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*E*2→*E*1 transition and Rb^+ release induced by Na^+ in the Na^+/K^+-ATPase. Vanadate as a tool to investigate the interaction between Rb^+ and *E*2 ☆

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ABSTRACT

This work presents a detailed kinetic study that shows the coupling between the *E*2→*E*1 transition and Rb^+ deocclusion stimulated by Na^+ in pig-kidney purified Na,K-ATPase. Using rapid mixing techniques, we measured in parallel experiments the decrease in concentration of occluded Rb^+ and the increase in eosin fluorescence (the formation of *E*1) as a function of time. The *E*2→*E*1 transition and Rb^+ deocclusion are described by the sum of two exponential functions with equal amplitudes, whose rate coefficients decreased with increasing [Rb^+]. The rate coefficient values of the *E*2→*E*1 transition were very similar to those of Rb^+-deocclusion, indicating that both processes are simultaneous. Our results suggest that, when ATP is absent, the mechanism of Na^+-stimulated Rb^+ deocclusion would require the release of at least one Rb^+ ion through the extracellular access prior to the *E*2→*E*1 transition. Using vanadate to stabilize *E*2, we measured occluded Rb^+ in equilibrium conditions. Results show that, while Mg^{2+} decreases the affinity for Rb^+, addition of vanadate offsets this effect, increasing the affinity for Rb^+. In transient experiments, we investigated the exchange of Rb^+ between the *E*2-vanadate complex and the medium. Results show that, in the absence of ATP, vanadate prevents the *E*2→*E*1 transition caused by Na^+ without significantly affecting the rate of Rb^+ deocclusion. On the other hand, we found the first evidence of a very low rate of Rb^+ occlusion in the enzyme–vanadate complex, suggesting that this complex would require a change to an open conformation in order to bind and occlude Rb^+.

1. Introduction

The Na^+/K^+-ATPase, as other P-type pumps, couples the hydrolysis of ATP to the exchange of intracellular for extracellular cations. The catalytic cycle, described by the Albers–Post model, comprises four basic steps: (1) binding of ATP and 3 Na^+ to the inner face of the enzyme in the *E*1 conformation, followed by phosphorylation of the pump and trapping of the ions; (2) the conformational transition of the phosphorylated enzyme and release of the 3 Na^+ to the exterior; (3) the binding of 2 K^+ at the exterior face of the pump which leads to the dephosphorylation and occlusion of the 2 K^+ in the *E*2 dephosphoform; and (4) the *E*2→*E*1 conformational change releases the occluded potassium ions to the cell interior, a reaction strongly activated by ATP acting at a regulatory site [1].

Since the *E*2→*E*1 transition is the main rate-limiting step in the enzyme cycle, a number of studies have been directed to investigate the correlation between this transition and K^+ deocclusion [2,3]. Although some degree of coupling between both processes has been found, it has not been possible until now to achieve a thorough kinetic comparison due to the inability to obtain millisecond scale sampling of the K^+-occluded forms [3]. While it is assumed that the *E*2→*E*1 transition precedes the release of K^+ to the cell interior when ATP is bound to the regulatory site, there is no evidence that this is also the case when this site is unoccupied.

In this work we perform a detailed characterization of the correlation between the time courses of the *E*2→*E*1 conformational change and the release of Rb^+ due to Na^+ addition, and provide evidence for interpreting the mechanism by which the enzyme returns to the *E*1 state in the absence of ATP.

As it was previously shown, vanadate inhibits the P-type-ATPase-reaction cycle by arresting the enzyme in an *E*2-like state [4]. Using vanadate to prevent the transition to *E*1, we investigated the exchange of Rb^+ from the *E*2-occluded state, which should take place through the extracellular access of the pump. Since K^+ occlusion occurs in the *E*2 intermediate of the Na,K-ATPase, vanadate has been used by us and by other research groups for stabilizing the K^+-occluded form in this and other P-type ATPases [5–10]. Here, we find that vanadate prevents the *E*2→*E*1 transition caused by Na^+ in the absence of ATP,

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without significantly affecting the rate of Rb^+ release from *E*2. Additionally, we show the first evidence of a very low rate of Rb^+ occlusion in the enzyme–vanadate complex.

2. Materials and methods

2.1. Enzyme and reaction conditions

Na^+/K^+-ATPase was partially purified from the outer medulla of pig kidney according to Jensen et al. [11]. The specific activity at the time of preparation was 23–25 (μmol P_i) min^{-1} (mg protein)$^{-1}$ measured under optimal conditions (150 mM NaCl, 20 mM KCl, 3 mM ATP and 4 mM $MgCl_2$ in 25 mM imidazol-HCl, pH 7.4 at 37 °C). The concentration of ADP-binding sites for this preparation is 2.4 to 2.7 nmol (mg protein)$^{-1}$. All incubations were performed at 25 °C in media containing 25 mM imidazol-HCl (pH 7.4 at 25 °C), 0.25 mM ethylene diamine tetracetic acid (EDTA) and the concentrations of Rb^+ (which in all cases was used in lieu of K^+) given in Results.

2.2. Reagents

[^{86}Rb]RbCl was from PerkinElmer Life Science. To obtain the orthovanadate solution, Na_3VO_4 from Sigma Chemical Co. (USA) was freed of Na^+ by passing solutions of this reagent through a column containing a cation exchange resin (Bio Rad AG MP-50) equilibrated with Tris–HCl pH = 7.4. Final concentration of Na^+ in the eluate, measured by flame photometry, was less than 0.05% of the vanadate concentration on a mol to mol basis. The stock solution contained 3.5 mM vanadate in imidazol-HCl pH 7.4 at 25 °C. All other reagents were of analytical grade.

