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The sided action of Na^+ on reconstituted shark Na^+/K^+-ATPase engaged in Na^+–Na^+ exchange accompanied by ATP hydrolysis. II. Transmembrane allosteric effects on Na^+ affinity

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The objective of the present investigation was to characterize the ATP-dependent Na^+–Na^+ exchange, with respect to cation sensitivity on the two aspects of the Na^+/K^+-pump protein. In order to accomplish this, we used Na^+/K^+-ATPase reconstituted with known orientation in the proteoliposomes. Activation by cytoplasmic Na^+ shows cooperative interaction between three sites. The apparent intrinsic site constants displayed transmembrane dependence on the extracellular Na^+ concentration. However, the apparent $K_{0.5}$ for cytoplasmic Na^+ is independent of the extracellular Na^+ concentration. The activation by extracellular Na^+ at a fixed cytoplasmic Na^+ concentration is biphasic with a component which saturates at a concentration of about 1–2 mM extracellular Na^+, a plateau phase up to 20 mM, and another component which tends to saturate at about 80 mM followed by a slight deactivation at higher concentrations of Na^+. The apparent $K_{0.5}$ value for extracellular Na^+ is also found to be independent of the Na^+ concentration on the opposite side of the membrane. The activation by extracellular Na^+ can be explained by the negative cooperativity in the binding of extracellular Na^+, but positive cooperativity in the rate of dephosphorylation of enzyme species with one and three sodium ions bound extracellularly. Na^+ bound to E_2-PNa has a transmembrane effect on the cooperativity between binding of cytoplasmic Na^+, and E_2-PNa_2 does not dephosphorylate. $K_{0.5}/V_m$ for cytoplasmic as well as for extracellular Na^+ decreases with an increase in the *trans* Na^+ concentration in the non-saturating concentration range. The experiments indicate that at a step in the reaction simultaneous binding of extracellular and cytoplasmic Na^+ occurs.

Introduction

Phospholipid vesicles reconstituted with Na^+/K^+-ATPase from shark rectal glands engage in ATP hydrolysis-dependent Na^+–Na^+ exchange which is not stimulated by ADP but rather on the contrary, is inhibited. The stoichiometry of the exchange is similar to that for the Na^+–K^+ exchange, and the exchange is electrogenic, i.e., extracellular Na^+ has K^+-like effects [1]. The activity is about 6% of the activity of the Na^+–K^+ exchange. This opens the possibility of investigating the sided activation by Na^+ and the transmembrane effect of Na^+ on the activation under conditions where the enzyme turn-over rate is low, but where the reaction pathway is the same as that for the Na^+–K^+ exchange.

Previous experiments with reconstituted Na^+/K^+-ATPase from kidney have shown that non-transported extracellular Na^+ in the presence of extracellular K^+ has an effect, on the sigmoidicity of the activation by cytoplasmic Na^+. A Hill plot

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analysis suggested that extracellular Na^+ effects the cooperativity between binding of cytoplasmic Na^+, an allosteric effect of extracellular Na^+ [2]. With extracellular Na^+ in the absence of extracellular K^+, cytoplasmic Na^+ activates along a hyperbolic curve in ATP hydrolysis-dependent, as well as in ADP-dependent, Na^+–Na^+ exchange.

Preliminary experiments [3] have shown that with the reconstituted shark enzyme, cytoplasmic Na^+ activates along an S-shaped curve with extracellular Na^+ also in the absence of extracellular K^+. In the presence experiments, the sided, as well as the transmembrane, effect of Na^+ on ATP hydrolysis-dependent Na^+–Na^+ exchange was tested. The curves have been analyzed using the general treatment of Adair-Pauling for allosteric enzymes [4] which enables the calculation of values for the site constants.

Methods

Proteoliposome preparation, detection of symmetry and pump orientation and measurement of Na^+–Na^+ exchange parameters have been described in preceding papers [5,6], as have the curve fitting procedures by computer and the advantages of the Eadie plot for detection of biphasic activation curves.

The ligand effects were tested by measuring the influx of Na^+ into the proteoliposomes (cellular efflux), as described previously, and the ATP hydrolysis was tested using [^{32}P]ATP and analyzed according to Lindberg and Ernster [7]. The fraction of the enzyme which is not incorporated (n-o) is inhibited by preincubation with 1 mM ouabain in the presence of inorganic phosphate (P_i) and Mg^{2+}. The blank is the activity in the presence of digitoxigenin which penetrates the proteoliposomes and inhibits both non-oriented (n-o) and inside-out (i:o)-oriented enzyme molecules. The terms influx and efflux refer to the cellular situation.

