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Calmodulin Activation of Red Blood Cell (Ca^{2+} + Mg^{2+})-ATPase and Its Antagonism by Phenothiazines

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SUMMARY

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Plasma membranes prepared from human red blood cells exhibit a Ca^{2+}-activated, Mg^{2+}-dependent adenosine triphosphatase activity which is a biochemical expression of the Ca^{2+} pump. The activity of this (Ca^{2+}+Mg^{2+})-ATPase was increased severalfold by the Ca^{2+}-binding protein, calmodulin, whether purified or present in a crude red blood cell hemolysate. The phenothiazines chlorpromazine (CPZ) and trifluoperazine (TFP) antagonized calmodulin activation of (Ca^{2+}+Mg^{2+})-ATPase in an apparently competitive manner. CPZ and TFP were less effective in antagonizing (Ca^{2+}+Mg^{2+})-ATPase activity in the absence of added calmodulin. TFP antagonized the calmodulin-dependent portion of (Ca^{2+}+Mg^{2+})-ATPase activity relatively specifically at 10 and 30 μM. Up to 100 μM neither haloperidol nor *d* - or *l*-butaclamol significantly antagonized calmodulin-induced activation of the (Ca^{2+}+Mg^{2+})-ATPase. Basal ATPase activity was also not inhibited. In contrast to the lack of inhibition by nonphenothiazine neuroleptics, ruthenium red nonspecifically antagonized both basal and calmodulin-activated (Ca^{2+}+Mg^{2+})-ATPase activity. It was concluded that phenothiazines antagonize activation of the (Ca^{2+}+Mg^{2+})-ATPase by binding to calmodulin. Ruthenium red appears to have a different mechanism of action. Because the potent neuroleptic nonphenothiazines, haloperidol and butaclamol, fail to antagonize calmodulin activation of red blood cell (Ca^{2+}+Mg^{2+})-ATPase it appears that general anti-calmodulin activity does not correlate with neuroleptic activity.

INTRODUCTION

Transport of calcium across the human red blood cell (RBC)[1] membrane is associated with the activity of membrane-bound (Ca^{2+} + Mg^{2+})-ATPase (1, 2). The present work, which is part of our search for a selective and potent inhibitor of plasma membrane Ca^{2+} transport, was prompted by two observations. First, a protein in the human RBC was demonstrated to activate the (Ca^{2+} + Mg^{2+})-ATPase (3–5). This protein was recently shown to be identical with calmodulin (6), also known as cyclic nucleotide phosphodiesterase activator, modulator protein, or calcium-dependent regulator protein (CDR). Calmodulin was also shown to stimulate the RBC membrane Ca^{2+} pump (7–9). These observations may imply a functional role for calmodulin in Ca^{2+} transport. Second, Levin and Weiss (10) found that trifluoperazine (TFP) and other phenothiazines bind to calmodulin and, thereby, inhibit calmodulin-induced activation of beef brain cyclic nucleotide phosphodiesterase. These authors also studied the antagonism of calmodulin-induced activation of beef brain phosphodiesterase by a series of neuroleptic drugs (11). Correlation between neuroleptic potency and anticalmodulin activity was reported. It was implied that antagonism of calmodulin may form the basis for neuroleptic activity. Such an idea is especially attractive in light of results which implicate calmodulin in the regulation of dopamine-sensitive adenylate cyclase of striatal membranes (12). Since the therapeutic effect of phenothiazines and other neuroleptic drugs is generally thought to depend on their anti-dopaminergic activity, the present work sought to examine the influence of several more or less specific neuroleptic drugs on the activation of the (Ca^{2+} + Mg^{2+})-ATPase of RBC membranes by calmodulin. Although lacking neuroleptic system specificity per se, RBC (Ca^{2+} + Mg^{2+})-ATPase has been used as a calmodulin-sensitive system in which anti-calmodulin drug action can be readily detected (13, 14). Calmodulin affects both bovine brain cyclic nucleotide phosphodiesterase and RBC (Ca^{2+} + Mg^{2+})-ATPase similarly with respect to enzyme activation, Ca^{2+} sensitivity,

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[1] Abbreviations used: RBC, red blood cell; TFP, trifluoperazine; CPZ, chlorpromazine; EGTA, ethyleneglycol bis (β-aminoethyl ether) *N*, *N'*-tetraacetic acid.

