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Steady-state kinetic analysis of the Na^+/K^+-ATPase. The activation of ATP hydrolysis by cations

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We studied the interactions between pairs of cations during activation of the steady-state hydrolysis of ATP of the Na^+/K^+-ATPase. Non-linear regression was used to obtain empirical equations that describe quantitatively the behaviour of the system. The curve relating activity to Na^+ concentration was describable by a Hill equation with $n_H = 2$ and not by the more frequently used expression based on rapid-equilibrium binding of Na^+ to three identical and non-interacting sites. At non-limiting concentrations of the other ligands, changes in the concentration of Na^+ or of Mg^{2+} modified in the same proportion the maximum effects and the apparent affinities of K^+, revealing the operation of either ping-pong or of ordered sequential mechanisms with irreversible steps separating the additions of each ligand. In contrast with this, changes in the concentration of Mg^{2+} altered only the maximum effect of Na^+, indicating that a ternary complex between the cations and the enzyme has to be formed and that certain particular relations have to hold among the rate constants of the system. The interactions described in this paper, together with those previously reported, allowed us to derive a general equation that adequately predicted the response of the Na^+/K^+-ATPase to the concentration of any pair of ligands at non-limiting concentrations of the rest. Confrontation of this equation with computer simulations of the behaviour of the Albers-Post model shows that this model predicts the interactions in which K^+ participates and perhaps also the interaction between Mg^{2+} and Na^+, but seems unable to predict the interactions between pairs of ligands that do not include K^+.

Introduction

In previous communications [1,2] we described the steady-state kinetic behaviour of the Na^+/K^+-ATPase in what regards the interactions between ATP and Na^+, ATP and K^+ and ATP and Mg^{2+} at concentrations of the nucleotide at which only effects on the low-affinity component of the substrate curve are to be expected. This paper extends this study to the interactions between Na^+ and K^+, Na^+ and Mg^{2+} and K^+ and Mg^{2+} during steady-state ATP hydrolysis. The quantitative description of the interactions between Na^+ and K^+ required the previous development of a descriptive equation for Na^+ activation. This proved to be very difficult because of the non-hyperbolic shape of the activation curves, the inhibition by excess Na^+ and the existence of ATP hydrolysis in the absence of either K^+ or Na^+. The problem would have been practically insoluble without the use of non-linear regression procedures.

The results of this paper allowed us to complete the information previously obtained and to develop an equation which describes the steady-state response of the Na^+/K^+-ATPase to any pair of activating ligands at non-limiting concentrations of the rest. This equation was used to confront the actual behaviour of the Na^+/K^+-ATPase with the predictions of the Albers-Post model. Results seem to indicate that the interactions in which K^+ participates are consistent with the current models whereas, with the probable exception of the interaction between Na^+ and Mg^{2+}, those in which K^+ does not participate are not.

Preliminary reports of some of the results presented here have been published [2,3].

Materials and Methods

These as well as the procedures used for the analysis of the results and their limitations have been described in the previous paper of this series [4].

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Results

The kinetics of Na^+ activation

Na^+/K^+-ATPase activity was measured as a function of Na^+ concentration from 0 to 80 mM in media containing 0.5 mM ATP, 0.7 mM $MgCl_2$ (0.3 mM free Mg^{2+}) and 10 mM K^+. Results in Fig. 1 show that the activity raised with the concentration of Na^+ along an S-shaped curve that tended to saturation above 50 mM Na^+.

If each of the possible states of occupation of the three binding sites for Na^+ [5] resulted in an enzymatically active ATPase, the initial velocity as a function of $[Na^+]$ would be given by:

$$v = V_0 + \frac{(V_3 - V_0) + \frac{(V_2 - V_0)K_3}{[Na^+]} + \frac{(V_1 - V_0)K_2K_3}{[Na^+]^2}}{1 + \frac{K_3}{[Na^+]} + \frac{K_2K_3}{[Na^+]^2} + \frac{K_1K_2K_3}{[Na^+]^3}} \quad (1)$$

where K_1, K_2 and K_3 are apparent dissociation constants and V_0, V_1, V_2 and V_3 have units of velocity.

As $[Na^+]$ approaches zero the ratio $(v - V_0)/[Na^+]$ will approach the value of the derivative of v with respect to $[Na^+]$. In Fig. 2 this ratio, measured in media containing 0.05 to 1 mM K^+, is plotted against $[Na^+]$. It can be seen that the points were reasonably well fitted by a straight line whose intercept (0.2 ± 0.13 (μmol · mg^{-1} · min^{-1} · mM^{-1})) seems to be not significantly different from zero. This suggests that it is a good approximation to take as zero the value of $dv/d[Na^+]$ at $[Na^+] = 0$. This would imply that $V_1 \approx V_0$ and hence that activation needs the binding of more than one Na^+ a requirement that fits with the observation that three Na^+ are transported in each hydrolysis cycle [6]. In this case activation would need the binding

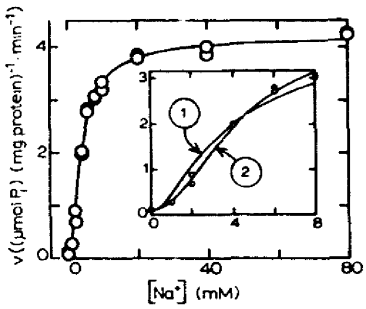

Fig. 1. A plot of Na^+/K^+-ATPase activity as a function of the concentration of Na^+ in media containing 0.5 mM ATP, 0.7 mM $MgCl_2$ and 10 mM K^+. The continuous line is the graphical representation of Eqn. 3 where each parameter was replaced by its best-fitting value. These (±S.E.) were: $V_0 = 0.103 \pm 0.062$ (μmol P_i)·mg^{-1}·min^{-1}, $V_m = 4.03 \pm 0.05$ (μmol P_i)·mg^{-1}·min^{-1}, $K_{Na} = 18.2 \pm 1.4$ mM^2. Inset: The initial part of the activation curve. Curve 1 is the graphical representation of Eqn. 2 where each parameter was replaced by its best-fitting value. Curve 2 has the same meaning for Eqn. 3.

