The K^+-activation of the Mg^{2+}- dependent cycle of Na, K-ATPase

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In the OPM régime ($Mg^{2+}_{free}/ATP_{free} \geq 2$, MgATP ≤ 1.85) dephosphorylation of the Mg^{2+}-bound phosphorylated form of Na, K-ATPase occurs. Under these conditions, the number of Na^+-bound sites of essential activating nature is 3 when $[K^+] \leq 100$ mM, and 4 when $[K^+] > 125$ mM. On the other hand, investigation of the K^+-dependent stage of dephosphorylation shows that the number of K^+-bound activation sites may be 0, 1 or 2. This alteration is not affected by the Na^+ concentration. However, an increase of Na^+ concentration changes the K^+-affinity of the enzyme. The experimental data show that in the OPM régime Mg^{2+}_{free} ions are essential activators of Na, K-ATPase system.

Keywords: essential activators, Na,K-ATPase, Na -ATPase

1. INTRODUCTION

The main function of the Na,K-ATPase system is the creation of Na^+ and K^+ ion gradients, on the basis of which the transmembrane electrical potential difference is built. However, Na,K-ATPase, due to its own electrogenicity, can directly generate an electrical potential. It is believed [7] that the stoichiometry of this process is: $3Na^+ : 2K^+ : 1$MgATP. However, in an excess of free Mg^{2+} ions ($[Mg^{2+}] >> [ATP_{free}]$ and [MgATP] ≤ 1.8 mM), when the Na,K-ATPase works in the special so-called OPM régime and the phosphorylated form of the Mg^{2+}-bound enzyme is dephosphorylated, this stoichiometry is violated [3, 4]. It was found that in the OPM régime at relatively high K^+ concentration 4 instead of 3 Na^+ ions are required to be bound [6].

Here we investigate the K^+-dependent Na,K-ATPase system in order to fully clarify possible alterations of the number and types of Na,K-ATPase K^+-binding sites.

2. METHODS

The subcellular fraction of albino rat brain synaptosomal plasma membrane served as material. Fractions were obtained between the 1.2–0.9 M sucrose layers [2]. The Na,K-ATPase activity has been measured as the ouabain-sensitive part of total ATPase, according to the method described earlier [6]. The incubation solution for Mg-ATPase contained: 1.7–1.8 mM MgATP, 0.2 mM ouabain, 140 mM KCl, 50 mM Tris/HCl buffer, pH=7.7. The incubation solution for total ATPase was: 1.7–1.8 mM MgATP, 50 mM Tris/HCl buffer, pH = 7.7 and different concentrations of $[Na^+]$, $[K^+]$, $[Mg^{2+}]$, and $[ATP_{free}]$. Calculation of free Mg^{2+}, free ATP and the MgATP complex was made using a dissociation constant $K_d = 0.085$ mM.

3. RESULTS

Activity of Na,K-ATPase in the OPM régime is ensured by the following substrate/modifyer ratio: [MgATP] ≤ 1.85 mM and $[Mg^{2+}] / [ATP_{free}] \geq 2$ [4, 6]. Thus, in all experiments the reaction media were chosen in accordance with these conditions.

When investigating the dependence of Na,K- ATPase velocity on the concentration of K^+ ions, first of all it is necessary to examine the impact of the absence of K^+ ions on the enzyme. As it is shown in Fig. 1, in the absence of K^+ ions the ouabain-sensitive Na,K-ATPase activity is maintained. The activity of Na,K-ATPase firstly increases while raising the Na^+ ion concentration and reaches its maximum at $[Na^+] = 20$ mM (Fig. 1, B). It should be noted that at even higher concentrations of Na^+ the activity drops sharply (Table 1). The table shows that Na, K-ATPase activity depends on the $[Mg^{2+}] / [ATP_{free}]$ ratio. At the low Na^+ concentration ($[Na^+] = 23.65$ mM), no statistically significant alteration of activity was observed, while at $[Na^+] = 133.6$ mM, activity increased reliably with increasing Mg^{2+} concentration. With the method of analysis of geometrical shapes of kinetic curves [2, 6], according to the data presented in Fig. 1, we can determine the number of Na-binding sites necessary for activation. Indeed, it was found that as a result of a qualitative transformation, the function $U(r) = \sqrt[r]{1/V} = a + b/[Na^+]$,

