Biochimica et Biophysica Acta, 981 (1989) 105–114
Elsevier

BBA 74399

Steady-state kinetic analysis of the Na^+/K^+-ATPase. The inhibition by potassium and magnesium

Rolando C. Rossi and Patricio J. Garrahan *

Instituto de Química y Fisicoquímica Biologicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Buenos Aires (Argentina)

(Received 23 January 1989)

Key words: ATPase, Na^+/K^+-; Sodium pump; Kinetics; Potassium ion; Magnesium ion

At micromolar ATP, low (< 5 mM) concentrations of K^+ activate the Na^+/K^+-ATPase to an extent that is substantially reduced compared to that observed at more physiological concentrations of the nucleotide. At higher concentrations of K^+, activation is replaced by partial inhibition. Inhibition is not due to the displacement of Na^+ by K^+, its main causes being a decrease of the V_m of the high-affinity component and the increase in the apparent K_m of the low-affinity component of the substrate curve of the Na^+/K^+-ATPase. The apparent affinity for inhibition by K^+ is highly dependent on Mg^{2+}. In the presence of an excess K^+, Mg^{2+} decreases towards zero the V_m of the high-affinity component and acts as a dead-end inhibitor of the low-affinity component of the substrate curve of the Na^+/K^+-ATPase. These results can be explained assuming that binding of an additional K^+ to the E_2 conformer of the Na^+/K^+-ATPase allows low-affinity binding of Mg^{2+} with the formation of a dead-end complex. In the case of Na^+-ATPase activity and for concentrations of ATP within the range of the substrate curve of this activity (0–2.5 μM), Mg^{2+} in concentrations up to 60 mM has no effect on ATPase activity at high (100 mM) $[Na^+]$. At lower $[Na^+]$, Mg^{2+} becomes a low-affinity inhibitor of Na^+-ATPase. Inhibition follows a pattern that is different from inhibition of Na^+/K^+-ATPase activity and is consistent with a mechanism in which Mg^{2+} acts both as a dead-end and as product inhibitor.

Introduction

It is currently believed that at low concentrations of ATP, K^+ inhibits the Na^+/K^+-ATPase through the same mechanism which at more physiological concentrations of ATP leads to activation [1,2]. The first experiments of this paper reexamine this question comparing Na^+-ATPase activity with the high-affinity component of the Na^+/K^+-ATPase activity. The results indicate that activation by K^+ albeit drastically reduced is still present at low conentrations of ATP and that inhibition by K^+ is the expression of a different mechanism than that which leads to activation.

Experiments in this paper also analyze the inhibition by excess K^+ of the low affinity component of the substrate curve and the effects of Na^+, Mg^{2+} and ATP on this phenomenon. A partial inhibition by excess K^+ is described. The inhibition is independent of Na^+ concentration, highly dependent on Mg^{2+} and surmountable by ATP. Finally inhibition of excess Mg^{2+} of Na^+-ATPase activity is also studied.

Correspondence: P.J. Garrahan, IQUIFIB, Facultad de Farmacia y Bioquímica, Junin 956, 1113 Buenos Aires, Argentina.

Materials and Methods

These as well as the procedures used for the analysis of the results and their limitations have been described in the first paper of this series [3].

Results

The effect of K^+ on the initial part of the substrate curve of the Na^+/K^+-ATPase

The experiment shown in Fig. 1, compares the response of the ATPase to very low concentrations of ATP in media with either 0, 2.5 or 30 mM K^+. The data were obtained at the same time and on the same enzyme preparation. It can be seen that even at the lowest ATP concentration tested (0.034 μM), the activity in media with 2.5 mM K^+ was higher than that in the absence of K^+ (Na^+-ATPase). In contrast with this in media with 30 mM K^+ the activity was less than Na^+-ATPase activity at ATP concentrations below 1 μM ATP and lower than the activity in media with 2.5 mM K^+ at all ATP concentrations tested.

While Na^+-ATPase activity follows Michaelis-Menten kinetics, the substrate curve of the Na^+/K^+-

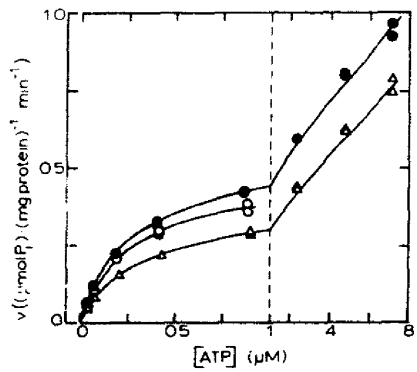

Fig. 1. A plot of ATPase activity as a function of the concentration of ATP in media containing 130 mM NaCl, 0.3 mM $MgCl_2$ and either 0 (○), 2.5 (●) or 30 (△) mM KCl. The ionic strength was kept constant at 160 mM with choline chloride. The continuous lines are the graphical representation of either a Michaelis-Menten equation (○) or of Eqn. 3 (●, △), for the values of the parameters given in Table I. Notice that the concentration scale changes at 1 μM.

ATPase is biphasic and can be described by a rational expression of degree two in [ATP] (see Ref. 4 and Eqn. 14 in Ref. 5). As we have shown in detail elsewhere [6] it is mathematically equivalent to convert the rational expression into the sum of two Michaelis-Menten equations or into:

$$v = \frac{V_{m1}}{1 + K_{m1}/[ATP] + [ATP]/K_{m2}} + \frac{V_{m2}}{1 + K_{m2}/[ATP](1 + K_{m1}/[ATP])} \quad (1)$$

A physical interpretation of Eqn. 1 in terms of the Albers-Post model is given in the first part of Discussion. The relationship between Eqn. 1 and the sum of two Michaelis-Menten equations can be easily grasped if we realize that both become rational expressions of degree two in [ATP] when their right-hand side terms are added by taking common denominator.

TABLE I

Comparison between the best-fitting values of V_m and K_m for the Na^+-ATPase and V_{m1} and K_{m1} for the high-affinity component of the Na^+/K^+-ATPase obtained from the data in Fig. 1

Values given as means ± S.E.

