Procedures to define kinetic mechanisms from catalytic activity measurements that obey the Michaelis-Menten equation are well established. In contrast, analytical tools for enzymes displaying non-Michaelis-Menten kinetics are underdeveloped, and transient-state measurements, when feasible, are therefore preferred in kinetic studies. Of note, transient-state determinations evaluate only partial reactions, and these might not participate in the reaction cycle. Here, we provide a general procedure to characterize kinetic mechanisms from steady-state determinations. We described non-Michaelis-Menten kinetics with equations containing parameters equivalent to $k_{\text{cat}}$ and $K_m$ and modeled the underlying mechanism by an approach similar to that used under Michaelis-Menten kinetics. The procedure enabled us to evaluate whether Na$^+$/K$^+$-ATPase uses the same sites to alternatively transport Na$^+$ and K$^+$. This ping-pong mechanism is supported by transient-state studies but contradicted to date by steady-state analyses claiming that the release of one cationic species as product requires the binding of the other (ternary-complex mechanism). To derive robust conclusions about the Na$^+$/K$^+$-ATPase transport mechanism, we did not rely on ATPase activity measurements alone. During the catalytic cycle, the transported cations become transitorily occluded (i.e. trapped within the enzyme). We employed radioactive isotopes to quantify occluded cations under steady-state conditions. We replaced K$^+$ with Rb$^+$ because $^{42}$K$^+$ has a short half-life, and previous studies showed that K$^+$- and Rb$^+$-occluded reaction intermediates are similar. We derived conclusions regarding the rate of Rb$^+$ deocclusion that were verified by direct measurements. Our results validated the ping-pong mechanism and proved that Rb$^+$ deocclusion is accelerated when Na$^+$ binds to an allosteric, nonspecific site, leading to a 2-fold increase in ATPase activity.

Regardless of the kinetic mechanism involved, under steady-state conditions, both catalytic activities and concentrations of enzyme reaction intermediates can be expressed as rational functions of the concentration of the reactants (1). For instance, we can express the rate of formation of a product $P$ as a function of the concentration of the varying-reactant $A$ as follows (2).

$$v_0([A]) = \frac{\sum_{j=0}^{n} \alpha_j [A]^j}{1 + \sum_{j=1}^{n} \beta_j [A]^j}$$

(Eq. 1)

Michaelis-Menten kinetics is obeyed when the maximum exponent on the concentration of the varying reactant is 1, i.e. $\alpha_j = 0$ and $\beta_j = 0$ for every $j > 1$. This holds for reaction schemes where the varying reactant binds to only one enzyme reaction intermediate. Non-Michaelis-Menten kinetics occurs, for instance, when the varying reactant is both substrate and inhibitor (substrate inhibition) or participates in alternative productive pathways or, with some exceptions, when its stoichiometric coefficient is >1.

Most reported enzymes seem to follow Michaelis-Menten kinetics. An enlightening literature search of kinetic studies performed in the period 1965–1976 (3) found non-Michaelis-Menten kinetics in ~800 enzymes (i.e. 20% of the total known at that time). Nevertheless, we believe that what was pointed out by the authors then remains valid today: Michaelis-Menten kinetics is usually not verified, and the proportion of enzymes with non-Michaelis-Menten kinetics is therefore probably much higher.

The analysis of kinetic mechanisms in enzymes with Michaelis-Menten kinetics has been widely described (4, 5). Briefly, the Michaelis-Menten equation is fitted to data of catalytic activity measured varying the concentration of one substrate (variable substrate) at different fixed concentrations of one of the others (changing fixed substrate) while keeping all other substrates, if there are any, at constant concentration. Then fitted values of parameters $k_{\text{cat}}/K_m$ and $K_m$ are plotted against the concentration of the changing fixed substrate. The patterns obtained give insights into the kinetic mechanism involved. For instance, for a bi-substrate enzyme, it is possible to realize whether both substrates must bind to the enzyme before giving any products (ternary-complex mechanism) or not (ping-pong mechanism). When products are absent, if $K_m$ tends to zero as the concentration of the changing fixed substrate tends to zero, then the mechanism is ping pong. Otherwise, it is a ternary-complex mechanism.

Reaction schemes for enzymes with non-Michaelis-Menten kinetics are generally assembled from transient-state results.
Analysis of non-Michaelis-Menten kinetics

Figure 1. A simplified version of the Albers–Post model for the physiological functioning of Na\(^+\)/K\(^+\)-ATPase. Intermediates in the reaction scheme (left) have been cartooned (right) for better comprehension. Subscripts \(i\) and \(e\) are for intracellular and extracellular, respectively. For the diagram on the left, occluded cations are shown in parentheses. For the diagram on the right, each intermediate is represented with its cytoplasmic side (in) facing up. Under physiological conditions, the reaction cycle proceeds clockwise.

because there is no validated procedure to do this from steady-state determinations. Transient-state studies provide very valuable information but are more difficult to perform, and they evaluate only partial reactions, which might not be part of the reaction cycle. Here we provide a general procedure to characterize enzymes with non-Michaelis-Menten kinetics by an approach similar to that used under Michaelis-Menten kinetics. The procedure enabled us to evaluate whether Na\(^+\)/K\(^+\)-ATPase uses the same sites to alternatively transport Na\(^+\) and K\(^+\).

