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INHIBITION OF RED CELL Ca^{2+}-ATPase BY VANADATE

GUY H. BOND and PATRICIA M. HUDGINS

Department of Physiology, Kirksville College of Osteopathic Medicine, Kirksville, MO 63501 (U.S.A.)

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Summary

1. The Mg^{2+}- plus Ca^{2+}-dependent ATPase (Ca^{2+}-ATPase) in human red cell membranes is susceptible to inhibition by low concentrations of vanadate.

2. Several natural activators of Ca^{2+}-ATPase (Mg^{2+}, K^+, Na^+ and calmodulin) modify inhibition by increasing the apparent affinity of the enzyme for vanadate.

3. Among the ligands tested, K^+, in combination with Mg^{2+}, had the most pronounced effect on inhibition by vanadate.

4. Under conditions optimal for inhibition of Ca^{2+}-ATPase, the $K_{1/2}$ for vanadate was 1.5 μM and inhibition was nearly complete at saturating vanadate concentrations.

5. There are similarities between the kinetics of inhibition of red cell Ca^{2+}-ATPase and ($Na^+ + K^+$)-ATPase prepared from a variety of sources; however, ($Na^+ + K^+$)-ATPase is approx. 3 times more sensitive to inhibition by vanadate.

Introduction

Pentavalent vanadium (vanadate) is a potent inhibitor of ($Na^+ + K^+$)-ATPase [1]. Inhibition of this enzyme by vanadate is unusual because it is influenced by all ligands which are required for activity: inhibition is facilitated by Mg^{2+} and K^+ (Refs. 1—4), and antagonized by Na^+ (Ref. 4) and ATP [5]. Vanadate is present in mammalian tissues at concentrations which might inhibit ($Na^+ + K^+$)-ATPase in vivo, and it has been suggested that vanadate might function as an endogenous regulator of ($Na^+ + K^+$)-ATPase [1—3]. This question remains

Abbreviations: $K_{1/2}$, the concentration of ligand required for half-maximal inhibition (a measure of apparent affinity); EGTA, ethyleneglycol bis(β-aminoethylether)-*N,N'*-tetraacetic acid.

unresolved but has led to an interest in possible effects of vanadate on other ATPases.

Josephson and Cantley [2] reported that vanadate has no effect on Ca^{2+}-ATPase from sarcoplasmic reticulum, mitochondrial F_1-ATPase or actomyosin-ATPase. On the other hand, dynein-ATPase is inhibited by vanadate at concentrations comparable to those which inhibit ($Na^+ + K^+$)-ATPase [6,7], and the present study demonstrates that the same is true of Ca^{2+}-ATPase in human red cell membranes.

Inhibition of red cell Ca^{2+}-ATPase by vanadate exhibits a complex dependence on the concentrations of a number of natural activators of this enzyme: Mg^{2+}, K^+ or Na^+, and a soluble cytoplasmic protein activator present in red cell hemolysate. Mg^{2+} is an essential requirement for the activity of Ca^{2+}-ATPase [8], and either K^+ or Na^+ can further activate the enzyme approx. 1.5-fold [9,10]. The soluble cytoplasmic protein produces marked activation [11]. This protein appears to be identical to the Ca^{2+}-dependent regulator protein which is widely distributed in mammalian tissues, and mediates the Ca^{2+}-dependent control of a number of enzymes [12—14]. This protein has recently been named 'calmodulin' [15]. These activators all facilitate inhibition by promoting the binding of vanadate. A preliminary report of this work has appeared [16].

Methods

Preparation of membranes. Membranes were prepared from freshly outdated human blood as previously described [10]. Briefly, washed red cells were lysed in ice-cold 1 mM Tris-EDTA (pH 7.8 at 23°C) and then washed repeatedly in the same medium to remove visible traces of hemoglobin. Protein was measured by using the method of Lowry et al. [17].

Source of calmodulin. The supernatant fluid from the first centrifugation after lysis was dialyzed against deionized water at 4°C until no Na^+ or K^+ could be detected by flame photometry. This membrane-free hemolysate was used as the source of cytoplasmic protein activator of Ca^{2+}-ATPase [11], or calmodulin. Since calmodulin is the only activator of Ca^{2+}-ATPase that has been isolated from hemolysate [18], the effect of hemolysate must be attributable to its content of calmodulin.

