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Vanadate inhibits vacuolar H^+-ATPase-mediated proton transport in chicken kidney microsomes by an ADP-dependent mechanism

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Abstract

Recent reports indicate that vacuolar-type proton ATPases from chicken osteoclasts (Chatterjee et al. (1992) Proc. Natl. Acad. Sci. USA 89, 6257–6261), yeast vacuoles and chromaffin granules (Beltran and Nelson (1992) Acta Physiol. Scand. Suppl. 607, 41–47) can be inhibited by vanadate, albeit at a concentration much higher than that required to inhibit P-type ATPases. We have characterized the mechanism by which vanadate inhibits vacuolar-type ATPase-mediated proton transport by chicken kidney microsomes. The initial rate of proton transport is somewhat less sensitive to vanadate than the total acidification, with IC_{50} values of 1.58 mM and 0.78 mM vanadate, respectively. The inhibition of both the initial rate and total acidification is noncompetitive with respect to ATP. The inhibition is abolished when ADP is removed by an ATP-regenerating system, and the addition of exogenous ADP increases the vanadate inhibition of proton transport in a synergistic manner, thus demonstrating that inhibition by vanadate is dependent on the presence of ADP and explaining the lower effect of vanadate on the initial rate of acidification. Phosphate protects proton transport activity from inhibition by vanadate. These effects of ADP and phosphate suggest that inhibition by vanadate may involve the formation of a complex with ADP at a nucleotide binding site, possibly at the catalytic site of the enzyme.

Keywords: Vanadate; Acidification; Vacuolar H^+-ATPase; ATPase, H^+-, vacuolar; Proton transport; (Kidney)

1. Introduction

Active transport of protons across membranes is carried out by three families of H^+-ATPases: vacuolar (V-type), mitochondrial (F_0F_1-type), and the gastric H^+/K^+ (P-type) ATPases, which are distinguished by differences in subunit composition, catalytic mechanism, and pharmacology. V-type ATPases catalyze the acidification of various intracellular compartments in all eukaryotic cells, including Golgi, clathrin-coated vesicles, lysosomes, endosomes and chromaffin granules [1,2], and are also present in the apical plasma membrane of cells that specialize in proton secretion, such as the epithelial renal intercalated cell [3,4], and the osteoclast [5–8]. They are distinguished pharmacologically from the other types of H^+-ATPase by their high sensitivity to bafilomycin A1 [9] and to *N*-ethylmaleimide (NEM), which interacts with a conserved cysteine in the ATP-binding domain of the A subunit of V-type ATPases that is absent in the F-type ATPases, making them virtually completely resistant to NEM [1,10]. While the F-type ATPases are sensitive to azide and oligomycin, these compounds have much lower effects on the V-type ATPases [1,11]. The P-type ATPases are poorly inhibited by NEM, bafilomycin A1, azide or oligomycin, but are considered to be uniquely sensitive to vanadate ($IC_{50} < 10$ μM) [1,11,12].

We and others have, however, found that the V-type ATPases present in the osteoclast ruffled-border membrane, yeast vacuoles and chromaffin granules are sensitive to vanadate, with an IC_{50} of 100–500 μM [8,13], thereby raising the possibility that sensitivity to vanadate in the high micromolar to millimolar range could be a general and as yet unrecognized feature of all V-type ATPases. Understanding the mechanism by which vanadate inhibits the V-type ATPases can further contribute to our understanding of the catalytic cycle of V-type ATPases. We have therefore undertaken to characterize the inhibitory effect of vanadate on V-type H^+-ATPases.

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2. Materials and methods

2.1. Chemicals

Acridine orange was obtained from Molecular Probes, creatine phosphate and creatine kinase were from Boehringer Mannheim and sodium orthovanadate (Na_3VO_4), vanadate-free ATP and ADP (potassium salt from yeast) were from Sigma. All other materials were purchased from Baker.

Vanadate was freshly prepared on a weekly basis. Sodium orthovanadate was dissolved in water and slowly titrated to pH 10 with concentrated HCl solution, resulting in the appearance of a rusty yellow color which disappeared when the solution was boiled. The cleared solution was further boiled for 4 min in order to reduce the content of various oxyvanadates. After cooling, the solution was brought to its final volume to yield a 200 mM stock solution. Prior to any experiment, the vanadate solution was kept for 3 h at 37°C to eliminate any decavanadate complexes that might have formed [14]. Addition of vanadate from the above stock solution to the acidification buffer, up to 2 mM final concentration, did not change the pH of the solution.

