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Na,K-ATPase in artificial lipid vesicles. Comparison of Na,K and Na-only pumping mode

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Na,K-ATPase from rabbit kidney outer medulla was reconstituted in large unilamellar lipid vesicles by detergent dialysis. Vesicles prepared in the presence or absence of potassium allowed to study two different transport modes: the (physiological) Na,K-mode in buffers containing Na^+ and K^+ and the Na-only mode in buffers containing Na^+ but no K^+. The ATP hydrolysis activity was obtained by determination of the liberated inorganic phosphate, P_i, and the inward directed Na^+ flux was measured by ^{22}Na-tracer flux. Electrogenic transport properties were studied using the membrane potential sensitive fluorescence-dye oxonol VI. The ratio $v(Na,K)/v(Na)$ of the turnover rates in the Na,K-mode and in the Na-only mode is 6.6 ± 2.0 under otherwise identical conditions and nonlimiting Na^+ concentrations. Strong evidence is found that the Na-only mode exhibits a stoichiometry of $3Na^+_{cyt}/2Na^+_{ext}/1ATP$, i.e. the extracellular (= intravesicular) Na^+ has a potassium-like effect. In the Na-only mode one high-affinity binding side for ATP ($K_M \cong 50$ nM) was found, in the Na,K-mode a high- and low-affinity binding side with equilibrium dissociation constants, K_M, of 60 nM and 13 μM, respectively. The sensitivity against the noncompetitively inhibiting ADP ($K_I = 6$ μM) is higher by a factor of 20 in the Na-only mode compared to the Na,K-mode. From the temperature dependence of the pumping activity in both transport modes, activation energies of 160 kJ/mol for the Na,K-mode and 110 kJ/mol for the Na-only mode were determined.

Introduction

The Na,K-ATPase is a protein of the plasma membrane which is found in nearly all animal cells. Under physiological conditions it transports sodium ions outward and potassium ions inward against their electrochemical gradients. The Na,K-ATPase is characterized by its requirement of ATP, Mg^{2+} and Na^+ on the cytoplasmic side of the protein and of K^+ at the extracellular side [1–5]. The functions of the Na,K-ATPase can be modified by variation of the ionic composition at both membrane interfaces. To prove that the resulting different flux modes are due to the action of the Na,K-ATPase, experiments can be repeated in the presence of specific blockers like ouabain or related cardioactive steroids [6,7] which abolish the functions of the Na,K-ATPase. For an extensive discussion of the different flux modes see Ref. 5.

The transport cycle of the pump, known as Post-Albers cycle, contains a series of reaction steps (Fig. 1A) which take into account the binding and/or dissociation of the different substrates and conformational changes. In the case of potassium free solutions the pumping cycle is modified and different pathways have been observed according to Fig. 1B. In the case of the potasisum free mode the rate-limiting step is the transition $E_2 \rightarrow E_1$ [8–10] with a rate constant in the range of 1 s^{-1}. The different transport modes depend on the substrate conditions. Electroneutral Na-Na exchange occurs via $3Na + E_1 \cdot ATP \rightleftharpoons Na_3E_1ATP \rightleftharpoons (Na_3)E_1\text{-}P \rightleftharpoons P\text{-}E_2Na_2 \rightleftharpoons P\text{-}E_2 + 3Na$ in the presence of ADP and high extracellular sodium [11–13]. In this mode no net hydrolysis of ATP is observed. When no ADP is present the Na-Na exchange is inhibited and Na exchange is observed where extracellular Na^+ ions have a slight K^+-like effect in accelerating hydrolysis of E_2P [14–16]. In the absence of extracellular Na^+ 'uncoupled' Na extrusion is reported [8–10]. Both pathways are shown in Fig. 1B. The latter two transport modes show electrogenic properties. If not otherwise specified in this paper

