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VANADATE OLIGOANIONS INTERACT WITH THE PROTON EJECTION BY THE Ca^{2+} PUMP OF SARCOPLASMIC RETICULUM

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Decameric vanadate differs from other oligomeric vanadate species in inhibiting Ca^{2+} uptake and H^+ ejection promoted by sarcoplasmic reticulum ATPase. A decavanadate solution, 2 mM in total vanadium, containing about 200 μM decameric species, inhibits by about 50% the uptake of Ca^{2+} and by 75% the H^+ ejection, whereas 2 mM nominal monovanadate slightly increases the uptake of Ca^{2+} and inhibits the ejection of H^+ by 25%. Moreover, decavanadate linearly increases the Ca^{2+}/H^+ ratio, whereas monovanadate mimicks decavanadate behavior only at concentrations up to 1.2 mM. For higher concentrations of monovanadate, this effect is reversed probably due to the formation of metavanadates, namely tetravanadate. It is concluded that Ca^{2+} uptake is tightly coupled to proton ejection through molecular events that are sensitive to the interaction of vanadate species. Apparently, the stoichiometry is variable and modulated by molecular events involved in vanadate interaction suggesting alterations in the energetic coupling associated with Ca^{2+} translocation.

Sarcoplasmic reticulum (SR) regulates the Ca^{2+} distribution inside the muscle cells (1). Ca^{2+} is actively translocated at expense of ATP usage by the SR Ca^{2+}-pump ATPase (2,3). The Ca^{2+}-ATPase enzyme has distinct domains for the interaction of ATP and the binding of Ca^{2+} (4). The molecular mechanism of interaction between these domains, reflecting the coupling between ATP usage and the Ca^{2+} translocation, is still a matter requiring understandable interpretation and clarification.

Enzymes involved in phosphotransferase and phosphohydrolase reactions can accept orthovanadate as an analog of orthophosphate, with complex formation between the enzyme and vanadate leading to the inhibition of enzymatic activity of the P-type ATPases, such as the SR Ca^{2+}-ATPase (5). The inhibitory effect of vanadate towards the SR ATPase activity is not clearly understood, but depends on the conformational state of the enzyme, with a putative block in the E2 conformation (6,7). However, several species of vanadate normally present in solution (8) interact with the SR ATPase (9,10) even when the E1 or the E1P conformation is favorable to the enzyme (10-12). Furthermore, decameric species of vanadate clearly differs from other oligomeric species in preventing the uptake of Ca^{2+} by SR ATPase, possibly due to interactions with the

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enzyme at a conformation other than E2 (10). Additionally, the decameric species mediates SR photocleavage (13), induces the formation of SR ATPase dimmers (14) and inhibits the hydrolysis of ATP by the SR ATPase (15) more strongly than oligomers present in nominal monovanadate solutions (10).

During the uptake of Ca^{2+} by the SR ATPase, H^+ are ejected (16-19) probably due to an early coupling event of the pump activity (16,17). It is definitely established that the ejection of H^+ is a normal operation of the calcium pump (20,21). However, information relating the coupling of H^+ fluxes and the Ca^{2+} accumulation is scarce.

In the present work we report the effects of different anionic species of vanadium (V) on the uptake of Ca^{2+} and the coupled H^+ ejection. Decavanadate strongly inhibits both activities, but monovanadate solutions, containing a mixture of several oligomeric vanadate species (monomeric, dimeric, tetrameric and pentameric vanadate) slightly increase the Ca^{2+} uptake with a limited inhibition of H^+ ejection.

MATERIALS AND METHODS

Monovanadate and decavanadate stock solutions (0.1 M total vanadium) were prepared according to Csermely et al (9) from sodium orthovanadate. The pH was adjusted to 7.0 with NaOH 5 N or HCl 5 N and the solutions kept in ice before use, as described elsewhere (10). During the pH adjustment, the monovanadate solution was heated until the yellow/orange color indicative of decameric vanadate species vanished. The presence of the vanadate oligomeric species was ascertained by ^{51}V-NMR as previously described (10).