2.2. Kidney microsomal preparation

Kidney microsomes were prepared from whole chicken kidneys. After removing the capsule, the tissue was minced (about 2 g/kidney) and diluted 1:7 (w/v) with cold homogenization buffer (20 mM Hepes-KOH, 1 mM EDTA, 250 mM sucrose (pH 7.4) at 4°C). Homogenization was performed on ice by eight strokes of a motor-driven Teflon homogenizer at 800 rpm. The homogenate was centrifuged for 7 min at 7000 rpm in an SS-34 Beckman rotor at 4°C. The pellet was discarded and the supernatant was centrifuged for 40 min at 18000 rpm in the same rotor. The supernatant was discarded and the pellet contained a dark tightly packed center covered with white fluffy membranes. The fluffy layer, containing the kidney-derived microsomes, was recovered by gentle vortexing in the presence of 1 ml of buffer A (150 mM KCl, 20 mM Hepes-KOH, 2 mM DTT (pH 7.4)) and glycerol was added to the suspension to 25% of the final volume. The microsomes were aliquoted and stored at −20°C, conditions under which the original proton-transport activity was maintained for at least 1 month. Protein determination was done by Bradford's method [15] using the Bio-Rad protein assay kit, with bovine serum albumin as a standard.

2.3. Vesicle acidification assay

The acidification assay is a modification of the one described elsewhere [16]. Unless otherwise specified, 35–45 μg of chicken kidney-derived membrane vesicles were incubated for 8 min in 2 ml acidification buffer (150 mM KCl, 20 mM Hepes-KOH, 5 mM $MgCl_2$ (pH 7.4), 5 μM acridine orange, 1.25 μM valinomycin) at 25°C. Proton transport was initiated by addition of potassium ATP, and monitored as the quench of acridine orange fluorescence (Ex. 490 nm, Em. 520 nm, with slit width of 10 and 20 nm, respectively, 400 V using a Hitachi F-2000 fluorescence spectrophotometer). The assay was stopped after 300 s by addition of 1 μM nigericin. A 10 s interval sampling record was generated, from which the initial rate (ΔF/min) was determined by averaging the change in fluorescence of the first 3 or 4 sampling intervals and extrapolated for a full minute. Total fluorescence change after addition of nigericin (ΔF) were also determined from this record.

2.4. Calculations

Linear and nonlinear regression analysis were done using the program Enzfitter (Elsevier-BIOSOFT). The results are given in mean parameter values ± standard error.

3. Results

3.1. Vanadate inhibits vacuolar H^+-ATPase-mediated proton transport in kidney microsomes

For the acidification assay, kidney microsomes (40–50 μg) were pre-incubated in acidification buffer (i.e., in the

