Eosin Fluorescence Changes during Rb^+ Occlusion in the Na^+/K^+-ATPase†

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ABSTRACT: We used suspensions of partially purified Na^+/K^+-ATPase from pig kidney to compare the effects of Rb^+, as a K^+ congener, on the time course and on the equilibrium values of eosin fluorescence and of Rb^+ occlusion. Both sets of data were collected under identical conditions in the same enzyme preparations. The incubation media lacked ATP so that all changes led to an equilibrium distribution between enzyme conformers with and without bound eosin and with and without bound or occluded Rb^+. Results showed that as Rb^+ concentration was increased, the equilibrium value of fluorescence decreased and occlusion increased along rectangular hyperbolas with similar half-maximal values. The time courses of attainment of equilibrium showed an initial phase which was so quick as to fall below the time resolution of our rapid-mixing apparatus. This phase was followed by the sum of at least two exponential functions of time. In the case of fluorescence the fast exponential term accounted for a larger fraction of the time course than in the case of occlusion. Comparison between experimental and simulated results suggests that fluorescence changes express a process that is coupled to Rb^+ occlusion but that is completed before occlusion reaches equilibrium.

Under physiological conditions, the Na^+/K^+-ATPase of plasma membranes couples the hydrolysis of ATP into ADP and P_i to the exchange of three intracellular sodium ions for two extracellular potassium ions. It is generally accepted (*1*) that this takes place in a series of steps which include the following: (a) the $(Na^+ + Mg^{2+})$-dependent phosphorylation by ATP of the E_1 conformer of the enzyme leads to the E_1P phosphoenzyme (during phosphorylation, three sodium ions are taken up from the cytosol and occluded in E_1P); (b) the $E_1P \rightarrow E_2P$ transition of the phosphoenzyme takes place (during this, occluded Na^+ is released into the extracellular medium); (c) extracellular K^+ activates the dephosphorylation of E_2P (during this, two potassium ions are taken up from the extracellular medium and occluded in E_2); and (d) finally E_2 returns to E_1, occluded K^+ is released into the cytosol, and thus the transport cycle is completed. K^+ or its congeners can also be occluded in media lacking ATP and Na^+ ("direct route"). This occluded state is kinetically identical to that which appears during dephosphorylation in the course of the physiological operation of the Na^+/K^+-ATPase, but, in contrast with this, it can occlude both one and two Rb^+ per enzyme molecule (*2*).

It has been reported (*3*) that, under equilibrium conditions, there is correlation between cation binding and the effects of Rb^+ (acting as a K^+ congener) on the extrinsic fluorescence of the Na^+/K^+-ATPase. Glynn and co-workers (*4*) used fluorescent probes to measure the rate of the $E_2 \rightarrow E_1$ transition and to compare it with the rate of release of occluded Rb^+. Their results were compatible with the hypothesis that the same conformational transition was the cause of the change in fluorescence and of the release of Rb^+. This result agrees with observations by Esmann (*5*).

In contrast with the studies on deocclusion, there is little or no quantitative information in the literature on the correlation between the rates of *occlusion* of K^+ or its congeners and the $E_1 \rightarrow E_2$ transition. This is mainly due to the technical difficulties of measuring the rate of occlusion with time resolutions of the order of milliseconds. These difficulties have recently been superseded by the rapid cooling and filtering technique developed by Rossi et al. (*6*). This procedure was applied in this paper to compare the kinetics of Rb^+ occlusion through the direct route with that of the $E_1 \rightarrow E_2$ transition measured by means of the decay in eosin fluorescence (*7*). Our previous studies on the interactions between eosin and the enzyme holding occluded Rb^+ (*8*) allowed us to optimize the experimental procedures used in the present paper.

MATERIALS AND METHODS

Na^+/K^+-ATPase partially purified from the outer medulla of pig kidney according to Jensen et al. (*9*) was a kind gift of the Department of Biophysics of the University of Århus, Denmark. The specific activity at the time of preparation was 23−25 (μmol P_i) min^{-1} (mg of protein)$^{-1}$ measured under optimal conditions (150 mM NaCl, 20 mM KCl, 3 mM ATP, and 4 mM $MgCl_2$ in 25 mM imidazole-HCl, pH 7.4 at 37 °C). This corresponds to an ADP-binding site concentration of 2.4−2.7 nmol (mg of protein)$^{-1}$.

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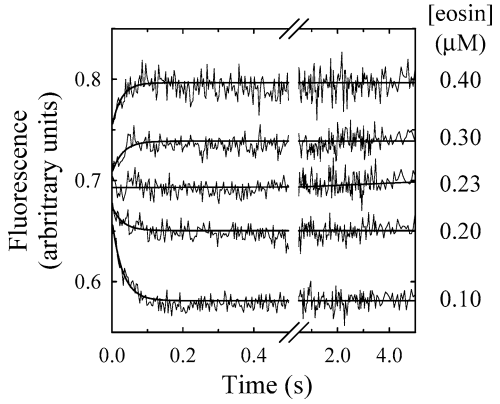

FIGURE 1: Time course of eosin fluorescence after dilution of a suspension of Na^+/K^+−ATPase equilibrated with 0.4 μM eosin with an equal volume of buffer containing the concentrations of eosin indicated in the graph. The final enzyme concentration was 50 μg/mL. Except when 0.23 μM eosin was present, the continuous lines represent the plot of a single-exponential function of time plus a constant term.

All incubations were performed at 25 °C in media containing 25 mM imidazole-HCl (pH 7.4 at 25 °C), 0.25 mM ethylenediamine tetracetic acid, and the concentrations of Rb^+ (which in all cases was used in lieu of K^+) and of eosin given in Results. The enzyme was kept in the dark throughout the experiments with eosin. No correction for ionic strength was made when different [Rb^+] were used, since it is known that the small changes in Rb^+ concentration of our experiments do not significantly affect either conformational changes (*10*) or eosin affinity (*11*).