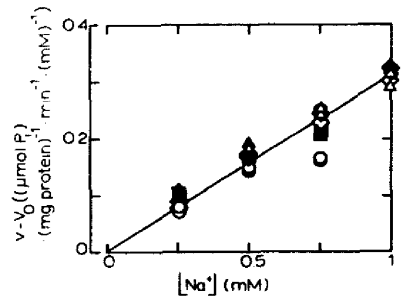

Fig. 2. A plot of the ratio: $(v - V_0)/[Na^+]$ vs. $[Na^+]$, in media containing 0.05 (○), 0.1 (■), 0.25 (△), 0.5 (●) or 1 (◇) mM K^+. The values were calculated from the data in Fig. 3.

of at least 3 Na^+ so that not only V_1 but also V_2 would be equal to V_0.

For these reasons we adjusted to the data an equation like [1] but lacking the terms containing V_1 and V_2. When we did this some of the parameters acquired values that were either negative or not different from zero, indicating that some of them were superfluous. One of the ways of reducing the number of coefficients is to take the denominator of the equation as the expansion of a binomial expression, i.e.:

$$v = V_0 + \frac{V_3 - V_0}{(1 + K_{Na}/[Na^+])^3} \quad (2)$$

Equations like Eqn. 2 with various values for the exponent have been used, both by us and by others, for describing the activation of the Na^+/K^+-ATPase by cations and that of other enzymes by diverse ligands (for examples see Refs. 7–16). Their apparent rightness was taken as evidence of mechanisms requiring the simultaneous occupation of several identical and noninteracting sites. When Eqn. 2 was fitted, the adjustment was biased, the experimental values falling below the best-fitting curve at low $[Na^+]$ and above this curve at high $[Na^+]$ (curve 1 in inset to Fig. 1). The bias disappeared when the exponent was freed to be adjusted by regression, however, under these conditions the best-fitting value of the exponent became 76 and hence devoid of any physical meaning.

After the failure of Eqn. 2, we used Eqn. 1, with $V_1 = V_0$, and systematically omitted some of this terms before fitting it to the data. Best fit was obtained canceling the last two terms of the numerator and the second and fourth terms of the denominator (continuous curve in Fig. 1 and curve 2 in inset to Fig. 1). In this case the exponent remained no significantly different from 2 when freed to be adjusted by the regression procedure. Hence, the following expression was taken as the best description of the activation by Na^+ of the ATPase:

$$v = V_0 + \frac{V_m - V_0}{1 + K_{Na}/[Na^+]^2} \quad (3)$$

Fig. 3. (A and B) A plot of Na^+/K^+-ATPase activity as a function of the concentration of Na^+ in media containing 0.05 (●), 0.1 (□), 0.25 (▲), 0.5 (○) or 1 (▽) mM K^+, 2 mM ATP and of 2 mM $MgCl_2$. The ionic strength was kept constant at 150 mM with choline chloride. The continuous lines are the graphical representations of Eqn. 7 where V_0 was replaced by Eqn. 5 whose parameters were fixed at the best-fitting values given in Fig. 4 and the other parameters were replaced by their best-fitting values. These (±S.E.) were: $V_m = 2.58 \pm 0.05$ (μmol P_i)·mg^{-1}·min^{-1}, $K_{K1} = 0.12 \pm 0.03$ mM, $K_{iNa1} = 3.9 \pm 6.9$ mM, $K_{K2} = 0.0054 \pm 0.0022$ mM, $K_{iNa2} = 1.6 \pm 1.0$ mM, $K_{Na} = 6.98 \pm 0.22$ mM^2. For the sake of clarity the plots were divided into two graphs and the curve with 0.05 mM K^+ was repeated to facilitate the comparison.

where V_0 and V_m are the velocities at zero or saturating $[Na^+]$, respectively, and K_{Na} is an apparent dissociation constant whose relation with K_2K_3 in Eqn. 1 is difficult to ascertain.

Interaction between Na^+ and K^+

In the experiment shown in Fig. 3A and B, Na^+/K^+-ATPase activity was measured as a function of Na^+ concentration, in media containing from 0.05 to 1 mM K^+, and non-limiting concentrations of ATP and of free Mg^{2+}. It can be seen that in all cases Na^+ activated along S-shaped curves, and that at the lower K^+ concentrations activity passed through a maximum and then decreased. The data at each $[K^+]$ were adequately fitted by a modification of Eqn. 3 in which an additional term was included to account for the decrease in activity at high $[Na^+]$, i.e.:

$$v = V_0 + \frac{V_{m,app} - V_0}{1 + K_{Na}/[Na^+]^2 + [Na^+]/K_{iNa}} \quad (4)$$

where $V_{m,app}$ is the maximum rate attainable for a given $[K^+]$ if there were no inhibition by excess Na^+.

In Figs. 4A and B the best-fitting values of the parameters of Eqn. 4 are plotted vs. $[K^+]$. V_0 was a biphasic function of $[K^+]$ expressible as (Fig. 4A):

$$V_0 = v_0 + \frac{(v_1 - v_0)K_2[K^+] - v_0[K^+]^2}{K_1K_2 + K_2[K^+] + [K^+]^2} \quad (5)$$

where the meaning of the v_i's and the K_j's is analogous to that of V_i's and the K_j's in Eqn. 1. Since the plot of K_{iNa} vs. $[K^+]$ approaches a straight line (Fig. 4A) it can be considered as a limiting case of:

$$K_{iNa} = K_{iNa0}(1 + [K^+]/K_K) \quad (6)$$

which would describe the effects of K^+ if inhibition by excess Na^+ were caused by the displacement of K^+.

