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VANADATE INHIBITION OF THE Ca^{2+}-ATPase FROM HUMAN RED CELL MEMBRANES

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Summary

(1) VO_3^- combines with high affinity to the Ca^{2+}-ATPase and fully inhibits Ca^{2+}-ATPase and Ca^{2+}-phosphatase activities. Inhibition is associated with a parallel decrease in the steady-state level of the Ca^{2+}-dependent phosphoenzyme.

(2) VO_3^- blocks hydrolysis of ATP at the catalytic site. The sites for VO_3^- also exhibit negative interactions in affinity with the regulatory sites for ATP of the Ca^{2+}-ATPase.

(3) The sites for VO_3^- show positive interactions in affinity with sites for Mg^{2+} and K^+. This accounts for the dependence on Mg^{2+} and K^+ of the inhibition by VO_3^-. Although, with less effectiveness, Na^+ substitutes for K^+ whereas Li^+ does not. The apparent affinities for Mg^{2+} and K^+ for inhibition by VO_3^- seem to be less than those for activation of the Ca^{2+}-ATPase.

(4) Inhibition by VO_3^- is independent of Ca^{2+} at concentrations up to 50 μM. Higher concentrations of Ca^{2+} lead to a progressive release of the inhibitory effect of VO_3^-.

Introduction

Since the discovery that commercial ATP obtained from equine muscle contains VO_3^- as an impurity many studies on its effects on the (Na^+ + K^+)-ATPase have appeared (for references see Ref. 1). VO_3^- inhibits (Na^+ + K^+)-ATPase with high affinity through a process which shows complex interactions with ATP, Mg^{2+}, Na^+ and K^+ [2—4]. In a preliminary report Bond and Hudgins [5] stated that the Ca^{2+}-ATPase of red cell membranes is sensitive to VO_3^-. This finding has been recently extended to Ca^{2+}-activated ATPase from sarcoplasmic reti-

culum (Ref. 6 and Barrabin, H. and de Meis, L., personal communication) and ascites cells [6] and to the active transport of calcium out of squid axons [7]. Studies of the effects of VO_3^- are useful because they may help in the understanding of the mechanism of hydrolysis of ATP by cation transport systems and give information on the physiological role of VO_3^- as a regulator of active transport of cations in vivo.

In this paper we present a study of the effects of VO_3^- on the Ca^{2+}-ATPase of human red cell membranes. Results show that VO_3^- inhibits with high affinity the Ca^{2+}-ATPase and that the inhibition is modulated by Mg^{2+} and K^+ in a way which resembles in many aspects the effects of these cations during inhibition of the $(Na^+ + K^+)$-ATPase by VO_3^-.

Materials and Methods

Materials. Fragmented membranes from human red blood cells were prepared as described previously [8]. This procedure yields membranes which are almost free of calmodulin [9]. [^{32}P]ATP was prepared according to the method of Glynn and Chappell [10] using enzymes and cofactors from Sigma Chemical Co. (U.S.A.). ^{32}P-labelled orthophosphate was provided by Comisión Nacional de Energía Atómica, Argentina. ATP prepared from yeast was purchased from Boehringer-Mannheim GmbH and used as the disodium salt. Vanadate solutions were prepared by dissolving NH_4VO_3 in a solution of 50 mM Tris-HCl (pH 7.7). All other reagents were of analytical reagent grade.