Na\(^+\)/K\(^+\)-ATPase is a transmembrane enzyme (6–9) that under physiological conditions couples the hydrolysis of one intracellular ATP molecule (into ADP and orthophosphate) to the exchange of three intracellular Na\(^+\) (Na\(_{i}^{+}\)) for two extracellular K\(^+\) (K\(_{e}^{+}\)). During the catalytic cycle, the enzyme undergoes phosphorylation and dephosphorylation, and the transported cations become transitorily occluded (trapped within the enzyme). The currently accepted Albers–Post model (10) poses that the binding of 3 Na\(_{i}^{+}\) to the intermediate E1ATP triggers its phosphorylation, leading to E1P(Na\(_{3}^{+}\)), which spontaneously undergoes a conformational change to give E2P, thereby releasing Na\(^+\) cations to the extracellular side. The binding of 2 K\(_{e}^{+}\) to E2P leads to its dephosphorylation and the concomitant occlusion of the cations. The K\(^+\)–occluded intermediates are very stable, making the release of K\(^+\) toward the intracellular side the rate-limiting step of the reaction cycle.

The Albers–Post model poses that Na\(^+\) and K\(^+\) are alternatively transported by the same set of sites (10–12). This ping-pong nature of the transport is supported by few studies on partial reactions of the catalytic cycle (13–16). However, many other studies evaluating the global functioning of the pump by steady-state measurements supported ternary-complex mechanisms (17–29), with at least one reaction intermediate with Na\(_{i}^{+}\) and K\(_{e}^{+}\) simultaneously bound to their transport sites. As far as we know, the ping-pong mechanism has not been corroborated to date by steady-state measurements.

ATPase activity measurements at varying concentrations of the transported cations would give enough information to address the question of the transport mechanism. However, we perceived that the complexity of the kinetic mechanism of Na\(^+\)/K\(^+\)-ATPase demanded complementary assays to derive robust conclusions and explain some unexpected results. Therefore, we included steady-state and transient-state determinations of the amount of the K\(^+\) congener Rb\(^+\) (15) that is trapped within enzyme reaction intermediates. Briefly, we proceeded as follows. We fitted rational functions of the concentration of Na\(^+\) (variable substrate) to measurements of Na\(^+\)/K\(^+\)-ATPase activity and steady-state amounts of occluded Rb\(^+\), at different fixed concentrations of Rb\(^+\) (changing fixed substrate). Fitted values of parameters equivalent to \(k_{cat}/K_m\) and \(K_m\) were then plotted versus the concentration of Rb\(^+\) to conclude, from the pattern obtained, whether the transport responds to a ping-pong or a ternary-complex mechanism. We did this analysis either in the absence or in the presence of 2 mM ADP. Results in the absence of ADP served as a control because under this condition, the system must give the ping-pong pattern, regardless of the transport mechanism involved (see “Theory”). Moreover, fitted values of other parameters allowed us to predict values for the velocity constants of Rb\(^+\) deocclusion. The predicted values agreed with those obtained by direct measurements, giving consistency to the whole analysis.

Our results validated the ping-pong nature of the transport mechanism from steady-state determinations. Additionally, we found that Rb\(^+\) deocclusion is accelerated when Na\(^+\) binds to an allosteric, nonspecific site, leading to a 2-fold increase in ATPase activity. It is very likely that the effects of the binding of Na\(^+\) to this allosteric site have been wrongly attributed to the occupancy of its transport sites, i.e. a ternary-complex transport mechanism.

Results and discussion

Theory

Here, we will illustrate how ping-pong and ternary-complex mechanisms are differentiated under Michaelis-Menten and non-Michaelis-Menten kinetics.

Fig. 2 shows representative models for the transport of Na\(^+\) and the K\(^+\) congener Rb\(^+\) by Na\(^+\)/K\(^+\)-ATPase. Panel A of Fig. 2 shows a ping-pong model based on that of Albers and Post, whereas panels B and C show two ternary-complex models that are compatible with the empirical evidence that originally led to the Albers–Post model (i.e. (i) Na\(^+\) is required for enzyme ATP-dependent phosphorylation and (ii) K\(^+\) is required for enzyme dephosphorylation (26)).

When the stoichiometry for transport is set to 1 Na\(_{i}^{+}\) for 1 Rb\(_{e}^{+}\) and the concentration of the products Na\(_{o}^{+}\) and Rb\(_{o}^{+}\) is set
to zero, all models follow Michaelis-Menten kinetics, and for a fixed concentration of Rb\textsuperscript{+}, the velocity of formation of the product P\textsubscript{i} per unit of total enzyme concentration (molar catalytic activity) can be expressed as follows,

\[
\text{Act}([Na^+]_i) = \frac{1}{K_{mNa}} \frac{d[P_i]}{dr} = \frac{[Na^+]_i}{K_{catNa} + [Na^+]_i} 
\]

(Eq. 2)

where \(k_{catNa}\) is the catalytic constant and \(K_{mNa}\) is the Michaelis-Menten constant.

As summarized in Table 1, the dependence of the ratio \(k_{catNa}/K_{mNa}\) on the concentration of the fixed substrate Rb\textsuperscript{+} varies with the model considered (30). For the ping-pong model, as [Rb\textsuperscript{+}] approaches zero, the ratio \(k_{catNa}/K_{mNa}\) tends to a positive value, whereas \(K_{mNa}\) becomes zero. Unless both ADP and P\textsubscript{i} are absent (this is discussed below), ternary-complex models exhibit a different pattern; when [Rb\textsuperscript{+}] approaches zero, the ratio \(k_{catNa}/K_{mNa}\) tends to zero, whereas \(K_{mNa}\) does not. This kind of analysis was originally proposed by Cleland (4, 5) and is part of what is known as Cleland’s rules.