Measurements of the time course of fluorescence were carried out with a stopped-flow reaction analyzer from Applied Photophysics (U.K.), which exhibits dead times of less than 2 ms. The procedure was based on that of Skou and Esmann (*12*), using eosin-Y from Sigma Chemical Co., USA. One syringe of the stopped-flow device was filled with Na^+/K^+-ATPase (100 μg of protein/mL) suspended in reaction media with 0.4 μM eosin. The other syringe was filled with reaction media having 0.23 μM eosin and enough Rb^+ to reach the concentrations indicated in Results. These concentrations of eosin and of enzyme prevented artifacts due to dilution (see comments to Figure 1). Each syringe delivered 75 μL per shot. The excitation wavelength was 520 nm. The emitted light was filtered through a cutoff filter at 550 nm. The band-pass was set at 2.3 nm. In each experiment, 3000 data points were collected. Between 5 and 10 experimental traces were averaged to evaluate each time course.

In the equilibrium fluorescence experiments Na^+/K^+-ATPase (80 μg of protein/mL) was incubated in the dark in reaction media containing 0.32 μM eosin and the concentrations of Rb^+ indicated in Results. Fluorescence was measured in an Aminco-Bowman/Series 2 spectrofluorimeter at 25 °C with a band-pass of 4 nm. The excitation wavelength was 520 nm, and emission was measured at 540 nm.

The time course of formation of occluded Rb^+ was measured with [^{86}Rb]RbCl (from Perkin-Elmer NEN Life Sciences, USA) by means of a rapid-mixing apparatus (SFM4/Q from Bio-Logic, France) and stopping the reaction by cooling, dilution, and filtering according to Rossi et al. (*6*). The method, which preserves the structural integrity of the enzyme, is able to stop deocclusion reactions that proceed with rate constants of up to 25 s^{-1} without significant loss of Rb^+. We have already shown (*2*) that the amount of radioactivity retained after a run without enzyme is the same as that retained after a run with heat-denatured enzyme. This indicates that our washing procedure eliminates essentially all [^{86}Rb]Rb^+ nonspecifically bound to the enzyme preparation. To perform the experiments, Na^+/K^+-ATPase (100 μg of protein/mL) was suspended in the incubation media with 0.4 μM eosin and mixed with an equal volume of a medium having 0.23 μM eosin and enough [^{86}Rb]Rb^+ to yield the concentrations given in Results. After different incubation times the reaction was stopped.

Theoretical equations were adjusted to the results by nonlinear regression based on the Gauss−Newton algorithm using commercial programs (Excel and Sigma-Plot for Windows). This procedure yields the best-fitting values of the parameters of the equation together with their respective standard errors.

To choose among different equations, we applied the second-order Akaike information criterion, that is,

$$\mathrm{AICc} = N \ln(S/N) + 2K\,N/(N - K - 1) \qquad (1)$$

where N is the number of data, S is the sum of weighted square errors of residuals, and K is the number of parameters of the fitted function plus one (*13*). We chose the equations that gave the lower value of AICc.

Numerical simulations of theoretical models were performed using Mathematica for Windows (version 5).

RESULTS

Effects of Dilution on Fluorescence. Since the stopped-flow apparatus requires the mixing of two solutions to yield the final incubation media, it became necessary to analyze the changes in fluorescence caused by the changes in concentration resulting from mixing. To do this, one of the two syringes of the stopped-flow apparatus was filled with a suspension of Na^+/K^+-ATPase in buffer containing 0.4 μM total eosin, while the other was filled with the same buffer with either 0.1, 0.2, 0.23, 0.3, or 0.4 μM eosin. After mixing, fluorescence was monitored during at least 5 s. Results in Figure 1 show that when the second syringe had either 0.4 or 0.3 μM eosin, fluorescence increased and when 0.1 or 0.2 μM eosin was present, fluorescence dropped. In both cases new constant values of fluorescence were reached in less than 0.2 s, in agreement with the observations of Smirnova and Faller (*14*). In contrast with this, when the second syringe contained 0.23 μM eosin, fluorescence remained independent of the incubation time. It is therefore possible to avoid artifacts caused by dilution that would interfere with the measurement of the effects of Rb^+ on fluorescence change. Additionally, the invariance of the fluorescence signal allowed us to estimate the equilibrium constant for the dissociation of eosin from the ATPase. This can be calculated considering that the absence of a fluorescence change is reached when the concentration of eosin present in the second syringe equals that of free eosin in the first syringe. Knowing the total concentrations of eosin (0.4 μM) and enzyme (0.25 μM assuming a single ATP-binding site per enzyme molecule) and the concentration of free eosin (0.23 μM) in the first syringe, assumption of a single site