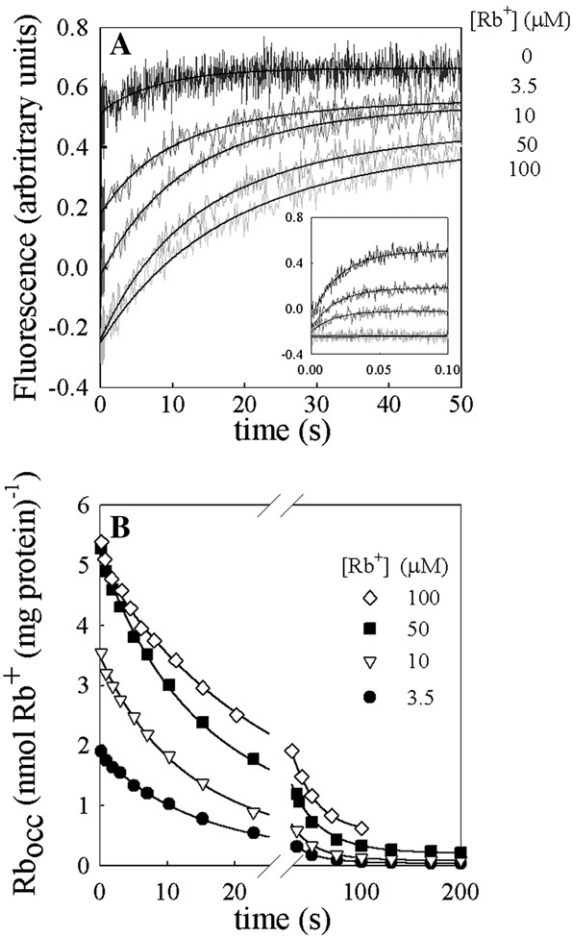

Fig. 1. Eosin fluorescence increase (A) and Rb^+ release (B) after Na^+ addition. One volume of the enzyme (50 μg/ml) with 0, 3.5, 10, 50 and 100 μM RbCl was mixed (time = 0) with one volume of a medium containing 12 mM NaCl and 0.8 μM eosin. The inset in Fig. 1A shows the first 0.1 s of the time courses of fluorescence change.

2.3. Measurements of occluded Rb^+

These were performed according to Rossi et al. [12]. The procedure uses a rapid-mixing apparatus (RMA) (SFM4 Bio-Logic, France) connected to a quenching and washing chamber. Quenching of occlusion reactions was attained by means of the quick drop in temperature, ligand concentrations and free [^{86}Rb]RbCl. Occluded Rb^+ was considered equal to that retained by the enzyme after washing with at least 300 ml of an ice-cold washing solution. Blanks were estimated from the amount of $^{86}Rb^+$ retained by the filters when the enzyme was omitted. To evaluate $^{86}Rb^+$ occlusion in equilibrium conditions, 30–50 μg of Na^+/K^+-ATPase were incubated during 15 to 120 min in reaction media with [^{86}Rb]RbCl.

2.4. E2→E1 conformational changes

The rise in *E*1 was measured as an increase in the fluorescence of eosin Y, a probe which binds with high affinity to this conformer [13,14]. The enzyme was kept in the dark throughout the experiments with eosin.

3. Results

3.1. Eosin fluorescence changes and Rb^+ deocclusion

We followed the time courses of occluded Rb^+ (Rb_{occ}) decrease and eosin fluorescence rise (*E*2→*E*1 transition) induced by Na^+, for Rb^+ concentrations ranging from 0 to 100 μM (Fig. 1). The enzyme incubated with rubidium was mixed with a medium containing NaCl and eosin. A control without Rb^+ was included. For Rb^+ 3.5 and 10 μM, best fit to fluorescence signal (continuous lines in Fig. 1A) was obtained with the following equation:

$$F = F_0 + F_{eo}\left(1-e^{-k_{eo}t}\right) + F_1\left(1-e^{-k_1 t}\right) + F_2\left(1-e^{-k_2 t}\right) \quad (1)$$

with $F_1 = F_2$. For 50 and 100 μM Rb^+ the rapid component, $F_{eo}(1-e^{-k_{eo}t})$, vanishes. When the enzyme was incubated in the absence of Rb^+, the fluorescence increase was fitted by a bi-exponential function presenting the rapid component plus a single slow one, $F_1(1-e^{-k_1 t})$.

For Rb^+ deocclusion experiments, best fit was obtained with (continuous lines in Fig. 1B):

$$Rb_{occ} = Rb_{occ\infty} + Rb_{occ1}e^{-k_1 t} + Rb_{occ2}e^{-k_2 t} \quad (2)$$

where $Rb_{occ1} = Rb_{occ2}$.

Best fitting values of k_1 and k_2 in Eqs. (1) and (2), shown in Fig. 2A, are almost superimposable and decrease with Rb^+ concentration. In order to facilitate the comparison between the time courses, the results in Fig. 1 were plotted together as a percentage of the total change (Fig. 3). It can be seen that the curves almost perfectly overlap for 50 and 100 μM Rb^+ where the great majority of the enzyme is in the *E*2(Rb_2) occluded form at time = 0. For lower Rb^+ concentrations, the additional fast fluorescence component (k_{eo} about 50 s^{-1}) becomes apparent, revealing the rapid binding of eosin to that fraction of the enzyme present as *E*1 [3].

