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Effects of Microtubular Inhibitors on Plasma Membrane Calmodulin-Dependent Ca^{2+}-Transport ATPase

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SUMMARY

Several microtubular inhibitors were examined for their effects on human red blood cell calmodulin-dependent Ca^{2+}-transport ATPase. The Vinca alkaloids vinblastine, vindesine, and vincristine were found to be inhibitors of the calmodulin-dependent fraction of red blood cell Ca^{2+}-transport ATPase, with IC_{50} values of 35, 100, and 220 μM, respectively. However, in the concentration range of 1–10 μM all three agents inhibited 10–14% of the calmodulin-dependent ATPase activity. The Vinca alkaloids were far less effective in antagonizing Ca^{2+}-transport ATPase activity in the absence of added calmodulin. In contrast, the potent microtubular inhibitors, nocodazole and colchicine, failed to inhibit significantly the calmodulin-dependent Ca^{2+}-transport ATPase. The concentration of vinblastine required for 50% inhibition of the calmodulin-dependent fraction of Ca^{2+} transport into inside-out vesicles of red blood cells was very similar to that found for the Ca^{2+}-ATPase. Kinetic analysis revealed that the activation of Ca^{2+}-transport ATPase induced by calmodulin is inhibited by vinblastine according to a "mixed-type" mechanism, although the mechanism is mostly of a competitive nature. The mechanism of action of vinblastine on calmodulin-dependent Ca^{2+}-transport ATPase was further elucidated by means of binding studies with radioactively labeled vinblastine. Vinblastine was found to bind to calmodulin as well as to Ca^{2+}-transport ATPase of red blood cells. Binding of vinblastine to calmodulin required the presence of Ca^{2+}, whereas binding to Ca^{2+}-transport ATPase occurred Ca^{2+}-independently. Calmodulin has two classes of vinblastine binding sites: one site with a high affinity for vinblastine ($K_D = 2$ μM) and one additional site with a lower affinity for the drug ($K_D = 10$ μM). With respect to other ATPases of the red blood cell membrane, the action of vinblastine on Ca^{2+}-transport ATPase seems to be specific since Mg^{2+}-ATPase and ($Na^+ + K^+$)-transport ATPase were hardly affected at concentrations up to 300 μM. The antagonism of calmodulin effects by Vinca alkaloids may be of general importance for all calmodulin-regulated parameters.

INTRODUCTION

Low intracellular Ca^{2+} concentrations in human RBCs[3] are maintained by an active Ca^{2+}-transport mechanism (1). Plasma membrane preparations from RBCs contain a Ca^{2+}-ATPase (1) which is the biochemical expression of the Ca^{2+}-pump mechanism. The identity of Ca^{2+}-ATPase and Ca^{2+}-pump protein clearly was demonstrated by reconstitution of the purified ATPase in artificial liposomes which were capable of active Ca^{2+} transport (2). Thus, we use the term Ca^{2+}-transport ATPase to refer to Ca^{2+}-ATPase.

The discovery of CaM has given a great impetus to investigations concerning the role of Ca^{2+} on cellular functions (3, 4). In dependence on the intracellular Ca^{2+} flux, CaM, a ubiquitous Ca^{2+}-binding protein, mediates many of the intracellular effects of Ca^{2+} by the reversible formation of a CaM-Ca^{2+} complex which regulates a multitude of important Ca^{2+}-dependent cell functions and enzyme systems (3, 4). CaM not only mediates the effect of an elevated intracellular Ca^{2+} concentration but also terminates the function of the CaM-Ca^{2+} complex by regulating the intracellular free Ca^{2+} concentration (3). It appears to activate Ca^{2+}-transport ATPases—responsible for controlling the concentration of Ca^{2+} in the cytosol—in plasma membrane (5, 6) and in sarcoplasmic reticulum membrane (7).

It has long been known that microtubules—the constituents of the mitotic spindle—are sensitive to Ca^{2+}, although the concentration of Ca^{2+} required for disassembly of microtubules has been somewhat controversial (8, 9). In the light of recent findings it seems likely that a Ca^{2+} concentration in the low micromolar range is suffi-

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[3] The abbreviations used are: RBC, red blood cell; CaM, calmodulin; VBL, vinblastine; MOPS, 4-morpholinopropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid.

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cient for disassembly of microtubules (10, 11). However, cyclic AMP has an adverse effect in that it promotes microtubule assembly at concentrations between 1 and 100 μM (12). Several agents causing depolymerization of microtubules are thought to mediate their antimitotic effect through their binding to tubulin (13). Since Ca^{2+} and cyclic AMP concentrations, both of which are regulated by CaM, and CaM itself play a role in the assembly-disassembly process of microtubules and during mitosis (4, 12), we wondered whether there was a connection between the action of microtubular inhibitors and the function of CaM. In particular, our experiments were aimed at testing the hypothesis that microtubular inhibitors mediate their effects on microtubules simply via an elevation of the intracellular Ca^{2+} concentration by inhibition of the function of CaM and plasma membrane Ca^{2+}-transport ATPase. As an appropriate enzyme system for our experiments we chose the Ca^{2+}-transport ATPase of human RBCs, an easily accessible enzyme dependent on CaM. Recently it was reported that VBL is an inhibitor of the CaM-dependent fraction of brain phosphodiesterase (14) and RBC Ca^{2+}-transport ATPase activity (15).

In the present work several microtubular inhibitors were tested for their ability to antagonize the activation of RBC Ca^{2+}-transport ATPase by CaM. The mechanism of action by which VBL inhibits the CaM-dependent Ca^{2+}-transport ATPase of RBCs has been studied in detail. A preliminary report of these investigations has been presented (16).

EXPERIMENTAL PROCEDURES

Materials. All reagents were of highest purity available. Vindesine, vincristine, and VBL were kindly provided by Eli Lilly Company (Indianapolis, Ind.). [^{3}H]-VBL was purchased from Amersham Buchler (Braunschweig, Germany). Colchicone was obtained from Serva (Heidelberg, Germany), and nocodazole from Janssen Pharmaceutica (Beerse, Belgium). Vinca alkaloid and colchicine solutions were prepared daily with distilled, deionized water. Nocodazole was dissolved in dimethyl sulfoxide and added to the assay medium under vigorous mixing. The final concentration of dimethyl sulfoxide in the assay medium, including the controls, in all cases was 0.5% (v/v).

Preparation of CaM. CaM from bovine brain was purified to homogeneity essentially according to the method described by Watterson *et al.* (17).

