The Occlusion of Rb\(^+\) in the Na\(^+\)/K\(^+\)-ATPase

II. THE EFFECTS OF Rb\(^+\), Na\(^+\), Mg\(^{2+}\), OR ATP ON THE EQUILIBRIUM BETWEEN FREE AND OCCLUDED Rb\(^+\)

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We used the direct route of occlusion to study the equilibrium between free and occluded Rb\(^+\) in the Na\(^+\)/K\(^+\)-ATPase, in media with different concentrations of ATP, Mg\(^{2+}\), or Na\(^+\). An empirical equation, with the restrictions imposed by the stoichiometry of ligand binding was fitted to the data. This allowed us to identify which states of the enzyme were present in each condition and to work out the schemes and equations that describe the equilibria between the ATPase, Rb\(^+\), and ATP, Mg\(^{2+}\), or Na\(^+\). These equations were fitted to the corresponding experimental data to find out the values of the equilibrium constants of the reactions connecting the different enzyme states. The three ligands decreased the apparent affinity for Rb\(^+\) occlusion without affecting the occlusion capacity. With [ATP] tending to infinity, enzyme species with one or two occluded Rb\(^+\) seem to be present and full occlusion seems to occur in enzymes saturated with the nucleotide. In contrast, when either [Mg\(^{2+}\)] or [Na\(^+\)] tended to infinity, no occlusion was detectable. Both Mg\(^{2+}\) and Na\(^+\) are displaced by Rb\(^+\) through a process that seems to need the binding and occlusion of two Rb\(^+\), which suggests that in these conditions Rb\(^+\) occlusion regains the stoichiometry of the physiological operation of the Na\(^+\) pump.

In media in which Rb\(^+\) is the only ligand of the Na\(^+\)/K\(^+\)-ATPase both the kinetics of direct occlusion and deocclusion and the equilibrium distribution between free and occluded Rb\(^+\) seem to indicate that either one or two Rb\(^+\) can be occluded per Na\(^+\)/K\(^+\)-ATPase molecule (1). This contrasts with the experimental evidence that under physiological conditions occlusion takes place only when two Rb\(^+\) are trapped per enzyme molecule. This contradiction may be caused because in physiological conditions other pump ligands are also present. This was analyzed in the experiments in this paper by means of a quantitative study of the effects of ATP, Mg\(^{2+}\), or Na\(^+\) on the equilibrium between free and occluded Rb\(^+\) formed by the direct route. Results show that occlusion of either one or two Rb\(^+\) persists in enzymes saturated with ATP but not in enzymes fully bound to Mg\(^{2+}\) or Na\(^+\). Although in all cases Rb\(^+\) was able to displace the second ligand and to reach maximal occlusion, in media with either Na\(^+\) or Mg\(^{2+}\) the displacement required the binding of two Rb\(^+\) to the enzyme suggesting that Na\(^+\) or Mg\(^{2+}\) have to be present to allow Rb\(^+\) occlusion to take place with a fixed stoichiometry of two.

EXPERIMENTAL PROCEDURES

The enzyme preparation, incubation conditions, reagents, methods for the determination of occluded Rb\(^+\) as well as the statistical procedures applied to the data are described in the previous paper of this series (1). ATP was purchased as the disodium salt and freed of Na\(^+\) by passing a 100 mM solution of ATP followed by 1 ml of 200 mM imidazole-HCl (pH 7.4 at 25 °C) through a column containing 1 ml of a cation exchange resin (Bio-Rad AG MP-50). Contaminant [Na\(^+\)] in the eluate, measured by flame photometry, was less than 0.05% of the [ATP] on a weight basis. Free Mg\(^{2+}\) was taken as equal to [MgCl\(_2\)] minus [EDTA]. In all experiments, occlusion equilibrium was attained by incubating enzyme during 15 min at 25 °C in media of the desired composition.

Model Selection—Regression procedures permitted to define the goodness of fit of a given equation to the experimental results and to choose among different models, by using the AIC criterion (2) which, as mentioned in the previous paper (1), is defined as $AIC = N \ln(SS) + 2P$, with $N$ = number of data, $P$ = number of parameters, and $SS$ = sum of weighted square residual errors. Statistical weights were 1 in all cases. To test if a parameter included in a given equation was significantly different from 0, $AIC$ was calculated either adjusting the parameter or fixing its value to 0 (thus decreasing $P$ by 1), and the equation with the lower AIC value was chosen.