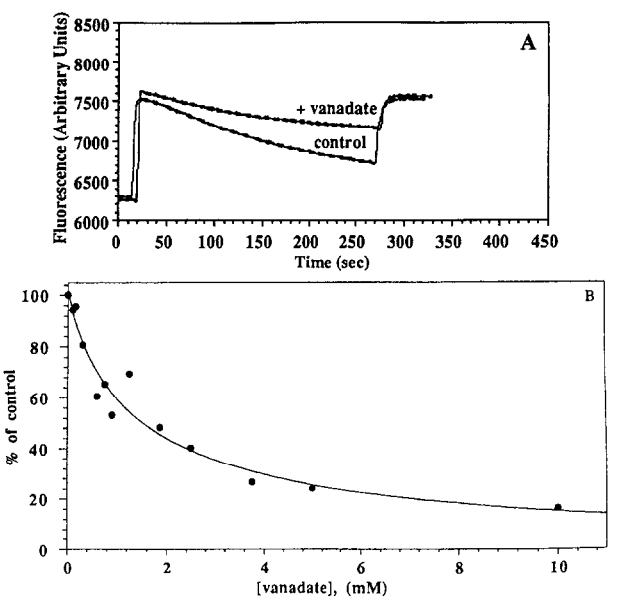

Fig. 1. Dose-dependent inhibition of proton transport by vanadate. (A) A typical set of assays of control (lower trace) and 750 μM vanadate (pre-incubated for 8 min) (upper trace) is shown. The acidification assay was started by adding 1.5 mM ATP. Vanadate inhibits the initial rate by 33% and the ΔF by 51%. (B) Kidney microsomes (50 μg) were pre-incubated with increasing concentrations of vanadate (100 μM–10 mM vanadate) in acidification buffer for 8 min. The acidification assay was initiated with 1.5 mM ATP. A dose-dependent inhibition of the initial rate of acidification was observed. The continuous line is that using the best fit to a model of ligand binding to a single site. Vanadate inhibited the initial rate of proton transport with an $IC_{50} = 1.58 \pm 0.52$ mM vanadate.

presence of 5 mM Mg^{2+}) in the presence or absence of 750 μM vanadate for 8 min. Fig. 1A (bottom trace) presents a typical trace obtained in the absence of vanadate. The proton transport was initiated by the addition of 1.5 mM ATP, a concentration that supports maximum proton transport activity [17]. The initial rate of proton transport, derived from the slope generated by the first 30 s of the acidification assay after the addition of ATP, is expressed as change of fluorescence units per minute (ΔF units/min). After a period of 250–300 s, a time at which the maximal acidification was usually reached, the system was uncoupled by the addition of 1.5 μM nigericin to monitor the total fluorescence change achieved due to the acidification of the microsomes (ΔF). Vanadate reduced the initial rate by 33% while decreasing the ΔF by 51% (Fig. 1A, upper trace). The inhibition of the initial rate by vanadate was dose-dependent, with an IC_{50} of 1.58 ± 0.52 mM vanadate (Fig. 1B). Vanadate was consistently found to a have a more potent inhibitory effect on the ΔF with an IC_{50} of 0.78 ± 0.11 mM (not shown).

The inhibition of proton transport by 750 μM vanadate was assayed over a range of ATP concentrations (from 62.5 to 1000 μM). The initial rates (Fig. 2A) and total change in fluorescence (ΔF) after 300 s (Fig. 2B) were measured. Regardless of whether the determination was based on the initial rate or on ΔF, the results are consistent with a noncompetitive inhibition by vanadate with respect to ATP. Both the maximal initial rate and the maximal ΔF were reduced in the presence of vanadate. (Maximal initial rate equals 187 ± 13 units/min without vanadate and 127 ± 5 units/min with vanadate; maximal ΔF equals 776 ± 73 units without vanadate and 389 ± 23

