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Plasma Membrane Ca^{2+} Transport: Antagonism by Several Potential Inhibitors

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Summary. Inside-out vesicles prepared from human red blood cells took up Ca^{2+} by an active transport process. Membranes from the same red blood cells displayed Ca^{2+}-activated, Mg^{2+}-dependent adenosine triphosphatase activity. Both the initial rate of Ca^{2+} transport and the $(Ca^{2+}+Mg^{2+})$-adenosine triphosphatase activity were increased approximately twofold by the calcium binding protein, calmodulin. Activities in the absence of added calmodulin were termed basal activities. Calmodulin-activated Ca^{2+} transport and adenosine triphosphatase activities could be antagonized in a relatively selective fashion by the phenothiazine tranquilizer drug, trifluoperazine. High concentrations of trifluoperazine also inhibited basal Ca^{2+} transport and adenosine triphosphatase activity. By contrast, calmodulin binding protein from beef brain selectively antagonized the effect of calmodulin on Ca^{2+} transport with no inhibition of basal activity. Ruthenium red antagonized calmodulin-activated and basal activity with equal potency. The results demonstrate that although phenothiazines can act as relatively selective antagonists of calmodulin-induced effects, other effects are possible and cannot be ignored. Calmodulin-binding protein may be a useful tool in the analysis of calmodulin functions. Ruthenium red probably interacts with Ca^{2+} pump adenosine triphosphatase at a site not related to calmodulin.

Low intracellular Ca^{2+} concentration in human red blood cells (RBCs) is maintained by an active extrusion mechanism (Schatzmann & Vincenzi, 1969; Schatzmann, 1975). Isolated plasma membrane preparations from RBCs exhibit a Ca^{2+}-activated, Mg^{2+}-dependent adenosine triphosphatase activity, or $(Ca^{2+}$-$Mg^{2+})$-ATPase, which presumably is the biochemical expression of this calcium pump mechanism (Schatzmann & Vincenzi, 1969).

Calmodulin (CaM) is a ubiquitous Ca^{2+} binding protein found in most eukaryotic cells including the RBC (Bond & Clough, 1973; Jarrett & Penniston, 1978). CaM regulates a number of Ca^{2+}-dependent enzyme systems (Cheung, 1980). CaM can activate the $(Ca^{2+}+Mg^{2+})$-ATPase of RBC membranes and the transport of calcium into inside-out vesicles (IOVs) made from RBCs (Hinds, Larsen & Vincenzi, 1978; Larsen & Vincenzi, 1979) in a concentration-dependent fashion. The main purpose of the present work was to demonstrate the activity of the calcium pump and its sensitivity to several potential inhibitors. To this end, three known inhibitors of the $(Ca^{2+}+Mg^{2+})$-ATPase were compared for their effects upon the Ca^{2+} pump of IOVs and on the plasma membrane $(Ca^{2+}+Mg^{2+})$-ATPase of the same cells. As a corollary it will be shown that the Ca^{2+} pump is a functional manifestation of the $(Ca^{2+}+Mg^{2+})$-ATPase.

Phenothiazines antagonize CaM activation of both the Ca^{2+}-dependent cyclic AMP phosphodiesterase of bovine brain (Levin & Weiss, 1978*a*; Weiss & Levin, 1978) and the $(Ca^{2+}+Mg^{2+})$-ATPase of RBCs (Levin & Weiss, 1978*b*; Vincenzi et al., 1978). In the presence of Ca^{2+} phenothiazines bind to CaM in the ratio of two drug molecules per CaM (Levin & Weiss, 1977). This drug-protein complex is inactive in stimulating CaM-dependent enzymes such as phosphodiesterase. Apparently by the same mechanism these drugs inhibit the CaM-activated portion of $(Ca^{2+}+Mg^{2+})$-ATPase.

Wang and Desai (1977) isolated from beef brain a protein they called modulator binding protein. This protein, which could also be called CaM-binding protein (Cheung, 1980), antagonizes CaM-induced activation of phosphodiesterase (Wang & Desai, 1977) and plasma membrane $(Ca^{2+}+Mg^{2+})$-ATPase but does not affect its basal activity (Larsen et al., 1978*b*). This type of antagonism is functionally similar to the antagonism exhibited by phenothiazines. In both cases binding of the antagonist to CaM depends on Ca^{2+} and the CaM-antagonist complex is apparently unable to interact with the enzyme.

Ruthenium red (RR), an inorganic dye used in the staining of tissues, cells, and organelles (Luft, 1971), has been shown to be an inhibitor of calcium transport in mitochondria (Moore, 1971). RR was also shown to be a somewhat specific inhibitor of

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the RBC ($Ca^{2+}+Mg^{2+}$)-ATPase (Watson, Vincenzi & Davis, 1971). The effect of this substance on the basal and activated forms of the ($Ca^{2+}+Mg^{2+}$)-ATPase was recently reported (Raess & Vincenzi, 1980*b*). It was suggested that RR reacts with the ATPase enzyme (rather than CaM) because it caused proportionally equivalent amounts of inhibition of both the CaM-activated and basal activities. Therefore, we sought to confirm this effect and extend the observation by measuring Ca^{2+}-pump activities and ($Ca^{2+}+Mg^{2+}$)-ATPase activities of the same membrane preparations. Preliminary results of some of these data have been presented (Raess & Hinds, 1979).