In order to examine models for cooperative binding of ligands to proteins, the so-called Hill equation is often used [8], which describes the degree of cooperativity by a single parameter namely the Hill coefficient n_H:

$$Y = K\cdot(X)^{n_H}/(1+K\cdot(X)^{n_H})$$

A more general equation for cooperative ligand binding under conditions of equilibrium is given by Adair [9]:

$$nY = \frac{\sum_{i=1}^{n} i(EX)_i}{\sum_{i=0}^{n} (EX)_i}$$

Each step in the binding is characterized by a separate intrinsic association constant K_i' which relates to the thermodynamic constants (K_i) by statistical factors:

$$K_i = \left(\frac{n+1-i}{i}\right)K_i'$$

For a three-site model, the Adair-equation conforms to [10]:

$$Y = \frac{K_1'X + 2K_1'K_2'X^2 + K_1'K_2'K_3'X^3}{1 + 3K_1'X + 3K_1'K_2'X^2 + K_1'K_2'K_3'X^3}$$

The fractional saturation (Y) which cannot be measured can be estimated by v/V_m, where v is the measured velocity and V_m is the maximum velocity of the reaction studied. The data from the rate of ATP-hydrolysis versus ligand concentration are fitted to the Adair equation in which K_i' and i (the site index number) are varied by a multiple non-linear regression analysis and the best fit is distinguished for a varying number of sites (n) by comparing the variance of the fit.

By assigning the fractional saturation (Y) as v/V_m in the Adair equation we assume quasi-equilibrium conditions in order for the fitted polynomial coefficients to represent intrinsic association constants. This assumes that the reactions involving cation binding are in rapid equilibrium and the cooperative interaction affects only the affinity of the cations. These assumptions are probable, since the rate of dephosphorylation in the absence of extracellular K^+ is slow compared to the other steps in the overall reaction scheme. However, we will refer to the computed intrinsic association constants as apparent in order to indicate that their interpretation depends on the model in use.

Results

The activation by Na^+ of an unsided preparation of membrane bound Na^+/K^+-ATPase in the absence of K^+ is shown in Fig. 1A with 1 μM ATP and 1B with 25 μM ATP. $K_{0.5}$ for ATP is about 0.1 μM [6]. The figure shows that $K_{0.5}$ for Na^+ activation decreases from about 15 mM to about 1.7 mM with an increasing concentration of ATP. The Na^+ activation curves are non-hyperbolic; this can be clearly seen from the insets which are Eadie plots which give one straight line for a hyperbolic curve.

Cytoplasmic effect of Na^+

At a concentration of 130 mM extracellular Na^+ (intraliposomal), Na^+ on the cytoplasmic side activates ATP hydrolysis along a sigmoid curve. This is shown with 25 μM ATP in Fig. 2. The Eadie plot (inset) displays positive cooperativity characteristics. The best fit to the Adair equation of the data for cytoplasmic Na^+ activation is obtained assuming three cytoplasmic Na^+ sites exhibiting positive cooperative interaction. The apparent intrinsic association constants calculated using the Adair equation is used to construct the curve in Fig. 2 and in the inset.

As discussed above for the unsided preparation, $K_{0.5}$ for the Na^+ activation decreases when the ATP concentration increases. With the sided preparation, there is no significant difference in $K_{0.5}$ for activation by cytoplasmic Na^+ with 1 μM and with 25 μM ATP. The effect of ATP on $K_{0.5}$

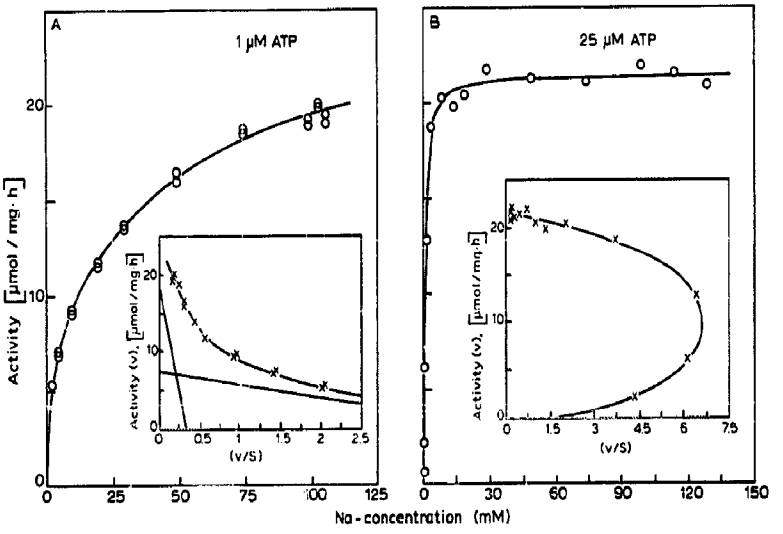


Fig. 1. The hydrolytic activity of membrane-bound (unsided) shark Na^+/K^+-ATPase as a function of the Na^+ concentration at 1 μM ATP (A) or at 25 μM ATP (B) in a 20 mM histidine-HCl buffer (pH 7.0) with 1 mM Mg^{2+} at 22° C. The continuous line in panel A is computed by a weighted non-linear regression analysis to a second-order rate equation