Preparation of CaM-deficient RBC membranes. Membrane-bound human RBC Ca^{2+}-transport ATPase was prepared on the basis of the iso-osmotic freeze-hemolysis procedure described by Gietzen *et al.* (18), modified as follows: RBCs were washed in a solution containing 150 mM KCl and 20 mM MOPS (pH 7.4). The buffer used for hemolysis contained 150 mM KCl, 10 mM MOPS (pH 7.4), 1 mM EDTA, 10 mM ascorbic acid, 10 mg of Tween 20/ml, 1 mM diisopropylphosphorofluoridate, and 1 mM 7-amino-1-chloro-3-L-tosylamidoheptane-2-one. The buffer used for the first three washes following hemolysis contained 150 mM KCl, 10 mM MOPS (pH 7.0), 1 mM EDTA, 10 mM ascorbic acid, and 0.5 mM diisopropylphosphorofluoridate. For the next three washes EDTA was omitted. After the last centrifugation the resulting pellet of RBC membranes was suspended in a 2-fold volume of 150 mM KCl/1 mM $MgCl_2$/750 mM sucrose/10 mM MOPS (pH 7.0) to give a final protein concentration of about 15 mg/ml.

Preparation of purified CaM-deficient RBC Ca^{2+}-transport ATPase. RBC Ca^{2+}-transport ATPase was purified to homogeneity using a CaM-Sepharose 4B affinity column as described by Gietzen *et al.* (19). The purified Ca^{2+}-transport ATPase was eluted from the affinity gel in a detergent/lipid system containing Tween 20 (0.5 mg/ml), Triton X-100 (0.5 mg/ml), and phosphatidylcholine (0.5 mg/ml).

Preparation of CaM-deficient inside-out RBC vesicles. Human RBCs were washed in an isotonic NaCl solution and lysed at 2° in a 15-fold volume of a medium consisting of 15.25 mM Tris-HCl (pH 7.75) and 1 mM Tris-EGTA. The mixture was centrifuged at 28,000 × *g* under refrigeration for 20 min and the membranes were washed four times with the same solution. Before the third and fourth centrifugations the membrane suspension was incubated for 30 min at 37°. The membranes were then washed once in 15.25 mM Tris-HCl (pH 7.75)/5 μM $CaCl_2$. To initiate vesiculation the white membranes were diluted in a 20-fold volume of a solution containing 0.5 mM Tris-HCl (pH 8.5) and 50 μM dithiothreitol. The mixture was first incubated for 30 min at 0° and then additionally for 15 min at 37° before the vesicles were pelleted at 28,000 × *g* in the cold for 20 min. The concentrated vesicle suspension was homogenized by passing it four times through a 26-gauge hypodermic needle, pelleted again, and resuspended in 140 mM KCl/20 mM Tris-HCl (pH 7.4) at 0° to give a final protein concentration of about 2.5 mg/ml.

The percentage of inside-out RBC vesicles in the preparation was tested by measuring acetylcholinesterase liberated by Triton X-100. According to this criterion, on average 65% of the material was made up by inside-out vesicles. Rightside-out vesicles do not interfere with the Ca^{2+} uptake assay since they are impermeable to ATP in the external medium.

Assay of ATPase activities. ATPase activities were determined at 30° as described previously (20). In the case of Ca^{2+}-transport ATPase the reaction was monitored continuously for 8 min, whereas in the case of Mg^{2+}-ATPase and (Na^+ + K^+)-transport ATPase the reaction was followed discontinuously over a period of 90 min. The assay medium contained, in a final incubation medium of 10 ml, approximately 70 μg of membrane protein per milliliter. The medium for Ca^{2+}-transport ATPase consisted of 10 mM Tris-maleate buffer (pH 7.0), 100 mM KCl, 0.2 mM ouabain, 1 mM ATP, 2 mM $MgCl_2$, and 36 μM Ca^{2+} [as a 0.4 mM Ca^{2+}/Mg^{2+}/EDTA buffer (21)]. Ca^{2+}-free controls contained 0.4 mM Mg^{2+}/EGTA instead of the Ca^{2+}/Mg^{2+}/EDTA buffer. These controls yielded simultaneously the Mg^{2+}-ATPase activity. The medium for (Na^+ + K^+)-transport ATPase consisted of 100 mM NaCl, 10 mM KCl, 30 mM imidazole-HCl (pH 7.2), 4 mM $MgCl_2$, 0.5 mM Tris-EGTA, and 2 mM ATP, with or without 0.2 mM ouabain. Before the reaction was started by the addition of ATP, the RBC membranes were preincubated with the corresponding drug for 10

min at 30° in the case of Mg^{2+}-ATPase and $(Na^+ + K^+)$-transport ATPase whereas in the case of Ca^{2+}-transport ATPase the RBC membranes were first preincubated for 10 min with the drug and additionally for 10 min in the presence (24 nM) or absence of CaM.

Ca^{2+}-uptake into inside-out RBC vesicles. Ca^{2+}-transport measurements were carried out as described previously (22). Before starting the transport process with ATP the vesicles were preincubated for 20 min with VBL and for 10 min with Ca^{2+} at 37° in a medium consisting of 130 mM KCl, 20 mM imidazole-HCl (pH 7.0), 2 mM $MgCl_2$, 0.2 mM EGTA, 0.19 mM $CaCl_2$ [plus $^{45}Ca^{2+}$ (0.1 μCi/ml)], yielding a free Ca^{2+} concentration of 4.65 μM, 2 mM Mg^{2+}-ATP. The assay medium contained 37 μg of vesicle protein per milliliter and when present 6 nM CaM. At 0, 3, and 6 min, 1-ml samples were filtered immediately through Millipore filters with a pore diameter of 0.45 μm, which retained all protein.

Binding studies. The interaction of VBL with CaM and Ca^{2+}-transport ATPase was investigated by the equilibrium binding technique of Hummel and Dreyer (23).

Protein determination. Protein was determined by the method of Bennett (24). Bovine serum albumin was used as a standard.

Statistics. Standard statistical methods were employed. Student's *t*-test was used as the test for significance of differences.

RESULTS

Effects of microtubular inhibitors on Ca^{2+}-transport ATPase activity. Figure 1 demonstrates the stimulation of RBC Ca^{2+}-transport ATPase by CaM and antagonism of the activation by some of the investigated microtubular inhibitors.

