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$(Na^+ + K^+)$-ATPase in artificial lipid vesicles: influence of the concentration of mono- and divalent cations on the pumping rate

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$(Na^+ + K^+)$-ATPase from kidney outer medulla was incorporated into artificial dioleoylphosphatidylcholine vesicles. Transport activity was induced by adding ATP to the external medium. A voltage-sensitive dye was used to detect the ATP-driven potassium extrusion in the presence of valinomycin. The observed substrate–protein interactions of the reconstituted $(Na^+ + K^+)$-ATPase largely agree with that from native tissues. An agreement between ATP hydrolysis and transport activity is given for concentration dependences of sodium, potassium, magnesium and calcium ions. The only significant deviations were observed in the influence of pH. Protons were found to have different influence on transport, enzymatic activity and phosphorylation of the enzyme. The transport studies showed a twofold interaction of protons with the protein: (1) competition with sodium at the cytoplasmic ion binding sites, (2) a non competitive inhibition of transport which is not correlated with protein phosphorylation.

Introduction

The $(Na^+ + K^+)$-ATPase is a plasma-membrane protein of animal cells that transports sodium ions outward and potassium ions inward against their electrochemical gradients [1]. The $(Na^+ + K^+)$-ATPase is characterized by its requirement of ATP, Mg^{2+} and Na^+ on the cytoplasmic side and of K^+ at the extracellular side [2–5].

An intermediate step in the pumping cycle is the phosphorylation of the protein. Magnesium is a cofactor required for phosphorylation [1,6] and for pumping activity [7,8]. At least in part of the pumping cycle the catalytic function of magnesium can be replaced by other divalent cations [9–12]. However, none of the substitutes is as effective as Mg^{2+}. Of special interest is the role of calcium which is the most common divalent cation in the cell besides magnesium. Calcium is reported to compete with magnesium [13,14].

Sodium as cofactor for the enzyme phosphorylation could not be replaced by other cations in the case of isolated enzyme [10] whereas lithium was found to substitute partly for sodium in experiments with $(Na^+ + K^+)$-ATPase in erythrocyte membranes [15]. Catalytic activity and sodium transport are concentration dependent [8,16–22] and are competitively inhibited by potassium on the cytoplasmic side of the membrane [15,23–25]. Recently an influence of pH on sodium binding was reported [26,46]. In contrast to the highly selective role of sodium at the periplasmic binding site the potassium ion can be replaced by a variety of other monovalent cations at the cytoplasmic binding site [15,16,25,27].

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In recent years reconstituted lipid vesicle containing purified $(Na^+ + K^+)$-ATPase have been used to study transport activity [28–39]. In most of the experiments carried out so far, the fluxes of Na^+ and K^+ have been studied with radioactive isotopes. The time resolution of the isotope technique is limited by the procedure for separating vesicles and medium.

Recently a fluorescence technique using a potential-sensitive dye was described which allows to monitor ATP-driven potassium extrusion continuously [37,40,41]. A statistical analysis of the time-course of the fluorescence signal taking into account the vesicle inhomogeneity and protein distribution yields the average density of functional pump molecules as well as the turnover rate of the pump [37,42,43]. In the following this method is used to investigate the transport activity of reconstituted $(Na^+ + K^+)$-ATPase as a function of ionic composition on the cytoplasmic (extravesicular) side of the membrane.

Materials and Methods

Dioleoylphosphatidylcholine was obtained from Avanti Polar Lipids, Inc., Birmingham, AL; ATP was from Boehringer, Mannheim (Sonderqualität). 1,3,3,1′,3′,3′-Hexamethylindodicarbocyanine (NK 529) was purchased from Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan. All other reagents were obtained from Merck (analytical grade). Dialysis tubing was purchased from Serva, Heidelberg.

$(Na^+ + K^+)$-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen [44], as described previously [37,45]. The specific activity was in the range of 1850–2100 μmol P_i per hour and mg protein at 37°C. The enzyme was solubilized in a solution of 23 mM sodium cholate in 'buffer H' (30 mM imidazole, 1 mM L-cysteine, 1 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM $MgSO_4$; the pH was adjusted to 7.2 with H_2SO_4). The enzyme solubilisate was mixed with dioleoylphosphatidylcholine solubilized in cholate buffer. The mixture (7.5 mg lipid and about 0.6 mg protein per ml) was dialyzed against buffer H containing 70 mM K_2SO_4 and 5 mM Na_2SO_4 [37,43]. The resulting vesicles had an average diameter of 96 nm with a half-width of the distribution of approx. 10 nm [37,40]. The $(Na^+ + K^+)$-ATPase used for the experiments in this paper was isolated form a different breeding of rabbits compared to previous papers [37,40,41]. From this enzyme preparations approx. 40% of enzymatic and transport activity was lost between microsomal and vesicle preparation. The remaining active pumps were unchanged in their properties.

