the vesicles. When acetylphosphate is used as a substrate instead of ATP, the uptake is somewhat slower and a better kinetic resolution is obtained even though maximal levels of calcium accumulation are reached within 2 min. It is shown in Fig. 1 that acetyl phosphate dependent calcium uptake by skeletal sarcoplasmic reticulum vesicles is inhibited by trifluroperazine in concentrations ranging between 25 and 200 μM. Identical results are obtained when ATP or other substrates are used, and if Ca^{2+} fluxes are measured by optical methods in the presence of metallochromic indicators. An important feature of the triofluoroperazine effect is that addition of the drug to vesicles filled with calcium as a consequence of active transport, causes immediate release of the accumulated calcium (Fig. 1). Inhibition of calcium uptake and activation of calcium release display an identical dependence on trifluoroperazine concentration.

The brief uptake following addition of ATP is significantly prolonged when oxalate is present in the reaction mixture, due to precipitation of calcium oxalate inside the vesicles which prevents 'back inhibition' of active transport by high intravesicular Ca^{2+}. Under these conditions then, it is possible to demonstrate that trifluoroperazine inhibits the steady state rates of calcium transport. It is shown in Fig. 2 that total inhibition is produced by 100–200 μM trifluoroperazine, independent of Ca^{2+} concentrations ranging between 0.3 and 18.0 μM. However, at lower trifluoroperazine concentrations, a stronger inhibition is obtained in the presence of 0.3 μM as compared with 18.0 μM Ca^{2+}.

We found that trifluoroperazine inhibits not only calcium transport, but also the ATPase activity coupled to calcium transport. For these experiments the sarcoplasmic reticulum vesicles were rendered 'leaky' by the addition of a calcium ionophore (A23187) to prevent net accumulation of Ca^{2+} and 'back inhibition'. Thereby, steady state measurements of P_i production were rendered possible. We then found that the inhibition produced by low (10 μM) concentrations of trifluoroperazine can be prevented by increasing the Ca^{2+} concentration (Fig. 3). We also found that the trifluoroperazine concentrations required to produce half maximal inhibition varied in proportion with the concentration of vesicles present in the reaction mixture (Fig. 4). When the same concentrations of vesicles was used, comparable concentrations of trifluoroperazine inhibited both net calcium transport and ATPase activity.

We also carried out rapid quench experiments and measured the formation of phosphorylated enzyme intermediate and the production of P_i within the first 200 ms following addition of ATP to sarcoplasmic reticulum vesicles (not shown). We found that in the presence of saturating (100 μM) Ca^{2+}, low concentrations (20–50 μM) of trifluoroperazine have little effect on phosphoenzyme formation, but inhibit P_i production. Higher trifluoroperazine concentrations inhibit phosphenzyme formation as well.