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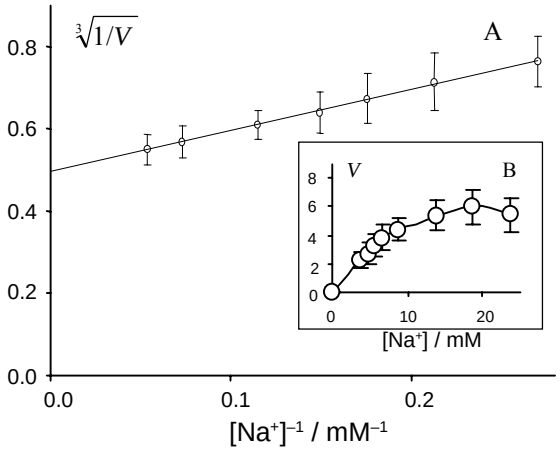

Figure 1. Dependence of the Na-ATPase activity (V) on Na^+-ion concentration in the absence of K^+.
[MgATP] = 1.8 mM, $[Mg^{2+}]$ = 3 mM and $[ATP_{free}]$ = 0.051 mM; $[K^+]$ = 0mM
A: $U = \sqrt[3]{1/V} = f(1/[Na^+])$; B: $V = f([Na^+])$

Table 1. Na-ATPase activity μM $P_i h^{-1}$ (mg protein)$^{-1}$
[MgATP] = 1.7 mM; $[K^+]$ = 0

$[ATP_{free}]$ / mM	$[Mg^{2+}]$ / mM	Na-ATPase activity (V_0)	
		$[Na^+]$=23.65 mM (fig. 2)	$[Na^+]$=133.6 mM (fig. 3)
0.19	0.79	10.41 ± 0.99	1.02 ± 0.60
0.15	0.98	10.21 ± 2.04	1.70 ± 0.79
0.08	1.94	11.90 ± 0.59	3.95 ± 0.64
0.05	2.90	10.78 ± 1.24	3.40 ± 0.80

where V is enzyme activity, at low concentrations of Na^+ and only when $r = 3$, has an asymptote (in other words, significant linearization of the function $U(r)$ is attained) (Fig. 1, A). In this case there is a discrepancy $(R–n) = –0.124$ [6] between the true number n of essential activators and R (the number of necessary activators) whose value calculated from Fig. 1 is 3.00 ± 0.39 (a = 0.496 ± 0.003 and b = 1.00 ± 0.02). According to the above mentioned it follows that for the reaction to run its course it is necessary to bind $3Na^+$ ions and at $[K^+]$=0 the ordinary Na-ATPase is functioning. On the basis of our data (Fig. 1 and Table 1) we can conclude that under conditions of fast equilibrium, in the most general case, the Na,K-ATPase activity (V) dependence on the K^+ concentration has the following analytical shape [1, 5]:

$$V = \frac{\sum_{i=0}^{p} \alpha_i [K^+]^i}{\sum_{i=0}^{s} \beta_i [K^+]^i}; \quad s = m + p \qquad (1)$$

where p is the number of K^+-binding sites required for modification possessing a partial effect; m is the number of sites for full inhibition; and α and β are positive permanent coefficients (n = 0, i.e. there are no essential activators).