The percentage of the amplitude of the slower phases relative to the total change, $[(F_1+F_2)/(F_{eo}+F_1+F_2)]100$, can be described by a hyperbolic function of Rb^+ concentration with a $K_{0.5}$ of 5.3 ± 0.7 μM and an ordinate value of 21 ± 2% (Fig. 2B). A similar value of $K_{0.5}$ (6.4 ± 0.8 μM) is obtained when a hyperbola passing through the coordinates' origin is fitted to the maximal changes of Rb^+ deocclusion

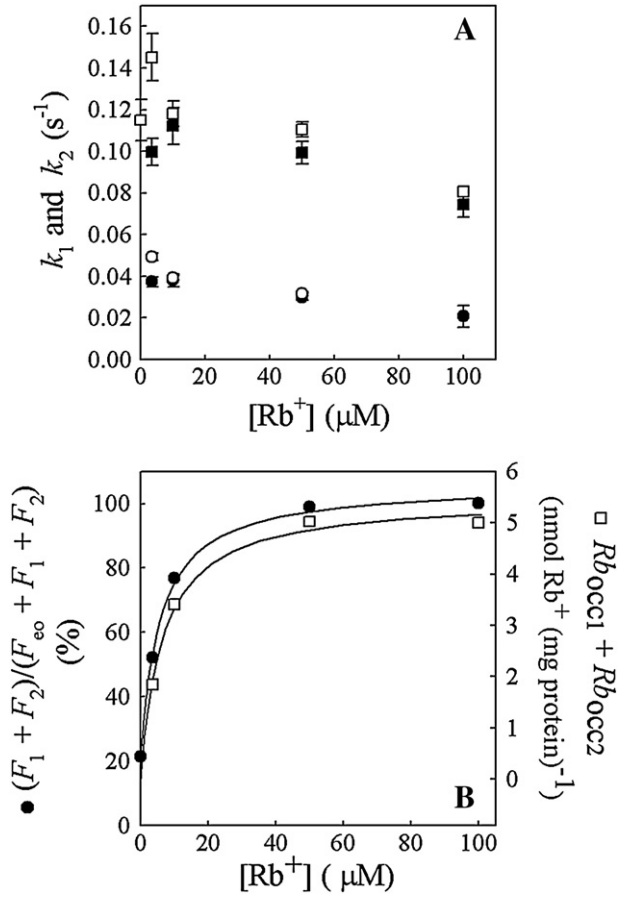

Fig. 2. (A) The best fitting values of k_1 (squares) and k_2 (circles) in Eq. (1) (open symbols) and Eq. (2) (filled symbols) as a function of [Rb^+]. (B) The percentage of the slower fluorescence phases, $(F_1+F_2)/(F_{eo}+F_1+F_2)\times 100$, and the amplitudes of Rb^+ deocclusion ($Rb_{occ1}+Rb_{occ2}$) obtained from the best fitting values of the parameters in Eqs. (1) and (2), respectively.

($Rb_{occ1}+Rb_{occ2}$) (see also references [14,15]). The ordinate value of 21% would represent the portion of the enzyme in the *E*2 state remaining at 25 mM imidazole-HCl. The influence of imidazole on the balance between *E*1 and *E*2 was tested in equilibrium experiments using Na,K-ATPase (35 μg/ml) plus eosin (0.4 μM) at various concentrations of imidazole-HCl at pH 7.4 (25 °C). Eosin fluorescence increased in a saturable manner with the concentration of imidazole, with a $K_{0.5}$ of about 9.5 mM (2.9 mM in terms of imidazolium concentration at the tested pH), which yielded an increase of 73% of the total change at 25 mM imidazole (7.7 mM imidazolium). Since high affinity binding of eosin occurs to the *E*1 conformer of the Na,K-ATPase, these results would indicate that, in the absence of added cations (other than imidazolium), more than 70% of the enzyme exists as *E*1 under the conditions of our experiments. Accordingly, the rapid fluorescence phase in Fig. 1A disappears at sufficiently high Rb^+ concentrations to shift the enzyme to the Rb^+-occluded form (*E*2 state of the enzyme).

The slower phases in the fluorescence changes are due to the *E*2→*E*1 conformational transition, which is simultaneous with the release of $^{86}Rb^+$ from the occluded form. The coupling between these two processes is evinced by the coincidence in the values of the rate coefficients, k_1 and k_2, these corresponding to components of equal amplitudes, as well as in the maximal changes in the slow fluorescence phases and Rb_{occ} for all the Rb^+ concentrations analyzed. Although Esmann [3] has shown that deocclusion of Rb^+ followed the fluorescence increase closely with time, the inability to measure occluded K^+ in the millisecond scale did not allow for an exhaustive kinetic analysis. Glynn et al. [2] also found that in the absence of ATP, the rate of release of occluded Rb^+ was usually somewhat faster than the rate of the fluorescence change, but they could not exclude the possibility that this was the consequence of systematic errors.

The analysis of the present results leads to the conclusion that the *E*2→*E*1 conformational transition and Rb^+ deocclusion induced by Na^+ are simultaneous processes.

3.2. Effect of vanadate on eosin fluorescence changes and Rb^+ deocclusion

The addition of vanadate, in the presence of Mg^{2+}, to the eosin-bound enzyme causes a decrease in the fluorescence signal to the same level as that obtained with 10 mM KCl or RbCl (not shown). The time course of this process (Fig. 4) was fitted with an exponential function with a rate coefficient of $0.320\pm0.001\ s^{-1}$, plus a linear term. Similar results were obtained when the enzyme was pre-incubated with Mg^{2+} proving that Mg^{2+} binds in rapid equilibrium to the protein. The fact that fluorescence does not decrease in the absence of $MgCl_2$ reflects the well known requirement of Mg^{2+} for the binding of vanadate [4,5].

In experiments similar to those presented in Fig. 1, the conformational change and Rb^+ deocclusion were measured in the presence of vanadate (Fig. 5). The enzyme with vanadate was equilibrated with rubidium and then exposed to a Na^+-containing medium. The plots of fluorescence and Rb_{occ} as a function of time show that vanadate abolished the eosin fluorescence change (Fig. 5A), whereas $^{86}Rb^+$ release still occurred at a significant rate, although the final level of Rb_{occ} was higher than in control conditions (Fig. 5B).

3.3. Vanadate and occluded Rb^+

The results presented here are consistent with the current idea that vanadate stabilizes the *E*2 conformation; however, the action of the inhibitor on *E*2 holding occluded Rb^+ has been poorly studied. We therefore performed experiments in equilibrium and pre-steady state conditions characterizing the effect of vanadate on the occluded state.