$[K^+]$ (mM)	V_{mNa} or V_{m1} (μmol·mg^{-1}·min^{-1})	K_{mNa} or K_{m1} (μM)	Slope (V_{m2}/K_{m2}) (μmol·mg^{-1}·min^{-1}·μM^{-1})
0	0.480 ± 0.029	0.253 ± 0.024	-
2.5	0.439 ± 0.013	0.189 ± 0.009	0.074 ± 0.003
30	0.279 ± 0.011	0.191 ± 0.012	0.069 ± 0.003

Since K_{m2} is around 200 μM, no significant error will be incurred in the experiment in Fig. 1 by omitting $[ATP]/K_{m2}$ from the first term and the first '1' from the second term of the right-hand side of Eqn. 1. This allows to rearrange Eqn. 1 as the sum of a Michaelis-Menten equation and a linear function of [ATP], i.e.:

$$v = \frac{V_{m1} - V_{m2}K_{m1}/K_{m2}}{1 + K_{m1}/[ATP]} + (V_{m2}/K_{m2})\,[ATP] \quad (2)$$

The data in the presence of K^+ were adjusted by Eqn. 2 and those in the absence of K^+ by a Michaelis-Menten equation. The best-fitting values of the parameters are given in Table I. It can be seen that at 2.5 mM K^+, V_{m1} was slightly lower than V_{mNa} and K_{m1} was significantly less than K_{mNa}. The weighted average of the ratio V_{m1}/V_{mNa}, calculated from the experiment in Fig. 1 and two additional independent experiments, was 1.058 ± 0.127 indicating that there was no significant difference between these parameters at 2.5 mM K^+. This together with the fact that K_{m1} is less than K_{mNa} and that the linear term in Eqn. 2 is always present, makes it clear that at 2.5 mM K^+, Na^+/K^+-ATPase activity will surpass Na^+-ATPase activity at any [ATP]. Table I also shows that, in the media with 30 mM K^+, V_{m1} is about 40% less than V_{mNa}, explaining why under

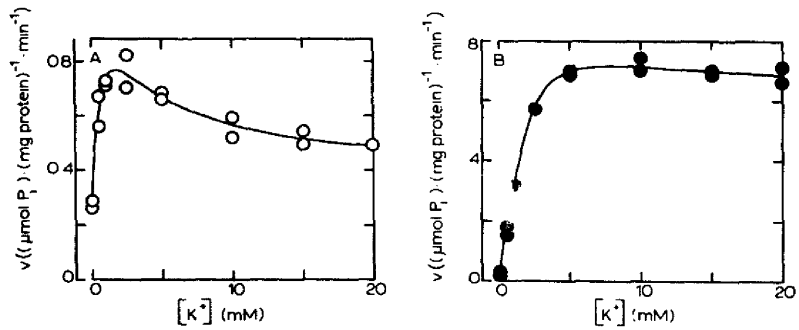

Fig. 2. A plot of ATPase activity as a function of the concentration of K^+ in media containing 130 mM NaCl and either 0.020 mM ATP and 0.3 mM $MgCl_2$ (0.285 mM free Mg^{2+}) (A) or 3 mM ATP and 3 mM $MgCl_2$ (0.470 mM free Mg^{2+}) (B). The ionic strength was kept constant at 151 mM with choline chloride. The continuous line for 0.020 mM ATP is the graphical representation of Eqn. 1 for the best-fitting values of its parameters. These (± S.E.) were: $V_{Na} = 0.27 \pm 0.03$ (μmol P_i)·mg^{-1}·min^{-1}, $V_{mK} = 1.30 \pm 0.50$ (μmol P_i)·mg^{-1}·min^{-1}, $V_r = 0.38 \pm 0.07$ (μmol P_i)·mg^{-1}·min^{-1}, $K_a = 0.97 \pm 0.79$ mM, $K_i = 2.57 \pm 2.73$ mM.

these conditions the activity was lower than those in media with either 0 or 2.5 mM K^+.

It would seem therefore that even at very low ATP, K^+ activates at low concentrations and that inhibition only becomes manifest at higher concentrations of the cation.

The inhibition of the low-affinity component of the substrate curve by K^+

In the experiments shown in Figs. 2A and B, Na^+/K^+-ATPase activity was measured as a function of K^+ concentrations in a 0–20 mM range, in media with either 0.02 or 3 mM ATP. In the media with 0.02 mM ATP (Fig. 2A), the effect of K^+ was clearly biphasic: activity first raised over that of the Na^+-ATPase and then decreased passing through a maximum. The following equation described the results:

$$v = \frac{V_{Na}K_aK_i + V_{mK}K_i[K^+] + V_r[K^+]^2}{K_aK_i + K_i[K^+] + [K^+]^2} \quad (3)$$

where K_a and K_i are apparent dissociation constants for activation and inhibition, respectively, V_{Na} is the activity in the absence of K^+ (Na^+-ATPase), V_{mK} the maximum effect of K^+ if there were no inhibition and V_r the activity when $[K^+]$ tends to infinity. The best-fitting values of these parameters are given in the legend of Fig. 2. It can be seen that V_r was significantly larger than zero strongly suggesting that K^+ is only a partial inhibitor. Confrontation of the results in Fig. 2A with those of Fig. 2B show that at 3 mM ATP activation by K^+ over Na^+-ATPase activity was much larger and inhibition by excess K^+ was almost absent.

To study in more detail the effect of ATP we measured Na^+/K^+-ATPase activity as a function of ATP concentration in a 0.090 to 0.760 mM range in media containing from 2.5 to 50 mM K^+. In all conditions $[ATP] \gg K_{m1}$ so that no appreciable error is committed if the terms: $K_{m1}/[ATP]$ in Eqn. 1 are canceled. Taking into account that $(1 + [ATP]/K_{m2})^{-1} = 1 - (1 + K_{m2}/[ATP])^{-1}$ the equation can be rearranged to approach the sum of a constant term and a Michaelis-Menten equation, i.e.:

$$v \approx V_{m1} + (V_{m2} - V_{m1})/(1 + K_{m2}/[ATP]) \quad (4)$$

Notice that at non-limiting [ATP] the activity will be independent of V_{m1}. In Fig. 3 the best-fitting values of V_{m1}, V_{m2} and K_{m2} for each curve are plotted vs. $[K^+]$. It can be seen that V_{m1} first increased and then decreased, a result which is the expected from the experiment in Fig. 1, whereas V_{m2} and K_{m2} increased along curves that reached saturation at 5 and 20 mM K^+, respectively. The increase of both parameters at low $[K^+]$ expresses the activating effect of the cation [7]. The rise in K_{m2} after V_{m2} has reached saturation will