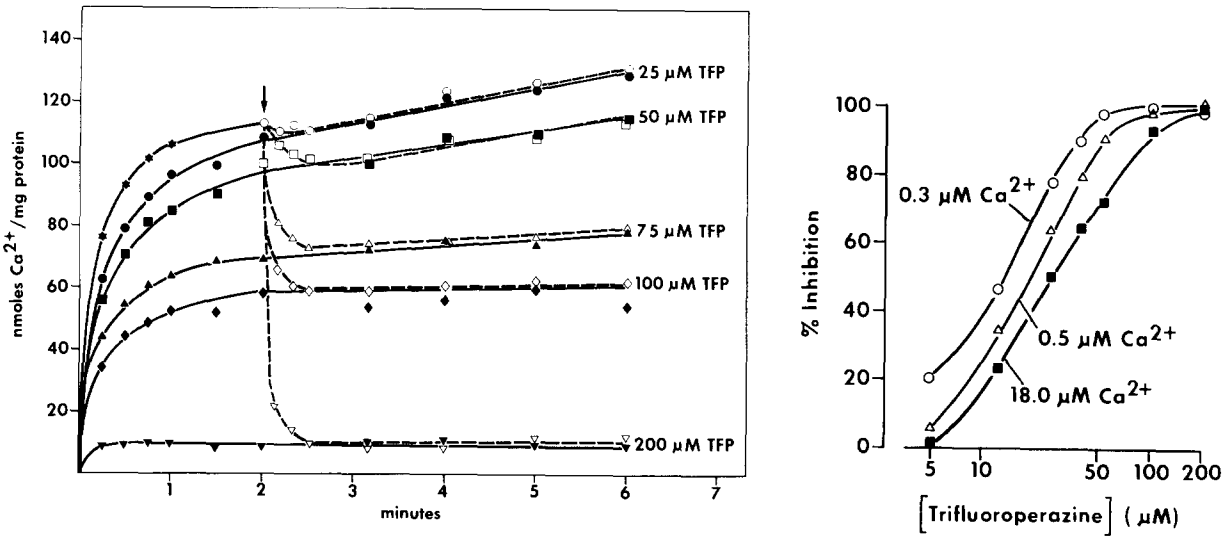

Fig. 1. Effect of trifluoroperazine on calcium uptake and release by skeletal sarcoplasmic reticulum vesicles. Calcium uptake was started by addition of 2 mM acetylphosphate to a reaction mixture containing 20 mM Tris-maleate (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 60 μM $^{45}CaCl_2$, and 0.3 mg sarcoplasmic reticulum protein/ml. The reaction was interrupted by filtration and the residual radioactivity in the filtrate was determined by scintillation spectrometry. Trifluoroperazine (TFP) was added either before (solid symbols) or 2 min after (open symbols) addition of 2 mM acetylphosphate.

Fig. 2. Trifluoroperazine inhibition of the velocity of calcium uptake by skeletal sarcoplasmic reticulum vesicles in the presence of oxalate. The reaction was started by addition of 1 mM ATP to a mixture containing 20 mM Tris-maleate (pH 6.8), 80 mM KI, 5 mM $MgCl_2$, $^{45}CaCl_2$ and EGTA to yield free Ca^{2+} as indicated on the figure, 50 μg protein/ml, 5 mM oxalate, and various concentrations of trifluoroperazine. The reaction was stopped at serial times by filtration, and the velocity of uptake obtained from linear plots of uptake as a function of time.

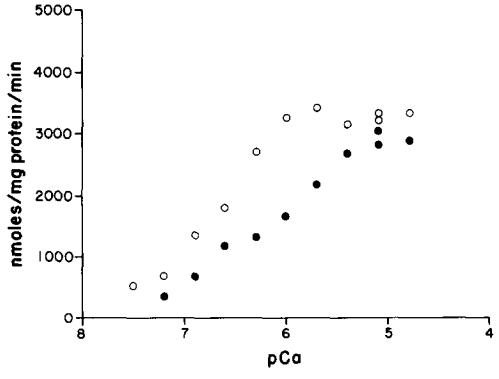

Fig. 3. Effect of low trifluoroperazine concentrations of the Ca^{2+} concentration dependence of sarcoplasmic reticulum ATPase. The reaction was started by addition of 1 mM ATP to a mixture containing 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, EGTA and $CaCl_2$ to yield various Ca^{2+} concentrations [11], 10 μM A23187 (a divalent cation ionophore, and 50 μg protein of sarcoplasmic reticulum (skeletal muscle vesicles) per ml. ○, control; ●, 10 μM trifluoroperazine.

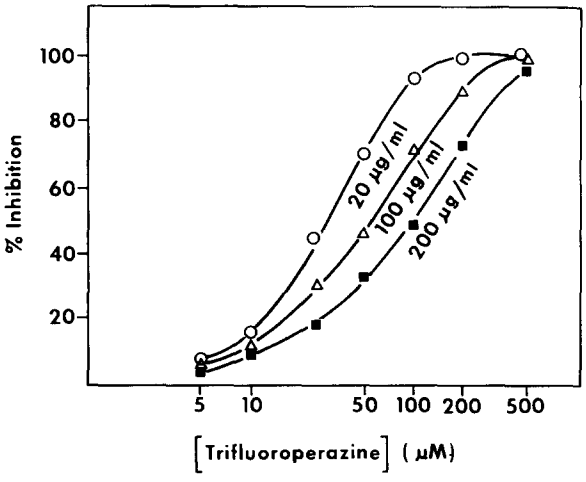

Fig. 4. Trifluoroperazine inhibition of Ca^{2+} dependent ATPase activity in skeletal sarcoplasmic reticulum vesicles, as a function of protein concentration. The reaction was started by addition of 1 mM ATP to a mixture containing 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 10 μM $CaCl_2$, 10 μM A23187 (a divalent cation ionophore), and various concentrations of trifluoroperazine and sarcoplasmic reticulum protein as indicated in the figure.