Fragmented sarcoplasmic reticulum vesicles were prepared from white skeletal rabbit muscles as described previously (22). Freshly isolated SR vesicles (50-60 mg/ml) suspended in 0.1 M KCl, 10 mM TRIS-Maleate pH 7.0 were diluted with equal volume of 2 M sucrose in small vials (0.3 ml) and frozen in liquid nitrogen before storage at -80 °C. These preparations retain full activities of Ca^{2+} uptake and ATP hydrolysis and good Ca^{2+}/ATP transport ratio (23). Protein concentration was determined by the method of biuret, using bovine serum albumin as standard (24). The amount of SR Ca^{2+}-ATPase routinely determined by densitometric analysis of SDS-polyacrylamide gel electrophoresis (25) was about 80% of total protein.

Ca^{2+} uptake measurements were performed by using a Ca^{2+} electrode (26) and the experiments were carried out at 25 °C in 2 ml assay medium containing 0.1 M KCl, 5 mM $MgCl_2$, 5 mM HEPES pH 6.0, 0.57 mg SR protein, 50 μM $CaCl_2$ and the reaction was initiated by adding 100 μM Mg-ATP. Vanadate nominal concentrations ranging from 0 to 4 mM "monovanadate" and 0 to 0.4 mM "decavanadate" (4 mM total vanadium) were added to the medium. Reaction initiation was within 2 min. after vanadate supplementation. At the end of each experiment, internal calibration was achieved by addition of standard $CaCl_2$ solutions.

H^+ ejection was monitored, at 25 °C, with a pH electrode in 2 ml assay medium containing 0.1 M KCl, 0.5 mM HEPES pH 6.0, 0.57 mg SR protein, 5 mM $MgCl_2$, 50 μM $CaCl_2$ and the reaction was initiated by adding 100 μM Mg-ATP (19). Experiments were also performed in a media containing 40 μM lasalocid. Vanadate nominal concentrations ranging from 0 to 4 mM "monovanadate" and 0 to 0.4 mM "decavanadate" were added to the medium before ATP addition. The reactions were initiated within 2-3 min after vanadate supplementation. The measurements were carried out at pH 6.0 to avoid scalar protons from ATP hydrolysis (release of orthophosphate and its acid-base behavior). However, the several features of Ca^{2+} transport remain similar to those observed at the more physiological pH of 7.0 (23). At the end of each experiment, internal calibration was achieved by addition of standard HCl solutions.

RESULTS

Decavanadate strongly inhibits the accumulation of Ca^{2+} and the associated ejection of H^+ by SR Ca^{2+}-pump whereas limited effects were observed for the monovanadate solutions