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and calmodulin-binding protein interaction. Drugs such as phenothiazines, which bind to calmodulin and thereby inhibit its interaction with phosphodiesterase, can be expected to affect (Ca^{2+} + Mg^{2+})-ATPase similarly.

For purposes of comparison, the anticipated specific anti-calmodulin effects of phenothiazines were assessed under the same conditions as the effects of ruthenium red. Calmodulin-activated and "basal" (no calmodulin added) activities of (Ca^{2+} + Mg^{2+})-ATPase were measured in each case. A preliminary report of these results has been presented (15).

METHODS

Outdated (21–35 days old), packed human RBCs from the local blood bank were used for plasma membrane ATPase preparations. The cells were washed three times in isotonic (154 mM) NaCl and the "buffy" coat was removed by aspiration. Hemolysis and subsequent steps for the preparation of membrane ghosts were carried out essentially as described previously (16). From the initial hemolysis step (1 vol of packed RBCs in 14 vol of 20 mM imidazole buffer; pH 7.4), the resulting supernate was saved, dialyzed against 50 vol of hemolyzing buffer for 12 h (dialysis tubing, Spectrapor 3, 3500 M_r cut off), and stored for subsequent addition to the ATPase assay medium. This is called hemolysate.

The ATPase assay medium contained (in a total volume of 3.0 ml) 18 mM histidine–imidazole buffer (pH 7.1 25°C), 3 mM adenosine triphosphate (Na_2ATP, pH 7.1, 25°C), 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, 0.1 mM ouabain, and 0.1 mM EGTA, with or without $CaCl_2$ to give a concentration of 1×10^{-5} M free Ca^{2+} as determined by a Ca^{2+}-selective electrode (Philips, No. IS561/Ca). Electrode measurements of free Ca^{2+} concentrations in the ATPase assay medium were performed at 37°C. Membrane protein concentrations ranged from 50 to 150 μg/ml. Membrane protein was determined by the method of Lowry *et al.* (17) or by a dye-binding assay (18) adapted to a Technicon autoanalyzer. Addition of ATP was used to start the reaction. Incubation periods were 60 or 90 min at 37°C in a Dubnoff metabolic shaking incubator. The reaction was terminated by the addition of 1.5 ml of 10% (w/v) sodium dodecyl sulfate. Inorganic phosphate determinations were performed on a Technicon Autoanalyzer using a modified Fiske–Subbarow (19) method. Details of the assay methods will be described elsewhere (20). Enzyme activities were operationally defined.

(Mg^{2+})-ATPase was that activity found in the presence of 0.1 mM EGTA and in the absence of added $CaCl_2$. (Ca^{2+} + Mg^{2+})-ATPase was defined as the additional ATP splitting obtained upon the addition of $CaCl_2$. "Basal" (Ca^{2+} + Mg^{2+})-ATPase activity was defined as that activity measured in the absence of added calmodulin, and "activated" (Ca^{2+} + Mg^{2+})-ATPase was defined as that activity measured in the presence of calmodulin. Calmodulin activation is defined as the difference between basal and activated (Ca^{2+} + Mg^{2+})-ATPase activity. From previous work it was assumed that calmodulin exerted no effect on the (Mg^{2+})-ATPase (16).