$$v = (a \cdot ATP^2 + b \cdot ATP)/(ATP^2 + c \cdot ATP + d)$$

with constants $a = 25.87$ μmol/mg per h, $b = 425.9$ mM, $c = 55.2$ mM, $d = 91.56$ mM^2 or to the equivalent sum of two Michaelis-Menten equations,

$$V = V_1 \cdot ATP/(K_1 + ATP) + V_2 \cdot ATP/(K_2 + ATP)$$

with constants $V_1 = 7.37$ μmol/mg per h, $K_1 = 1.71$ mM, $V_2 = 18.59$ μmol/mg per h, $K_2 = 53.5$ mM. The half-maximal activation constant $K_{0.5}$ is 14.5 mM. The inset in panel A shows the data replotted in an Eadie plot with the regression curve computed as explained above. The slopes of the two assymptotes are defined by the two K values which intersect the ordinate at V_1 and V_2. In panel B the inset shows the data replotted according to the Eadie plot. The data are fitted to a second-degree rate equation with coefficients: $a = 21.3$ μmol/mg per h, $b = 1.09$ mM, $c = 0.34$ mM and $d = 2.28$ mM^2. The data in panel B cannot be fitted to the sum of two Michaelis-Menten equations. The half-maximal activation constant $K_{0.5}$ is 1.7 mM.

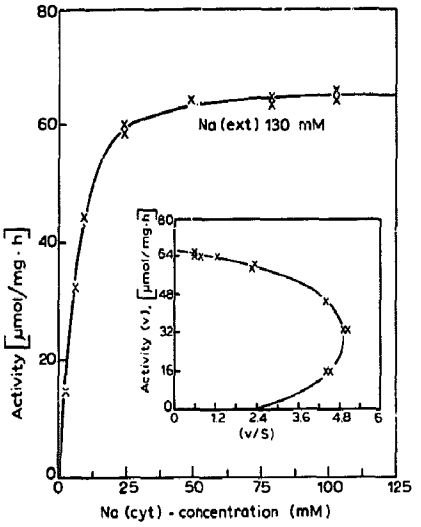


Fig. 2. The hydrolytic activity of reconstituted inside-out oriented shark Na^+/K^+-ATPase as a function of the cytoplasmic Na^+ concentration at 25 µM ATP in 20 mM histidine-HCl, 1 mM Mg^{2+} (pH 7.0 at 22° C). The extracellular Na^+ concentration is 130 mM and with no K^+. The inset depicts the data replotted as an Eadie plot. The curves are calculated by regression analysis using the Adair equation with three cooperative cytoplasmic Na^+ sites. The fitting parameters are: $K_1' = 0.036 \pm 0.008$ mM^{-1}, $K_2' = 0.19 \pm 0.07$ mM^{-1}, $K_3' = 0.47 \pm 0.13$ mM^{-1} and $V_m = 66.3 \pm 0.65$ µmol/mg i:o protein per h. The $K_{0.5}$ value is 6.7 mM.

