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TRIFLUOPERAZINE INHIBITION OF CALMODULIN-SENSITIVE Ca^{2+}-ATPase AND CALMODULIN INSENSITIVE ($Na^+ + K^+$)- AND Mg^{2+}-ATPase ACTIVITIES OF HUMAN AND RAT RED BLOOD CELLS

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Trifluoperazine dihydrochloride-induced inhibition of calmodulin-activated Ca^{2+}-ATPase and calmodulin-insensitive ($Na^+ + K^+$)- and Mg^{2+}-ATPase activities of rat and human red cell lysates and their isolated membranes was studied. Trifluoperazine inhibited both calmodulin-sensitive and calmodulin-insensitive ATPase activities in these systems. The concentration of trifluoperazine required to produce 50% inhibition of calmodulin-sensitive Ca^{2+}-ATPase was found to be slightly lower than that required to produce the same level of inhibition of other ATPase activities. Drug concentrations which inhibited calmodulin-sensitive ATPase completely, produced significant reduction in calmodulin-insensitive ATPases as well. The data presented in this report suggest that trifluoperazine is slightly selective towards calmodulin-sensitive Ca^{2+}-ATPase but that it is also capable of inhibiting calmodulin-insensitive ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase activities of red cells at relatively low concentrations. Thus the action of the drug is not due entirely to its interaction with calmodulin-mediated processes, and trifluoperazine cannot be assumed to be a selective inhibitor of calmodulin interactions under all cimcumstances.

Introduction

It is now clearly established that red cell Ca^{2+}-ATPase is one of several enzyme systems activated by a well characterized calcium binding protein, calmodulin [1–8]. A number of calmodulin-mediated enzymatic reactions and processes have been found to be inhibited by trifluoperazine [9–11]. Therefore this drug has been used by many investigators as a tool to demonstrate the role of calmodulin in various cellular processes [12–14]. Levin and Weiss [15] showed that trifluoperazine is a very potent and selective inhibitor of calmodulin-induced activation of Ca^{2+}-ATPase of rat red cell membranes but that concentrations of trifluoperazine up to 200 μM failed to inhibit basal Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of these membranes. On the basis of these data and the data obtained in other purified enzyme systems, namely cyclic AMP-phosphodiesterase and adenylate cyclase, Weiss and Wallace [16] suggested that trifluoperazine, and possibly other antipsychotics, produce their effects by directly blocking calmodulin action. Raess and Vincenzi [17] found that the trifluoperazine concentration required for 50% inhibition of calmodulin-induced activation of Ca^{2+}-ATPase in human red cell membranes was less than half that reported for rat red cell membranes by Levin and Weiss [15] (18 μM and 50 μM, respectively). The effect of trifluoperazine on basal Ca^{2+}-ATPase activity of human membranes was not clear from their report.

Neither of these reports provides data on the effect of trifluoperazine on calmodulin-insensitive ($Na^+ + K^+$)-ATPase of red cells.

In the present study we investigated the trifluoperazine inhibition of calmodulin-insensitive ($Na^+ + K^+$)- and Mg^{2+}-ATPase activities of red cells and re-examined the effect of trifluoperazine on calmodulin-sensitive Ca^{2+}-ATPase activities of rat and human red cells using whole hemolysates and isolated membranes. We found that trifluoperazine inhibits all the ATPase activities of red cells to a varying degree. Our data indicate that trifluoperazine is only slightly selective for calmodulin-activated Ca^{2+}-ATPase, and caution should be exercised in using trifluoperazine as a specific inhibitor of calmodulin-mediated functions under all circumstances.

Preliminary data on this study were presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics on August 16–20, 1981 at Calgary, Canada [23].

Materials

L-Histidine, Na_2-ATP, Trizma, EGTA and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO). Saponin was obtianed from Calbiochem (La Jolla, CA) and used without further purification. Chloroform and methanol were purchased from Mallinckrodt Chemical Works (St. Louis, MO). Trifluoperazine dihydrochloride (stelazine) was a generous gift from Smith, Kline& French Laboratory (Philadelphia, PA).