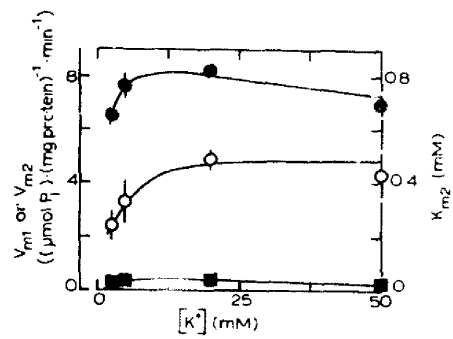

Fig. 3. A plot of the best-fitting values (± S.E., vertical bars) of V_{m1} (■), V_{m2} (●), and K_{m2} (○) (Eqn. 4) vs. the concentration of K^+. The data were obtained measuring Na^+/K^+-ATPase activity as a function of the concentration of ATP in a 0.090 to 0.760 mM range, in media containing 100 mM NaCl, enough $MgCl_2$ as to keep free $[Mg^{2+}]$ constant at 0.4 mM and either 2.5, 5, 20 or 50 mM KCl. The ionic strength was kept constant at 151 mM with choline chloride.

cause an ATP-surmountable inhibition of the low-affinity component of the substrate curve. The inhibition will be only partial since K_{m2} tends to a finite value instead of growing indefinitely as $[K^+]$ increases.

Figs. 4A and B show the results of an experiment in which Na^+/K^+-ATPase activity was measured as a function of the concentration of K^+ in media containing from 0.005 to 0.04 mM ATP (Fig. 4A). Eqn. 3 was adjusted to the data and the best-fitting values of K_i, V_{mK} and V_r were plotted vs. [ATP] (Fig. 4B). It can be seen that K_i was an hyperbolic and that V_{mK} and V_r were approximately linear functions of [ATP]. The response of K_i was the expected if inhibition by K^+ were exerted through a saturable increase in K_{m2} (see experiments in Fig. 3). On the other hand since $[ATP] \ll K_{m2}$, and at non-limiting [ATP] inhibition disappears: V_{mK}, V_r, and V_{m2} should have the same value and the slopes of the approximately straight lines that fit V_{mK} and V_r will approach the ratio V_{m2}/K_{m2}. Therefore the fact that the slope of V_i vs. $[K^+]$ is considerably less than that of V_{mK} vs. [ATP] implies that when $[K^+]$ tends to infinity K_{m2} acquires a finite value which is larger than that obtainable at optimal concentrations of $[K^+]$. This is also in agreement with the observation of the experiment in Fig. 3. Therefore the experiment in Fig. 4 provides independent evidence that K^+ at high concentrations is partial inhibitor acting on K_{m2}.

The effect of Na^+ on inhibition by K^+

In the experiment shown in Fig. 5A Na^+/K^+-ATPase activity was measured as a function of the concentration of Na^+ in media containing 20 μM ATP and either 2.5 or 20 mM K^+. It is clear from the constancy of the ratio between the activities at 20 and at 2.5 mM KCl (inset to Fig. 5A), the degree of inhibition by K^+ is independent of the concentration of Na^+. In the experiment shown in Fig. 5B ATPase activity was measured as

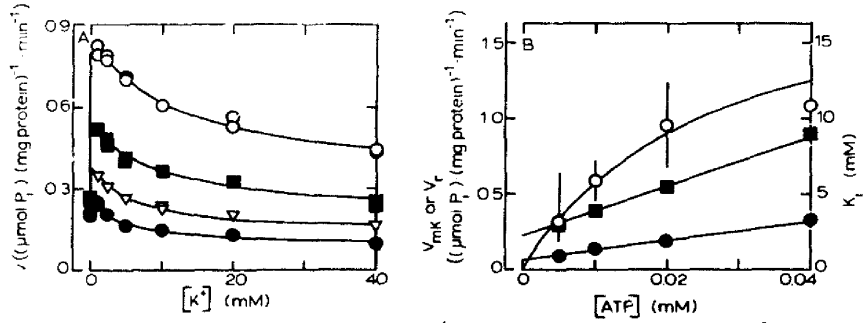

Fig. 4. (A) A plot of ATPase activity as a function of the concentration of K^+ in media containing 130 mM NaCl, 0.3 mM $MgCl_2$ and either 0.005 (●), 0.10 (▽), 0.20 (■) or 0.040 (○) mM ATP. The ionic strength was kept constant at 170 mM with choline chloride. The continuous lines for each ATP concentration are the graphical representation of Eqn. 1 for the best-fitting values of the parameters. (B) A plot the best-fitting values for K_i (○), V_{mK} (■) and V_r (●) (±S.E., vertical bars) as a function of the concentration of ATP.

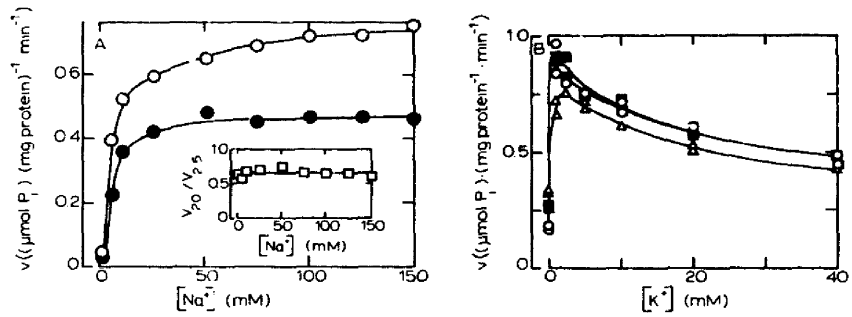

Fig. 5. (A) A plot of Na^+/K^+-ATPase activity as a function of the concentration of Na^+ in media containing 0.020 μM ATP, 0.3 mM $MgCl_2$ and either 2.5 (○) or 20 (●) mM KCl. The ionic strength was kept constant at 171 mM with choline chloride. Inset: A plot of the ratio between the activities at 20 and at 2.5 mM KCl. (B) ATPase activity as a function of the concentration of K^+ in media containing 0.010 mM ATP, 0.3 mM $MgCl_2$ and either 50 (○), 100 (■) or 150 (△) mM NaCl. The ionic strength was kept constant at 191 mM with choline chloride.

a function of the concentration of K^+ in media with 10 μM ATP and either 50, 100 or 150 mM Na^+. It can be seen that the K_i for K^+ (Eqn. 3) is independent of the concentration of Na^+.