The fluorescence of the indocyanine dye was measured as described previously [37]. The excitation wavelength was set to 620 nm (slit width 5 nm) and the emission wavelength to 680 nm (slit width 20 nm). The thermostated cell, equipped with a magnetic stirrer, was filled with 1 ml buffer H containing 5 mM K_2SO_4, 70 mM Na_2SO_4, 2.3 μM of the dye. 5 μl of the vesicle suspension containing approx. 10 mg lipid/ml was added to the solution in the cell. When the fluorescence signal had reached a steady level, valinomycin was added from a concentrated stock solution in ethanol to give a total concentration 20 nM. After activation of the pump by addition of ATP to the medium, the time-course of the fluorescence signal was recorded.

The analysis of the experimental data requires a calibration of the relative fluorescence change $\Delta F/F_0$ as a function of membrane voltage U (F_0 is the fluorescence signal prior to the addition of valinomycin). As described previously [37], the calibration was carried out by measuring $\Delta F/F_0$ at different ratios of internal and external K^+ concentrations in the presence of valinomycin, using the Nernst equation. Changing Na^+, K^+, Mg^{2+} and Ca^{2+} concentrations in the measuring buffers had no influence on the fluorescence-potential calibration. In the data analysis the size distribution of the vesicles was accounted for explicitely, as well as the distribution in the number of pump molecules per vesicle [37]. A theoretical curve was fitted to the observed time-course of $\Delta F/F_0$ with the turnover rate v_m and the average number χ of pump molecules per μm^2 as adjustable parameters [37], assuming that in the presence of valinomycin three Na^+ ions move inward and three K^+ ions outward per turnover. v_m is the turnover rate at time $t = 0$; since the initial intravesicular K^+ concentration is 140 mM, v_m is virtually identical with the maximum turnover rate

at saturating [K^+]. For a given vesicle preparation the experiments could always be fitted with a single value of χ, as expected. Variations in the concentrations of different ion species only affected the turnover rate v_m.

Since the buffer used in the experiments contained 1 mM EDTA, the free calcium concentration which was varied in a series of experiments was determined by a calcium-sensitive electrode (F2110 Ca, Radiometer SA, Copenhagen, Denmark; reference electrode: $Hg/HgSO_4$-electrode, Metrohm AG, Herisau, Switzerland).

The enzymatic activity was determined in parallel to the fluorescence experiments. At certain time periods aliquots of 80 μl containing 5 μg protein were taken from the vesicle suspension and the reaction was stopped in icecold perchloric acid. The content of free inorganic phosphate was determined by the method of Panusz et al. [47]. Contaminations with phosphate were determinated by an additional experiment in which all components were mixed at 0°C. The results were used for correction. Control experiments using vesicles without enzyme showed that spontaneous hydrolysis was negligible within the time of experiments (< 30 min). Experiments in the presence of 300 μM ouabain showed no significant difference in enzymatic activity, indicating a negligible fraction of not incorporated ($Na^+ + K^+$)-ATPase.

Results

Effects of extravesicular (cytoplasmic) sodium and potassium

Activation of the ($Na^+ + K^+$)-pump by ATP is demonstrated in Fig. 1. $\Delta F/F_0$ is the relatively change of fluorescence intensity, referred to the fluorescence intensity F_0 prior to the addition of valinomycin. At the beginning of the experiment the interior of the ($Na^+ + K^+$)-ATPase vesicles contained 140 mM K^+ and 10 mM Na^+, the medium 2.5 μM indocyanine, 10 mM K^+ and 140 mM Na^+ (lower trace). The only anion was sulfate. Valinomycin and ATP were added successively. The fluorescence change after addition of valinomycin reflects the establishment of the Nernst potential for K^+. When ATP is added to the medium, K^+ is extruded from the vesicles and the Nernst potential decreases. In a control experiment 5 μl vesicles dialyzed against 10 mM Na^+ were diluted into a Na^+ free medium, containing 140 mM Tris (tris(hydroxymethyl)aminomethane). Since ATP was added as Tris salt, the total external Na^+ concentration was 50 μM. As seen from Fig. 1 the pump activity is negligibly small in the presence of 50 μM Na^+. The extravesicular medium corresponds to the cytoplasm in the native state of the pump. The initial jump of fluorescence upon ATP addition is partly caused by dilution of the medium and (possibly) by an ad-

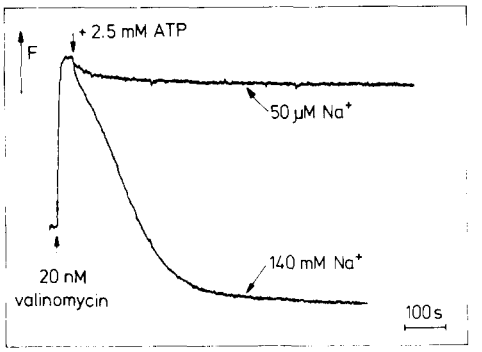

Fig. 1. Pumping activity of reconstituted ($Na^+ + K^+$)-ATPase detected by the change of fluorescence intensity F. ($Na^+ + K^+$)-ATPase vesicles were formed in buffer H containing 70 mM K_2SO_4 and 5 mM Na_2SO_4. 5 μl of the vesicle suspension (10 mg lipid/ml) were diluted into 1 ml buffer H containing 2.3 μM indocyanine dye and either 5 mM K_2SO_4 plus 70 mM Na_2SO_4 or 70 mM Tris sulfate. Thereafter 20 nM valinomycin and 2.5 mM ATP were added successively. The fast signal change after addition of ATP results from dilution of the medium. The temperature was 16.2°C.