Methods. Unless indicated, enzymatic assays were performed by incubating membranes at 37°C in media containing: 5 mM $MgCl_2$, 0.5 mM EGTA, 0.6 mM $CaCl_2$, 100 mM KCl, 50 mM Tris-HCl (pH 7.8) and various amounts of ATP, *p*-nitrophenylphosphate (ditris salt) and fragmented membranes. Ca^{2+}-dependent activities were taken as the difference between the activities in the above media and the activities measured in the same media except that $CaCl_2$ was omitted. When the concentration of ATP during the assays was more than 0.1 mM the concentration of membranes was 1 mg protein/ml and the orthophosphate liberated was estimated by a modification of the Fiske-Subbarow procedure [11]. When the concentration of ATP was less than 0.1 mM, Ca^{2+}-ATPase activity was measured following the procedure described previously [12] using [^{32}P]ATP as the substrate. Ca^{2+}-phosphatase activity was measured using 7 mM *p*-nitrophenylphosphate (ditris salt) in a medium with 1 mM MgATP and $1 \cdot 10^{-3}$ M ouabain according to the method of Rega et al. [13]. Ca^{2+}-dependent phosphoenzyme was measured after 30 s phosphorylation in media with 15 μM [^{32}P]ATP at 0°C following the procedure described previously [14]. Control experiments showed that at 37°C no pretreatment with VO_3^- was necessary for maximal inhibition but, since the onset of inhibition at 0°C is much slower than at 37°C, before phosphorylation the membranes were preincubated at 37°C during 10 min in the reaction media without ATP. Protein was estimated by using the method of Lowry et al. [15].

Results

Fig. 1 compares the effects of increasing concentrations of VO_3^- on the enzymic activities of the Ca^{2+}-ATPase from red cell membranes. ATPase activi-

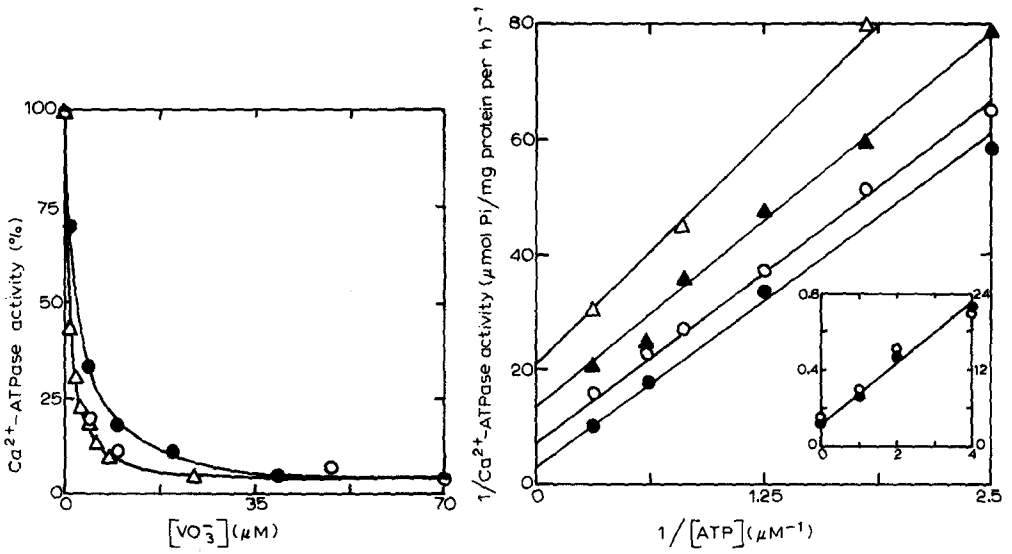


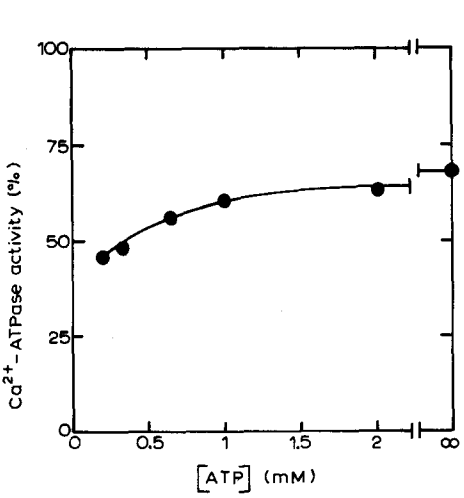
Fig. 1. The effects of VO_3^- on Ca^{2+}-ATPase activity measured at 15 (○) and 750 (●) μM ATP and on Ca^{2+}-phosphate activity (△) of human red cell membranes. Activities are expressed as percent of the activities at 0 μM VO_3^- which were (μmol/mg protein per h): Ca^{2+}-ATPase at 750 μM ATP, 1.05; Ca^{2+}-ATPase at 15 μM ATP, 0.27; and Ca^{2+}-phosphatase, 0.37.