Materials and Methods

Fresh blood was obtained from healthy adult volunteers. The blood was centrifuged at 2,000 rpm for 5 min (Sorvall SS-34 rotor) prior to removal of plasma and buffy coat by aspiration. The remaining cells were washed three times with equal volumes of cold isotonic (0.9% NaCl) saline under the same conditions.

IOVs were prepared from the washed RBCs by a method modified from that of Steck and Kant (1974), with omission of the density gradient step (Larsen & Vincenzi, 1979). The crude membrane mixture (IOVs and broken membranes) was suspended in 2–3 volumes of 40 mM tris-glycylglycine buffer (pH 7.1) with 0.1 mM $MgCl_2$, and centrifuged at 15,000 rpm for 30 min. The resulting pellet consisted of two distinct layers; a loose fluffy layer and a hard-packed button. The loose fluffy layer was removed by gentle swirling in a small volume of the same buffer. The hard pellet was gently resuspended in the same buffer and recentrifuged. Again, the loose fluffy material was removed. The hard pellet was rich in IOVs. With care we obtained up to 72–74% sealed IOVs. The remaining contaminant consisted solely of broken membranes. Membranes were stored at a concentration of approximately 3 mg/ml in the wash buffer. The loose fluffy membrane suspension which consisted mainly of broken membranes, was saved for the measurement of ($Ca^{2+}+Mg^{2+}$)-ATPase activities. This material usually contained less than 5% IOVs.

The percentage of IOVs was determined by the latency of acetylcholine esterase (AChE) activity in the presence and absence of Triton-X-100 (Steck, 1974). The percentage of right-side-out vesicles was determined by the latency of glyceraldehyde-3-phosphate dehydrogenase (G-3PD) in the presence and absence of Triton X-100 (Steck, 1974). Membrane protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) in the presence of 0.02% sodium dodecyl sulfate (SDS) using bovine serum albumin as a standard.

$^{45}Ca^{2+}$ transport experiments were performed at 37 °C with constant, gentle stirring in a final reaction volume of 1.0 ml. The standard reaction medium contained (final concentrations) 9.0 mM histidine-imidazole buffer (pH 7.1, 25 °C), 40 mM NaCl, 7.5 mM KCl, 3.0 mM $MgCl_2$, 0.1 mM ouabain, 3.0 mM ATP (pH 7.1), 0.1 mM $CaCl_2$ (sp act 0.68 or 1.62×10^7 cpm/μmol), 50–150 μg membrane protein, and other additions as indicated. The reaction mixtures were preincubated for 10 min and transport was initiated by the addition of ATP. At time zero and at specific time intervals, 100 μl of the transport reaction mixture were removed and quenched in 2.2 ml ice-cold 40 mM Tris glycylglycine buffer (pH 7.1) with 0.1 mM $MgCl_2$. The IOVs were rapidly collected by vacuum filtration on an Amicon microporous filter (25 mm diameter, 0.45 μm), and washed once with 2.2 ml of the same buffer. The filters were dissolved in 15 ml Aquasol® and were counted for $^{45}Ca^{2+}$.

Assays of ATPase activity of the fluffy membrane fraction were set up as previously described (Farrance & Vincenzi, 1977) with modifications as noted. The incubation medium contained in a final volume of 3.0 ml: approximately 100 μg of membrane protein; 3 mM ATP (pH 8.1); 18 mM histidine imidazole buffer (pH 7.1, 25 °C); 3 mM $MgCl_2$; 80 mM NaCl; 15 mM KCl; 0.1 mM ouabain; 0.1 mM ethyleneglycol-bis-(β-amino-ethyl) ether)N,N′-tetracetic acid (EGTA); without (for Mg^{2+}-ATPase activity) or with 0.2 mM $CaCl_2$ [for ($Ca^{2+}+Mg^{2+}$)-ATPase activity]. CaM was purified from outdated human red blood cells by the method of Jung (1978).

Membranes were incubated for 90 min at 37 °C in a Dubnoff metabolic shaking incubator. The reaction was terminated by the addition, with mixing, of 1.5 ml of 10% SDS. Inorganic phosphate (P_i) liberated was determined by the Fiske and Subbarow (1925) method adapted to an autoanalyzer (Raess & Vincenzi, 1980*a*).

Trifluoperazine (TFP) solutions were made up fresh before each assay. Care was taken to protect the stock solutions from light to prevent the formation of free radicals (Akera & Brody, 1969). TFP and ^{3}H-TFP were generous gifts from Smith Kline and French Laboratories, Philadelphia, Pa. TFP concentrations were measured from optical density measurements at 256 nm ($\varepsilon=3.26\times10^4\ M^{-1}\cdot cm^{-1}$).

CaM-binding protein (modulator binding protein) from beef brain was a generous gift from Dr. J. Wang (Winnipeg, Canada). It was washed three times in saline using an Amicon Centriflo Membrane Cone (CF-25) and concentrated to 200 μg/ml.

Commercially available RR (K&K Laboratories, Philadelphia, Pa.) was repurified by differential solubilization according to the method of Luft (1971). RR concentrations were determined from optical density measurements at 533 nm ($\varepsilon=6.77\times10^4\ M^{-1}\cdot cm^{-1}$).

A 0.1 mM stock solution of the calcium ionophore A23187 (a gift from Eli Lilly, Indianapolis, IN) was prepared in absolute ethanol. $^{45}Ca^{2+}$ was purchased from ICN (Irvine, CA) and ATP was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). All other reagents were reagent grade.

