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Inhibition of Calmodulin-Stimulated (Ca^{2+} + Mg^{2+})-ATPase Activity by Dimethyl Sulfoxide

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ABSTRACT. Membrane-bound (Ca^{2+} + Mg^{2+})-ATPase activity from human erythrocyte white ghosts in the calmodulin-activated state was inhibited by DMSO in concentrations of 3% (v/v) and above. At 10%, DMSO inhibited calmodulin activation by 47.7%, while basal, calmodulin-independent (Ca^{2+} + Mg^{2+})-ATPase and (Mg^{2+})-ATPase activity remained unaffected. (Na^+ + K^+)-ATPase activity was also reduced but exhibited a greater IC_{50}. Concentration-effect analyses showed the inhibition by 10% DMSO to be a reversible, non-competitive effect with regard to calmodulin, Ca^{2+}, and substrate activation. Calmodulin-stimulated processes may be more susceptible to inhibition by DMSO than related enzymatic catalysis, and thus may help explain the multitude of reported cellular events caused by the solvent. Furthermore, DMSO affected membrane-associated enzymatic mechanisms opposite to those reported for purified enzyme outside its native membrane environment. BIOCHEM PHARMACOL **57**;1:39–44, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. (Ca^{2+} + Mg^{2+})-ATPase activity; dimethyl sulfoxide; inhibition of calmodulin activation; membrane-associated enzymes; substrate activation

Evaluating drug action over a wide range of concentrations in pharmacological and toxicological assessments can present a problem if the aqueous solubilities of the compounds to be tested are limited. In this regard, DMSO can be a valuable tool in promoting dissolution of hydrophobic drugs. Furthermore, DMSO is used extensively as a cryoprotectant in cell culture methodology. However, DMSO is not without effects of its own on biologic systems in concentrations encountered as a drug vehicle, chemical solvent, and residual tissue culture component. For example, DMSO can cause substantial cardiotoxicity in mice [1]. On a cellular level, DMSO has been shown recently to promote apoptosis with inter-nucleosomal DNA fragmentation in a human leukemic cell line, implicating the involvement of unidentified protein kinase activity [2, 3]. Other studies report that DMSO causes growth inhibition of HeLa cells in culture [4], potentiation of TNF†-induced cytotoxicity in various human myeloid cell lines [5], and arrest of the human lymphoid cell line SKW6-CL4 in G1 and differentiation into IgM-secreting plasma cells [6]. At the subcellular level, DMSO is reported to interfere with the interconversion of the phosphorylated forms of (Na^+ + K^+)-ATPase activity from sheep kidneys [7], and to mimic the effects of calmodulin on purified erythrocyte (Ca^{2+} + Mg^{2+})-ATPase activity [8].

The impetus for the present study comes from investigations in this laboratory using DMSO as an *in vitro* drug vehicle for aqueous membrane ATPase assays, in conjunction with its less well understood properties as a free radical scavenger [1, 9–12]. Thus, we here characterize the effects of DMSO on human erythrocyte, membrane-bound ATPase activities with particular emphasis on the kinetics of inhibition of calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity.

MATERIALS AND METHODS

Preparation of Erythrocyte Plasma Membrane Fragments

White membrane ghosts were prepared from banked, outdated, washed human erythrocytes according to standard, previously reported methods [13]. Briefly, packed human red cells were washed three times in about 4 vol. of ice-cold 154 mM NaCl at 3000–5000 rpm (1090–3020 *g*). After each centrifugation, the supernate, comprised of white cells and platelet debris, was removed carefully by aspiration to give a homogenous sample of erythrocytes. The washed cells were hemolyzed with ice-cold 20 mM imidazole, 0.1 mM EGTA buffer (pH 7.4), and centrifuged at 18,000 rpm (39,000 *g*) for 20 min. The supernate was then discarded, and the pellets were washed two additional times with 20 mM imidazole buffer (pH 7.4) and once with 40 mM histidine, 40 mM imidazole, pH 7.1 (H/I). The resulting membrane fraction was suspended and stored on ice in an

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† *Abbreviations:* ATPase, adenosine triphosphohydrolase; Ca^{2+}, free calcium ion; and TNF, tumor necrosis factor.