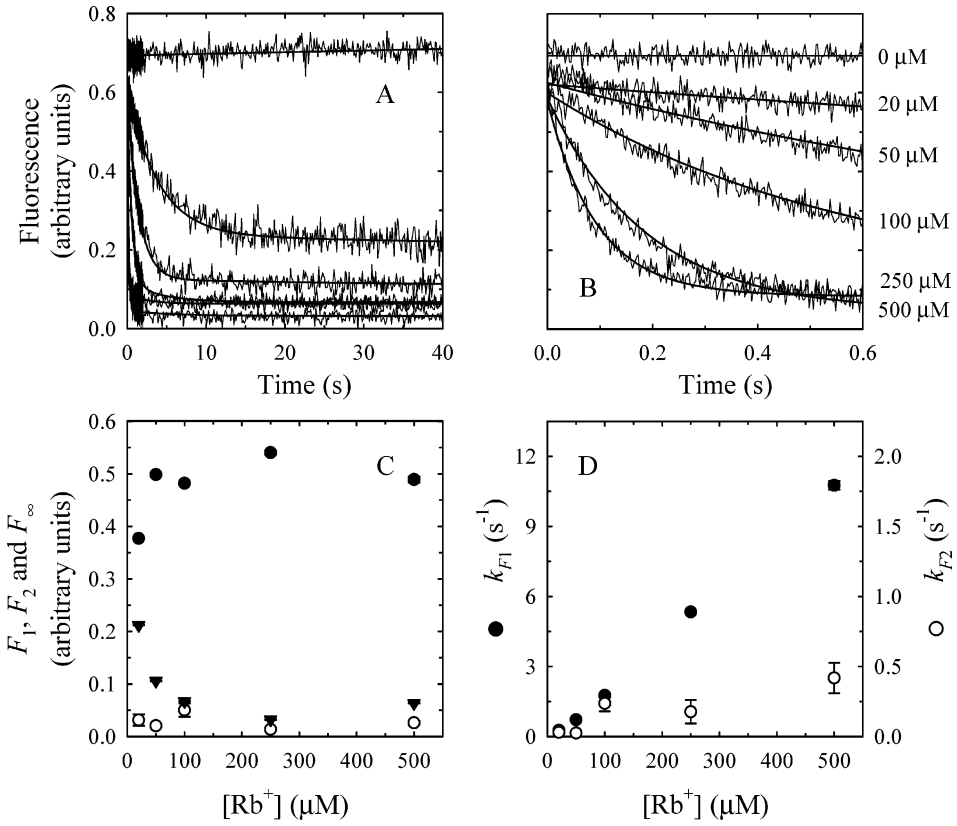

FIGURE 2: (A) Effect of Rb^+ concentrations on the time course of fluorescence change in Na^+/K^+−ATPase suspensions. Panel B shows the first 0.6 s of the time courses. Continuous lines are drawn according to eq 4. The values for F_1 (●), F_2 (○), and F_∞ (▼), and for k_{F1} (●), and k_{F2} (○), with their standard errors are plotted as a function of Rb^+ concentration (panels C and D, respectively). Values of F_1, k_{F1}, and F_∞ are the best fitting values using eq 4 in which F_2 and k_{F2} were considered equal to the values of best fit of F_3 and k_{F3} in eq 3.

for eosin in the ATPase will give a K_d for the eosin−ATPase complex:

$$K_d = \frac{([\text{enzyme}]_{\text{total}} - [\text{eosin}]_{\text{total}} + [\text{eosin}]_{\text{free}})[\text{eosin}]_{\text{free}}}{[\text{eosin}]_{\text{total}} - [\text{eosin}]_{\text{free}}} = 0.108\ \mu\text{M} \quad (2)$$

a value which is in the same range of our previous estimation of this parameter (*8*).

Effects of Rb⁺ on Fluorescence. Eosin-labeled enzyme suspended in incubation medium was mixed with an equal volume of the same medium containing 0.23 μM eosin and either no Rb^+ or enough Rb^+ to give a final concentration of 20, 50, 100, 250, or 500 μM. After mixing, fluorescence was measured during at least 100 s.

Results in Figure 2A and B show that, as in Figure 1, in the absence of Rb^+, fluorescence remained independent of time. The average value in this condition (F_B) was taken as the baseline for fluorescence measurements. In media with Rb^+, fluorescence decreased to a lower constant value. The rate of the fall of fluorescence increased while its asymptotic value decreased as the concentration of Rb^+ rose.

The highest fluorescence signal appeared in media without Rb^+. This is consistent with the idea that in this condition the equilibrium between E_1 and E_2 is poised toward E_1, as it is to be expected from the sodium-like effect of imidazole on the equilibrium between conformers (*15*).

The best fit to the results was obtained with the following equation

$$F = F_1 e^{-k_{F1}t} + F_2 e^{-k_{F2}t} + F_3 e^{-k_{F3}t} + F_\infty \quad (3)$$

However, some of the best fitting parameters of eq 3 showed a large degree of instability (scatter), notably those related to components whose rate coefficient had values that were not far enough from each other. To decrease the scatter and to facilitate the comparison of the time courses of fluorescence with those of occlusion (see below and comments under Discussion), we proceeded as follows. We first fitted eq 3 to the results. The best fitting values of the parameters of the slowest phase (F_3 and k_{F3}) were then fixed (and now called F_2 and k_{F2}) in the following equation:

$$F = F_1 e^{-k_{F1}t} + F_2 e^{-k_{F2}t} + F_\infty \quad (4)$$

Each of the curves (see continuous lines in Figure 2) was analyzed by nonlinear regression using eq 4 to find the best fitting values of F_1, k_{F1}, and F_∞.

Results in Figure 2 show that fluorescence is maximal at $t = 0$, when $F = F_0 = F_1 + F_2 + F_\infty$, and decreases toward F_∞ as time tends to infinity. Figure 2B also shows that F_0 is always smaller than fluorescence in the absence of Rb^+ (F_B). This indicates that the fluorescence changes induced by Rb^+ includes a phase whose maximal value is $F_B - F_0$ and which is not taken into account in eq 4 because it is completed at times below the dead time (2 ms) of our stopped-flow apparatus.

The best fitting values of the parameters of eq 4 are plotted in Figure 2C,D as a function of [Rb^+]. It can be seen (Figure 2C) that most of the fluorescence change can be accounted

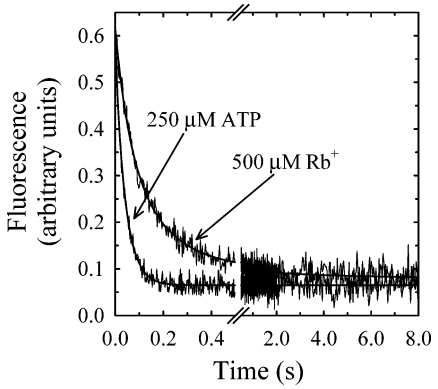

FIGURE 3: Time courses of the decay of eosin fluorescence in media with either 250 μM ATP or 500 μM Rb^+. Data were fitted by the sum of two exponential functions of time for the experiment with ATP (eq 4) and the sum of three exponential functions of time (eq 3) for the experiment with Rb^+.

for by F_1 so that F_2 represents a very small fraction of the overall change. As shown in Figure 2D, the rate coefficients increased linearly with [Rb^+], the values of k_{F1} being about 30-fold higher than those of k_{F2}. The saturation of the effects of Rb^+ on F_1 and F_2 contrasts with the lack of saturation of the effects of Rb^+ on the rate coefficients. The reason for this will be explained in relation with the experiments on the Rb^+ occlusion.