The results in Fig. 5A and 5B make it evident that neither the degree nor the apparent affinity for inhibition by excess K^+ is affected by Na^+. This allowed us to exclude displacement of Na^+ by K^+ from its activating sites as the cause of this phenomenon.

The effect of Mg^{2+} on the inhibition by K^+

Na^+/K^+-ATPase was measured as a function of the concentration of K^+ in media with 0.010 mM ATP and either 0.02, 0.3 or 5 mM free Mg^{2+} (Fig. 6). Notice that that the inhibitory effects of free Mg^{2+} are especially conspicuous at concentrations of Mg^{2+} at which practically all the ATP will be as MgATP, which makes it unlikely that the observed effects are caused by variations in the ratio between free ATP and MgATP. Eqn. 3

TABLE II

The effect of Mg^{2+} on the best-fitting values obtained from the regression of Eqn. 3 to the data in Fig. 6

Because of the distribution of experimental points as [Mg^{2+}] increased the precision of the estimates of K_i and V_r increased and that of K_a decreased. Notice that for 0.3 and 5.00 mM [Mg^{2+}] the K_i values are considerably smaller than 40 mM (the highest [K^+] tested in Fig. 6) which explains why for these concentrations V_r is similar as the observed velocities at 40 mM [K^+]. This is not the case for the experiment at 0.02 mM [Mg^{2+}] in which K_i is much larger than 40 mM. For this reason in this case V_r is much smaller than the velocity at 40 mM [K^+]. Values are given in $\mu mol \cdot mg^{-1} \cdot min^{-1}$, K values are given in μM.

[Mg^{2+}] (μM)	V_{Na}	V_{mK}	V_r	K_a	K_i
0.02	0.46±0.02	1.09±0.04	0.35±0.90	0.19± 0.09	93 ±169
0.30	0.42±0.02	1.30±0.12	0.70±0.04	0.55± 0.21	6.7± 3.6
5.00	0.43±0.01	1.50±3.0	0.28±0.01	9.7 ±32.7	0.6± 1.9

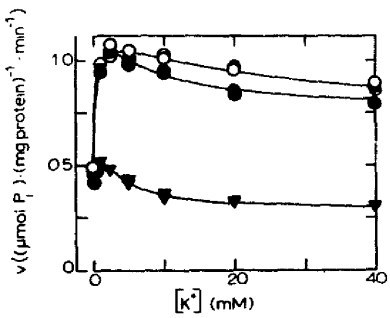

Fig. 6. A plot of ATPase activity as a function of the concentration of K^+ in media containing 130 mM NaCl, 0.010 mM ATP and either 0.02 (○), 0.3 (●) or 5 (▼) mM free Mg^{2+}. The ionic strength was kept constant at 170 mM with choline chloride. The continuous lines for each Mg^{2+} concentration are the graphical representation of Eqn. 3 for the values of the parameters given in Table II.

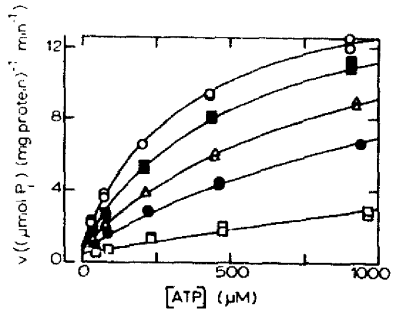

Fig. 8. A plot of Na^+/K^+-ATPase activity as a function of the concentration of ATP in media containing 130 mM NaCl, 40 mM K^+ and either 0.3 (○), 1 (■), 2.5 (△), 5 (●) or 20 (□) mM free Mg^{2+}. The ionic strength was kept constant at 230 mM with choline chloride. The continuous lines are the graphical representation of Eqn. 4 where V_{m1} and K_{m2} are the functions of the concentration of Mg^{2+} given in Eqns. 5 and 6, respectively, and where each parameter was replaced by its best-fitting value. These (±S.E.) were: $V_{m10} = 0.76 \pm 0.08$ (μmol P_i)·mg^{-1}·min^{-1}, $V_{m2} = 16.13 \pm 0.29$ (μmol P_i)·mg^{-1}·min^{-1}, $K_{m20} = 291 \pm 18$ μM, $K_{i1} = 33 \pm 14$ mM, $K_{i2} = 1.15 \pm 0.06$ mM.

was adjusted to the data, the best-fitting values of its parameters for each Mg^{2+} concentration are given in Table II. It is apparent that as $[Mg^{2+}]$ rises V_{Na} and V_{mK} change little, V_r first rises and then declines and K_a increases continuously. However, the most conspicuous effect of Mg^{2+} seems to be the modification of K_i for $[K^+]$ i.e.: as $[Mg^{2+}]$ goes from 0.020 to 5 mM, K_i declines from values high enough as to make inhibition negligible to around 1 mM. These results suggest that the cause of the dependence on Mg^{2+} of inhibition by $[K^+]$ lies mainly in the effect of Mg^{2+} on the K_i for K^+ and to a much lesser extent on its effect on the maximal inhibition by K^+. The findings in the experiment in Fig. 6 were confirmed by an independent experiment in which inhibition by K^+ was tested in media with either 0.3 or 20 mM free Mg^{2+} (results not shown).

In spite that regression of three curves in Fig. 6 gave similar standard deviations, Table II shows that the standard error of K_i was very large for the curve with 0.02 mM $[Mg^{2+}]$. Although this probably expresses the uncertainties of the estimation of a parameter whose value is much higher than the highest $[K^+]$ tested, it raises doubts about the significance of the value and hence about the soundness of our conclusions. For this reason we tried to find independent experimental evidence that inhibition by K^+ depends on Mg^{2+}. To do this we started from the prediction that if this were so, then K^+ would be required for inhibition by Mg^{2+}. This was explored in the experiment shown in Figs. 7A and B in which Na^+/K^+-ATPase activity at high (3 mM) [ATP] was measured as a function of K^+ concentration in media with either 0.3 or 20 mM free Mg^{2+}. It is clear that with 20 mM Mg^{2+} Na^+/K^+-ATPase activity was slowed down. When plotted as a function of $[K^+]$, the extent of inhibition, measured as the ratio between the activities with 20 and 0.3 mM Mg^{2+}, decreased along a function that started at near 1 (no inhibition) and tended to about 0.4 (Fig. 7B), showing that the inhibitory effect of high $[Mg^{2+}]$ was strictly dependent on K^+.