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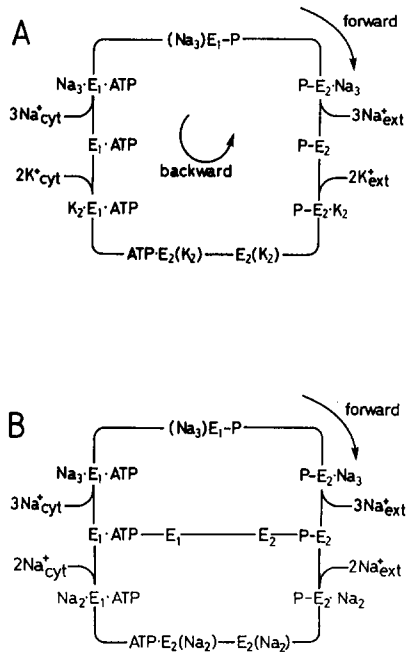

Fig. 1. (A) Post-Albers scheme for the pumping cycle of the Na,K-ATPase, E_1 and E_2 are conformations of the enzyme with ion binding sites exposed to the cytoplasmic and extracellular membrane interface, respectively. In the 'occluded' states $(Na_3)E_1$-P and $E_2(K_2)$ the ions are unable to exchange with the aqueous phase. Dashes indicate covalent bonds and dots indicate noncovalent bonds. (B) Pumping cycle of the Na,K-ATPase in the absence of potassium, based on the reaction scheme of part A of this figure. This cycle is referred as Na-only mode of the ion pump.

the term 'sodium-only mode' refers only to pumping action in the absence of potassium.

At physiological levels of intra- and extracellular ion concentrations the pump moves three Na^+ ions inward and two K^+ ions outward, so that the overall transport process is associated with a net translocation of one elementary charge. The analysis of the reaction steps have shown that the potassium translocating steps are electroneutral [10,17–19] and charge movements are only observed in the deocclusion of sodium $(Na_3)E_1$-P $\rightleftharpoons$ $P\text{-}E_2Na_3$ and/or association/dissociation of sodium at the external interface $P\text{-}E_2Na_3 \rightleftharpoons P\text{-}E_2 + 3Na^+$ [20–22].

A recent paper of Cornelius [45] dealt already with properties of the Na,K-ATPase in the Na-only mode. Na,K-ATPase from shark, reconstituted in lipid vesicles, showed a coupling ratio of 3 Na^+ transported from the cytoplasmic interface to the extracellular side to 1 ATP hydrolysed.

In this paper the properties of the Na,K-ATPase are investigated in reconstituted lipid vesicles with tracer flux and spectroscopical measurements. The behaviour of the ion pump under Na,K-exchanging and Na-only pumping conditions are observed and discussed in terms of the Post-Albers scheme.

Materials and Methods

Materials

Dioleoylphosphatidylcholine was obtained from Avanti Polar Lipids, Birmingham, AL, U.S.A.; Oxonol VI (bis(3-propyl-5-oxoisooxazol-4-yl)pentamethin oxonol) was from Molecular Probes, Junction City, OR, U.S.A.; ATP (Sonderqualität) and valinomycin from Boehringer-Mannheim, vanadate from Ventron, Karlsruhe. The phospholipid contents were determined by the Phospholipid B test from Wako Pure Chemical Industries, Ltd., Osaka, Japan. ^{22}Na was obtained from Amersham International, the scintillation fluid (Aqualuma Plus) from Baker Chemicals, the ion exchange resin (Bio-Rex 70) from Bio-Rad (München), all other reagents were from Merck (analytical grade). Dialysis tube was purchased from Serva, Heidelberg.

Vesicle preparations

Na,K-ATPase was prepared from outer medulla of rabbit kidneys using procedure C of Jørgensen [24], as described previously [23,25]. The specific activity was in the range of 1700–2100 μmol P_i per h per mg at 37°C. Reconstituted vesicles containing Na,K-ATPase were prepared by cholate removement in 'buffer H' containing 30 mM imidazole, 1 mM L-cysteine, 1 mM EDTA, 5 mM $MgSO_4$ and various amounts of Na_2SO_4 and K_2SO_4 as described in previous papers [23,25,26].

