The Occlusion of Rb⁺ in the Na⁺/K⁺-ATPase

I. THE IDENTIFICATION OF OCCLUDED STATES FORMED BY THE PHYSIOLOGICAL OR THE DIRECT ROUTES: OCCLUSION/DEOCCLUSION KINETICS THROUGH THE DIRECT ROUTE

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Occlusion of K⁺ or its congeners in the Na⁺/K⁺-ATPase occurs after K⁺-dependent dephosphorylation (physiological route) or in media lacking ATP and Na⁺ (direct route). The effects of P₁ or ATP on the kinetics of deocclusion of the K⁺-congener Rb⁺ formed by each of the above mentioned routes was independent of the route of occlusion, which suggests that both routes lead to the same enzyme intermediate. The time course of occlusion via the direct route can be described by the sum of two exponential functions plus a small component of very high velocity. At equilibrium, occluded Rb⁺ is a hyperbolic function of free [Rb⁺] suggesting that the direct route results in enzyme states holding either one or two occluded Rb⁺. Release of occluded Rb⁺ follows the sum of two decreasing exponential functions of time, corresponding to two phases with similar sizes. These phases are not caused by independent physical compartments. The rate constant of one of the phases is reduced up to 30 times by free Rb⁺. When Rb⁺ is the only pump ligand, the kinetics of occlusion and deocclusion through the direct route are consistent with an ordered-sequential process with additional independent step(s) interposed between the uptake or the release of each occluded Rb⁺.

The coupling of the hydrolysis of ATP to the active transport of Na⁺ and K⁺ in the Na⁺/K⁺-ATPase (EC 3.6.2.7) takes place through several elementary steps including: (i) the Na⁺-dependent phosphorylation of the ATPase by ATP, (ii) the K⁺-activated hydrolysis of the phosphoenzyme thus formed (1), (iii) conformational changes of both the phospho and the dephosphoenzymes, and (iv) the occlusions of Na⁺ and K⁺. When occluded, the access of Na⁺ or K⁺ to the bulk of the solvent is strongly restricted, probably because they are moving through the ATPase as part of their active transport. K⁺ can be replaced by Rb⁺, Cs⁺, Tl⁺, or NH₄⁺ in all the reactions in which it participates. Occlusion of K⁺ was first proposed on the basis of indirect evidences by Post and co-workers (1) and then confirmed by other researchers who showed that the K⁺-congener Rb⁺ (2–4) or Tl⁺ (5) became associated to the Na⁺/K⁺-ATPase in such a way that they are only slowly removed by cation exchange resins (2, 6) or by extensive washings (3–5).

A minimal sequence of steps for the formation and release of occluded K⁺ or its congeners and its coupling to cation transport (7) based on the currently accepted reaction scheme of the Na⁺/K⁺-ATPase (for references, see Ref. 4) shown in Fig. 1, would be as follows.

\[ E₁P + nK_{extracellular} \rightarrow E₂P-K⁺ \quad \text{(a)} \]
\[ E₁P-K⁺ \rightarrow E₂(K⁺)_{occluded} + P_{intracellular} \quad \text{(b)} \]
\[ E₂(K⁺)_{occluded} \rightarrow E₃ + nK_{extracellular} \quad \text{(c)} \]
\[ E₂(K⁺)_{occluded} + ATP_{extracellular} \rightarrow E₃(K⁺)_{occluded}ATP \quad \text{(d)} \]
\[ E₃(K⁺)_{occluded}ATP \rightarrow E₄ATP + nK_{intracellular} \quad \text{(e)} \]

**SCHEME 1**

The macroscopic distinction between intra- and extracellular location of ligands is lost in fragmented membrane preparations. Following Glynn and Karlish (7), we call E₁ the conformer of the pump with high affinity for ATP and for intracellular Na⁺, which catalyzes the reversible transfer of the terminal phosphate of ATP to the enzyme with formation of E₁P. E₂ is the conformer of the pump with high affinity for extracellular K⁺ and low affinity for ATP. E₃ catalyzes the reversible transfer of orthophosphate between E₂P and water. E₄ and E₅ also differ in their spectroscopic properties (8) and in their reactivity to proteolytic enzymes (9).

Extracellular K⁺ binds to the E₂P (Reaction a in Scheme 1) remaining exchangeable with the medium until P₁ is released leaving K⁺ occluded in E₂. In agreement with other authors (see Ref. 7) we call this the physiological route of occlusion because it not only requires the physiological operation of the pump but seems to be a necessary step of this operation (4). Occluded K⁺ is released into the intracellular medium (Reactions c-e in Scheme 1). This step is accelerated about 200 times after intracellular ATP binds to E₃(K⁺)_{occluded} (Reactions d-e in Scheme 1). This effect does not involve the hydrolysis of ATP and is exerted at a site whose affinity is much lower than that of the active site of the ATPase (3, 4, 8). Occlusion of K⁺ can also be attained by the reversal of Reaction c in Scheme 1 as

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in previous papers (see Ref. 7) we call this the direct route because it does not involve other intermediates than those formed between K\textsuperscript{+} and the enzyme. Occlusion through the direct route leads to the equilibrium distribution between free and occluded K\textsuperscript{+}, while the physiological route leads to a steady state, whose duration will depend on the supply of ATP and on the accumulation of P\textsubscript{i} and ADP (7).

Under physiological conditions, a small fraction of the pump units exchange intra- for extracellular K\textsuperscript{+} (K/\textsuperscript{K}/exchange (10)). This requires P\textsubscript{i} and needs but does not consume ATP and does not lead to net transport. It probably expresses the reversible shuttling of the ATPase between the states shown in Reactions a-e of Scheme 1. Hence, direct as well as physiological occlusion routes would be used during the normal operation of the pump. Scheme 1 and Fig. 1 also show that occlusion can follow the binding of K\textsuperscript{+} to E\textsubscript{1}ATP. This will not be considered here (but see Ref. 11).

During the physiological operation of the pump, the value of the coefficient “n” in Scheme 1 and Fig. 1 seems to be two (4, 5) since occlusion only takes place when two K\textsuperscript{+} (or Rb\textsuperscript{+}) ions are bound. This is consistent with the observation that 2 K\textsuperscript{+} ions are transported in each Na\textsuperscript{+}/K\textsuperscript{+}-ATPase cycle (12).

There is no a priori reason for positing that the two occlusion routes lead to the same enzyme states as it is assumed in Scheme 1 and Fig. 1. On the basis that in general, only identical intermediates having identical distribution will show the same kinetic behavior, we studied the kinetics of deocclusion in media containing different concentrations of P\textsubscript{i} or ATP. Studies of this kind have been performed by Forsbush (3) but because of his setting he could not discard the possibility that “different but related occluded states could interconvert.” This proviso does not apply to our experimental procedure (13). Moreover, the results presented here extend the conditions employed both by Forsbush and by ourselves (14), to a much wider range of ATP or P\textsubscript{i} concentrations. The second part of this paper is an analysis of the equilibrium distribution between free and occluded Rb\textsuperscript{+} and of the kinetics of formation and breakdown of enzyme states holding occluded Rb\textsuperscript{+} using the direct route.