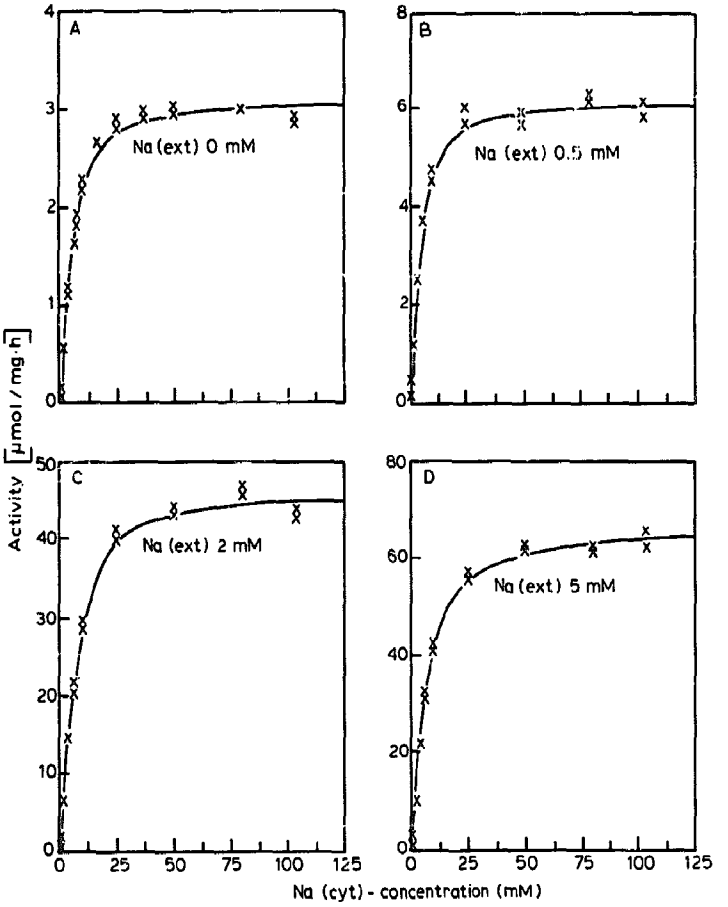


Fig. 3. Saturation plots as depicted in Fig. 2 but obtained at eight different fixed extracellular Na^+ concentrations. The fitting parameters (i.e., the intrinsic association site constants and V_m) used to construct the curves are calculated by regression analysis to the Adair equation with three sites. The fitting parameters transformed into dissociation constants are shown in Table I. The fitting

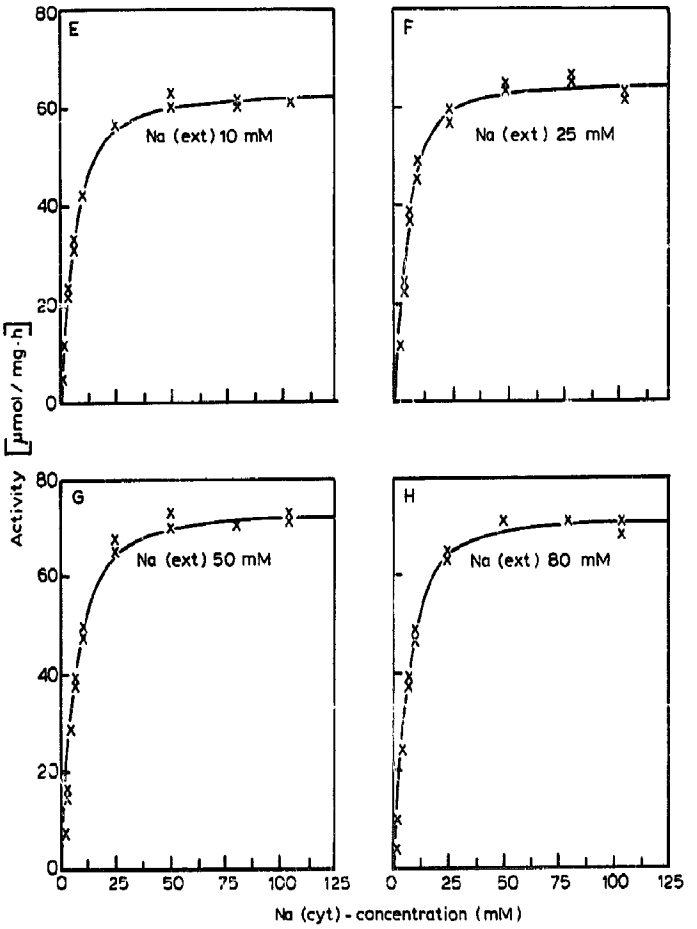
for Na^+ activation on the cytoplasmic side is not significant (data not shown).

The same results as those shown in Fig. 2 are obtained when the influx of Na^+ is used as a test parameter, but there is more scatter on the results. In the following, the results presented are based on measurements of hydrolysis.

Transmembrane Na^+-effect

The effect of the extracellular Na^+ concentration on the activation by cytoplasmic Na^+ of the hydrolytic activity is shown in Fig. 3. The experiments are performed at eight different fixed extracellular concentrations of Na^+ between 0 to 130 mM. The concentration of ATP is 25 μM in all experiments.

The experiment without extracellular Na^+ (Fig. 3A) corresponds to the uncoupled Na^+ efflux. It amounts to about 5–10% of the maximum Na^+–Na^+ exchange. Eadie plots (not shown) show that the activation curves are all sigmoid. Attempts to fit the data to either a simple hyperbolic or to the sum of two Michaelis-Menten equations were unsuccessfull. A plot of $[Na]/v^{(1/n)}$ versus [Na] gives linear plots for $n = 3$, but not for $n < 3$. The curves are the best fits to the Adair equation assuming a model with three cytoplasmic Na^+ sites which interact cooperatively. The resulting



parameters for the uncoupled efflux (the curve with zero extracellular Na^+) are: $K_1' = 0.047\ mM^{-1}$, $K_2' = 0.32\ mM^{-1}$, $K_3' = 0.29\ mM^{-1}$ and $V_m = 3.14$ μmol/mg (i:o)-protein per h. The $K_{0.5}$ values for the different activation curves are depicted in Fig. 4.

TABLE I

THE BEST-FITTING PARAMETERS COMPUTED USING A NON-LINEAR WEIGHTED REGRESSION ANALYSIS TO THE ADAIR EQUATION

Parameters are for three cooperative cytoplasmic Na^+ sites at different fixed extracellular Na^+ concentrations. The values are given as means ± S.E. for n different experiments.