When the concentration of the products Na\textsuperscript{+} and Rb\textsuperscript{+} is zero but the stoichiometry for transport is 3 Na\textsuperscript{+} for 2 Rb\textsuperscript{+}, as posed in Fig. 2, we obtain a rational equation of higher complexity.

\[
\text{Act}([Na^+]_i) = \frac{a_{Na13} [Na^+]_i^3}{K_{Na13} K_{Na12} K_{Na11} + [Na^+]_i^2 K_{Na12} K_{Na11} + [Na^+]_i K_{Na11} K_{Na12} K_{Na13}} 
\]

(Eq. 3)

Here, the parameters \(K_{Na11}, K_{Na12},\) and \(K_{Na13}\) are the apparent stepwise dissociation constants of the enzyme for Na\textsuperscript{+}, and, like \(K_{mNa}\), they have concentration units. The meaning of \(a_{Na13}\) is equivalent to that of \(k_{catNa}\) in Equation 2, although this is not always true, for instance, in branched models (see the supporting material). As in the case of \(k_{catNa}\), the units of \(a_{Na13}\) are those of the catalytic activity, Act.

In Equation 3, the dependence of \(a_{Na13}/(K_{Na13} K_{Na12} K_{Na11})\) and \(K_{Na11} K_{Na12} K_{Na13}\) on [Rb\textsuperscript{+}] is the same as that of \(k_{catNa}/K_{mNa}\) and \(K_{mNa}\), respectively, in Michaelis-Menten models (cf. Equations S1 and S2, S5 and S6, and S9 and S10 with Equations S13 and S14, S18 and S19, and S23 and S24).

The ping-pong pattern (see Table 1) results from the fact that the denominator in the steady-state equations lacks a term independent of the concentrations of both substrates, Na\textsuperscript{+} and Rb\textsuperscript{+}.

This occurs whenever the reaction scheme contains irreversible steps interposed between those in which the substrates bind (4). Consequently, in the absence of both ADP and P\textsubscript{i}, all three models in Fig. 2 will display the ping-pong pattern (31). When both ADP and P\textsubscript{i} are present and both products Na\textsuperscript{+} and Rb\textsuperscript{+} are absent, the only scheme in Fig. 2 showing the ping-pong pattern will be the ping-pong model in A. Therefore, to truly distinguish between ping-pong and ternary-complex mechanisms, ADP and/or P\textsubscript{i} must be present in the reaction.

When working with non-compartmentalized preparations, the concentrations of a transported substance as substrate and product are necessarily equal and cannot be manipulated independently. In our system, [Na\textsuperscript{+}] = [Na\textsuperscript{+}] = [Na\textsuperscript{+}] and [Rb\textsuperscript{+}] = [Rb\textsuperscript{+}] = [Rb\textsuperscript{+}]. The presence of products Na\textsuperscript{+} and Rb\textsuperscript{+} is thus unavoidable and will reverse the reaction steps that involve their release. Furthermore, Na\textsuperscript{+} and Rb\textsuperscript{+} compete for both intracellular and extracellular transport sites of the enzyme. Under the conditions described, equations for models in Fig. 2 change. In fact, even if stoichiometry for transport is set to 1 Na\textsuperscript{+} for 1 Rb\textsuperscript{+}, models no longer follow Michaelis-Menten kinetics. In the absence of ADP or P\textsubscript{i}, they give the following.

\[
\text{Act}([Na^+]_i) = \frac{[Na^+]_i}{K_{Na3} + \sum_{j=1}^{d} \frac{[Na^+]_i}{K_{Na3}^j}} 
\]

(Eq. 4)

where \(d = 2\) and \(3\) in ping-pong and ternary-complex models, respectively.

If stoichiometry for transport is 3 Na\textsuperscript{+} for 2 Rb\textsuperscript{+}, we obtain the following.

\[
\text{Act}([Na^+]_i) = \frac{a_{Na13} [Na^+]_i^3}{K_{Na3} K_{Na2} K_{Na1}} 
\]

(Eq. 5)
where $d = 6$ and $8$ in ping-pong and ternary-complex models, respectively.

Despite the differences in the form of the equations for non-compartmentalized systems (cf. Equations 2 and 3 and Equations 4 and 5), for $[\text{Rb}^+]$ tending to zero, the critical features in the patterns remain unchanged. In the ping-pong pattern, the ratios $a_{\text{Na}1}/K_{\text{Na}1}$ and $a_{\text{Na}2}/(K_{\text{Na}1} K_{\text{Na}2} K_{\text{Na}3})$ tend to non-zero values, whereas $K_{\text{Na}1}$ and $K_{\text{Na}1} K_{\text{Na}2} K_{\text{Na}3}$ tend to zero. Conversely, in the ternary-complex pattern, the ratios $a_{\text{Na}1}/K_{\text{Na}1}$ and $a_{\text{Na}2}/(K_{\text{Na}1} K_{\text{Na}2} K_{\text{Na}3})$ tend to zero, whereas $K_{\text{Na}1}$ and $K_{\text{Na}1} K_{\text{Na}2} K_{\text{Na}3}$ tend to zero (see Table 1; proof for this is given in the supporting material, Equations S3 and S4, S7 and S8, S11 and S12, S15 and S16, S20 and S21, and S25 and S26).

Therefore, ping-pong and ternary-complex mechanisms are still distinguishable, remarkably, despite the reversibility of the steps that involve the release of Na$^+$ and Rb$^+$ as products.