Our task was the investigation of the mechanisms of K^+-ion-induced activation of the Na,K-ATPase system. Thus it is expedient, instead of the $V = f(x)$ dependence, to study the dependence $1/(V - V_0) = f(1/[K^+])$ for low concentrations of K^+, where V is Na,K-ATPase activity; and V_0 is Na,K-ATPase activity at $[K^+]$ = 0. The results of these experiments are shown in Fig. 2, Fig. 3, and Table 2. Experiments were carried out at a constant concentration of substrate, i.e. [MgATP] = 1.7 mM, and at

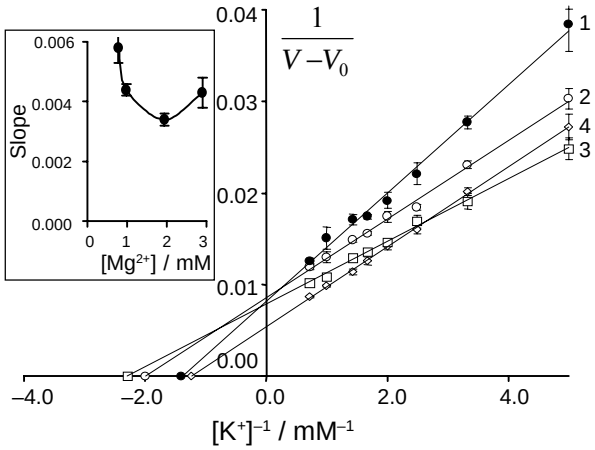

Figure 2. Dependence of the difference ($V–V_0$) on inverse K^+-concentration.
[MgATP] = 1.7 mM, $[Na^+]$ = 23.65 mM.
$[Mg^{2+}]$ / $[ATP_{free}]$ = 0.789 / 0.189 (curve 1); 0.981 / 0.147 (curve 2); 1.94 / 0.08 (curve 3); 2.90 / 0.05 (curve 4); (mM / mM)
Inset: Dependence of the slope of the regression lines on Mg^{2+}-concentration.

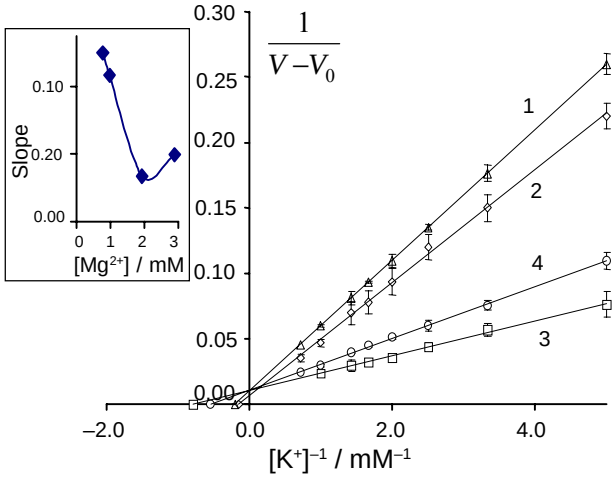

Figure 3. Dependence of the difference ($V–V_0$) on inverse K^+-concentration.
[MgATP] = 1.7 mM, $[Na^+]$ = 133.5 – 133.7 mM.
$[Mg^{2+}]$ / $[ATP_{free}]$ = 0.789 / 0.189 (curve 1); 0.981 / 0.147 (curve 2); 1.94 / 0.08 (curve 3); 2.90 / 0.05; (curve 4) (mM / mM)
Inset: Dependence of the slope of the regression lines on Mg^{2+}-concentration.

Table 2. Parameters of regression line ([MgATP] = 1.7 mM)