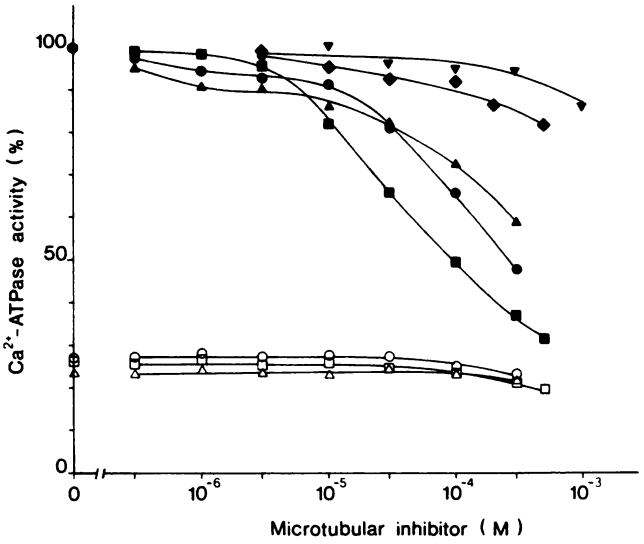

FIG. 1. *Effects of microtubular inhibitors on RBC CaM-dependent Ca^{2+}-transport ATPase*

Basal (*open symbols*) and CaM (24 nM)-activated (*closed symbols*) Ca^{2+}-transport ATPase activities were determined in the absence and in the presence of various concentrations of microtubular inhibitors (□, ■, VBL; ○, ●, vindesine; △, ▲, vincristine; ◆, nocodazole; ▼, colchicine). The data are plotted as a percentage of the activity in the presence of CaM and in the absence of the drugs (100% activity = 30–40 nmoles of P_i per milligram of membrane protein per minute. Each point represents the mean of 4–10 determinations (SD ≤ 3.5%).

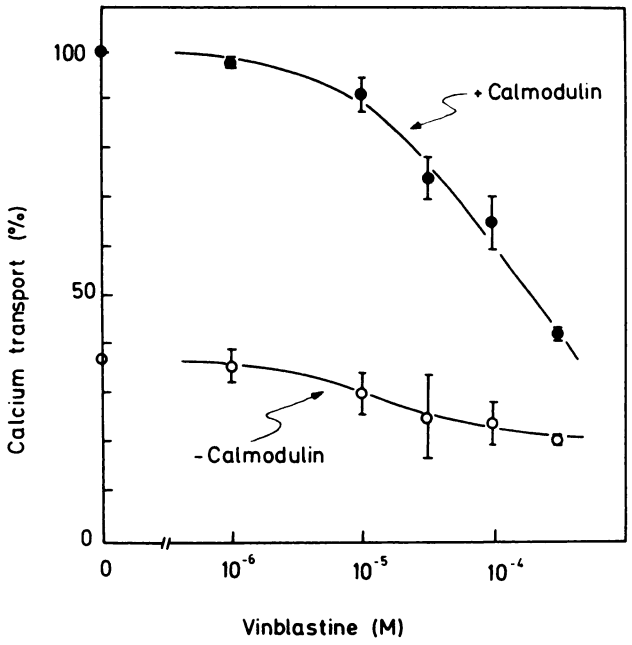

FIG. 2. *Inhibition of Ca^{2+} transport by VBL*

Concentration-dependent inhibition of CaM (6 nM)-activated (●——●) and basal (○——○) Ca^{2+} transport into inside-out RBC vesicles by VBL. Ordinate: relative initial rate (0–6 min after adding ATP) of $^{45}Ca^{2+}$ uptake into vesicles [100% activity = 4.13 ± 1.73 (SEM) nmoles of Ca^{2+} per milligram of vesicle protein per minute]. Each point represents the mean of three experiments with three different preparations ± 1 SEM. (Reproduced from ref. 16 by permission of Karger-Verlag.)

In the absence of drugs, as documented by the points on the ordinate, Ca^{2+}-transport ATPase of disrupted RBC membranes could be maximally stimulated by CaM 4- to 5-fold above the basal Ca^{2+}-transport ATPase activity ("basal" Ca^{2+}-transport ATPase activity was defined as that activity determined in the absence of added CaM). Human RBC membranes at 30° exhibited a specific activity of 7–9 nmoles of P_i per milligram of membrane protein per minute for the basal Ca^{2+}-transport ATPase. The addition of 24 nM CaM to the assay mixture increased the specific activity to a value of 30–40 nmoles per milligram of membrane protein per minute. This concentration of CaM was sufficient to provide near-maximal activation of Ca^{2+}-transport ATPase.

As can be seen from Fig. 1, the Vinca alkaloids VBL, vindesine, and vincristine were able to antagonize the CaM-induced activation of human RBC Ca^{2+}-transport ATPase, with IC_{50} values of 35, 100, and 220 μM, respectively. However, because of a biphasic inhibition, vincristine and vindesine inhibited the CaM-dependent fraction of Ca^{2+}-transport ATPase activity by 10–14% in the concentration range 1–3 μM, whereas VBL did not show this biphasic inhibitory characteristic and required 3–10 μM for the same inhibition. Statistical analysis of the data revealed that inhibition of Ca^{2+}-transport ATPase activity produced by vincristine and vindesine at 3 μM is significantly different from the control value ($p < 0.01$),

whereas VBL did not significantly inhibit the enzyme at this concentration (the mean of control and drug values each were obtained from 10 determinations). The Vinca alkaloids were far less effective in antagonizing basal Ca^{2+}-transport ATPase activity. A slight inhibition of basal Ca^{2+}-transport ATPase activity could be observed only at higher concentrations of these three drugs (IC_{50} > 500 μM).

In contrast to the Vinca alkaloids, the potent microtubular inhibitors, nocodazole and colchicine, failed to produce a pronounced inhibition of the CaM-stimulated Ca^{2+}-transport ATPase activity (Fig. 1).

Inhibition of Ca^{2+} transport into inside-out RBC vesicles by VBL. VBL, the most effective Vinca alkaloid for antagonism of CaM-stimulated ATPase activity, was investigated for its effects on Ca^{2+} transport into inside-out RBC vesicles in the absence and presence of added CaM (Fig. 2). Ca^{2+} transport of CaM-deficient inside-out RBC vesicles was found to be less responsive to added CaM (cf. points on the ordinate) as compared with Ca^{2+}-transport ATPase activity of disrupted RBC membranes (see Fig. 1). Obviously the CaM-dependent fraction of Ca^{2+}-transport activity of inside-out vesicles was inhibited half-maximally by VBL (IC_{50} = 50–60 μM) in the same concentration range as the ATPase activity. Ca^{2+}-transport activity of inside-out vesicles without added CaM declined slightly with increasing VBL concentrations.