Fig. 2. A Lineweaver-Burk plot of Ca^{2+}-ATPase activity as a function of ATP concentration measured in media with 0 (●); 1 (○); 2 (▲) and 4 (△) μM VO_3^-. The inset presents the reciprocal of K_m (○, (μM) left-hand site ordinate) and of V (●, (μmol/mg protein per h) right-hand side ordinate) obtained from the intercepts of the lines that fit the points in the figure, as a function of the concentration of VO_3^- (μM).

ties were measured at 15 and 750 μM ATP. Results show that Ca^{2+}-ATPase and Ca^{2+}-phosphatase activities are inhibited with high affinity. We have shown elsewhere that the Ca^{2+}-ATPase possesses high-affinity catalytic sites and low-affinity regulatory sites for ATP [12]. At 15 μM ATP only the catalytic sites of the ATPase are significantly occupied. Therefore, full inhibition at 15 μM ATP indicates that binding of ATP at the regulatory site is not required for VO_3^- to inhibit Ca^{2+}-ATPase activity.

Effects of VO_3^- at low [ATP]

The interactions of VO_3^- and ATP at the catalytic site were studied measuring the effects of different concentrations of VO_3^- on the substrate activation curve of the Ca^{2+}-ATPase at low (0.4 to 3.2 μM) ATP concentrations. Double-reciprocal plots of the activity vs. ATP concentration (Fig. 2) give straight lines for each of the VO_3^- concentrations tested. The lines are almost parallel to one another indicating that as the concentration of VO_3^- increases V and K_m decrease in a roughly proportional fashion. When the reciprocal of K_m and of V are plotted against the concentration of VO_3^- (inset in Fig. 2) the values can be fitted by straight lines of positive intercept and slope. This indicates that both K_m and V vary with VO_3^- concentration according to an equation containing a term $(1 + (VO_3^-)/K_i)^{-1}$. The value of K_i in this equation can be estimated from the intercept at the abscissa of the plot in the inset to Fig. 2 to be about 0.8 μM for both K_m and V.



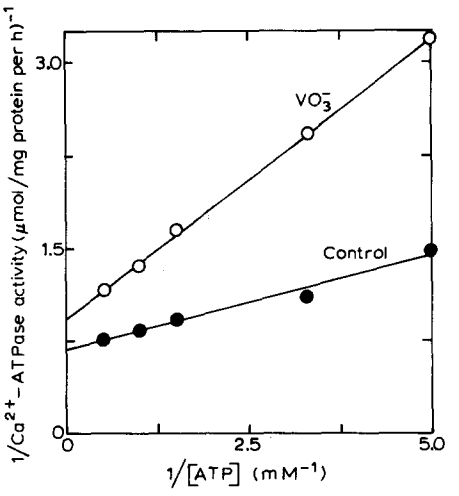


Fig. 3. Percentage of Ca^{2+}-ATPase activity remaining in the presence of 1 μM VO_3^- as a function of ATP concentration. The Ca^{2+}-ATPase at infinite ATP concentration was estimated by extrapolation of double-reciprocal plots of the activity vs. ATP concentration.

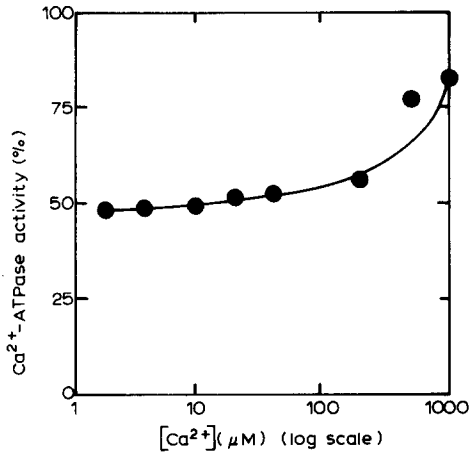
Fig. 4. A Lineweaver-Burk plot of Ca^{2+}-ATPase activity vs. ATP concentration from 0.2 to 2.0 mM in the presence and absence of 1 μM VO_3^-. The points represent the difference between total Ca^{2+}-ATPase activity and the maximum activity resulting from the occupation of the high-affinity site for ATP. The latter, calculated from double-reciprocal plots similar to those used for the experiment of Fig. 2, were 0.29 μmol/mg per h and 0.15 μmol/mg per h in the absence and presence of 1 μM VO_3^-, respectively.