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equal volume of H/I. Membrane protein, typically 7–10 mg/mL, was determined by the method of Lowry *et al.* [14] using bovine serum albumin as a standard. For pretreated membranes, previously prepared white membrane ghosts (0.2 mg/mL in H/I) were incubated with and without 10% DMSO at 37° for 1 hr. Pretreatment was terminated with the addition of an equal volume of ice-cold H/I and subsequent centrifugation at 18,000 rpm (39,200 *g*) for 20 min at 4°. The resulting membrane pellet was then washed three additional times with large volumes of ice-cold H/I and resuspended in an equal portion of the same to give a protein concentration of typically 7–10 mg/mL [14].

Plasma Membrane ATPase Activity Measurement

The standard incubation solution for determining the various ATPase activities at 37° consisted of 18 mM histidine, 18 mM imidazole, 0.2 mM $CaCl_2$, 0.1 mM ouabain, 15 mM KCl, 80 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 1 mM ATP, and a membrane protein concentration of 0.2 mg/mL. To assess (Mg^{2+})-ATPase activity, Ca^{2+} was omitted from the incubation mixture. Ouabain and Ca^{2+} were omitted to measure $(Na^+ + K^+)$-ATPase activity, while determination of calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activity involved the addition of up to 10^{-7} M calmodulin. Variations in additions are explained in the appropriate figure legends. Enzymatic reactions were terminated at 60 min by the addition of an equal volume of 2% SDS. Inorganic phosphate hydrolyzed by the various ATPase activities was determined by a modification of the colorimetric method of Fiske and Subbarow [15]. Specific ATPase activities were calculated on the basis of the phosphomolybdate complex formation and measured at a wavelength of 820 nm [15] or according to a method described by Sadrzadeh *et al.* [16] but read at 650 nm. Cross-evaluation of the two methods yielded identical results. Reported units of enzymatic activity are nanomoles P_i per milligram of membrane protein per minute. Unless otherwise indicated, data points shown represent means and standard error of the means of 3–5 independent experiments performed in duplicate. Where missing, error bars were smaller than symbol size. Significance of differences was analyzed by the Student's unpaired *t*-test.

Packed, outdated (0–35 days) human blood cells were obtained from two regional blood banks. DMSO (highest purity available) was purchased from the Eastman Kodak Co. Adenosine-5′-triphosphate, crystallized disodium salt, was obtained from Boehringer Mannheim. SDS (electrophoresis purity) was purchased from Bio-Rad. All other reagents, including human erythrocyte- and bovine testes-derived calmodulin, were obtained from the Sigma Chemical Co.

RESULTS

Figure 1 shows the concentration-effect relationship of DMSO on the various membrane-bound erythrocyte ATPase activities. Concentrations ranging from 0.01 to 10% (v/v) DMSO (1.4 mM to 1.4 M) did not affect either (Mg^{2+})-ATPase activity or basal $(Ca^{2+} + Mg^{2+})$-ATPase activity. However, calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activity exhibited inhibition by DMSO at concentrations above 3%, with 10% (1.4 M) resulting in a 34.8% reduction in activity. Extrapolation from a fitted curve ($r = 0.9997$) yielded an IC_{50} of 13.3% or 1.88 M DMSO for the calmodulin-stimulated portion of the $(Ca^{2+} + Mg^{2+})$-ATPase activity. $(Na^+ + K^+)$-ATPase activity was also inhibited, but to a lesser extent (26.1% at 10% DMSO with an IC_{50} of 2.37 M). DMSO at 30% (4.2 M) essentially eliminated all ATPase activities.