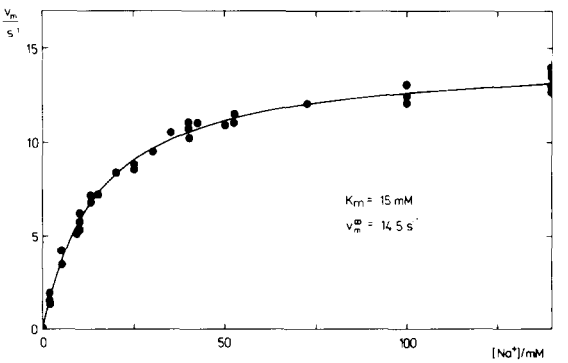

Fig. 2. Turnover rate v_m as a function of external Na^+ concentration; T 16°C. Except for the Na^+ concentration the experimental conditions were the same as indicated in Fig. 1. The theoretical curve represents a Michaelis-Menten plot according to Eqn. 1 with $K_m = 15$ mM, $v_m^\infty = 14.5$ s^{-1}.

ditional effect of adsorption of the negatively-charged Mg-ATP complex onto the vesicle membrane.

In Fig. 2 the turnover rate v_m is plotted as a function of external Na^+ concentration c_N. By addition of $Tris^+$ the total cation concentration ($Na^+ + Tris^+$) was kept constant at 140 mM. The values of v_m were determined according to the fitting procedure described in Ref. 37 with fixed values of the average vesicle diameter ($\bar{r} = 45$ nm), the variance of vesicle radii ($\sigma = 10$ nm), and of the average number n_p of pump molecules (ATP binding site facting outward) per vesicle. n_p varied for the different preparations between 4.5 and 7.2, depending on the initial protein concentration. The experimental values of v_m could be fitted by a Michaelis-Menten relation with a single binding constant according to

$$v_m = v_m^\infty \cdot \frac{c_N}{c_N + K_m} \quad (1)$$

with $K_m = 15$ mM. v_m^∞ is the turnover rate of the pump in presence of saturating Na^+ concentration c_N.

In another set of experiments the potassium concentration was varied in the external medium.

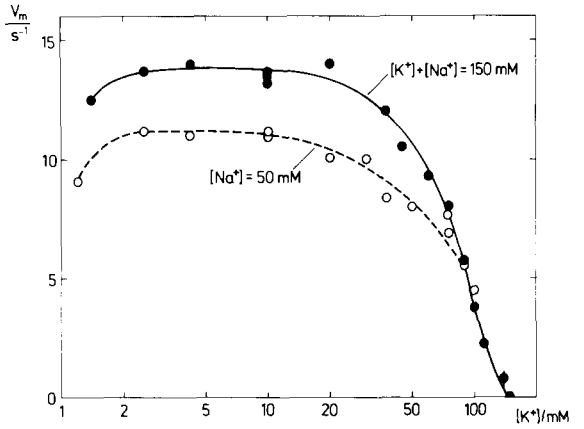

Fig. 3. Turnover rate v_m as a function of external K^+ concentration; T 16°C. The upper curve refers to experiments in which the sum of the K^+ and Na^+ concentrations was kept constant at 150 mM. The lower curve was obtained from experiments with a constant Na^+ concentration of 50 mM and variable K^+ concentration; $Tris^+$ was added in order to maintain the total cation concentration at 150 mM. All other conditions were the same as described in Fig. 1. The curves were drawn to guide the eye.

The turnover rate v_m as a function of the potassium concentration is shown in Fig. 3. Two different sets of experiments were performed. The upper curve in Fig. 3 describes experiments with different potassium concentrations in which the sum of Na^+ and K^+ concentration was kept at 150 mM. This series of experiments was carried out for comparison with data from literature [48]. In the second set the sodium concentration was kept constant at 40 mM and the potassium concentration was varied maintaining the ionic strength constant by addition of Tris. In both cases the pump is inhibited at potassium concentrations higher than 20 mM. A reduction of the turnover rate to 50% can be estimated to occur at 90–100 mM. In case of constant Na^+ concentration the inhibition can be described approximately by the empirical equation

$$v_m = v_m^* \frac{K_i}{K_i + C_K} \quad (2)$$

with $K_i = 95$ mM and $v_m^* = 12\ s^{-1}$ (at $c_N = 50$ mM).