Methods

Isolation of membranes and hemolysate

Blood was drawn from healthy human subjects by venipuncture and from male Sprague-Dawley rats by heart or tail artery puncture and collected in heparinized tubes. The red cells were washed in 0.172 M Tris-HCl buffer (pH 7.6) and hemoglobin-free membranes were prepared by hypotonic lysis as described previously [1]. To prepare hemolysate, washed cells were lysed in 0.172 M Tris-HCl buffer (pH 7.6) containing 0.1 mg saponin/ml as described previously [7]. To expose maximum ATPase activity, membranes were pretreated with saponin [7]. Red cells and isolated membranes were counted in a Model Z Coulter Counter using 100 μm and 70 μm apertures, respectively.

Preparation of red cell calmodulin

Human red cell calmodulin was prepared by the method of Luthra and Kim [18].

Measurement of ATPase activities

ATPase activities were measured in 0.1 ml volumes of hemolysate, membrane suspension, and membrane suspension plus calmodulin. The total assay volume was 0.7 ml and contained 3.1 mM $MgCl_2$, 2.1 mM disodium ATP, 68 mM NaCl, 28 mM KCl, 68.5 mM histidine (pH 7.5), and in appropriate cases 0.5 mM ouabain, 4.8 μM $CaCl_2$, 180 μM EGTA, and 2 μg calmodulin. The trifluoperazine concentration was varied from 0 to 300 μM. The reaction was always begun with the addition of membranes or lysate which was quickly followed by the addition of ATP. The reaction was terminated after 2 h at 37°C by the addition of 1.4 ml chloroform/methanol (2:1, v/v). The inorganic phosphate released from ATP was measured in the aqueous phase as described previously [1].

Activities were expressed as μmol inorganic phosphorus (P_i) released/10^{10} cells or ghosts. The activity obtained in the presence of 180 μM EGTA was considered to be ($Na^+ + K^+ + Mg^{2+}$)-ATPase and was substracted from the activity obtained in the presence of 4.8 μM Ca^{2+} to obtain basal Ca^{2+}-ATPase and calmodulin-stimulated Ca^{2+} ATPAse values. The activity obtained in the presence of 180 μM EGTA and 0.5 mM ouabain was defined as Mg^{2+} ATPase. The activity of ($Na^+ + K^+ + Mg^{2+}$)-ATPase minus that of Mg^{2+}-ATPase gave ($Na^+ + K^+$)-ATPase. The activity obtained in the presence of Ca^{2+} and absence of EGTA and calmodulin was referred to as ($Na^+ + K^+ + Mg^{2+} + Ca^{2+}$)-ATPase. The activity obtained in the absence of EGTA and presence of Ca^{2+} and calmodulin was referred to as total ATPase. Calmodulin activation of the Ca^{2+}-ATPase of isolated membranes was obtained by subtracting the value of ($Na^+ + K^+ + Ca^{2+} + Mg^{2+}$)-ATPase from total ATPase activity. Calmodulin activation could not be obtained in lysed cells, since calmodulin was already present in the cytoplasmic lysate.

Results

Fig. 1 shows the effect of varying concentrations of trifluoperazine on the ATPase activities of rat hemolysate. All the ATPase activities were inhibited by trifluoperazine. At low trifluoperazine concentrations the inhibition of Ca^{2+}-ATPase activity was slightly greater than the inhibition of other activities. 150 μM trifluoperazine produced more than 80% inhibition of both Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities, while Mg^{2+}-ATPase activity was reduced only by 30% to 40%.

Fig. 2 illustrates ATPase activities of human hemolysate and their inhibition by trifluoperazine. All ATPase activities of human hemolysate were lower than those of rat hemolysate, but the pattern of trifluoperazine inhibition was similar.