Assay conditions. All incubations were carried out at 38°C in a volume of 2 ml. Assay conditions common to all experiments were: 2 mM ATP (disodium or Tris salt), 63 mM Tris-HCl (pH 7.4 at 38°C), 200 μM Ca^{2+} and 0.25 mM ouabain. Other conditions are described in the figure legends. Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase activity from total ATPase activity measured in the presence of Ca^{2+}. Mg^{2+}-ATPase activity was measured in the presence of 0.25 mM EGTA and without Ca^{2+}. ATPase activity was assayed in terms of inorganic phosphate (P_i) production as described previously [10]. All assays were conducted in duplicate. Figures represent the average of three or more separate experiments.

Materials. The ATP used in these experiments was Sigma grade II ATP (Sigma Chemical Co., St. Louis, MO, U.S.A.). This ATP does not contain vanadate. All other chemicals were reagent grade. Cations were present as chloride salts; vanadate as the trisodium salt.

Kinetic analysis. Inhibition was analyzed kinetically by first calculating fractional inhibition (f_i) as a function of vanadate concentration under various conditions: activity in the presence of vanadate was expressed as a fraction of a control activity and this fraction was subtracted from 1.0. Fractional inhibition is thus expressed on a scale of 0 to 1.0, from no inhibition to complete inhibition, respectively. Curves describing fractional inhibition as a function of vanadate concentration were rectangular hyperbolae subject to analysis by standard graphical procedures used to evaluate the relationship between rate and substrate concentration in enzyme kinetics. We used modified Lineweaver-Burk plots in which vanadate concentration divided by f_i was plotted as a function of vanadate concentration. In this treatment f_i is analogous to rate and vanadate concentration is analogous to substrate concentration. These plots were linear, the abscissa intercept giving the $K_{1/2}$ for vanadate, and the reciprocal of the slope giving the maximum inhibition (I_{max}) at a saturating vanadate concentration [19]. $K_{1/2}$ and I_{max} values were obtained by linear regression analysis.

Results

Effects of Mg^{2+} concentration on inhibition by vanadate

Fig. 1A and B shows the influence of Mg^{2+} concentration on Ca^{2+}-ATPase activity and on inhibition by vanadate. In the absence of vanadate, Mg^{2+} concentrations above 6 mM were only slightly inhibitory. Fig. 1A shows that 2 μM vanadate was not inhibitory in the absence of either K^+ or Na^+. The apparent activation by vanadate at the lowest Mg^{2+} concentrations might be caused by the small amount of Na^+ added as Na_3VO_4. In the presence of K^+ (Fig. 1B), 2 μM vanadate inhibited substantially and this inhibition was more pronounced at higher Mg^{2+} concentrations. With Na^+ present, vanadate was less inhibitory,

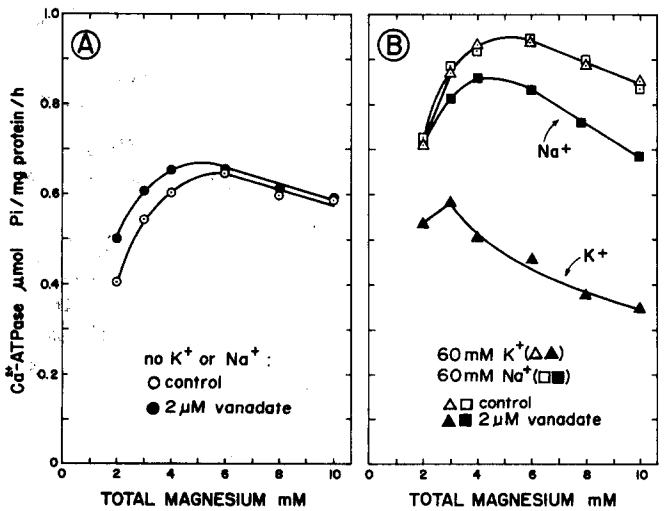

Fig. 1. Ca^{2+}-ATPase activity as a function of Mg^{2+} concentration with and without vanadate (2 μM). (A) No alkali metal cation was present. (B) Either 60 mM K^+ or 60 mM Na^+ was present. Other conditions are described in Methods. Higher control activities in B as compared to A reflect activation by Na^+ or K^+.