Results

In order to determine the time course of Ca^{2+} transport, experiments were run for various lengths of time in the presence and absence of CaM. It was found that the uptake of $^{45}Ca^{2+}$ became nonlinear after the first 5 min and would plateau at about 36 min in the absence of added CaM and at about 18 min in the presence of added CaM. If allowed to continue the absolute amount of Ca^{2+} transported into IOVs in the presence and absence of CaM was identical (*see* Table 1). It was concluded that the best time for determination of transport rates at 37 °C would be within the first three minutes. Within this time interval the amount of $^{45}Ca^{2+}$ taken up was a linear function of time. Regression lines of such data gave r values of 0.989–0.999.

As shown in Fig. 1, the initial rate of $^{45}Ca^{2+}$ transport was influenced by addition of CaM to the incubation medium. In the absence of added CaM the rate of Ca^{2+} transport was 14.4 nmoles/min/mg IOV protein. With increasing concentrations of added CaM the Ca^{2+} transport increased to 26.8 nmoles/

Table 1. Effects of trifluoperazine (TFP) on Ca^{2+} accumulation, Ca^{2+} transport and Ca^{2+} pump-ATPase activity of RBC membranes

[TFP] (M)	Ca^{2+} accumulation[a]	Ca^{2+} transport[b]	$(Ca^{2+}+Mg^{2+})$ ATPase[c]
0	7.40 (≡100%)	≡100%	≡100%
1×10^{-6}	7.52 (102%)	99%	104%
3×10^{-6}	7.71 (104%)	92%	98%
1×10^{-5}	7.77 (105%)	91%	93%
3×10^{-5}	8.07 (109%)	73%	76%
1×10^{-4}	5.53 (75%)	32%	30%
3×10^{-4}	0.07 (1%)	0	0

[a] mmoles Ca^{2+}/liter ionophore accessible volume associated with IOVs in the presence of 3.3 μg/ml CaM, at the end of 90 min. Accumulation of Ca^{2+} in the absence of TFP is defined as 100%.

[b] Percent of maximal (initial 0 to 3 min) rate of Ca^{2+} transport in the absence of TFP.

[c] Percent of maximal $(Ca^{2+}+Mg^{2+})$-ATPase activity in the absence of TFP. Reaction conditions are as described in Fig. 3*A* and 3*B*. For comparison, the accumulation, transport rate and ATPase activities determined in the absence of CaM and TFP were 8.76 mmoles Ca^{2+}/liter and 42 and 37% of the values obtained in the presence of CaM, respectively.

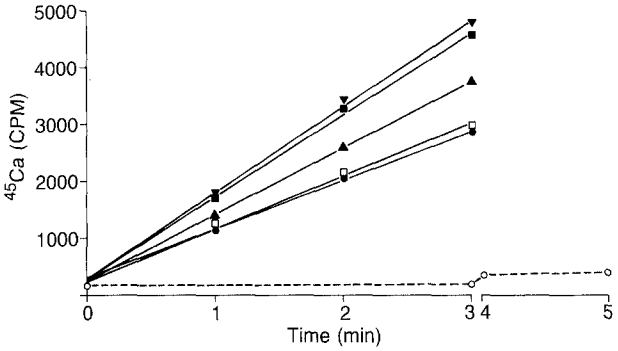

Fig. 1. Initial Ca^{2+} transport into IOVs. Points represent typical raw data values used to derive initial Ca^{2+} transport rates such as in Figs. 2, 3*A*, 4 and 5*A*. IOVs were preincubated at 37 °C as described in Materials and Methods and uptake was initiated by addition of ATP; except in the ATP-free control (○–○). Uptake was terminated by addition to ice-cold Trisglycylglycine buffer and rapid filtration. Filters were then washed once with the same medium. Uptake of $^{45}Ca^{2+}$ was measured in the absence (●–●) and in the presence of several concentrations of CaM: 0.11 (□–□); 0.33 (▲–▲); 1.1 (■–■); and 3.3 μg/ml (▼–▼). IOVs displayed ATP-dependent $^{45}Ca^{2+}$ uptake. Accumulated $^{45}Ca^{2+}$ rapidly lost upon addition (at time 3 min) of the Ca^{2+} ionophore, A23187 (not shown). As shown, the ionophore also caused a modest increase of $^{45}Ca^{2+}$ in IOVs in the absence of ATP. Incubation medium contained 131 μg membrane protein/ml. Membrane material was determined to be 64% sealed IOVs

min/mg IOV protein. This represents an 86% increase above the "basal" transport rate (the rate measured in the absence of added CaM). This degree of activation by CaM is slightly less than the value obtained previously in this laboratory under similar conditions (Larsen & Vincenzi, 1979). It should be noted that the uptake of $^{45}Ca^{2+}$ into IOVs is an active transport process. This uptake occurs against an electrochemical gradient. When the Ca^{2+} ionophore, A23187 (1.6×10^{-5} M) was added to the medium, accumulated Ca^{2+} was rapidly lost from the IOVs (data not shown).