**EXPERIMENTAL PROCEDURES**

**Enzyme—Na\textsuperscript{+}/K\textsuperscript{+}-ATPase** was partially purified from pig kidney (15). The specific activity at the time of preparation ranged from 19 to 28 \textmu m of P\textsubscript{i} min\textsuperscript{-1} (mg protein\textsuperscript{-1}) measured at 37 °C in media with 150 mM NaCl, 20 mM KCl, 3 mM ATP, 4 mM MgCl\textsubscript{2}, and 25 mM imidazole-HCl, pH 7.4. The variability in specific activity was reflected in the maximal amount of occluded rubidium obtained (Rb\textsubscript{occ, max}) but in all cases the molar ratio Rb\textsubscript{occ, max}/(ADP-binding sites) was not significantly different from 2.

**Reagents and Reaction Conditions**—[\textsuperscript{86}Rb]RbCl and [\textsuperscript{32}P]orthophosphoric acid were from PerkinElmer Life Science. [\gamma\textsuperscript{32}P]ATP was synthesized using the procedure of Glynn and Chappell (16), except that no unlabeled orthophosphate (P\textsubscript{i}) was added. All other reagents were of analytical grade. Incubations were performed at 25 °C in media containing 25 mM imidazole-HCl (pH 7.4 at 25 °C) and 0.25 mM EDTA. The concentrations of other components varied according to the experiments and are indicated under “Results.” Before starting a reaction, the components to be mixed were diluted in the reaction media. Control experiments (not shown) indicated that, under the conditions used, the enzyme retained its activity for up to 240 min long incubation.

**Measurement of Rubidium Occlusion—Following Rossi et al. (13),** quenching of occlusion reactions was attained by means of the quick drop in temperature, in ligand concentrations and in free [\textsuperscript{86}Rb\textsuperscript{+}]. Occluded Rb\textsuperscript{+} was considered equal to that retained by the enzyme after washing with at least 300 ml of an ice-cold washing solution containing 30 mM KCl and 20 mM imidazole-HCl (pH 7.4 at 0 °C) flowing at a rate of 40 ml/s. The procedure uses a rapid-mixing apparatus (RMA) (SFM4 from Bio-Logic, France) connected to a quenching and washing chamber. Depending on the incubation time, quenching reactions were performed either in a test tube or in the RMA, but in all cases the reaction was stopped injecting the enzyme suspension from the RMA into the quenching and washing chamber at a flow rate of 1–5 ml/s (13).

**Equilibrium Rb\textsuperscript{+} Occlusion through the Direct Route—70 to 150 \mu g/ml of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase were incubated during 15 to 120 min in media with [\textsuperscript{86}Rb\textsuperscript{+}]RbCl. Blanks were estimated from the amount of [\textsuperscript{86}Rb\textsuperscript{+}]Rb\textsuperscript{+} retained by the filters when the enzyme was diluted. Their values were similar to those obtained with heat-inactivated enzyme (30 min at 65 °C) or with native enzyme in media with 40 mM Na\textsuperscript{+}. Blank values, which were usually much lower than 10% of the amount of occluded [\textsuperscript{86}Rb\textsuperscript{+}Rb\textsuperscript{+}], were independent of the mass of enzyme and linearly related to the Rb\textsuperscript{+} concentration (13).

**Steady-state Rb\textsuperscript{+} Occlusion through the Physiological Route—95 to 110 \mu g/ml Na\textsuperscript{+}/K\textsuperscript{+}-ATPase was incubated with [\textsuperscript{86}Rb\textsuperscript{+}]RbCl in media containing NaCl, MgCl\textsubscript{2}, and micromolar ATP. Incubation lasted for 3 s to ensure steady-state conditions. Blanks were estimated from samples lacking ATP.

**The Time Course of Formation of Occluded Rubidium through the Direct Route—Enzyme suspension (222 \mu g of protein/ml) in 25 mM imidazole-HCl (pH 7.4 at 25 °C) and 0.25 mM EDTA, was mixed in the RMA with an equal volume of the same medium having different concentrations of [\textsuperscript{86}Rb\textsuperscript{+}]RbCl and incubated for different lengths of time. Then, 0.57 ml of the incubation mixture was quenched into the quenching and washing chamber.

**The Time Course of Rb\textsuperscript{+} Deocclusion—This was done looking at the decrease of occluded [\textsuperscript{86}Rb\textsuperscript{+}]Rb by isotope dilution of the [\textsuperscript{86}Rb\textsuperscript{+}]Rb. When the effects of ATP or P\textsubscript{i} were compared, 1 volume of the incubation suspension containing the occluded species was mixed with 1 volume of a solution having sufficient unlabeled Rb\textsuperscript{+} as to give a 100- or 200-fold decrease in the specific activity of [\textsuperscript{86}Rb\textsuperscript{+}]Rb and to set [Rb\textsuperscript{+}] at 10 mM. When the kinetics of deocclusion was studied using Rb\textsuperscript{+} as the only purine ligand, isotopic dilution was attained adding enough of a solution of identical composition as to cause a 20-fold decrease in the specific activity of [\textsuperscript{86}Rb\textsuperscript{+}]Rb.

**ATPase Activity in the Presence of Inorganic Orthophosphate—This** was measured as the amount of [\textsuperscript{32}P]P, released from [\gamma\textsuperscript{32}P]ATP, according to the method described by Schwarzbmaum et al. (17) slightly modified to quantitatively extract the P\textsubscript{i} present in the media. Incubation time was short enough as to prevent the hydrolysis of more than 10% of the ATP present and to ensure initial rate conditions. Enzyme concentration was 8 \mu g of protein/ml and blanks consistent in an assay were all the Na\textsuperscript{+} in the medium was replaced by K\textsuperscript{+}.

**Data Analysis and Development of Theoretical Models—Equations** were adjusted to the results by nonlinear regression using commercial programs (Excel 7.0 for Windows and Sigma-Plot 2.0 for Windows). The goodness of fit of a given equation to the experimental results was evaluated by the AIC criterion (18) defined as AIC = N ln(SS) + 2 P, with N = number of data, P = number of parameters, and SS = sum of weighted square residual errors. Unitary weights were considered in

\[ AIC = N \ln(SS) + 2P \]
all cases. It is obvious that AIC values may be positive or negative. The best equation was considered that which gave the lower value of AIC.

To test kinetic models we developed a procedure (19) for its use in Mathematica™ for Windows™ (version 4.1). This includes the following steps: (i) The set of differential equations that describe the model together with the corresponding conservation equations is worked out; (ii) initial values are assigned heuristically to the rate constants and to the total enzyme concentration (E<sub>T</sub>); then the numerical solutions of the set of differential equations is obtained; (iii) the solutions are compared with the experimental values and the rate constants and E<sub>T</sub> are corrected using a procedure based on the Gauss-Newton algorithm (20); (iv) the corrected values are used to obtain a new set of numerical solutions; and (v) steps iii and iv are repeated until the standard deviation of the residual errors reaches a minimal constant value.