The Quantitative Analysis of Rb\(^+\) Occlusion and Ligand Binding—Let us consider the transition from an occluded state with $i$ occluded Rb\(^+\) and $j$ bound X, $E(Rb)_iX_j$, to a non-occluded state, $E(Rb)_iX_j$, followed by the dissociation into its constituent components.

$$
T_u \quad E(Rb)_iX_j \rightarrow E(Rb)_iX_j = iRb^+ + jX + E
$$

SCHEME 1

In our experiments, $X$ is ATP, Mg\(^{2+}\), or Na\(^+\). This equilibrium is governed by a deocclusion constant, $T_u$, and a dissociation constant, $K_u$ (see Ref. 1). Note that the dissociation of $E(Rb)_iX_j$ in Scheme 1 could be split into its elementary reactions and that $K_u$ can accordingly be factored into the equilibrium constants of each step (see Scheme 2 below). According to Scheme 1, the equilibrium concentrations of every $E(Rb)_iX_j$ and $E(Rb)_iX_j$ can be written as follows.

$$
E(Rb)_iX_j = \frac{[Rb]_i[X]^j} {K_iT_u}, \quad E(Rb)_iX_j = \frac{[Rb]_i[X]^j} {K_i}
$$

(Eq. 1)

This allows us to express the amount of occluded Rb\(^+\) ($Rb_{occl}$) as follows.

$$
Rb_{occl} = \frac{\text{nmol of occluded Rb}^+}{\text{mg protein}} = E \sum_{i=1}^{p} \sum_{j=0}^{p} \frac{[Rb]_i[X]^j} {K_iT_u} (1 + T_u)
$$

(Eq. 2)
In Equation 2, \([E]\) cancels out because it appears as a factor of all the terms both in the numerator and in the denominator, and when \(i = j = 0\) then \(K_i = 1\). The equation includes four stoichiometric coefficients which measure the maximal numbers of occluded \(\text{Rb}^+\) \((p)\,\text{of X bound to the enzyme holding occluded \(\text{Rb}^+\) (q)},\text{of bound \(\text{Rb}^+\), either occluded or not (r)},\text{and of bound X (s)}\) For obvious reasons, the index \(i\) in the numerator of Equation 2 starts at one and not at zero. Notice that the numerator contains only terms that correspond to enzyme states holding occluded \(\text{Rb}^+\), so that \(p \leq q \leq s \leq s\). In this respect, it differs from a general binding equation, in which all bound states are measurable. For this reason the factor \(1 + T_i\) is present in each term of the denominator of Equation 2, where both occluded and not occluded states must be taken into account, and is absent from the terms in the numerator, where only occluded states are considered.

Equation 2 can be written as,

\[
\text{Rb}_{\text{obs}} = \frac{\sum_{i=1}^{p} \sum_{j=0}^{q} N_j \cdot [\text{Rb}^+] \cdot [X]}{\sum_{i=1}^{s} \sum_{j=0}^{q} D_j \cdot [\text{Rb}^+] \cdot [X]} \quad \text{(Eq. 3)}
\]

where

\[
N_j = \frac{E_j}{K_j T_j}, \quad D_j = 1 + T_j, \quad \text{(Eq. 4)}
\]

Notice that, from Equation 4 it follows that \(N_j/D_j = i E_j/(1 + T_j)\). This allows us to identify the relative distribution between states with bound \(\text{Rb}^+\) and states with bound and occluded \(\text{Rb}^+\) (which, to facilitate the reading, we hereafter shall call bound \(\text{Rb}^+\) and occluded \(\text{Rb}^+\), respectively). If the equilibrium between states with occluded and states with bound \(\text{Rb}^+\) is displaced toward the occluded states then \(T_j < 1\) and the ratio will become not significantly different from \(i E_j\). We have already discussed in detail the consequences of \(T_j < 1\) on the apparent dissociation constant for \(\text{Rb}^+\) (see comments to Scheme 2 in Reference 1, where \(T_o\) was denoted as \(K_{\text{occ}}\)).