Purification of calmodulin from RBC hemolysate (non-dialyzed) was carried out by a simple, three-step ion-exchange and gel filtration method. Briefly, 1 vol of washed RBCs was hemolyzed in 15 vol of 20 mM imidazole buffer containing 0.1 mM EGTA, pH 7.4. The resulting crude hemolysate was batched onto DEAE-cellulose, washed with hemolyzing buffer containing 300 mM NaCl, and then column-chromatographed off with hemolyzing buffer containing 1 M NaCl. The eluate was diluted to lower ionic strength (500 mOsM) as measured by vapor pressure osmometry and loaded onto a DEAE-Sephadex column. It was then eluted off with a NaCl gradient between 300 and 800 mM NaCl. The fractions were assayed for calmodulin activity using (Ca^{2+} + Mg^{2+})-ATPase. Fractions containing substantial activity were pooled, concentrated by Amicon filtration (PM 10 membrane), and then chromatographed on a gel filtration column (Ultrogel AcA 54). Fractions from this column were assayed for protein by the method of Bradford (18) and for calmodulin activity using (Ca^{2+} + Mg^{2+})-ATPase activity. Fractions containing calmodulin activity were concentrated in a dialysis bag (Spectrapor 3, 3500 M_r cutoff) against crystalline sucrose. This concentrated material was dialyzed extensively against 154 mM NaCl solution and stored in this form at −20°C. The protein concentration of this stock solution was 1.3 mg/ml as measured by the method of Lowry *et al.* (17). Appropriate dilutions for use in the experiments were made with distilled, deionized water.

Phenothiazine solutions were prepared daily just prior to addition to the incubation medium. During the assay preparation steps and the entire incubation period, tubes containing phenothiazines were protected from light. These precautions were taken to avoid formation of phenothiazine free radicals which are known to interfere with (Na^+ + K^+)-ATPase (22).

Ruthenium red was obtained from K&K Laboratories Inc. (Plainview, N. Y.). Impurities were separated by the method of Luft (21) and ruthenium red concentrations determined by spectrophotometric assay at 533 nM (ϵ=6.765 × 10^4 $M^{-1} \cdot cm^{-1}$).

Na_2ATP was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.). Chlorpromazine was obtained through Sigma Chemical Company (St. Louis, Mo.). The following drugs were kindly supplied by the manufacturers: butaclamol, Ayerst Laboratories (Montreal, Canada); haloperidol, Lot No. 7701128, McNeil Laboratories (Fort Washington, Pa.); and trifluoperazine, Lot No. 5-4 TFD, Smith, Kline and French Laboratories (Philadelphia, Pa.). For each drug an appropriate solvent control was included. Unless otherwise stated, these solvent controls did not affect enzyme activities. All other chemicals were standard reagent grade. Standard statistical methods were employed with Student's *t* test used as the test for significance of differences.

RESULTS

Figure 1 demonstrates the activation of (Ca^{2+} + Mg^{2+})-ATPase by hemolysate and antagonism of the activation by CPZ. In Fig. 1, as in the work of Bond and Clough (3), calmodulin was present in a crude hemolysate of RBCs. In the absence of added hemolysate RBC membranes

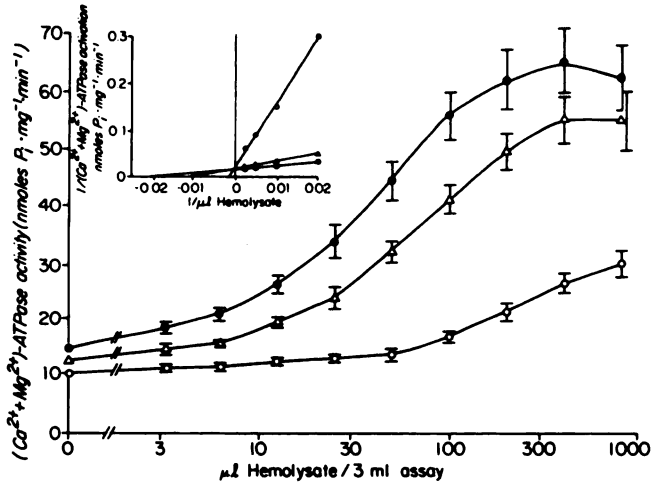

FIG. 1. *Antagonism of hemolysate-induced activation of $(Ca^{2+} + Mg^{2+})$-ATPase by chlorpromazine (CPZ)*