Fig. 4B shows that $V_{m,app}$ and K_{Na} can be fitted by the same saturable function of $[K^+]$ indicating that in the overall rate equation the functions describing activa-

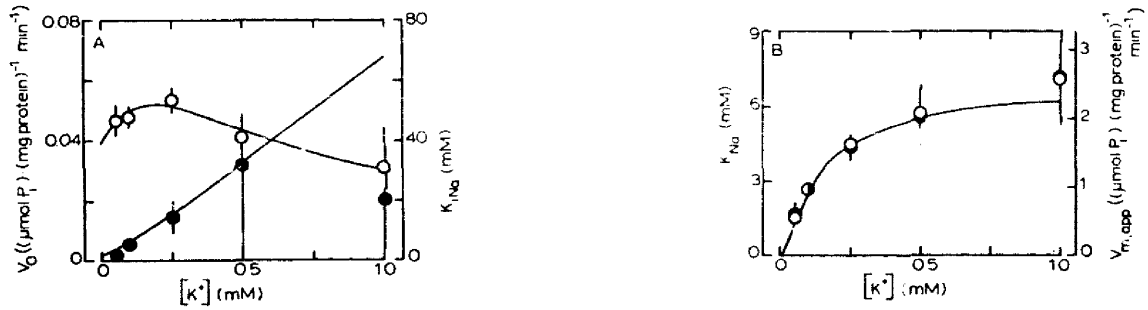

Fig. 4. A plot of the best-fitting values of V_0 (○) and K_{iNa} (●) (A), and of $V_{m,app}$ (○) and K_{Na} (●) (B) as functions of the concentration of K^+. The values were obtained by adjusting Eqn. 4 to each set of data in Fig. 3. The continuous curve that describes V_0 was obtained by adjusting Eqn. 5 to the individual values of V_0. The best-fitting values for the parameters ± S.E. were: $v_0 = 0.039 \pm 0.017$ (μmol P_i)·mg^{-1}·min^{-1}, $v_1 = 0.099 \pm 0.094$ (μmol P_i)·mg^{-1}·min^{-1}, $K_1 = 0.343 \pm 1.193$ mM, $K_2 = 0.411 \pm 0.668$ mM. In the case of V_m, K_{Na} and K_{iNa} the continuous lines were calculated from the best fitting values, obtained from the regression of Eqn. 7 to the whole set of data after reordering this equation to the form of Eqn. 4.

tion by each cation must be separable into different terms of a sum.

Using Eqn. 4, the information of the plots in Figs. 4A and B and including a term of degree 2 for activation by K^+ [17], we obtained the following expression for activity as a function of $[Na^+]$ and $[K^+]$ at non-limiting [ATP] and $[Mg^{2+}]$:

$$v = V_0 + \frac{V_m - V_0}{1 + \frac{K_{K1}}{[K^+]}\left(1 + \frac{[Na^+]}{K_{iNa1}}\right) + \frac{K_{K2}}{[K^+]^2}\left(1 + \frac{[Na^+]}{K_{iNa2}}\right) + \frac{K_{Na}}{[Na^+]^2}} \quad (7)$$

where K_{K1}, K_{K2}, K_{iNa1} and K_{iNa2} are apparent dissociation constants.

Eqn. 7 was fitted to the whole set of data in Fig. 3 using $[Na^+]$ and $[K^+]$ as independent variables. The effects of K^+ on V_0 were included employing as constant terms the best-fitting values of the parameters of Eqn. 5 (see legend to Fig. 4A and B). Otherwise five additional parameters would have had to be fitted to describe an activity whose value does not exceed 2% of the maximal. As it can be appreciated in the continuous lines in Figs. 3A and B and 4A and B, Eqn. 7 fitted not only to the experimental data (Figs. 3A and B) but also described adequately the effect of $[K^+]$ on the parameters of the Eqn. 4 (Figs. 4A and B).

As Eqn. 7 does not take into account the well known competitive effects of K^+ on activation by Na^+ [17], we tested the effect of multiplying K_{Na} by $(1 + [K^+]/K_i)$. No significant improvement was obtained because the best-fitting value of K_i was 45 mM, so that for the Na^+ and K^+ concentrations used in the experiment in Fig. 2 the competitive effects of K^+ were negligible.

Since Eqn. 7 has no terms containing the product of $[Na^+]$ and $[K^+]$ as activators, the following modification of Eqn. 7 was evaluated to test the possible existence of such terms:

$$v = V_0 + \{(V_m - V_0)/(1 + K_{Na}/[Na^+]^2)\} \times \left\{1 + \left[\frac{K_{K1}}{[K^+]}\left(1 + \frac{[Na^+]}{K_{iNa1}}\right) + \frac{K_{K2}}{[K^+]^2}\left(1 + \frac{[Na^+]}{K_{iNa2}}\right)\right] \times \frac{1 + K_{Na'}/[Na^+]^2}{1 + K_{Na}/[Na^+]^2}\right\}^{-1} \quad (8)$$

When $K_{Na'} = 0$, Eqn. 8 will become Eqn. 7 and when $K_{Na'} = K_{Na}$ the functions that contain $[Na^+]$ and $[K^+]$ as activators will become separable into a product, so that activation by each cation will be exerted only on the maximum effect of the other. Apart from these particular cases Eqn. 8 allows the regression procedure to select a continuous range of values of $K_{Na'}$ generating two terms including the product of $[Na^+]$ and $[K^+]$ as activators. This endows Eqn. 8 with an ability that Eqn. 7 lacks for detecting interactions between Na^+ and K^+. In spite of this, when we adjusted Eqn. 8 no improvement of the fit over that obtained with Eqn. 7 was achieved and the best-fitting value of $K_{Na'}$ was not significantly different from zero (0.27 ± 0.21 mM^2), showing that the regression procedure had spontaneously selected Eqn. 7.