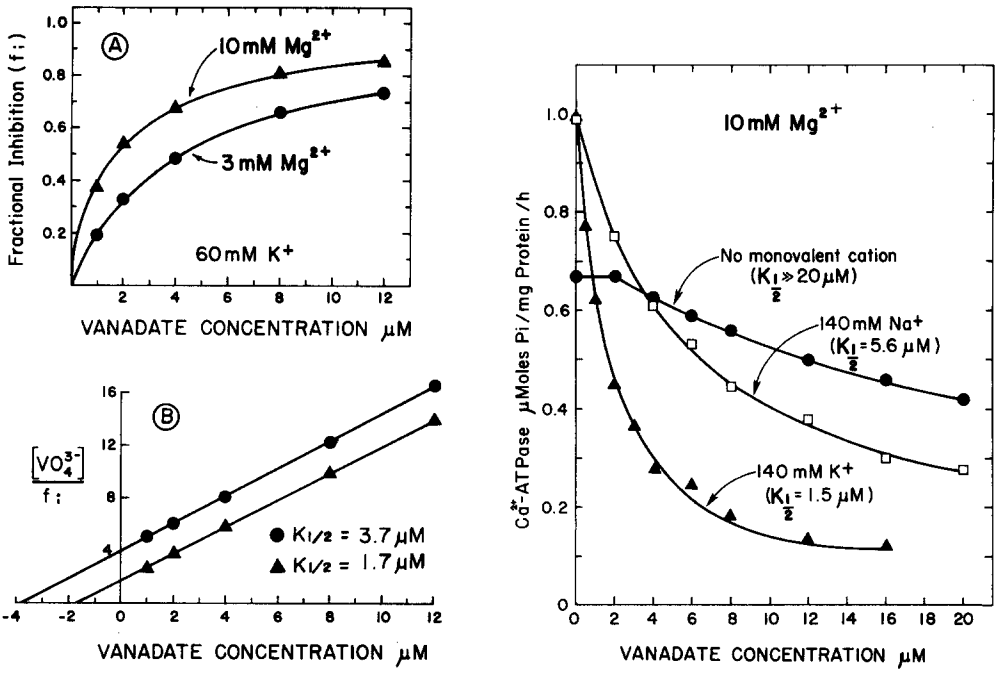

Fig. 2. (A) Fractional inhibition (f_i) of Ca^{2+}-ATPase as a function of vanadate concentration. The Mg^{2+} concentration was either 3 or 10 mM and K^+ was 60 mM. Fractional inhibition was calculated as described in Methods. (B) A kinetic plot of the data in A. This is a modified Lineweaver-Burk plot in which f_i rather than rate is treated as the dependent variable. See Methods for other details. Kinetic constants are shown on the figure.

Fig. 3. Ca^{2+}-ATPase activity as a function of vanadate concentration in the presence and absence of K^+ or Na^+ (140 mM). The Mg^{2+} concentration was 10 mM. $K_{1/2}$ values for vanadate are shown on the figure. $K_{1/2}$ in the absence of K^+ or Na^+ was estimated visually in other experiments extended to higher vanadate concentrations. $K_{1/2}$ in the presence of K^+ or Na^+ was obtained from kinetic plots similar to that in Fig. 2B. These plots were linear. I_{max} was 0.96 with either K^+ or Na^+. The curves were constructed from the indicated kinetic constants by multiplying the activity in the absence of vanadate by $(1 - f_i)$, where f_i is given by: $0.96[V]/(K_{1/2} + [V])$.

but an Mg^{2+}-dependent inhibition was still evident (Fig. 1B). These results show that Mg^{2+}, K^+ and Na^+ all facilitate inhibition by vanadate.