Effects of crude hemolysate (1:15 dilution of cell contents) in the absence and presence of two concentrations of CPZ. Hemolysate-induced activation of human RBC membrane $(Ca^{2+} + Mg^{2+})$-ATPase control in the absence of CPZ (●), and in the presence of CPZ at 3×10^{-5} M (△) and 1×10^{-4} M (○). ATPase activity assayed at 37°C as outlined under Methods. Free Ca^{2+} was 10^{-5} M. Basal $(Ca^{2+} + Mg^{2+})$-ATPase activities, i.e., activities in the absence of added hemolysate, are represented by points on the ordinate. In this and all subsequent figures, each point represents the mean value obtained from three independent membrane preparations, obtained from different blood samples. Bars represent the SEM. Inset: Double-reciprocal plot of nmol P_i·mg membrane protein^{-1}·min^{-1} versus activation ($1/V$) due to 50, 100, 200, and 400 µl hemolysate.

exhibited $(Ca^{2+} + Mg^{2+})$-ATPase activity, designated as basal $(Ca^{2+} + Mg^{2+})$-ATPase activity, of 14.67 ± 0.37 (SEM) nmol P_i·mg membrane protein^{-1}·min^{-1} (points on the ordinate). $(Ca^{2+} + Mg^{2+})$-ATPase activity increased to a maximum of 64.70 ± 5.47 (SEM; n=3) nmol P_i·mg membrane protein^{-1}·min^{-1} upon the addition of 400 µl of hemolysate to the reaction mixture (final volume = 3.0 ml). Approximately 38 µl of hemolysate assay was required for half-maximal activation. In the presence of 3×10^{-5} and 1×10^{-4} M CPZ, basal $(Ca^{2+} + Mg^{2+})$-ATPase activity was inhibited by 14 and 30%, respectively. In addition, the concentration–effect curve of hemolysate was shifted to the right in the presence of these two concentrations of CPZ. With 3×10^{-5} M CPZ the amount of hemolysate required for half-maximal activation was increased by approximately 2.5-fold. With 1×10^{-4} M CPZ, effects of up to 50 µl of hemolysate were completely antagonized. The lines in the double-reciprocal plot (inset) represent computer-fitted linear regressions based on data obtained with addition of 50, 100, 200, and 400 µl of hemolysate. The double-reciprocal plot yielded estimates of half-maximal activation by 46.8, 87.5, and 573.9 µl of hemolysate/assay in the absence and in the presence of the two concentrations of CPZ, respectively. Extrapolated maximal specific activities were 57.7, 52.3, and 41.7 nmol P_i·mg membrane protein^{-1}·min^{-1}, respectively. The data are compatible with the interpretation that CPZ competitively antagonizes calmodulin present in the hemolysate.

Apparent competitive antagonism between TFP and purified calmodulin is demonstrated in Fig. 2. As in Fig. 1, the phenothiazine exerted some inhibition of the basal activity, being significant ($P < 0.05$) only with 10^{-4} M TFP. More importantly, it can be seen that TFP exerted a major effect on the calmodulin activation of the $(Ca^{2+} + Mg^{2+})$-ATPase by shifting the concentration–effect curve to the right (1×10^{-5} M TFP) and abolishing it (10^{-4} M TFP). At 1×10^{-5} M, TFP shifted the apparent K_d for calmodulin from 0.05 to 0.26 µg/ml) (equivalent to 3.0×10^{-9} to 1.6×10^{-8} M, assuming a M_r of 16,723. This represents an approximate fivefold decrease in the apparent affinity of calmodulin for the $(Ca^{2+} + Mg^{2+})$-ATPase. It should be noted that at this concentration of TFP basal $(Ca^{2+} + Mg^{2+})$-ATPase activity was not significantly inhibited.

An estimate of the degree of purification of calmodulin can be made by comparing the two concentration–effect curves of Fig. 1 and 2 in the absence of any added drug. The amount of hemolysate required for half-maximal activation of the $(Ca^{2+} + Mg^{2+})$-ATPase was 12.83 µl/ml. This amount of hemolysate contained 268 µg of protein, mainly hemoglobin. From this and the amount of calmodulin needed for half-maximal activation (Fig. 2; 0.05 µg/ml) an approximate 5400-fold purification can be calculated. The potency of the final calmodulin preparation in the activation of $(Ca^{2+} + Mg^{2+})$-ATPase is comparable to that reported by Jarrett and Penniston (5).