The equilibrium level of Rb_{occ} as a function of [Rb^+] (Fig. 6A) shows that Mg–vanadate decreases the affinity for the cation with respect to that obtained in the absence of Mg–vanadate. However, vanadate increases the affinity with respect to the control with Mg^{2+}. In addition, excess Mg^{2+} concentrations decrease the Rb_{occ} level in the presence of vanadate (Fig. 6B) as well as in its absence [15].

When measuring the time course of Rb^+ occlusion of the enzyme incubated with Mg–vanadate (Fig. 7), the initial rate was 500 times lower than that measured in the absence of both Mg^{2+} and vanadate, and about 4 times lower than that obtained in a control medium with Mg^{2+} alone.

The time courses of $^{86}Rb^+$ release from occluded states formed in the presence or absence of Mg–vanadate were measured after a 20-fold isotopic dilution of $^{86}Rb^+$ in a medium with Na^+, allowing a better evaluation of the effects of the inhibitor on the stability of the Rb^+ occluded form. Fig. 8 shows that both time courses are similar, being the initial velocity (nmol Rb^+ (mg prot)$^{-1}$ s^{-1}) 1.50 ± 0.22 (enzyme + Mg–vanadate) and 0.40 ± 0.03 (enzyme + Mg^{2+}). It is worth mentioning that the difference in the final equilibrium levels of Rb_{occ} observed in Fig. 5B, is minimized in this experiment due to the large isotopic dilution of the $^{86}Rb^+$.

At first sight, the marginal effect of vanadate on the initial rate of deocclusion and the low velocity of occlusion seems difficult to reconcile with the higher equilibrium level of Rb_{occ} as compared with the values found in media with Mg^{2+} alone. However, attention should be paid to the fact that more than two species are involved in these processes, i.e. while occlusion in media with Mg^{2+} alone would involve mainly the binding of Rb^+ to *E*1, followed by the transition to *E*2(Rb), both occlusion and deocclusion in media with Mg–vanadate would occur exclusively among states in the *E*2 conformation. To clarify this point, we show in Fig. 9 a simplified model describing the Na,

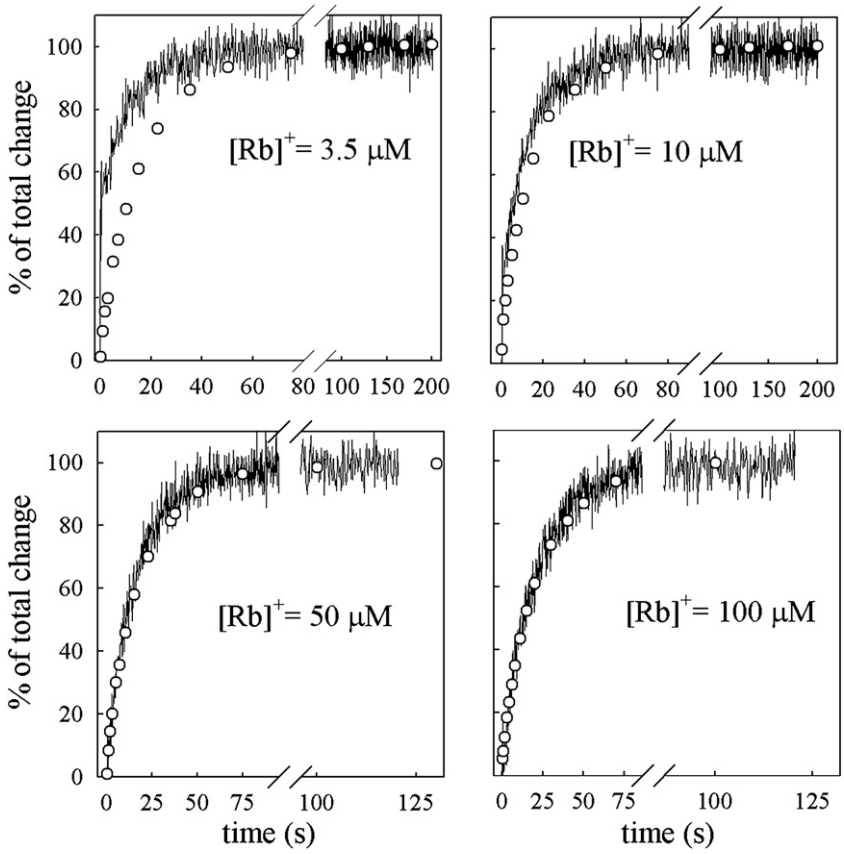

Fig. 3. Normalized time courses of fluorescence changes (continuous traces) and Rb^+ deocclusion (open circles) from Fig. 1A and B, plotted for each Rb^+ concentration.

K-ATPase–vanadate interaction and the effect of the transported cations. The enzyme exists in two main conformations, *E*1 and *E*2, being able to exchange ions through an intracellular or extracellular access, respectively. In the scheme, X represents the transport sites which can be occupied by Na^+ or K^+, or their congeners.

4. Discussion

This work presents a detailed kinetic study that shows the coupling between the *E*2→*E*1 transition and Rb^+ deocclusion stimulated by Na^+. Taking advantage of the fact that vanadate stabilizes the *E*2 conformer of the Na,K-ATPase, we investigated the kinetics of exchange of Rb^+ between the medium and the *E*2–vanadate complex. We found that, while the rate of Rb^+ deocclusion is only marginally affected by vanadate, Rb^+ occlusion into the *E*2–vanadate complex is a very slow process.