RESULTS

Effects of the Route of Occlusion on the Kinetics of Deocclusion

We measured the amount of occluded [86Rb]Rb<sup>+</sup> (Rb<sub>occ</sub>) remaining at different times after isotopic dilution in media with different concentrations of either ATP or P<sub>i</sub>. A good description of the time course of deocclusion was obtained with the sum of two exponential functions of time plus a time-independent term (Equation 1 below). Regression analysis to fit exponentials can yield strong statistical correlation between rate coefficients (k values) and amplitudes (A values). This leads to high standard errors of the parameters, which may affect the evaluation of the significance of differences between two sets of values. To circumvent this, we also compared the data by means of a graphical procedure. This was based on the fact that, if the kinetics of deocclusion were the same for the enzyme states formed via the two routes of occlusion then the time courses of Rb<sup>+</sup> loss from both kind of intermediates should differ only in a constant factor contained in the initial amount of occluded Rb<sup>+</sup>. The effect of this factor can be canceled out by dividing each data value by the initial amount of occluded Rb<sup>+</sup> (Rb<sub>occ,0</sub>). This procedure is simpler and relies on less assumptions than that we had used before, which took into account Rb<sub>occ</sub> when time tends to infinity (14). We calculated Rb<sub>occ,0</sub> extrapolating the function that we had used during regression.

Effect of ATP on the Loss of Occluded [86Rb]Rb<sup>+</sup>

Fig. 2 shows the time course of [86Rb]Rb<sub>occ</sub> formed by either the physiological (panel A) or the direct (panel B) routes and incubated for deocclusion in media of identical composition containing 0 (only in panel B), 10 or 100 μM ATP. The value of Rb<sub>occ,0</sub> obtained by the direct route was larger than that obtained by the physiological route (cf. panels A and B) because during steady-state hydrolysis of ATP at limiting [Rb<sup>+</sup>], enzyme states not containing occluded Rb<sup>+</sup>, such as phosphoenzymes, will represent a significant fraction of the total amount of enzyme. It is clear that ATP induced a large increase in the rate of loss of occluded [86Rb]Rb<sup>+</sup>. As already mentioned, a good description of the whole set of results was obtained using the sum of two exponential functions of time plus a time-independent term corresponding to the amount of Rb<sup>+</sup> that remains occluded when the time after dilution tends to infinity, i.e.,

\[ \text{Rb}_{\text{occ}} = A e^{-k_1 t} + A e^{-k_2 t} + A_0. \]  

(Eq. 1)

The best fitting values of the parameters of Equation 1 are given in Table I and were used to calculate the continuous lines in Fig. 2. For comparative purposes, Table I also includes the values of the parameters obtained by fitting to the same data a single exponential function of time plus a constant term; these were used to draw the dotted lines in Fig. 2 (panels A and B). As judged from the values of sum of squares (SS) and AIC in Table I it is apparent that Equation 1 gives a better description of the results. As mentioned above we also performed a graphical comparison of the two set of data. Plots of the time course of deocclusion normalized for Rb<sub>occ,0</sub> in media with 10 or 100 μM ATP are given in panels C and D, respectively. It can be seen that the experimental points for the two occlusion routes are almost superimposable. It is noteworthy that the value of A<sub>c</sub> cannot be fully explained by the new isotopic equilibrium reached after dilution of [86Rb]Rb<sup>+</sup>. If this were true, then Rb<sub>occ,0</sub> (i.e., A<sub>c</sub> + A<sub>D</sub>) whereas as shown in Table I, the actual values of A<sub>c</sub> were significantly higher.

We also measured the release of occluded Rb<sup>+</sup> into media containing from 0 to 2500 μM ATP. A single exponential function of time plus a constant term was adjusted to the data for each ATP concentration, since this gave sufficient quantitative information for performing paired comparisons of deocclusion rates. The best fitting values of the rate coefficients are plotted as a function of [ATP] in Fig. 3. The continuous curves in the figure show that the effect of ATP on them can be adequately described by a hyperbolic function.

\[ k = \frac{(k_1 - k_0) [\text{ATP}]}{[\text{ATP}] + K_{\text{ATP}}} + k_0 \]  

(Eq. 2)

The results in Fig. 3 make it clear that the parameters of Equation 2 are the same regardless of the route followed to occlude Rb<sup>+</sup>.

Effect of P<sub>i</sub> on the Loss of Occluded [86Rb]Rb<sup>+</sup>

We looked at the time course of loss of occluded [86Rb]Rb<sup>+</sup> formed either through the direct or the physiological routes in media containing from 0 to 8 mM P<sub>i</sub>. All media also contained 2.5 μM ATP to ensure equal exposure to the nucleotide in all samples.
Regression analysis of the results in Fig. 1 using Equation 1 or a single exponential plus a constant

| Equation 1 | 10 μM ATP | 100 μM ATP |
| --- | --- | --- |
| Direct | Physiological | Direct | Physiological |
| **A** (nmol/mg) | 3.15 ± 0.13 | 1.53 ± 0.36 | 3.04 ± 0.27 | 2.01 ± 0.18 |
| **k** (s⁻¹) | 1.151 ± 0.089 | 2.14 ± 0.79 | 9.1 ± 1.1 | 9.3 ± 1.4 |
| **A** (nmol/mg) | 1.24 ± 0.14 | 1.11 ± 0.41 | 1.66 ± 0.29 | 0.62 ± 0.20 |
| **k** (s⁻¹) | 0.136 ± 0.021 | 0.36 ± 0.15 | 0.72 ± 0.30 | 1.27 ± 0.58 |
| **A** (nmol/mg) | 0.510 ± 0.025 | 0.402 ± 0.050 | 0.058* | 0.292 ± 0.038 |
| SS | 0.0330 | 0.0264 | 0.0365 | 0.0180 |
| AIC | −54.80 | −27.06 | −35.3 | −42.21 |

The best fitting values of **k**₀, **k**ᵣ, and **K**₀.₅ of Equation 3 given in Table II show that the **K**₀.₅ for **P** is the same for **k**₁ and **k**₂ and that **k**₂, for **k**₂ has comparable values as that for **ATP**-stimulated deocclusion (c.f. Table II and legend to Fig. 3). All the parameters of **P**-stimulated deocclusion were practically independent of the route of occlusion. Therefore, as in the case of **ATP**, the kinetics of the deocclusion accelerated by **P** was the same regardless of the route followed to reach occlusion.

To check this, we looked at the effect of **P** on the **ATP**ase activity. Inhibition of the **ATP**ase by **P** is a necessary, but not sufficient, condition for attributing the activation of deocclusion by **P** to the reversal of **B** in Scheme 1. Fig. 6 shows the results of our measurements of steady-state **ATP** hydrolysis as a function of [**P**] in media of identical composition and temperature as those used in the deocclusion experiments. **P** acted as a partial inhibitor of the **ATP**ase reducing its activity to about half when [**P**] tended to infinity. Inhibition took place along a hyperbola that was half-maximal at 0.86 ± 0.12 mM **P** (continuous line in Fig. 6). The figure also shows that the effect of **P** is enhanced by increasing [Mg²⁺].