Mechanism of action of VBL on CaM-dependent Ca^{2+}-transport ATPase. To obtain stronger evidence for the involvement of CaM in the inhibition of RBC Ca^{2+}-transport ATPase by VBL, the influence of the CaM concentration on the antagonistic effect of VBL was investigated. In the absence of the drug, approximately 2.5 nM CaM was required for half-maximal activation of the CaM-dependent fraction of ATPase activity (Fig. 3A). In the presence of 25 and 50 μM VBL the concentration-effect curves of CaM were shifted to the right. With 25 μM VBL the concentration of CaM required for half-maximal activation was increased by approximately 2-

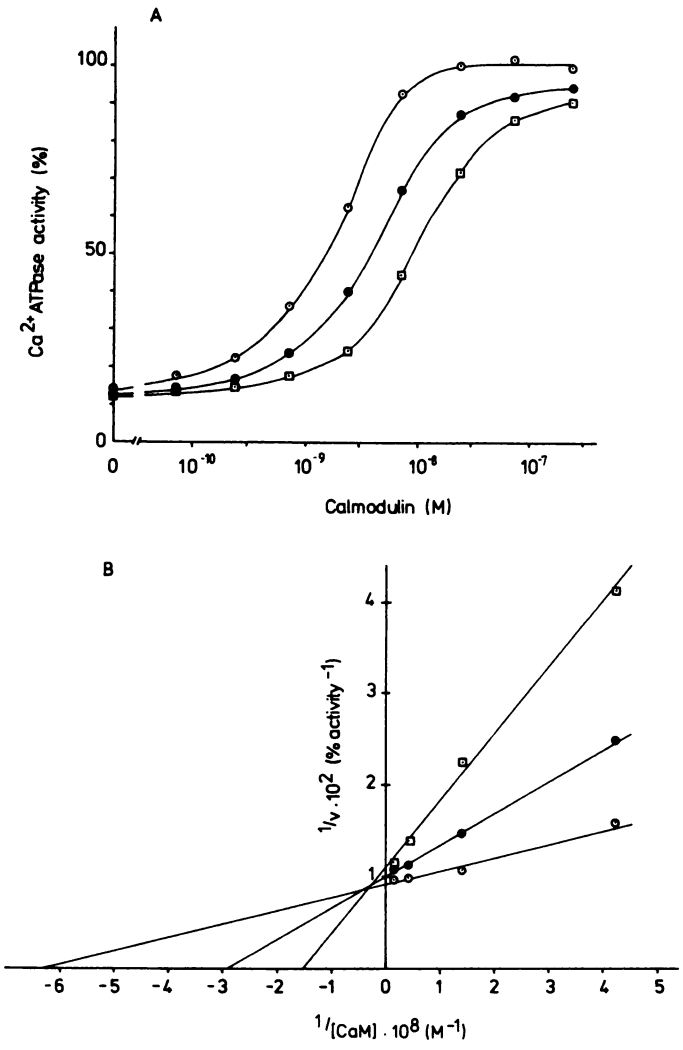

FIG. 3. *Antagonism of CaM-induced activation of Ca^{2+}-transport ATPase by VBL*

A. Effects of CaM on RBC Ca^{2+}-transport ATPase in the absence of VBL (○) and presence of 25 μM (●) and 50 μM (□) VBL. The ATPase activity is related to the activity in the presence of 60 nM CaM and in the absence of the drug. Points on the ordinate represent the basal Ca^{2+}-transport ATPase activity, i.e., the activity obtained in the absence of added CaM. Each point represents the mean of six determinations obtained with three different RBC membrane preparations.

B. Double-reciprocal plot of dependence of rate (v) of Ca^{2+}-transport ATPase reaction on CaM (2.4, 7.2, 24, and 72 nM). The lines of best fit were drawn by linear regression analysis.

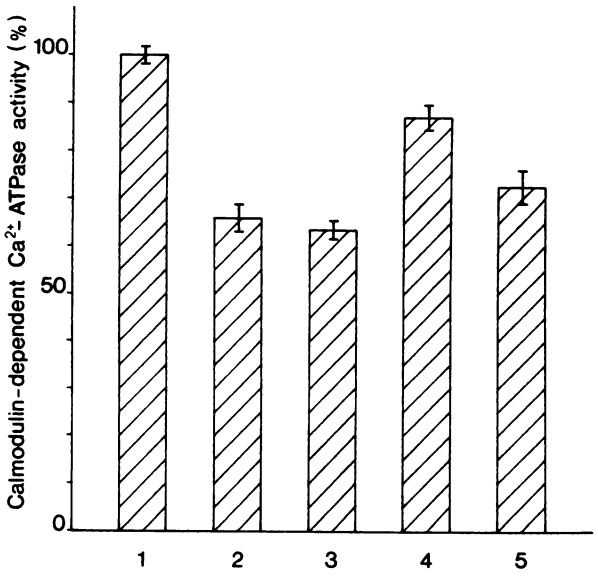

FIG. 4. *Influence of the modus of preincubation upon the inhibitory potency of VBL*

The CaM-dependent fraction of Ca^{2+}-transport ATPase was determined in the absence and presence (24 nM) of CaM. The sequence of preincubation of the different components (ATPase, CaM, and VBL) was varied. Column *1*: ATPase and CaM were preincubated for 20 or 30 min at 30° before starting the assay. The CaM-dependent fraction of the ATPase activity represents the control and equals 100%. Column *2*: ATPase plus VBL (25 μM) were preincubated for 10 min, and CaM was added; the mixture was preincubated for 20 min more before assay. Column *3*: CaM plus VBL were preincubated for 10 min, and ATPase was added; the mixture was preincubated for 20 min more before assay. Column *4*: ATPase plus CaM were preincubated for 10 min, and VBL was added; the mixture was preincubated for 20 min more before assay. Column *5*: ATPase plus CaM plus VBL were mixed at the same time and preincubated for 30 min before assay. Each value represents the mean ± standard deviation of six determinations.

fold. With 50 μM VBL the concentration of CaM needed for half-maximal activation again was approximately 2 times higher as compared with 25 μM VBL. The lines in the double-reciprocal plot (Fig. 3B) represent computer-fitted linear regressions based on data obtained with 2.4, 7.2, 24, and 72 nM CaM. The double-reciprocal plot for these CaM concentrations yielded estimates of half-maximal activation by 1.6, 3.4, and 6.5 nM CaM in the absence and in the presence of the two concentrations of VBL, respectively. With increasing VBL concentrations the extrapolated maximal activities decreased slightly. The data are compatible with the interpretation that the activation of Ca^{2+}-transport ATPase induced by CaM is inhibited according to a "mixed-type" mechanism, although the mechanism is mostly of a competitive nature.