Figures 3 and 4 present data obtained when $(Ca^{2+} + Mg^{2+})$-ATPase activities were determined in the absence and in the presence of 0.565 µg/ml of calmodulin. This amount was sufficient to provide maximal activation of the $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 2). Data are expressed as percentage of maximal activity in the presence of calmodulin and the absence of drug. Five different drugs were tested for anti-calmodulin activity in this manner. As may be seen in Figs. 3A, and B, there was no significant inhibition of the basal $(Ca^{2+} + Mg^{2+})$-ATPase activity by TFP up to 1×10^{-4} M ($P > 0.05$). Likewise, CPZ did not significantly inhibit basal activity over the range

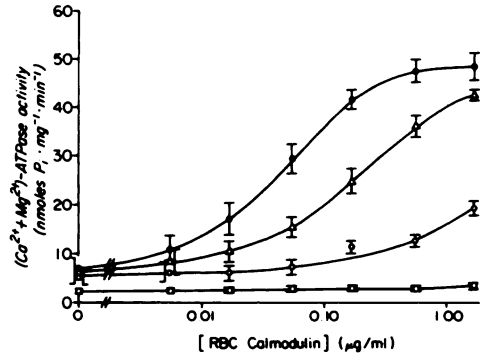

FIG. 2. *Antagonism of calmodulin-induced activation of $(Ca^{2+} + Mg^{2+})$-ATPase by trifluoperazine*

Effects of purified calmodulin in the absence and presence of three different concentrations of trifluoperazine (TFP). Calmodulin-induced activation of human RBC membrane $(Ca^{2+} + Mg^{2+})$-ATPase in the absence of TFP (●), and in the presence of TFP at 1×10^{-5} M (△), 3×10^{-5} M (○), and 1×10^{-4} M (□). ATPase was assayed as outlined under Methods. Free Ca^{2+} was 10^{-5} M. Points on the ordinate represent basal activity in the absence of added calmodulin.

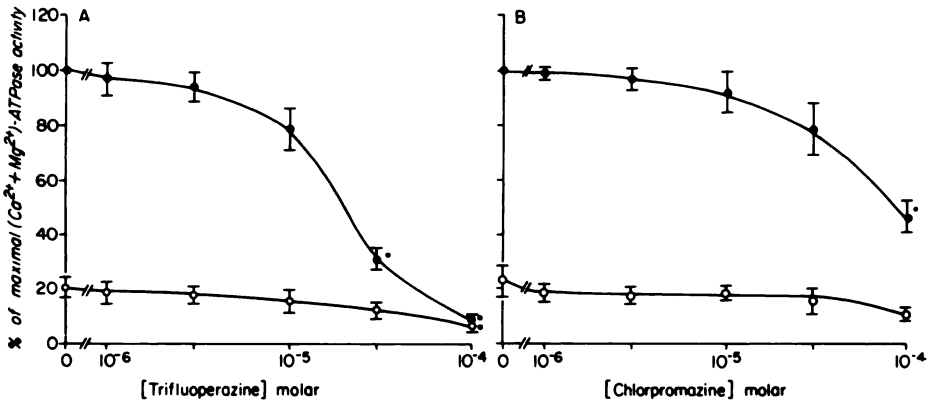

FIG. 3. *Effects of phenothiazine neuroleptics on basal and calmodulin-activated (Ca^{2+} + Mg^{2+})-ATPase*