Equations 2 and 3 take into account that every \(E(\text{Rb},[X])\) and \(E(Rb,X)\) states permitted by the stoichiometry of binding of \(\text{Rb}^+\) and of \(X\) are present. This may not be always the case and, in a given experimental situation, one or more of these states may not exist and their corresponding terms have to be eliminated from Equations 2 and 3. Although it is not possible to know beforehand which states are absent, these can be identified adjusting an "empirical" equation of the form of Equation 3 to the data, without the restrictions imposed by Equation 4. When this is done, the coefficients of terms that express the concentration of absent states will become not significantly different from zero. On this basis, when fitting this empirical equation to our data we discarded those terms whose coefficients became negligible and considered as nonexistent the enzyme states described by them, provided that this did not affect the goodness of the fit and diminished the value of the AIC criterion. We thus obtained a "reduced" empirical equation. Additionally, by evaluating the ratios \(N_j/D_j\) as described above, we were able to define which of the \(E(Rb,[X])\) and \(E(Rb,X)\) states were actually present and use this information to write down the minimal scheme that describes the equilibria among them. Once a minimal equilibrium diagram was established, an equation was derived in terms of \(E_j\) and the equilibrium constants of the scheme (for obvious reasons this equation will have the same form as the reduced empirical equation). In general, these equilibrium constants differed from the \(K_i\) in Scheme 1 because:

(i) stepwise dissociation constants like those governing the following equilibria,

\[
K_i \quad \text{ERbX} \rightleftharpoons \text{ERb} \cdot \cdot \cdot \cdot \cdot \cdot [\text{Rb}^+] + X
\]

\[
K_i \quad \text{ERb} \rightleftharpoons \cdot \cdot \cdot \cdot \cdot \cdot [\text{Rb}^+] \cdot \cdot \cdot \cdot \cdot \cdot X
\]

Scheme 2

were considered, so that the coefficients \(K_i\) of Equation 2 were expressed as the product of these constants, (ii) when information was not enough as to identify them, some of the stepwise equilibrium constants were grouped together into constants that included deocclusion of \(\text{Rb}^+\) and dissociation of ligands. Thus, using the equation derived from the scheme, the values of the equilibrium constants were obtained by means of regression analysis.
ATP. As none of the \( \frac{N_i}{D_{ij}} \) ratios were significantly different from \( i E_T \) (see Equation 4 and Scheme 1 for \( T_{ij} \ll 1 \)), it follows that all states with bound \( \text{Rb}^+ \) were mostly in the occluded form. These states and the equilibria among them are given in the scheme in Fig. 2. From what we have already mentioned, it is obvious that the equation derived from this scheme will have the same form as Equation 6. The dependence of its coefficients with the equilibrium dissociation constants of the scheme in Fig. 2 is given in Table I and the best fitting values of the constants are shown in Table II. These values were used to draw the continuous lines that fit the data in Fig. 1.

The following are relevant properties of the scheme in Fig. 2:

1) For all ATP concentrations,

\[
\lim_{\text{[Rb]}} \frac{\text{Rb}_{\text{sec}}} = 2E_T \tag{Eq. 7}
\]

which indicates that, for the reasons discussed in comments to Scheme 1 and Equations 2–5, even for the ATP-bound enzyme, the equilibrium between bound and occluded \( \text{Rb}^+ \) is sufficiently shifted toward occlusion as to allow complete saturation of the two occlusion sites in the ATPase. Since the \( K_{\text{ATP}} \) shifted toward occlusion as to allow complete saturation of the ATPase, \( \text{Rb}_{\text{sec}} \) was measured in media containing \( 0.8 \) (●), \( 3 \) (○), \( 8 \) (▲), \( 24.7 \) (■), \( 98.7 \) (■), \( 250 \) (□), and \( 500 \) (●) \( \mu M \text{Rb}^+ \), as a function of the concentration of ATP (panel A). Panel B shows the initial part of the plot in panel A. In panel C \( \text{Rb}_{\text{sec}} \) is plotted as a function of the concentration of \( \text{Rb}^+ \) in media containing \( 0 \) (●), \( 20 \) (○), \( 60 \) (▲), \( 200 \) (■), \( 600 \) (■), \( 1200 \) (□), or \( 2000 \) (●) \( \mu M \text{ATP} \). Panel D is the plot of the initial part of the same curve. The continuous lines are the plot of Equation 6 with its coefficients replaced by their meaning in terms of equilibrium constants of the scheme in Fig. 2 (see Table I) with the best fitting values given in Table II.