$[Na^+]$ / mM	Free [ATP] / mM	Free $[Mg^{2+}]$ / mM	Minimal power of numerator $(V\text{-}V_0)=f([K^+])$	$\frac{1}{V-V_0}=A+\frac{B}{[K^+]}$ A	B	Correlation coefficient
23.65 (fig. 2)	0.19	0.79	1.000 ± 0.009	0.0087 ± 0.0004	0.0058± 0.0002	0.9976
	0.15	0.98	1.000 ± 0.005	0.0054 ± 0.0001	0.0044 ± 0.0001	0.9990
	0.08	1.94	1.000 ± 0.006	0.0078 ± 0.0002	0.0034 ± 0.0001	0.9992
	0.05	2.90	1.000 ± 0.005	0.0086 ± 0.0001	0.0043 ± 0.0002	0.9997
133.6 (fig.3)	0.19	0.79	1.007 ± 0.042	0.0101 ± 0.0021	0.0501 ± 0.0035	1.0000
	0.15	0.98	1.005 ± 0.038	0.0063 ± 0.0021	0.0433 ± 0.0008	0.9992
	0.08	1.94	1.000 ± 0.015	0.0105 ± 0.0009	0.0134 ± 0.0003	0.9992
	0.05	2.90	1.001 ± 0.017	0.0108 ± 0.0005	0.0198 ± 0.0002	0.9997

different $[Na^+]$ concentrations: 23.65 mM (Fig. 2) and 133.6 mM (Fig. 3). The $[Mg^{2+}]$ / ATP_{free} ratio was high (> 4). In this case the concentration of ATP_{free} was low enough, compared to $[Mg^{2+}]$ and [MgATP], to be disregarded (thus ensuring Na,K-ATPase system activity in the Mg^{2+}_{free} surplus régime). As shown in Figs 2, and 3, and Table 2, in every case the $1/(V-V_0)=f(1/[K^+])$ dependence, within 0.25 mM ≤ $[K^+]$ ≤ 1.4, has a reliable linearity. However, at both low (Fig. 2) and high concentrations of Na^+ the slope of the curves changes with increasing Mg^{2+} concentration (Figs 2 and 3). During the increase of $[Mg^{2+}]$ the slope firstly decreases, then at $[Mg^{2+}]$ > 2 mM it increases. During the increase of the permanent concentration of Na^+ ions from 23.65 to 133.6 mM, the apparent dissociation constant of activation for the K^+ ions reliably decreases.

According to the above experiments it could be definitely concluded that Mg^{2+} ions produce a significant effect on the kinetic parameters of the activating influence of K^+ ions on the Na,K-ATPase system working in the OPM régime. Analogous results are expected for Na^+ ions as well.

4. DISCUSSION

Let us introduce the following notation: [MgATP] = *S*, $[Mg^{2+}]$ = *M*, $[Na^+]$ = *X*, $[K^+]$ = *Y*, $t = 1/y$, V_0 is the Na,K-ATPase activity when $[K^+] = 0$, $\Delta V = V - V_0$, and $\Delta U = 1/\Delta V$.

According to the equation (1), let us evaluate the $\Delta U = f(t)$ function

$$\Delta U=\frac{\beta_0\sum_{i=0}^{s}\beta_i t^{-i}}{\sum_{i=0}^{p}D_{i0}t^{-i}-\alpha_0\sum_{i=0}^{s}\beta_i t^{-i}} \qquad (2)$$

where $D_{i0}=(\alpha_i\beta_0-\alpha_0\beta_i)$, while $V_0=\alpha_0/\beta_0$. Analysis of this relation shows that the $\Delta U = f(t)$ function has an asymptote only when $D_{10} \neq 0$. Therefore the asymptote ($\Delta U = a + bt$) coefficients could be assessed:

$$a=\lim_{t\to\infty}(\Delta U-t\Delta U')=\frac{\beta_0(D_{10}\beta_1-D_{20}\beta_0)}{D_{10}^2}$$
$$b=\lim_{t\to\infty}\Delta U'=\frac{\beta_0^2}{D_{10}} \qquad (3)$$