(A) Basal (open symbols) and calmodulin (0.565 μg/ml)-activated (closed symbols) (Ca^{2+} + Mg^{2+})-ATPase activities were determined in the absence and in the presence of various concentrations of trifluoperazine (TFP). The data are plotted as a percentage of the activity in the presence of calmodulin and the absence of TFP. This is represented by the upper symbol on the ordinate which had an absolute value of 38.15 ± 7.99 (SEM) nmol P_i·mg membrane protein^{-1}·min^{-1}. All points with asterisks indicate values which differ significantly ($P < 0.05$) from the respective drug-free control value. (B) The same information applies as in (A) with the exception of chlorpromazine (CPZ) as the drug used. The absolute value for calmodulin-activated (0.565 μg/ml) control (Ca^{2+} + Mg^{2+})-ATPase in the absence of CPZ was 38.69 ± 6.93 (SEM) nmol P_i·mg membrane protein^{-1}·min^{-1}. All points with asterisks indicate values which differ significantly ($P < 0.05$) from the respective drug-free control value.

of concentrations tested ($P > 0.05$). By contrast, TFP and CPZ significantly decreased activity in the presence of calmodulin ($P < 0.05$). TFP (Fig. 3A) exhibited an apparent IC_{50} of approximately 18 μM and was, therefore, found to be more potent than CPZ which had an apparent IC_{50} of approximately 75 μM. At 1×10^{-4} M, TFP significantly inhibited both basal and calmodulin-activated (Ca^{2+} + Mg^{2+})-ATPase. In Fig. 4A and B it can be seen that over the same range of concentrations the nonphenothiazine neuroleptic drugs *d*-butaclamol (Fig. 4A) and haloperidol (Fig. 4B) were less potent in antagonizing calmodulin activation of the (Ca^{2+} + Mg^{2+})-ATPase. Furthermore, the nonneuroleptic isomer *l*-butaclamol (Fig. 4A) antagonized the activation by calmodulin to the same extent as the neuroleptically active *d*-isomer.

Ruthenium red was previously reported to inhibit the (Ca^{2+} + Mg^{2+})-ATPase of RBC membranes (23). Figure 5 shows that ruthenium red antagonized both basal and calmodulin-activated (Ca^{2+} + Mg^{2+})-ATPase. The IC_{50} values for the basal and activated (Ca^{2+} + Mg^{2+})-ATPase were estimated to be 9 and 7 μM, respectively.

DISCUSSION

Results of the present study confirm the activation of (Ca^{2+} + Mg^{2+})-ATPase by calmodulin (3–5). Calmodulin stimulated (Ca^{2+} + Mg^{2+})-ATPase when present in crude hemolysate or when added to the enzyme assay in purified form. Purified calmodulin used in this study was obtained by a relatively simple three-step procedure which yielded an approximately 5400-fold purification. The apparent K_d for calmodulin activation was 3.0×10^{-9} M. This value is in good agreement with the apparent K_d value of 4.4×10^{-9} M previously reported for activation of transport into inside-out vesicularized RBC membranes (9).

The present results confirm previous reports (15, 24) of phenothiazine antagonism of calmodulin-activated RBC (Ca^{2+} + Mg^{2+})-ATPase. The results are entirely

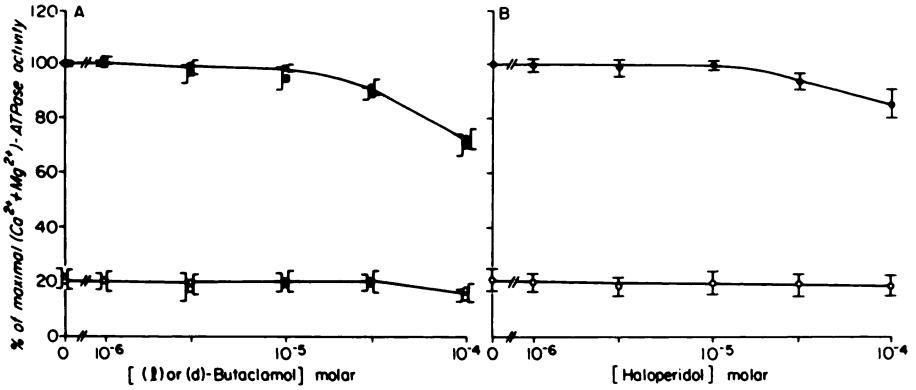

FIG. 4. *Effects of nonphenothiazine neuroleptics on basal and activated (Ca^{2+} + Mg^{2+})-ATPase*