Effects of VO_3^- at high [ATP]

Results in Fig. 3 show that the ATPase activity remaining in the presence of 1 μM VO_3^- increases from 48 to 70% in going from 0.2 mM to non-limiting concentrations of ATP. The fact that inhibition by VO_3^- is partially surmountable by ATP at concentrations at which the catalytic site is fully occupied indicates that VO_3^- interacts with the regulatory sites for ATP. To study this, activation by ATP at the regulatory site of the Ca^{2+}-ATPase was measured in media with and without 1 μM VO_3^- (Fig. 4). VO_3^- reduces by about 40% the maximum activating effect of ATP and increases from 230 to 480 μM the K_m value of the regulatory site for ATP (see Fig. 2). Since at low ATP concentration 1 μM VO_3^- inhibits 50% of the Ca^{2+}-ATPase, the reduction in the maximum activating effect of high ATP concentration can be fully accounted for by the effects of VO_3^- at the catalytic site. The decrease by VO_3^- of the affinity for ATP is paralleled by a similar effect of ATP on the affinity for VO_3^- since, as can be seen in Fig. 1, when ATP concentration is raised from 15 to 750 μM the concentration of VO_3^- for half maximal inhibition is doubled. It seems, therefore, that between the sites for VO_3^- and the regulatory sites for ATP there are negative interactions in affinity. These may explain the partial release of inhibition that is observed at high concentrations of ATP (Fig. 3).

The effects of Ca^{2+}

Inhibition of the Ca^{2+}-ATPase by 1 μM VO_3^- was measured at Ca^{2+} concentrations ranging from 2 to 1000 μM (Fig. 5). At concentrations of Ca^{2+} up to 40 μM the inhibition by VO_3^- remains constant, indicating that there are no



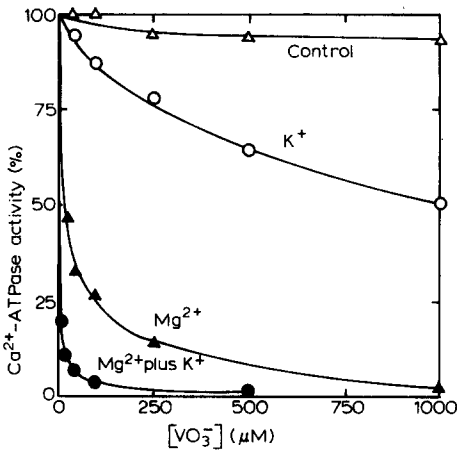


Fig. 5. Percentage of Ca^{2+}-ATPase activity remaining in the presence of 1 μM VO_3^- as a function of the concentration of Ca^{2+} in media with 1 mM ATP. All media contained 3 mM Ca^{2+}-EDTA buffer and the concentration of Ca^{2+} was calculated according to the method of Wolff [18].

Fig. 6. The effects of VO_3^- on Ca^{2+}-ATPase activity. The activity was measured in media containing 0 mM $MgCl_2$ and 0 mM KCl (△); 100 mM KCl (○); 5 mM $MgCl_2$ (▲) or 5 mM $MgCl_2$ and 100 mM KCl (●). The concentration of ATP was 15 μM. At this concentration of ATP the Ca^{2+} pump catalyzes a significant hydrolysis of ATP in the absence of added Mg^{2+} [19]. Activities are expressed as % of the activity at 0 mM VO_3^- which were (μmol/mg protein per h): 0 mM $MgCl_2$ and 0 mM KCl, 0.029; 100 mM KCl, 0.042; 5 mM $MgCl_2$, 0.17 amd 5 mM $MgCl_2$ and 100 mM KCl, 0.25.

interactions between the high-affinity sites for Ca^{2+} and the sites for VO_3^- of the Ca^{2+}-ATPase. When the Ca^{2+} concentration rises above 100 μM inhibition by VO_3^- decreases progressively.