Another important aspect to consider is that Na$^+$/K$^+$-ATPase also hydrolyzes ATP by reaction pathways other than the physiological one (10, 11, 32, 33): (i) X/X-ATPasic cycling mode, in the absence of alkaline cations (34, 35); (ii) X/Rb$^+$-ATPasic cycling mode, when Rb$^+$ is transported in the absence of Na$^+$ (33, 36, 37); (iii) Na$^+$/Na$^+$-ATPasic cycling mode, when the enzyme exchanges Na$^+$ for Na$^+$ (22, 33, 38, 39); and (iv) other reaction pathways where Na$^+$ triggers enzyme phosphorylation but is followed by spontaneous dephosphorylation (uncoupled sodium efflux) (35, 40–43) or by dephosphorylation stimulated by a single Rb$^+$ (44). Equations from models containing alternative cycling modes show that some of these affect the parameters to the point that they can make both $a_{\text{Na}3} / (K_{\text{Na}1} K_{\text{Na}2} K_{\text{Na}3})$ and $K_{\text{Na}1} K_{\text{Na}2} K_{\text{Na}3}$ tend to non-zero values as $[\text{Rb}^+]$ tends to zero (cf. Equation S15 with Equation S27 and Equation S21 with Equation S32), thus undermining the possibility to discriminate ping-pong from ternary-complex mechanisms using the criteria in Table 1. We will show that the influence of alternative cycling modes can be neglected in our system.

**The roles of ADP**

As mentioned before, to truly distinguish between ping-pong and ternary-complex mechanisms, the products ADP and/or P$_i$ must be present in the reaction to avoid irreversible steps between the binding of Na$^+$ and Rb$^+$ as substrates. It can be shown that to make the distinction between the ping-pong model in Fig. 2A and the ternary-complex model in Fig. 2B, the presence of ADP is required (see Equation S21), whereas if the ternary-complex model is that in Fig. 2C, the presence of P$_i$ is required (see Equation S25). Nevertheless, the model shown in Fig. 2C assumes that binding of Na$^+$ is essential for the release of Rb$^+$, which contrasts with the evidence shown both in the literature (15) and later in this work (see Fig. 5). For this reason, we will only consider the ternary-complex model in Fig. 2B as an alternative to the ping-pong model.

Because ADP is needed to distinguish between these mechanisms, we must take into consideration that this nucleotide plays a dual role: not only as product of the ATP-dependent phosphorylation step but also as a competitor of ATP for the nucleotide-binding sites in E1 and E2(Rb$^+$) (15, 31, 45).

**Analysis of non-Michaelis-Menten kinetics**

In Fig. 3A we show the dependence of Na$^+$-ATPase activity on [ATP] when measured in the presence of 2 mM ADP. When [ATP] is higher than around 1 mM, Na$^+$-ATPase activity almost reaches its maximum, meaning that most of the ADP has been displaced by ATP from the nucleotide-binding sites in E1. The effect of 2 mM ADP on ATPase activity measured at 2.5 mM ATP is therefore mainly due to the reversion of the phosphorylation step.

Both ATP and ADP bind to Rb-occluded intermediates, leading to an increase in the rate of Rb$^+$ deocclusion, but ADP is not as effective as ATP and binds with lower affinity (15). Fig. 3B shows that at 2.5 mM ATP, the apparent velocity constant of Rb$^+$ deocclusion, $k_{\text{app-deocc}}$, decreases slightly with the concentration of ADP.

Based on these results, ADP effects on the reaction cycle other than the reversion of the phosphorylation step are minimized when using ATP and ADP concentrations of 2.5 and 2 mM, respectively.

**ATPase activity and levels of occluded Rb$^+$; general properties of the catalytic mechanism**

We performed parallel measurements of ATPase activity (Act) and steady-state levels of occluded Rb$^+$ (Occ) as a function of [Na$^+$] at different fixed [Rb$^+$] values. Reaction media contained 2.5 mM ATP, 0 or 2 mM ADP, and choline chloride to keep constant the ionic strength. For each [Rb$^+$], Act and Occ were adequately described by rational equations (see Equations 11 and 12) with the same denominator, as expected if both measurements expressed steady-state properties of the same enzyme (e.g. see Refs. 1 and 46 and the supporting material).

Here, we will make a qualitative analysis of representative Act and Occ curves at Rb$^+$ concentrations arbitrarily classified as low (approximately between 0.03 and 0.3 mM) and high (approximately >1 mM). We will focus on the predominant cycling modes of the enzyme under the experimental conditions tested.

Fig. 4A shows the results of a typical experiment performed at low [Rb$^+$]. In the absence of Na$^+$, both Act and Occ have small but significant non-zero values as a result of the X/Rb$^+$-ATPasic cycling mode (33–37), where the enzyme couples the hydrolysis of ATP to the occlusion and transport of Rb$^+$.
[Na\(^+\)] increases, Act and Occ curves exhibit a correlated increase due to the transition toward the physiological Na\(^+\)/Rb\(^+\)-ATPasic cycling mode. Further increments in [Na\(^+\)] causes a correlated decrease in these curves, which is easily explained by posing that Na\(^+\) displaces Rb\(^+\) from its transport sites in E2P, reducing both the level of occluded Rb\(^+\) and the rate of enzyme dephosphorylation. When [Na\(^+\)] is increased even more, the Na\(^+\)/Na\(^+\)-ATPasic cycling mode (exchanging 3 Na\(^+\) for 2 Na\(^+\)) starts to prevail (22, 33, 39), resulting in an increase in Act, whereas Occ tends to zero.