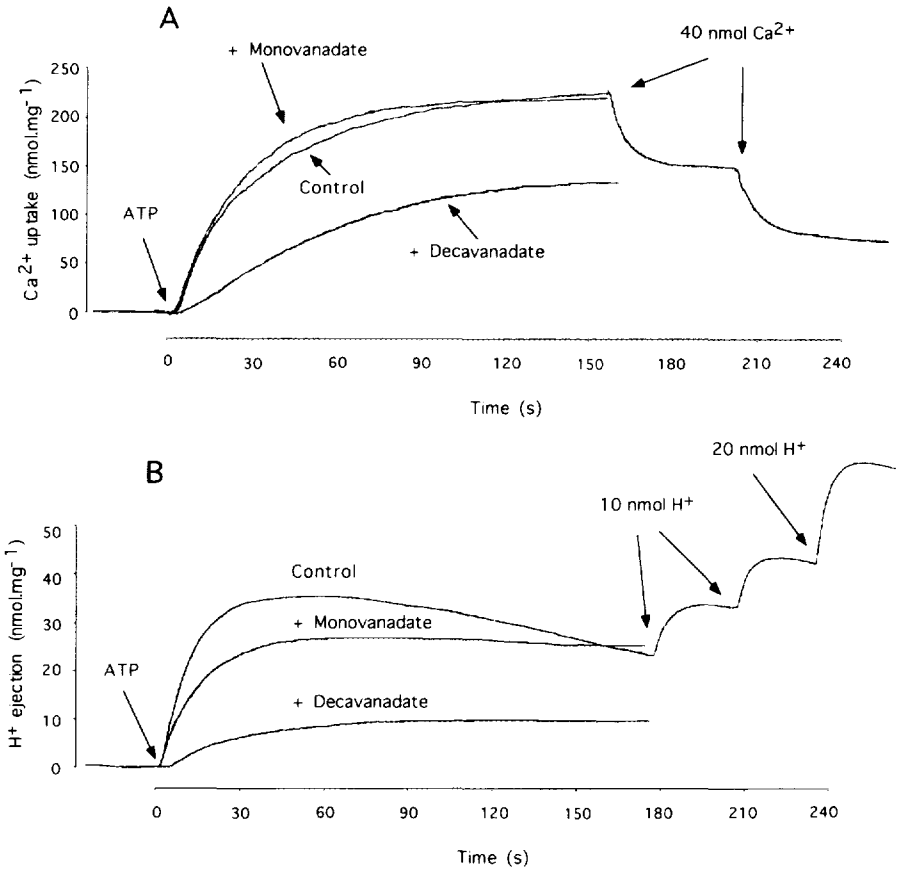

Fig. 1. Ca^{2+} accumulation (panel A) and H^+ ejection (panel B) profiles promoted by SR Ca^{2+}-pump in the absence and in the presence of nominal 2 mM monovanadate or 0.2 mM decavanadate solutions. The Ca^{2+} accumulation profiles were obtained by using a Ca^{2+} electrode and the studies followed at 25 °C, in 2 ml assay medium containing 0.1 M KCl, 5 mM $MgCl_2$, 50 µM $CaCl_2$, 0.57 mg protein and 5 mM HEPES pH 6.0. Internal calibration was performed by adding 40 nmol of Ca^{2+} twice at the end of each experiment. The H^+ ejection profiles were obtained by using a pH electrode and the studies followed at 25 °C, in 2 ml assay medium containing 0.1 M KCl, 5 mM $MgCl_2$, 50 µM $CaCl_2$, 0.57 mg protein and 0.5 mM HEPES pH 6.0. Internal calibrations were performed by adding 10 nmol and 20 nmol of standard HCl at the end of each experiment. The reactions were started by adding 100 µM MgATP.

(Fig.1). About 50% of the Ca^{2+} is taken up by the SR in the first 30 s period (Fig. 1A). Decavanadate 0.2 mM (2 mM in total vanadium) delays the beginning of the reaction and the uptake proceeds at a slow rate. Equivalent vanadium concentration in the form of monovanadate (2 mM) slightly increases the rate of Ca^{2+} accumulation. Fig. 1B shows that the peak of H^+ ejection occurs in the first period of 30 s, followed by a plateau and a slow phase of alkalization. Decavanadate strongly affects the H^+ ejection, and as also observed for monovanadate, it suppresses the slow of alkalization phase. Monovanadate also prevents the ejection but to a smaller extent as compared with decavanadate. It should be noted that the presence of vanadate affects the calibration of Ca^{2+} and H^+ traces. Therefore, the curves were always normalized against the control to correct for these marginal effects of vanadate solutions.