Figure 4 demonstrates the influence of the sequence of preincubation of the different components that participate in the inhibitory process (i.e., ATPase, CaM, and VBL). Preincubation of the ATPase and CaM in the absence of the drug yielded the fraction of CaM-dependent Ca^{2+}-transport ATPase activity that represented the reference value of 100% (Column *1*). There was no detectable difference in the resulting ATPase activity if the ATPase was preincubated with CaM for 20 or 30 min. Incubating the ATPase first with VBL and adding CaM afterward gave an inhibitory effect of VBL (Column *2*) upon the CaM-dependent fraction of Ca^{2+}-transport ATPase activity similar to that obtained by first incubating CaM and VBL and then adding the ATPase (Column *3*). In contrast, VBL had a reduced inhibitory potency when it was added to a mixture of ATPase and CaM (Column *4*). When all three components were mixed at the same time, an intermediate inhibitory effect was obtained (Column *5*). Statistical analysis of the data revealed that the value of each column is significantly different from the other ($p < 0.005$) except for Column *2* as compared with Column *3*.

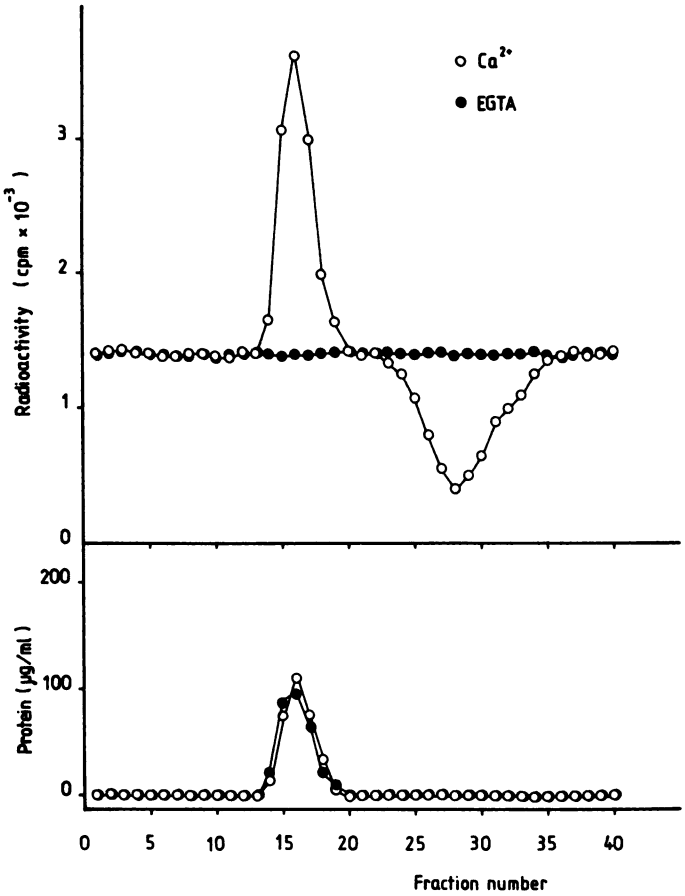

FIG. 5. *Elution pattern for the determination of VBL binding by CaM*

A Sephadex G-50 column (0.9 × 30 cm) was pre-equilibrated with a buffer similar to that used for the assay of Ca^{2+}-transport ATPase: 10 mM Tris-maleate (pH 7.0)/100 mM KCl/2 mM $MgCl_2$/1 μM VBL [plus [^{3}H]VBL (7.5 nCi/ml)]/36 μM Ca^{2+} (○) as a 0.4 mM Ca^{2+}/Mg^{2+}/EDTA buffer (21) or 0.4 mM Mg^{2+}/EGTA (●). Purified CaM (200 μg = 12 nmoles) was dissolved in the equilibration buffer, incubated at room temperature for 10 min, and then applied to the column. Gel filtration was carried out at 22° with the above-mentioned buffer at a flow rate of 15 ml/hr, and 0.6-ml fractions were collected. Samples of 0.2 ml of each fraction were counted for radioactivity in 5 ml of Instagel (Packard) scintillation fluid containing in addition 0.6 ml of 1.7% (w/v) sodium dodecyl sulfate. Samples of 0.4 ml of each fraction were used for protein determination.

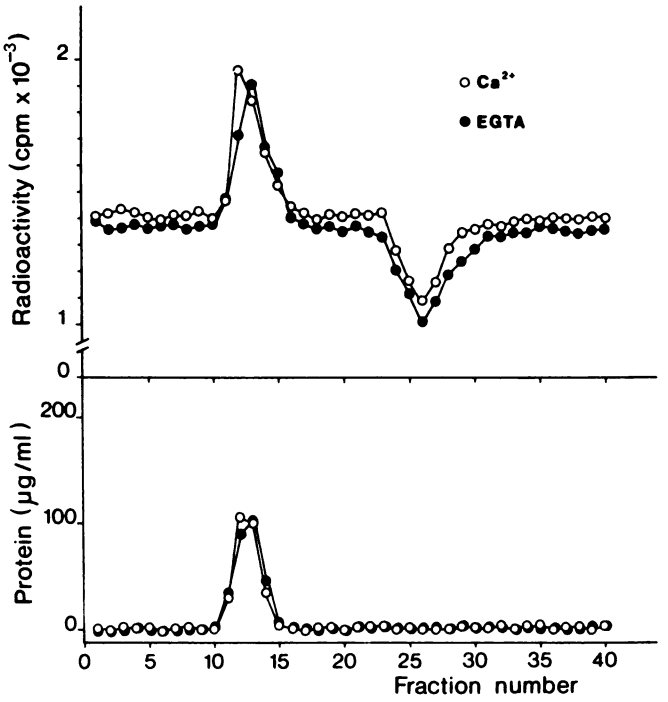

FIG. 6. *Elution pattern for the determination of VBL binding by Ca^{2+}-transport ATPase*

A Sephadex G-50 column (0.9 × 30 cm) was pre-equilibrated with a buffer containing 10 mM Tris-maleate (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$, 1 μM VBL [plus [^{3}H]VBL (7.5 nCi/ml)], phosphatidylcholine (0.5 mg/ml), Tween 20 (0.5 mg/ml), Triton X-100 (0.5 mg/ml), and 36 μM Ca^{2+} (○) as a 0.4 mM Ca^{2+}/Mg^{2+}/EDTA buffer or 0.4 mM Mg^{2+}/EGTA (●). Purified RBC Ca^{2+}-transport ATPase [150 μg = 1.07 nmoles, assuming a molecular weight of 140,000 (19)], dissolved in the equilibration buffer, was applied to the column. Gel filtration was carried out at 4°. All other experimental details were the same as described in the legend to Fig. 5. Since the Ca^{2+}-transport ATPase, as obtained from the preceding purification step, was already solubilized in a phospholipid/detergent system identical with that of the equilibration buffer, the equilibration buffer serves as an internal control for unspecific binding of VBL by detergents and phospholipid.