Extracellular Na^+ concn. (mM)	Apparent intrinsic dissociation constants (mM)			Apparent dissociation constant (mM) ($K_{0.5}$)	Maximum hydrolytic activity (μmol/mg (i:o)protein per h) (V_m)	Number of experiments (n)
	K_1'	K_2'	K_3'			
2	26.9±0.8	3.9±0.3	4.0±0.2	6.92±0.14	45.7±1.4	3
5	23.2±1.7	4.4±0.8	4.3±0.8	6.75±0.03	64.0±1.8	4
10	13.5±1.4	7.5±0.6	2.4±0.2	6.42±0.09	61.6±2.4	8
25	14.6±0.9	9.9±1.0	1.6±0.3	6.41±0.09	66.1±1.5	8
50	15.2±2.1	7.0±1.5	3.5±0.6	6.74±0.12	72.2±1.7	5
80	24.8±3.2	4.7±1.3	3.6±0.5	6.22±0.11	86.1±3.6	4
130	40.2±2.5	3.5±0.9	3.0±0.5	6.70±0.11	73.6±1.3	4

fitting parameters (K_1', K_2' and K_3') converted to apparent dissociation constants are shown in Table I. As indicated, the apparent intrinsic dissociation constants are related thus: $K_1' > K_2' > K_3'$, i.e., the three cytoplasmic Na^+ sites exhibit positive cooperative interaction.

The calculated values for the three cytoplasmic apparent dissociation constants depend on the *trans* concentration of Na^+. However, although the extracellular Na^+ concentration has an effect on the apparent intrinsic site constants, the Na^+ value for half-maximal activation, $K_{0.5}$, is about 6 mM, independent of the extracellular concentration of Na^+ (Table I and Fig. 4).

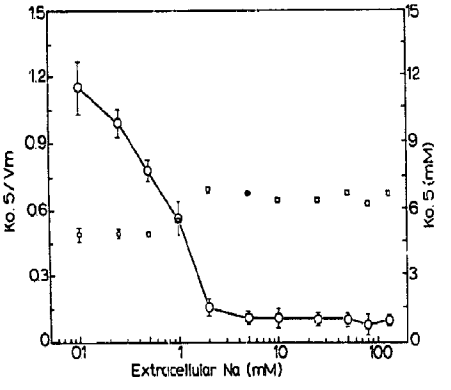


Fig. 4. $K_{0.5}$ (small circles) and the ratio $K_{0.5}/V_m$ (large circles) for cytoplasmic Na^+ as a function of the *trans* -concentration of extracellular Na^+. Vertical bars indicate ± 1 S.E. ATP, 25 μM; Mg^{2+}, 1 mM; 20 mM, histidine-HCl (pH 7.0, 22° C).

The ratio $K_{0.5}/V_m$ for cytoplasmic Na^+ increases as the extracellular concentration of Na^+ decreases. The effect is seen with lower non-saturating concentrations of extracellular Na^+ (Fig. 4).

Extracellular effect of Na^+

The results of the experiments shown in Fig. 3 are replotted in Fig. 5 in order to show the activation by extracellular Na^+ at different fixed cytoplasmic Na^+ concentrations. The activity is shown as a function of the extracellular Na^+ concentration with the cytoplasmic concentration held con-

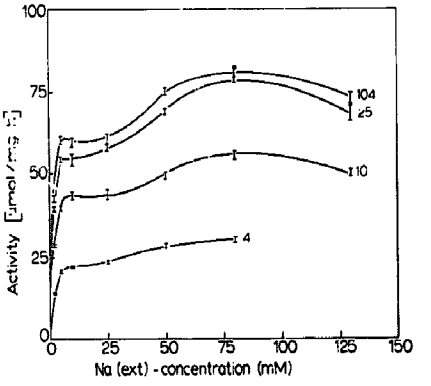


Fig. 5. The hydrolytic activity of (i:o)-oriented enzyme molecules as a function of extracellular Na^+ at four fixed concentrations of cytoplasmic Na^+, as indicated on the curves. The concentration of ATP is 25 μM, Mg^{2+}, 1 mM; histidine-HCl, 20 mM (pH 7.0, 22° C). The curves were drawn by eye. Bars indicate ± 1 S.E. for six experiments.