To obtain further insight on the effects of Rb^+, we compared the time course of the drop of fluorescence elicited by 500 μM Rb^+ with that caused by 250 μM ATP, a concentration of nucleotide that should fully displace bound eosin from its site in E_1 (Figure 3). In this condition, the remaining equilibrium eosin fluorescence signal will only express the fluorescence of free eosin plus that of eosin nonspecifically bound to membrane components, as detected by Skou and Esmann (*7*). It can be seen that the equilibrium fluorescence signal in media with enough [Rb^+] to fully drive the enzyme into the E_2 state is almost the same as that observed when all eosin has been displaced from the enzyme. This contrasts with the calculated equilibrium value of bound eosin at 500 μM Rb^+, which is still about 7% of that obtained in the absence of the cation (*8*).

Effects of Rb^+ on Occlusion. We measured the time course of occlusion of Rb^+ under identical conditions as those we used to measure fluorescence, in media containing eosin and [^{86}Rb]Rb^+ in concentrations ranging from 20 to 500 μM.

Results are shown in Figure 4A,B as plots of occluded Rb^+ (Rb_{occ}) vs incubation time. It can be seen that Rb_{occ} increased with time, tending to a constant value. Both the rate of formation (Figure 4B) and the asymptotic values of Rb_{occ} increased with the concentration of Rb^+. The best fit to the time courses of occlusion (continuous lines in Figure 4A,B) was obtained with the following equation:

$$Rb_{occ} = Rb_{occ0} + Rb_{occ1}(1 - e^{-k_{occ1}t}) + Rb_{occ2}(1 - e^{-k_{occ2}t}) \quad (5)$$

which shows that as time goes from zero to infinity, Rb_{occ} goes from Rb_{occ0} to a maximal value along two exponential functions of time which are governed by the rate coefficients k_{occ1} and k_{occ2} and whose maximal changes are Rb_{occ1} and Rb_{occ2}, respectively. The best fitting values of the parameters of eq 5 are plotted in Figure 4C,D as a function of Rb^+ concentration. It can be seen that in all cases $Rb_{occ0} > 0$ (Figure 4C), which indicates that, as in the case of fluorescence, occlusion includes a component that is faster than the temporal resolution of our measuring procedure. Figure 4C also shows that Rb_{occ1} increased tending to saturation, whereas Rb_{occ2} slightly decreased with [Rb^+]. As in the case of the effects of Rb^+ on fluorescence, the two rate coefficients increased with the concentration of the cation, showing no tendency to saturation (Figure 4D). This is due to the fact that the rate coefficients are only affected by the affinity of the binding of Rb^+, whereas those of Rb_{occ1} and Rb_{occ2} include also the affinity of the transition between bound and occluded Rb^+. As we have shown previously (*2*), most of the bound Rb^+ is driven into the occluded state, which acts as a "sink" that traps Rb^+, thus increasing the overall apparent affinity for the cation.

Comparison of the Effects of Rb^+ on the Kinetics of Fluorescence Changes and of Occlusion. The parameters that give the best fit to the time courses of fluorescence (Figure 2C,D) and occlusion (Figure 4C,D) show that the rate coefficients for both processes are not significantly different. In contrast with this, the rapid phase accounted for more than 94% of the total fluorescence change, while it only contributed between 65 and 85% of the total occlusion change. To look at this in more detail, we proceeded as follows.

We first redefined the changes in fluorescence to transform them into increasing functions of time as

$$\Delta F = F_B - F = \Delta F_0 + \Delta F_1(1 - e^{-k_{F1}t}) + \Delta F_2(1 - e^{-k_{F2}t}) \quad (6)$$

where F_B is the value of the fluorescence in the absence of Rb^+, F is defined in eq 4, and ΔF_0 is the maximal change of the very fast component of the time course of fluorescence.

We then plotted the time courses of fluorescence and occlusion after normalizing them with respect to their fast components. This leads, in the case of fluorescence, to

$$\text{relative } \Delta F(t) = \frac{\Delta F_0 + \Delta F_1(1 - e^{-k_{F1}t}) + \Delta F_2(1 - e^{-k_{F2}t})}{\Delta F_0 + \Delta F_1} \quad (7)$$

and in the case of occlusion:

$$\text{relative } Rb_{occ}(t) = \frac{Rb_{occ0} + Rb_{occ1}(1 - e^{-k_{occ1}t}) + Rb_{occ2}(1 - e^{-k_{occ2}t})}{Rb_{occ0} + Rb_{occ1}} \quad (8)$$

In this kind of normalization, as time tends to infinity, both equations will tend to a value that will exceed 1 to an extent that will depend on the contribution of the slow phase of each time course to its respective total change.