If reversal of Reaction **B** in Scheme 1 were the cause of acceleration of deocclusion by **P**, then it should be accompanied by the phosphorylation of the enzyme. Our attempts to measure **EP** formation from **P** at the same conditions as those used in the experiments shown in Fig. 6 yielded phosphoenzyme levels, which were a small fraction of the blanks and that therefore gave too much scatter as to allow reliable conclusions (experiments not shown). Low levels of **EP** under analogous conditions as those used by us has been reported by others (22) and could be explained if **E**₂**P** were formed from **E**₂**Rb** with an overall rate constant (20 s⁻¹, see values of **k**₂ in Fig. 5, panel C, and in Table II), which is much slower than the rate constant for breakdown of **E**₂**P** at nonlimiting concentration of **Rb**⁺ (at least 230 s⁻¹ (4)).

Equilibrium and Kinetic Properties of the Direct Occlusion Route

The Time Course of Equilibration between Free and Occluded **Rb**⁺—We measured occlusion and deocclusion of **Rb**⁺ via the
direct route with Rb\(^+\) as the only pump ligand. Na\(^+\)/K\(^+-\)ATPase in media containing Rb\(^+\) concentrations going from 3 to 228 \(\mu\)M was incubated at 25 °C for periods ranging from 0.037 to 180 s and then Rb\(_{\text{occ}}\) was measured.

The results are given in Fig. 7 as plots of Rb\(_{\text{occ}}\) versus incubation time for each of the [Rb\(^+\)] tested. It can be seen (panel A) that equilibrium was approached along curves whose maximal values increased with Rb\(^+\) concentration tending to saturation. The first 2 s of the time courses of occlusion are plotted in panel B. This “blow up” of the initial part of the curves shows that no time lag was detectable during the build-up of occluded Rb\(^+\), indicating that occlusion is much faster than the reactions that precede it. Results in panel B also show that the curves that fit the experimental points intersect the ordinate at positive values that increase with [Rb\(^+\)]. Control experiments (not shown) indicated that this could not be accounted for by incomplete washing of free [\(^{86}\)Rb]Rb\(^+\) or by the presence of a compartment not pertaining to the enzyme. The initial occlusion could therefore be part of the kinetic process of the formation of occluded Rb\(^+\).

Best fit to each of the curves in Fig. 7, panels A and B, was attained by the following function of time.

\[
\text{Rb}_{\text{occ}} = A_1 + A_2 \left(1 - e^{-k_2 t}\right) + A_3 \left(1 - e^{-k_3 t}\right)
\]  

(Eq. 4)

The continuous lines in Fig. 7 are plots of Equation 4 for the best fitting values of its parameters. The initial rate of occlusion (\(v_o\)) was calculated, disregarding the rapid phase represented by \(A_0\) in Equation 4, by means of the mathematical trick of fitting the points in the vicinity of zero (less than 0.5 s) by the third-order polynomial: \(A_0 + v_0 t + C t^2 + D t^3\). The first derivative of this function at \(t = 0\) will be equal to \(v_0\). The inset to panel B shows that \(v_0\) increases with [Rb\(^+\)] along a rectangular hyperbola (continuous curve in the inset). The \(K_{0.5}\) of this hyperbola is sufficiently high for the range of [Rb\(^+\)] tested, to make a linear approximation a reasonable description of \(v_0 = f(\text{Rb}^+)\) as shown by the dashed line in the inset to Fig. 7B.

Fig. 8 shows plots of the best fitting values of the five parameters of Equation 4 as a function of the concentration of Rb\(^+\). \(A_1\) and \(A_2\) (panel A) were adequately described by increas-

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**Fig. 4.** Loss of occluded [\(^{86}\)Rb]Rb\(^+\) formed either by the direct (panel A) or physiological (panel B) routes, at different concentrations of P\(_r\). Occlusion media contained 2.3 mM MgCl\(_2\) (2.05 mM free Mg\(^2+\)) and (physiological route) 100 \(\mu\)M [\(^{86}\)Rb]RbCl, 150 mM NaCl, and 5 \(\mu\)M ATP or (direct route) 75 \(\mu\)M [\(^{86}\)Rb]RbCl. Deocclusion media contained 10 mM RbCl, 1.15 mM MgCl\(_2\) (0.9 mM free Mg\(^2+\)), 75 mM NaCl, 2.5 \(\mu\)M ATP and either 0 (●), 0.15 (○), 1 (▲), 4 (▼), or 8 (■) mM Pi. Panels C-G show the same results, from 0 to 8 mM P\(_r\), respectively, scaled according to the procedure described in the main text, for states formed through either the physiological (●) or direct (○) route of occlusion. Continuous lines are plots of Equation 1 with \(A_1 = A_2 = A\) for the best fitting values of the parameters shown in Fig. 5.

**Fig. 5.** Best fitting values of the parameters \(k_1\) (panel A), \(k_2\) (panel B), \(A_1\) (panel C), and \(A_2\) (panel D) of Equation 1 as a function of [Pi]. These were obtained by fitting Equation 1, with \(A_1 = A_2 = A\), to the results in Fig. 4, panels A and B, for either the physiological (●) or direct (○) route of occlusion. Vertical bars are ± 1 S.E. Continuous lines in panels A and B are plots of Equation 3 using the best fitting values shown in Table II.
Occlusion and Deocclusion Kinetics of Rb⁺ in Na⁺/K⁺-ATPase

TABLE II

| Parameter | Panel A (k₁) | Direct | Panel B (k₂) | Direct |
|-----------|--------------|--------|--------------|--------|
| k₀ (s⁻¹)  | 0.5 ± 0.3    | 0.5 ± 0.5 | 0.15 ± 0.05  | 0.15 ± 0.02 |
| k₅ (s⁻¹)  | 20 ± 2       | 2.9 ± 0.8 | 3.4 ± 0.1    | 3.05 ± 0.09 |
| K₅₅ (mM)  | 2.2 ± 0.3    | 2.9 ± 0.8 | 2.4 ± 0.3    | 2.9 ± 0.3 |

Effect of P, on the rate of [²²⁶Rb]Rb⁺ deocclusion

Best-fitting values (±1 S.E.) of the parameters in Equation 3 used to draw the continuous lines in Fig. 5.

Taking hyperbolic functions of the concentration of Rb⁺ of the shape,

\[ A_i - A_{i,max}[Rb^+] + K_{i,5} \]  \( \text{(Eq. 5)} \)

where \( A_i \) and \( A_{i,max} \) are either \( A_1 \) and \( A_{1,max} \) or \( A_2 \) and \( A_{2,max} \). The values of \( K_{i,5} \) and of \( A_{i,max} \) of the two exponential terms were not significantly different from each other and when a single coefficient \( A \) was used to adjust both exponential terms in Equation 4, the best fitting values obtained for this parameter were not different from those obtained from the independent adjustment of \( A_1 \) and \( A_2 \).

Panel B in Fig. 8 shows that Equation 4 adequately described the total amount of occluded Rb⁺ in equilibrium with free Rb⁺ (Rb_{oc,eq}), calculated as \( A_0 + A_1 + A_2 \) (Equation 4). The best fitting value of \( K_{0,5} \) was nonsignificantly different from those which adjusted \( A_1 \) and \( A_2 \). Panel B also shows that \( A_2 \) followed a saturable function of [Rb⁺]. In view of its small relative size (it did not exceed 15% of Rb_{oc,eq}) no attempt was made to fit an equation to these data.