Effects of Mg^{2+} and Ca^{2+}

It is known that Mg^{2+}, a necessary cofactor for ($Na^+ + K^+$)-ATPase [6], can be replaced by other divalent cations which, however, activate phosphorylation by ATP with less effectivity [9,11]. For physiological reasons the most important other divalent ion besides Mg^{2+} is Ca^{2+}. In Fig. 4A the pump rate v_m is shown for different concentrations of Mg^{2+} and Ca^{2+} which were added simultaneously to the vesicle suspension. Two significant observations can be made from these results: (1) In the absence of Mg^{2+} pumping activity is negligible, even with 20 mM Ca^{2+} (not shown in Fig. 4A). (2) Increasing concentrations of Ca^{2+} decrease the pump rate. In order to test the interaction between Mg^{2+} and Ca^{2+}, the data were replotted in the form of a Lineweaver-Burk diagram in Fig. 4B, which clearly reveals a competitive interaction between Ca^{2+} and Mg^{2+}. The inhibition constant of calcium was determined to be 35 μM. The pump rate v_m^∞ at saturating Mg^{2+} concentration is 15.6 s^{-1} under the conditions of Fig. 4. To test whether Ca^{2+} influences sodium binding the Ca concentration dependence was

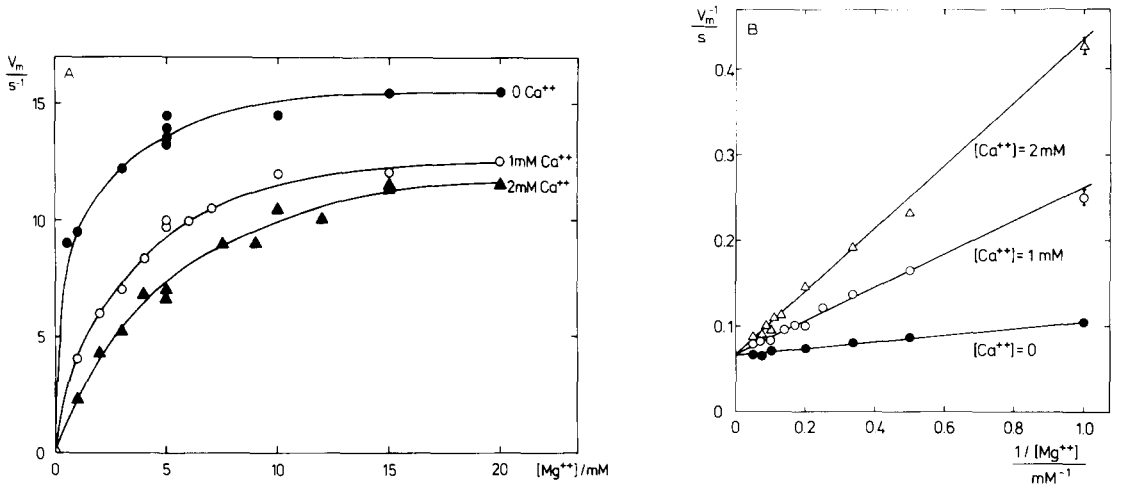

Fig. 4. Influence of Mg^{2+} and Ca^{2+} ions on cation transport of the $(Na^+ + K^+)$-ATPase. (A) Turnover rate v_m as a function of external Mg^{2+} concentration in the absence or presence of 1 or 2 mM Ca^{2+}. Besides the concentration of divalent cations all other concentrations were as indicated in Fig. 1. (B) Data redrawn in the form of a Lineweaver-Burk plot. The intersection of all three lines is found to be at $v_m^{\infty} = 15.6\ s^{-1}$.

measured for two different sodium concentrations (140 mM and 40 mM). The results are shown in Fig. 5. Since the electrolyte used in these experiments contained 1 mM EDTA, the free concentration of Ca^{2+} was determined with a Ca^{2+}-sensitive electrode. According to these measurements the nominally Ca-free buffer still contained 2–3 μM Ca^{2+}, which is in the expected range of Ca^{2+} contamination in the pro analysis $MgSO_4$.

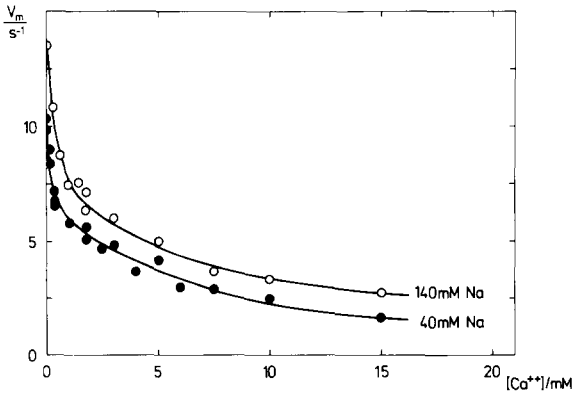

Fig. 5. Turnover rate v_m as a function of external Ca^{2+} concentration for 140 mM and 40 mM Na^+ concentration. In the experiments with 40 mM Na^+, 100 mM $Tris^+$ was added in order to maintain a constant ionic strength. All other conditions were as indicated in Fig. 1.