Fig. 4B shows the results of a typical experiment performed at high [Rb\(^+\)]. At null [Na\(^+\)], there is a substantial occlusion of Rb\(^+\) through the direct route (i.e. without formation of phosphoenzyme). The strong accumulation of intermediates occluding Rb\(^+\) inhibits the ATPase activity given by the X/Rb\(^+\)-ATPasic cycling mode (33, 36, 37). When [Na\(^+\)] is raised, Act and Occ curves exhibit a correlated increase due to the transition toward the physiological Na\(^+\)/Rb\(^+\)-ATPasic cycling mode. Further increments in [Na\(^+\)] lead to a decrease in Occ that is less steep than that observed at low [Rb\(^+\)] and is correlated not to a decrease but to a considerable increase in ATPase activity. These effects of Na\(^+\) cannot be attributed to a transition toward the Na\(^+\)/Na\(^+\)-ATPasic cycling mode (expected to take place at much higher [Na\(^+\)]) but to the fact that Na\(^+\) binds with low affinity to the Rb-occluded intermediate and promotes Rb\(^+\) deocclusion, which is the rate-limiting step of the Na\(^+\)/Rb\(^+\)-ATPasic cycling mode (see Fig. 5 and Refs. 15, 47, and 48).

The continuous lines that describe the results of ATPase activity in Fig. 4 respond to equations of the form of Equation 11 with n = d (see “Experimental procedures”). When the denominator (denom) is distributed among the numerator terms, Act can be expressed as follows,

\[
\text{Act}([\text{Na}^+]) = \sum_{j=0}^{n} v_{\text{Na}j} \tag{Eq. 6}
\]

with n equal to 5 or 4 for low and high [Rb\(^+\)], respectively, and

\[
v_{\text{Na}j} = \frac{[\text{Na}^+]^{j} \Pi_{j-1} K_{\text{Na}j}}{\text{denom}} \tag{Eq. 7}
\]

\(v_{\text{Na}j}\) terms estimate the flux of the reaction through specific cycling modes and are plotted in Fig. 4 (C and D). At low [Rb\(^+\)], ATPase activity is mostly given by \(v_{\text{Na}3}\), and at high [Rb\(^+\)], it is given by the sum of \(v_{\text{Na}3}\) and \(v_{\text{Na}4}\). As we show under “Comparison of global and partial reactions,” the terms \(v_{\text{Na}3}\) and \(v_{\text{Na}4}\)
mainly reflect the contribution to Act of the Na+/Rb+-ATPase cycling mode that takes place, respectively, without or with the low-affinity binding of Na+ to the Rb-occluded intermediate. The sum vNa⁰ + vNa₁ + vNa₂ = vNa₁₂ is given by cycling modes where the enzyme is phosphorylated through pathways other than the physiological one, whereas vNa₃ is mostly given by the Na⁺/Na⁺-ATPase cycling mode, i.e. exchange of 3 Na⁺ for 2 Na⁺.

Comparison of global and partial reactions

To evaluate whether the binding of Na⁺ to the Rb-occluded intermediate can account for the low-affinity increase in ATPase activity observed in Fig. 4B (vNa₁ in Fig. 4D), we measured the time course of Rb⁺ deocclusion at different concentrations of Na⁺ and obtained the apparent velocity constant of the process (kapp-deocc). Fig. 5 shows (empty circles) that kapp-deocc increases from 10⁻²⁻⁻⁻⁻ in the absence of Na⁺ to about 24 s⁻¹ at 100 mM Na⁺ and then decreases very slightly with further increments in the cation concentration. We have included the results of a similar experiment performed in the presence of 5 mM RbCl (Fig. 5, filled circles). Our results suggest that Na⁺ and Rb⁺ compete for the same site(s) because their effects on kapp-deocc are not additive (the effect of Rb⁺ tends to disappear as [Na⁺] increases). As reported previously (15), not only Na⁺ but also other alkali cations, including Rb⁺, increase kapp-deocc to a similar extent, supporting the allosteric nature of the binding site.

We have solved models like those in Fig. 2 (A and B) but including both alternative cycling modes (e.g. X/X- + X/Rb⁺ - or Na⁺/Na⁺ -ATPase cycling modes) and the binding of Na⁺ to E2(Rb⁺) ATP as a putative activator of Rb⁺ deocclusion, like in the scheme in Fig. 5B. The models lead to more complex equations with the form of Equations 11 and 12. The solutions are given in the supporting material (see models PP1 and TC2). When alternative cycling modes are canceled, both models predict that in the absence of ADP (see Equations S29 and S30 and Equations S34 and S35),

\[ a_{Na3}(o_{Na3}/2)_{ADP} = k_{deocc} \]  
(Eq. 8)

and

As these equalities are met only when cycling modes other than the physiological one are neglected, they are a good test for the contribution of such modes to the terms vNa₁ and vNa₂ above.

Values for aNa₃ and oNa₃ were obtained from the fitting of Equations 11 and 12 to the whole set of results of Act and Occ as described under “Empirical equations.” Fig. 6 shows that fitted curves reproduced very well the results (parameter values are given in Table 2). From results obtained in the absence of ADP, aNa₃/oNa₃ = 10.1 ± 0.2 s⁻¹ regardless of [Rb⁺], whereas aNa₃/oNa₃ was 22.1 ± 0.6 s⁻¹ at 5 mM Rb⁺. These values are in very good agreement with those of kdeocc (10.4 ± 0.9 s⁻¹) and kdeoccna (23.4 ± 0.8 s⁻¹), respectively, given by the model in Fig. 5B when fitted to direct measurements of kapp-deocc (see continuous line in Fig. 5A; model parameter values are listed in Table 3).

The analysis above confirms, at least for measurements performed at zero [ADP], that the contribution of alternative cycling modes to vNa₁ is negligible for all [Rb⁺] tested, whereas this is true for vNa₃ only at high [Rb⁺].