Panels C and D show plots of the rate coefficients \( k_1 \) and \( k_2 \) of Equation 1. It is apparent that \( k_1 \) was about 10 times larger than \( k_2 \) and increased in an approximately linear fashion with [Rb⁺] (panel C), whereas \( k_2 \) remained practically unaffected by changes in the concentration of the cation (panel D). The linear response of \( k_1 \) to [Rb⁺] was confirmed in independent experiments covering Rb⁺ concentrations not used in the experiment in Fig. 7.

The Shape of the Rb_{oc,eq} Versus [Rb⁺] Curve—The hyperbolic response of Rb_{oc,eq} to [Rb⁺] (panel B in Fig. 8) is difficult to harmonicize with the evidence that indicates that occlusion only takes place when two Rb⁺ are trapped per ATPase molecule since this would yield sigmoidal and not hyperbolic curves (for review, see Refs. 4 and 6, but also see Refs. 23–25). In view of this, we investigated more thoroughly the equilibrium distribution between occluded and free Rb⁺ in media containing Rb⁺ in concentrations spread along a 0.05 to 472 μM range and placing sufficient points at very low concentrations of Rb⁺, where sigmoidicity would be more manifest. Results in Fig. 9, show that under these conditions the Rb_{eq,vers} (Rb⁺) curve was still hyperbolic, even at the lowest [Rb⁺] tested (inset).

The Release of Occluded Rb⁺—In a preliminary experiment, we found that the 20-fold dilution of the enzyme associated to the isotopic dilution to measure the release of occluded [²²⁶Rb]Rb⁺ had no effect on the equilibrium distribution between free and occluded Rb⁺. Therefore, the procedure allowed us to determine the loss of occluded [²²⁶Rb]Rb⁺ under conditions in which the equilibrium between free and occluded Rb⁺ is preserved.

We studied the kinetics of the loss of occluded Rb⁺ using ATPase preparations equilibrated during 15 min in media containing from 2 to 500 μM [²²⁶Rb]Rb⁺. As shown in Fig. 10, like the experiments in which ATP or P, were present, the loss followed a double exponential function as Equation 1. The results also show that the release of Rb⁺ was markedly slowed down as the concentration of Rb⁺ in the media was increased. This effect of Rb⁺ is shown more clearly in the inset to panel A of Fig. 10 in which the results of the experiments with the lowest and highest [Rb⁺] tested (2 and 500 μM) were scaled dividing Rb_{oc,eq} at any incubation time by Rb_{oc,eq,0}. The plot strongly suggests that only one of two exponential components of the time course is involved in the inhibition by Rb⁺. This was confirmed plotting the best fitting values of the rate coefficients \( k_1 \) and \( k_2 \) of each of the curves in Fig. 10 against the concentration of Rb⁺. It is clear (Fig. 11) that as [Rb⁺] increased, one of the constants (arbitrarily designated as \( k_2 \)) dropped to a very low value while the other remained unaffected by Rb⁺. Inhibition of the loss of Rb⁺ was not mimicked by either 1 mM Na⁺ or 0.7 mM free Mg²⁺ (not shown). In media with no Rb⁺, \( k_1 \) becomes sufficiently near to \( k_2 \) as to make the time course of loss of Rb⁺ describable by a single exponential function.

Regression analysis showed that the inhibitory effect of Rb⁺ on \( k_2 \) was exerted along a decreasing hyperbolic function of [Rb⁺], i.e. as follows.

\[ k_2 = \frac{(k_{20} - k_{2,2}K_{I}) + k_{2,2}}{[Rb^+] + K_{I}} \]  \( \text{(Eq. 6)} \)

The continuous line that fits the experimental points in panel B of Fig. 11 is a plot of this equation for the best fitting values of the parameters (see legend to Fig. 11). The effect of Rb⁺ on \( k_2 \) is exerted with a \( K_{I} \) of about 7.5 μM, which is similar to that obtained for the effect of Rb⁺ on the equilibrium level of Rb_{oc,eq} (cf. Fig. 9). Although \( k_{20} \) is only about 3% of \( k_{20} \), our results strongly suggest that \( k_{2,2} \) is significantly different from zero since its value is about 10 times larger than that of its standard error and remained different from zero when [Rb⁺] was raised to 10 mM (experiment not shown).
In the experiment shown in Fig. 12 the incubation leading to occlusion was carried out in media containing 200 \( \mu M \) Rb\(^+\) during only 0.34 s and the time course of deocclusion was measured and compared with that of enzyme in which occlusion was allowed to reach equilibrium. According to the results in Figs. 7 and 8, and Equation 4, at 200 \( \mu M \) Rb\(^+\) occlusion via the faster exponential term would have reached 94% of the maximum, whereas occlusion via the slower term would only have reached about 4% of the maximum. Panel A in Fig. 12 shows that for the enzyme incubated during 0.34 s, Rb\(^+\) loss again followed a biphasic time course, with phases of similar amplitudes. After scaling (panel B), this time course and that for the enzyme that had reached equilibrium between free and occluded Rb\(^+\) were superimposable, indicating that they both follow similar kinetics.

![Image](http://www.jbc.org/DownloadedFrom)
values are expressed taking as 1 the amount of Rb, corresponding to
or long enough (15 min (27)) to ensure equilibrium. For both curves the
dilution of \([\text{86Rb}] \text{Rb}^+\) the proposal by Sachs (27) that inhibition by \(\text{Pi}\) requires the
which were (27) as a function of the concentration
Continuous line
is a plot of Equation 6 for the best fitting values of its param-
Continuous lines
A
is given in the main text. Panel B shows the initial 60 s of the same time courses.
The continuous lines are the plot of Equation 1 for the values of the parameters that gave best fit to the experimental results (see Fig. 11). In these
curves, the values of \(A_0\) corresponded very well to those expected from calculations based on the isotopic dilution.

The Complex Kinetics of the Release of \(\text{Rb}^+\)—In all conditions
tested the kinetics of \(\text{Rb}^+\) release was describable by the sum of
two exponential functions of time, plus a time-independent term (\(A_0\) in Equation 1) (see also Refs. 3, 23, and 28). As
mentioned under “Results,” and in contrast with what happen when \(\text{Rb}^+\) is the only pump ligand, \(A_0\) is higher than that predicted by the isotopic equilibrium reached after dilution of
\([\text{86Rb}] \text{Rb}^+\). Therefore, it is likely that additional and much slower deocclusion phases, not matched by exponential functions of time in Equation 1, are present forcing the regression procedure to increase the value of \(A_0\).

The interpretation of the multiple phases in the deocclusion curves is likely to depend on the deocclusion conditions. The kinetics of this process in the case of \(\text{Pi}\) is very similar to that
when \(\text{Rb}^+\) is the only pump ligand. It does not seem to be far fetched to explain the behavior in the presence of \(\text{Pi}\) by adapting
to it the treatment developed below for deocclusion in this condition (see also Refs. 21, 28, and 29).