These cations could act either by increasing the affinity of the enzyme for vanadate, or by increasing the extent of inhibition after vanadate has bound. An experiment designed to distinguish between these possibilities in the case of Mg^{2+} is shown in Fig. 2A and B. In Fig. 2A, fractional inhibition of Ca^{2+}-ATPase is plotted as a function of vanadate concentration at two Mg^{2+} concentrations. Vanadate was more inhibitory at the higher Mg^{2+} concentration (10 mM), and the curve was shifted to the left of that with 3 mM Mg^{2+}. Clearly, Mg^{2+} acted at least in part by increasing the apparent affinity of Ca^{2+}-ATPase for vanadate. Fig. 2B shows a modified Lineweaver-Burk plot of these data (see Methods). Linearity of these plots indicates that vanadate binds to a single class of inhibitory sites. Maximum inhibition (I_{max}) at a saturating vanadate concentration was 0.96 at both Mg^{2+} concentrations. Inhibition is, therefore,

nearly complete when vanadate has bound, and Mg^{2+} acts only to increase the apparent affinity for vanadate. In most subsequent experiments, the Mg^{2+} concentration was 10 mM.

Effect of K^+ and Na^+ on inhibition by vanadate

The effects of K^+ and Na^+ on inhibition were examined more fully in Fig. 3, where Ca^{2+}-ATPase activity is plotted as a function of vanadate concentration. In the absence of K^+ or Na^+, inhibition was just detectable at 4 μM vanadate; thereafter, activity declined gradually. This curve did not fit a consistent kinetic pattern, and there was a considerable variation between experiments. Fig. 3 shows that either K^+ or Na^+ produced an equivalent activation of Ca^{2+}-ATPase in the absence of vanadate. Both cations facilitated inhibition, but K^+ was more effective than Na^+. Kinetic plots similar to those shown in Fig. 2B were constructed from these data. These plots were linear, and gave the $K_{1/2}$ values for inhibition by vanadate shown next to the curves with K^+ and Na^+ (Fig. 3). I_{max} was 0.96 in both cases. These results show that K^+ and Na^+ both act by increasing the apparent affinity for vanadate.

Concentrations of Li^+ up to 140 mM did not activate Ca^{2+}-ATPase and did not facilitate inhibition by vanadate (data not shown). Therefore, the binding of vanadate appears to be independent of ionic strength, and the effects of Mg^{2+}, K^+ and Na^+ must be attributed to specific interactions at sites on the enzyme.

Figs. 4 and 5 compare the dependence of inhibition on the concentrations of K^+ and Na^+, respectively. The upper curves in these figures depict normal patterns of activation of Ca^{2+}-ATPase by these cations. In the presence of 1 μM vanadate, K^+ activated only slightly at low concentrations, and then inhibited (Fig. 4). At higher vanadate concentrations, however, no K^+-dependent activity was evident, and the only effect of K^+ was to facilitate inhibition. The site occupied by K^+ to facilitate inhibition was saturated at approx. 40 mM K^+ as activity declined to a plateau (Fig. 4). When K^+ was saturating, inhibition was limited by the vanadate concentration.

In the presence of vanadate, Na^+ was a poor activator (Fig. 5). Some Na^+-dependent inhibition was seen at the highest vanadate concentrations, but this inhibition was small compared to that observed with K^+ (Fig. 4). It appeared from this experiment that vanadate interfered with activation of Ca^{2+}-ATPase by Na^+.

The interaction between K^+ and vanadate was examined further in another series of experiments similar to those in Fig. 4, in which vanadate concentration was varied at several fixed concentrations of K^+. Inhibition was then expressed as a function of the concentrations of either vanadate or K^+ at fixed concentrations of the other ion. The data were analyzed kinetically as described in Methods, and $K_{1/2}$ values for inhibition by vanadate and K^+ are plotted in Fig. 6A and B, respectively. The results show that each ion facilitates binding of the other. These findings are consistent with a model in which vanadate binds preferentially to enzyme complexed with K^+ (and Mg^{2+}). This model also predicts that inhibition will approach completion when vanadate concentration increases at fixed K^+ but is less than complete when K^+ concentration increases at fixed vanadate concentrations, as in Fig. 4.