In Figs 2 and 3 the relations of $\Delta U = f(t)$ to various concentrations (*M* and *X*) are demonstrated. At low values of the argument all have a reliable linear dependence. Thus, it could be definitely concluded that the function $\Delta U = f(t)$ has an asymptote with positive slope. Likewise, because $\beta_0/D_{10}>0$, then $D_{10}>0$ and, concomitantly, $\alpha_1\neq 0$; $D_{10}=(\alpha_1\beta_0-\alpha_0\beta_1)$; ($\alpha_i > 0$, $\beta_i > 0$). Besides, there are Na-ATPase points at $\alpha_0 \neq 0$. According to all the above mentioned it could be concluded that the value of the numerator parameter in equation (1) is $p \geq 1$. On the other hand, according to the reference data [7], it could be supposed that the maximal number of K^+-binding sites equals 2. Therefore, equation (1) could be made more specific as follows:

$$V=\frac{\alpha_0+\alpha_1 y+\alpha_2 y^2}{\sum_{i=0}^{s}\beta_i y^i}; \quad s = 2 + m \qquad (4)$$

Considering the complexity of the geometrical shapes of possible kinetic curves, the molecular mechanism of the Na,K-ATPase system is based on the "minimal model" principle. According to this principle, such a minimal number of ligand-binding sites and intermediates is chosen, which are sufficient for coincidence of the geometrical shapes of the theoretically and experimentally obtained curves. Further, on the basis of the analysis of the relation $V = f(X)$, at various constant concentrations *S*, *M*, and *Y*, it was earlier concluded that the analytical shape of the velocity equation [6] is:

$$V=\frac{[N_X]\cdot[N_Y]}{[N_Y]\cdot\left(1+\frac{S}{K_S}+\frac{M}{K_{IM}}\right)\left(1+\frac{Y}{K'_Y}\right)\left(1+\frac{X}{K'_X}\right)\left[\left(1+\frac{X}{K_{AX}}\right)^3+\frac{2Y}{K_{IY}}+\frac{Y^2}{(K_{IY})^2}\right]+[N_X]\cdot\left(1+\frac{M}{K_{AM}}\right)\left(1+\frac{X}{K''_X}\right)\left(1+\frac{Y}{K''_Y}\right)\left[\left(1+\frac{X}{K_{IX}}\right)^3+\frac{2Y}{K_{AY}}+\frac{Y^2}{(K_{AY})^2}\right]} \quad (5)$$

where K_S; K_{AM}; K_{IM}; K_{AX}; K_{IX}; K'_X; K''_X; K_{AY}; K_{IY}; K'_Y; K''_Y are the apparent dissociation constants of the ligands identified by subscripts, and the single and double primes refer to inside and outside respectively[6],

$$N_X=\frac{S}{K_S}\left(\frac{X}{K_{AX}}\right)^3\left(k_0^A+k_{XY}^A\frac{XY}{K'_XK'_Y}\right) \quad \text{and}$$

$N_Y=f(Y,X,M)$, where k^A_0 and k^A_{XY} are the phosphorylation catalyzing coefficients.

According to our experimental conditions and data obtained, this equation (5) could be made specific and hence simplified:

1) because the reaction medium did not contain extremely low concentrations of K^+ (0.2 mM ≤ [K^+] ≤ 1.4 mM), and yet [K^+] < 100 mM and $k^A_{xy}\frac{xy}{K'_xK'_y}=0$ [3, 4], we can consider that $Y/K'_Y=0$ and $Y/K_{IY}=0$ (K_{IY} is the K^+- dissociation constant on the inside of the membrane);
2) considering that in our case S = const and X = const, the respective terms can be taken as constants;
3) in the OPM régime the Mg^{2+}-bound phosphorylated intermediate is dephosphorylated; therefore one multiplier of N_y may be M/K_{AM}. Besides, as was shown in equation (4) $\alpha_0 \neq 0$ and $\alpha_i \neq 0$. Concomitantly, $N_y = M(c_0 + c_1Y + c_2 Y^2)$, where the constants c_0, c_1, and c_2 depend on neither Y, M nor S and may be a function of X ([Na^+]).