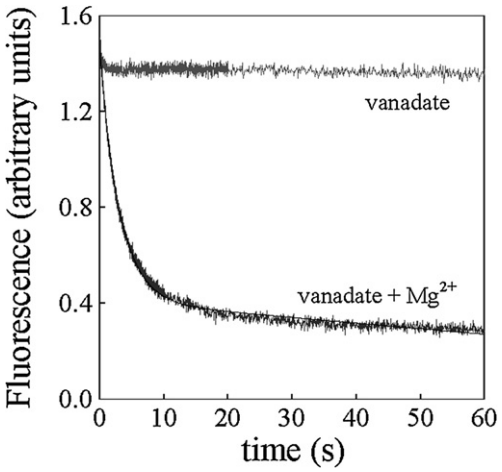

Fig. 4. Fluorescence decrease due to vanadate addition. The enzyme in the presence of eosin was mixed with vanadate or vanadate plus $MgCl_2$. Final media contained 50 μg/ml protein, 0 or 2 mM $MgCl_2$, 0.1 mM vanadate and 0.4 μM eosin.

4.1. *E*2→*E*1 transition and Rb^+ deocclusion

Our results demonstrate that, unlike for the *E*1→*E*2 conversion, which is completed before occlusion of Rb^+ reaches equilibrium [14], one can use fluorescence changes to monitor Na^+-induced Rb^+ deocclusion. It is worth noting that the simultaneity between the *E*2→*E*1 transition and Rb^+ deocclusion does not necessarily imply that the latter occurs as a result of the former (see below), since the alternative possibility that the transition takes place *after* the release of Rb^+ from *E*2 could as well be true. In any case, the simultaneity requires the second reaction to be much faster than the first one.

The total amplitude of the slow fluorescence changes increase with [Rb^+] along a hyperbolic function with an ordinate value of about 20%. Skou and Esmann [3,16] have proposed that this ordinate value could be explained by the presence of contaminant K^+. In our case this seems unlikely, since the concentration of contaminant K^+ measured in our solutions was less than 0.1 μM. In our view, the ordinate value would rather correspond to the fraction of Na,K-ATPase in the *E*2 conformation present in a medium with 25 mM imidazole without added cations. This is consistent with both our results and those of Fedosova and Esmann [17], who measured the K_d for nucleotides as a function of the concentration of the imidazolium cation. Under the conditions of our experiments (25 mM imidazole and pH 7.4) the concentration of imidazolium should be around 7.7 mM, enough to favor the prevalence of the *E*1 conformation although leaving a significant proportion (20%) of the enzyme in *E*2.

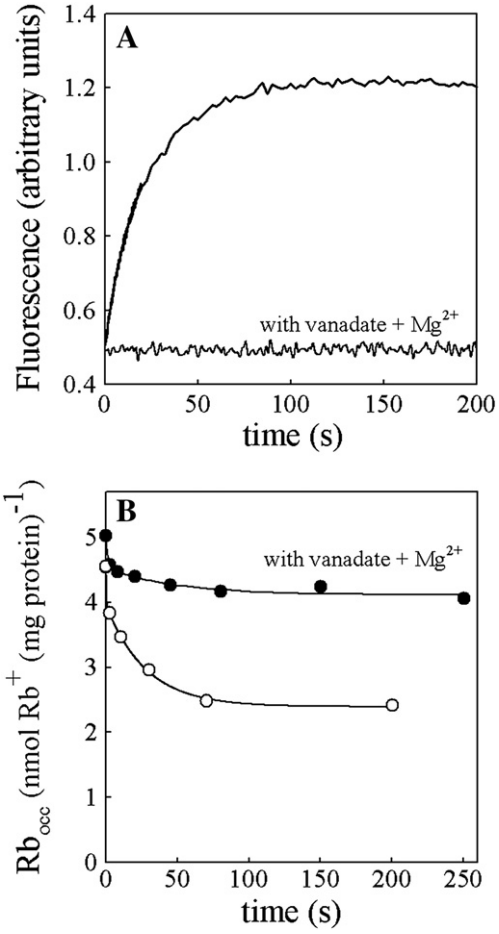

Fig. 5. Effect of vanadate on eosin fluorescence (A) and Rb^+ release (B) after Na^+ addition. One volume of the enzyme (50 μg/ml) with 500 μM RbCl incubated with or without Mg–vanadate was mixed (time = 0) with one volume of a medium containing NaCl and eosin. Final media contained 6 mM NaCl, 250 μM RbCl, 0.4 μM eosin and, when present, 0.1 mM vanadate plus 2 mM $MgCl_2$.

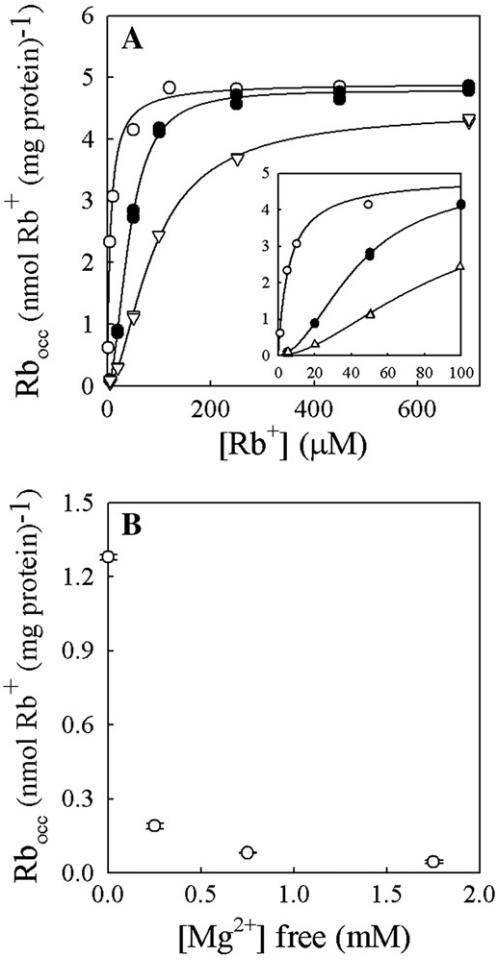

Fig. 6. Equilibrium levels of occluded Rb^+ (Rb_{occ}). (A) Na,K-ATPase (50 μg/ml) was incubated with Rb^+ for a sufficient length of time as to reach equilibrium in the absence (○) or in the presence of 2 mM $MgCl_2$ (▽) or 2 mM $MgCl_2$ plus 0.1 mM vanadate (●). (B) Effects of Mg^{2+} on the equilibrium levels of Rb_{occ} in the presence of 50 μg/ml protein, 3 μM RbCl and 0.1 mM vanadate.