Fluorescence measurements

Fluorescence experiments were carried out in a Perkin Elmer 650-40 fluorescence spectrophotometer. The thermostatically controlled cuvette holder was equipped with a magnetic stirrer. If not otherwise indicated, the excitation wavelength was set of 580 nm (slit width 20 nm) and the emission wavelength to 660 nm (slit width 20 nm). The Oxonol VI stock solution contained 1 mM dye in ethanol. From this stock solution dilutions were prepared daily by mixing with ethanol/water (1 : 9, v/v). The final concentration in the cuvette was chosen to be 30 nM.

Measurements were performed as described previously [26,27] at 18°C if not indicated otherwise. Since Oxonol VI is a well-understood and successfully applied potential probe, the fluorescence signals can be transformed into potential curves [26]. The initial slope of the potential change after starting the pump by ATP addition is proportional to the product of pumping rate and amount of transport active Na,K-ATPase, averaged over all vesicles [27].

Tracer flux experiments

To determine active or passive sodium flux through vesicle membranes, tracer-flux experiments were performed. Vesicles were incubated with or without 20 mM Mg-ATP in buffer H containing 75 mM Na_2SO_4 or 5

mM K_2SO_4 + 70 mM Na_2SO_4 and up to $3.7 \cdot 10^4$ Bq ^{22}Na. After a certain incubation time, aliquots of the vesicle suspension were taken and added into ice-cold saccharose solution of equal osmolarity which is used as stopping buffer. The aliquots (volume V_a) contained approx. 18 μg lipid and 900 Bq ^{22}Na. The external radioactivity was removed by a method derived from Cornelius and Skou [28]. Vesicles and stopping buffer were flushed within 30 s through a microcolumn filled with 400 μl cation exchange resin Bio-Rex 70 with 1 ml of isoosmolar saccharose solution into scintillation vials containing 5 ml scintillation fluid (Aqualuma Plus, Baker). All radioactivity values obtained from the same incubation were referred to the radioactivity of an equal volume (V_a) of the incubation buffer ('external' ^{22}Na).

Determination of inorganic phosphate

The content of free inorganic phosphate was determined by the method of Panusz et al. [29]. Contaminations with phosphate were determined by an additional experiment in which all components were mixed at 0 °C where the action of the ATPase can be neglected. The results were used for correction. Control experiments using vesicles without enzyme showed that spontaneous hydrolysis of ATP was negligible within the time of experiments (< 30 min). Experiments in the presence of 250 μM ouabain exhibited no significant difference in enzymatic activity, indicating that only a negligible fraction of not incorporated Na,K-ATPase contributes to ATP hydrolysis.

Results

Switching from Na,K-mode to Na-only mode

In experiments with Na,K-ATPase reconstituted in lipid vesicles only inside-out oriented pump molecules are activated since ATP is added to the external medium and does not diffuse through the vesicle membrane in the time range of the experiments. In the Na,K-pumping mode of the enzyme the decrease of the intravesicular potassium concentration has to be taken into account [23]. During the course of an experiment starting with a high intravesicular K^+ concentration the potassium will be extruded almost completely and the pump has to switch from the Na,K-mode into a Na-only mode.

In Fig. 2 the hydrolysis of ATP in terms of liberated inorganic phosphate, P_i, is shown for vesicles containing 140 mM K^+ + 10 mM Na^+ (open circles) and vesicles containing 150 mM Na^+ (filled circles) in the beginning. In buffer with a ionic composition of 140 mM Na^+ + 10 mM K^+ or 150 mM Na^+, respectively, vesicles were incubated to get a concentration of 700 μg/ml lipid. The temperature was 12 °C. The reaction was started by addition of ATP (20 mM) and valinomycin (final concentration 40 nM to avoid fast changes of membrane potential by the electrogenic effect of the pump [23]).