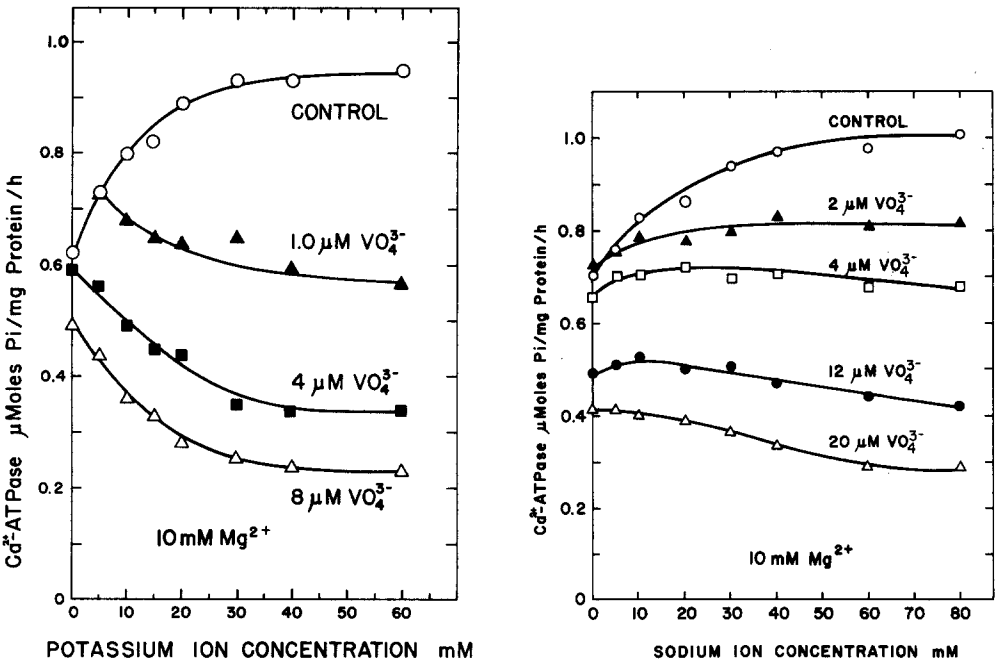

Fig. 4. Ca^{2+}-ATPase activity as a function of K^+ concentration, with and without vanadate. The Mg^{2+} concentration was 10 mM.

Fig. 5. Ca^{2+}-ATPase activity as a function of Na^+ concentration, with and without vanadate. The Mg^{2+} concentration was 10 mM.

Effect of calmodulin on inhibition by vanadate

Ca^{2+}-ATPase activity is a saturable function of calmodulin concentration [11], and the 100 μl of hemolysate used in these experiments provided maximal activation. The Mg^{2+} concentration was 3 mM as compared to 10 mM in most other experiments because we have observed that Mg^{2+} can interfere with activation of Ca^{2+}-ATPase by calmodulin.

With 60 mM K^+ (Fig. 7A), or 60 mM Na^+ (Fig. 7B), there was marked activation by calmodulin in the absence of vanadate. In both cases, vanadate was more inhibitory in the presence of calmodulin. Kinetic analysis of these curves showed that $K_{1/2}$ for vanadate was lower in the presence of calmodulin. I_{max} was 0.96 in both cases. Calmodulin reduced the $K_{1/2}$ value for vanadate by a factor of approx. 2, and $K_{1/2}$ with Na^+ was still higher than with K^+. Calmodulin also facilitated inhibition in the absence of an alkali metal ion (not shown).

We measured $K_{1/2}$ for inhibition of (Na^+ + K^+)-ATPase in one experiment under conditions which were the same as those in Fig. 7A, except that 34 mM Na^+ was present and Ca^{2+} and ouabain were omitted. The $K_{1/2}$ value for vanadate was 0.5 μM, whether calmodulin was present or not. Therefore, (Na^+ + K^+)-ATPase is approx. 3 times more sensitive than Ca^{2+}-ATPase to inhibition by vanadate under similar assay conditions and in the presence of calmodulin, as in Fig. 7A. This comparison of relative sensitivities is of interest because it was