(A) Basal (open symbols) and calmodulin (0.565 μg/ml)-activated (closed symbols) (Ca^{2+} + Mg^{2+})-ATPase activities were determined in the absence and in the presence of various concentrations of *d*-butaclamol (□) and its neuroleptically inactive isomer *l*-butaclamol (○). Experimental conditions were identical to those described in Fig. 3A. (B) Basal (open symbols) and calmodulin (0.565 μg/ml)-activated (closed symbols) (Ca^{2+} + Mg^{2+})-ATPase activites were determined in the absence and in the presence of various concentrations of haloperidol.

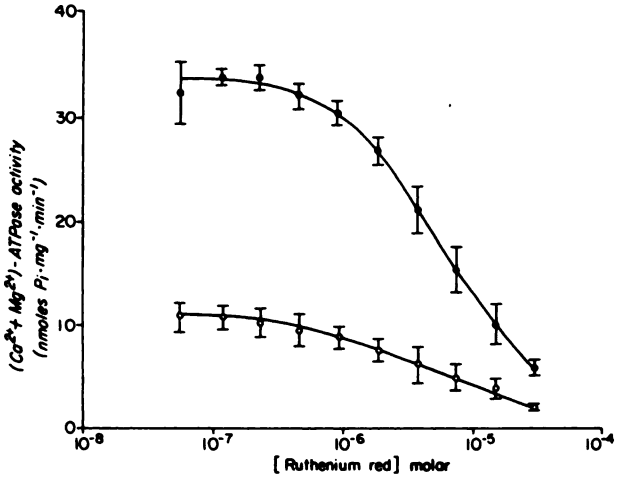

FIG. 5. *Effect of ruthenium red on basal and calmodulin-activated ($Ca^{2+}+Mg^{2+}$)-ATPase activities*

Basal (open symbols) and calmodulin (0.565 μg/ml)-activated (closed symbols) (Ca^{2+} + Mg^{2+})-ATPase activities were determined in the presence of various concentrations of ruthenium red. Under the same conditions (Mg^{2+})-ATPase (6.82 ± 0.33 (SEM) nmol P_i·mg membrane protein^{-1}·min^{-1} (not shown)) was inhibited on the average by 27 ± 3.8% (SEM) at a 3×10^{-5} M ruthenium red concentration.

consistent with the interpretation that these drugs act by binding to calmodulin, a phenomenon documented by Levin and Weiss (10). Phenothiazines bind to calmodulin only in the presence of Ca^{2+} (13). By definition, Ca^{2+} is always present during the assessment of (Ca^{2+} + Mg^{2+})-ATPase, whether calmodulin is added or not. This is not necessarily the case for the assay of cyclic nucleotide phosphodiesterases, a system more widely investigated for its calmodulin dependence (25). Farrance and Vincenzi (16) showed reversible, Ca^{2+}-dependent binding of calmodulin to RBC membranes. It was suggested that calmodulin binds to the ATPase. Evidence for such binding has recently been put forth (14). Phenothiazines probably prevent binding of calmodulin to the ATPase, although this has not been directly demonstrated.

The phenomenon of phenothiazine antagonism of calmodulin is functionally identical to the previously demonstrated (26) antagonism by calmodulin-binding protein (modulator-binding protein). It was shown that calmodulin-binding protein antagonized calmodulin activation of (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} transport (13), but exerted no effect on basal (Ca^{2+} + Mg^{2+})-ATPase activity. In the present work phenothiazines were found to inhibit calmodulin activity and, to a lesser extent, the basal (Ca^{2+} + Mg^{2+})-ATPase activity.