The effects of Mg^{2+} and K^+

Fig. 6 shows the effects of increasing concentrations of VO_3^- on Ca^{2+}-ATPase activity in media with and without 100 mM KCl in the presence and absence of 5 mM $MgCl_2$. In the absence of Mg^{2+} and K^+, VO_3^- is almost ineffective. In media with Mg^{2+} alone, half-maximal inhibition is attained at 25 μM VO_3^-. This value is lowered to 1.2 μM by the inclusion of K^+ together with Mg^{2+}. The effect of K^+ is strongly dependent on Mg^{2+}, since with K^+ alone half-maximal inhibition is only reached at 1 mM VO_3^-.

Fig. 7A shows a series of Dixon plots relating the reciprocal of Ca^{2+}-ATPase activity to VO_3^- concentrations, measured at a series of Mg^{2+} levels and in the presence of 100 mM KCl. The plots are straight lines, suggesting that for all the Mg^{2+} concentrations tested VO_3^- combines at a single class of site the occupation of which leads to total inhibition of the Ca^{2+}-ATPase. The inset in Fig. 7A shows that as Mg^{2+} concentration increases the concentration of VO_3^- for half-maximal inhibition drops along a curve which tends to saturation at 20 mM Mg^{2+}. When the reciprocal of the percent inhibition is plotted against the reciprocal of the concentration of Mg^{2+} at different VO_3^- concentrations, straight lines are obtained (Fig. 7B), indicating that Mg^{2+} increases the affinity for VO_3^- by combination at a single class of site. The concentration of Mg^{2+} for half-maximal effect drops from 5.3 to 0.8 mM as VO_3^- increases from 0.4 to 3.6 μM.

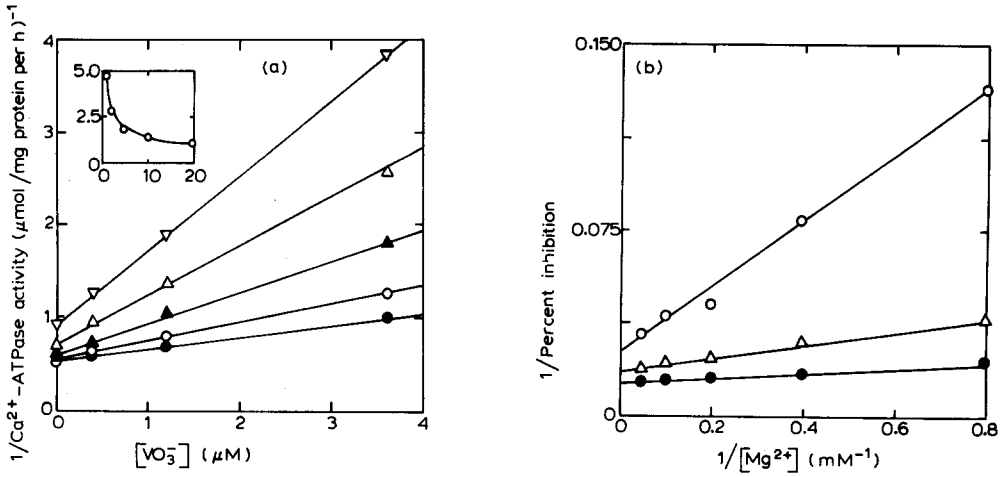


Fig. 7 (A). A plot of the reciprocal of Ca^{2+}-ATPase activity as a function of VO_3^- concentration in media containing 0.5 mM ATP and 1.25 (●); 2.5 (○); 5.0 (▲); 10.0 (△), or 20 (▽) mM $MgCl_2$. The inset represents the concentration of VO_3^- for half-maximal inhibition (μM) calculated from the lines that fit the experimental points, as a function of the concentration of Mg^{2+} (mM). (B) A reciprocal plot of the percent inhibition of the Ca^{2+}-ATPase by 0.4 (●); 1.2 (△) and 3.6 (○) μM VO_3^- as a function of the reciprocal of the concentration of Mg^{2+} in media with 0.5 mM ATP.