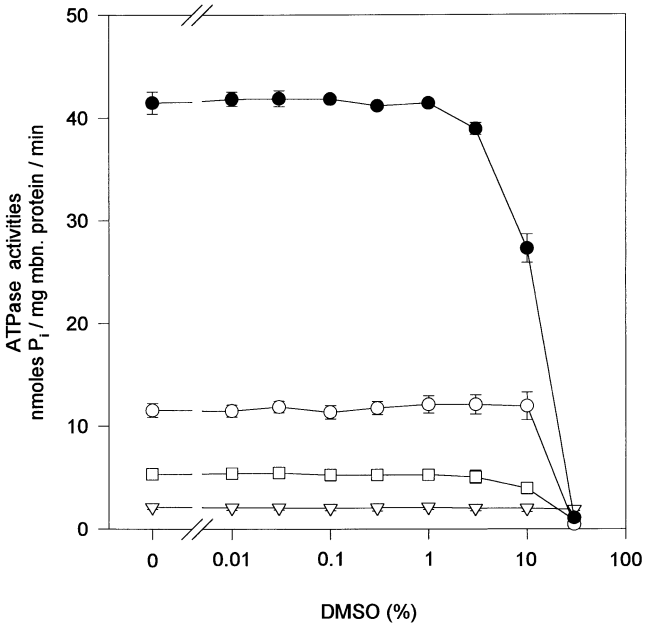

FIG. 1. Effects of DMSO on erythrocyte plasma membrane ATPase activities. (Mg^{2+})-ATPase (▽), $(Na^+ + K^+)$-ATPase (□), basal, calmodulin-independent $(Ca^{2+} + Mg^{2+})$-ATPase (○), and 10^{-8} M calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase (●) activities in the absence and presence of 1.4 mM to 4.2 M (0.01 to 30%) DMSO. Values are means ± SEM of three experiments measured in duplicate.

To further explore the nature of the inhibitory actions of DMSO on calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activity, Ca^{2+} activation and substrate dependency profiles were determined. Figure 2 depicts the effects of 10% DMSO on the calmodulin concentration-effect relationship on $(Ca^{2+} + Mg^{2+})$-ATPase activity at constant ATP and Ca^{2+} concentrations. DMSO at 10% resulted in a marked reduction of hydrolytic activity at concentrations of calmodulin above 10^{-9} M. Maximal calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activity at 10^{-7} M calmodulin was inhibited by 29.7% without an appreciable change in the affinity of calmodulin for the $(Ca^{2+} + Mg^{2+})$-ATPase (apparent $K_D = 1.0$ nM vs 1.2 nM control). This indicates a type of non-competitive inhibition by DMSO with respect to calmodulin.

Similarly, Fig. 3 shows that substrate activation of the basal enzyme activity by ATP was not affected by 10% DMSO. However, the same concentration of DMSO in-

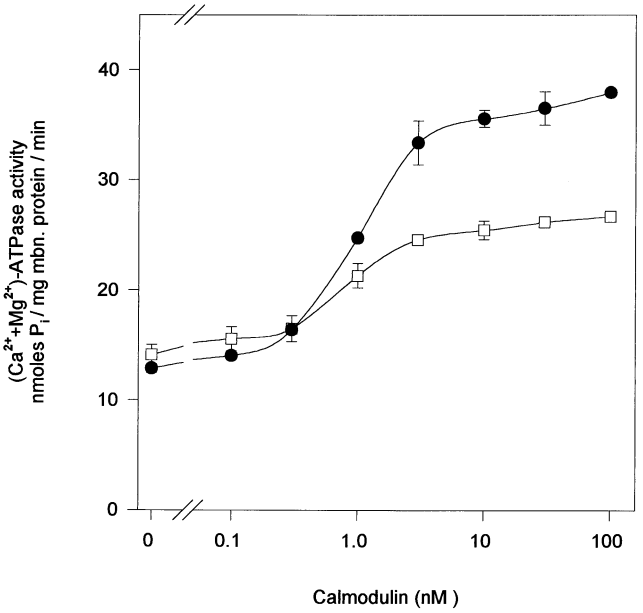

FIG. 2. Effect of DMSO on the concentration-effect relationship of calmodulin and $(Ca^{2+} + Mg^{2+})$-ATPase activity. Calmodulin stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase activity in the absence (●) and the presence (□) of 10% DMSO. Values are means ± SEM (N = 3).

hibited maximal enzyme velocity of the calmodulin-stimulated enzyme by as much as 45% in the presence of 3 mM ATP. Again, this is without an appreciable change in substrate affinity, further supporting the concept of a relatively selective inhibitory effect on calmodulin-stimulated enzyme.