Figure 5 shows the results of our experiments plotted according to eqs 7 and 8. Each panel represents one concentration of Rb^+ and includes an inset showing the initial parts of the time courses, where it can be seen that, for a given Rb^+ concentration, these were not significantly different from each other. In contrast, fluorescence reached an

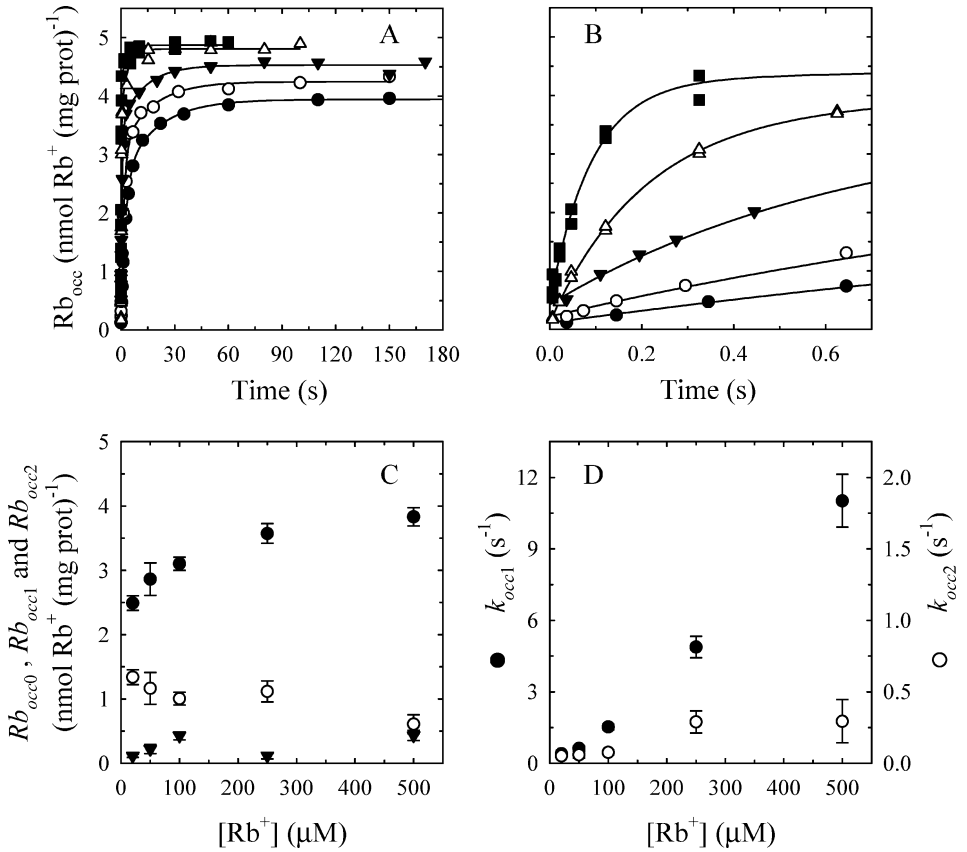

FIGURE 4: (A) The time courses of Rb^+ occlusion in media containing 20 (●), 50 (○), 100 (▼), 250 (△), or 500 (■) μM Rb^+. Continuous lines are the plots of eq 5 for the best fitting values of its parameters. (B) Plot of the first 0.65 s of the time courses shown in panel A. The best fitting values of Rb_{occ0} (▼), Rb_{occ1} (●), and Rb_{occ2} (○), and of k_{occ1} (●) and k_{occ2} (○) in eq 5, are plotted as a function of Rb^+ concentration (panels C and D, respectively).

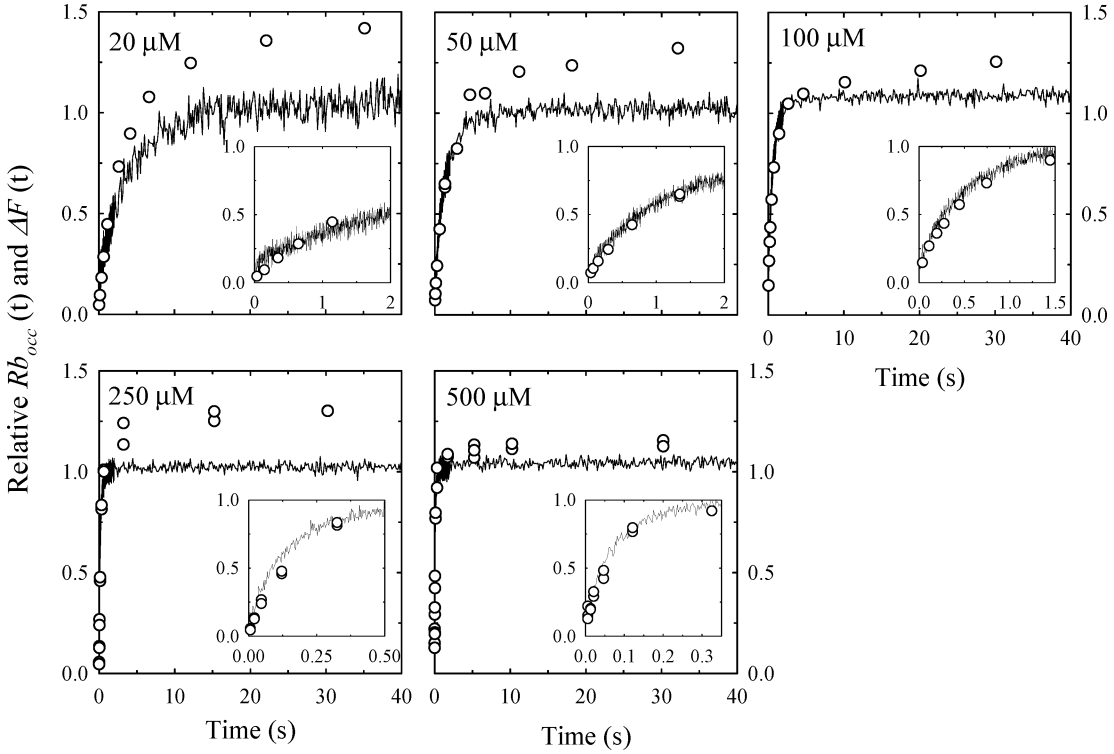

FIGURE 5: Plots of the time courses of fluorescence changes (continuous trace) and of Rb^+ occlusion (open circles) for the concentrations of Rb^+ indicated. The inset in each panel is the initial part of the time courses. The data were taken form the results in Figures 2 and 4 and normalized according to eqs 7 and 8 for fluorescence and occlusion, respectively.

asymptotic value which was near 1, whereas occlusion tended to completion at values significantly higher than unity.