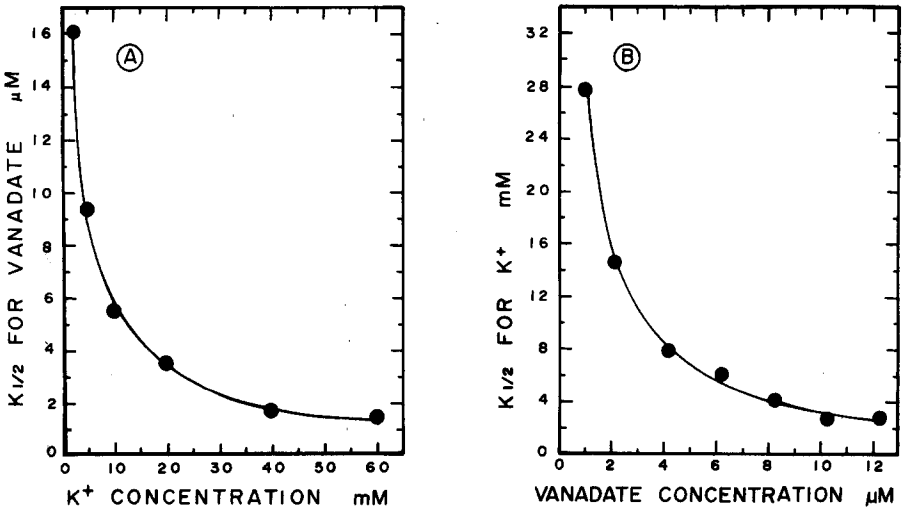

Fig. 6. (A) $K_{1/2}$ for inhibition by vanadate as a function of K^+ concentration. At each K^+ concentration shown on the abscissa, f_i was calculated as a function of the vanadate concentration as described in Methods. Each $K_{1/2}$ value represents the average of four or more separate experiments carried out at a fixed K^+ concentration and a range of vanadate concentrations similar to that in Fig. 3. (B) $K_{1/2}$ for inhibition by K^+ as a function of vanadate concentration.

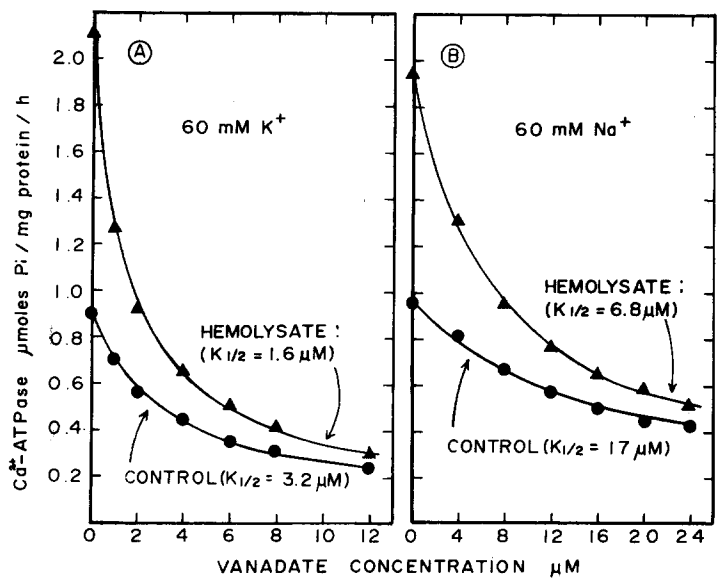

Fig. 7. Ca^{2+}-ATPase activity as a function of vanadate concentration, with and without hemolysate (100 μM). This volume of hemolysate contributed a saturating concentration of calmodulin. The Mg^{2+} concentration was 3 mM. In (A) 60 mM K^+ was present and (B) 60 mM Na^+. $K_{1/2}$ values are shown on the figure. I_{max} was 0.96 in both A and B. The solid lines through the experimental points were calculated as described in the legend of Fig. 3.

made under conditions approximating the intracellular composition of red cells, except for the high Ca^{2+} concentrations used for the assay of Ca^{2+}-ATPase.

Discussion

The present study has shown that Mg^{2+}, K^+ or Na^+, and calmodulin, all activators of Ca^{2+}-ATPase, also facilitate inhibition by vanadate. They act by increasing the apparent affinity of an inhibitory site for vanadate, and vanadate binds more readily when all activation sites are occupied by their respective ligands. The extent of inhibition once vanadate has bound is nearly complete in all cases.