Influence of pH

The influence of pH is shown in Fig. 6. In the pH range between 6 and 8.5 the pH was adjusted by addition of concentrated H_2SO_4 or NaOH to the buffer solution and below 6 by addition of citric acid. The pH values were measured before and after the experiment with a miniature pH electrode which was immersed in the fluorescence cell. pH variations during an experiment were always less than 0.1 pH unit. Since the calibration of fluorescence change as a function of transmembrane voltage may depend on pH, calibrations were carried out at different pH values by addition of valinomycin in the presence of a potassium gradient. Stable fluorescence signals after valinomycin addition were observed between pH 3.5 and 9.7 with protein-free vesicles. The relative fluorescence change for a calculated Nernst potential of -66 mV (vesicle inside negative) varied linearly between 0.37 (pH 3.5) and 0.29 (pH 9.7). In the data analysis of these experiments shifts were taken into account. With reconstituted $(Na^+ + K^+)$-ATPase vesicles, voltage-induced fluorescence signals could be measured between pH 4.2 and 8.7. Below pH 4.2 the signal declined to zero within 2 min. A similar, but much slower decrease of the fluorescence signal was observed between pH 8.6 and 8.9.

In order to investigate the possibility of a com-

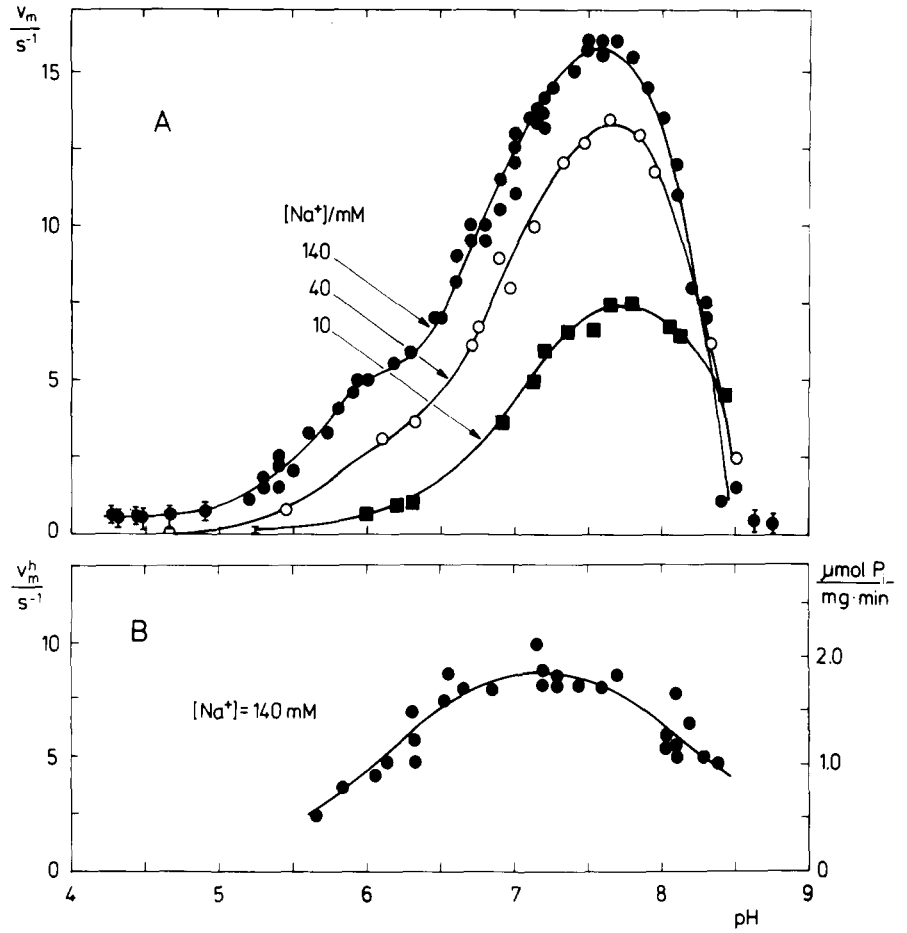

Fig. 6. (A) Turnover rate v_m as a function of external pH determined for three different external Na^+ concentrations. In the experiments with 10 and 40 mM Na^+ the ionic strength was kept constant by addition of $Tris^+$. All other conditions were the same as indicated in Fig. 1. The lines were drawn to guide the eye. T 16°C. (B) ATP hydrolysis rate v_m^h, referred to a single pump molecule, in the presence of valinomycin. 140 mM external Na^+, T 12°C; the other conditions were the same as in Fig. 6A.

petition between Na^+ and H^+, the pH dependence of transport rate v_m was measured at three different Na^+ concentrations (10, 40 and 140 mM). As seen from Fig. 6A, the pH at which v_m is maximal shifts from 7.8 (at 10 mM Na^+) to 7.6 (at 40 mM Na^+) and 7.5 (at 140 mM Na^+).