ATP poses a different problem in the analysis of deocclusion kinetics. The rate constant for the dissociation of ATP from the
occluded state is much larger than the constant of deocclusion of \(\text{Rb}^+\) (4) indicating that ATP binds in rapid equilibrium to
this state. Under these conditions, ATP would promote the simultaneous release of two occluded \(\text{Rb}^+\) (3), yielding a single exponential for the deocclusion curve. This contrasts with our
results (see also Ref. 3), which show that two phases are always present and that the rate coefficient of the slower phase is considerably lower than the value that would make it kine-
tically compatible with the turnover of the pump. It seems therefore inescapable to conclude that, in media with ATP, the slow phases represent deocclusion pathways unrelated to the phys-
ological operation of the pump. Likely candidates for these pathways are the release of \(\text{K}^+\) from extracellular instead than
from intracellular sites, as suggested for the route of sponta-
neous deocclusion by Forbush (Ref. 28, and see also Ref. 7),
and/or the presence of heterogeneous or nonfunctional enzyme
units in our preparations.

The Quantitative Behavior of Equilibrium Occlusion When
\(\text{Rb}^+\) Is the Only Pump Ligand: The Equilibrium Constant for
Direct Occlusion—It seems reasonable to posit that occlusion comprises at least two steps: (i) the binding of \(\text{Rb}^+\) on sites from
which it is exchangeable with free Rb\(^+\), and (ii) the occlusion of the bound Rb\(^+\). This view is supported by the observation (inset to Fig. 7, panel B) that initial velocity of occlusion follows hyperbolic kinetics as a function of the concentration of free Rb\(^+\). In what follows, we shall call “bound” the Rb\(^+\) that is not occluded, to distinguish it from the occluded Rb\(^+\) (which is also bound). Assuming, for the sake of simplicity, that only one Rb\(^+\) binds and becomes occluded per enzyme, a two-step occlusion reaction can be written as follows.

\[
\text{Scheme 2}
\]

\[
K_{\text{deocc}} = \frac{[E[Rb^+]]}{[ERb^+_H]} \quad \text{and} \quad K_{\text{diss}} = \frac{[E][Rb^+]}{[ERb^+_H]} \quad (\text{Eq. 7})
\]

The amount of occluded Rb\(^+\) in equilibrium with free Rb\(^+\) will follow a rectangular hyperbola.

\[
ERb_{\text{occ}} = \frac{E_T}{1 + K_{\text{diss}} \left( 1 + K_{\text{deocc}} \left[ Rb^+ \right] \right)} \quad (\text{Eq. 8})
\]

Equation 8 shows that \(K_{\text{app}}\) and \(ERb_{\text{occ,max}}\) will depend on \(K_{\text{deocc}}\) and/or \(K_{\text{diss}}\) as follows,

\[
K_{\text{app}} = K_{\text{diss}} \left( \frac{K_{\text{deocc}}}{1 + K_{\text{diss}}} \right) \quad (\text{Eq. 9})
\]

\[
ERb_{\text{occ,max}} = \frac{E_T}{1 + K_{\text{diss}}} \quad (\text{Eq. 10})
\]

so that, as \(K_{\text{deocc}}\) goes from infinity to zero, the fraction of enzyme holding occluded Rb\(^+\) will go from zero to one and \(K_{\text{app}}\) will go from \(K_{\text{diss}}\) to zero.

The properties of Scheme 2, which can be extended to the binding and occlusion of more than one Rb\(^+\) and to processes having more than two steps, show that \(K_{\text{app}} = K_{\text{diss}}\) only when \(K_{\text{deocc}}\) tends to infinity, i.e. when there is no occlusion. In any other condition \(K_{\text{app}} < K_{\text{diss}}\). For the same reason, at saturating concentrations of free Rb\(^+\), \(ERb_{\text{occ}}\) will approach \(E_T\) only if \(K_{\text{deocc}}\) tends to zero. Therefore the observation that occluded Rb\(^+\) when \([\text{Rb}^+]\) tends to infinity is very close to twice \(E_T\) (see “Experimental Procedures” and Refs. 4 and 5) strongly suggests that \(K_{\text{deocc}}\) is low enough as to displace almost completely the equilibrium toward occlusion. In our experiments \(K_{\text{app}}\) was about 5 \(\mu\text{M}\), whereas the \(K_{0.5}\) for the effect of Rb\(^+\) on the initial rate of Rb\(^+\) uptake (inset to Fig. 7, panel B), which should estimate (probably as a lower limit) \(K_{\text{diss}}\) was around 85 \(\mu\text{M}\). On the basis of Scheme 2, \(K_{\text{deocc}}\) would be about 0.06. If these numbers were correct, Equation 10 would indicate that \(ERb_{\text{occ,max}}\) is actually close to \(2 \times E_T\).

The Shape of the Rb\(^+\) Occlusion Curve and the Apparent Affinity for Rb\(^+\) Occlusion—The experimental data indicating that occlusion only takes place when 2 mol of Rb\(^+\) are bound per mole of enzyme (see for instance, Refs. 4–6), imply that the curve relating the equilibrium between free and occluded Rb\(^+\) should be sigmoidal instead of hyperbolic as in the experiments presented in this paper (see also, Refs. 23–25). It is possible to force models requiring more than one Rb\(^+\) for occlusion, to approach a hyperbolic response by assigning to one of the Rb\(^+\)-binding and occluding sites a sufficiently high affinity as to be occupied (but not in the occluded state) by Rb\(^+\) at all concentrations tested (23). In this case the Rb\(_{\text{deocc}}\) versus [Rb\(^+\)] function would depend on the titration with Rb\(^+\) of only the site with lower affinity in the enzyme. Although this hypothesis has the appeal of not modifying the stoichiometry of occlusion, at this stage of our knowledge it is no more than an \textit{ad hoc} way to conciliate findings that seem to be contradictory. It seems therefore more reasonable to consider that the hyperbolic response observed in our experiments indicates that during direct occlusion, and when Rb\(^+\) is the only pump ligand, forms holding either one or two occluded Rb\(^+\) participate in the equilibrium between free and occluded Rb\(^+\). Independent support for this hypothesis is provided by the biphasic time courses of formation and release of occluded Rb\(^+\).

Another striking feature of the Rb\(_{\text{occ}} = f([\text{Rb}^+])\) curve is its very high affinity for the cation. In fact using the nomenclature of Scheme 2, \(K_{\text{app}}\) was about 5 \(\mu\text{M}\) and \(K_{\text{diss}}\) was around 85 \(\mu\text{M}\). It is difficult to conceive that with these values for affinity, occluded Rb\(^+\) (or K\(^+\)) will be released efficiently into the cytosol whose concentration of K\(^+\) is about 150 \(\mu\text{M}\). Similar considerations apply to the inhibition of deocclusion by free Rb\(^+\) which is also exerted with an affinity (K\(_{\text{app}}\) about 7.5 \(\mu\text{M}\)) which would make it difficult the rate of release of Rb\(^+\) into the cytosol. In this respect it is noteworthy that Forbesh (21, 28) studying the release of occluded Rb\(^+\) in media with P\(_i\) and Mg\(^{2+}\) found a high affinity inhibition by Rb\(^+\) and concluded that the release was through extracellular sites. He also suggested (28) that extracellular sites mediated the release of occluded Rb\(^+\) in the absence of other pump ligands.