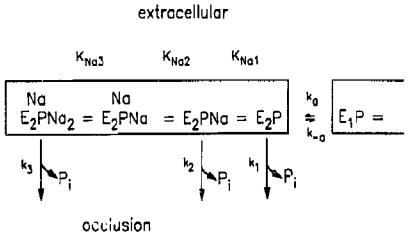


Fig. 6. A model for activation by extracellular Na^+ of dephosphorylation. The enzyme species enclosed in boxes are assumed to be in rapid equilibrium. K_{Na} is the dissociation constant and k the rate constant. The model envisages that an extracellular sodium ion is bound at a transport site on E_2P to form E_2PNa. This species can dephosphorylate with a rate constant of k_2. An allosteric site is then occupied, but the species formed, E_2PNa_2, is not supposed to dephosphorylate before the second transport site is occupied. Dephosphorylation of E_2P without Na^+ bound is given by k_1 and corresponds to the uncoupled efflux which is very small, about 5% of the maximum Na^+–Na^+ exchange.

stant between 4 mM and 104 mM. The activation by extracellular Na^+ gives a complicated biphasic curve: extracellular Na^+ activates with a high affinity, this is followed by a plateau with concentrations of sodium between 5 and 20 mM, and at higher concentrations of Na^+, there is a further activation with an optimum around 80 mM followed by a slight decrease in activity with higher concentrations of Na^+. The $K_{0.5}$ value is about 2 mM.

Levitzki and Koshland [11] have analyzed substrate curves which exhibit a decrease in activity at intermediary substrate concentrations followed by an activation at higher concentrations. They demonstrated that this can be explained from a negative cooperativity in ligand binding combined with positive cooperativity in the rate of catalysis. In Fig. 6, a model is shown for extracellular Na^+ activation based on the ideas of Levitzki and Koshland. With no Na^+, E_2-P dephosphorylates with a rate constant, k_1, which is low; this is the uncoupled Na^+ efflux which amounts to about 5–10% of V_m for Na^+–Na^+ exchange. At low Na^+ concentrations, Na^+ binds to the first transport site and E_2-PNa is formed with a high affinity for Na^+ (dissociation constant K_{Na1}) and with a rate constant of k_2 for dephosphorylation. At higher Na^+ concentrations, a second, allosteric Na^+ site is filled, with the dissociation constant K_{Na2} ($K_{Na2} > K_{Na1}$). E_2-PNa_2 cannot dephosphorylate, and it is the formation of this species which gives rise to the plateau phase in the saturation curve. At higher Na^+ concentrations, the second transport site is filled and E_2-PNa_3 is formed. This form dephosphorylates with a rate

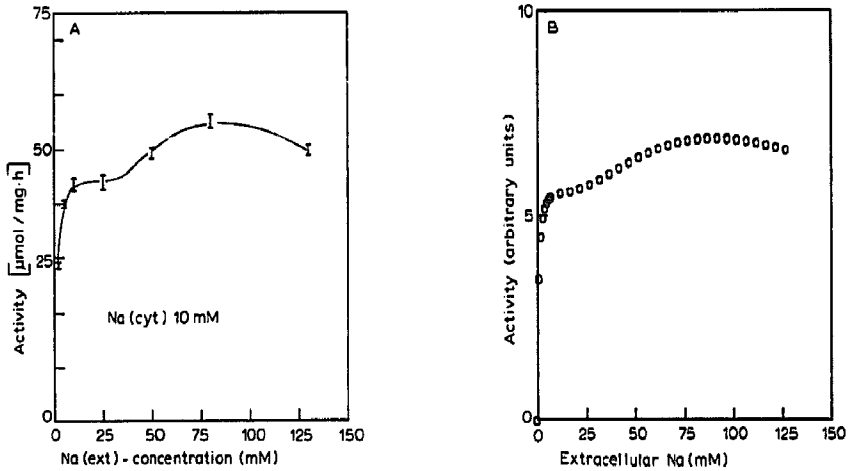


Fig. 7. (A) The hydrolytic activity of reconstituted inside-out shark Na^+/K^+-ATPase as a function of extracellular Na^+ with 10 mM cytoplasmic Na^+. ATP, 25 µM; Mg^{2+}, 1 mM; histidine-HCl, 20 mM (pH 7.0, 22°C). (B) Computer simulation of the curve in A using the model described in Fig. 6. The dissociation constants used for the E_2P pool are: $K_{Na1} = 1$ mM, $K_{Na2} = 60$ mM and $K_{Na3} = 80$ mM. The dissociation constant for the backward reaction $E_1P + Na^+ = E_1PNa$ is 25 mM. The rate constants are: $k_a = 300$ s^{-1}, $k_{-a} = 5$ s^{-1}, $k_2 = 7.5$ s^{-1} and $k_3 = 22$ s^{-1}. The ATP concentration is 25 µM. The rate constants used for the turnover steps not shown are high enough that in steady state practically all the enzyme is in the phosphoform.

constant of k_3, which is higher than k_2, and the dephosphorylation gives rise to occlusion and transport of $2Na^+$ bound at the transport sites while the Na^+ bound to the allosteric site is not transported.

Using Cha's method [12], the rate equations equivalent to the activation by extracellular Na^+ according to the model is solved and the saturation curve is simulated on a microcomputer. The slight deactivation at Na^+ concentrations higher than 80 mM is simulated by assigning a dissociation constant for the backward reaction E_1-P + $Na^+ = E_1$-PNa which is lower than K_{Na3}. The result is given in Fig. 7. Comparison with the experimental curve in panel A shows that it is possible with this model to simulate quite accurately the experimental curve for the activation by extracellular Na^+.