Fig. 3 shows the effect of varying concentrations of trifluoperazine on the ATPase activities of membranes isolated from rat red cells. Comparison of Figs. 1 and 3 demonstrates that all ATPase activities were substantially lower in membranes than hemolysates. As expected, the addition of calmodulin activated Ca^{2+}-ATPase and, as in hemolysates, all ATPase activities were inhibited by trifluoperazine. At low trifluoperazine concentrations, inhibition of the calmodulin-activated portion of Ca^{2+}-ATPase was greater than the inhibition of basal Ca^{2+}-ATPase, $(Na^+ + K^+)$-ATPase, and Mg^{2+}-ATPase activities. At 150 μM trifluoperazine, $(Na^+ + K^+)$-ATPase and calmodulin activation of Ca^{2+}-ATPase were almost completely abolished. On the other hand, at the same concentration of drug, only 50% inhibition of Mg^{2+}-ATPase activity was obtained. A similar pattern of data was obtained in the case of human red cell membranes (Fig. 4). For example, at 150

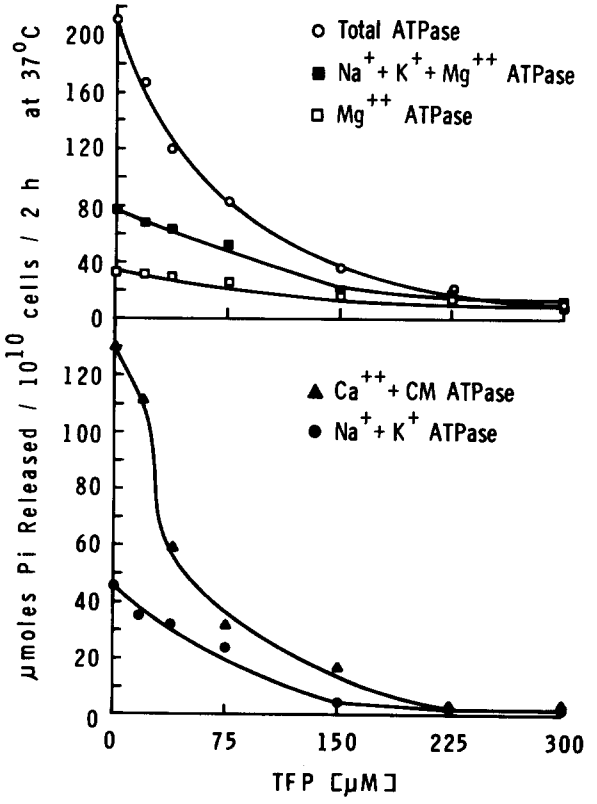

Fig. 1. Effect of varying concentrations of trifluoperazine (TFP) on ATPase activities of rat hemolysate. CM, calmodulin.

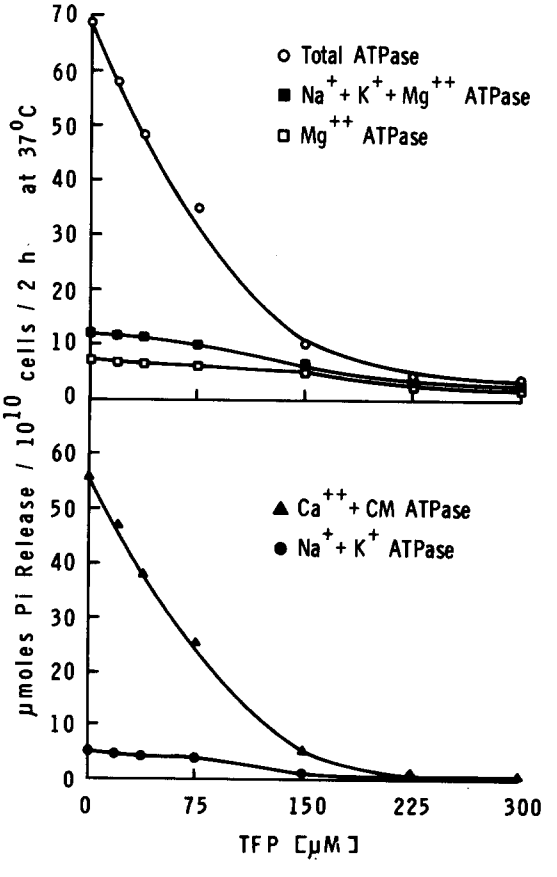

Fig. 2. Effect of varying concentrations of trifluoperazine (TFP) on ATPase activities of human hemolysate. CM, calmodulin.