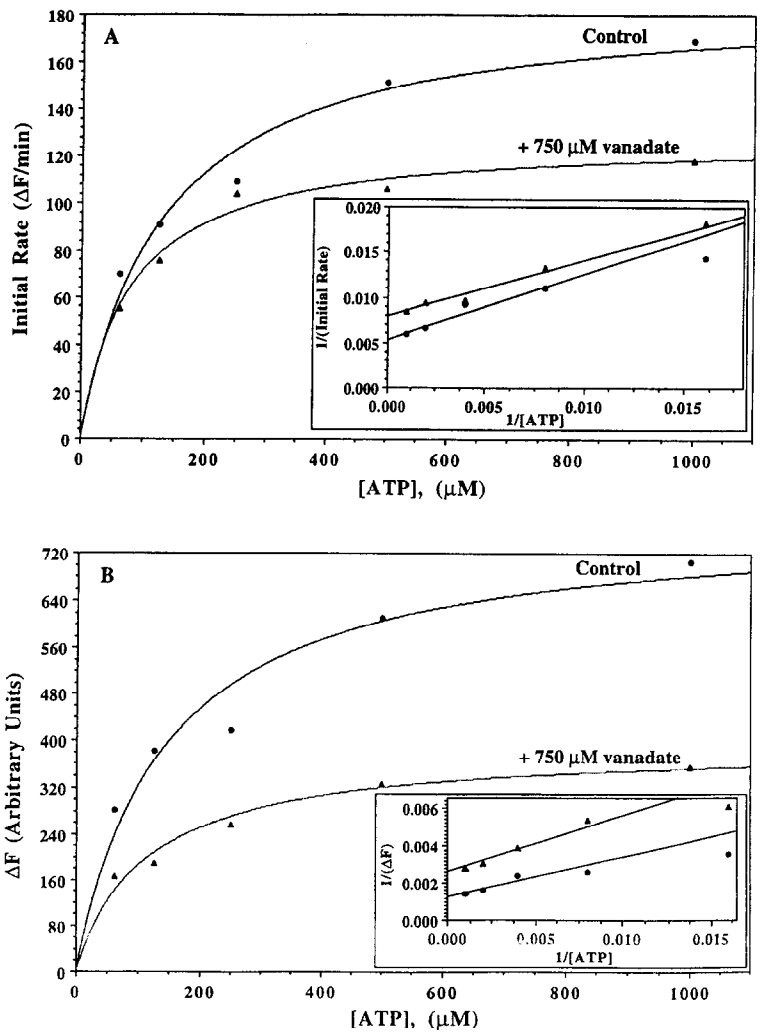

Fig. 2. Effect of ATP on vanadate inhibition. Proton transport (45 μg protein) as a function of ATP concentration (62.5–1000 μM ATP) in the absence of vanadate (●) or in the presence of 750 μM vanadate, 8 min pre-incubation (▲): (A) on the initial rate of proton transport, (B) on the total acidification. The continuous lines are the best fit to Michaelis-Menten-type kinetics. The V_{max} values of the initial rates were found to be 187 ± 13 in control and 127 ± 5 ΔF units/min in the presence of vanadate, while the K_m values were 131 ± 30 and 79 ± 13 μM ATP, respectively. The maximal acidification of the control was found to be 776 ± 73 arbitrary units, compared with 389 ± 23 arbitrary units in the presence of vanadate; the K_m values were 142 ± 42 μM ATP and 111 ± 23 μM ATP, respectively. The insets represent the Lineweaver-Burk transformations of the data, with the corresponding data points with the same symbols.

units with vanadate.) The apparent K_m for ATP was actually higher in the absence of vanadate than in its presence (131 ± 30 vs. 79 ± 13 μM ATP, respectively, for the initial rate and 142 ± 42 vs. 111 ± 23 μM ATP, respectively, for the ΔF), since the percent inhibition by vanadate was lower at the lower ATP concentrations.

3.2. The inhibition of proton transport by vanadate is dependent on the presence of ADP

Our observation that vanadate inhibits the total acidification to a greater extent than the initial rate suggested that the accumulation of one or both of the reaction products might be potentiating the inhibitory effect of vanadate. We first determined whether preventing the accumulation of ADP during the acidification assay by including an ATP regenerating system would affect the degree of vanadate inhibition. Microsomes (50 μg) were pre-incubated in the presence and absence of 1 mM vanadate for 8 min, with or without 25 units creatine kinase/ml and 20 mM phosphocreatine. In the absence of the ATP regenerating system, vanadate inhibited the initial rate of proton transport by 26% and the ΔF by 45% (Fig. 3A). The presence of the ATP regenerating system increased the initial rate of proton transport in the vanadate-free assay from 742 to 792 ΔF/min, suggesting that the microsomal preparation contained trace amounts of ADP, and the total acidification from 920 to 1029 ΔF units (compare the lower traces in Fig. 3A to Fig. 3B), consistent with the accumulation of ADP during the assay when the ATP regenerating system is omitted. In the presence of the ATP regenerating system, vanadate did not inhibit the initial rate, and the inhibition of total acidification was markedly reduced, from 45% to 8% (Fig. 3B), demonstrating that the inhibitory effect of vanadate indeed depends on the accumulation of ADP in the assay. In addition, this experiment excludes the possibility that the presence of ATP and Mg^{2+} are sufficient for the vanadate inhibition to occur, and thus demonstrates that the inhibition of V-type ATPases and P-type ATPases by vanadate must proceed by different mechanisms, since the presence of Mg^{2+} alone is sufficient for vanadate inhibition of the P-type ATPases [18].