It is noteworthy that the agreement between the values of aNa₃/oNa₃ and aNa₃ at zero [Na⁺] (kdeocc in Fig. 5B) indicates that the binding of Na⁺ to the Rb-occluded intermediate is not essential for the release of Rb⁺ during the ATP-driven exchange of 3 Na⁺ for 2 Rb⁺. In other words, Na⁺-independent deocclusion of Rb⁺ is compatible with the turnover rate of the enzyme. Moreover, the simple assumption of the existence of an allosteric site for Na⁺ in E2(Rb⁺)ATP explains qualitatively and quantitatively both the activation of Rb⁺ deocclusion and the significant contribution of vNa₃ at high [Rb⁺].

Validation of the ping-pong mechanism for the transport of Na⁺ and K⁺ by steady-state determinations

To validate the ping-pong mechanism, we evaluated the pattern obtained for parameters aNa₃(KNa₁ KNa₂ KNa₃) and KNa₁ KNa₂ KNa₃ as a function of [Rb⁺]. In one set of experiments, we omitted both ADP and P₃, as a control of the ping-pong pattern. In another set, media contained enough ADP to reverse the phosphorylation step, bringing to a minimum its effects as a competitor of ATP for the nucleotide-binding sites in E1 and E2(Rb⁺) (see Fig. 3). Fig. 6 shows the fitting of empirical equations to the complete set of results for ATPase activity and occluded Rb⁺ as a function of [Na⁺] at different Rb⁺ concentrations, as described under “Experimental procedures.” The addition of 2 mM ADP to the reaction medium causes ATPase activity to decrease by nearly one-half, indicating the reversion of the ATP-driven phosphorylation step.

Fig. 7 shows the dependence of the fitted values of the parameters aNa₃(KNa₁ KNa₂ KNa₃) and KNa₁ KNa₂ KNa₃ on [Rb⁺] in the absence and in the presence of ADP. The results clearly validate the ping-pong nature of the transport mechanism of Na⁺/K⁺ -ATPase. Notably, the product KNa₁ KNa₂ KNa₃ tends to zero as [Rb⁺] tends to zero (Table 1).
A model to describe the results

Fig. 8 shows a ping-pong model for the active transport of three Na\(^+\) for two Rb\(^+\) cations. The model includes alternative cycling modes that were extensively studied elsewhere in our system (33). The continuous lines in Fig. 9 are the plot of model equations fitted to the results shown in Fig. 6. The values for the parameters are listed in Table S1.

The physiological cycling mode is given by the reaction cycle 

\[ E_1ATP \rightarrow E_1P(Na^3) \rightarrow E_2P \rightarrow E_2(Rb^2)ATP \rightarrow E_1ATP. \]

The ping-pong nature of the model becomes evident by noting that binding of Na\(^+\) (Rb\(^+\)) to transport sites is not mandatory for Rb\(^+\) (Na\(^+\)) to be released from the enzyme as product. We included in the model the allosteric binding of one Na\(^+\) ion to the Rb-occluded state, 

\[ E_2(Rb^2)ATP. \]

This binding increases the velocity of Rb\(^+\) release because \( k_{doccNa} \) and \( k_{deoc} \) (see Table S1) and is responsible for an important fraction of the ATPase activity given by the physiological cycling mode at [Na\(^+\)] \( \approx 50 \) mM.

Dotted lines in Fig. 9 show the contribution of the physiological cycle to ATPase activity, which is calculated as \( k_{4rb2}f_{p2b2}[E2P] \) (see Fig. 8). As [Rb\(^+\)] tends to infinity, this contribution approaches ATPase activity (i.e. alternative cycling modes tend to vanish).

Conclusion

We characterized the kinetic mechanism of Na\(^+\)/K\(^+\)-ATPase from steady-state determinations. The analytical procedure that we implemented here is also valid for other membrane transport ATPases. Of note, we used a non-compartmentalized enzyme preparation because the transported substances as products do not interfere with the analysis.

To describe non-Michaelis-Menten kinetics, we found it very useful to reparameterize the equation recommended by the International Union of Biochemistry (IUB, now IUBMB), which has not been revised since 1981 (49). We proposed parameters that facilitate predicting the trace of the curve from their values and vice versa. For example, parameter \( a_{Na3} \) in Equation 3 equals the limiting value of Act when [Na\(^+\)] tends to infinity. An approximate value of parameter \( a_{Na3} \) is therefore easy to guess from visual inspection of the results. This facilitates the selection of the equation that best reproduces the results. In contrast, the equation recommended by the IUBMB (Equation 1) contains the parameter \( \alpha_p \), which equals \( a_{Na3}/(K_{Na1}K_{Na2}K_{Na3}) \) and, as a consequence, is difficult to guess an approximate value for.