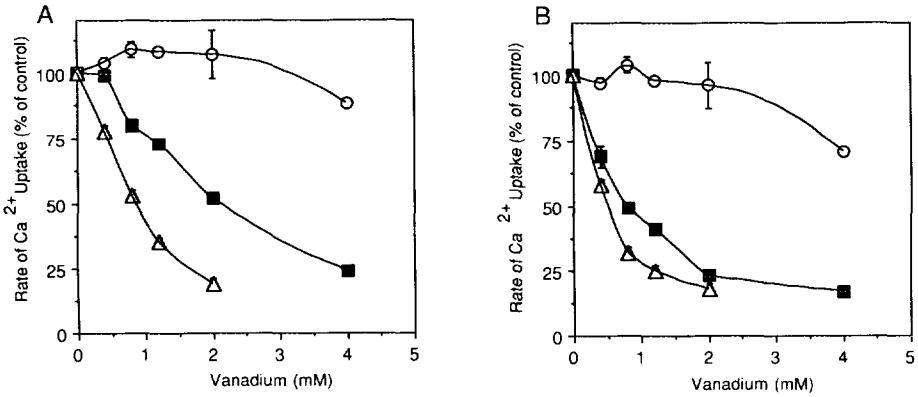

Fig. 2. Effect of monovanadate (O), decavanadate (■) and both (Δ) on the uptake of Ca^{2+} by SR. The reactions were followed as described in the legend of Fig. 1. The rates of Ca^{2+} are the amounts of Ca^{2+} taken up 1 min. (A) and 15 s (B) after MgATP addition. Each data point is the average of 4 independent experiments.

The rates of Ca^{2+} uptake and H^+ ejection are represented in Figs. 2A and 3A as the amount of ions detected after an elapsed period of 1 min. from the beginning of reactions. These rates approach the asymptotic values of Ca^{2+} uptake and H^+ ejection. The control Ca^{2+} uptake activity regarding the Ca^{2+} uptake was 185±13 nmol $Ca^{2+}.mg^{-1}.min^{-1}$ and the for the H^+ ejection was (n=20), 34±5 nmol $H^+.mg^{-1}.min^{-1}$, reaching to a Ca^{2+}/H^+ ratio of 5.4, essentially in agreement with values previously observed (18,19,21). Specific rates were also calculated after an elapsed period of 15 s as represented in Figs. 2B and 3B. These values represented more closely the initial reaction rates that could not be measured with the described methodologies. The estimated specific rates in this case are 391±13 nmol $Ca^{2+}.mg^{-1}.min^{-1}$ and 85±14 nmol $H^+.mg^{-1}.min^{-1}$, giving a Ca^{2+}/H^+ ratio of 4.6. At kinetic resolution of 30 s the values are 287±13 nmol $Ca^{2+}.mg^{-1}.min^{-1}$ and 60±8 nmol $H^+.mg^{-1}.min^{-1}$, reaching to a Ca^{2+}/H^+ ratio of 4.8.

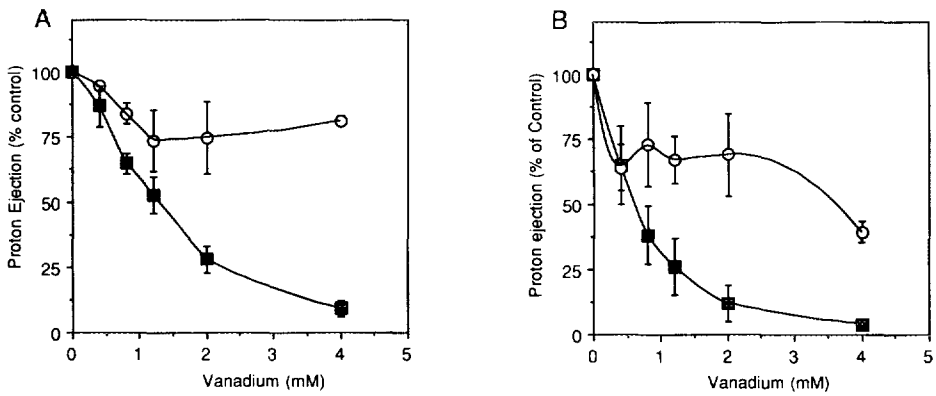

Fig. 3. Effect of monovanadate (O) and decavanadate (■) solutions on the proton ejection by SR. The reactions were followed as described for Fig. 1. The rates of H^+ ejection are the amounts of H^+ released upon 1 min. (A) and 15 s (B) after MgATP addition. Each data point is the average of 6 to 10 independent experiments.