4.2. Mechanism for the effects of Na^+

Since the Albers–Post model proposes that the *E*1 and *E*2 conformers respectively expose the transport sites to the intracellular and extracellular faces of the membrane, one would be tempted to accept that the ions are rapidly released to the inner face of the pump only after the slow *E*2→*E*1 transition has occurred. This view would find support in the fact that the slow phase of fluorescence change obtained after adding Na^+ to the enzyme in the absence of Rb^+, which could be taken as an expression of the conformational transition in the enzyme carrying no ions, shows a rate coefficient whose value is very similar to that extrapolated from k_1 at zero [Rb^+]. There is however a problem with this interpretation regarding the roles normally assigned to Na^+ and K^+ in the *E*2→*E*1 transition: it has been proposed [18,19] that Na^+ exerts its effects by competing with K^+ for the sites in *E*1, thus preventing the binding of K^+ and its occlusion in *E*2. As noted by Esmann [3], see also Faller et al. [19] and Humphrey et al. [20], if this were so, the rate coefficient of occlusion as well as that of deocclusion of Rb^+ (K^+) should increase with the concentration of this cation and decrease with that of Na^+ in a competitive manner. Our results show instead, a decrease in the rate coefficients k_1 and k_2 with [Rb^+] indicating that this cation is being released *before* the *E*2→*E*1 transition takes place, not *after*. Regarding the role of Na^+, in the absence of nucleotides Glynn and Richards [5] observed no influence of (approximately) 45 mM Na^+ on the rate of deocclusion, and Forbush found a similar lack of effect at Na^+ concentrations up to 50–100 mM (see Fig. 9 in ref. [21]). Therefore, that Rb^+ deocclusion occurs after the binding of Na^+ at a concentration of 6 mM seems very unlikely. On the other hand, Forbush [10] has shown that Na^+ in fact reverses in a competitive manner the blocking effects of K^+ observed in media with MgPi, *i.e.* when the enzyme is in the *E*2 conformation and the transport sites are exposed to the extracellular face of the membrane [22]. This leads us to propose the hypothesis that, when ATP is absent, the mechanism of Na^+-stimulated Rb^+ deocclusion would require the release of at least one Rb^+ ion through the extracellular access prior to the binding of Na^+ and the subsequent *E*2→*E*1 transition. In this case, the rate limiting step would be the opening of the extracellular access (the *E*2(X)→*E*2X step in Fig. 9) which, if it were independent of the degree of occupancy of the transport sites, could also explain the rate of the *E*2→*E*1 transition observed in the absence of Rb^+. The model shown in Fig. 9 supports this interpretation since it assumes that *E*2 can exist in two subconformations, a closed (occluded) one, *E*2(X), and an open one (to the extracellular side), *E*2X, and that both subconformations as well as *E*1 can exist either combined to Rb^+ and/or Na^+, or carrying no ions.

4.3. Effects of vanadate

It has been shown that Mg–vanadate increases the affinity of the Na,K-ATPase for K^+ [23]. In this work we present evidence that Mg–

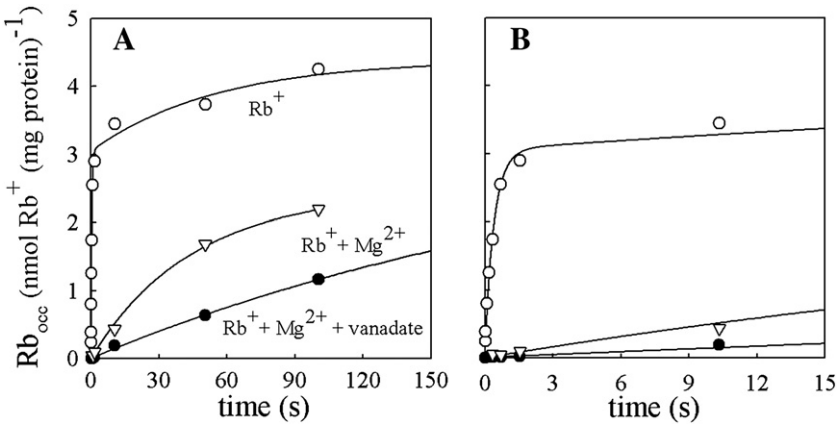

Fig. 7. The time course of Rb^+ occlusion. The enzyme with no added ligands (○) or incubated with $MgCl_2$ (▽) or with $MgCl_2$ plus vanadate (●), was mixed with $^{86}Rb^+$ for different lengths of time. Final media contained 50 μg/ml protein, 100 μM Rb^+ and, when present, 2 mM $MgCl_2$ with or without 0.1 mM vanadate. (B) The first 15 s of the time courses is shown.

vanadate increases the affinity for Rb^+ to form the occluded state with respect to the control with Mg^{2+}alone, albeit this affinity is lower than that observed in the absence of both ligands. According to results from our laboratory [15], Mg^{2+} seems to compete with Rb^+ preventing the occlusion of a single Rb^+, which could explain the decrease in the affinity for the cation. Although vanadate offsets the decrease in Rb^+ affinity caused by Mg^{2+}, we cannot ascertain whether the inhibitor enables the enzyme to occlude a single Rb^+.