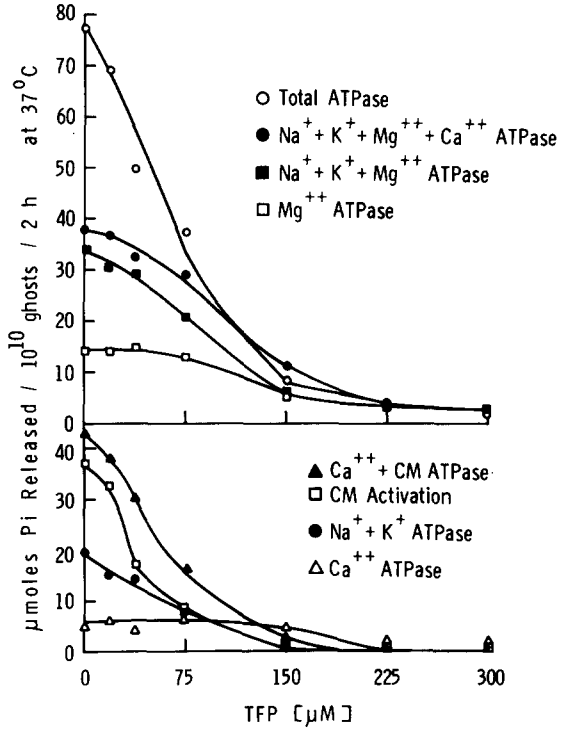

Fig. 3. Effect of varying concentrations of trifluoperazine (TFP) on ATPase activities of rat red cell membranes. CM, calmodulin.

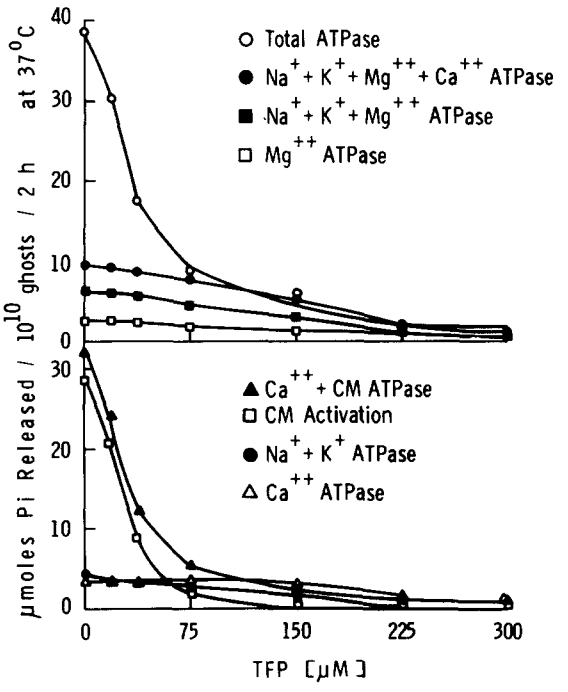

Fig. 4. Effect of varying concentrations of trifluoperazine (TFP) on ATPase activities of human red cell membranes. CM, calmodulin.

μM trifluoperazine, more than 80% inhibition of calmodulin activation of Ca^{2+}-ATPase and ($Na^+ + K^+$)-ATPase was observed, while Mg^{2+}-ATPase was reduced less than 50%.

Table I summarizes data on the inhibition of Mg^{2+}-ATPase, ($Na^+ + K^+$)-ATPase, and (Ca^{2+} + calmodulin)-ATPase in hemolysates and isolated membranes from five human subjects and five rats at 100 μM trifluoperazine. Inhibition is greatest for (Ca^{2+} + calmodulin)-ATPase, slightly less for ($Na^+ + K^+$)-ATPase, and least for Mg^{2+}-ATPase. These relationship held true in isolated membranes and hemolysates of both human and rat red cells. At this concentration of trifluoperazine one particular ATPase activity was never completely inhibited without some inhibition of others as well.