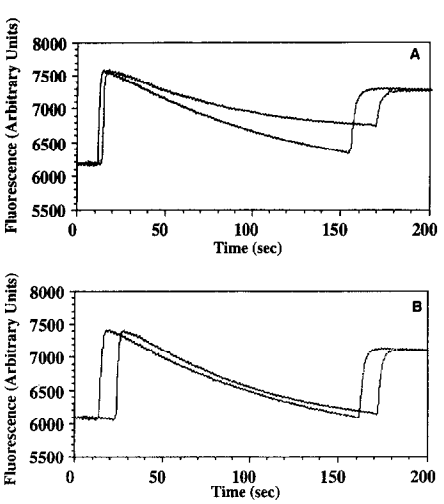

Fig. 3. Vanadate has no effect in the presence of an ATP-regenerating system. The pre-incubation and acidification assay were performed in the absence (A) and the presence (B) of an ATP-regenerating system. The acidification assay was initiated with 1.5 mM ATP. (A) Proton transport, in the absence of ATP-regenerating system, in the absence (lower trace) and presence (upper trace) of 1 mM vanadate (8 min pre-incubation). (B) Proton transport, with ATP-regenerating system (25 units/ml creatine kinase and 20 mM phosphocreatine) in the absence (lower trace) and presence (upper trace) of 1 mM vanadate.

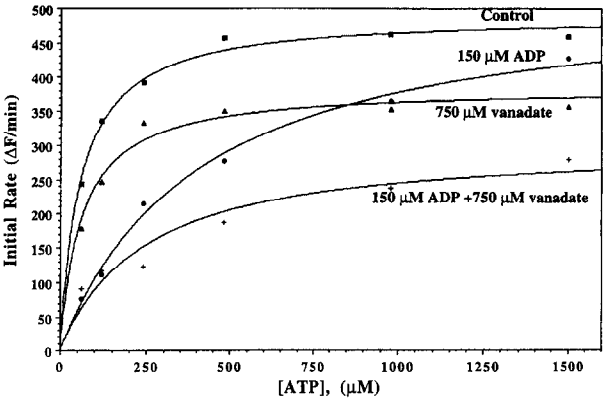

Fig. 4. ADP and vanadate interact to inhibit proton transport. Kidney microsomes (50 μg protein) were pre-incubated for 8 min with either no additions (■), in the presence of 150 μM ADP (●), in the presence of 750 μM vanadate (▲) or in the presence of 150 μM ADP plus 750 μM vanadate (+). For each condition, the dose–response curve for ATP (60–1500 μM ATP) was generated. The continuous lines are the best fit of the data to Michaelis-Menten type kinetics. The V_{max} values were 495 ± 92 ΔF/min for control, 520 ± 25 ΔF/min in the presence of ADP, 385 ± 13 ΔF/min in the presence of vanadate and 303 ± 32 ΔF/min in the presence of ADP and vanadate, and the K_m values were 57 ± 6 μM ATP for control, 404 ± 51 μM ATP in the presence of ADP, 61 ± 10 μM ATP in the presence of vanadate and 240 ± 81 μM ATP in the presence of ADP and vanadate.

To further evaluate the role of ADP in the vanadate inhibition, we tested the effect of including 150 μM ADP in the pre-incubation medium, either alone or together with vanadate (750 μM) and for the same amount of time (8 min). An ATP dose–response curve was generated for each of the treatments and fitted to a Michaelis-Menten kinetic model. The fitted curves of the initial rate values are presented in Fig. 4. As we have shown before [17], ADP alone competitively inhibited proton transport, increasing the apparent K_m for ATP (from 57 ± 6 to 404 ± 51 μM ATP) without affecting the V_{max} (520 ± 25 and 495 ± 92 ΔF/min, respectively). In contrast, the effect of ADP was more complex in the presence of vanadate, with characteristics of both competitive and noncompetitive inhibitors. Thus, the apparent K_m for ATP increased from 61 ± 10 μM ATP when only vanadate was present to 240 ± 81 μM ATP when both vanadate and ADP were present, an effect characteristic of a competitive inhibitor. However, the V_{max} obtained when ADP was included with