In Fig. 6B the rate v_m^h of ATP hydrolysis is given as a function of pH for 140 mM Na^+ in the extravesicular medium. v_m^h was determined at 12°C under otherwise the same conditions as the experiments of Fig. 6A by measuring the amount of inorganic phosphate (P_i) liberated in the vesicle suspension. v_m^h is defined as the number of phosphate ions liberated per second, divided by the number N of active pump molecules (ATP-binding site facing outward) in the vesicle suspension; N was obtained from the density χ of functionally oriented pump molecules per unit membrane area (see above) and from the total amount of lipid in the suspension. In six different vesicle preparations used in the experiments of Fig. 6B, χ varied between 160 and 290 μm^{-2}, corresponding with $M = 280000$ g/mol [37] to 0.12–0.22 mg active enzyme per ml. The hydrolysis rate was found to be almost constant for about 5 min after addition; at larger times v^h decreases as a consequence of K^+ depletion in the vesicle interior. The pH for optimum enzymatic activity was found to be 7.2 in accordance with literature values [16,26]. The results of similar experiments with 10 mM Na^+

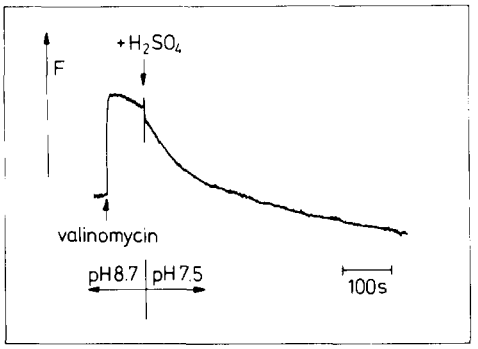

Fig. 7. Effects of a pH jump on the pumping activity of reconstituted ($Na^+ + K^+$)-ATPase. At the beginning the pH of the buffer was adjusted by NaOH to 8.6. Thereafter vesicles and 2.5 mM ATP were added. After maintaining the Nernst-potential by addition of valinomycin a slow decrease of fluorescence is observed. Addition of H_2SO_4 induces a small immediate jump in fluorescence intensity and an increase of pumping activity. At the end of the experiment the pH was determined to be 7.5. The other conditions are given in Fig. 1.

(+130 mM $Tris^+$) and 40 mM Na^+ (+100 mM $Tris^+$) coincide completely with the data for 140 mM Na^+ shown in Fig. 6B.

In order to check for reversibility of the pH effects, the following test was carried out. A normal experiment was started at pH 8.5 at which only a small transport activity was observed in the presence of ATP. By injection of protons (15 μl of 2 M H_2SO_4) the pH was quickly changed to 7.3. This resulted in a rapid increase of turnover rate, as shown by Fig. 7. Similar experiments in which the pH was raised from 5 to 7 by addition of NaOH gave comparable results. This means that an irreversible change of pumping activity can be excluded in the pH range between 5 and 8.5.

Discussion

Dependence of pumping rate on cytoplasmic sodium concentration

The dependence of pumping rate on sodium concentration in the external medium which corresponds to the cytoplasmic phase (Fig. 2) can be described empirically by a Michaelis-Menten equation for a single binding site with an apparent binding constant of 15 mM. This result suggests that the turnover rate is controlled by binding of a single sodium ion.

Under physiological conditions the coupling ratio of the Na^+/K^+ pump is close to 1 ATP: 3 Na: 2 K [2,3,6,39,50] and the number of binding sites per phosphorylation site is three for sodium and two for potassium [29,33,51–53]. If the three sodium binding sites had comparable affinity a third order Michaelis-Menten kinetics would be expected. Investigating the ouabain-sensitive sodium-extrusion from barnacle muscle, Nelson and Blaustein observed a distinct sigmoidal Na^+-concentration dependence with a Hill coefficient of approx. 3.5 [19]. In other presentations a much weaker dependence of transport rate on Na^+ concentration was observed. Sachs [18] analyzed the ouabain sensitive potassium efflux from erythrocytes as a function of internal Na^+ concentration and obtained Hill coefficients in the range of 1.4 to 1.6. Studies with inside-out human red cell membrane vesicles performed by Blostein [54] exhibited a Na^+ concentration dependence without pronounced sigmoidal behaviour. Brand and Whittam investigated the ouabain-sensitive K^+ influx in pig red blood cells as function of internal Na^+ concentration and found agreement with single site Michaelis-Menten kinetics for Na^+ [55]. Karlish and Stein [22] using reconstituted ($Na^+ + K^+$)-ATPase vesicles, studied Na^+ uptake as a function of (cytoplasmic) Na^+ concentration. They determined Hill coefficients between 1.2 and 1.9 for the dependence of transport rate on extravesicular Na^+ concentration and estimated binding constants for Na^+. Their result, $K_1 = K_2 = 0.78$ mM, $K_3 = 78$ mM is consistent with a dependence of transport rate on the first power of Na^+ concentration.