As expected for an active transport process, the uptake of $^{45}Ca^{2+}$ into IOVs is dependent upon ATP. As shown in Fig. 1, there was no uptake of $^{45}Ca^{2+}$ into IOVs in the absence of ATP. When 1.6×10^{-5} M A23187 was added to the ATP-free control incubation medium there was a small increase in $^{45}Ca^{2+}$ associated with the IOVs. This demonstrates that IOVs exclude $^{45}Ca^{2+}$ in the absence of ATP and also allows one to estimate the intravesicular volume. We have previously termed this volume the "ionophore accessible volume" (Hinds et al., 1978). In the experiment depicted in Fig. 1, the intravesicular volume estimated by this method was equal to 21 μl/mg protein. Considering the specific activity of $^{45}Ca^{2+}$ in this system the internal Ca^{2+} concentration at the end of 3 min of transport was 2.5 mM in vesicles exposed to the highest concentration of CaM. This surprisingly high intravesicular Ca^{2+} concentration is the result of the very small volume/surface relationship of IOVs and the high capacity of the Ca^{2+} pump. It should be noted that the vesicular volume is small compared to the total incubation medium. Thus, in the example above, when the average internal IOV $[Ca^{2+}]$ was 2.5 mM the $[Ca^{2+}]$ in the incubation medium had decreased from its nominal value of 0.1 mM by only 7%.

The data in Fig. 2 show the concentration effect relationship of CaM on $^{45}Ca^{2+}$ transport and the influence of three concentrations of TFP thereon. This curve depicts the typical response of the Ca^{2+} pump to activation by CaM (Larsen and Vincenzi, 1979) with an apparent K_d for CaM of 18.5 nM. In the absence of added TFP, CaM produced maximum activation of $^{45}Ca^{2+}$ transport of 86% above the basal rate. In the presence of 1×10^{-5} M TFP the CaM concentration-effect curve was shifted to the right, suggestive of competitive antagonism. The basal activity (no CaM) was depressed somewhat but at this TFP concentration inhibition was not always seen (e.g., *see* Fig. 3*A*). At 3×10^{-5} M TFP, the concentration-effect curve was further displaced to the right with the highest concentration of CaM (3.3 μg/ml) eliciting only a 13% activation of Ca^{2+} transport above the basal. At 1×10^{-4} M TFP, there was no CaM-dependent activation of $^{45}Ca^{2+}$ transport. A significant decrease (38%) in basal activity was observed at this drug concentration. Considering the lipophilic nature of phenothazines, inversion of or

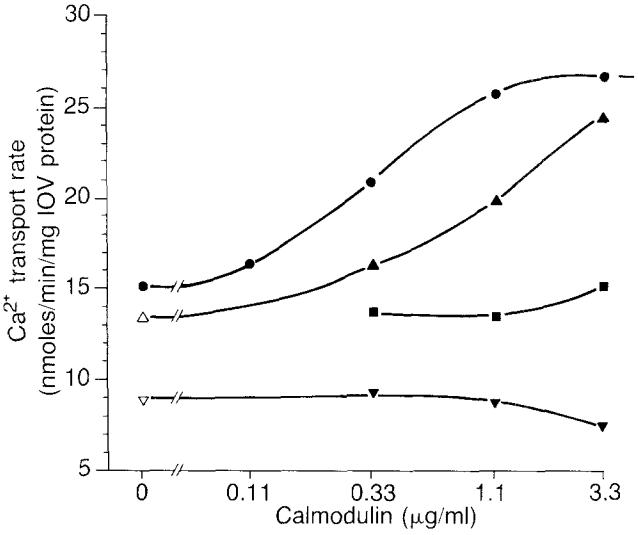

Fig. 2. CaM activation of Ca^{2+} transport: concentration effect relationships in the absence and presence of TFP. The net Ca^{2+} transport rate is plotted as a function of log [CaM]. Data in the absence of TFP (●–●) are taken from Fig. 1. Calcium transport was inhibited with TFP at 1×10^{-5} (▲–▲), 3×10^{-5} (■–■), and 1×10^{-4} M (▼–▼), respectively