**Figure 1.** The effects of ATP on the equilibrium distribution between free and occluded \( \text{Rb}^+ \). \( \text{Rb}_{\text{sec}} \) was measured in media containing \( 0.8 \) (●), \( 3 \) (○), \( 8 \) (▲), \( 24.7 \) (■), \( 98.7 \) (■), \( 250 \) (□), and \( 500 \) (●) \( \mu M \text{Rb}^+ \), as a function of the concentration of ATP (panel A). Panel B shows the initial part of the plot in panel A. In panel C \( \text{Rb}_{\text{sec}} \) is plotted as a function of the concentration of \( \text{Rb}^+ \) in media containing \( 0 \) (●), \( 20 \) (○), \( 60 \) (▲), \( 200 \) (■), \( 600 \) (■), \( 1200 \) (□), or \( 2000 \) (●) \( \mu M \text{ATP} \). Panel D is the plot of the initial part of the same curve. The continuous lines are the plot of Equation 6 with its coefficients replaced by their meaning in terms of equilibrium constants of the scheme in Fig. 2 (see Table I) with the best fitting values given in Table II.

**Table I**

| Coefficient | Meaning | Unit | S.E. |
|-------------|---------|------|------|
| \( N_{11} / E_T \) | \( K_1 K_{\text{ATP}} \) | \( \mu M \) | 0.015 nmol (mg protein)⁻¹ |
| \( N_{20} / E_T \) | \( 2 K_2 K_{\text{ATP}} \) | \( \mu M \) | 0.015 nmol (mg protein)⁻¹ |
| \( N_{10} / E_T \) | \( K_1 K_{\text{ATP}} \) | \( \mu M \) | 0.015 nmol (mg protein)⁻¹ |
| \( N_{21} / E_T \) | \( K_2 K_{\text{ATP}} \) | \( \mu M \) | 0.015 nmol (mg protein)⁻¹ |
| \( D_{11} \) | \( K_{\text{ATP}} \) | \( \mu M \) | 0.015 nmol (mg protein)⁻¹ |
| \( D_{21} \) | \( K_{\text{ATP}} \) | \( \mu M \) | 0.015 nmol (mg protein)⁻¹ |
| \( D_{21} \) | \( K_{\text{ATP}} \) | \( \mu M \) | 0.015 nmol (mg protein)⁻¹ |

**Table II**

The best fitting values of the equilibrium constants of the scheme in Fig. 2

\( K_{\text{ATP}} \) and \( K_{\text{ATP}} \) were calculated as \( K_1 K_{\text{ATP}} K_1 \) and \( K_2 K_{\text{ATP}} K_2 \), respectively, using the thermodynamic equivalence of pathways, and propagating the error of the estimations of the fitted constants. \( E_T \) was 2.844 ± 0.015 nmol (mg protein)⁻¹.

Equation 8 indicates that the value of \( K_{0.5} \) for ATP will go from \( K_{\text{ATP}} \) when \([\text{Rb}^+] = 0 \) to \( K_{\text{ATP}} \) when \([\text{Rb}^+] \) tends to infinity. At \([\text{Rb}^+] \) between these two limits \( K_{0.5} \) for ATP will be a combination of \( K_{\text{ATP}} \) and \( K_{\text{ATP}} \), that is, of the equilibrium constants for the dissociation of ATP from the enzyme having either none, one or two occluded \( \text{Rb}^+ \), respectively. Table II shows that \( K_{\text{ATP}} > K_{\text{ATP}} > K_{\text{ATP}} \) indicating that increases in \([\text{Rb}^+] \) will increase \( K_{0.5} \). It is noteworthy that the best fitting values for \( K_{\text{ATP}} \) and \( K_{\text{ATP}} \) are comparable with the equilibrium constants for the dissociation of ATP from the catalytic or regulatory sites for the nucleotide, which are usually supposed to be present in the \( E_1 \) or \( E_2 \) conformers of the ATPase, respectively (5, 15–17). A definition of \( E_1 \) and \( E_2 \) is given in Ref. 1.