Decavanadate 0.2 mM (2 mM of total vanadate) decreases by 48% the rate of Ca^{2+} accumulation at 1 min., whereas monovanadate nominally 2 mM moderately increases the uptake of Ca^{2+} and only for 4 mM a slight inhibition is observed (Fig.2A). The monovanadate solutions containing several vanadate species, namely monomeric, dimeric, tetrameric and pentameric species, as observed by ^{51}V-NMR spectroscopy (not shown), synergistically increase the Ca^{2+} uptake inhibition promoted by decameric species (Fig.2A). Thus, in the presence of 2 mM monovanadate, that per se does not inhibit the Ca^{2+} uptake, the inhibitory effect of 0.2 mM decavanadate increases from 48% to 81%, at 1 min. of Ca^{2+} uptake.

Ejection of H^+ as a consequence of Ca^{2+} pumping, as observed by protonometry at pH 6.0 to avoid scalar H^+ from ATP hydrolysis (release of orthophosphate and its acid-base behavior), is strongly inhibited by decavanadate solutions. In fact, the decavanadate concentration (0.2 mM) that promotes 50% inhibition of Ca^{2+} uptake rate (1 min. reaction) inhibits by 75% (n=10) the release of H^+ (Fig.3A). On the other hand, 2 mM monovanadate only inhibits the ejection of H^+ by 25% (n=10) and no additional inhibition is observed at higher concentrations. Rather than, the effect of 4 mM monovanadate is slightly lower as compared with the effect of 2 mM (Fig.3A). However, significative inhibition is observed when measurements were performed 15 s after reaction starting (Fig.3B).

When the Ca^{2+} accumulation inside the vesicles is prevented by the ionophore lasalocid, the similar distinct effects described above for monovanadate and decavanadate solutions were observed (not shown). Under these conditions, for 0.2 mM decavanadate, the release of H^+ is inhibited by 58%, whereas no inhibitory effects were observed for the presence of equivalent vanadium concentration in the form of monovanadate (2 mM). As previously pointed out (21), the ionophore prevents Ca^{2+} accumulation but not the translocation probably occurring at a faster rate than the Ca^{2+}/H^+ exchange promoted by the ionophore. Therefore, H^+ ejection is detected also in the presence of ionophore although it occurs at a decreased rate and amount.

The asymptotic Ca^{2+}/H^+ ratio linearly increases as a function of decavanadate concentration (Fig.4). Monovanadate also increases the Ca^{2+}/H^+ ratio but only at concentrations below 1.2

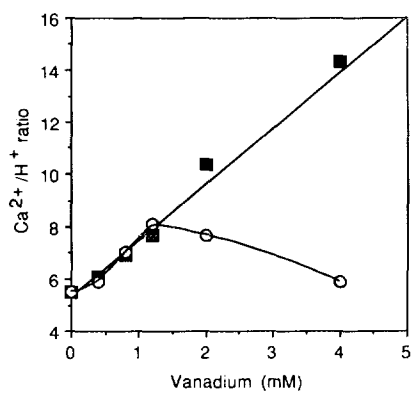

Fig. 4. Ca^{2+}/H^+ ratio as a function of monovanadate (O) and decavanadate (■) concentrations. This ratio is calculated with the amounts of Ca^{2+} accumulated and H^+ released in 1 min. per mg protein.

mM. Higher concentrations have an opposite result with no effect detected at 4 mM (Fig.4). This surprising monovanadate effect may be due to the presence of tetrameric vanadate since it increases linearly and significantly in relation to other oligomeric species in solution when the nominal monovanadate concentration ranges from 1 to 5 mM (10).