Considering all the above we obtain:

$$V=\frac{M\left(c_0+c_1Y+c_2Y^2\right)}{M\left(c_0+c_1Y+c_2Y^2\right)\cdot\Theta_0+\left(d_0+d_1Y+d_2Y^2+d_3Y^3\right)\cdot\Theta_1} \quad (6)$$

where $\Theta_0=\left(1+\frac{K_S}{S}+\frac{MK_S}{SK_{IM}}\right)\left(1+\frac{X}{K'_X}\right)\frac{1}{k_0^A}$;

$\Theta_1=\left(1+\frac{M}{K_{AM}}\right)\left(1+\frac{X}{K''_X}\right)$;

$d_0=\left(1+\frac{X}{K_{IX}}\right)^3$; $d_1=\left(\frac{d_0}{K''_Y}+\frac{2}{K_{AY}}\right)$;

$d_2=\left(\frac{1}{(K_{AY})^2}+\frac{2}{K''_YK_{AY}}\right)$; $d_3=\frac{1}{K''_Y(K_{AY})^2}$.

Comparing equations (4) and (6) , and according to equation (3) , we can determine the asymptote deflexion as a function of M.

$$b=\frac{\beta_0^2}{D_{10}}=\frac{[\Theta_0c_0M+\Theta_1d_0]^2}{M\Theta_1[(X)]}=\frac{[\lambda_0+\lambda_1M+\lambda_2M^2]^2}{M(\mu_0+\mu_1M)} \quad (7)$$

where $(X)=d_0(c_1-c_0/K''_Y)-2c_0/K_{AY}$, λ_i and μ_i are positive, do not depend on M and Y and hence are constants. Experimental results (Figs 2 and 3) show that b>0, besides $\lambda_i > 0$ and $\mu_i > 0$, then it is clear that $\varphi(x)$>0. Let us determine limits for the derivative $b'=\frac{db}{dM}$:

$$\lim_{M\to0}(b')=-\frac{\lambda_0^2}{\mu_0\varphi(X)}\lim_{M\to0}M^{-2}=-\infty$$
$$\lim_{M\to\infty}(b')=\frac{2\lambda_2^2}{\mu_1\varphi(X)}\lim_{M\to\infty}M=+\infty \quad (8)$$

Insofar as the derivative's limits have different signs, then the function $b = f(M)$ has at least one turning point ($b' = 0$) and represents a concave curve. Thus, the calculated geometrical shape of $b' = f(M)$ completely fits the experimentally obtained curve shapes (Fig. 2, inset and Fig. 3, inset). However, if we consider that $N_Y=(c_0+c_1YM+c_2Y^2M)$ or $N_Y=(c_0M+c_1Y+c_2Y^2M)$, then the geometrical shapes of the calculated and experimental curves $b = f(M)$ will not coincide. This means that one of the multipliers of the numerator of equation (4) is M and, consequently, the Mg^{2+} ions should be considered as essential activators of Na,K-ATPase in the OPM régime.

In the velocity equation (4), for the enzyme working in the OPM régime $\alpha_0 \neq 0$ (there is activity of Na-ATPase) and $\alpha_1 \neq 0$ ($\Delta U = f(t)$ has an asymptote). Therefore, depending on the K^+ concentration, the number of K^+-bound activating sites may change. Although alteration of these sites' number is not dependent on the Na^+ concentration, Table 2 shows that at both low ([Na^+] = 23.5 mM) and high ([Na^+] = 133.6 mM) concentrations of Na^+ the minimal value of the numerator of the function $\Delta V = f$([K^+]) does not change and in both cases equals unity.

5. CONCLUSIONS

Na,K-ATPase may work in the OPM régime, even in the absence of K^+. According to the K^+ concentration, the number of K^+-bound activation sites may change and because 0,1 or 2. Earlier it was shown that in the OPM régime the number of Na^+ ions acting as essential activators, at low concentrations of K^+ ($[K^+]<100$ mM), equals 3, while at high K^+ concentration ($[K^+]\geq 125$ mM) it equals 4.

The classical notion of the molecular mechanism of Na,K-ATPase considers that the hydrolysis of one MgATP molecule requires binding of three Na^+ and two K^+ ions, and it is believed that this stoichiometry is constant, but our data clearly demonstrate that in the OPM régime the stoichiometry is degraded.

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