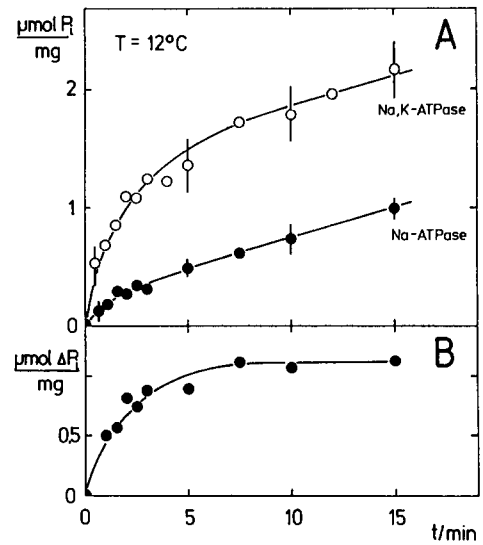

Fig. 2. ATP hydrolysis determined in μmol P_i per mg protein. (A) The data points are accumulated amounts of inorganic phosphate from start until the time t. Intravesicular cation composition at time zero: 140 mM K^+ + 10 mM Na^+ (open circles), 150 mM Na^+ (filled circles). The extravesicular medium contained 140 mM Na^+ + 10 mM K^+ or 150 mM Na^+, respectively. (B) Difference of hydrolysis activity of Na,K-mode and Na-only mode. The finding that ΔP_i is constant for times greater than 7 min shows that the enzymatic activity is equal for both sets of experiments.

Immediately after mixing, the time zero sample was taken by removing an aliquot of 8 μl (containing ≅ 0.6 μg protein) which was mixed with ice-cold stopping buffer containing perchloric acid. Further samples were taken at different times. The values given in Fig. 2 are average values of 11 series of experiments testing the Na,K-mode and 4 series testing the Na-only mode. The error bars indicate the standard deviation from the mean values. The initial curvature in the phosphate-release of the Na-only mode is probably caused by a voltage-dependent reduction of the pumping rate [22,27]. Fig. 2B represents the difference of the liberated inorganic phosphate, ΔP_i, between vesicles starting with and without intravesicular potassium. A distinct saturation is observed, revealing that the enzymatic activity of the potassium-depleted vesicles is equal to that of the Na-only pumping vesicles.

Another set of experiments was performed to study the influence of intravesicular (extracellular) potassium on the electrogenic pumping of the reconstituted enzyme. Oxonol VI was used to indicate the membrane potential as described previously [26,27]. From the same batch of dialysed vesicles aliquots were incubated for 24 to 36 h in buffers containing different concentrations of K^+ ions. As shown below, this time is sufficient to equilibrate the extra- and intravesicular ion composition. To 1 ml of buffer H with 75 mM Na_2SO_4, Oxonol VI was injected to a final concentration of 30 nM and subsequently 1 μl of vesicle preparation (4 μg lipid).

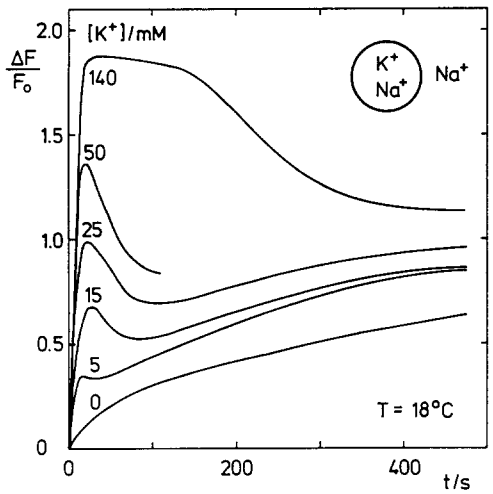

Fig. 3. Membrane potential generated by the pumping activity of the Na,K-ATPase measured as a change of Oxonol VI fluorescence. Vesicles were reconstituted in buffer H containing 150 mM Na^+ and afterwards equilibrated in buffers with different concentrations of K^+. $[K^+]+[Na^+]=150$ mM was maintained constant. Into 1 ml of 150 mM Na^+ buffer 30 nM Oxonol VI and vesicles (10 μg/ml lipid) were added to give the fluorescence signal F_o. At time $t=0$ the pumping was started by addition of 250 μM ATP. ΔF is the change of fluorescence from the F_o level. The temperature was 18° C.

After reaching a stable fluoresence signal, ATP was injected to a final concentration of 0.5 mM.