Figs. 5 and 6 show the effect of saponin on

TABLE I

INHIBITION OF ATPase ACTIVITIES BY 100 μM TRIFLUOPERAZINE

ATPase activities are expressed as percent of the activities obtained without the addition of trifluoperazine from five rats and five normal human subjects.

	Percent activity ± S.D.		
	Mg^{2+}-ATPase	($Na^+ + K^+$)-ATPase	(Ca^{2+} + calmodulin)-ATPase
Rat hemolysate	75.5 ± 5.5	47.5 ± 2.5	25.0 ± 7.0
Rat membranes	92.5 ± 5.5	46.0 ± 10.0	20.0 ± 8.0
Human hemolysate	84.5 ± 7.5	77.0 ± 5.0	32.5 ± 10.0
Human membranes	82.5 ± 15.0	73.0 ± 7.5	15.0 ± 5.0

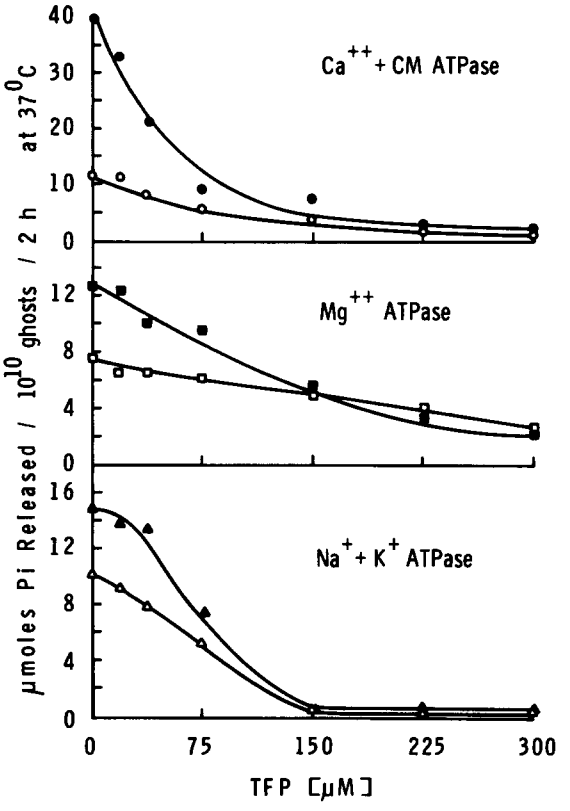

Fig. 5. Effect of varying concentrations of trifluoperazine (TFP) on ATPase activities of rat red cell membranes in the presence (closed symbols) and absence (open symbols) of saponin. Saponin was added at a concentration of 0.1 mg/ml. CM, calmodulin.

ATPase activities in the rat red cell membrane system at various concentrations of trifluoperazine.