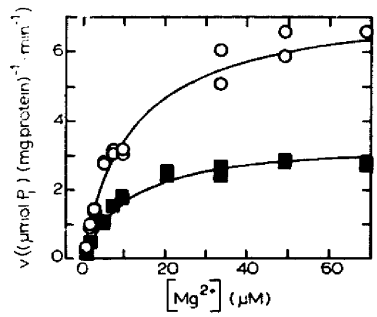

Fig. 5. A plot of Na^+/K^+-ATPase activity as a function of the concentration of free Mg^{2+} in media containing 1 mM ATP, 10 mM K^+ and either 2 (■) or 20 (○) m*M* Na^+. The continuous line is the graphical representation of Eqn. 10 where each parameter was replaced by its best-fitting value. These (± S.E.) were: $V_{max} = 8.45 \pm 0.37$ (μmol P_i)·mg^{-1}·min^{-1}, $K_{Mg} = 11.8 \pm 1.7$ μM, $K_{Na} = 2.78 \pm 0.26$ mM, $[Mg]_0 = 11.1 \pm 4.4$ μM.

It seemed therefore reasonable to conclude that a good description of the interactions between K^+ and Na^+ is provided by an expression lacking terms containing both $[K^+]$ and $[Na^+]$ as activators.

Interaction between Mg^{2+} and Na

Fig. 5 shows the results of an experiment in which Na^+/K^+-ATPase activity was measured as a function of free Mg^{2+} in a 0 to 70 μM concentration range and in media containing non-limiting concentrations of ATP and K^+, and either 2 or 20 mM Na^+. Although the activity is plotted against the concentration of free ionic magnesium (Mg^{2+}), the regression procedure was performed using the total concentration of added magnesium as an independent variable and the concentration of contaminant magnesium as a parameter to be fitted. These were related to free $[Mg^{2+}]$ through:

$$[Mg^{2+}] = \left\{-b + \left[b^2 + 4K_{dATPMg}([Mg]_0 + [Mg]_{add})\right]^{1/2}\right\}/2 \quad (9)$$

where $[Mg]_{add}$ and $[Mg]_0$ are the total concentrations of added and contaminant magnesium, respectively, K_{dATPMg} is the dissociation constant of ATPMg and $b = [ATP] + K_{dATPMg} - [Mg]_{add} - [Mg]_0$. The equation that gave the best fit to the results was:

$$v = \frac{V_m}{(1 + K_{Mg}/[Mg^{2+}])(1 + K_{Na}/[Na^+])} \quad (10)$$

where $[Mg^{2+}]$ must fulfill Eqn. 9, and K_{Mg} and K_{Na}

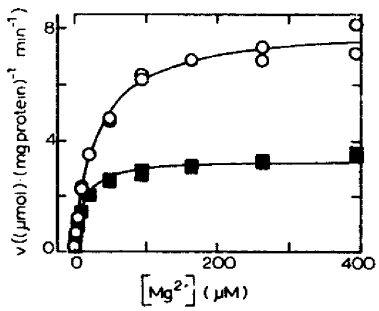

Fig. 6. A plot of Na^+/K^+-ATPase activity as a function of the concentration of free Mg^{2+} in media containing 1 mM ATP, 130 mM Na^+ and either 0.5 (■) or 5 (○) mM K^+. The continuous lines are the graphical representation of Eqn. 11 where each parameter was replaced by its best-fitting value. These (±S.E.) were: $V_m = 9.68 \pm 0.20$ (μmol P_i)·mg^{-1}·min^{-1}, $K_{Mg} = 36.2 \pm 2.7$ μM, $K_K = 0.948 \pm 0.049$ mM, $[Mg]_0 = 13.5 \pm 4.4$ μM.

are the apparent dissociation constants for each ligand. Hyperbolic activation by Na^+ was assumed, however since only two concentrations were tested, any other increasing function of $[Na^+]$ would have fitted equally well.

In Eqn. 10 the functions of the concentration of free Mg^{2+} and of Na^+ are separated into different factors so that changes in the concentration of one of the cations will only influence the maximum effect of the other leaving unaltered its apparent affinity.

Interaction between K^+ and Mg^{2+}

Fig. 6 shows the results of an experiment in which Na^+/K^+-ATPase activity was measured as a function of free Mg^{2+} in a 0 to 400 μM concentration range, in media with either 0.5 or 5 mM K^+, non-limiting concentrations of ATP and Na^+. As in Eqn. 10, the total concentration of magnesium was used as an independent variable and the contaminant magnesium concentration was taken as a parameter to be fitted. The equation that gave best fit was:

$$v = \frac{V_m}{1 + K_{Mg}/[Mg^{2+}] + K_K/[K^+]} \tag{11}$$

where free $[Mg^{2+}]$ must fulfill Eqn. 9 and where K_{Mg} and K_K are the apparent dissociation constant for each ligand. As in the experiment in Fig. 5, hyperbolic activation by K^+ was assumed since only two concentrations of the cation were tested.

In Eqn. 11 the functions of the concentration of free Mg^{2+} and K^+ appear in different terms of a sum so that the same function of the concentration of one of the cations will influence the maximum effect and the apparent affinity of the other.

Discussion

The rate equation for activation by Na^+

Results in this paper suggest that activation by Na^+ is not adequately described by equations that assume that the main species involved in Na^+/K^+-ATPase activity must bind Na^+ in rapid-equilibrium to three identical and non interacting sites [7–12,16].