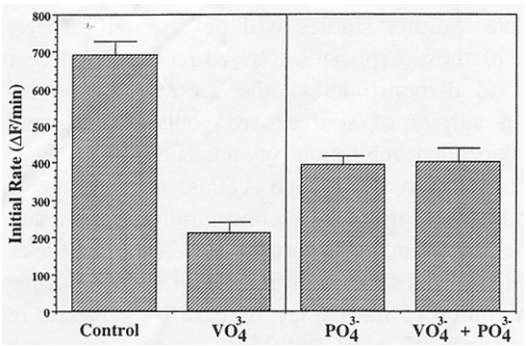

Fig. 5. Phosphate protects against vanadate inhibition. Kidney microsomes (50 μg protein) were pre-incubated for 8.5 min in the absence or presence of 1.5 mM vanadate and/or 12.5 mM phosphate. The average initial rate and standard error of quadruplicate determinations are presented. The average initial rates were 690 ± 37 ΔF/min for control, 211 ± 31 ΔF/min in the presence of vanadate alone, 392 ± 24 ΔF/min in the presence of phosphate alone and 400 ± 38 ΔF/min in the presence of phosphate and vanadate together.

vanadate (303 ± 32 ΔF/min) was lower than the V_{max} with vanadate alone (385 ± 13 ΔF/min), despite the fact that the maximum ATP concentration was 1.5 mM, a concentration where ADP alone has little inhibitory effect. These results indicate that ADP and vanadate interact, since in the absence of such interaction we would have expected a competitive-type curve for vanadate plus ADP, converging with the curve obtained with vanadate alone.

3.3. Phosphate protects the enzyme from vanadate inhibition

Phosphate, the other product of ATP hydrolysis, is a noncompetitive inhibitor with respect to ATP ($K_i = 10$ mM) [17]. The effect of phosphate on vanadate inhibition was assessed by including 12.5 mM phosphate during the 8.5 min pre-incubation period in the presence and absence of 1.5 mM vanadate (Fig. 5). Vanadate alone reduced the initial rate of proton transport from 690 ± 37 ΔF/min to 211 ± 31 ΔF/min, an inhibition of 70%, while 40 mM phosphate alone reduced the initial rate to 392 ± 24 ΔF/min, an inhibition of 43%. In vesicles pre-incubated with 1.5 mM vanadate in the presence of 12.5 mM phosphate, the initial rate of proton transport was 400 ± 38 ΔF/min (42% inhibition), a value which is essentially identical to the inhibition achieved by phosphate alone. Thus, phosphate appears to protect the enzyme against vanadate inhibition.

4. Discussion

Although sensitivity to vanadate in the low micromolar range is a characteristic of P-type ATPases, we and others have reported that V-type ATPases from chicken osteoclasts, yeast vacuoles and chromaffin granules are inhibited by vanadate, with IC_{50} values ranging from approximately 100–500 μM [8,13]. Taken together, these results suggest that all V-type ATPases may be susceptible to inhibition by vanadate, albeit at significantly higher vanadate concentrations than those that inhibit P-type ATPases.

In the present report, we have demonstrated that the chicken kidney V-type ATPase, which was not previously studied, is also sensitive to vanadate, and used this system to characterize the mechanism of this inhibition. Proton transport into chicken kidney microsomes was inhibited by vanadate in a dose-dependent manner when saturating amounts of ATP were used in the assay. The increase in the inhibitory effect of vanadate as the acidification assay progressed suggested the possibility that the presence of ADP, produced by the V-type ATPase during the assay, favored the action of vanadate. In fact, ADP appears to be required for vanadate inhibition, as demonstrated by the fact that including an ATP regenerating system during the preincubation and assay abolished the vanadate-induced inhibition of the initial rate of acidification and markedly reduced the inhibition seen at the end of the assay period. Further demonstration that inhibition by vanadate involves an interaction with ADP was provided by the experiment showing that the V_{max} obtained when both vanadate and ADP were present in the assay is much less than that seen with vanadate alone. If vanadate and ADP were acting independently to inhibit the ATPase, one would expect that the presence of ADP would not affect the V_{max}, since ADP is a competitive inhibitor of ATP, and thus it does not inhibit proton transport at high ATP concentrations [17].