The possible formation of a CaM-VBL complex as well as an ATPase-VBL complex, as suggested by the data of Fig. 4, was investigated by the equilibrium binding technique introduced by Hummel and Dreyer (23) on a Sephadex G-50 gel filtration column by means of tritium-labeled VBL. Figures 5 and 6 show elution patterns of typical binding experiments. The appearance of [^{3}H]VBL peaks and troughs in the elution patterns provided evidence for the binding of [^{3}H]VBL to CaM (Fig. 5) and to purified RBC Ca^{2+}-transport ATPase (Fig. 6). The radioactivity peak coincides with the protein peak of CaM (Fig. 5). However, the radioactivity peak was not observed when EGTA instead of Ca^{2+} was present (Fig. 5), suggesting that the binding of VBL to CaM is dependent on the presence of Ca^{2+}. In contrast, the radioactivity peak coincided with the protein peak of purified ATPase whether elution was carried out in the presence of Ca^{2+} or EGTA (Fig. 6). Obviously, binding of VBL to Ca^{2+}-transport ATPase occurs Ca^{2+}-independently. At a free concentration of 1 μM VBL, 0.52 mole of VBL is bound per mole of Ca^{2+}-transport ATPase, as may be calculated from the radioactivity peak shown in Fig. 6.

The binding of VBL to CaM was measured in the presence of calcium using a wide range of drug concentrations. The binding characteristics of VBL to CaM are shown in Fig. 7. The stoichiometry of the interaction between VBL and CaM and the dissociation constant for the complex were determined from a Scatchard plot (Fig. 7B) (25). The Scatchard plot consists of two linear regions with different slopes. The results suggest that there are two classes of VBL binding sites on CaM with respect to their affinities. There is one high-affinity site per mole of CaM with a dissociation constant of 2 μM, and one additional binding site with lower affinity (K_D = 10 μM).

Effect of VBL on (Na^+ + K^+)-transport ATPase and Mg^{2+}-ATPase. The influence of VBL on other ATPases of human RBCs also was investigated (Fig. 8). For comparison the data obtained for basal and CaM-stimulated Ca^{2+}-transport ATPase were plotted in the same graph. Mg^{2+}-ATPase and (Na^+ + K^+)-transport ATPase were only slightly affected by VBL in the concentration range investigated (≤ 300 μM). At a concentration of 300 μM VBL, Mg^{2+}-ATPase and (Na^+ + K^+)-transport ATPase were inhibited about 6% and 15%, respectively.

DISCUSSION

The results of the present study demonstrate that the Vinca alkaloids can antagonize the activation of RBC Ca^{2+}-transport ATPase by CaM. In contrast, the potent microtubular inhibitors nocodazole and colchicine were shown to have only a minor effect on the same system in concentration ranges up to 0.5 mM and 1 mM, respectively.

The action of VBL on CaM-dependent Ca^{2+}-transport ATPase seems to be rather specific with respect to other ATPases of the RBC membrane, since Mg^{2+}-ATPase and (Na^+ + K^+)-transport ATPase are far less susceptible to the drug (Fig. 8). In the present work the Vinca alkaloids were found to inhibit preferentially the CaM-dependent fraction of the Ca^{2+}-ATPase activity and, to a far lesser

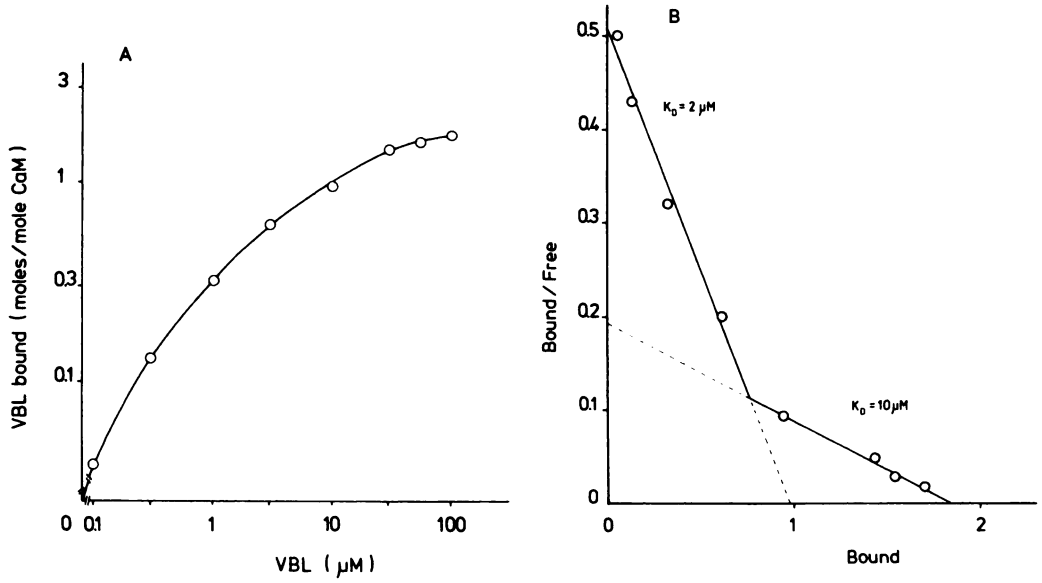

FIG. 7. *Binding of VBL to CaM*

A. Concentration-dependent binding of VBL to CaM was determined by the equilibrium binding technique of Hummel and Dreyer (23). Experimental details were identical with those described in the legend to Fig. 5 except that (a) varying concentrations of VBL [0.1–100 μM VBL plus [^{3}H]VBL (7.5 nCi–100 nCi/ml)] were used, (b) 500 μg of CaM (30 nmoles) were applied to the column, (c) fractions of 1 ml were collected, and (d) aliquots of 0.8 ml of each fraction were counted for radioactivity. CaM as well as [^{3}H]VBL were from different batches than those used for the experiments shown in Figs. 5 and 6. The moles of VBL bound per mole of CaM for each VBL concentration were calculated from the area under the peak region of the radioactivity profile.

B. Data were plotted according to the Scatchard equation. The lines of best fit were drawn by linear regression analysis. The dissociation constant (K_D) was calculated from the inverse slope of the lines, and the number of binding sites per molecule of CaM was estimated from the *X*-intercept. *Bound*, moles of VBL bound per mole of CaM; *Free*, concentration (micromolar) of VBL in the buffer with which CaM is in equilibrium during the binding experiments.