The experiments described above demonstrate that trifluoroperazine is an inhibitor of ATPase activity and calcium transport in isolated sarcoplasmic reticulum vesicles. In order to determine whether a similar inhibition is produced on the sarcoplasmic reticulum in situ, and if interference with sarcoplasmic reticulum function affects regulation of contractile function, we then extended our investigation to include dissociated cardiac myocytes as an experimental system. Our preparation of dissociated ('chemically skinned') myocytes contains a homogeneous population of cells which are permeable to Ca^{2+} and other small electrolytes, but retain cytoplasmic proteins and enzymes. When exposed to ATP and/or phosphocreatine in the presence of oxalate, the sarcoplasmic reticulum of the dissociated myocytes accumulates large amounts of calcium which can be demonstrated by methods analogous to those used for isolated sarcoplasmic reticulum vesicles (Fig. 5A). With this type of measurement we found that calcium transport by the sarcoplasmic reticulum of myocytes in situ is inhibited by trifluoroperazine in concentrations similar to those effective in isolated vesicles of skeletal muscle sarcoplasmic reticulum (Fig. 5B).

An interesting feature of the dissociated myocytes is that they undergo phasic contractile activation under conditions permitting sarcoplasmic reticulum function. Such an activation which results in rythmic contractions of the myocytes is strictly dependent on Ca^{2+} release from sarcoplasmic reticulum. If a divalent cation ionophore is added to prevent calcium accumulation by sarcoplasmic reticulum (and thereby release), no contractile activation is noted and the myocytes remain relaxed [5]. As expected, we found that trifluoroperazine concentrations producing total inhibition of sarcoplasmic reticulum function, also prevented activation and phasic contractions of the myocytes. On the other hand, low concentrations of trifluoroperazine which produce partial inhibition of calcium uptake by sarcoplasmic reticulum, were found to increase the frequency of phasic contractions of the myocytes (Fig. 5B). It is possible that this effect is related to trifluoroperazine interference with other regulatory mechanisms of cardiac fibers, such as those which are dependent on calmodulin.

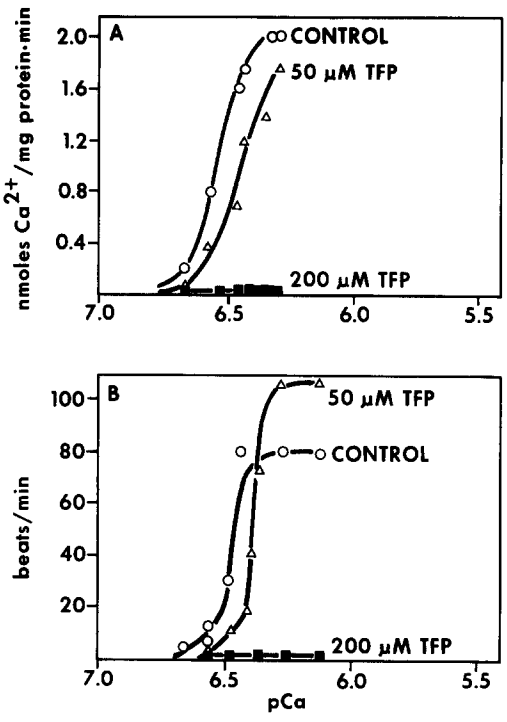

Fig. 5. Effect of trifluoroperazine on calcium uptake by sarcoplasmic reticulum in situ (A), and phasic contractile activity (B) of cardiac myoxytes. Calcium uptake (A) was measured in a reaction mixture containing the components of the cells suspension medium and 10 mM pyruvate, 10 mM glutamate, 10 mM maleate, 5 mM oxalate, 6 mM phosphocreatine, 1.2 mM ATP, 6 μg oligomycin and 12 μM FCCP (to avoid mitochondrial activity), 227 μM EGTA and $^{45}CaCl_2$ to yield the experimental p*Ca*, and 2 mg cell protein/ml. The reaction was stopped at serial times by filtration, and the radioactive activity on the washed filters was determined. The velocity of calcium uptake was obtained from linear plots of uptake vs. time. Phasic contractile activity (B) was observed on a Zeiss light microscope and recorded on a video tape. The contractile behavior of a large number of myocytes was then studied by viewing the tape on a video screen. The reaction mixture was identical to that in (A).