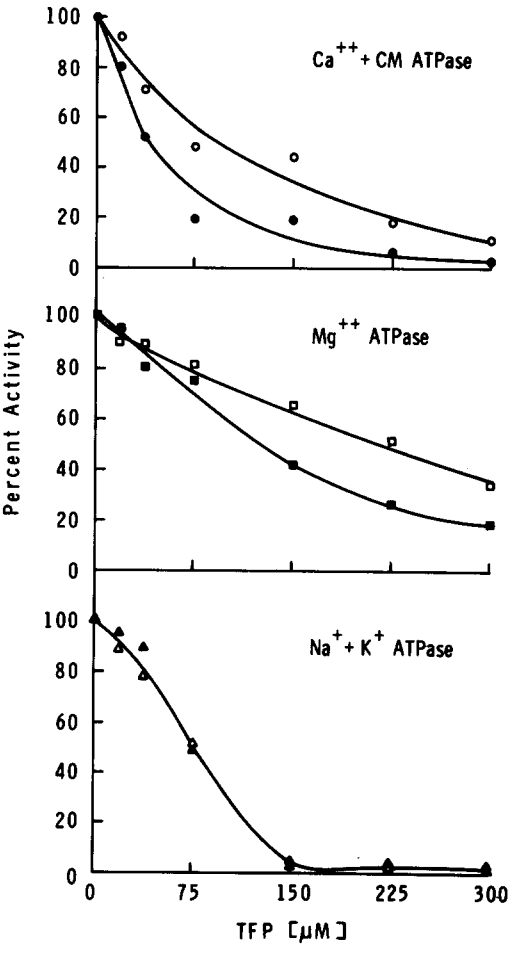

Fig. 6. Comparison of the inhibition of ATPase activities of rat red cell membranes by trifluoperazine (TFP) in the presence (closed symbols) and absence (open symbols) of saponin. ATPase activities are shown as percents of activities in samples without trifluoperazine. CM, calmodulin.

TABLE II

TRIFLUOPERAZINE INHIBITION OF ATPase ACTIVITIES OF HUMAN AND RAT RED CELL MEMBRANES

ATPase activity	I_{50}[a] (μM)(±S.E.)	
	Human ($n=8$)	Rat ($n=8$)
Ca^{2+} + CM[b]	34.1±2.0	40.2±1.8
$Na^{+}+K^{+}$	108 ±9.4	76.8±6.5
Mg^{2+}	175.6±6.0	136.9±6.6

[a] Concentration of trifluoperazine necessary for 50% inhibition of ATPase activity.
[b] Concentration of calmodulin (CM) used was 2 μg/0.7 ml incubation medium.

Discussion

Calmodulin activation of Ca^{2+}-ATPase of red cells has been reported to be inhibited by trifluoperazine [15,17], and the data presented in this report have confirmed these previous observations. The trifluoperazine concentration required to inhibit 50% of calmodulin activation (I_{50}) was found to be 40 ± 1.8 μM in rat red cell membranes (Table II), a value lower than that reported previously by Levin and Weiss [15]. The I_{50} in human red cell membranes was even lower, 34.1 ± 2.0 μM, but was higher than the value reported by Raess and Vincenzi [17]. The Ca^{2+}-ATPase activities of both isolated membranes and hemolysates of human and rat cells were substantially higher in our studies than those of other investigators [15,17]. For example, rat membrane (Ca^{2+} + calmodulin (CM))-ATPase activity in our hands was approx. 3-times higher than that reported by Levin and Weiss [15] when appropriate corrections were made for the difference in expression of the activities. As reported previously, Ca^{2+}-ATPase activity of human red cell membranes was increased if membranes were treated with saponin prior to the assay (Ref. 7). A similar observation was made in the case of rat red cell membranes (Fig. 5). It appears that the presence of saponin contributed to the low I_{50} value obtained in rat red cell membranes in our studies (Fig. 6). I_{50} values obtained in the absence of saponin were similar of those obtained by Levin and Weiss [15].

The I_{50} values for ($Na^+ + K^+$)-ATPases were 76.8 ± 6.0 μM and 108.5 ± 9.4 μM for rat and human red cells, respectively, and these values were higher than those of calmodulin-activated Ca^{2+} ATPase both in rat and human red cell membranes (Table II). The I_{50} values for Mg^{2+}-ATPase of human and rat red cell membranes were 175.6 ± 6.0 μM and 136.9 ± 6.6 μM, respectively, and these values were also higher than ($Na^+ + K^+$)-ATPase and calmodulin-activated Ca^{2+}-ATPase. 150 μM trifluoperazine almost completely inhibited (Ca^{2+} + calmodulin)-ATPase and ($Na^+ + K^+$)-ATPase activities of rat hemolysate and membranes and inhibited Mg^{2+}-ATPase by 50–60%.