In addition, we identified parameters that report key features that the reaction scheme must include in order to reproduce the results. The parameters exhibit the same patterns as those of \( k_{cat}/K_m \) and \( K_m \) in the classical Michaelis-Menten equation. Therefore, it is possible to use the same general approach to analyze both Michaelis-Menten and non-Michaelis-Menten kinetics. Of note, the analytical procedure that we described here is a "model-free" strategy (different from posing various models and selecting the one that fits the results).
Analysis of non-Michaelis-Menten kinetics

Table 2
Fitted values ± S.E. of parameters in empirical equations (Equations 11 and 12) to the results obtained at different [Rb+] and [ADP]

| Parameter | Value ± S.E. |
|-----------|-------------|
| K<sub>Na2</sub> | 0.004 ± 0.001 |
| K<sub>Na3</sub> | 0.1 ± 0.2 |
| K<sub>Na4</sub> | 132 ± 40 |
| K<sub>Na5</sub> | 2.3 ± 0.8 |
| K<sub>Na6</sub> | 3 ± 1.2 |
| K<sub>Na7</sub> | 0.2 ± 0.1 |

Table 3
Parameter | Value ± S.E. |
|-----------|-------------|
| a<sub>1</sub> | 0.003 ± 0.002 |
| a<sub>2</sub> | 0.1 ± 0.1 |
| a<sub>3</sub> | 1.1 ± 0.4 |
| a<sub>4</sub> | 0.2 ± 0.2 |

These results provide insights into the kinetic mechanisms of the transport process, highlighting the role of monovalent cations in modulating the activity of the transport system. The data suggest a complex interplay between Na<sup>+</sup> and K<sup>+</sup> in determining the transport kinetics, which is essential for understanding the physiological functions of the transport system. Further studies are needed to elucidate the full extent of these interactions and their implications in vivo.
a binding cavity at the boundary between the P-domain and the transmembrane domain. Other authors also found kinetic evidence for an allosteric site for Na⁺, although they claim that it is on the extracellular face of the pump (48).

Because the allosteric modulation has a significant impact on the catalytic activity of the enzyme, studies of ligands for the allosteric site might have relevant pharmacological interest, whereas detailed kinetic studies in patients with some mental disorders may also lead to insights into the disease. In this sense, it is worth noting that Li⁺, a cation that inhibits Rb⁺ deocclusion (15), leads to an up-regulatory response in the number of pump units in lymphocyte membranes of healthy

Figure 7. Dependence of the parameters $a_{Na^3}/(K_{Na^1} K_{Na^2} K_{Na^3})$ (top) and $K_{Na^1} K_{Na^2} K_{Na^3}$ (bottom) with the fixed concentration of Rb⁺ in the absence (empty symbols) and in the presence of 2 mM ADP (filled symbols). Error bars, S.E. The insets show more clearly the results obtained at low Rb⁺ concentrations.
patients, and this is not observed in patients with maniac-depressive psychosis (50–52). Whether the difference in these patients is due to alterations on the allosteric site requires further investigation.

Experimental procedures

Enzyme

We used a purified preparation of Na\(^+\)/K\(^+\)-ATPase inserted in open membrane fragments. This was obtained from pig kidney red outer medulla by treatment of the microsomal fraction with SDS and extensive washing (53) based on the method of Jorgensen (54). The preparation was kindly provided by the Department of Biophysics, University of Århus, Denmark. Enzyme was quantified by measuring occluded Rb\(^+\) under thermodynamic equilibrium in media containing 250 mM \([\text{RbCl}], 0.25 \text{ mM EDTA, and 25 mM imidazole-}HCl (\text{pH 7.4 at 25 }\circ\text{C}). Under this condition, it is a good approximation to consider that every enzyme molecule occludes two Rb\(^+\) ions.

Using this stoichiometry, we obtained an average value of 2.51 ± 0.09 (nmol of enzyme/mg of protein) from four independent experiments.

Reagents and reaction conditions

\([^{86}\text{Rb}]\text{RbCl}\) and \([\gamma-^{32}\text{P}]\text{ATP}\) were from PerkinElmer Life Sciences. All other reagents were of analytical grade. Experiments were performed at 25 °C in media containing 2.5 mM ATP, 0.25 mM EDTA, 0 or 2 mM ADP, enough MgCl\(_2\) to give 0.5 mM free Mg\(^{2+}\), 25 mM imidazole-HCl (pH 7.4 at 25 °C), and different concentrations of RbCl (instead of KCl), NaCl, and choline chloride so that the total concentration of the last three salts was always 170 mM.

ATPase activity (Act)

We determined ATPase activity from the time course of the release of \([^{32}\text{P}]\text{P}\) from \([\gamma-^{32}\text{P}]\text{ATP}\), as in Monti et al. (33). Briefly, after the incubation time, we quenched the reaction,
extracted the $^{32}$P$_i$ and measured radiation due to the Čerenkov effect in a scintillation counter. Although we avoided hydrolysis of $>10\%$ of the ATP initially present, we always checked that the release of $P_i$ was linear with time to ensure initial rate conditions. These steady-state determinations were made in media containing a protein concentration of 20 $\mu$g/ml and incubation times ranging from 0 to 30 min. Reaction blanks were determined by measuring ATP hydrolysis in media containing 170 mM K$^+$, giving values not significantly different from those at 170 mM Na$^+$ and 1 mM ouabain (a specific inhibitor of Na$^+$/K$^+$-ATPase).

Specific concentration of occluded Rb$^+$ (Occ)

The methodology is described by Rossi et al. (55). Briefly, we employed a rapid-mixing apparatus (SFM4 from Bio-Logic, Seyssinet-Pariset, France) to start, incubate, and then squirt the reaction mixture at 2.5 ml/s into a quenching-and-washing chamber. There, the reaction was stopped by sudden dilution and cooling with an ice-cold solution of 30 mM KCl in 5 mM imidazole-HCl (pH 7.4 at 0 °C) flowing at 40 ml/s through a Millipore-type filter, where the enzyme is retained and washed. We measured the nonspecific $^{86}$Rb$^+$ binding to the filter by omitting the enzyme from the reaction medium. These values were similar to those obtained in the presence of enzyme inactivated either by heat (30 min at 65 °C) or by dilution 1:44 (final concentrations: 36 $\mu$g/ml protein, 6 mM sucrose, 25 mM imidazole-HCl, 0.25 mM EDTA, pH 7.4, at 25 °C) and exposure to a freeze ($-18$ °C, overnight) and thaw cycle. Steady-state determinations were made in media containing a protein concentration of 30–40 $\mu$g/ml after an incubation time of 7 s.