If we take for granted the fixed 3:1 stoichiometry, the equation that describes activation by Na^+ (Eqn. 3) can be considered a particular case of Eqn. 1, in which not only $V_1 - V_0$ and $V_2 - V_0$ are near zero but also the terms of degree one and three can be ignored because their apparent dissociation constants are very small relative to that of the term of degree two. One, but certainly not the only, explanation for this is provided by the effects of the Na^+-independent steps of the ATPase reaction on the kinetics of activation. If these are included explicitly as a parameter K_t such that when $[Na^+]$ tends to infinity the fraction of the enzyme that is in the state that needs to bind Na^+ becomes $1/(1 + K_t)$ (for a more complete discussion of this see Ref. 10) the equation becomes:

$$v = V_0 + \frac{(V_3 - V_0)}{(f([Na^+]) + K_t)} \tag{12}$$

where $f([Na^+])$ is the denominator of Eqn. 1. It can be shown that as K_t rises the influence of terms of higher degree will progressively increase and that in the limit Eqn. 12 will tend to a Hill equation with an apparent dissociation constant equal to $K_1K_2K_3/(1 + K_t)$ and n_H equal to the number of sites. Although this can be demonstrated analytically, to prove it we used the much simpler procedure of performing graphical simulations of Eqn. 12 for increasing values of K_t (results now shown).

During equilibrium binding, n_H only approaches the number of sites in the limiting case of 'infinite cooperativity'. In our case 'infinite cooperativity' is a kinetic effect of the fixed stoicheiometry which, by allowing only the state with 3 Na^+ bound to be transformed, drives the sates with lesser degree of occupation by Na^+ into the fully occupied state.

Graphical simulations also show that for finite values of K_t, the terms of degree 2 and 3 in $[Na^+]$ in Eqn. 1 will predominate over that of degree one. To check how this operated in our experimental conditions we adjusted to the data of Fig. 1 and equation like Eqn. 2 but with K_t added to the denominator as a parameter to be fitted. The adjustment was as good as that obtained with Eqn. 3 and the best fitting value of K_t was 7. Hence it seems likely that in our system and for the experimental value of K_t, the term of degree two in $[Na^+]$ predominates to such an extent as to become able to describe by itself the results.

A theoretical estimation of the value of K_t can be obtained solving the Albers-Post model for the steady-state concentration of E_1ATP at non limiting $[Na^+]$. When this is done using the scheme in Fig. 5A and the numerical values of Table II of the preceding paper of this series [4] the calculated value of K_t results to be around 7. This suggest that the regression generated a physically reasonable value.

It seems therefore plausible that effects mediated by the Na^+-independent steps of the ATPase reaction may be the cause of the ability of Eqn. 3 to describe the data. This implies also that an identical and non-interacting site hypothesis may still hold for activation by Na^+ but that it has to be modified to take into account the effects of states of the ATPase that do not need Na^+.In the treatment given in Results Eqn. 3 was preferred over Eqn. 12 because the apparent dissociation constants would be implicit functions of K_t, so that Eqn. 3 avoids the endorsement of a yet unproven hypothesis without rejecting the possibility of its existence.

The interactions of activating ligands with the Na^+/K^+-ATPase

In previous studies [1,2] of low-affinity activation of the Na^+/K^+-ATPase by ATP we have shown that K^+ alters in the same proportion V_m and K_m (see also Refs. 18 and 19), while Na^+ and Mg^{2+} only affect the maximum effect of ATP and do not modify the apparent affinity for the nucleotide, ATP exerting a similar effect on the kinetics of activation by Na^+ or Mg^{2+}. It can be demonstrated (Rossi and Garrahan, unpublished) that six different functions of ATP, Na^+, K^+ and Mg^{2+} describe equally well these effects. This indetermination has been removed by the experiments reported in this paper which provide sufficient information as to select among the six equations the only one which accounts for the interactions between any pair of activating ligands at non-limiting concentrations of the rest, during low-affinity activation of the Na^+/K^+-ATPase by ATP, this is:

$$v = V_1 + \{V_{m2}\}\{(1 + K_{m2}/[ATP])(1 + K_{mg}/[Mg^{2+}])$$

$$\times(1 + f_2([Na^+])) + f_1([K^+])\}^{-1} \quad (13)$$

where K_{m2} is the K_m of the low-affinity component of the substrate curve at non-limiting concentrations of the activating ligands, $f_1([K^+])$ and $f_2([Na^+])$ are the functions of these cations in the denominator of Eqn. 7. Except at very low [ATP], V_1 is a minute fraction of the total activity which includes the maximum activity of the high-affinity component of the substrate curve and the activities in the absence of K^+ and/or Na^+. Save for the dead-end effects of Na^+ on K^+ activation, that are included in $f_1([K^+])$, Eqn. 13 does not take into account the inhibition by excess Na^+, K^+ and Mg^{2+} [20,21] and is therefore valid only when these effects are negligible.

The interactions between ligands in the Albers-Post model. To analyze this we performed computer simulations of the effects of activating ligands on the kinetic parameters of this model. For this we employed the version of the Albers-Post model shown in Fig. 5A of the preceding paper of this series [4]. The steady-state rate equation is therefore identical as Eqn. 5 of the preceding paper [4] when $[AN] = 0$ (see also Ref. 22):

$$v = \frac{a[ATP] + b[ATP]^2}{c[ATP]^2 + d[ATP] + e} \quad (14)$$

The meaning of the coefficients is given in Table I of the preceding paper [4]. In terms of these, the parameters that define low-affinity activation by ATP, i.e., V_m and K_{m2} will be b/c and d/c, respectively. In general both will be functions of $[Na^+]$, $[K^+]$ and $[Mg^{2+}]$, for this reason in what follows they will be called $V_{m'}$ and $K_{m2'}$, reserving the expressions without apostrophes for their values at non-limiting cation concentration.

The simulations were run keeping [ATP] high enough as to make negligible the pathways in which k_1 or k_{-1} participate. Following the Albers-Post model, Na^+ and Mg^{2+} were assumed to be exclusive ligands of E_1 or E_1ATP where they promote phosphorylation and K^+ of E_2P where it promotes dephosphorylation. Since we were looking at the nature of the interactions rather than at quantitative predictions, two simplifying assumptions were made. One was that cations bind in rapid equilibrium, this allowed us express their effects multiplying k_2 by increasing functions $[Na^+]$ and $[Mg^{2+}]$, k_4 by an increasing function of $[K^+]$ and k_{-3} and k_{-7} by decreasing functions of the appropriate cation. The other was to consider these functions as hyperbolae that start at or tend to zero and that are half-maximal at 2 mM for Na^+ and K^+ and at 0.1 mM for Mg^{2+}. This ignores the sigmoidicity of some of the responses and the slow phosphorylation and dephosphorylation in the absence of Na^+ or K^+, respectively.