Among the ligands tested, K^+ and Na^+ had the most pronounced influence on inhibition. These cations, at saturating concentrations, produced an equivalent activation of Ca^{2+}-ATPase (Figs. 1A, B and 3), and the two cations together have no greater effect than either alone [10]. These observations suggest that K^+ and Na^+ activate at the same site by the same mechanism. This site has a higher affinity for K^+ than for Na^+ [9,20], but no other difference between these cations as activators has been described. Nevertheless, in terms of facilitating inhibition by vanadate, K^+ was markedly more effective than Na^+ (Fig. 3). The greater efficacy of K^+ as compared to Na^+ is clearly unrelated to the absolute rate of catalysis. If K^+ and Na^+ activate by the same mechanism, it appears that Ca^{2+}-ATPase can exist in two different, but equally active conformational states depending on which cation is bound, and this difference influences the binding of vanadate.

Ca^{2+}-ATPase activity in the presence of K^+ or Na^+ fell below that in the absence of either cation as the vanadate concentration increased (Fig. 3). This result shows that vanadate did not selectively inhibit the K^+- or Na^+-dependent components of activity; rather, all the measurable activity was more susceptible to inhibition by vanadate in the presence of K^+ or Na^+. These observations support the interpretation that there is a single population of Ca^{2+}-ATPase in red cell membranes which is uniformly activated by K^+ or Na^+, and uniformly inhibited by vanadate.

Calmodulin increased Ca^{2+}-ATPase activity more than did either K^+ or Na^+ (Fig. 7A and B). Nevertheless, at saturation, calmodulin decreased the $K_{1/2}$ value for vanadate by a factor of only approx. 2 in the presence of K^+ (Fig. 7A) or Na^+ (Fig. 7B). Again, it is clear that the effect these ligands have on vanadate binding is not directly related to the catalytic rate. The effect of calmodulin could be superimposed on that due to K^+ or Na^+ and Mg^{2+}, indicating that the occupation of binding sites of all these ligands caused a qualitatively similar and additive effect on the conformation of a vanadate binding site.

Although calmodulin appeared to increase the affinity of an inhibitory site for vanadate, the results are open to another interpretation. Activity in the presence of calmodulin was never lower than in its absence when the vanadate concentration increased (Fig. 7A and B), and it could be argued that vanadate interfered with activation by calmodulin. This interpretation requires two classes of site for vanadate : one at which vanadate inhibits Ca^{2+}-ATPase, and another at which it interferes with activation by calmodulin. If the latter site

had a higher affinity for vanadate than the former, it could appear that calmodulin decreased $K_{1/2}$ for vanadate, as observed. Linearity of kinetic plots argues against two classes of site, but departures from linearity can be difficult to detect unless the sites have substantially different affinities.

The present experiments revealed a striking similarity in the kinetics of inhibition by vanadate of red cell Ca^{2+}-ATPase, and $(Na^+ + K^+)$-ATPase obtained from numerous sources, including red cells. Mg^{2+} and K^+ also facilitate inhibition of $(Na^+ + K^+)$-ATPase by promoting the binding of vanadate [1—5]. Thus, the mechanism of inhibition of both enzymes by vanadate is generally similar in the sense that interactions between sites for Mg^{2+}, K^+ and vanadate have similar end results.

Josephson and Cantley [2] were not able to show that vanadate, at low concentrations, inhibited Ca^{2+}-ATPase from sarcoplasmic reticulum. On the other hand, since our paper was submitted for publication, two reports have appeared describing an inhibition of Ca^{2+}-ATPAse by vanadate. O'Neal et al. [21] reported that vanadate inhibits a number of ATPase including Ca^{2+}-ATPase from sarcoplasmic reticulum and from red cells. No Mg^{2+}- or K^+-dependence was described and $K_{1/2}$ for vanadate was higher than we found. Wang et al. [22] described a K^+-dependent inhibition of Ca^{2+}-ATPase from sarcoplasmic reticulum with a $K_{1/2}$ value for vanadate of 5 μM. This enzyme, like the one from red cells, requires Mg^{2+} for activity and is activated by K^+, Na^+ and certain other alkali metal cations (cf. Refs. 23 and 24).

Acknowledgements

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