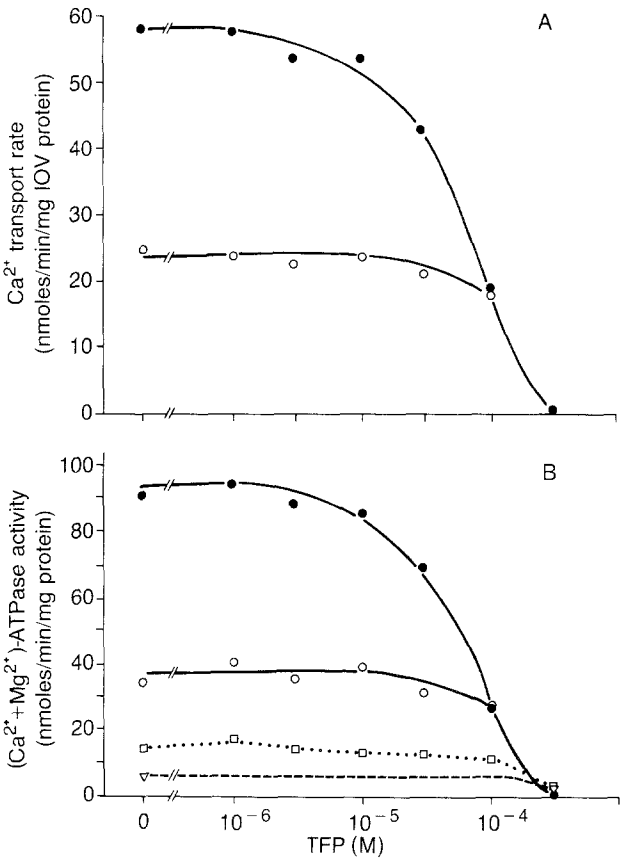

Fig. 3. Trifluoperazine: concentration-effect relationships on Ca^{2+} transport and $(Ca^{2+}+Mg^{2+})$-ATPase. (*A*) Concentration-dependent inhibition of CaM-activated (●–●) and basal (○–○) Ca^{2+} transport by TFP. Membrane protein concentration was 102 μg/ml; 50.5% was present as IOVs. When present, CaM was 3.3 μg/ml, a concentration sufficient for maximal activation. (*B*) Concentration-dependent inhibition of three operationally defined membrane ATPases by TFP: CaM-activated (3.3 μg CaM/ml) $(Ca^{2+}+Mg^{2+})$-ATPase (●–●), basal, $(Ca^{2+}+Mg^{2+})$-ATPase (○–○), $(Na^{+}+K^{+}+Mg^{2+})$-ATPase (□–□), Mg^{2+}-ATPase (▽–▽). Membrane protein concentration was 34 μg/ml. For ATPase assay, membranes were incubated at 37 °C for 90 min. The reaction was terminated by the addition of SDS and inorganic phosphate was assayed as described in Materials and Methods

disruption of the IOVs to right-side-out vesicles was considered as a possible artifact. For example, inversion would prevent substrate access and reduce all activities and disruption would increase the amount of broken membranes. However, sidedness assays showed that the membranes used in the experiments shown in Fig. 2 were 64% inside-out at the beginning and after approximately 20 min at 37 °C with gentle stirring they were 58% inside-out. At 3×10^{-4} M TFP there was no transport of Ca^{2+} (Table 1).

The concentration dependence of the effects of TFP on both the basal and activated pump rates was examined in the presence and absence of 3.3 μg/ml CaM, respectively. Figure 3*A* shows the effects of increasing TFP concentrations upon the basal and CaM-activated rates. As expected from Fig. 2, the major effect with lower concentrations of TFP was upon the CaM-activated form of the enzyme, with little effect upon the basal pump activity, up to about 3×10^{-5} M TFP. At this concentration, CaM-activated transport was antagonized by 45%, whereas the basal transport was decreased by only 9%. At 1×10^{-4} M TFP, CaM-activated transport was totally abolished, while basal transport decreased by 26.5%. At 3×10^{-4} M TFP, no Ca^{2+} transport was detected.

Activity of the RBC Ca^{2+} pump has been associated with the activity of membrane-bound $(Ca^{2+}+Mg^{2+})$-ATPase. ATPase activities of the fluffy membrane fraction obtained during the preparation of IOVs were therefore examined over the same range of TFP concentrations. For comparison, and as a control for the specificity for the effect of TFP upon the $(Ca^{2+}+Mg^{2+})$-ATPase, two other operationally-defined ATPase activities were also examined (Fig. 3*B*). In Fig. 3*B*, as in Fig. 3*A*, solid lines represent CaM-activated and basal activities, respectively. The dotted line represents $(Na^{+}+K^{+}+Mg^{2+})$-ATPase and the dashed line represents Mg^{2+}-ATPase activity. The effects of TFP on the basal and activated $(Ca^{2+}+Mg^{2+})$-ATPase activities were identical to those on the Ca^{2+} pump. At 3×10^{-5} M TFP, the CaM-activated ATPase activity decreased by 44% and basal activity decreased by 12%. At 1×10^{-4} M TFP, there was total loss of CaM activation of the enzyme while the basal activity was depressed by

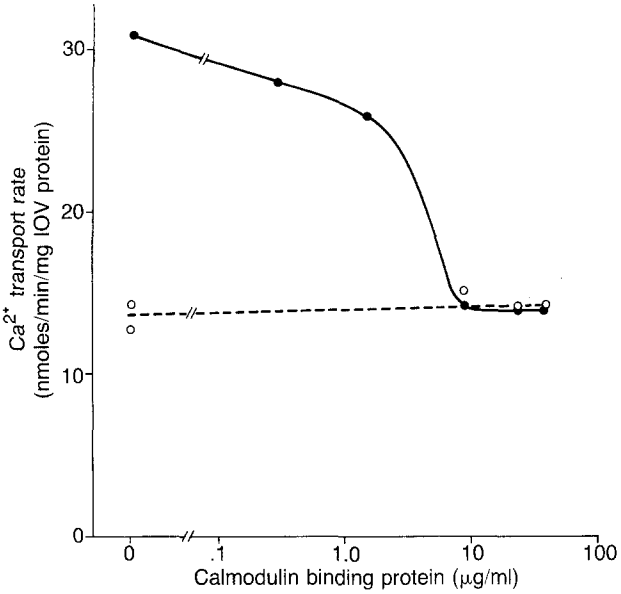

Fig. 4. Inhibition of CaM-activated Ca^{2+} transport by CaM-binding protein. Ca^{2+} transport into IOVs was measured as in Fig. 1 in the absence (o–o) and presence (•–•) of CaM. When added, CaM was present at 2.2 μg/ml. CaM-binding protein antagonized CaM activation of Ca^{2+} transport but did not inhibit basal transport

about 22%. The $(Na^+ + K^+ + Mg^{2+})$-ATPase activity was fairly constant up to 1×10^{-4} M TFP and was inhibited at 3×10^{-4} M.