We obtained further information on the kinetics of inhibition by Mg^{2+} by measuring Na^+/K^+-ATPase activity as a function of ATP concentration in a 35–960 μM range, in media containing 40 mM K^+ and from 0.3 to 20 mM free Mg^{2+} (Fig. 8). The whole set of data was

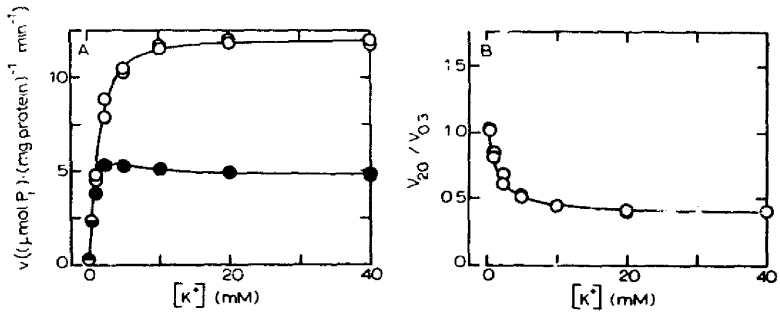

Fig. 7. (A) A plot of Na^+/K^+-ATPase activity as a function of the concentration of K^+ measured in media with 3 mM ATP, 130 mM NaCl and either 0.3 (○) or 20 (●) mM free Mg^{2+}. The ionic strength was kept constant at 230 mM with choline chloride. (B) A plot of the ratio between the activities at 20 and at 0.3 mM free Mg^{2+} from experiment in (A) against K^+ concentration.

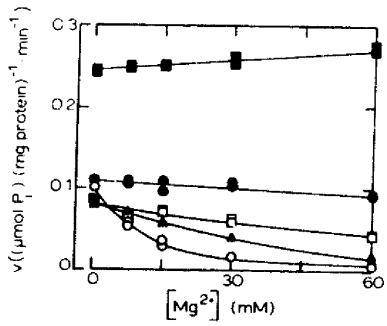

Fig. 9. A plot of Na^+-ATPase activity as a function of the concentration of free Mg^{2+} in media containing 0.01 mM ATP and either 1 (○), 5 (▲), 10 (□), 25 (●) or 100 (■) mM NaCl. The ionic strength was kept constant at 280 mM with choline chloride.

adjusted by multiple regression using [ATP] and $[Mg^{2+}]$ as the independent variables. Best fit was obtained using Eqn. 4 with V_{m2} independent of $[Mg^{2+}]$ and:

$$V_{m1} = V_{m10} K_{i1} / ([Mg^{2+}] + K_{i1}) \quad (5)$$

and

$$K_{m2} = K_{m20}(1 + [Mg^{2+}]/K_{i2}) \quad (6)$$

These results indicate that increasing $[Mg^{2+}]$ decreased V_{m1} hyperbolically towards zero and that Mg^{2+} acted as a dead-end inhibitor on the low-affinity component of the substrate curve of the ATPase increasing linearly K_{m2}.

The inhibition by Mg^{2+} of the Na^+-ATPase

In the experiment shown in Fig. 9, Na^+-ATPase activity was measured as a function of the concentration of Mg^{2+} from 0.3 to 60 mM, in media containing from 1 to 100 mM NaCl and 0.01 mM ATP. It can be seen that in media with 100 mM Na^+ inhibition was absent and in media with 1 mM Na^+ inhibition was practically complete at 60 mM Mg^{2+}. Lack of inhibition at high $[Na^+]$ in media without K^+ is congruent with the experiments presented above.

We studied the kinetics of the inhibition by Mg^{2+} at low $[Na^+]$ measuring Na^+-ATPase activity as a function of ATP concentration in media containing 10 mM Na^+ and either 0.3, 10, 25 and 50 mM free Mg^{2+} (Figs. 10A and B). A Michaelis-Menten equation was fitted to each individual curve and the values of V_{mNa} and K_{mNa} thus obtained were plotted vs. the concentration of free Mg^{2+}. This gave the following expressions for the effects of free Mg^{2+} on these parameters.

$$V_{mNa} = V_{m0}/(1 + [Mg^{2+}]/K_{iv}) \quad (7)$$

and

$$K_{mNa} = K_{m0}\frac{1 + [Mg^{2+}]/K_{i01} + [Mg^{2+}]^2/K_{i02}}{1 + [Mg^{2+}]/K_{iv}} \quad (8)$$

Eqns. 7 and 8 allowed us to adjust the whole set of data by means of multiple regression using [ATP] and $[Mg^{2+}]$ as the independent variables (continuous lines in Figs. 10A and B). The same parameter (K_{iv}) was used in the denominators of V_{mN} and K_{mNa} because when two independent parameters were adjusted their values became not significantly different and the standard deviation of the regression increased.

It is clear therefore that the kinetics of inhibition by Mg^{2+} of Na^+-ATPase activity is substantially different from that of the Na^+/K^+-ATPase activity. In addition comparison of the values of K_{iv}, K_{i01} and K_{i02} (legend to Fig. 10) with K_{i1} and K_{i2} for inhibition of Na^+/K^+-ATPase (legend to Fig. 8) shows that the apparent affinity for Mg^{2+} as inhibitor of the Na^+-