The fluorescence responses of the vesicle suspensions upon activation of the pump are shown in Fig. 3. With increasing potassium concentration the transient high electrogenic activity raises with steeper initial slope over the monotonous signal of the Na^+-only mode. The biphasic signals in the presence of K^+ are generated by the increased turnover rate of the Na,K-mode in the beginning and successively by the reduction of the turnover rate when the concentration of K^+ approaches zero. The higher membrane voltage leads to increased leakage currents until the stationary conditions of the Na-only mode are reached. For long times, $t > 1000$ s, all fluorescence signals converge at the same value, independent of the initial potassium concentration. The steady-state value depends only on the enzymatic activity of the vesicle preparation. When the potassium concentration in the extravesicular medium is changed in the range between 0 and 25 mM the fluorescence signal is only insignificantly altered over the total time range of the experiment.

The establishment of a transmembrane potential for such long time ranges, independent of the concentration of potassium, proves the electrogenic pump action of the Na-only ATPase. Addition of vanadate as a competitor for the ATP binding site leads to a decay of the membrane potential at any time point of the experiment.

These results show that the reduction of the intravesicular (i.e., extracellular) potassium concentration by the action of the Na,K-pump has significant influence on the observed transport phenomena. To analyse the two modes of pumping we decided to compare the initial slope of the fluorescence signals in the oxonol experiments where the intravesicular potassium concentration is still high compared to the (extracellular) binding constant for potassium.

The fluorescence signals can be calibrated into a membrane potential [26]. But since the vesicle preparations are inhomogeneous with respect to vesicle size and number of transport-active pumps [23,30], different vesicles have at the same time different membrane potentials. Fluorescence signals therefore can be transformed only into average potentials, U_{AV}. Performing such a transformation for the vesicle preparation shown in Fig. 3, it is found that the initial slope of U_{AV} in the presence of intravesicular potassium is 3–12 mV/s depending on the concentration of potassium (5 mM–140 mM). In absence of potassium the initial slope is determined to be 600 μV/s.

Comparison of Na-only and Na,K mode

(a) Tracer flux experiments with ^{22}Na

Fig. 4 shows a ^{22}Na-influx experiment performed at 25° C. A vesicle preparation containing 7.2 mg/ml lipid and 0.8 mg/ml protein with an enzymatic activity of 211 μmol P_i/h per mg protein was split in two equal fractions and dialysed for another 36 h in buffers containing 150 mM Na^+ or 140 mM $K^+ + 10$ mM Na^+. The enzymatic activity was not changed by this procedure. The uptake of sodium was measured in the presence and absence of ATP for both fractions of vesicles. In the absence of ATP the leakage effects were de-

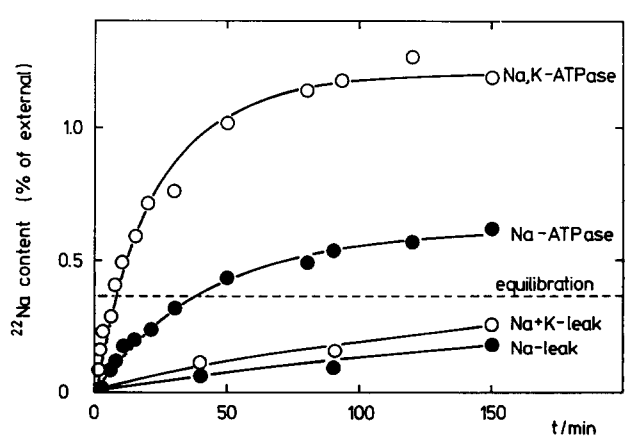

Fig. 4. Na^+ influx generated by Na,K-ATPase in reconstituted vesicles. At $t=0$, 20 mM Mg-ATP were added. The temperature was 25° C. Open circles represent data of vesicles containing 140 mM $K^+ + 10$ mM Na^+ at $t=0$. Filled circles represent data of vesicles in the Na^+-only mode of the Na,K-ATPase. The lower curves, marked 'leak', were obtained in the absence of ATP. The ordinate is the ^{22}Na content of the vesicles referred to the total ^{22}Na content of the vesicle suspension (in percent).