It should be noted that concentrations of trifluoperazine mentioned here must be considered nominal only. We anticipated that a significant fraction of TFP might bind to the membranes and/or to the walls of the test tube. Using both a spectrophotometric method and ^{3}H-TFP we concluded that concentrations of free TFP were 43% of the total between 1×10^{-6} M and 1×10^{-4} M. The fraction bound appeared to be independent of the total concentration. The bound TFP appeared to be approximately equally distributed between the membranes and glassware.

The effect of CaM-binding protein on the Ca^{2+} pump is demonstrated in Fig. 4. Where added, CaM was present at 1.3×10^{-7} M. CaM-binding protein decreased the CaM-activated portion of the Ca^{2+} pump activity but did not inhibit basal pump activity. Even with 39 μg/ml CaM-binding protein (4.6 times greater than the concentration needed for complete antagonism of exogenous CaM) we were unable to show any significant inhibition of the basal Ca^{2+} pump rate. This suggests that IOVs are free of endogenous CaM; or at least CaM which is accessible to CaM-binding protein.

Concentration-effect relationships of RR are displayed in Fig. 5. In contrast to TFP and CaM-binding protein, RR did not selectively antagonize CaM. In

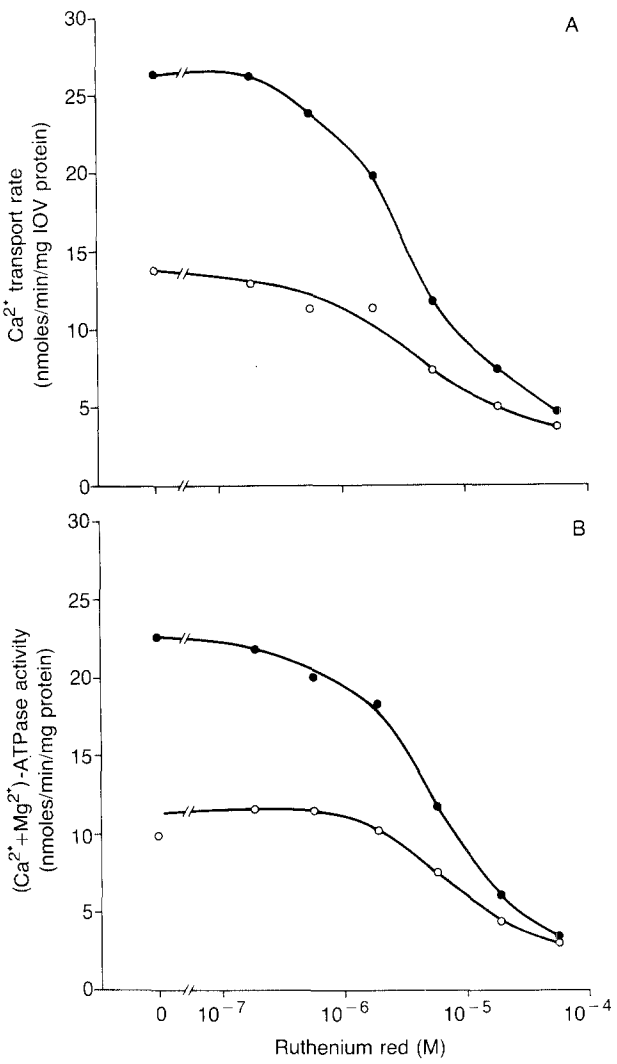

Fig. 5. Ruthenium Red (RR): Concentration-effect relationships on Ca^{2+} transport and $(Ca^{2+}+Mg^{2+})$-ATPase. (*A*) Concentration-dependent inhibition of CaM-activated (•–•) and basal (o–o) Ca^{2+} transport by RR. Membrane protein concentration was 109 μg/ml; 72.2% was present as IOVs. When present, CaM was 3.3 μg/ml; a concentration sufficient for maximal activation. (*B*) Concentration-dependent inhibition of CaM-activated (3.3 μg/ml) $(Ca^{2+}+Mg^{2+})$-ATPase (•–•), and basal $(Ca^{2+}+Mg^{2+})$-ATPase (o–o) by RR. Membrane protein concentration was 22 μg/ml. ATPase assay was as in Fig. 3

Fig. 5*A*, it can be seen that RR decreased both basal and activated Ca^{2+} pump rates with equal IC_{50} values of about 4×10^{-6} M. In Fig. 5*B*, similar effects on basal and activated $(Ca^{2+}+Mg^{2+})$-ATPase ativities are shown with approximately equivalent IC_{50} values of 5×10^{-6} M.

Discussion

Results presented in this paper confirm that IOVs of RBC membranes actively transport Ca^{2+} (Weiner

& Lee, 1972). Transport depended on the presence of ATP (Fig. 1) and could be short circuited by the Ca^{2+} ionophore A23187. In addition, and as reported previously, the rate of transport could be increased in a concentration-dependent fashion by purified CaM (Hinds et al., 1978; MacIntyre & Green, 1978). The rates of basal and CaM-activated Ca^{2+} transport were 14.4 and 26.8 nM/min/mg IOV protein, respectively. These values compare well with values calculated on the basis of membrane protein from intact RBCs. For example, using ionophore-loaded RBCs, Sarkadi, Szász, Gerlóczy & Gárdos (1977) measured a maximum efflux rate of about 85 μmoles Ca^{5+}/min/liter cells. Assuming red blood cell volume of 100 fl and a membrane protein content of 6.6×10^{-13} g per RBC ghost (Dodge, Mitchell & Hanahan, 1963), we estimate this rate to be equivalent to 12.9 nmol/min/mg membrane protein. Differences in the rate of Ca^{2+} pump of the RBC membrane in various laboratories have been considered by Roufogalis (1979) and Vincenzi and Hinds (1980).