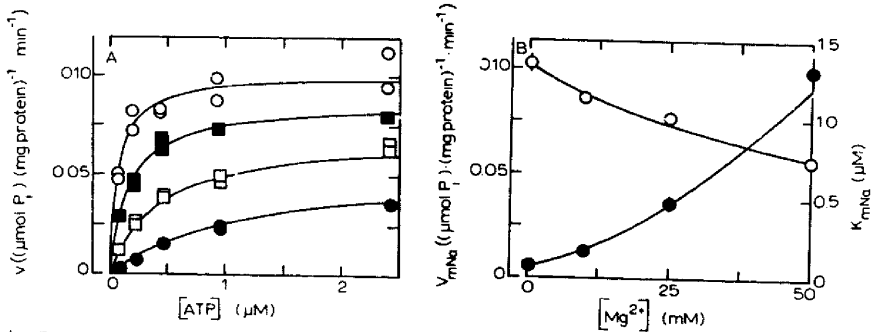

Fig. 10. (A) A plot of Na^+-ATPase activity as a function of ATP concentration in media containing 0.3 (○), 10 (■), 25 (□), or 50 (●) mM free Mg^{2+}, 10 mM NaCl and enough choline chloride as to keep the ionic strength constant at 170 mM. The continuous lines are the graphical representation of a Michaelis-Menten equation where V_{mNa} and K_{mNa} are the functions of the concentration of Mg^{2+} given in Eqns. 7 and 8, respectively, and where each parameter was replaced by its best-fitting value. These (±S.E.) were: $V_{m0} = 0.102 \pm 0.003$ (μmol P_i)·mg^{-1}·min^{-1}, $K_{m0} = 0.073 \pm 0.09$ μM, $K_{iv} = 62 \pm 12$ mM, $K_{i01} = 13 \pm 7$ mM, $K_{i02} = 101 \pm 22$ mM^2. (B) A plot of the best-fitting apparent values (±S.E., vertical bars) of V_{mNa} (○) and K_{mNa} (●) for each concentration of Mg^{2+} in (A) as a function of the concentration of Mg^{2+}. The continuous lines are the graphical representation of Eqns. 5 and 6 for the values of the parameters given in (A).

ATPase is considerable less than that for inhibition of the Na^+/K^+-ATPase.

Discussion

Pctassium activates at low ATP concentrations

Inhibition by K^+ of Na^+/K^+-ATPase activity in media with micromolar concentrations of ATP has been reported by several authors (see Ref. 1 and 8). On the basis of the Albers-Post model this phenomenon was interpreted, by Post et al. [1] as an evidence that ATP activates with low affinity the $E_2K \rightarrow E_1 + K^+$ reaction (see Ref. 5) so that at sufficiently low [ATP] acceleration of dephosphorylation by K^+ is more than offset by the slowdown of the $E_2K \rightarrow E_1 + K^+$ transition. Notice that since this conjecture involves the same effect of K^+ in activation and inhibition, it necessarily implies that at constant [ATP] the effect of K^+ will be either continuous activation, lack of effect or continuous inhibition, depending on the level of the nucleotide In contrast with this results in this paper show that at sufficiently low concentrations K^+ activates even at the smallest [ATP] tested, inhibition being only apparent at higher [K^+]. A biphasic response to K^+ is also apparent in the data of Post et al. which show that in media with 0.9 μM ATP, as [K^+] raised activity first increased and then dropped (Fig. 1 in Ref. 1).

Since a single effect cannot explain the biphasic response to K^+, it seems that a more fitting explanation of the effects of the cation at low [ATP] is to propose that the level of ATP only regulates the extent of activation and that activation and inhibition by K^+ take place through two different mechanisms.

The turnover of the ATPase at low ATP. The assignments that the Albers-Post model makes for the effect of cations [5] on the Na^+/K^+-ATPase imply that at the concentrations of Na^+ and Mg^{2+} used in the experiments reported here the $E_2 \rightarrow E_1$ and the $E_2ATP \rightarrow E_1ATP$ transitions will be virtually irreversible. Taking this for granted, it is east to show (Rossi and Garrahan, unpublished) that the first term of Eqn. 1 will measure the rate through of the pathway that does not use the $E_2ATP \rightarrow E_1ATP$ reaction and that V_{m1} and K_{m1} will be very close to the V_m and K_m of an enzyme with the reaction mechanism as the Na^+/K^+-ATPase but lacking the ATP-dependent pathway. This makes it meaningful to compare the kinetic parameters of the Na^+-ATPase with those of the high-affinity component of the Na^+/K^+-ATPase. Hence the fact that in media with 2.5 mM K^+, the values of V_{m1} and K_{m1} are very near to those of V_{mNa} and K_{mNa}, would mean that the rate-limiting steps of the Na^+-ATPase and of the Na^+/K^+-ATPase in the absence of the $E_2ATP \rightarrow E_1ATP$ transition will have similar magnitude. Published data based on measurements of partial reactions at room temperature give for the rate constant for dephosphorylation in the absence of K^+, i.e.: the limiting step of the Na^+-ATPase a value (2.5 s^{-1}, [9]) which is about 10-fold higher than that for the $E_1 \rightarrow E_1$ transition (0.26 s^{-1} [10]), i.e.: the limiting step of the Na^+/K^+-ATPase in the absence of activation by ATP. If these differences persisted at 37°C, then, in contrast with the experimental observations in this paper, V_{m1} and K_{m1} would be ten times lower than V_{mNa} and K_{mNa}. As it is improbable that rising the temperature will increase more than ten times one rate constant with respect to the other it seems that the estimated values of one of the rate constants is wrong. Since the mentioned value for the rate constant of dephosphorylation allows a correct estimate of K_{mNa} [6], it would seem that the reported value of the rate contant for the $E_2K \rightarrow E_1 + K^+$ transition is an underestimation.

A mechanism for the inhibition of the Na^+/K^+-ATPase by K^+ and Mg^{2+}

The inhibition by K^+ described in this paper was not caused by displacement of Na^+. The possibility that K^+ acted as product inhibitor reversing the reaction that releases it from the ATPase can be ruled out since it would have given total instead of the observed partial inhibition.

The low-affinity component. The features of the inhibition by K^+ and Mg^{2+} of the low-affinity component of the substrate curve are predicted by the scheme shown in Fig. 11. Its basic assumption is that K^+ acting at sites different from those from which it activates (activation is not shown in Fig. 11) enables Mg^{2+} to form a ternary complex with K^+ and the enzyme (EKMg) that is unable to bind ATP. The solution of the scheme, assuming rapid-equilibrium and lack of interac-

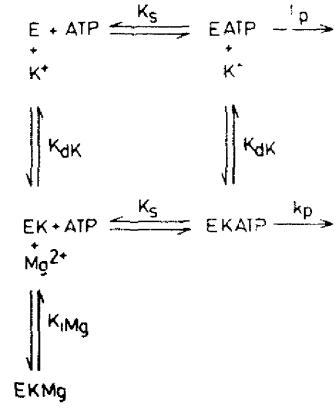

Fig. 11. A kinetic scheme for inhibition by K^+ and Mg^{2+}. Activation by K^+ is considered to be independent from inhibition an is not shown. Except for k_P (the rate constant for catalysis), the other constants are equilibrium dissociation constants of the corresponding steps. Notice that neither k_P nor K_s are modified by K^+.

tions for the binding of the ligands, gives for initial velocity:

$$\frac{v}{V_m} = \left[1 + \frac{K_s}{[ATP]}\left[1 + \frac{[Mg^{2+}][K^+]}{K_{iMg}([K^+] + K_{dK})}\right]\right]^{-1} \quad (9)$$

and for $K_{i,app}$ for inhibition by K^+ (K_{iK}):

$$K_{iK} = \frac{K_{dK}K_{iMg}(1 + [ATP]/K_s)}{[Mg^{2+}] + K_{iMg}(1 + [ATP]/K_s)} \quad (10)$$

where K_{dK} and K_{iMg} are the equilibrium constant for the dissociation of K^+ and Mg^{2+}, respectively.