This suggests that the apparent constant for the dissociation of Mg^{2+} from its sites decreases with VO_3^-.

Control experiments (not shown) indicated that under the conditions of Fig. 7A and B and in the absence of VO_3^-, activation of the Ca^{2+}-ATPase by Mg^{2+} reaches saturation at 0.6 mM and that at concentrations of Mg^{2+} greater than 5 mM, activity progressively declines. The kinetics of the stimulatory effect of K^+ on inhibition by VO_3^- are essentially similar to those of Mg^{2+}. Dixon plots of the effects of VO_3^- on Ca^{2+}-ATPase activity at different K^+ concentrations give straight lines (Fig. 8A). The concentration of VO_3^- giving half-maximal inhibition decreases as a function of K^+ concentration, tending to a value of about 1 μM (inset in Fig. 8A). Plots of the reciprocal of percent inhibition against the reciprocal of K^+ concentration give straight lines for each of the VO_3^- concentrations tested. The concentration of K^+ giving half-maximal effect increases from 17 to 83 mM as VO_3^- increases from 0.6 to 10 μM. In a parallel experiment, the concentration of K^+ for half-maximal activation of the Ca^{2+}-ATPase in the absence of VO_3^- was found to be 25 mM.

The effects of Na^+ and Li^+

Na^+ activates the Ca^{2+}-ATPase with an apparent affinity 6-fold lower than that of K^+. Li^+ does not activate the enzyme [16]. In the experiment in Fig. 9, the effect of K^+ on inhibition by VO_3^- was compared with that of Na^+ and Li^+. Results show that Li^+ does not stimulate inhibition by VO_3^-. Under conditions in which K^+ lowers by 30-fold the concentration of VO_3^- for half-maximal inhibition, Na^+ lowers it 5-fold. Hence Na^+ is 6-fold less effective than K^+ during inhibition by VO_3^-.