The preparations used in this study showed variability in specific activity and degree of CaM activation. However, on the same preparation the (Ca^{2+} + Mg^{2+})-ATPase activity was normally greater than or equal to the Ca^{2+} transport rate. Discrepancies between the (Ca^{2+} + Mg^{2+})-ATPase activity and Ca^{2+} transport rate in a given preparation could be due to differences in the experimental conditions. On the other hand, this may be a reflection of the fact that the Ca^{2+} pump is tightly coupled whereas the (Ca^{2+} + Mg^{2+})-ATPase is uncoupled. It should also be noted that different membrane fractions obtained from the IOV preparation were employed in the Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase assays (Figs. 3*A* and *B*, and 5*A* and *B*). If ATPase and transport were measured simultaneously using the same IOVs, one might be able to address the controversy of pump stoichiometry (Sarkadi et al., 1977; Larsen, Hinds & Vincenzi, 1978*a*; Roufogalis, 1979). Since this is not the case, these experiments cannot be used for a definitive assessment of pump stoichiometry. Thus, the data in Figs. 3 and 5 are compatible with, but do not prove, a stoichiometry of 1.

Data in Fig. 1 also demonstrate that IOVs are not "leaky" to Ca^{2+} in the absence of ATP. A definite increase in vesicle-associated $^{45}Ca^{2+}$ occurred upon the addition of A23187. Assuming equal $^{45}Ca^{2+}$ distribution across its vesicular membrane, the increase in vesicle-associated $^{45}Ca^{2+}$ was used to calculate vesicular volume. Using this value (21 μl/mg membrane protein) the intravesicular Ca^{2+} concentration was estimated to be 2.5 mM at the end of 3 min incubation in the presence of CaM (Fig. 2). The remarkably rapid accumulation of Ca^{2+} in vesicles is a product of the high pump rate and the small volume of the vesicles. Based upon preliminary calculations considering initial rates of Ca^{2+} transport and ATP hydrolysis, intravesicular volume and Ca^{2+} concentrations there appear to be more than sufficient amounts of substrates available. Therefore, the decrease in Ca^{2+} transport resulting in a plateau (zero net accumulation) could be due to high intravesicular Ca^{2+} concentrations causing either an increased passive efflux of Ca^{2+} or slowing of a tightly coupled pump.

Figure 2 demonstrates the dependence of Ca^{2+} transport on CaM. The apparent K_D CaM for activation of transport was 18.5 nM. This value is similar to that reported for the increase in activity of RBC membrane (Ca^{2+} + Mg^{2+})-ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977; Larsen & Vincenzi, 1979). It should be noted that the apparent K_D for pump or ATPase activation is much less than the RBC content of CaM (Vincenzi & Hinds, 1980).

Figure 2 also demonstrates TFP inhibition of Ca^{2+} transport. The main antagonism occurred against the CaM-activated portion of transport, but inhibition of the basal transport was also apparent. It is presumed that antagonism of CaM occurs as a result of Ca^{2+}-dependent binding of TFP to CaM as demonstrated by Levin and Weiss (1977). The fact that high concentrations of CaM could overcome 1×10^{-5} M TFP (Fig. 2) is consistent with this interpretation.

Before further discussing the effects of TFP it seems appropriate to emphasize that the concentrations of TFP in the present work represent the total amount of drug added. Somewhat over half of the drug was bound to the reaction vessel and to the biological membranes in our system. Such binding was anticipated, but was less than expected from published information. In any event, the concentrations of TFP represent only nominal values. Obviously the extent of phenothiazines binding may be subject to a number of variables and may be different in other systems. It will be obvious at the outset that the concentrations of TFP in the present work are high. We have already demonstrated that neuroleptic activity of various drugs does not correlate with antagonism of CaM in our system (Raess & Vincenzi, 1980*b*). Thus, the present work was aimed at investigating various means of antagonizing the Ca^{2+} pump, rather than implicating such effects as a potential therapeutic approach.

The concentration-dependence of the effects of TFP on Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity is demonstrated in Fig. 3*A* and *B*, respective-

ly. Results are compatible with the interpretation that TFP antagonizes CaM-induced activation of Ca^{2+} transport by antagonizing CaM-induced activation of the ATPase. The results also show some inhibition of basal transport, especially at high concentrations, as well as abolition of transport and all membrane ATPase activities with 3×10^{-4} M TFP. We interpret this to mean that there is little, if any, interaction between TFP and the Ca^{2+} pump itself up to 1×10^{-4} M. At 1×10^{-4} M TFP, CaM-induced activation of both ATPase and transport was essentially abolished while basal activity was reduced by approximately one-fourth. It seems reasonable to suggest that drug-induced disruption of the membrane lipid environment occurs with these extremely high concentrations of the drug. This could inhibit basal ATPase and pump activities whether they were dependent on endogenous CaM or not. Indirect evidence for a disruption of membrane function comes from another kind of observation. It may be noted in Fig. 3 that the *initial* rate of Ca^{2+} uptake in the absence of CaM and in the presence of CaM plus 10^{-4} M TFP was about the same. However, when IOVs were allowed to transport for 90 min, steady-state (plateau) $^{45}Ca^{2+}$ uptake in the presence of the drug was less (Table 1). If the vesicles are normally "tight" for Ca^{2+} then at least some of the vesicles were probably disrupted in the presence of the drug, an effect independent of CaM-binding or inhibition of the enzyme.