Concerning the effects of Mg^{2+} on k_2, we have already mentioned [23], that if its only activating effect were the promotion of the phopshorylation by ATP acting on k_2 and if, as it seems likely, free ATP and MgATP bound equally well to E_1, then the assumptions stated above would be valid both if the site for Mg^{2+} in E_1ATP were provided by bound ATP or pertained to the enzyme itself. A similar consideration have been recently formulated by Sachs [24].

The interactions in which K^+ participates. The calculated values of V'_m, K'_{m2} (Fig. 7A) and of the $K_{0.5}$ and the maximum effect of Mg^{2+} (Fig. 7B) were plotted vs. $[K^+]$. It is apparent that except at very low concentrations, $[K^+]$ affects in the same proportion the maximum effects and the apparent affinities so that their ratios

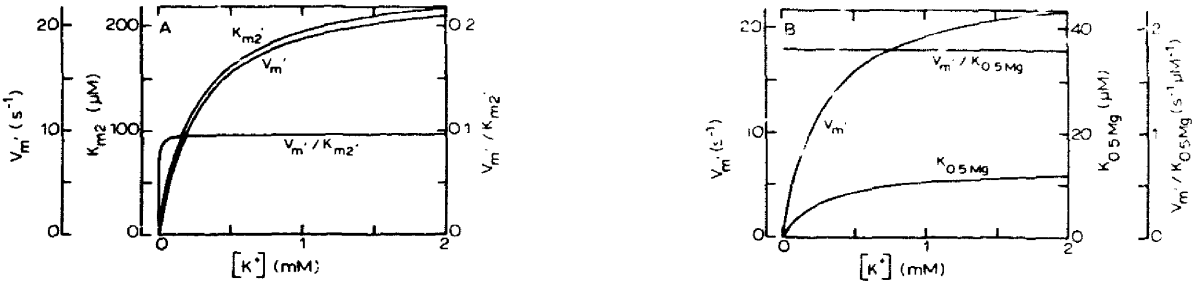

Fig. 7. Simulations of the Albers-Post model. (A) The effect of K^+ on V'_m and K'_{m2}, and on the ratio V'_m/K'_{m2}. (B) The effect of K^+ on the apparent values of V'_m and the $K_{0.5}$ for free Mg^{2+}, and on the ratio $V'_m/K_{0.5}$, assuming that the rest of the ligands are saturating.

remain independent of $[K^+]$. Similar results (not shown) were obtained plotting the maximum effect, and the $K_{0.5}$ for Na^+. The deviation of V'_m/K'_{m2} from a constant value at very low $[K^+]$ (Fig. 7A) expresses the existence of an ATP-independent pathway for the $E_2 \rightarrow F_1$ transition which is postulated by the model. Since this pathway is not operative at saturating [ATP] the deviation is not seen in the simulation of the interactions between K^+ and Mg^{2+} (Fig. 7B). It seems therefore that the Albers-Post model adequately predicted the experimental behaviour (Eqn. 13) for the interactions between K^+ and any of the other ligands tested.

The behaviour simulated in Figs. 7A and B occurs when ligands bind in an obligatory order in steps separated by irreversible reaction(s). This may happen in 'ping-pong kinetics', in which the release of one ligand precedes the addition of the other or in 'ordered sequential kinetics' in which a ternary complex is formed [25]. In the latter case a rate equation for initial velocity in the absence of products which is not distinguishable from that of ping-pong kinetics will be obtained if an additional irreversible step is interposed between the reversible ordered addition of two ligands to form the ternary complex. This case is not treated explicitly in Ref. 25.

The Albers-Post model assumes ping-pong kinetics when it postulates that Na^+ is released before K^+ is added. In the absence of ADP and inorganic phosphate the binding of these cations is separated by irreversible steps. However the experimental and the simulated results are also consistent with mechanisms in which activation by Na^+ and K^+ occurs via the sequential ordered formation of a ternary complex between Na^+, K^+ and the ATPase. Mechanisms of this kind have been postulated by several authors [16,26].

Mechanisms in which ternary complexes participate are assumed by the Albers-Post model for the addition of K^+ and Mg^{2+} [27] and of K^+ and ATP [19]. In these the binding of K^+ and Mg^{2+} is separated by the same irreversible steps as the binding of Na^+ and K^+ whereas the irreversible steps that separate the binding of K^+ and of ATP are those governed by k_4 which becomes irreversible in the absence of P_i and by k_7 which becomes irreversible in the presence of saturating concentrations of Na^+ and Mg^{2+}.

It is important to notice that the agreement between the predicted and the experimental behaviour does not rely on the numerical values of rate and equilibrium constants and is therefore independent of the actual values of the constants and hence not submitted to the uncertainties mentioned in Materials and Methods of Ref. 4.

It could be argued that our kinetic treatment, which is based on equations for initial velocity in the absence of products, is not suitable for describing our system because as it is 'unsided' 'products', that is the cations acting after being released will be always present at the same concentration as the 'substrates', that is the cations that activate the reaction. This argument, however, seems to be wrong. Products modify the kinetic behaviour if they convert into reversible those steps that are irreversible in their absence. In the treatment of the preceding paragraphs the steps that have to be irreversible to comply with our reasoning are so because of the absence or ADP or P_i and/or because of the displacement between conformers and not because of the absence of cations as 'products'.