It could be argued that, instead of the procedure used for the plots in Figure 5, the correct normalization procedure

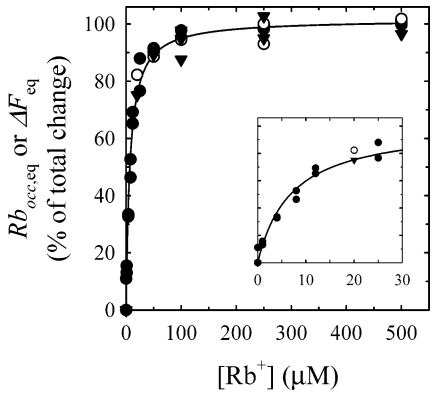

FIGURE 6: Comparison of the effects of Rb^+ on the equilibrium levels of eosin fluorescence (●, ○) and of Rb^+ occlusion (▼). Some of the values (●) were obtained from direct measurement of equilibrium fluorescence. For each [Rb^+], the quantities $Rb_{occ,eq}$ (equal to $Rb_{occ0} + Rb_{occ1} + Rb_{occ2}$) and ΔF_{eq} (equal to $F_B - F_\infty$) for Rb^+ occlusion and fluorescence change, respectively, were normalized taking as 100% the best fitting value of Y_{max} in ($Rb_{occ,eq}$ or ΔF_{eq}) = $Y_{max}[Rb^+]/(K_{0.5} + [Rb^+])$.

should be to take as a 100% the maximum changes of fluorescence and occlusion. Notice however that if this normalization were applied, it would have led to the less economical interpretation that the initial rate of fluorescence was higher than that of Rb^+ occlusion, the difference between them requiring one to invoke the presence of a hypothetical intermediate interposed between the species whose fluorescence decreases after addition of Rb^+ and the species which occludes this cation.

Effects of Rb^+ on the Equilibrium Levels of Fluorescence and Occlusion. The absolute values of the total changes of fluorescence (Figure 2A) and of Rb^+ occlusion (Figure 4A) were normalized and plotted as a function of [Rb^+]. In addition, and to cover a wider range of [Rb^+], we also plotted the normalized fluorescence change obtained after incubating the ATPase in media with eosin and different Rb^+ concentrations during at least 5 min to ensure equilibrium. As shown in Figure 6, the normalized values for fluorescence and occlusion were practically superimposable and a single hyperbola with a $K_{0.5} = 7.01 \pm 0.45$ μM (continuous line in Figure 6) satisfactorily described the whole series of data.

DISCUSSION

The experiments reported in this paper compare for the first time the transient kinetics of Rb^+ occlusion and of eosin fluorescence changes and allow therefore drawing conclusions on the correlation between occlusion and conformational transitions of the Na^+/K^+-ATPase.

All our experiments were initiated with Na^+/K^+-ATPase suspended in imidazole buffer, lacking ATP. In these conditions, the enzyme will settle in an equilibrium in which most of it will be in its E_1 conformation and have high affinity for eosin. We have shown previously (*8*) that eosin dissociates from E_1 with a $K_d = 0.25$ μM. As mentioned in Results, this value is consistent with the observation in this paper that the fluorescence signal is independent of dilution when the concentration of free eosin is kept at 0.23 μM (Figure 1). As Rb^+ is added, the enzyme will progressively tend to its E_2 state and occlude Rb^+. At sufficiently high concentrations of Rb^+, most of the enzyme will be in its E_2 state, hold two occluded Rb^+ per enzyme molecule and have low affinity for eosin (K_d about 8.8 μM; see ref *8*). Using this value and that for the K_d of E_1 given above, it can be calculated that at saturating [Rb^+] the fluorescence signal would drop to about 7% of the value it has in the absence of Rb^+. This seems to be at variance with our observation that saturating [Rb^+] almost fully switches off the fluorescence signal due to bound eosin (see comments to Figure 3). It would seem therefore that Rb^+ not only lowers the affinity for eosin but also decreases the fluorescence signal of the eosin that remains bound to the states of the enzyme occluding Rb^+, a conclusion which is in agreement with that of Smirnova and Faller (*14*).

The time courses of the approach of occlusion and of eosin fluorescence to equilibrium values after addition of Rb^+ follow the sum of a constant term plus at least two exponential functions of time. Rb^+ occlusion curves were better fitted by the sum of two increasing exponential functions of time, whereas the sum of three decreasing exponential functions of time yielded a better fit to the fluorescence curves. Notice that the rate coefficient of the extra exponential needed to fit the time course of fluorescence was close to that of the other fast component (not shown). This caused a large degree of instability (scatter) in the parameters that described the fast phases of fluorescence. Reducing to two the number of exponential functions helped to solve the instability of the parameters, but it introduced some bias in the fitting that affected those components whose accuracy was critical for a proper comparison between time courses of fluorescence and those of occlusion. It can be shown that if a two-exponential function is fitted to data that are better described by the sum of three exponentials, the bias introduced causes the underestimation of the parameters of the faster component at the expense of an overestimation of those of the slower component. Therefore, as a compromise to comply with the goals of performing a correct comparison and showing a set of parameters with meaningful values, fluorescence time courses were fitted to the sum of two decreasing exponential functions of time but setting the parameters that describe the slower phase at the values obtained from fitting three exponential functions of time.

It is difficult to discard the possibility that the difference in the number of exponentials required for best fit of the fluorescence and occlusion time courses is caused by the much larger number of experimental points available for fitting the fluorescence curves. In this respect, it is interesting to notice that if only the values at the same time points as those of the occlusion experiments are used, the best fit to the time course of fluorescence is obtained with only two exponential functions (results not shown). An alternative explanation would be to posit that the third exponential represents a residual release of bound eosin due to dilution of the eosin−ATPase complex, not exactly canceled by the concentration of eosin added in the second syringe.