The Kinetics of Occlusion and Deocclusion Make Unstable Models Having Two Independent Rb\(^+\)-occluding Sites per ATPase Molecule—Our experiments in Fig. 12 showed that the time courses of Rb\(^+\) release from enzyme that had not reached occlusion equilibrium exhibited slow and fast components with the same size as those of enzymes in which Rb\(_{\text{occ}}\) had reached equilibrium with free Rb\(^+\). This rules out the possibility that the biphasic response is caused by the presence of two independent compartments since a single exponential curve should be apparent if only one of the two compartments were significantly occupied by the cation. The inadequacy of this hypothesis is also apparent in its inability to explain why the loss of Rb\(^+\) from one of the compartments is almost fully arrested by sufficiently high concentrations of Rb\(^+\) in the incubation media.

The Leaky Single File Model for Rb\(^+\) Occlusion May Explain the Results—In equilibrium conditions, an alternative to the independent site model is to postulate that only one Rb\(^+\)-binding site is accessible, through which two Rb\(^+\) transit sequentially into the occluded state. A similar mechanism was postulated by Glynn et al. (29) and Forbesh (21, 28) to explain Rb\(^+\) deocclusion in the presence of Mg-P. The simplest reaction for this process is as follows.

\[
\text{Scheme 3}
\]

In this scheme, the value of the rate constants that govern the equilibrium between Rb\(^+\)-bound states that are occluded or not occluded are supposed to be strongly poised toward the occluded states, so that intermediates with bound Rb\(^+\) were omitted. Moreover, the entry and occlusion of the second Rb\(^+\) requires a change in the position of the Rb\(^+\) that is already
Occlusion and Deocclusion Kinetics of Rb⁺ in Na⁺/K⁺-ATPase

The procedure used is described in “Data Analysis and Development of Theoretical Models,” under “Experimental Procedures.” The value of total enzyme (Eₗ ± 1 S.E.) in nmol (mg protein)⁻¹ obtained from the fitting was 1.86 ± 0.02 for the data in Figs. 7 and 9, or 2.7 ± 0.02 for the data in Fig. 10. This was due to the variability in specific activity between the two different lots of enzyme used in the experiments, as described under “Experimental Procedures.”

| Rate constants | Values ± 1 S.E. | Units |
|----------------|-----------------|-------|
| k₁ and k₂      | 0.025 ± 0.00003 | s⁻¹ μM⁻¹ |
| k₁ - k₂        | 0.058 ± 0.0025  | s⁻¹    |
| k₂            | 0.064 ± 0.0063  | s⁻¹    |
| k₃            | 0.099 ± 0.0031  | s⁻¹    |
| k₄, p₁ and p₂  | 0.0002 ± 0.00001 | s⁻¹ μM⁻¹ |
| k₅            | 2 ± 39          | s⁻¹ μM⁻¹ |
| k₆            | 596 ± 2         | s⁻¹    |
| k₇            | 0.014 ± 0.052   | s⁻¹    |
| k₈            | 0.006 ± 0.021   | s⁻¹    |
| k₉            | 337 ± 1667     | s⁻¹    |
| k₁₀           | 0.09 ± 0.11     | s⁻¹    |

* Calculated according to the restrictions given in the main text (Equation 12).

There is no structural information to support the idea of a “pocket” in the enzyme. We have postulated such a mechanism to facilitate the visualization of the occlusion kinetics but any structure which imposes the restrictions of Scheme 3 will yield the same kinetics as the leaky single file model.

To analyze to what extent the leaky single file model is able to account quantitatively for the results presented in this paper we simulated the behavior of the reaction scheme in Fig. 13. This scheme includes Scheme 3 as the states connected by means of continuous lines but additional transitions were incorporated, not only to explain the leak at [Rb⁺] tending to infinity but also the existence of a rapid initial occlusion (the term A₁ in Equation 4), which was explored including the steps connected by dotted lines and incorporating the conformers E₁ and E₂ of the ATPase. Since both the Scheme 3 and the scheme in Fig. 13 consider Rb⁺ binding and occlusion as a single-step process, in both the initial rate of occlusion will be a linear function of the concentration of Rb⁺. Since (inset to Fig. 7) for the range of [Rb⁺] used in our experiments, the deviation of the initial slope from linearity is small, to avoid further complications of the scheme in Fig. 13 we did not include the additional steps necessary to account for the saturable increase in the initial rate of occlusion with [Rb⁺] (see inset to panel B in Fig. 7 and Refs. 21 and 30).

To simulate the behavior of the model in Fig. 13 we applied the method described under “Experimental Procedures,” including the following restrictions. (a) The rate constants of occlusion and deocclusion of Rb⁺ for a given site were considered independent of the occupancy of the other site, i.e., as follows.

\[ k₁ = k₂; \quad k₃ = k₄; \quad k₅ = k₆; \quad k₇ = k₈; \quad k₉ = k₁₀ \]  

Equation 11

(b) The equivalence between the different pathways connecting the same initial and final states places the following additional restrictions on the values of the rate constants.

\[ k₉₁ = k₉₂k₉₃/k₉; \quad k₈₁ = k₈₂k₈₃/k₈; \quad k₇₁ = k₇₂k₇₃/k₇ \]  

Equation 12

(c) When Rb⁺ occlusion was simulated, a time-independent parameter, which does not belong to the scheme in Fig. 13, was added to the equations to account for the eventual presence of

The rate of release of occluded Rb⁺ from the slow compartment even when free [Rb⁺] tends to infinity. This leak is not predicted by Scheme 3, but is represented in Fig. 13 by the off rate constants of the steps in dashed lines. A similar “leak” was found by Forbush (28) when studying the blocking effect of high concentrations of K⁺ and its congeners in media containing Mg-Pi.
a term such as that symbolized by \( A_0 \) in Equation 4 of “Results.” (d) In agreement with the usually held views on the properties of the \( E_1 \) and \( E_2 \) conformers of the \( \text{Na}^+/\text{K}^-\text{-ATPase} \) (Ref. 7, and see also comments to Scheme 1 and legend to Fig. 1), we assumed that only \( E_2 \) is capable to occlude \( \text{Rb}^+ \).

Table III shows the best fitting values for each of the constants of the scheme in Fig. 13. The following comments seem to be pertinent: (i) \( k_1, k_{-1}, k_2, k_{-2}, k_T, \) and \( k_{-T} \) are at least 1 order of magnitude larger than the rate constants governing the transitions between the other states of \( E_2 \). Therefore, the main pathway for occlusion and deocclusion is identical to that of Scheme 3. (ii) The transfer of occluded \( \text{Rb}^+ \) at very low rates governed by the coefficients of \( k_{-p_1} \) and \( k_{-p_2} \) (cf. those with \( k_1 \) and \( k_{-1} \) from its deep position to the media) would explain why the loss of occluded \( \text{Rb}^+ \) from the slow compartment is not zero at \([\text{Rb}^+]\) tending to infinity. (iii) The very large values of the constants of the reactions that lead to occlusion after the binding of \( \text{Rb}^+ \) to \( E_1 \) are not enough to explain the time-independent component of the occlusion curves.