In agreement with the experimental results, Eqns. 9 and 10 predict that: (i) inhibition needs both K^+ and Mg^{2+}, (ii) neither K^+ nor Mg^{2+} will affect V_m, (iii) as $[K^+]$ goes from zero to infinity, the apparent value of K_m rises hyperbolically (iv) the apparent value of K_m rises linearly with $[Mg^{2+}]$, (v) the activity decreases with the concentration of K^+ along an hyperbola tending to a level that will depend on the concentrations of ATP and of Mg^{2+}, (vi) as $[Mg^{2+}]$ inceases from zero to infinity K_{iK} decreases from K_{dK} to zero, and (vii) as [ATP] increases from zero to infinity K_{iK} rises hyperbolically.

To incorporate the scheme in Fig. 11 into the version of the Albers-Post model shown in Fig. 5 of Ref. 3 we assumed that $E_2(K)$ can exist associated with either 'n' or '$n+1$' K^+ and that the state with '$n+1$' K^+ binds Mg^{2+} forming a dead-end complex. The effect of the $(n+1)$th K^+ can be included in the overall equation multiplying the rate constants of the transitions of $E_2(K)$ (k_6 and k_5) by the fraction (f) of this state that does not bind the inhibitory Mg^{2+}, i.e.:

$$f = \left\{1 + [Mg^{2+}][K^+]/[K_{iMg}([K^+] + K_{dK})]\right\}^{-1} \quad (11)$$

The fraction (g) of E_2P with either 2 or 3 K^+ bound will be:

$$g = [3(K^+/K_{dK})^2 + (K^+/K_{dK})^3]/(1 + K^+/K_{dK})^3 \quad (12)$$

The above considerations permitted us to express the effect of K^+ on dephosphorylation and on the E_2P to E_1P transition multiplying k_4 by g and k_{-3} by $(1 + K^+/K_{dK})^{-3}$.

Eqns. 11 and 12 allowed us to generate the simulated curves in Fig. 12 (A and B). Fig. 12A shows that the model qualitatively predicted the observed response of Na^+/K^+-ATPase activity to $[K^+]$ at low ATP and at different concentrations of Mg^{2+} (cf. Figs. 8 and 12A). Fig. 12B shows that, in compliance with the experimental results (cf. Figs. 3 and 12B) as K^+ concentration increases V_{m2} and K_{m2} increase along curves that tend to saturation but that for K_{m2} this effect is reached at higher concentrations of K^+ so that the ratio V_{m2}/K_{m2} decerases as $[K^+]$ increases. This effect might seem to be inconsistent with the simulations of Fig. 7A of the preceding paper of this series [5]. However, the discrepancy is only apparent since in the preceding paper we only simulated the activation effects of K^+.

To the best of our knowledge there is no experimental evidence against our idea of the binding of an additional K^+ to E_2P. Since K^+ binding to E_2P is usually assumed to take place on extracellular sites, it is interesting to point that in a preliminary communication Beaugé et al. [12] reported that in red cell ghosts containing low [ATP] activation by external K^+ is followed by inhibition at higher concentrations of the cation. The lack of sideness of our preparation did not allow us to pursue further this analogy.

As judged by its apparent affinity, the site at which Mg^{2+} inhibits is either different from that at which Mg^{2+} activates the Na^+/K^+-ATPase or is the same site on a different conformation of the enzyme. It is tempting to identify the inhibitory site with the site that releases Mg^{2+} during a hydrolysis cycle. This is supported by experiments that show that Mg^{2+} is discharged from the pump during dephosphorylation [13] and by work by Pedemonte and Beaugé [14,15] on the kinetics of the interactions between Mg^{2+} and inorganic phosphate during inhibition of the Na^+/K^+-ATPase by Mg^{2+}. In this case also a more thorough examination of this point would require a 'sided' preparation.

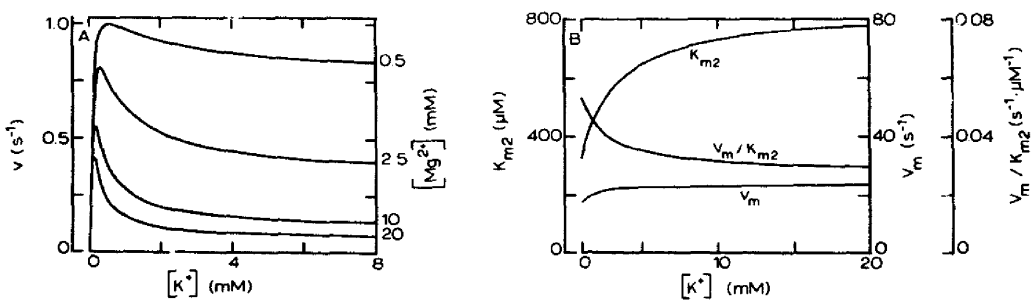

Fig. 12. (A) A simulation of the effects of K^+ on Na^+/K^+-ATPase activity in media containing 1 μM ATP, non-limiting concentrations of Na^+ and the concentrations of free Mg^{2+} given at the right of the graph. Na^+-ATPase activity was not considered. (B) A simulation of the effects of K^+ on V_m, K_{m2} and the ratio V_m/K_{m2}, for free $[Mg^{2+}] = 1$ mM. K_{dK} and K_{iMg} were taken as 2 and 1 mM, respectively.