3) The initial slope of the \( \frac{\text{Rb}_{\text{sec}}}{f} \) curve,

\[
\frac{1 + \frac{\text{[ATP]}}{K_{\text{ATP}}}}{K_{\text{ATP}} / K_{\text{ATP}}} \tag{Eq. 9}
\]

will go from \( E_1 / K_1 \) to \( E_2 / K_2 \) as \([\text{ATP}] \) goes from zero to infinity, so that, when \([\text{ATP}] \) tends to infinity, the initial part of the \( \frac{\text{Rb}_{\text{sec}}}{f} \) curve
Effects of Rb⁺ and ATP, Mg2⁺, or Na⁺ on Occluded Rb⁺ in Na⁺/K⁺-ATPase

The effects of Mg²⁺ on the equilibrium distribution between free and occluded Rb⁺, Rbocc, was measured as a function of [Mg²⁺] in media containing 2.4 (○), 5 (□), 12 (▼), 50 (◇), 125 (■), or 250 (▲) μM Rb⁺. Panel B is a plot of the initial part of the curves in panel A. The continuous lines are the plot of Equation 10 where its coefficients were replaced by their meaning in terms of the equilibrium constants of the scheme in Fig. 4 (see Table III), using the best fitting values given in Table IV. This procedure was also used to plot the calculated values of Rbocc as a function of [Rb⁺] (panels C and D) for [Mg²⁺] (read from left to right) 0, 0.01, 0.045, 0.1, 0.45, 1, and 4.5 mM, since there were not enough experimental values for each of these curves.

\[ Rb_{occ} = \frac{N_{Mg}[Rb^+] + N_{Mg}^2[Rb^+]^2}{D_{Mg} + D_{Mg}[Rb^+] + D_{Mg}[Rb^+]^2} \] (Eq. 10)

It can be seen that Equation 10 has no terms containing [Mg²⁺] in the numerator and that only a first order term in [Rb⁺] [Mg²⁺] appears in the denominator.

The states of the enzyme with Mg²⁺ and/or Rb⁺ and the equilibria among them are given in the scheme in Fig. 4. In this scheme, Rbocc will obey an equation like Equation 10 whose coefficients will depend on the equilibrium dissociation constants as shown in Table III and whose best fitting values are given in Table IV. These values were used to draw the continuous lines in Fig. 3.

The following considerations are relevant to the scheme in Fig. 4. 1) Since Rb⁺ competes with Mg²⁺, as [Rb⁺] tends to infinity, Rbocc will tend to 2 \( E_T / K_2 \) as in the absence of Mg²⁺. 2) At constant [Rb⁺], as [Mg²⁺] tends to infinity, Rbocc will tend to zero along rectangular hyperbolas which will become half-maximal when,

\[ K_i = K_{Mg} \] (Eq. 11)

so that \( K_i \) will increase without bounds as [Rb⁺] increases. This is consistent with the scheme in Fig. 4 which shows that Rb⁺ fully displaces Mg²⁺ from the enzyme and necessarily means that Mg²⁺ will also fully displace Rb⁺ from the ATPase. 3) As [Mg²⁺] increases, the plots of the theoretical values of Rbocc vs [Rb⁺] (panels C and D in Fig. 3) evince an increasing sigmoidicity and a drop in the apparent affinity for Rb⁺. 4) The initial slope of Rbocc = \( f([Rb^+]) \).

\[ E_T / K_{Mg} \] (Eq. 12)

will tend to zero as [Mg²⁺] tends to infinity. 5) Although the scheme in Fig. 4 includes an enzyme state holding only one occluded Rb⁺, the concentration of this state will be negligible when [Mg²⁺] tends to infinity so that, in this condition, displacement of Mg²⁺ will need the binding of two Rb⁺. Addition-
ally, the scheme includes a Mg$^{2+}$-bound state where Rb$^+$ is bound, but not occluded (a unique case in this paper).

The Effects of Na$^+$ on the Equilibrium Distribution between Occluded and Free Rb$^+$—The equilibrium level of occluded Rb$^+$ was measured after incubating Na$^+$/K$^+$-ATPase preparation in media containing from 0 to 10 mM Na$^+$ and from 0.74 to 248 µM Rb$^+$. Results in Fig. 5 are plotted as a function of [Na$^+$] (panels A and B, and [Rb$^+$] panels C and D). It can be seen that at constant [Rb$^+$], Na$^+$ decreased the equilibrium level of occlusion along sigmoidal curves that tended to zero as [Na$^+$] raised and which were displaced to the right as [Rb$^+$] increased (panel A). The initial part (0 to 0.5 mM Na$^+$) of the Rb$^+$ versus [Na$^+$] curves (panel B) shows that the slope of these curves approached zero as [Na$^+$] tended to zero and [Rb$^+$] tended to either zero or infinity.