The interaction between vanadate and the Na,K-ATPase is explained by the scheme in Fig. 9 as follows: (i) vanadate is assumed to be an exclusive ligand of *E*2 (as currently accepted), and (ii) in equilibrium conditions with no added cations the concentration of the closed form, *E*2(X)V, predominates with respect to that of the open one, *E*2XV. The fact that Mg–vanadate can combine to the enzyme in the absence of Na^+ and/or Rb^+ is demonstrated by the eosin-fluorescence drop observed and the absolute requirement of Mg^{2+} for this process to take place.

Since vanadate exclusively binds to *E*2, the model explains (i) why the inhibitor cancels the Na^+-promoted increase in eosin fluorescence, and (ii) the higher equilibrium levels of occluded Rb^+ reached in the presence of vanadate in media with Na^+ and Mg^{2+}. According to the model, the Na^+-dependent release of Rb^+ from the *E*2–vanadate complex would mainly occur via Na^+ competing with Rb^+ for the (extracellular) sites in *E*2, as proposed by Forbush for the MgPi–*E*2 complex [10].

The rate of Rb^+ release from the vanadate-inhibited enzyme is not very different from, and in fact somewhat higher than the rate of Rb^+

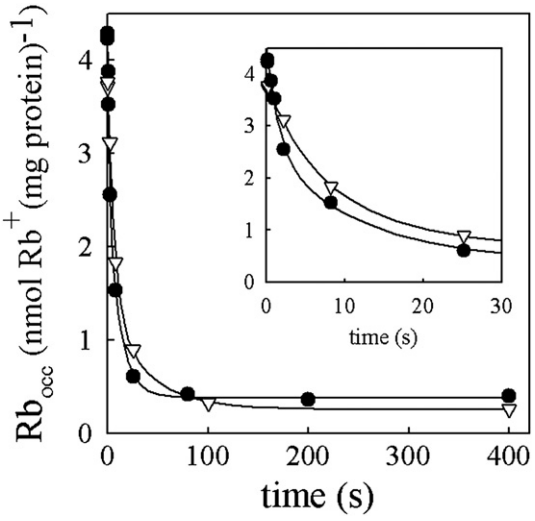

Fig. 8. The time course of Rb^+ release after Na^+ addition. Rb_{occ} remaining after a 20-fold reduction in the specific activity of [^{86}Rb]Rb^+ in a medium with Mg^{2+} (▽) or Mg–vanadate (●). Occluded Rb^+ was formed incubating the enzyme with Rb^+ and Mg^{2+} in the presence (●) or in the absence of vanadate (▽). Final media contained 50 μg/ml protein, 250 μM Rb^+, 6 mM NaCl, 2 mM $MgCl_2$ and 0 or 0.1 mM vanadate. The inset shows the first 30 s of the time courses.

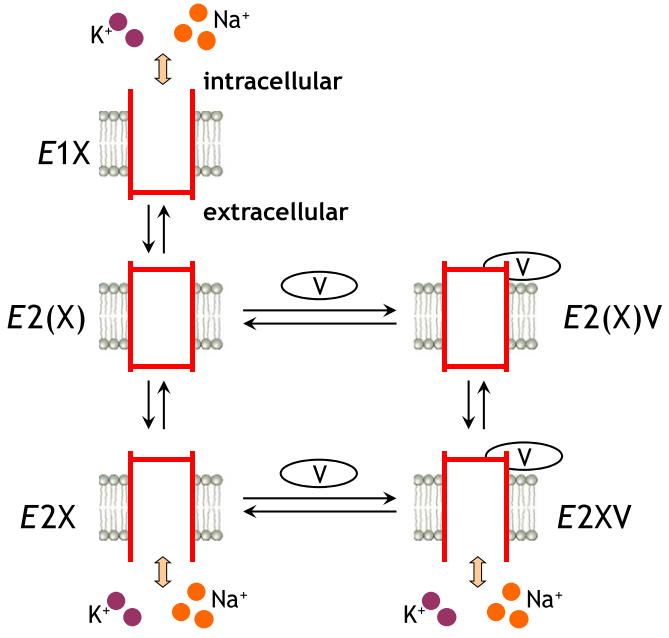

Fig. 9. A scheme for the interaction between Na,K-ATPase, vanadate and ligands. The enzyme, embedded in the phospholipid bilayer, exists in two main conformations *E*1 and *E*2, which are able to exchange ions at the internal or external side of the membrane, respectively. X represents the transport sites that could be empty or occupied by Na^+ or K^+. *E*2 can be present in a closed, *E*2(X), or open, *E*2X, conformation. Mg–vanadate (V) can bind to the enzyme forming the open (*E*2XV) or closed (*E*2(X)V) complexes.

release observed for the control without inhibitor. Both Glynn [5] and Forbush [10], who showed that vanadate dramatically inhibits the large increase in the rate constant for Rb^+-deocclusion caused by ATP and ADP, also found only marginal effects of vanadate on this rate coefficient in the absence of nucleotides. This would also speak in favor of the hypothesis posited above that, in the absence of vanadate (and nucleotides), the $E2 \rightarrow E1$ transition would occur after the release of Rb^+ through the extracellular access.

The fact that occlusion of Rb^+ into the vanadate–enzyme complex is very slow as compared to that observed in the absence of the inhibitor is explained in the model where Rb^+ occlusion takes place from the *E*2 conformer, in the case of the enzyme–vanadate complex, and from *E*1 in the controls without vanadate. The "closed" *E*2–vanadate state, *E*2(X)V, would require a change to an "open" conformation in order to bind and occlude Rb^+. Since the release of vanadate followed by the transition to *E*1 seems very improbable (the rate constant of vanadate release is $4\,h^{-1}$ at 4 °C, [23]), the easiest explanation would imply that this open conformation is *E*2XV and exposes the cation sites to the extracellular aspect of the enzyme. Accordingly, Forbush has shown that like in the case of the MgPi–*E*2 complex, deocclusion of Rb^+ from the vanadate-inhibited enzyme displays blocking effects by K^+ [10]. The difficulty for *E*2-vanadate to occlude Rb^+ evokes the "K^+-insensitive-*E*2P" state, formed from MgPi in the absence of transported cations, found by Post et al. [24]. Similarly, ouabain, which is thought to stabilize the unphosphorylated enzyme in the *E*2 conformation, prevents Rb^+ occlusion if the Rb^+ is added after the ouabain has bound [25].