termined. Data points were taken up to 3000 min. All uptake curves could be described by a monoexponential function of time with a time constant, τ, and a saturation value, N^{∞}. In the absence of ATP, N^{∞} was independent of the intravesicular ion composition. The saturation value of $N^{\infty} = 0.36\%$ can be interpreted as internal volume, V_i, compared to the external. The theoretical value of entrapped volume would be 0.78% assuming spherical vesicles with an internal radius, r_i = 40 nm, a membrane thickness, $d = 5$ nm, a density of the lipid, $\sigma = 1$ g/ml, and lipid concentration in the incubation medium of 3.24 mg/ml [23]. (The 2-fold smaller experimental value of V_i may result from the presence of leaky vesicles or from nonvesicular lipid aggregates. This finding is in agreement with published data [23,25,31].) The initial passive influx $I_1 = N^{\infty}/\tau$ is for potassium containing vesicles higher by a factor of 1.6 (± 0.4 for different vesicle preparations).

In Fig. 4 the pumping activity in the Na-only mode leads to $N^{\infty} = 0.62\%$ and $\tau = 44$ min; in the Na,K-mode one obtains $N^{\infty} = 1.2\%$ and $\tau = 21$ min. In comparison with the leak experiments the final intravesicular Na^+ concentration (which is proportional to N^{∞}) saturates in the ratio 1 : 2 : 4 for leakage, Na-only and Na,K-mode. This ratio is significantly influenced by the enzymatic activity of the vesicle preparation. The ratio of initial pumping activities, $I_{p,0} = N^{\infty}/\tau$, was found to be $I_{p,0}(\text{Na,K})/I_{p,0}(\text{Na-only}) = 4.1 \pm 1.5$.

(b) Cytoplasmic sodium concentration

Transport-active vesicle preparations, prepared in 150 mM Na^+-buffer were split and dialysed another 36 h in

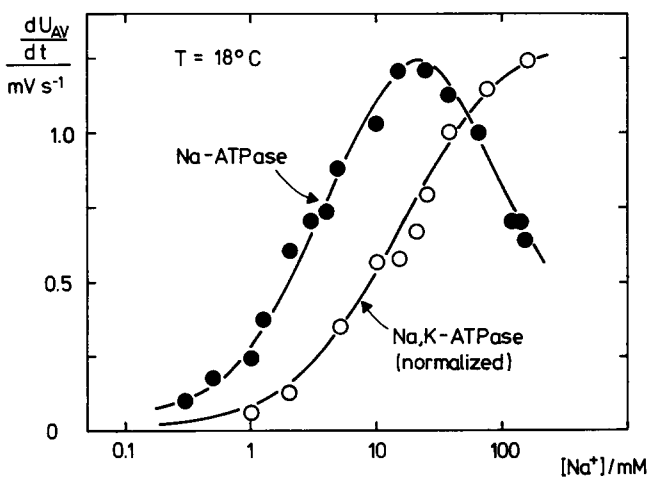

Fig. 5. Initial increase of membrane potential dU_{AV}/dt as a function of the (cytoplasmic) Na^+-concentration. $T = 18°C$. Open circles: vesicles containing 140 mM $K^+ + 10$ mM Na^+, the line drawn to connect the data represents a Michaelis-Menten plot according to Eqn. 1 with $K_M = 15$ mM. The values of dU_{AV}/dt are reduced by a factor of 10 to get them into an appropriate range compared to the other data. Filled circles: vesicle containing 150 mM Na^+, the line represents a kinetic behaviour as shown in Eqn. 2 with $K_M = 8$ mM, $K_I = 140$ mM.

buffers containing 150 mM Na^+ or 140 mM $K^+ + 10$ mM Na^+. Fluorescence experiments in the presence of 30 nM Oxonol VI were performed in buffers with various concentrations of Na^+. Tris-H_2SO_4 containing buffers were used to maintain the cation concentration at 150 mM. The fluorescence signals were digitized and transformed into average membrane potentials, U_{AV}, by an appropriate calibration curve. From the initial slope of the potential signal, built up after addition of ATP, dU/dt was calculated which is proportional to the membrane current of an average vesicle [27]. In Fig. 5 the results for both pumping modes are plotted together. The data for vesicles working in the Na,K-mode the actual number are presented by 10-fold reduction to get them into a comparable range. The observed shape of concentration dependence was obtained by all four different vesicle preparations used for these experiments.