As discussed above $K_{0.5}/V_m$ for cytoplasmic Na^+ activation increases with a decrease in the extracellular Na^+ concentration in the non-saturating range (Fig. 4). Similarly, $K_{0.5}/V_m$ for extracellular Na^+ increases with a decrease in the concentration of cytoplasmic Na^+ into the non-saturating range (not shown).

Discussion

In red cells, sigmoid dependence on cytoplasmic Na^+ concentration has been demonstrated by Garay and Garrahan [13] and by Blostein [14] for both Na^+–K^+ and for Na^+–Na^+ exchange. In proteoliposomes, Karlish and Stein found sigmoid activation by cytoplasmic Na^+ of Na^+–K^+ exchange, but quasi-hyperbolic activation of the ATP hydrolysis- as well as of the ADP-dependent Na^+–Na^+ exchange [2]. Recently, Apell and Marcus [15] found hyperbolic activation by cytoplasmic Na^+ of Na^+/K^+-ATPase activity of reconstituted rabbit kidney enzyme.

In the present investigation, the kinetic data can be interpreted as indicating positive cooperative activation by cytoplasmic Na^+ of Na^+–Na^+ exchange accompanied by ATP hydrolysis. In evaluating the binding constants for cytoplasmic Na^+, we take the rate equation (v/V_m) as proportional to the fractional saturation (Y). Therefore, it is implicitly assumed that all three enzyme forms with bound cytoplasmic Na^+ contribute to catalysis, their rate per binding site being equal. However, a model in which only enzyme forms with three sodium ions bound contribute to catalysis would be equally probable. Distinction, between such models could be possible by analyzing stoichiometry at low and high Na^+ concentrations. With these assumptions and at saturating extracellular Na^+ concentrations the three apparent intrinsic site dissociation constants are about 40 mM, 3.5 mM and 3 mM for the first, second and third site, respectively. The $K_{0.5}$ value is found to be about 6 mM.

With reconstituted kidney enzyme and using a Hill plot to analyze the results, Karlish and Stein [2] showed that extracellular Na^+ influences the degree of sigmoidicity of the curves for cytoplasmic Na^+ activation of Na^+–K^+ exchange. The experiments lead to the conclusion that non-transported extracellular Na^+ has an effect on the cooperativity for binding of cytoplasmic Na^+, an allosteric effect of extracellular Na^+. This is observed in the Na^+–K^+ exchange reaction but not in Na^+–Na^+ exchange. In contrast to this, the present experiments with ATP hydrolysis-dependent Na^+–Na^+ exchange show that extracellular Na^+ in the absence of extracellular K^+ influences the cooperativity between the binding of three sodium ions on the cytoplasmic side of the system. It is not possible to determine whether these differences in results are due to different preparations and/or to the more sensitive analysis using the more general approach of Adair-Pauling rather than the Hill equation.

Although extracellular Na^+ is found to influence the cooperativity between the binding of the three cytoplasmic sodium ions, there is practically no effect on the $K_{0.5}$ value for cytoplasmic Na^+ activation. Neither is there any effect of cytoplasmic Na^+ on the $K_{0.5}$ for activation by extracellular Na^+.

An increase in the ATP concentration from 1 μM to 25 μM, which decreases the $K_{0.5}$ for Na^+ activation of the unsided preparation has no effect on the $K_{0.5}$ value for activation by cytoplasmic Na^+, suggesting that it is due to an effect on the $K_{0.5}$ value for Na^+ activation on the extracellular side.

With the unsided preparation of Na^+/K^+-ATPase, a biphasic activation with Na^+ in the

absence of K^+ is observed in accordance with previous investigations [16–20,22]. The present experiments suggest that this is mainly due to an effect of extracellular Na^+.

With sided preparations, red blood cells, extracellular Na^+ in low concentrations in the absence of extracellular K^+ inhibits an uncoupled Na^+ efflux with a flux minimum at about 5 mM Na^+; at higher concentrations of extracellular Na^+, the efflux is stimulated [21]. A similar effect of extracellular Na^+ is observed on the Na-ATPase activity [22,23].