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References

[1] I.M. Glynn, All hands to the sodium pump, J. Physiol. 462 (1993) 1–30.
[2] I.M. Glynn, Y. Hara, D.E. Richards, M. Steinberg, Comparison of rates of cation release and conformational change in dog kidney Na, K-ATPase, J. Physiol. 383 (1987) 477–485.
[3] M. Esmann, Influence of Na^+ on conformational states in membrane-bound renal Na,K-ATPase, Biochemistry 33 (1994) 8558–8565.
[4] S.J. Karlish, L.A. Beaugé, I.M. Glynn, Vanadate inhibits (Na^+/K^+)ATPase by blocking a conformational change of the unphosphorylated form, Nature 282 (5736) (1979) 333–335.
[5] I.M. Glynn, D.E. Richards, Occlusion of rubidium ions by the sodium–potassium pump: its implications for the mechanism of potassium transport, J. Physiol. 330 (1982) 17–43.
[6] M.R. Montes, A.J. Spiaggi, J.L. Monti, F. Cornelius, C. Olesen, P.J. Garrahan, R.C. Rossi, Rb^+ occlusion stabilized by vanadate in gastric H+/K+-ATPase at 25 °C, Biochim. Biophys. Acta 1808 (2011) 316–322.
[7] K. Abe, Unique structures and conserved conformational changes found in gastric (1723) H,K-ATPase, Na,K-ATPase and related P-ATPases: structure, biology and medicine, 13th International P-type ATPase Meeting, 2011.
[8] E.C. Rabon, K. Smillie, V. Seru, R. Rabon, Rubidium occlusion within tryptic peptides of the H, K-ATPase, J. Biol. Chem. 268 (1993) 8012–8018.
[9] J.D. Clausen, D.B. McIntosh, D.G. Woolley, J.P. Andersen, Modulatory ATP binding affinity in intermediate states of *E*2P dephosphorylation of sarcoplasmic reticulum Ca2+-ATPase, J. Biol. Chem. 286 (2011) 11792–11802.
[10] B. Forbush, Rapid release of 42K or 86Rb from two distinct transport sites on the Na, K-pump in the presence of Pi or vanadate, J. Biol. Chem. 262 (23) (1987) 11116–11127.
[11] J. Jensen, J.G. Norby, P. Ottolenghi, Binding of sodium and potassium to the sodium pump of pig kidney evaluated from nucleotide-binding behaviour, J. Physiol. 346 (1984) 219–241.
[12] R.C. Rossi, S.B. Kaufman, R.M. González-Lebrero, J.G. Nørby, P.J. Garrahan, An attachment for nondestructive, fast quenching of samples in rapid-mixing experiments, Anal. Biochem. 270 (1999) 276–285.
[13] J.C. Skou, M. Esmann, Eosin, a fluorescent probe of ATP binding to the (Na^+–K^+)-ATPase, Biochim. Biophys. Acta 647 (1981) 232–240.
[14] M.R. Montes, R.M. González-Lebrero, P.J. Garrahan, R.C. Rossi, Eosin fluorescence changes during Rb^+ occlusion in the Na^+/K^+-ATPase, Biochemistry 45 (2006) 13093–13100.
[15] R.M. González-Lebrero, S.B. Kaufman, P.J. Garrahan, R.C. Rossi, The occlusion of Rb^+ in the Na,K-ATPase II, J. Biol. Chem. 277 (2002) 5922–5928.
[16] J.C. Skou, M. Esmann, The effects of Na^+ and K^+ on the conformational transitions of ($Na^+ + K^+$)-ATPase, Biochim. Biophys. Acta 746 (1983) 101–113.
[17] N.U. Fedosova, M. Esmann, Nucleotide-binding kinetics of Na,K-ATPase: cation dependence, Biochemistry 43 (2004) 4212–4218.
[18] S.J. Karlish, Characterization of conformational changes in (Na,K) ATPase labeled with fluorescein at the active site, J. Bioenerg. Biomembr. 3–4 (1980) 111–136.
[19] L.D. Faller, R.A. Scheiner-Bobis, R.A. Farley, Temperature dependence of the rates of conformational changes reported by fluorescein 5′-isothiocyanate, Biochemistry 30 (14) (1991) 3503–3510.
[20] P.A. Humprey, C. Lupfert, H. Apell, F. Cornelius, R. Clarke, Mechanism of the rate-determining step of the Na^+,K^+-ATPase pump cycle, Biochemistry 41 (2002) 9496–9507.
[21] B. Forbush III, Rapid release of ^{42}K and ^{86}Rb from an occluded state of the Na,K-pump in the presence of ATP or ADP, J. Biol. Chem. 262 (1987) 11104–11115.
[22] B. Forbush III, Occluded ions and Na,K-ATPase, Progress in Clinical and Biological Research, 258, Alan R. Liss, Inc., New York, 1988.
[23] L.C. Cantley, L.G. Cantley, L. Josephson, A characterization of vanadate interactions with the (Na,K)-ATPase, J. Biol. Chem. 253 (20) (1984) 7361–7368.
[24] R.L. Post, G. Toda, F.N. Rogers, Phosphorylation by inorganic phosphate of sodium plus potassium ion transport adenosine triphosphatase, J. Biol. Chem. 250 (1975) 691–701.
[25] I.M. Glynn, S.J. Karlish, Occluded cations in active transport, Annu. Rev. Biochem. 59 (1990) 171–205.