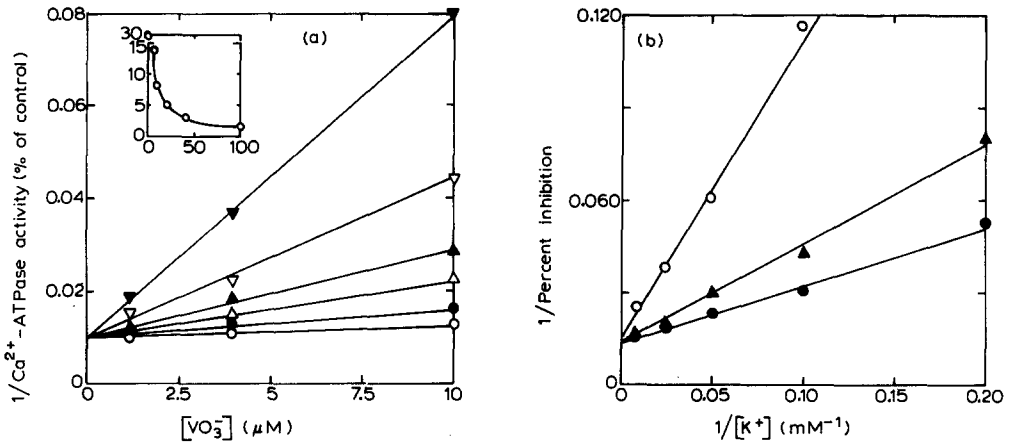


Fig. 8 (A). A plot of the reciprocal of Ca^{2+}-ATPase activity as a function of VO_3^- concentration measured in media with 0.5 mM ATP and 0 (○); 5 (●); 10 (△); 20 (▲); 40 (▽) and 100 (▼) mM KCl. For each K^+ concentration used, Ca^{2+}-ATPase activity is expressed as percent of the activity in the absence of VO_3^-. The inset represents the concentration of VO_3^- for half-maximal inhibition (μM), calculated from the lines that fit the experimental points, as a function of the concentration of K^+ (mM). (B) A reciprocal plot of the percent inhibition of the Ca^{2+}-ATPase by 0.6 (●); 4.0 (▲) and 10.0 (○) μM VO_3^- as a function of the reciprocal of the concentration of K^+ in media with 0.5 mM ATP.

The effect of VO_3^- on Ca^{2+}-dependent phosphoenzyme

Red cell membranes were preincubated during 10 min at 37°C in media containing different concentrations of VO_3^- with and without 100 mM KCl. After this the membranes were phosphorylated with [^{32}P]ATP at 0°C. Results in

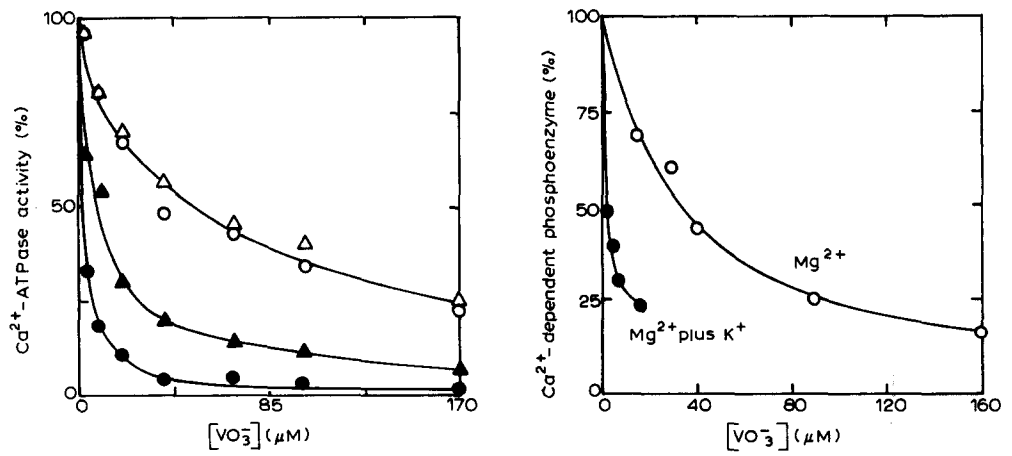


Fig. 9. The effect of VO_3^- on Ca^{2+}-ATPase activity measured at 0.75 mM ATP in media containing 100 mM LiCl (○); NaCl (▲) or KCl (●). 100 mM Tris-HCl replaced the monovalent cations in the control (△). Activities are expressed as percent of the activities in the absence of VO_3^-.

Fig. 10. The effect of VO_3^- on the level of Ca^{2+}-dependent phosphoenzyme formed in the presence of 15 μM [^{32}P]ATP in media with (●) and without (○) 100 mM KCl. The amount of phosphoenzyme is expressed as percent of the amount of phosphoenzyme measured in the absence of VO_3^- which was 1.65 pmol/mg protein in media with K^+ and 0.96 pmol/mg protein in media without KCl.

Fig. 10 show that the level of phosphoenzyme decreases with VO_3^-. Half-maximal effect of VO_3^- is reached at 35 μM in the presence of Mg^{2+} and at 2 μM in the presence of Mg^{2+} plus K^+. These values are essentially similar to the concentration of VO_3^- for half-maximal inhibition of the Ca^{2+}-ATPase, suggesting that inhibition of phosphorylation and of ATP hydrolysis is caused by the combination of VO_3^- at the same sites.

Discussion

Results in this paper show that VO_3^- inhibits the Ca^{2+}-ATPase of red cell membranes with high affinity.