Caution must be emphasized in interpreting data obtained in the presence of high concentrations of TFP. Nevertheless, results show that TFP can be used as an inhibitor of the CaM-dependent plasma membrane Ca^{2+} pump. This is in agreement with Schatzmann (1970) who reported that 1×10^{-4} M chlorpromazine decreased, but did not abolish Ca^{2+} transport from resealed ghosts. It seems likely that chlorpromazine antagonized the CaM-activated Ca^{2+} transport while the Ca^{2+} transport related to basal $(Ca^{2+} + Mg^{2+})$-ATPase activity was not inhibited. The results also show that TFP at 3×10^{-4} M can disrupt other membrane ATPases. A current trend in the literature is to suggest that TFP be used to probe CaM effects in complex systems. The presumption is that TFP specifically antagonizes CaM and that TFP inhibition of a process implicates CaM in that process. Results of the present work show that extreme caution must be used in such an approach. If that approach were applied to the data in Figs. 2 and 3, one might conclude that endogenous CaM is involved in basal $(Ca^{2+} + Mg^{2+})$-ATPase and the basal Ca^{2+} pump as well as the Mg^{2+}-ATPase and $(Na^{+} + K^{+} + Mg^{2+})$-ATPase. We consider this rather unlikely.

Data in Fig. 4 demonstrate the CaM-binding protein selectively antagonizes the CaM-activated portion of Ca^{2+} transport. No decrease in basal transport was observed with as much as 39 μg/ml CaM-binding protein. These results confirm the previously reported selective antagonism of CaM activation of $(Ca^{2+} + Mg^{2+})$-ATPase in this system (Larsen et al., 1978*a*). The finding is consistent with the interpretation advanced by Wang and Desai (1977) and others that CaM binding protein binds to CaM, thereby competing in the activation of various CaM-dependent enzymes. Results in Fig. 4 show that CaM-binding protein can serve as a functional regulator of CaM-induced plasma membrane Ca^{2+} transport. Considering the multiple roles of CaM, the potential significance (Cheung, 1980) of CaM-binding protein in cellular regulation seems obvious, but has not yet been widely investigated.

The lack of inhibition of basal activities by CaM-binding protein may be taken to mean that basal transport and ATPase (Larsen et al., 1978*a*) are not dependent on endogenous CaM or that CaM-binding protein cannot gain access to endogenous enzyme-bound CaM if present. Considering the method of preparing IOVs and that most CaM is easily removed from RBC membranes (Farrance & Vincenzi, 1977), we favor the former interpretation. The lack of inhibition of basal Ca^{2+} transport and basal $(Ca^{2+} + Mg^{2+})$-ATPase activities by CaM-binding protein (Larsen et al., 1978*b*; present study) is in direct contrast with results and interpretation by Lynch and Cheung (1979). In their experiments CaM-binding protein abolished $(Ca^{2+} + Mg^{2+})$-ATPase activity completely. Since their methods do not specify any details on the CaM-binding protein used, it is not possible to comment on this discrepancy.

Results in Fig. 5*A* and *B* demonstrate that RR inhibits both basal and CaM-activated portions of both Ca^{2+} transport and $(Ca^{2+} + Mg^{2+})$-ATPase. Unlike TFP and CaM-binding protein, no selectivity against CaM was apparent. This is in agreement with the results reported by Raess and Vincenzi (1980*b*) on the inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase by RR. It seems likely that RR exerts an inhibitory effect on Ca^{2+} transport and the $(Ca^{2+} + Mg^{2+})$-ATPase whether CaM is present or not since both activated and basal activities exhibit identical IC_{50} values for RR. Similarities in shape and IC_{50}s between Ca^{2+} transport inhibition curves (Fig. 5*A*) and $(Ca^{2+} + Mg^{2+})$-ATPase inhibition curves (Fig. 5*B*) suggests a common mode of inhibition by RR on these two processes. From previous reports on the inhibition of the $(Ca^{2+}$-$Mg^{2+})$-ATPase by RR (Watson et al., 1971; Raess & Vincenzi, 1980*b*) it is not clear as on which side of the membrane RR exerts its effects.

Inhibition of Ca^{2+} transport into IOV by RR indicates that the cytoplasmic face of the membrane contains site(s) of interaction. External face sites are not eliminated. RR could be used as a Ca^{2+} pump inhibitor in well-defined systems, but because it also inhibits other membrane ATPases, and mitochondrial Ca^{2+} transport (Moore, 1971) it shows little promise as a selective inhibitor of either the plasma membrane pump or CaM-activated functions in complex biological systems.

In conclusion, we have presented evidence that TFP in moderate concentrations can specifically antagonize the CaM-mediated portion of Ca^{2+} transport and the $(Ca^{2+}+Mg^{2+})$-ATPase activity in red blood cell membranes, and higher concentrations of TFP apparently have a detrimental effect upon these membrane processes. Therefore, caution should be observed when using TFP to probe CaM-mediated functions.

The demonstration of both CaM activation of Ca^{2+} transport and the $(Ca^{2+}+Mg^{2+})$-ATPase as well as the antagonism by the different inhibitors tested provide further evidence that both of these entities are physicochemical manifestations of the same enzyme.

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