The present data on Mg^{2+}-ATPase of human red cell membrane were similar to those of Raess and Vincenzi [17]. However, the data on rats are not in agreement with the data of Levin and Weiss [15] in which Mg^{2+}-ATPase was shown to be inhibited insignificantly even in the presence of 250 μM trifluoperazine. When trifluoperazine inhibition was investigated in saponin-treated and untreated membranes, Mg^{2+}-ATPase activity was higher in saponin-treated membranes (Fig. 5). Saponin treatment also enhanced trifluoperazine inhibition to some extent (Fig. 6). The percent inhibition of Mg^{2+}-ATPase was still more than 40–50% at 150 μM trifluoperazine, a value significantly higher than that reported by Levin and Weiss [15]. Since Mg^{2+} ATPase was measured in the presence of ouabain, it was conceivable that ($Na^+ + K^+$)-ATPase was not completely inhibited by 0.5 mM ouabain as used in this study. That could have influenced the observed inhibition of Mg^{2+}-ATPase. However, increasing concentration of ouabain to 2.6 mM did not produce any significant difference between trifluoperazine inhibition of ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase in hemolysates or membranes of rat red cells (data not shown). Thus the reason for the difference between the data of Levin and Weiss [15] and our data on Mg^{2+}-ATPase in rat red cells remains unknown. It may be speculated that the Mg^{2+}-ATPase activity obtained in our studies is different from theirs due to differences in the techniques of membrane isolation. Membranes prepared by our method show Mg^{2+}-ATPase activity approx. 3-times higher than those of Levin and Weiss when expressed as μmol P_i released/h per g protein. Perhaps a portion of Mg^{2+}-ATPase activity in rat membranes is susceptible to trifluoperazine and was lost in their membrane preparations.

Weiss and Wallace have recently reviewed the effects of interaction between antipsychotic agents and calmodulin [16]. It is clear that trifluoperazine has a very high binding affinity to calmodulin compared to many other drugs [16]. Binding of trifluoperazine requires calmodulin to be in its active form as a calmodulin-calcium complex. It is not clear, however, whether Ca^{2+} binds to calmodulin and then to trifluoperazine, or Ca^{2+} bound to calmodulin exposes some regions on calmodulin for its interaction with trifluoperazine. Direct interaction of trifluoperazine with various calmodulin-dependent enzymes was ruled out by

Weiss and Wallace, since the calmodulin-dependent form of cyclic AMP phosphodiesterase failed to bind radioactive trifluoperazine [16]. On the basis of data on purified cyclic AMP phosphodiesterase, Weiss and Wallace concluded that trifluoperazine also inhibited calmodulin-stimulated ATPase by selectively dissociating calmodulin from Ca^{2+}-ATPase [16]. Most investigators have assumed that trifluoperazine is a specific inhibitor of (Ca^{2+} + calmodulin)-ATPase and other calmodulin-sensitive enzymes. However, the data presented in this report show that trifluoperazine is capable of inhibiting both calmodulin-sensitive and calmodulin-insensitive and Ca^{2+}-dependent and Ca^{2+}-independent ATPase activities of red cell membranes. This report strongly suggests that trifluoperazine is capable of binding to membrane ATPases either directly or through other membrane components which regulate ATPase activities independent of Ca^{2+} and calmodulin. Thus there are at least two different mechanisms of trifluoperazine action, one involving the binding of calmodulin in the presence of Ca^{2+} and the other involving direct interaction of trifluoperazine with membrane bound ATPase molecules and possibly other activators such as phospholipids which are known to stabilize various ATPase activities when added to purified enzyme [19]. Thus trifluoperazine can inhibit ATPase activities which are not Ca^{2+}-dependent but require membrane phospholipids for their activity [20–22]. However, the interaction or trifluoperazine with other membrane components, such as phospholipids or ATPases, is probably not as strong as that with calmodulin. Thus the I_{50} for calmodulin-activated ATPases is lower than the I_{50} for other ATPases. Although trifluoperazine is slightly selective for calmodulin-activated ATPase, it must be emphasized that it was not possible to inhibit all the calmodulin-dependent ATPase activity without inhibiting calmodulin-insensitive ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase activities significantly. Therefore pharmacological action of trifluoperazine in tissues other than red cells may not be entirely due to its interaction with calmodulin-mediated processes. Furthermore, trifluoperazine cannot be regarded as a selective and specific inhibitor of calmodulin-mediated phenomenon under all circumstances.

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