The constant terms at $t = 0$ in eqs 3 and 4 (ΔF_0), and 5 (Rb_{occ0}) indicate that the time courses include a phase that is so fast that it exceeds the time resolution of our instruments. We have already detected this phase in the time course of Rb^+ occlusion through the direct route (*2*, *8*) and that Rb_{occ0} disappears after preincubating the Na^+/K^+-ATPase during 5 min in media with 60 μM unlabeled Rb^+ but not with 400 μM Mg^{2+} (*17*). Moreover, the fast phase is not apparent when

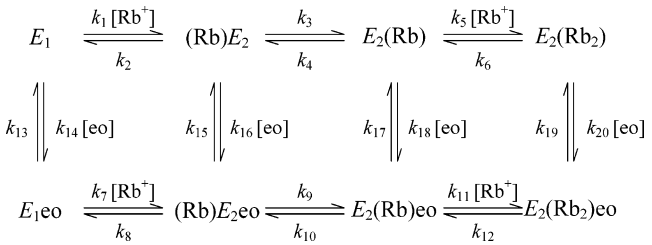

FIGURE 7: Minimal model, based on that of ref *2*, for the effects of Rb^+ on the time courses of occlusion and of eosin fluorescence changes. The model implies that all bound Rb^+ is occluded and disregards the presence of the very fast component of fluorescence and occlusion.

measuring the time course of occlusion via the physiological route (*16*). Results in this paper show that this phase also appears during fluorescence, whose measurement does not involve manipulation of the incubation mixture. The very fast phases of occlusion and fluorescence might be due to a process not related to the operation of the Na^+/K^+-ATPase. These could include, in the case of occlusion, the nonspecific binding of Rb^+ and, in the case of fluorescence, the release of eosin nonspecifically bound to the enzyme preparation or a Rb^+-induced change in turbidity. However, in view of the efficiency of our washing procedure (see Materials and Methods) and of the evidence given above regarding occlusion experiments, we cannot discard the possibility that ΔF_0 and Rb_{occ0} are genuine components of the processes that lead to Rb^+ occlusion via the direct route.

A trivial explanation of the multiexponential time courses observed by us would be to posit the existence of multiple states of the enzyme which either do not interconvert or they do it at a much slower rate than those of fluorescence changes and of occlusion. However, we have shown that multiexponential time courses can be obtained from a single enzyme state, provided that Rb^+ occlusion follows a sequential ordered kinetics, both in the absence (*2*) and in the presence of eosin (*8*). Results in this paper show that the changes in fluorescence also follow multiexponential kinetics but indicate that the contribution of the slow phase to the time course of fluorescence change is considerably less than that of Rb^+ occlusion.

To see if a single sequential ordered mechanism is able to explain the kinetic features of occlusion and of fluorescence, we simulated the evolution toward equilibrium of the minimal model shown in Figure 7. The model incorporates the binding of eosin to the sequential ordered mechanism developed by us to account for the kinetics of Rb^+ occlusion via the direct route (*2*) and considers that the only species that contributes to fluorescence is E_1eo. Notice that the model does not intend to explain the very rapid phase of occlusion and fluorescence changes.

Simulations were run for Rb^+ concentrations going from 50 to 500 μM and for incubation times going from 0 to 200 s. The simulated results were plotted after normalizing them, following the same procedure as that for the results shown in Figure 5.

To perform the simulations of the evolution to equilibrium after the addition of Rb^+, we proceeded as follows:

1. We used the numerical solutions of the differential equations that describe the time course of the scheme in Figure 7 to find the set of the values of the rate constants that gave a good fit to the experimental results.

2. These values were used to numerically integrate the differential equations in order to obtain the concentrations of enzyme forms as a function of time and to calculate the time courses of Rb_{occ} and of F according to eqs 9 and 10 which follow, respectively.

Occlusion and fluorescence were calculated as (see Figure 7):

$$Rb_{occ} = [(Rb)E_2] + [E_2(Rb)] + [(Rb)E_2eo] + [E_2(Rb)eo] + 2([E_2(Rb_2)] + [E_2(Rb_2)eo]) \quad (9)$$

$$F = \alpha[E_1eo] \quad (10)$$

where "eo" is eosin and α is a proportionality constant. Equation 10 considers that contribution to fluorescence of the species holding occluded Rb^+ is negligible.