Best fit of the experimental data of Rb$^+$ versus [Rb$^+$] and [Na$^+$] was obtained with the following reduced empirical equation.

$$\text{Rb}_{\text{occ}} = \frac{N_{\text{Rb}}[\text{Rb}] + N_{\text{Na}}[\text{Na}] + N_{\text{Na}}[\text{Na}]^2}{D_{\text{Rb}}[\text{Rb}] + D_{\text{Na}}[\text{Na}] + D_{\text{Na}}[\text{Na}]^2}$$

(Eq. 13)

Equation 13 lacks those terms in which the value of the sum of the exponents of [Rb$^+$] and [Na$^+$] exceeds 3, which indicates that no enzyme forms exist holding more than three ions. As in the case of ATP, none of the $N/D$ ratios were significantly different from 1 $E_p$, indicating that all states with bound Rb$^+$ were mostly in the occluded form.

The possible states of the enzyme holding Rb$^+$ and/or Na$^+$ and the equilibria among them are given in the scheme in Fig. 6. In this scheme, $\text{Rb}_{\text{occ}} = f([\text{Rb}^+]), [\text{Na}^+]$ will obey an equation like Equation 13 with coefficients depending on the equilibrium dissociation constants as shown in Table V and whose best fitting values are given in Table VI. These values were used to draw the continuous lines that fit the data in Fig. 5.

The following comments seem pertinent to the scheme in Fig. 6. 1) At any Na$^+$ concentration, as [Rb$^+$] tends to infinity, $\text{Rb}_{\text{occ}}$ will tend to 2 $E_p$. Therefore as in the cases of ATP and Mg$^{2+}$, full occlusion is attainable in the presence of Na$^+$. 2) The initial slope of $\text{Rb}_{\text{occ}} = f([\text{Na}^+])$ when [Rb$^+$] tends to infinity will be as follows.

$$\frac{N_{\text{Na}}D_{\text{Rb}} - N_{\text{Rb}}D_{\text{Na}}}{D_{\text{Rb}}}$$

(Eq. 14)

Inspection of Table V shows that $D_{\text{Rb}} = N_{\text{Rb}}2E_p$ and $D_{\text{Na}} = N_{\text{Na}}2E_p$ so that the two terms in the numerator will be equal and Equation 14 will be zero. Hence, as [Rb$^+$] tends to infinity, the initial slope of the $\text{Rb}_{\text{occ}}$ versus [Na$^+$] curves tends to zero. 3) The initial slope of the $\text{Rb}_{\text{occ}} = f([\text{Rb}^+])$ is equal to,

$$\frac{N_{\text{Rb}}[\text{Rb}] + N_{\text{Na}}[\text{Na}] + N_{\text{Na}}[\text{Na}]^2}{D_{\text{Rb}}[\text{Rb}] + D_{\text{Na}}[\text{Na}] + D_{\text{Na}}[\text{Na}]^2}$$

(Eq. 15)

which will tend to zero as [Na$^+$] tends to infinity. 4) Although states with a single occluded Rb$^+$ are considered in the scheme in Fig. 6, their concentration will become negligible as [Na$^+$] tends to infinity.

General Considerations and Conclusions—We have shown that ATP, Mg$^{2+}$, or Na$^+$ decrease the equilibrium level of $\text{Rb}_{\text{occ}}$ lowering the apparent affinity of the ATPase for Rb$^+$ during occlusion by the direct route. Mg$^{2+}$ or Na$^+$ are able to draw the apparent affinity for Rb$^+$ to zero. In contrast with this, as [ATP] tends to infinity the affinity for Rb$^+$ tends to a lower but not zero, value. Regardless of the model used, these results indicate that the ATPase can simultaneously bind ATP and occlude Rb$^+$ whereas enzymes fully occupied by Na$^+$ or Mg$^{2+}$ cannot occlude Rb$^+$. The effects of ATP, Mg$^{2+}$, or Na$^+$ on $\text{Rb}_{\text{occ}}$ are completely surmountable by [Rb$^+$] so that, in the presence of any of these ligands, the maximal occlusion reaches 2 mol/
Effects of Rb⁺ and ATP, Mg²⁺, or Na⁺ on Occluded Rb⁺ in Na⁺/K⁺-ATPase