In parallel experiments designed to explore the possible participation of slow calcium channels in the cyclic activation of the dissociated myocytes, we used verapamil, nefedipine and dietiazem up to concentrations (10 μM) higher than those producing depression of slow currents in whole fibers [15]. We found no effects at these concentrations, either on Ca^{2+} uptake or on Ca^{2+} release by sarcoplasmic reticulum, as revealed by measurements of calcium transport or by the characteris-

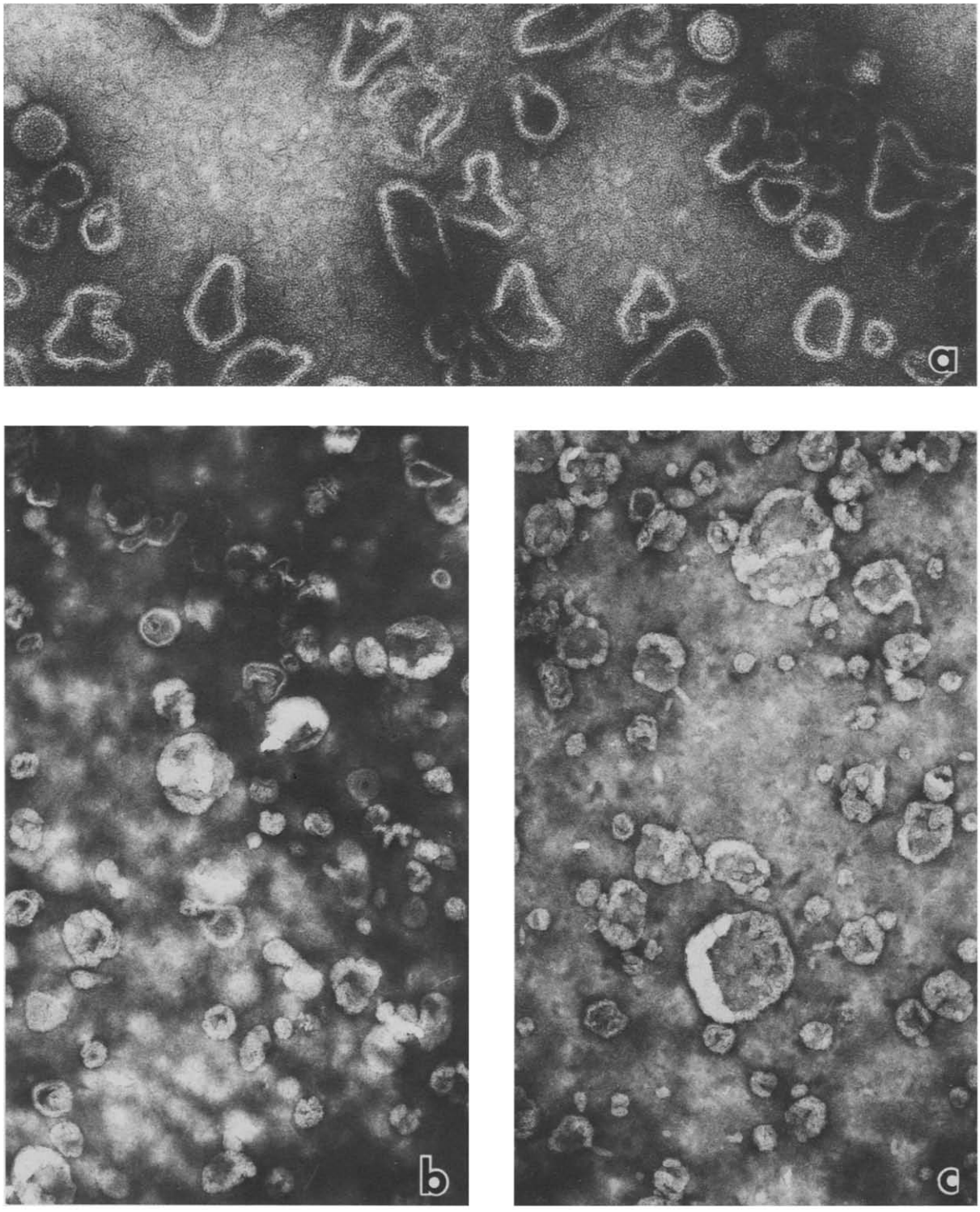

Fig. 6. Electron microscopic appearance of negatively stained sarcoplasmic reticulum vesicles. (a) Control ($\times$ 132000); (b) 50 μM trifluoroperazine ($\times$ 59200); (c) 200 μM trifluoroperazine ($\times$ 76000).

tics of contractile activation in the dissociated myocytes.

Discussion

In agreement with previous reports [1–4], we found that trifluoroperazine inhibits calcium uptake and ATPase activity of skeletal sarcoplasmic reticulum vesicles (Figs. 1–4). Furthermore, we were able to show that trifluoroperazine inhibits calcium accumulation by cardiac sarcoplasmic reticulum in situ, and interferes with its role in activation of contractile events as revealed by the behavior of dissociated myocytes. Therefore the effects first observed with subcellular particles are specifically relevant to functional mechanisms of the whole cell. Trifluoroperazine inhibits equally well skeletal and cardiac sarcoplasmic reticulum.

Inhibition of calcium uptake and ATPase activity by trifluoroperazine has been attributed to interaction of the drug with a glycoprotein component of the sarcoplasmic reticulum membrane [4], or to interaction with calmodulin and inhibition of calmodulin-dependent phosphorylation of a 60 kDa protein component [3]. We find that the concentration dependence of trifluoroperazine inhibition varies with the concentration of sarcoplasmic reticulum in the reaction mixture (Fig. 4). This indicates a fairly large partitioning of trifluoroperazine into the sarcoplasmic reticulum membrane, as independently demonstrated by ultrastructural alterations consisting of membrane thickening and loss of granular detail on the outer surface of the vesicles (Fig. 6). Therefore, the total amount of drug is a limiting factor when the concentration of sarcoplasmic reticulum is increased. It is then apparent that a structural perturbation produced