With the reconstituted shark enzyme, the uncoupled Na^+ efflux in the absence of extracellular Na^+ is low, 5–10% of the Na^+–Na^+ exchange. Extracellular Na^+ has no inhibitory effect: the activity in the presence of Na^+, tested at concentrations of 0.1 mM and more is higher than that in the absence of Na^+ and increases with the Na^+ concentration. This means that E_2-P has a lower rate of dephosphorylation than E_2-PNa and that in order to explain the biphasic activation by Na^+ E_2-PNa_2 does not dephosphorylate, while E_2-PNA_3 does and with a higher rate than E_2-PNa (the model in Fig. 6). For methodological reasons it is difficult to measure the stoichiometry of the Na^+–Na^+ exchange at low extracellular Na^+ concentrations and thereby to determine whether dephosphorylation of E_2-PNa results in Na^+ transport. With saturating concentrations of Na^+ on both sides of the membrane, the stoichiometry of the Na^+ transport (efflux-influx) is 2.8–1.8 per ATP hydrolysed [1]. This indicates that one of the three sodium ions bound to E_2-PNa_3 is not transported. This is in agreement with previous suggestions of a modifying effect of a non-transported extracellular sodium ion [22,24,25] and the observations of Karlish and Stein [2] that there is an effect of extracellular Na^+ which is not transported, and of Sachs [26] that non-transported extracellular Na^+ has a protective effect against the enhancement, by low concentrations of extracellular K^+, of the inhibition by vanadate. The inhibition by vanadate probably occurs with the K^+-occluded form of the enzyme, suggesting a transmembrane effect of extracellular Na^+. The affinity for Na^+ relative to K^+ at the Na^+-protective site is high enough for the site to be occupied by Na^+ at physiological concentrations of extracellular Na^+ and K^+ [26].

The inhibitory effect of low concentrations of extracellular Na^+ on the Na^+ efflux [21] and on the Na^+-ATPase activity [22,23] with red blood cells and the stimulation by higher concentrations of extracellular Na^+ can be explained from the model in Fig. 6, assuming that E_2-PNa in the red blood cells has a lower rate of dephosphorylation than E_2-P.

Unless the enzyme can 'remember' through a turnover that there has been Na^+ on the extracellular side, the effect of extracellular Na^+ on the cooperativity for binding of cytoplasmic Na^+ means a simultaneous binding of extracellular Na^+ on an allosteric site and of Na^+ on transport sites on the cytoplasmic side. The observation that the $K_{0.5}/V_m$ for Na^+ decreases with an increase in non-saturating transmembrane concentrations of Na^+ also points towards a simultaneous binding af cytoplasmic and extracellular Na^+ (see Ref. 27).

In previous experiments with red blood cells, Sachs [28] found that the $K_{0.5}/V_m$ for extracellular K^+ decreases with an increase in cytoplasmic Na^+, in agreement with simultaneous binding of Na^+ and K^+ at a given step in the reaction. However, using a model for Na^+–K^+ exchange which included an uncoupled Na^+ efflux, Sachs [28] showed that the observed decrease in $K_{0.5}/V_m$ can be explained by the uncoupled Na^+ efflux i.e., with corrections made for the uncoupled efflux, $K_{0.5}/V_m$ is constant, compatible with a ping-pong model and not with simultaneous binding of Na^+ and K^+.

In the present experiments, $K_{0.5}$ for extracellular Na^+ as well as for cytoplasmic Na^+ is practically independent of a variation in the transmembrane concentration of Na^+. With a correction for the uncoupled efflux of Na^+ which is only 5–10% of the Na^+–Na^+ exchange, there is still a decrease in $K_{0.5}/V_m$ for cytoplasmic Na^+ with an increase in non-saturating transmembrane concentrations of Na^+, in agreement with simultaneous, but not consecutive, binding of extracellular and cytoplasmic Na^+. This does not necessarily mean simultaneous transport.

Based on the analysis of sequential models by Cleland [27], Hoffman and Tosteson [29] concluded that ping-pong-bi-bi mechanisms, like the consecutive Albers-Post model [30,31], were unable to explain their experimental findings in sheep red cells that the $K_{0.5}$ for activation of the *cis*-ion

is independent of the concentration of the *trans*-ion, and they suggested a simultaneous exchange of Na^+ and K^+.

With three sodium ions bound extracellularly but only two transported, it seems reasonable to assume that it is one of those three sodium ions which causes the allosteric effect, but which one? A guess would be the one which blocks dephosphorylation [24], the second one bound in the model shown in Fig. 6. But what is the nature of this site? Is it a site which does not take part in transport, or is it the one of the three sites which takes part in the transport of Na^+ from the cytoplasmic to the extracellular side, but which does not transport Na^+ in the opposite direction. With an allosteric effect of extracellular Na^+ on the binding of cytoplasmic Na^+ the latter cannot be the case in a consequtive reaction but in a simultaneous: in the translocation step the allosteric extracellular Na^+ on the third site is not transported but leaves to the extracellular side [32].

Simultaneous binding of extracellular and cytoplasmic Na^+, assuming it is only non-transported Na^+ with an allosteric effect which is bound extracellularly, has implications for the transport model. It is, however, necessary to know what the effect of K^+ is on the Na^+ effect and vice versa on each of the two sides of the membrane to which the model is applied. We shall return to this problem in a subsequent paper.

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