The meaning of the coefficients of Equation 13 in terms of the equilibrium constants of the scheme in Fig. 6

| Coefficient | Meaning |
|-------------|---------|
| N⁺/E⁺ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| N₂⁺/E⁺ | 2\(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| N₁⁺/E⁺ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| N₁⁻/E⁻ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| D₂₀ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| D₂₀ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| D₁₀ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| D₂₀ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| D₂₀ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| D₂₀ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |
| D₂₀ | \(K_0 \cdot K_1 \cdot K_{Rb} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na} \cdot K_{Na}\) |

The best fitting values of the equilibrium constants of the scheme in Fig. 4

| Constant | \(\mu \pm S.E.\) | Best fitting value |
|----------|-----------------|-------------------|
| \(K_0\)  | 2.661 ± 0.023   | \(10.645 ± 0.092\) |
| \(K_1\)  | 9.8 ± 1.6       | \(64 ± 15\)       |
| \(K_2\)  | 261 ± 66        | \(451 ± 73\)      |
| \(K_{Na}\)| 1680 ± 220     | \(10200 ± 2500\) |
| \(K_{Rb}\)| 181 ± 43       | \(4800 ± 1400\)  |
| \(K_{Na}\)| 1430 ± 300     | \(Na⁺\)           |

mol of ATPase as it happens in media with Rb⁺ alone, indicating that in all conditions tested the equilibrium between bound and occluded Rb⁺ is strongly shifted toward occlusion. This conclusion is trivial for the case of Mg²⁺ because enzymes holding occluded Rb⁺ are unable to bind this cation, but not for the cases of ATP and Na⁺ since these ligands do bind to the Rb⁺-occluded states (according to our analysis in Figs. 2 and 6: 1 ATP or 1 or 2 Na⁺). For instance (and in contrast to what we observed in this paper), Shani et al. (4) obtained only 75% of the maximal occlusion measuring direct occlusion at 0 °C in media with 9.6 mM ATP. This might mean that in their conditions, the equilibrium between occluded and bound Rb⁺ is poised as to leave a considerable fraction of the bound Rb⁺ freely exchangeable with the medium. (Note also that the technique of cation-exchange resin columns they used could underestimate Rb⁺occlusion in media with high [ATP], because of the high rate of Rb⁺ loss during the measurement.) With regard to Na⁺, we found no other reports in the literature on whether this cation might affect the equilibrium between bound and occluded Rb⁺.

ATP, Mg²⁺, or Na⁺, apart from lowering the apparent affinity for Rb⁺ change from hyperbolic to sigmoid the shape of the Rb⁺occlusion = \(f([Rb⁺])\) curve. This may happen either because these ligands induce the appearance of positive cooperativity for the occlusion of Rb⁺ or because they force Rb⁺ occlusion to take place only when two Rb⁺ are bound to the same enzyme molecule. Both processes require more than one Rb⁺ to be bound to the enzyme and therefore both will be inoperative as [Rb⁺] tends to zero, since in this condition the probability of having more than one Rb⁺ bound to the same enzyme molecule vanishes. For this reason, if interactions in affinity were the cause of sigmoidicity, the initial part of the Rb⁺occlusion = \(f([Rb⁺])\) curve would be a straight line of positive slope corresponding to the saturation of the first site on different enzyme units and would have zero slope if sigmoidicity were due to a stoichiometric requirement for two Rb⁺. To select one of these two alternatives it is mandatory to examine the initial part of the Rb⁺occlusion = \(f([Rb⁺])\) curve when the concentrations of the ligand that caused the sigmoidicity tends to infinity. This ensures that all enzyme molecules are equally affected by this ligand.

In the case of ATP, the criterion of the initial slopes strongly suggests that the nucleotide allows the occlusion of one or two Rb⁺ and induces sigmoidicity promoting positive interactions in this process. This view agrees with the equilibrium model discussed above and gains independent support from our observations that, with saturating ATP, the equilibrium constant for the release of Rb⁺ from the enzyme holding two occluded Rb⁺ is smaller than that holding one occluded Rb⁺ (cf. \(K_2\) in Table II), while it should have been four times larger to yield a hyperbolic response (see comments to Equation 5).