What becomes very difficult in an 'unsided' preparation is to use the kinetics of product inhibition to

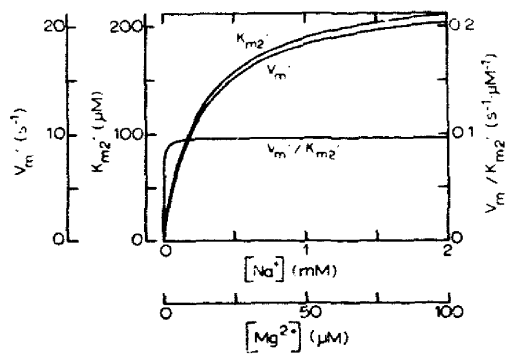

Fig. 8. Simulations of the Albers-Post model. The effect of either Na^+ or Mg^{2+} on V'_m and K'_{m2} and on the ratio V'_m/K'_{m2}, assuming that the rest of the ligands are saturating.

discriminate between ping-pong and ordered sequential mechanisms [25]. In fact if the effects of P_i and/or ADP were tested we would have to deal not only with product inhibition by these metabolites but also with product inhibition by cations with the additional complication that in the case of the cations the concentration of 'products' cannot be manipulated independently of the concentration of 'substrates'. For these reasons we did not attempt to use product inhibition to discriminate among mechanisms.

The interactions in which K^+ does not participate. In Fig. 8 the calculated values of V'_{m2} and K'_{m2} were plotted against $[Na^+]$ or $[Mg^{2+}]$. It is apparent that the ratio V'_{m2}/K'_{m2} is independent of $[Na^+]$ or $[Mg^{2+}]$ indicating that the Albers-Post model predicts that maximum effects and apparent-affinities are altered in the same proportion, the deviation at low Na^+ or Mg^{2+} having the cause already mentioned in connection with Fig. 7A. This behaviour results from the postulates of the model that the binding of Na^+ and Mg^{2+} at E_1 is separated from that of ATP at E_2 by the irreversible steps governed by k_2, k_4 and k_7. It is therefore independent of the actual values of the constants.

The predictions of the Albers-Post model on the interactions between Mg^{2+}, Na^+ and ATP are in sharp disagreement with the experimentally observed behaviour in which only maximum effects are modified and apparent affinities remain constant (Eqn. 13). In most cases lack of interactions between pairs of ligands requires the reversible formation of a ternary complex between the enzyme and the ligands, the absence of products and a series of additional restrictions which are detailed in the Appendix. The formation of ternary complexes between Na^+, Mg^{2+} and the E_1 conformer of the ATPase is a postulate of the Albers-Post model. Current experimental evidence does not allow to determine if the additional restrictions described in the Appendix are satisfied. Therefore it is not yet possible to decide whether lack of interactions between Na^+ and Mg^{2+} generates contradictions with the postulates of the Albers-Post model. For this reason these interactions were not simulated.

Regarding the interactions between Mg^{2+} or Na^+ with ATP, they would seem to require a link between the ATP-dependent release of K^+ from E_2K and complexes of this conformer with ATP, Na^+ and Mg^{2+}. This is not contemplated by the Albers-Post model. Both Plesner and Plesner [16] and Nörby [26] have proposed kinetic schemes that approach these requirements assuming that the ATP-dependent release of occluded K^+ occurs in a Na^+-bound form of the enzyme. This view however seems not to be supported by studies on elementary steps which show that neither Na^+ nor Mg^{2+} are needed for the release of occluded K^+ by ATP (Ref. 28, but see Ref. 29). We have proposed elsewhere [1] that this process might not require by itself the binding of Na^+ and Mg^{2+}, but that only when the $Na^+ + Mg^{2+}$ bound forms participate, the resulting products will be in conditions to undergo the further transitions needed for net turnover. Since there is no experimental proof for or against this view it remains an open question whether or not the incapability of the Albers-Post scheme to explain the interactions between Na^+ and ATP and Mg^{2+} and ATP represents an intrinsic flaw of the model or can be corrected modifying its usual assumptions for the cation requirements of the elementary steps.

Appendix

In complex systems like the Na^+/K^+-ATPase a detailed quantitative knowledge of the equilibrium and rate constants involved in the reactions is required to define the causes for the constancy in apparent affinities. In this respect mechanisms that generate this behaviour differ from those that give proportional modifications in maximum effects and apparent affinities which are definable knowing the order of addition of ligands and which steps are irreversible.

The absence of interactions in apparent affinities

In single-step rapid-equilibrium kinetics the meaning of the constancy in affinities is obvious but in more complex reactions explanations may be very intricate. Since the general analysis of this is beyond the scope of this paper we will only examine schemes (Figs. 9A and B) in which two ligands (A and B) bind to the enzyme which then passes through a state E(AB) whose transitions are independent of the ligands in the media. These schemes can be considered simplified versions of the interactions of between pairs of ligands with the ATPase and are therefore applicable to those interactions that take place without changes in apparent affinities (i.e., Na^+/ATP and Mg^{2+}/ATP).

It can be proved that there is no general way to keep constant the apparent affinities if one of the ligands is released before the addition of the other so that the

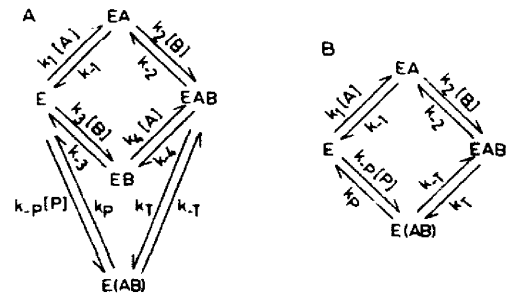

Fig. 9. (A) A random and (B) a sequential mechanism for the binding of two ligands (A and B) to an enzyme which before catalysis passes through a state (E(AB)) which does not require ligands in the solution.

EAB complex is not formed. Hence in these systems the formation of ternary complexes as shown in Figs. 9A and B is mandatory.