Comparison between the Predicted and Observed Kinetics for

**Occlusion and Release of \( \text{Rb}^+ \).**—The best-fitting values of the rate constants and numerical solution of the differential equations describing the scheme in Fig. 13 were used to calculate the time course of occlusion and release of \( \text{Rb}^+ \) for the same ranges of incubation times and \( \text{Rb}^+ \) concentration as those employed in the experiments in this paper. The same values of rate constants were used to calculate the equilibrium level of \( \text{Rb}_{\text{occ}} \) versus \([\text{Rb}^+]\).

The simulated time courses and equilibrium levels of \( \text{Rb}_{\text{occ}} \) versus \([\text{Rb}^+]\) are shown as the continuous curves in Fig. 14 together with the experimental points taken from Figs. 7, 9, and 10. It is apparent that, except for a slight overestimation of the rate of the slow phase of \( \text{Rb}^+ \) occlusion at 228 \( \mu \text{M} \) \( \text{Rb}^+ \) (panel A), there is a satisfactory agreement between the experimental and the simulated values for the whole range of \([\text{Rb}^+]\) and incubation times tested.

We also looked if the scheme in Fig. 13 was able to predict the distribution between the fast and slow components of the release of occluded \( \text{Rb}^+ \) when the incubation leading to occlusion was short enough as to leave almost empty the slow compartment (see Fig. 12). To do this we used the best fitting values in Table III to generate discrete points of \( \text{Rb}_{\text{occ}} \) corresponding to the same incubation times and \( \text{Rb}^+ \) concentrations as those used in Fig. 12.

Fig. 15 shows that the discrete values generated by the simulation are adequately fitted by Equation 1 (continuous lines). The legend to Fig. 15 shows that the best fitting value of \( A_2 \) was about 40% of \( A_1 + A_2 \), which is near to what it was observed in the experimental values shown in Fig. 12.

**Final Remarks.**—Results in this paper show that, in the absence of other ligands, \( \text{Rb}^+ \) occlusion and deocclusion via the direct route follow biphasic time courses and that this behavior is not due to the presence of independent compartments. This phenomenon can be quantitatively explained positing the existence of enzyme states with one and two occluded \( \text{Rb}^+ \) formed through an ordered-sequential addition and release of the cation. The biphasic deocclusion kinetics and the micromolar apparent dissociation constant for \( \text{Rb}^+ \) for blocking the release of half of the occluded \( \text{Rb}^+ \) are in agreement with results presented here and by other authors for \( \text{P}^- \)-stimulated deocclusion (28, 29). This may indicate that the direct route of occlusion takes place through external, rather than internal sites of the \( \text{Na}^+/\text{K}^-\text{-ATPase} \) (see also Ref. 28), a view that facilitates to
account for the micromolar apparent dissociation constant observed for the Rb$\text{occlusion}$ versus [Rb$^+$] curve in our equilibrium experiments. The participation of external sites in Rb$^+$ deocclusion in the absence of other ligands is not considered by Scheme 1 and Fig. 1 and if it existed, it would require additional steps apart from those postulated in these schemes.

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REFERENCES
1. Post, R. L., Hegyvary, C., and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540
2. Beaugé, L. A., and Glynn, I. M. (1979) Nature 280, 510–512
3. Forbush, B., III (1987) J. Biol. Chem. 262, 11104–11115
4. Kaufman, S. B., González-Lebrero, R. M., Schwartzbaum, P. J., Nerby, J. G., Garrahan, P. J., and Rossi, R. C. (1999) J. Biol. Chem. 274, 20779–20790
5. Rossi, R. C., and Nerby, J. G. (1993) J. Biol. Chem. 268, 12579–12590
6. Glynn, I. M., and Richards, D. E. (1982) J. Physiol. (Lond.) 330, 17–43
7. Glynn, I. M., and Karlish, S. J. D. (1990) Annu. Rev. Biochem. 59, 171–205
8. Karlish, S. J. D., and Yates, D. W. (1978) Biochem. Biophys. Acta 527, 115–130
9. Joergensen, P. L. (1975) Biochim. Biophys. Acta 401, 399–415
10. Karlish, S. J. D., and Stein, W. D. (1982) J. Physiol. 328, 295–316
11. González-Lebrero, R. M., Kaufman, S. B., Garrahan, P. J., and Rossi, R. C. (2002) J. Biol. Chem. 277, 5922–5928
12. Garrahan, P. J., and Glynn, I. M. (1967) J. Physiol. (Lond.) 192, 217–235
13. Rossi, R. C., Kaufman, S. B., González-Lebrero, R. M., Nerby, J. G., and Garrahan, P. J. (1999) Anal. Biochem. 270, 276–285
14. González-Lebrero, R. M., Kaufman, S. B., Nerby, J. G., Garrahan, J. G., and Rossi, R. C. (2000) Excerpta Med. Int. Congr. Ser. 120, 433–436
15. Jensen, J., Nerby, J. G., and Ottolenghi, P. (1984) J. Physiol. (Lond.) 346, 219–241
16. Glynn, I. M., and Chappell, J. B. (1964) Biochem. J. 90, 147–149
17. Schwartzbaum, P. J., Kaufman, S. B., Rossi, R. C., and Garrahan, P. J. (1995) Biochim. Biophys. Acta 1253, 33–40
18. Yamaoka, K., Nakagawa, T., and Uno, T. (1978) J. Pharmacokin. Biopharm. 6, 165–175
19. González-Lebrero, R. M. (2001) Caracterización cinética del fenómeno de occlusión de K$^+$ en la Na$^+$/K$^+$-ATPasa. Ph.D. thesis, University of Buenos Aires
20. Fraser, R. D. B., and Suzuki, E. (1973) in Physical Principles and Techniques of Protein Chemistry: Part C (Leach, S. J., ed) pp. 301–355, Academic Press, New York
21. Forbush, B., III (1988) in The Na$^+$/K$^+$-Pump. Part A, Molecular Aspects (Skou, J. C., Nerby, J. G., Maunsbach, A. B., and Esmann M., eds) pp. 229–248 Alan Liss, Inc., New York
22. Post, R. L., Taniguchi, K., and Toda, G. (1974) Ann. N. Y. Acad. Sci. 242, 80–91
23. Shani, M., Goldschleger, R., and Karlish, S. J. D. (1987) Biochim. Biophys. Acta 904, 13–21
24. Jensen, J., and Nerby, J. G. (1989) Biochim. Biophys. Acta 985, 248–254
25. Esmann, M. (1994) Biochemistry 33, 8558–8565
26. Pedemonte, C. H., and Beauge, L. A. (1983) Biochim. Biophys. Acta 748, 245–253
27. Sachs, J. R. (1988) J. Physiol. (Lond.) 400, 545–574
28. Forbush, B., III (1987) J. Biol. Chem. 262, 11116–11127
29. Glynn, I. M., Howland, J. L., and Richards, D. E. (1985) J. Physiol. 368, 453–469
30. Hasenauer, J., Huang, W-H., and Askari, A. (1993) J. Biol. Chem. 268, 3289–3297
The Occlusion of Rb+ in the Na+/K+-ATPase: I. THE IDENTITY OF OCCLUDED STATES FORMED BY THE PHYSIOLOGICAL OR THE DIRECT ROUTES: OCCLUSION/DEOCCLUSION KINETICS THROUGH THE DIRECT ROUTE
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