In contrast with the effects of ATP, the initial slope of the Rb⁺occlusion = \(f([Na⁺])\) curves becomes zero in media in which \([Mg²⁺]\) or \([Na⁺]\) tend to infinity, strongly suggesting that sigmoidicity caused by either of these ligands takes place because only occluded states with two Rb⁺ per ATPase will exist, as it is posited by the equilibrium schemes discussed above. Therefore, Mg²⁺ or Na⁺, but not ATP seem to be necessary for the ATPase to operate with a fixed stoichiometry of two Rb⁺ for occlusion that holds under physiological conditions (6, 17). The experiments in this paper do not allow us to know the molecular mechanism by which ATP generates cooperativity while Mg²⁺ or Na⁺ induce fixed stoichiometry for Rb⁺ occlusion.

The analysis of the value of the initial slope can also be applied to find out the mechanism that leads to the sigmoidal shape of the curves that describe the displacement of Rb⁺ by Na⁺. Our results show that the initial slope of the Rb⁺occlusion = \(f([Na⁺])\) curves tend to zero as [Rb⁺] tends to infinity. For the reasons already explained, this strongly suggests that more than one Na⁺ has to be bound for the displacement of occluded Rb⁺ to take place. In our equilibrium scheme in Fig. 6, 3 Na⁺ are necessary. This fits nicely with the number of Na⁺ ions translocated in each Na⁺/K⁺ exchange cycle catalyzed by the Na⁺/K⁺-ATPase (12).

It is tempting to postulate that the effects of Mg²⁺ or Na⁺ on the apparent affinity for Rb⁺ and on the shape of the Rb⁺occlusion = \(f([Rb⁺])\) curve are caused because either cation stabilizes the ATPase in a conformer (presumably \(E_1\)) which is unable to occlude Rb⁺ and that two Rb⁺ are needed to displace the system to a state (presumably \(E_2\)) in which they become occluded. In this process, Mg²⁺ or Na⁺ would be released from the enzyme. It is generally accepted that Na⁺ is an exclusive ligand of \(E_1\), but in terms of scheme in Fig. 6, there are states containing occluded Rb⁺ (and therefore presumably in the \(E_2\) form) that are able to bind Na⁺. In fact, only the states with no Rb⁺ occluded could correspond to the \(E_1\) form, notably those with the higher number of Na⁺ bound. This would still fit to the results found by other authors on the differential properties of \(E_1\) and \(E_2\) (13, 18, 19). The case for Mg²⁺ is more difficult to sustain, as the cation is known to act on both \(E_1\) and \(E_2\) (5, 7, 20). However, the values of the dissociation constants for Mg²⁺ found in our results (see Table IV) are more consistent with the high affinity effects that Mg²⁺ exerts on \(E_1\) than with the low affinity effects displayed on \(E_2\).

The effects of ATP on the apparent affinity for Rb⁺ and on
the shape of the $Rb_{oc} = f([Rb^+]$) curve are substantially different from those of $Mg^{2+}$ or $Na^+$. A plausible cause for this can be found in the fact that, in contrast with the complexes of the enzyme with $Mg^{2+}$ or fully saturated with $Na^+$, the enzyme-ATP complex is able to occlude $Rb^+$ with the same capacity as the free enzyme. Although its affinity for $E_1$ is much higher, ATP is known also to be a ligand of $E_2$. Since occlusion only takes place in the $E_2$ conformer, if the $E_2$-ATP complex were able to bind and occlude $Rb^+$, the occlusion that follows binding would displace the equilibrium between $E_1$ and $E_2$. ATP acting on $E_2$ induces a large increase in the rate of release of occluded $Rb^+$ (1, 5, 17, 21, 22). Therefore, either this increase is unable to significantly poise the equilibrium between $E_1$ and $E_2$, or the postulate of occlusion only $E_2$ATP has to be accompanied by the additional proposal that ATP induces an increase in the rate of formation of occluded $Rb^+$ large enough as to keep the maximal amount of $Rb_{oc}$ independent of the concentration of the nucleotide. This latter possibility was proposed by Hasenauer et al. (23).

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