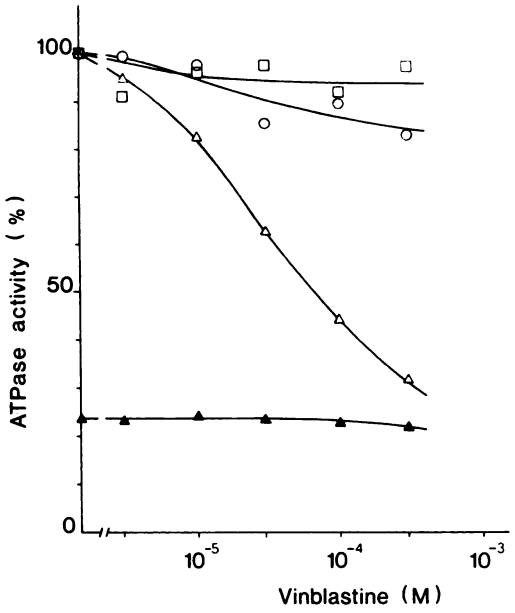

FIG. 8. *Effect of VBL on RBC ATPase activities*

$(Na^+ + K^+)$-transport ATPase (○), Mg^{2+}-ATPase (□), basal (▲), and CaM (24 nM)-stimulated (△) Ca^{2+}-transport ATPase activities were determined in the absence and in the presence of various concentrations of VBL. $(Na^+ + K^+)$-transport ATPase (100% activity = 7–10 nmoles of P_i per milligram of membrane protein per minute) activities are related to the enzyme activities in the absence of VBL. Ca^{2+}-transport ATPase activity is related to the CaM-activated enzyme in the absence of the drug. Each point represents the mean of at least four determinations.

extent at considerably higher concentrations, the basal Ca^{2+}-ATPase activity (Fig. 1). The concentration of VBL producing half-maximal inhibition of the CaM-dependent fraction of Ca^{2+} transport into inside-out RBC vesicles (Fig. 2) was found to be slightly higher (IC_{50} = 50–60 μM) as compared with the corresponding ATPase activity (IC_{50} = 35 μM). This small discrepancy may be due to the different methods of preincubation used in the two assays (see Experimental Procedures) as can be derived from the results dealing with the sequence of preincubation (Fig. 4). The decline of Ca^{2+} transport into inside-out vesicles (without added CaM) to a seemingly steady value with increasing VBL concentration might be due to some residual CaM in the preparation (Fig. 2). This view is supported by the fact that the decline occurs in the same concentration range as found for Ca^{2+} transport of inside-out vesicles in the presence of CaM.

Antagonism of CaM-induced activation of Ca^{2+}-transport ATPase by Vinca alkaloids seems to be ruled by a complex mechanism (Fig. 3). The concentration of CaM required for half-maximal activation of Ca^{2+}-transport ATPase was increased by VBL, and the V_{max} for Ca^{2+}-transport ATPase activity was slightly decreased as the drug concentration was increased. A double-reciprocal plot of the data suggests a "mixed-type" mechanism for the inhibition, although the mechanism is mostly of a competitive nature.

The results of the experiments dealing with the sequence of preincubation of the Ca^{2+}-transport ATPase with CaM and VBL (Fig. 4) showed that VBL seems to be less effective in inhibiting the Ca^{2+}-transport ATPase if the ATPase-CaM complex already exists. Moreover, the data obtained are consistent with the interpretation that VBL may bind to CaM as well as to the Ca^{2+}-transport ATPase. Binding studies with radioactively labeled VBL confirmed the latter interpretation (Figs. 5 and 6). Binding of VBL to CaM required the presence of Ca^{2+}, whereas binding to Ca^{2+}-transport ATPase occurred Ca^{2+}-independently. Presumably VBL binds to CaM according to the same mechanism operative in several neuroleptics.

It is thought that CaM-interacting agents bind to a specific hyrophobic site whose exposure is caused by the binding of Ca^{2+} to CaM (26). Scatchard analysis (Fig. 7) revealed that CaM displays one high-affinity binding site for VBL (K_D = 2 μM) and one additional binding site with lower affinity (K_D = 10 μM). The results presented here do not allow a definite statement concerning the proportion of specific (related to CaM-induced activation) or unspecific binding of VBL to Ca^{2+}-transport ATPase. From the fact that the sequence of preincubation (of the components ATPase, CaM, and VBL) strongly influenced the inhibitory potency of the drug on the CaM-dependent fraction of Ca^{2+}-transport ATPase (Fig. 4), it can be concluded that binding of VBL to the ATPase is at least in part specific.

Despite considerable binding of VBL to Ca^{2+}-transport ATPase at 1 μM (Fig. 6), only negligible inhibition of basal Ca^{2+}-transport ATPase was observed at concentrations higher than 100 μM (Fig. 1). This observation is compatible with the interpretation that specific binding of VBL to Ca^{2+}-transport ATPase occurs at a site that antagonizes the effect of CaM, i.e., the stimulation of ATPase activity. VBL may bind at the CaM binding site of ATPase or at another site by which binding of CaM to Ca^{2+}-transport ATPase is suppressed.

The Vinca alkaloids VBL, vindesine, and vincristine differed in their inhibitory potency on the CaM-dependent fraction of Ca^{2+}-transport ATPase activity with observed IC_{50} values of 35, 100, and 220 μM, respectively (Fig. 1). This order of potency inversely correlates with therapeutic doses and side effects (e.g., neurotoxicity) of the Vinca alkaloids (27). However, in the low micromolar range the order of inhibitory potency correlates with therapeutic doses and side effects of the Vinca alkaloids. Because of a biphasic inhibition, vincristine and vindesine significantly inhibited (10–14%) CaM-dependent Ca^{2+}-transport ATPase in the concentration range of 1–3 μM, whereas VBL did not show this biphasic inhibitory characteristic and required 3–10 μM for the same inhibition (Fig. 1).

The parallel inhibition of Ca^{2+} transport (Fig. 2) and Ca^{2+}-ATPase (Fig. 1) by VBL is not surprising since recently the Ca^{2+}-ATPase and Ca^{2+}-pump protein was identified (2). Consequently, the inhibition of the CaM-dependent Ca^{2+}-transport ATPase by Vinca alkaloids may lead to an elevated intracellular Ca^{2+} concentration. However, even if the partially inhibitory effect (10–14%) of Vinca alkaloids on the CaM-dependent fraction of Ca^{2+}-transport ATPase in the lower concentration range (Fig. 1) is sufficient to alter the intracellular Ca^{2+} concentration, it seems doubtful that Vinca alkaloids exert their microtubule-disrupting effects via an elevated intracel-

lular Ca^{2+} concentration or via inhibition of other CaM-regulated functions. Binding of Vinca alkaloids to tubulin occurs at considerably lower concentrations as compared with CaM, and microtubular depolymerization and antimitotic effects are observed at concentrations of 10^{-7} M (28).