The binding constant $K_m = 15$ mM determined in this paper cannot be compared directly with other data, since the binding constant is dependent of the extracellular ion composition [22]. In the above mentioned papers the $K_{0.5}$ values vary between 5 mM [55] and 20 mM [19].

A molecular interpretation of this result was proposed by Karlish and Stein [22] who suggested a neutral and two negatively charged sodium binding sites. In this case the binding sites are not equivalent, and for electrostatic reasons the affinity of sodium at the neutral site could be considerably reduced.

Inhibition by cytoplasmic potassium

The analysis of turnover rate as a function of

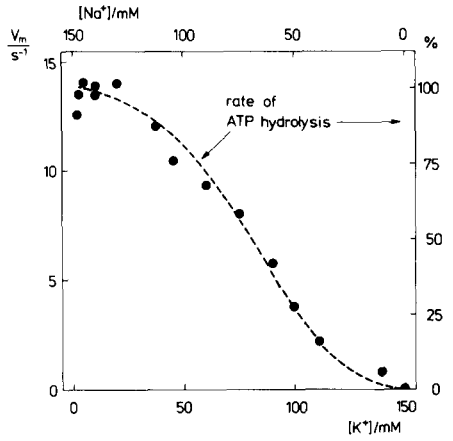

Fig. 8. Comparison of pump rate and hydrolysis rate of the $(Na^+ + K^+)$-ATPase. Data points are redrawn from Fig. 3 (full circles). The line is redrawn from Fig. 1 of Ref. 24. $(Na^+ + K^+)$-ATPase from salt gland of *Squalus acanthias*; 30 mM histidine buffer (pH 7.4); T 37°C; [ATP] 10 μM).

extravesicular (cytoplasmic) potassium concentration (Fig. 3) reveals an inhibitory effect of K^+ ions. This suggests that under physiological conditions the pump never reaches its maximum possible turnover rate. Intravesicular (extracellular) potassium concentrations greater than 1 mM do not influence the pumping rate [2].

An inhibitory effect of potassium on transport was observed in red blood cells [56,15]; a similar inhibition on ATP hydrolysis was reported for microsomal preparations of different tissues [57, 23–25]. Fig. 8 shows perfect agreement between pumping rate of Fig. 3 and rate of ATP hydrolysis measured by Skou with a microsomal preparation from *Squalus acanthias* [24].

Cytoplasmic magnesium and calcium concentration

Magnesium ions activate pumping activity of the $(Na^+ + K^+)$-ATPase with a concentration dependence which can be described by an empirical equation according to Eqn. 1 with a half saturation constant of 0.6 mM in the absence of other divalent cations. Since ATP, which was present in a concentration of 2.5 mM, forms Mg-ATP complexes this number cannot be directly applied to physiological situations. In the absence of magnesium no pump activity could be observed. In transport experiments magnesium cannot be replaced by calcium, even at concentrations of 20 mM.

If magnesium and calcium are present simultaneously, the catalytic function of magnesium is inhibited by calcium. A competition between sodium and calcium at the sodium binding site can be excluded according to Fig. 5. However, the results shown in Fig. 4B clearly indicate competitive inhibition between magnesium and calcium. Previous investigations of the effects of divalent ions have been mainly carried out by studying phosphorylation, where Mg^{2+} can be replaced with low effectivity by Ca^{2+} [9–11]. ATPase activity was found to be reduced in the presence of Ca^{2+} in experiments with rat myometrical microsomes [58], with red blood cells [59], and with kidney microsomes [11]. Competition between Mg^{2+} and Ca^{2+} was also observed in studies of ATP hydrolysis with bovine brain enzyme [60]. A comparison of these findings leads to the conclusion that the replacement of magnesium by calcium in partial reactions like phosphorylation is not sufficient for the role of magnesium in the complete pumping cycle.

Influence of pH on pumping rate

As far as it is known, protons are not transported by the $(Na^+ + K^+)$-ATPase. Nevertheless the pump activity is influenced by the pH of the aqueous phase. In Fig. 6 the pH dependence is shown to be different for different Na^+ concentrations, the optimum pumping rate shifting to higher proton concentration with increasing Na^+ concentration. The reduction of pump activity at high pH values is not significantly dependent on Na^+ concentration; this can be explained by deprotonation of acidic groups in parts of the protein involved in conformational changes. In contrast, a clear Na^+ concentration dependence is observed in the pH range 5–7.5. The analysis of the data in the pH range 6.4–7.4 indicates that the inhibition is of a mixed type with a significant component of competitive inhibition.