Phenothiazine-induced inhibition of basal (Ca^{2+} + Mg^{2+})-ATPase may be a reflection of endogenous calmodulin in the RBC membranes. Phenothiazines gaining access to the membrane could presumably antagonize tightly bound calmodulin. It is possible that calmodulin may exist as a subunit of the (Ca^{2+} + Mg^{2+})-ATPase in much the same way it exists in skeletal muscle phosphorylase kinase (28). Although the ATPase has been solubilized and reconstituted as a pump (27) no information on its subunit structure is available at this time. An alternative and very likely explanation for the decreased basal activity caused by high concentrations of phenothiazines is that these highly lipophilic drugs exert a nonspecific membrane-perturbing effect. In any event, phenothiazines can reduce the activated (Ca^{2+} + Mg^{2+})-ATPase to or below basal activity. With this result, and the results of Schatzmann (29), it may be inferred that both the basal and the activated (Ca^{2+} + Mg^{2+})-ATPase activities are capable of promoting Ca^{2+} transport. Schatzmann (29) found that CPZ slowed, but did not stop, the extrusion of Ca^{2+} from resealed RBC ghosts.

An apparent difference remains between our results and those of Weiss and Levin (11). We found minimal antagonism of (Ca^{2+} + Mg^{2+})-ATPase (basal or activated) by the relatively selective and potent dopamine antagonists haloperidol or *d*-butaclamol. By contrast, using calmodulin-induced activation of cyclic nucleotide phosphodiesterase from beef brain Weiss and Levin (11) found an IC_{50} value for haloperidol of 60 μM. Of course, it is possible that various calmodulin-mediated effects (e.g., ATPase versus phosphodiesterase) could differ. That is, calmodulin could have different sites for interaction with cyclic nucleotide phosphodiesterase on the one hand, and the Ca^{2+} pump ATPase on the other hand. One would then have to postulate that the site which activates phosphodiesterase, but not that which activates the Ca^{2+} pump ATPase, is sensitive to the binding of neuroleptic drugs. We consider this to be unlikely in view of the fact that respective IC_{50} values for phenothiazine inhibition of calmodulin-dependent phosphodiesterase, on one hand, and Ca^{2+} pump ATPase, on the other hand, are very similar. In any event, since haloperidol and *d*-butaclamol are both more potent and more specific neuroleptics than TFP and CPZ and since the nonneuroleptic *l*-isomer of butaclamol behaves identically with the neuroleptically active isomer, we must conclude that neuroleptic activity does not correlate with antagonism of (Ca^{2+} + Mg^{2+})-ATPase activation.

It must be noted that drug concentrations, and therefore IC_{50} values, in this study are nominal only. There is considerable binding of these drugs to glassware and membranes. Preliminary results (not shown) from radiometric and spectrophotometric measurements of TFP indicate that only about 40% of the total drug was free at any given reported concentrations. According to these preliminary measurements, the remaining fraction of total TFP was associated with the membrane and the glass test tube in approximately equal fractions. Therefore, in accordance with the recommendations of Seeman (30), we do not consider that the IC_{50} values represent K_i values for the various drugs. In spite of the quantitative difficulties which have been mentioned, relative potencies can be compared and qualitative differences can be appreciated. Thus, we anticipate that relatively selective dopamine antagonists such as haloperidol will not be found to be particularly potent in antagonizing calmodulin-mediated effects. It seems likely that the multiple side effects of the phenothiazines (and perhaps to some extent certain therapeutic effects) may be related to widespread antagonism of calmodulin. This would indirectly modify many cellular processes which calmodulin presumably controls (25).

Ruthenium red an inhibitor of mitochondrial transport (31) inhibited both basal and calmodulin-activated (Ca^{2+}

+ Mg^{2+})-ATPase activities of RBC membranes. Thus, one would predict that ruthenium red would also inhibit Ca^{2+} transport across RBC plasma membranes. The site and mechanism of ruthenium red action on the (Ca^{2+} + Mg^{2+})-ATPase are unknown. Since ruthenium red exhibits a similar inhibitory effect on both the basal and calmodulin-activated Ca^{2+} pump ATPase, neither competition at the calmodulin binding sites nor interaction with calmodulin itself is likely. We interpret this to mean that ruthenium red does not act on calmodulin like phenothiazines, but rather at another site; probably on the catalytic portion of the ATPase in the membrane. Considering its molecular structure, it seems reasonable to suggest that ruthenium red competes with Ca^{2+} for pump sites.

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