by trifluoroperazine partitioning into the membrane is likely to be involved in the mechanism of ATPase inhibition. Partitioning and surface properties were previously pointed out with regards to the effects of chlorpromazine on sarcoplasmic reticulum [1], and the effects of several phenothiazines on $(Na^+ + K^+)$-ATPase [10]. From the experimental stand point, it is required that comparison of various effects of trifluoroperazine be made not only at the same drug concentration, but also at the same concentration of sarcoplasmic reticulum vesicles.

As previously noted [4], we also observed that inhibition by low trifluoroperazine concentration is prevented by increasing the Ca^{2+} concentration in the medium (Figs. 2 and 3). At higher concentrations, the inhibition is of a more general character. In this regard, it should be pointed out that trifluoroperazine inhibits the erythrocyte Ca^{2+}-ATPase not only under conditions of calmodulin activation, but also under conditions of activation by acid phospholipids or controlled proteolysis [15].

Another finding of our studies is that addition of trifluoroperazine to sarcoplasmic reticulum vesicles filled with calcium by active transport (in the absence of oxalate), produces sudden release of calcium (Fig. 1). We also found that the dependences of ATPase inhibition and calcium release on trifluoroperazine concentration are identical (Fig. 1), as if the two effects were produced by a common mechanism of enzyme and membrane perturbation. It should be also noted that not all mechanisms of ATPase inhibition produce rapid release of accumulated calcium. For instance, it is possible to produce total and sudden ATPase inhibition by adding EGTA or La^{3+} [6], which is followed by slow or no significant release. On the other hand, the trifluoroperazine induced efflux is rapid and very prominent, being comparable in this respect to the effect of divalent cation ionophores [17].

In conclusion, our experiments indicate that the effect of trifluoroperazine is principally due to structural perturbations produced by partitioning of the drug into the sarcoplasmic reticulum membrane. The effect is manifested first by a decreased affinity of the ATPase for Ca^{2+}, then by inhibition of hydrolytic activity, and finally by reduction of phosphoenzyme formation.

Acknowledgments

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