The non-competitive part of the pH dependence has not necessarily to result from interactions of protons at the cytoplasmic interface of the protein, since control experiments with ATPase vesicles containing fluorescein-dipalmitoylphosphatidylethanolamine as pH indicator in the membrane [61] demonstrated a significant proton conductivity (unpublished results).

Since the optimum pH determined by ATP hydrolysis [25] differed from that detected by transport studies in this paper, the experiments were repeated at 12°C under otherwise identical conditions to determine hydrolysis activity of the reconstituted $(Na^+ + K^+)$-ATPase as function of pH. In agreement with Ref. 25 maximum hydrolysis activity was found at pH 7.2. Since the shape of pH dependence coincides with data taken at 37°C [25] and 12°C the temperature dependence of the pH effects may be neglected.

Control experiments were performed with lower Na concentration (40 mM and 10 mM) in the pH range between 6 and 8.5. No significant dependence of hydrolysis rate on sodium concentration was observed. These findings suggest that enzymatic activity and transport are affected differently by protons and sodium ions.

The influence of pH on different activities of the pump is summarized in Fig. 9. In addition to ion pumping and ATP-hydrolysis rate, phosphorylation of $(Na^+ + K^+)$-ATPase from rabbit kidney, taken from Ref. 49, is plotted for comparison. The transport activity obviously exhibits the most pronounced pH dependence, whereas pH has only a weak influence on phosphate binding. The lower pH sensitivity of hydrolysis and phosphorylation compared to transport at pH 6 to 7 restricts possible explanations of the non-competitive inhibition in this pH range as indicated above. Since pH-effects on phosphorylation of the pump can be ruled out as a source of inhibition according to

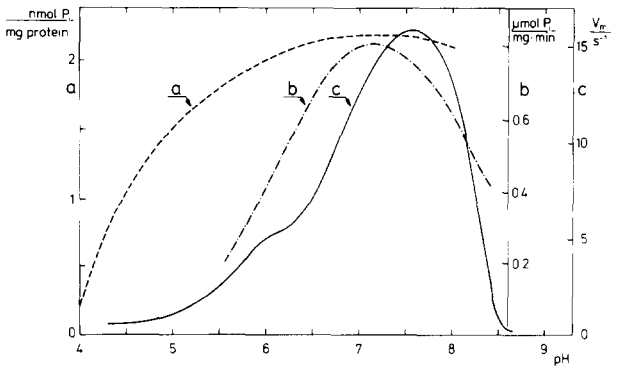

Fig. 9. Comparison of influence of pH on (a) phosphorylation, (b) ATP hydrolysing activity and (c) turnover rate of ion transport. Curve (a) is taken from Ref. 49. Curves (b) and (c) from this paper.

Fig. 9, a possible explanation consists in the assumption that protonation of basic groups of the protein may hinder motion of parts of the enzyme involved in conformational changes.

The enzymatic activity at pH 7.2 varied in the range 1.8–2.1 μmol P_i/mg min at 12°C. At this temperature under the given experimental conditions the turnover rate (as determined from K^+ flux experiments) was 10 s^{-1} [37], which corresponds to an enzymatic activity of 2.1 μmol P_i/mg min ($M = 280\,000$ g/mol). For experimental reasons the liberated P_i had to be collected over a period of 5 min; therefore a more precise comparison has to take into account vesicle inhomogeneity, because smaller vesicles with a large number of functionally oriented pumps get depleted of internal potassium within a short time. As a consequence the average pumping rate v of these vesicles is reduced. A simulation based on the model described in Ref. 43 with $\chi = 175$ μm^{-2}, $v_m = 10$ s^{-1}, 1 ATP hydrolyzed per cycle yields in an average enzymatic activity of 1.7 μmol P_i/mg min during the first 5 min. This value agrees with the experimentally observed activity.

The results of this paper demonstrate that the kinetic properties of the $(Na^+ + K^+)$-ATPase reconstituted in lipid vesicles largely agree with the properties of the native enzyme. The advantages of the vesicle system compared to membrane fragments are mainly the well separated membrane interfaces, which allow to discriminate between 'inside' and 'outside', and the easy reproducibility and analysis of transport experiments. In conclusion it can be stated that a comparison of our data with previously published transport studies demonstrates an extensive agreement with respect to substrate-protein interactions for $(Na^+ + K^+)$-ATPase from different tissues. A similar agreement was found in substrate-protein interactions when results of ATP hydrolysis studies were compared with our transport studies. The only significant deviations were observed in the influence of protons which are not known to be a necessary cofactor for the protein function. It was shown that the range of high activity is different for transport, ATP hydrolysis and phosphorylation of the protein. Protons presumably have a twofold effect on the pumping protein by competing for Na binding sites on one hand and on the other

hand by protonating basic groups at low pH and deprotonating acidic groups at high pH parts of the protein restricting motions associated with conformational changes.

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