DISCUSSION

This article reports the original observation that decavanadate promotes the coupled inhibition of Ca^{2+} transport and H^+ ejection by SR Ca^{2+}-pump. This inhibitory effect induced by vanadate depends on specific vanadate oligomers, being decavanadate the strongest inhibitor.

At the experimental conditions used, less than 10% of the decameric species is converted to monomeric vanadate (10); typically, a decavanadate solution 2 mM in vanadium contains about 190 μM of decameric and 100 μM of monomeric species. Clearly, the decameric species is the promoter of Ca^{2+} transport inhibition since monovanadate has no significant effect (Fig. 2). However, the presence of monovanadate synergistically increases the Ca^{2+} transport inhibition of decavanadate; therefore, a combined effect of decameric and monomeric species is possible. This observation suggests that, upon binding to the Ca^{2+}-ATPase, monomeric vanadate may favor the interaction of decameric species with the enzyme.

In parallel with the inhibition of Ca^{2+} uptake, decavanadate also prevents the associated ejection of H^+ (Figs. 1B and 3) indicating that the events are coupled. Decavanadate 0.2 mM inhibits by 75% the ejection of protons. A similar profile of inhibition has been observed for the hydrolysis of ATP by the SR calcium pump in the same experimental conditions (10). Therefore, decavanadate equally blocks the ejection of H^+ and the ATP hydrolysis coupled to the accumulation of calcium meaning that the ejection of H^+ by SR is a major feature of the calcium pump. Conversely to the coupled system, decavanadate also inhibits the ejection of H^+ of the uncoupled ATPase by the presence of a Ca^{2+} ionophore, whereas, under these conditions, monovanadate does not inhibit the ejection of H^+. It was previously observed (10) that the presence of the ionophore does not prevent the inhibition of SR Ca^{2+}-ATPase activity by decavanadate. Therefore, the inhibition of H^+ ejection by decavanadate suggests that the ejection of H^+, detected in the presence of ionophore, is associated with Ca^{2+} translocation.

The linear increase of the Ca^{2+}/H^+ ratio as a function of decavanadate concentration suggests that the decameric species affects primarily the H^+ ejection associated with the Ca^{2+} transport. Monovanadate, although not effective on Ca^{2+} uptake, it affects to a limited extent the Ca^{2+}/H^+ ratio for concentrations up to 1.2 mM (Fig.4). The effect fades out as the concentration increases beyond this limit and essentially no effect is detected at 4 mM. This failure of effect is possibly be due to the formation of metavanadates that may interact with the enzyme in such a way that the effect of monomeric species is canceled.

The limited but positive effect of monovanadate in depressing 25% of H^+ ejection at 1.2 mM, indicates that some of the ejected protons are not directly coupled to Ca^{2+} transport. This interpretation is corroborated by the fact that decavanadate (0.2 mM) decreases by 75% the H^+ ejection but only by 50% the Ca^{2+} uptake. Therefore, we are tempted to suggest that the fraction

of released protons not associated with the Ca^{2+} transport is possibly related to a partial energetic uncoupling of the pump in the sense that some ejected protons result from energy lost not profitable for the Ca^{2+} transport. However, further experiments will be required to understand the role of the proton ejection not directly involved in the Ca^{2+} transport.

Oligomeric species of vanadate have different domains of interaction with the SR ATPase (12,13,15) and the inhibitory effect of vanadium is still not clearly understood. Decameric vanadate binds to the SR ATPase at the ATP binding site (15) but it may also bind specifically to a place responsible for blocking the movement of Ca^{2+} and H^{+}. Some of the interactions, viz. decameric species, disrupt the energetic coupling and the enzyme turnover. However, other interactions of vanadium, viz. monomeric species, may be without effect or even improve the coupling of Ca^{2+} pumping.

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