Inhibition of the Ca^{2+}-ATPase is associated with a decrease in the steady-state level of the Ca^{2+}-dependent phosphoenzyme. This contrasts with the lack of effect of VO_3^- on the phosphorylation of the $(Na^+ + K^+)$-ATPase [4]. The decrease in the level of phosphoenzyme does not necessarily imply that VO_3^- blocks the phosphorylation reaction. Phosphoenzyme will also decrease if VO_3^- inhibits other partial reactions of the Ca^{2+}-ATPase, like for instance the E_2-to-E_1 conversion [14] as seems to be the case for the $(Na^+ + K^+)$-ATPase [17]. In fact, if we assume that VO_3^- binds to the E_2 conformer of the Ca^{2+}-ATPase to block the E_2-to-E_1 conversion, steady-state kinetics give equations that predict both a kinetic pattern similar to that of Fig. 2 and a parallel decrease of phosphoenzyme level and of Ca^{2+}-ATPase activity (Barrabin, H., unpublished results).

VO_3^- lowers the apparent affinity of the regulatory site for ATP but inhibition persists at non-limiting concentrations of ATP. Thus, the site for VO_3^- in the Ca^{2+}-ATPase seems to be different from the regulatory site for ATP. It would seem, therefore, that inhibition of the Ca^{2+}-ATPase by VO_3^- can be accounted for by its effects on the catalytic site and that these effects are exerted from sites that show negative interactions in affinity with the regulatory sites for ATP. This mechanism is different to that proposed for the $(Na^+ + K^+)$-ATPase by Cantley et al. [2] who suggested that in this system VO_3^- competes with ATP at its low-affinity site.

Results also show that there are no interactions between the sites for VO_3^- and the high-affinity sites for Ca^{2+} of the Ca^{2+}-ATPase. Ca^{2+} at high concentration decreases inhibition by VO_3^-, an effect which has also been observed in the Ca^{2+}-ATPase from sarcoplasmic reticulum (Ref. 6 and Barrabin, H. and de Meis, L., personal communication). Results in this paper do not allow us to conclude whether the release from inhibition at high Ca^{2+} concentrations is caused by: (i) Ca^{2+} at a low-affinity site; (ii) displacement of Mg^{2+} or (iii) the formation of an ineffective Ca-vanadate complex.

The Ca^{2+}-ATPase possesses sites for Mg^{2+} and for K^+ which show positive interactions in affinity with the site for VO_3^-. The interactions among these sites explain fully the large enhancement of the inhibitory effect of VO_3^- by Mg^{2+} and K^+. The apparent affinities of the sites of Mg^{2+} and K^+ for inhibition by VO_3^- seem to be higher than those of the sites for activation of Ca^{2+}-dependent ATP hydrolysis. This make it difficult to identify these sites. In spite of this, the relative effectiveness of Li^+, Na^+ and K^+ as activators of the Ca^{2+}-ATPase is preserved when they stimulate inhibition by VO_3^-. The effects of

Mg^{2+} and K^+ on VO_3^- inhibition of the Ca^{2+}-ATPase are essentially similar to those reported by other authors for the $(Na^+ + K^+)$-ATPase [3]. In this system, also the affinities of Mg^{2+} and K^+ for promotion of inhibition of the $(Na^+ + K^+)$-ATPase by VO_3^- have been reported to be less than those for stimulation of ATP hydrolysis [3]. An exception to the above-mentioned similarities is that in the $(Na^+ + K^+)$-ATPase, Na^+ antagonizes the effect of K^+ [3,4]. It is likely that this is a consequence of the fact that in the $(Na^+ + K^+)$-ATPase, Na^+ is not a congener of K^+ for activation, whereas in the Ca^{2+}-ATPase Na^+ replaces K^+ in this respect [16].

In view of the similarities between the effects of VO_3^- on the Na^+ and the Ca^{2+} pumps, it seems that caution must be exerted when interpreting the interactions of K^+ and VO_3^- with the Na^+ pump in terms of the molecular mechanism of the active transport of K^+ catalyzed by this system.

Acknowledgements

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