It is interesting that the concentration of VBL causing 50% inhibition of the CaM-stimulated fraction of Ca^{2+}-transport ATPase is similar to that reported by Watanabe *et al.* (14) for phosphodiesterase of bovine brain. Therefore it seems likely that all CaM-regulated systems may be targets of the Vinca alkaloids. Since a considerable accumulation of Vinca alkaloids in different tissues has been demonstrated recently (29), it is tempting to speculate that Vinca alkaloid antagonism of the effects of CaM could account for some of the side effects rather than being the primary mechanism of microtubular depolymerization and antimitotic action (see above). Antagonism of CaM effects may be relevant to the neurotoxic properties of Vinca alkalids, as it seems to be established that CaM plays an important role in neurotransmission (30). However, further investigations are necessary before the role of antagonism of CaM effects by Vinca alkaloids is known.

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REFERENCES

1. Schatzmann, H. J. Dependence on calcium concentration and stoichiometry of the calcium pump in human red cells. *J. Physiol. (Lond.)* **235**:551–569 (1973).
2. Niggli, V., E. S. Adunyah, J. T. Penniston, and E. Carafoli. Purified (Ca^{2+}-Mg^{2+})-ATPase of the erythrocyte membrane. *J. Biol. Chem.* **256**:395–401 (1981).
3. Cheung, W. Y. Calmodulin plays a pivotal role in cellular regulation. *Science (Wash. D. C.)* **207**:19–27 (1980).
4. Means, A. R., and J. R. Dedman. Calmodulin—an intracellular calcium receptor. *Nature* (Lond.) **285**:73–77 (1980).
5. Gopinath, R. M., and F. F. Vincenzi. Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+} + Mg^{2+})-ATPase. *Biochem. Biophys. Res. Commun.* **77**:1203–1209 (1977).
6. Sobue, K., S. Ichida, H. Yoshida, R. Yamazaki, and S. Kakiuchi. Occurrence of a Ca^{2+}- and modulator protein-activatable ATPase in the synaptic plasma membranes of brain. *F. E. B. S. Lett.* **99**:199–202 (1979).
7. Katz, S. and M. A. Remtulla. Phosphodiesterase protein activator stimulates calcium transport in cardiac microsomal preparations enriched in sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* **83**:1373–1379 (1978).
8. Olmstead, J. B., and G. G. Borisy. Ionic and nucleotide requirements for microtubule polymerization in vitro. *Biochemistry* **14**:2996–3005 (1975).
9. Weisenberg, R. C. Microtubule formation in vitro in solutions containing low calcium concentrations. *Science (Wash. D. C.)* **177**:1104–1105 (1972).
10. Kiehart, D. P. Studies on the in vivo sensitivity of spindle microtubules to calcium ions and evidence for a vesicular calcium-sequestering system. *J. Cell Biol.* **88**:604–617 (1981).
11. Schliwa, M., U. Euteneuer, J. C. Bulinski, and J. G. Izant. Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins. *Proc. Natl. Acad. Sci. U. S. A.* **78**:1037–1041 (1981).
12. Steiner, M. 3′,5′-cyclic AMP binds to and promotes polymerization of platelet tubulin. *Nature (Lond.)* **272**:834–835 (1978).
13. Dustin, P. Microtubule poisons, in *Microtubules*. Springer-Verlag, Berlin, 167–225 (1978).
14. Watanabe, K., E. F. Williams, J. S. Law, and W. L. West. Specific inhibition of a calcium dependent activation of brain cyclic AMP phosphodiesterase activity by vinblastine. *Experientia (Basel)* **35**:1487–1489 (1979).
15. Gietzen, K., and H. Bader. Effects of vinblastine and colchicine on calmodulin-dependent Ca^{2+}-transport ATPase of human erythrocytes. *IRCS Med. Sci.* **8**:396–397 (1980).
16. Gietzen, K., A. Wüthrich, A. Mansard, and H. Bader. Effects of Vinca alkaloids on calmodulin-dependent Ca^{2+}-transport ATPase, in *Proceedings of the International Vinca Alkaloid Symposium: Vindesine*. Karger-Verlag, Basel, 16–26 (1981).
17. Watterson, D. M., W. G. Harrelson, P. M. Keller, F. Sharief, and T. C. Vanaman. Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase and actomyosin ATPase. *J. Biol. Chem.* **251**:4501–4513 (1976).
18. Gietzen, K., S. Seiler, S. Fleischer, and H. U. Wolf. Reconstitution of the Ca^{2+}-transport system of human erythrocytes. *Biochem. J.* **188**:47–54 (1980).
19. Gietzen, K., M. Tejcka, and H. U. Wolf. Calmodulin affinity chromatography yields a functional purified erythrocyte (Ca^{2+} + Mg^{2+})-dependent adenosine triphosphatase. *Biochem. J.* **189**:81–88 (1980).
20. Arnold, A., H. U. Wolf, B. P. Ackermann, and H. Bader. An automated continuous assay of membrane-bound and soluble ATPases and related enzymes. *Anal. Biochem.* **71**:209–213 (1976).
21. Wolf, H. U. Divalent metal ion buffers with low pH-sensitivity. *Experientia (Basel)* **29**:241–249 (1973).
22. Wüthrich, A., and H. J. Schatzmann. Inhibition of the red cell calcium pump by quercetin. *Cell Calcium* **1**:21–35 (1980).
23. Hummel, J. P., and W. J. Dreyer. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta.* **63**:530–532 (1962).
24. Bennett, T. P. Membrane filtration for determining protein in the presence of interfering substances. *Nature (Lond.)* **213**:1131–1132 (1967).
25. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
26. LaPorte, D. C., B. M. Wierman, and D. R. Storm. Calcium-induced exposure of a hydrophobic surface on calmodulin. *Biochemistry* **19**:3814–3819 (1980).
27. King, K. L., and G. B. Boder. Correlation of the clinical neurotoxicity of the vinca alkaloids vincristine, vinblastine, and vindesine with their effects on cultured rat midbrain cells. *Cancer Chemother. Pharmacol.* **2**:239–242 (1979).
28. Bhattacharyya, B., and J. Wolff. Tubulin aggregation and disaggregation: mediation by two distinct vinblastine-binding sites. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2375–2378 (1976).
29. Owellen, R. J., M. Blair, A. Van Tosh, and F. C. Hains. Determination of tissue concentrations of Vinca alkaloids by radioimmunoassay. *Cancer Treat. Rep.* **65**:469–475 (1981).
30. Roufogalis, B. D. Calmodulin: its role in synaptic transmission. *Trends Neurosci.* **3**:238–241 (1980).

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