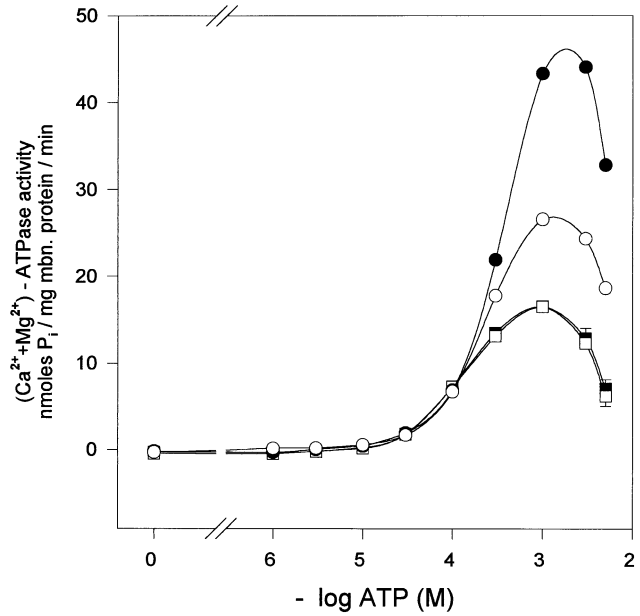

FIG. 3. Effects of DMSO on basal and calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase substrate activation. ATP activation of 10^{-8} M calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase without (●) and with (○) 10% DMSO. Basal $(Ca^{2+} + Mg^{2+})$-ATPase in the absence of calmodulin, without (■) and with (□) 10% DMSO. Values are the means of duplicate measurements from one experiment (●, ○) or the means ± SD of two experiments measured in duplicate (■, □).

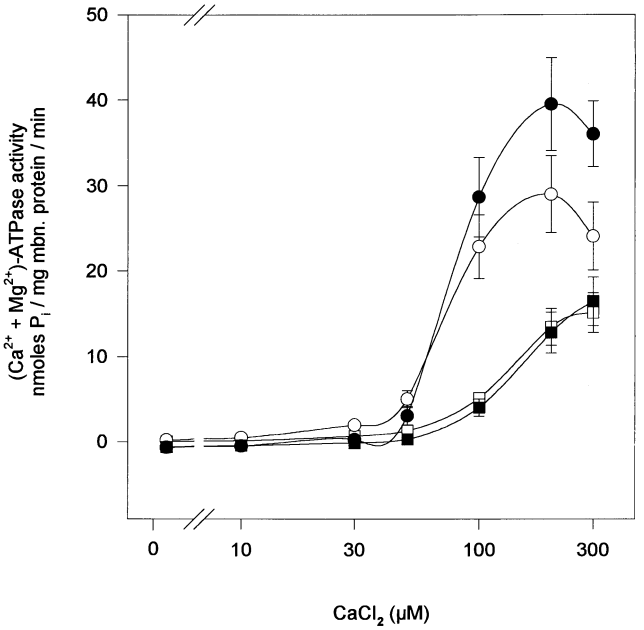

FIG. 4. Effects of DMSO on Ca^{2+} activation of $(Ca^{2+} + Mg^{2+})$-ATPase. Basal $(Ca^{2+} + Mg^{2+})$-ATPase (squares) and calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase (10^{-7} M, circles) in the absence (filled symbols) and the presence (open symbols) of 10% DMSO (1.4 M). The abscissa refers to $CaCl_2$ added to the incubation medium. Values are means ± SEM (N = 3).

Figure 4 depicts the Ca^{2+} activation curve of basal and calmodulin-stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activities in the presence and the absence of 10% DMSO. In the absence of DMSO, control curves (filled symbols) show the typical decrease in K_D for Ca^{2+} and an increase in the V_{max} of the $(Ca^{2+} + Mg^{2+})$-ATPase to 266% of basal activity in the presence of calmodulin (10^{-7} M). With the addition of 200 μM $CaCl_2$ resulting in 20 μM free Ca^{2+} according to ion-selective calcium electrode measurements [13], 10% DMSO selectively interfered with the calmodulin-activated enzyme, resulting in a 25% decrease in activity (from 42.8 ± 4.9 to 32.3 ± 4.0 nmol P_i/mg membrane protein/min).

Pretreatment of membranes (200 μg/mL, see Materials and Methods) at 37° for 1 hr with 10% DMSO and subsequent removal of the solvent resulted in membrane ATPase activities, including $(Ca^{2+} + Mg^{2+})$-ATPase, that were essentially indistinguishable from sham-pretreated control membranes. A noted exception to this was for the highest concentration of calmodulin (100 nM), which showed a small but significant decrease of 7.9% (Table 1). Pretreatment of 10^{-5} M calmodulin for 60 min at 37° with 10% DMSO, and subsequent final dilution of the DMSO and calmodulin in the assay incubation medium to 0.1% and 10^{-7} M, respectively, also had no effect on the ability of calmodulin to stimulate $(Ca^{2+} + Mg^{2+})$-ATPase compared with controls (Table 1).