Determination of the apparent velocity constant of Rb$^+$ deocclusion ($k_{app-deocc}$)

This was done by following the time course of Rb$^+$ deocclusion and fitting the equation,

$$\text{Occ}(t) = \text{Occ}_{0} - q e^{-k_{app-deocc}(t+\theta)}$$

(Eq. 10)

where $\theta$ is the time required to quench the reaction ($8.0 \pm 0.9$ ms) and $t$ is the incubation time. We obtained the Rb-occluded intermediates through the direct route of occlusion by incubation of the enzyme at 25 °C in a medium containing 0.10 mM $[^{86}$Rb] RbCl, 0.25 mM EDTA, and 25 mM imidazole-HCl, pH 7.4, for at least 20 min to achieve thermodynamic equilibrium. With the aid of the rapid mixing apparatus, we mixed 1 volume of the suspension (time 0) with 1 volume of media lacking $^{[86}$Rb]RbCl for different incubation times. Final concentrations were 2.5 mM ATP, 0.25 mM EDTA, 0.5 mM free Mg$^{2+}$, 25 mM imidazole-HCl, pH 7.4, 0.05 mM (the minimal concentration attainable after the dilution) or 5 mM RbCl, and different concentrations of NaCl, using choline chloride to keep constant the ionic strength. At the end of the incubation period, we quantified the Rb$^+$ that remained occluded. To determine the Rb$^+$ occluded at time 0, the suspension was diluted in a medium with 0.25 mM EDTA in 25 mM imidazole-HCl, pH 7.4, a condition where Rb$^+$ deocclusion is very slow.
Analysis of non-Michaelis-Menten kinetics

Empirical equations

For each [Rb+], we described steady-state measurements of ATPase activity and occluded Rb+ by empirical equations of the form,

\[
\frac{\text{Act}(\text{Na}^+)}{\text{denom}} = a_{\text{Na}0} + \sum_{j=1}^{n} a_{\text{Na}j} \frac{[\text{Na}^+]^j}{\prod_{g=1}^{j} K_{\text{Na}g}} \quad \text{(Eq. 11)}
\]

and

\[
\frac{\text{Occ}(\text{Na}^+)}{\text{denom}} = a_{\text{Na}0} + \sum_{m=1}^{m} a_{\text{Na}m} \frac{[\text{Na}^+]^m}{\prod_{g=1}^{m} K_{\text{Na}g}} \quad \text{(Eq. 12)}
\]

where

\[
\text{denom} = 1 + \sum_{j=1}^{d} \frac{[\text{Na}^+]^j}{\prod_{g=1}^{j} K_{\text{Na}g}} \quad \text{(Eq. 13)}
\]

and the \(K_{\text{Na}g}\) parameters are apparent stepwise dissociation constants expressed in concentration units, whereas the \(a_{\text{Na}j}\) and \(a_{\text{Na}m}\) parameters are coefficients with units of Act and Occ, respectively. All parameters where simultaneously fitted to the results by weighted nonlinear regression based on the Gauss-Newton algorithm using commercial programs (Excel, SigmaPlot, and Mathematica for Windows). Weighting factors were calculated as the reciprocal of the variance of experimental data. We performed a systematic search of the equations that best describe the results for each [Rb+]. First, we determined the degree of the polynomials in the numerator and the denominator. We restricted \(n \leq d\) and \(m \leq d\), to conform to steady-state equations for enzymes. Second, we evaluated the contribution of terms of intermediate degree by fixing some coefficient values to zero or establishing mathematical constraints between two or more coefficients. The goodness of fit of these equations was assessed by the corrected asymptotic information criterion (56), defined as follows,

\[
\text{AIC}_c = nd \ln \left( \frac{SS_r}{nd} \right) + \frac{2(np + 1)nd}{(nd - np - 2)} \quad \text{(Eq. 14)}
\]

where \(nd\) is the number of data points, \(np\) is the number of parameters to be fitted, and \(SS_r\) is the sum of weighted square residual errors. We selected the equation that gave the minimum \(\text{AIC}_c\) value because it is the one that describes the results using the minimum number of parameters.

We minimized the errors of the parameters by introducing constraints to their fitting values. For a given [Rb+] and [ADP], we obtained good fitting results by setting \(a_{\text{Na}0} = a_{\text{Na}1} = a_{\text{Na}2} (= a_{\text{Na}012})\), \(a_{\text{Na}0} = a_{\text{Na}1} = a_{\text{Na}2} (= a_{\text{Na}012})\), and \(K_{\text{Na}1} = K_{\text{Na}2} (= K_{\text{Na}12})\). These constraints are purely empirical. Additionally, for a given [ADP], we forced all equations to have the same ratio \(a_{\text{Na}3}/(a_{\text{Na}3}/2)\). This constraint did not affect the capacity of equations to reproduce the results. It is based on equations from models such as those in Fig. 2 (see Equations S17 and S22 or Equations S29 and S34) and is independent of the nature of the transport mechanism.

Author contributions—J. L. E. M. contributed to the development of the analytical procedure used here to address non-Michaelis-Menten kinetics, designed and performed the experiments, analyzed the results, and wrote the paper. M. R. M. contributed to the discussion of the results. R. C. R. conceived the study, contributed to the development of the analytical procedure used here to address non-Michaelis-Menten kinetics, analyzed the results, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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