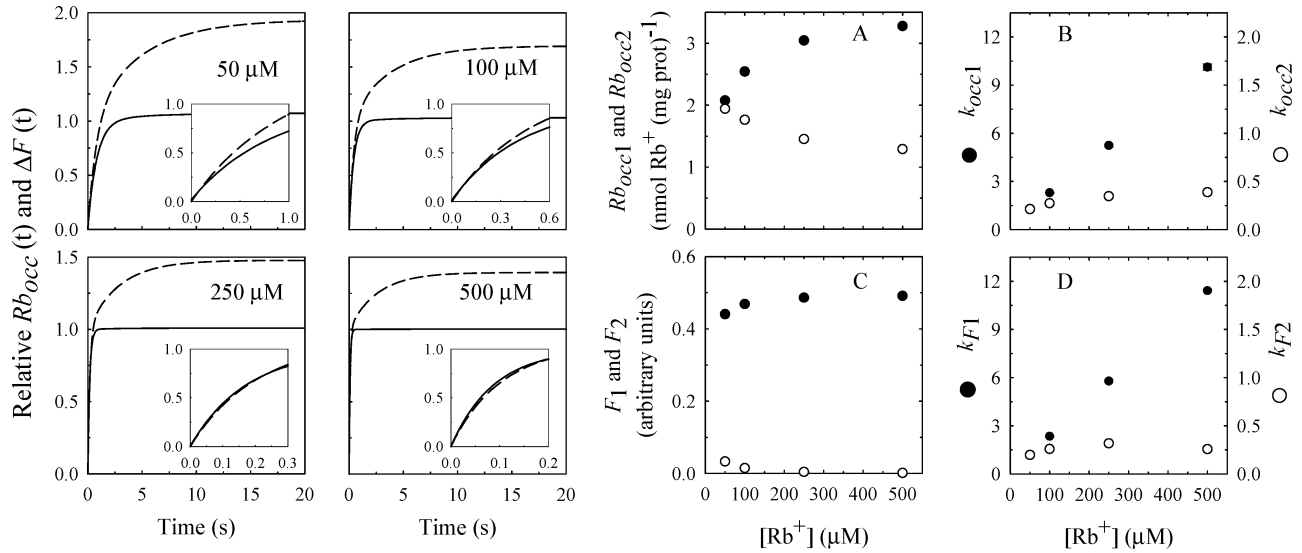

FIGURE 8: (Left four panels) Normalized time courses of fluorescence (continuous lines) and of occlusion (dashed lines) simulated with the model in Figure 7 for the concentrations of Rb^+ indicated in the graphs. (Panels A, C) Best fitting values of the maximum absolute changes of the two components of fluorescence (F_1 and F_2) and Rb^+ occlusion (Rb_{occ1} and Rb_{occ2}) plotted as a function of [Rb^+]. (Panels B, D) Best fitting values of the two rate coefficients of fluorescence (k_{F1} and k_{F2}) and Rb^+ occlusion (k_{occ1} and k_{occ2}) plotted as a function of [Rb^+].

3. The simulated results thus obtained were fitted to the sum of two exponential functions of time and normalized with respect to the size of the faster exponential component as for the experimental data of Figure 5.

The values of the rate constants and of the total concentrations of enzyme and eosin employed in the simulations were as follows: $k_1 = 0.03\ \mu M^{-1}\ s^{-1}$; $k_2 = 0.0668\ s^{-1}$; $k_3 = 0.0273\ s^{-1}$; $k_4 = 0.136\ s^{-1}$; $k_5 = 0.025\ \mu M^{-1}\ s^{-1}$; $k_6 = 0.061\ s^{-1}$; $k_7 = 0.0209\ \mu M^{-1}\ s^{-1}$; $k_8 = 1.50\ s^{-1}$; $k_9 = 1.53 \times 10^5\ s^{-1}$; $k_{10} = 4.14 \times 10^5\ s^{-1}$; $k_{11} = 0.246\ \mu M^{-1}\ s^{-1}$; $k_{12} = 1.56\ s^{-1}$; $k_{13} = 11\ s^{-1}$; $k_{14} = 55\ \mu M^{-1}\ s^{-1}$; $k_{15} = 9.00\ s^{-1}$; $k_{16} = 1.40\ \mu M^{-1}\ s^{-1}$; $k_{17} = 4.04\ s^{-1}$; $k_{18} = 1.16\ \mu M^{-1}\ s^{-1}$; $k_{19} = 37.6\ s^{-1}$; $k_{20} = 4.18\ \mu M^{-1}\ s^{-1}$; $[\text{enzyme}]_{\text{total}} = 0.125\ \mu M$; $[\text{eosin}]_{\text{total}} = 0.315\ \mu M$.

As shown in Figure 8, the simulated time courses qualitatively predict the experimental behavior because of the following:

1. The time courses of both fluorescence and occlusion follow the sum of two exponential functions of time. In contrast with the experimental results, in this case the sum of three exponentials does not improve the fit. This seems to agree with the possibility stated above that the third exponential of fluorescence is due to a residual release of eosin from the eosin−ATPase complex.

2. The initial part of the time courses of occlusion and fluorescence are superimposable.

3. Fluorescence changes are completed before occlusion, and its fast component occupies a larger fraction of the time course. This is a consequence of the fact that in the model in Figure 7 fluorescence disappears with [E_1eo], while additional steps are required to complete occlusion.

4. The response to [Rb^+] of the parameters of the equations that fit the simulated results is not very different from the behavior detected experimentally (cf. Figures 2C,D and 4C,D with Figure 8A−D).

5. All the values of rate and equilibrium constants that best fit the model have the same order of magnitude as those proposed by us and by other authors (for references see *2* and *8*).

6. In addition (results not shown) the simulated values of fluorescence and occlusion at equilibrium can be approximately fitted to single rectangular hyperbolas, whose $K_{0.5}$ (3.3 μM for F and 8.1 μM for Rb_{occ}) are comparable to those measured experimentally. This fits with our previous experimental observation on the hyperbolic response of equilibrium Rb^+ occlusion to Rb^+ concentration (*2*).

The results of the simulation also show that the rate constants for the binding of eosin to the enzyme states holding occluded Rb^+ are somewhat lower than the binding of eosin to E_1. Since this may be a limitation of the minimal model in Figure 7, it is not yet possible to decide if the difference in the values of the rate constants is a genuine feature of the Na^+/K^+-ATPase. Note also that, for the step involving the change in the position of the occluded Rb^+, we arrived at values of rate constants which are more than a million times larger in the presence of eosin (k_9 and k_{10}) than in its absence (k_3 and k_4). This could be reflecting a nucleotide-like effect of eosin (*8*), in the sense that this step in the eosin bound enzyme is not playing a role in determining the rate of occlusion and deocclusion of the second Rb^+.

The time course of eosin fluorescence therefore seems to be a good marker only of the initial part of the time course of occlusion. This is a strong indication of the fact that the conformational changes reported by fluorescence express the process that leads to Rb^+ occlusion. However, our results suggest the existence of enzyme states that occlude Rb^+ and bind eosin but in which fluorescence is quenched. Hence, not all the changes in fluorescence will be caused by binding or release of eosin from the ATPase. This is not only of mechanistic importance but also indicates that caution has to be exerted in taking fluorescence changes as a marker of occlusion.

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