The apparent equilibrium constant (K_{mA}) for the dissociation of A during steady-state enzymic activity can be expressed as:

$$K_{mA} = [A]_{free} \times \frac{([E]+[EB]+[EA']+[EAB']+[E(AB)'])}{[EA]+[EAB]+[E(AB)]-([EA']+[EAB']+[E(AB)'])} \quad (15)$$

where the terms in which the ligands appear within parenthesis are the states of the enzyme whose transitions do not need free ligands, and the terms with apostrophes are the contribution to the concentration of the corresponding species of the pathways not requiring the binding of A. Eqn. 15 contains all conceivable states of binding of E with A and B. Depending on the actual reaction mechanism some of these states may be absent. Obviously K_{mA} will be independent of [B] if the ratio in Eqn. 15 also fulfills this condition. An analogous expression can be derived for K_{mB} and in general for each K_m in any multi-reactant system in which the K_m values are independent of the concentration of their corresponding ligands (Rossi and Garrahan, unpublished).

As shown in Figs. 9A and B the EAB complexes may arise by random or by ordered binding of ligands. Among the former only non-equilibrium random binding can be excluded beforehand, since in it the shape of activation curve of one of the ligands will depend on the concentration of the other [25].

(a) Random rapid-equilibrium binding of both ligands. Using Eqn. 15 for the scheme in Fig. 9A:

$$K_{mA} = K_{sA}\frac{1+[B]/K_{sB}+k_{-P}[P]/(k_P+k_{-T})}{1+\{1+k_T/(k_{-T}+k_P)\}[B]/(\alpha K_{sB})} \quad (16)$$

where α measures the change in each K_s when the other ligand binds to the enzyme. Since only a singular value of [P] could make K_{mA} independent of [B], the general treatment applies when there are no effects of products, i.e.: [P] = 0 or $k_{-P} = 0$. Eqn. 16 shows that if $\alpha = 1$ (no interactions), as the concentration of the other ligand goes from zero to infinity each K_m will decline from K_s to $K_s/[1 + k_T/(k_{-T} + k_P)]$ [23]. Hence for the ratio of each ligand to be independent of the concentration of the other, $k_T \ll k_{-T} + k_P$, that is the steady-state concentration of E(AB) must be negligible. On the other hand, if $\alpha = 1 + k_T/(k_{-T} + k_P) > 1$ (negative interactions) the terms containing [B] in Eqn. 16 will be canceled out, there will be no interactions in apparent affinities and each K_m will become equal to its respective K_s. The expression will be more complicated when several intermediates follow the formation of the ternary complex. To yield independence in apparent affinities negative interactions have to be indirect so as to increase the value of the dissociation constants from K_s to αK_s. This excludes competitive effects in which K_m values increase without bounds.

(b) Random rapid-equilibrium binding of one of the ligands. If the addition of only one of the ligands (say A) did not take place in rapid-equilibrium and if the binding of B did not affect the rate constants for the addition and release of A, the following expression for K_{mA} can be derived from Eqn. 15 when [P] or k_{-P} are 0:

$$K_{mA} = K_{sA}\frac{1+[B]/K_{sB}+[k_T/(k_{-T}+k_P)]k_P/k_{-1}}{1+[B]/K_{sB}+k_T/(k_{-T}+k_P)} \quad (17)$$

If $k_P = k_{-1}$, K_{mA} will become independent of [B] and equal to K_{sA}, in spite of the fact that A does not bind in equilibrium. Under these conditions:

$$K_{mB} = K_{sB}(k_P + k_{-T})/(k_T + k_P + k_{-T}) \quad (18)$$

which shows that the K_m of the ligand that binds in rapid-equilibrium will be smaller than its K_s by a factor that is proportional to the relative abundance of E(AB).

We have shown elsewhere [23] that a mechanism like that described by Eqns. 17 and 18 explains both the lack of interactions between Mg^{2+} and ATP and the high apparent affinity for activation by Mg^{2+} of the Na^+-ATPase activity.

(c) Ordered addition of the ligands. In this case (Fig. 9B) both ligands may bind away from equilibrium but no irreversible step must separate their addition. Using Eqn. 15 with [EB] = 0, K_{mA} when [P] or k_{-P} are 0 will be:

$$K_{mA} = K_{sA}\frac{k_T k_P + k_{-2}(k_{-T}+k_P) + k_2[B]k_T k_P/k_{-1}}{k_T k_P + k_{-2}(k_{-T}+k_P) + k_2[B](k_T + k_{-T} + k_P)} \quad (19)$$

If $k_T k_P/k_{-1} = k_T + k_{-T} + k_P$ then K_{mA} will become independent of [B] and equal to K_{sA}. If the steady-state distribution heavily favored E(AB) ($k_T \gg k_{-T} + k_P$), it would suffice that k_P be equal to k_{-1}, for K_{mA} to be equal to K_{sA}. In this case the requisites for non-interaction in the sequential model become akin to those of the random model with steady-state binding of one of the ligands.

Under conditions that give no interactions the K_{mB} will be:

$$K_{mB} = K_{sB}(1 + k_{-T}/k_P + k_T/k_{-2})/(1 + k_{-T}/k_P + k_T/k_P) \quad (20)$$

which shows that depending on whether k_{-2} is smaller, equal or greater than k_P, K_{mB} will be greater, equal or smaller than K_{sB}, respectively.

The distinction among mechanisms

If detailed information were available, the following experimental criteria would be sufficient to discriminate among the mechanisms analyzed above:

(i) In the cases analyzed under (a) and (c) the conditions would be valid for any number of intermediates. For (b) lack of interactions in apparent affinities requires the existence of only one form of E(AB) or, as an approximation, if more than one form were involved that the steady-state distribution among them to strongly favour that which gives the products.

(ii) In the case analyzed in (a) both K_m values will be equal to their K_s values. In the two other K_{mA} will be equal to its K_s, but in case (b) K_{mB} will always be smaller than K_{sB} and in case (c) there will be no general rules.

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