TABLE 1. Reversibility of DMSO inhibition of calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase

Membrane/Calmodulin pretreatment	ATPase activity*	
	Sham control	10% DMSO
(Mg^{+})-ATPase activity	1.88 ± 0.08	1.83 ± 0.14
(Na^{+} + K^{+})-ATPase activity	4.72 ± 0.73	4.11 ± 0.14
Basal (Ca^{2+} + Mg^{2+})-ATPase	10.42 ± 0.14	10.02 ± 0.09
+3 nM CaM	31.60 ± 0.25	30.34 ± 0.36
+100 mM CaM	42.53 ± 0.28	39.18 ± 0.37†
100 mM Pretreated CaM	44.38 ± 0.69‡	44.32 ± 0.37‡

*Values are means ± SEM from four sets of determinations from two independent membrane pretreatment experiments, except where noted. The source of calmodulin in these experiments was from beef testes, which activates erythrocyte (Ca^{2+} + Mg^{2+})-ATPase identically to human erythrocyte-derived calmodulin used in all other experiments (results not shown). Unless indicated otherwise, there was no significant differences (at the $P < 0.05$ level) between control and 10% DMSO values.

†$P = 0.004$.

‡Means ± SD from four sets of determinations from one calmodulin pretreatment experiment.

DISCUSSION

In human erythrocytes, (Ca^{2+} + Mg^{2+})-ATPase activity is the biochemical expression of the plasma membrane Ca^{2+}-pump [17]. For these cells, it is the only mechanism to extrude Ca^{2+} from the inside and therefore can be used to estimate the ability of the cells to maintain low intracellular Ca^{2+}, a pivotal prerequisite to normal function in all cells [17, 18]. Xenobiotics, as well as endogenously generated compounds, that interfere with this ion-regulatory mechanism are destined to have an impact on a range of cellular functions, the most likely of which is cell death.

DMSO, a prevalent drug dissolution vehicle and cryoprotectant for cell and tissue culture purposes, was shown in the present work to interfere considerably with calmodulin stimulation of (Ca^{2+} + Mg^{2+})-ATPase activity in erythrocyte plasma membrane fragments. Calmodulin stimulation of the Ca^{2+}- pump is thought to play a crucial role in activating the Ca^{2+} extrusion process when the cell is stressed, either by chemical or physical means, to the point where intracellular Ca^{2+} accumulates to physiologically unacceptable levels. Thus, results from the experiments presented here suggest that high concentrations of DMSO can interfere with the Ca^{2+} extrusion process and its stimulation by calmodulin in particular. By inference, other calmodulin-stimulated processes, such as activation of cyclic nucleotide phosphodiesterases [19], nitric oxide synthase [20], and certain phosphatases [21] in more complex cells, may also be affected by the solvent. On the other hand, the present results clearly showed that high purity DMSO up to 10% (v/v) was remarkably ineffective in altering non-calmodulin-stimulated enzyme activities in a native membrane environment. Under present experimental conditions, 30% DMSO (v/v) was required to effectively eliminate all membrane-bound (Ca^{2+} + Mg^{2+})-ATPase and (Na^{+} + K^{+})-ATPase activities in a standard 60 min *in vitro* enzyme assay (Fig. 1).