Measuring the effect of ATP on the rate constant for the release of Rb^+ from $E_2(Rb)$ at different concentrations of Mg^{2+}, Forbush [11] observed that Mg^{2+} decreased both the apparent affinity and the maximum effect of the nucleotide. Our results agree with these observations in what respects the effects on apparent affinity but do not reproduce in the overall reaction the reported action on the maximal effect of ATP. The inhibitory effects of Mg^{2+} on red cell Na^+ pump have been examined recently by Sachs [16,17]. This author proposed like us that E_2 is the species involved in inhibition. Sachs found that Mg^{2+} increased K_m and decreased V_m for MgATP. The effect on K_m is in harmony with our results. Whether the discrepancy with our results in what regards the effect on V_m is genuine or is caused by the different preparation and/or the concentration variable used remains an open question.

The high-affinity component. Our results show that excess K^+ decreases both V_{m1} and K_{m1} and that in this condition high concentrations of Mg^{2+} diminishes V_{m1} towards zero. These findings are compatible with the model we have just analyzed, since this assumes that after binding Mg^{2+}, E_2 not only becomes unable to combine with ATP but also of undergoing that $E_2 \rightarrow E_1$ transition. If we identify the high-affinity component with that part of the Na^+/K^+-ATPase which does not occur through the $E_2ATP \rightarrow E_1ATP$ pathway, inhibition of the $E_2 \rightarrow E_1$ transition would not be surmountable by ATP, thus explaining the effect on V_{m1}.

Inhibition by Mg^{2+} in the Na^+-ATPase

At high (130 mM) concentrations of Na^+ inhibition by Mg^{2+} needs K^+, but when the concentration of Na^+ decreases, Mg^{2+} becomes able to inhibit in the absence of K^+, albeit with much less apparent affinity and following a different kinetic pattern than in the presence of the cation. Inhibition by Mg^{2+} of Na^+-ATPase activity at low Na^+ concentration has also been reported by Beaugé and Campos [18].

To understand the physical meaning of the effects of Mg^{2+} on V_{mNa} and K_{mNa} described in Results, we solved the currently accepted kinetic scheme of the Na^+-ATPase (Fig. 13) as a function of the concentration of ATP. The steady-state rate equation in the absence of ADP and P_i predicts Michaelis-Menten behaviour with:

$$V_m = k_4/D \quad (13)$$

$$K_m = [k_4/k_1(1 + k_{-5}/k_5)(1 + k_{-1}/k_2)]/D \quad (14)$$

where:

$$D = 1 + k_4(1/k_2 + 1/k_3 + 1/k_5) + k_{-3}/k_3 \quad (15)$$

If Mg^{2+} reacted with one or more enzyme forms to yield dead-end complexes then the rate constants that govern the transitions of the affected states would be multiplied by $(1 + [Mg^{2+}]/K_{iMg})$, so that their reciprocals would be linear functions of $[Mg^{2+}]$. Hence if k_2, k_3 or k_5 depended in this way on $[Mg^{2+}]$, the denominators of V_m and K_m (Eqns. 14 and 15) would be the same linear function of the concentration of the cation. The reciprocals of k_2 and k_5 are also present in the denominator of the Eqn. 14. Therefore if k_{-1} or k_{-5} were linear functions of $[Mg^{2+}]$, the numerator of K_m (Eqn 14) would become a parabolic function of $[Mg^{2+}]$. The linear dependence of a reverse rate constant with $[Mg^{2+}]$ implies that the cation is acting through product inhibition. This would occur if Mg^{2+} were cyclically added and released during hydrolysis, a fact that is supported by current experimental evidence [13].

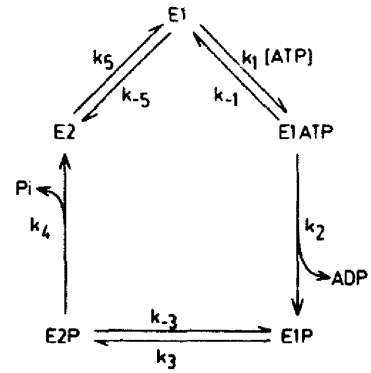

Fig. 13. A reaction scheme for the Na^+-ATPase activity.

It seems therefore reasonable to conclude that in the absence of K^+ and at low $[Na^+]$, Mg^{2+} seems to inhibit acting both as a product and as a dead-end inhibitor.

Acknowledgements

This work was supported by grants from CONICET, Universidad de Buenos Aires, and Fundación Antorchas, Argentina. P.J.G. is an established investigator from CONICET.

References

1 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540.
2 Glynn, I.M. (1985) in The Enzymes of Biological Membranes, 2nd Edn. (Martonosi, A., ed.), pp. 33–114, Plenum Press, New York.
3 Rossi, R.C. and Garrahan, P.J. (1989) Biochim. Biophys. Acta 981, 85–94.
4 Moczydlowski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2357–2366.
5 Rossi, R.C. and Garrahan, P.J. (1989) Biochim. Biophys. Acta 981, 95–104.
6 Rossi, R.C. and Garrahan, P.J. (1985) in The Sodium Pump, Proceedings of the 4th International Conference on Na,K-ATPase (Glynn, I.M. and Ellory, C.J., eds.), pp. 443–455, The Company of Biologists, Cambridge.

7 Garrahan, P.J., Rossi, R.C. and Rega, A.F. (1982) Ann. N.Y. Acad. Sci. 402, 239–251.
8 Glynn, I.M. and Karlish, S.D.J. (1976) J. Physiol. 256, 465–496.
9 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 448–463.
10 Karlish, S.J.D. and Yates, D.W. (1978) Biochim. Biophys. Acta 527, 115–130.
11 Forbush, B., III (1987) J. Biol. Chem. 262, 11104–11115.
12 Beaugé, L.A., Glynn, I.M. and Richards, D.E. (1979) J. Physiol. 295, 88P.
13 Richards, D.E. (1987) in Proceedings of the 5th International Conference on Na,K-ATPase, Aarhus, Denmark, Abstr. No. 63.
14 Pedemonte, C.H. and Beaugé, L.A. (1983) Biochim. Biophys. Acta 748, 245–253.
15 Pedemonte, C.H. and Beaugé, L.A. (1986) Arch. Biochim. Biophys. 244, 596–606.
16 Sachs, J.R. (1988) J. Physiol. 400, 545–574.
17 Sachs, J.R. (1988) J. Physiol. 400, 575–591.
18 Beaugé, L.A. and Campos, M.A. (1986) J. Physiol. 375, 1–25.