The requirement for ADP explains the relationship of vanadate inhibition to ATP concentration. As more ATP is added to the assay system, more ADP is generated as the acidification assay proceeds. Thus, the ADP concentration at the time at which ΔF is measured is significantly higher than at the beginning of the experiment, and the increase in ADP concentration during the assay is greater at higher ATP concentrations, explaining why the effects of vanadate on total acidification are more pronounced than the effects on the initial rate where only residual or low ADP concentrations are present.

We previously reported that phosphate inhibits the initial rate of proton transport in kidney microsomes in a noncompetitive manner, with a K_i of 10 mM [17]. However, inclusion of phosphate with vanadate resulted in a degree of inhibition comparable to that induced by phosphate alone and less than that induced by vanadate. This antagonism by phosphate and the requirement for ADP together suggest that vanadate interacts with the phosphate binding region of a nucleotide binding site, possibly the catalytic site itself. This hypothesis is supported by our observation that omitting Mg^{2+} from the preincubation reduces vanadate's inhibition of the initial rate of acidification by about half (data not shown), which implies that Mg^{2+} is acting as a cofactor for the binding of the residual ADP in the vesicle preparations. If ADP, Mg^{2+} and

vanadate indeed interact at a common site on the V-type ATPase, they may form a transition state-like complex that mimics the transition state of the catalytic cycle of the V-type ATPases, $Mg^{*}ADP^{*}P_i$, although we cannot rule out other possible mechanisms at this time. While the transition state of the catalytic cycle seems to be short-lived and phosphate is rapidly released [17], the transition state-like structure that we suggest is formed in the presence of vanadate ($Mg^{*}ADP^{*}VO_4$) is apparently more stable, leading to inhibition of the catalytic cycle of the V-type ATPase. In any case, our results in the present study clearly demonstrate that the mechanism of inhibition of V-type ATPases by vanadate is distinctly different from that described for P-type ATPases. Specifically, inhibition of P-type enzymes does not require ADP, and the presence of Mg^{2+} is both necessary and sufficient for vanadate inhibition [18], whereas our experiment with the ATP-regenerating system demonstrates that ADP is required and that Mg^{2+} is not sufficient to support vanadate inhibition of V-type enzymes.

It has recently been reported that vanadate (up to 5 mM) could not inhibit the activity of purified V-type ATPase from chicken osteoclast preparations [19], an observation that disagrees with some previous reports [8,13] and with our current observations. Since all the reports demonstrating vanadate inhibition of V-type ATPase activity were based on a proton transport assay using crude microsomal preparations, this discrepancy could lead to the conclusion that the inhibition of V-type ATPase-mediated proton transport by vanadate is indirect, that is, there may be another protein in the preparation that regulates the pump activity and is sensitive to vanadate in an ADP-dependent manner. However, there are two differences in the experimental conditions used with the two systems that could explain the failure to observe inhibition of the purified ATPase by vanadate. First, we omit dithiothreitol from our acidification assays, since we have found that it markedly reduces the inhibitory effect of vanadate on proton transport by osteoclast-derived vesicles and, to a lesser extent, by kidney vesicles. The presence of dithiothreitol in the assays of the purified ATPase could therefore account in part for the failure to detect inhibition by vanadate. Second, as we report here, the inhibition by vanadate is entirely dependent on the presence of ADP. Crude preparations of microsomes likely contain contaminating ATPases and phosphatases that will produce, in a time-dependent manner, high enough concentrations of ADP to support the inhibition of proton transport by vanadate. Purified ATPase preparations, on the other hand, have significantly lower total ATPase activity [19], and are unlikely, therefore, to produce amounts of ADP that are sufficient to support the inhibition of proton transport by vanadate. Further studies will be required to determine which of these explanations is correct, and, more importantly, to demonstrate whether or not V-type ATPases from a variety of sources are generally susceptible to ADP-dependent inhibition by vanadate.

In conclusion, the V-type ATPase from chicken kidney is inhibited by millimolar concentrations of vanadate, as has been previously reported for V-type ATPases from osteoclasts, yeast and chromaffin granules. Furthermore, the inhibition of the kidney ATPase by vanadate requires the presence of ADP and Mg^{2+} and is antagonized by phosphate, suggesting that ADP, Mg^{2+} and vanadate may interact at a nucleotide binding site, possibly the catalytic site, and may form a transition state-like complex that is responsible for the inhibition.

Acknowledgements

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