From Fig. 2, it is clear that the inhibitory effect of 10% (v/v) DMSO (1.4 M) resulted from a decrease in enzymatic velocity without an appreciable change in the apparent affinity of calmodulin for the ATPase. This inhibition seemed to occur over a relatively narrow spectrum of concentrations, with 3% DMSO having a relatively small effect on the calmodulin activation, 10% DMSO inhibiting about half of the calmodulin-activated (Ca^{2+} + Mg^{2+})-ATPase, and 30% DMSO eliminating all membrane ATPase activities non-selectively (Fig. 1). While the inhibitory effect of DMSO was non-competitive with calmodulin, it appeared to be selective for the calmodulin-stimulated portion of the enzymatic activity at concentrations of <10% (v/v). Similarly, Fig. 3 and 4 show that basal, calmodulin-independent, substrate activation was completely unaffected by 10% DMSO. However, the calmodulin-stimulated enzyme was reduced markedly at ATP concentrations at or above the K_m (Fig. 3), as was the situation in the Ca^{2+} substrate activation curves shown in Fig. 4. The observed, relatively modest inhibition of (Na^{+} + K^{+})-ATPase activity by DMSO is in good agreement with previous reports on the inhibitory effects of the solvent on the Na^{+}-pump in bovine corneal endothelial cell cultures [22] and in sheep kidney medulla [7]. In the latter, DMSO is thought to change water activity to such an extent as to interfere with the interconversion of the phosphorylated forms of the enzyme [7]. Overall, our data suggest that the inhibitory effects of DMSO are directed toward the calmodulin-activated state of the enzyme and that DMSO does not appear to compete with either substrate activation or positive effector activation by Ca^{2+} or Mg^{2+}. Many apparently non-competitive, insurmountable inhibition kinetics are due to irreversible, covalent modification of the enzyme, its activators, and/or the membrane environment [13, 23]. To rule out an irreversible direct effect of DMSO on the (Ca^{2+} + Mg^{2+})-ATPase or its membrane environment, pretreatment of membranes with 10% DMSO, and subsequent removal of DMSO by extensive washing, returned membranes to their original calmodulin sensitivity (Table 1). This ruled out an irreversible or covalent modification of the calmodulin binding site (or any other site) on the (Ca^{2+} + Mg^{2+})-ATPase activity and suggested a reversible, non-competitive inhibition of the calmodulin-activated enzyme. Similarly, pretreatment of calmodulin itself with 10% DMSO had no effect on its subsequent stimulation of (Ca^{2+} + Mg^{2+})-ATPase activity, which also supported the concept of reversible, non-competitive interference with calmodulin stimulation. Thus, from a number of potential sites for drug interference (e.g. the Ca^{2+} binding or other sites on calmodulin or the hydrophobic calmodulin-binding domain of the (Ca^{2+} + Mg^{2+})-ATPase), the membrane-enzyme interface emerges as the most likely site for DMSO action. Unfortunately, in the absence of binding data, and direct measurements of membrane phase and fluidity changes, the exact mechanism and site of action of DMSO remain unknown. However, given the non-competitive nature of the inhibi-

tion, the hydrophobic enzyme-calmodulin interaction site is an unlikely candidate in contrast to the situation with higher-affinity calmodulin antagonists such as trifluoperazine, calmidazolium, and the naphthalenesulfonamide W-7 [24–26].

It is interesting to note that with (Ca^{2+} + Mg^{2+})-ATPase from human erythrocyte plasma membranes purified by calmodulin affinity chromatography, DMSO can have opposite effects from those reported in this study. Chiesi *et al.* [27] and others suggest that DMSO, in concentrations similar to those used in this work, enhances E_2 conformation competency, therefore promoting calmodulin-like activation of the enzyme [8, 28–30]. While some calmodulin antagonists, trifluoperazine and compound 48/80, are unable to inhibit DMSO-activated (Ca^{2+} + Mg^{2+})-ATPase, another antagonist, calmidazolium, inhibits all calmodulin-independent, calmodulin- and DMSO-activated non-membrane associated (Ca^{2+} + Mg^{2+})-ATPase activities [8]. More recently, Freire *et al.* [30], using purified pig erythrocyte (Ca^{2+} + Mg^{2+})-ATPase to hydrolyze the pseudo substrate *p*-nitrophenylphosphate, which is activated extensively by DMSO in the absence of Ca^{2+}, also show a concentration-dependent inhibition of (Ca^{2+} + Mg^{2+})-ATPase activity by DMSO similar to the inhibition we report in Fig. 4 [30]. We concur with these authors that the actions of DMSO cannot be attributed to a "simple calmodulin-like" effect reported by others, but rather result in a shift in enzyme conformation that is less sensitive to calmodulin. A plausible explanation for this may be the lowering of water activity by the solvent in or near the calmodulin binding domain, and at higher concentrations in the phosphoenzyme hydrolysis reaction step, $E_2P + H_2O \rightarrow E_2 + P_i$. This step is accelerated by ATP, dependent upon Mg^{2+} (to form the active MgATP complex), and stimulated by calmodulin [31]. To pinpoint the exact mechanism, however, studies comparing shifts in water activity of membrane-bound and vesicle reconstituted enzyme would be crucial. A lowered water activity could also explain the inhibition seen here with (Na^+ + K^+)-ATPase, which is in line with previously published reports involving the sodium pump [32–34].

Early reports of both inhibitory and stimulatory effects of DMSO on various sarcoplasmic reticular membrane activities [35] and purified human erythrocyte (Ca^{2+} + Mg^{2+})-ATPase [28–30, 36], in conjunction with data from this report, serve as a reminder for caution when interpreting kinetic parameters of enzymatic mechanisms in non-native membrane environments. Moreover, these results can be used to provide guidelines for using the solvent as a drug vehicle and a tissue culture tool, as well as to emphasize the importance of appropriate vehicle controls.

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