The concentration dependence of the pumping activity is different for both pumping modes. In the Na,K-mode the experimental values of $dU/dt = \dot{U}$ can be fitted by a first order Michaelis-Menten relation

$$\dot{U} = \dot{U}_{\infty} \cdot \frac{C_{Na}}{C_{Na} + K_M} \tag{1}$$

with $K_M = 15$ mM. The binding constant is in accordance with a previously published value determined under comparable conditions [32]. $\dot{U}_{\infty}$ is the initial slope in the presence of saturating Na^+ concentrations. The value of $\dot{U}_{\infty}$ is 14 mV/s. In the Na-only mode a more complex behaviour is observed. The experimental data can be described by the kinetical behaviour of an enzyme with a single binding site and an allosteric inhibition of Na^+ at high concentration:

$$\dot{U} = \dot{U}_{\infty} \cdot \frac{C_{Na}}{C_{Na} + K_M} \cdot \frac{K_I}{C_{Na} + K_I} \tag{2}$$

with $K_M = 8$ mM, an inhibitor binding constant, K_I = 140 mM and a concentration independent constant, $\dot{U}_{\infty}$ with a value of 2.0 mV/s. The experimentally determined ratio of $\dot{U}(140\ K^+ + 10\ Na^+)/\dot{U}(150\ Na^+)$ is 18.5 ± 1.0 at an extravesicular sodium concentration of 150 mM and 6.4 ± 0.3 at 20 mM extravesicular sodium, respectively.

(c) Nucleotide concentration dependence

In Fig. 6 the initial slope of the membrane potential, dU_{AV}/dt, is plotted as a function of ATP concentration, C_T. Since at low ATP concentration (< 20 nM in the Na-only mode, < 100 nM in the Na,K-mode) the consumption of ATP by the pump significantly changes the ATP concentration within 10 s, the determination of the initial slope, $dU_{AV}/dt \approx \dot{U}$ becomes tentative at low ATP concentrations. In the Na-only mode the experi-

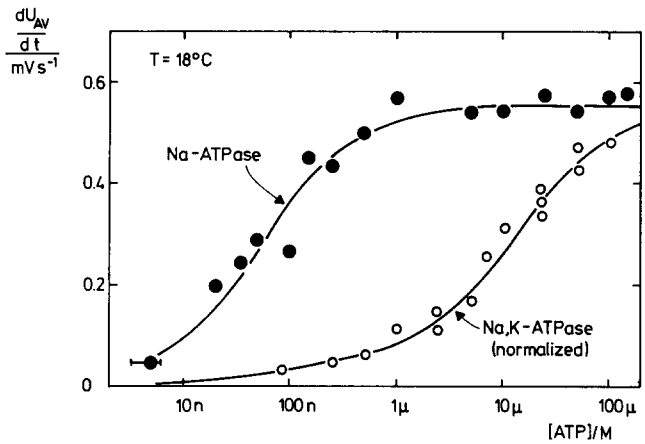

Fig. 6. Initial increase of membrane potential dU_{AV}/dt as a function of ATP concentration. Virtually no ADP is present. The filled circles show the concentration dependence of the pump working in the Na-only mode. The solid line follows a kinetic behaviour according to Eqn. 1 with $K_M = 54$ nM and a maximum dU_{AV}/dt of 0.55 mV/s. The open circles show the concentration dependence of the pump working in the Na,K-mode (normalized to the maximum dU_{AV}/dt value of the Na-only mode with a factor close to 0.1) and is represented by Eqn. 3 with $K_{M,1} = 60$ nM and $K_{M,2} = 13$ μM.

mental values of $\dot{U}$ can approximately fitted by the empirical equation given in Eqn. 1 as a first-order Michaelis-Menten function with an ATP equilibrium dissociation constant, K_M, of 54 nM.

In the case of the Na,K-mode a more complex concentration dependence is observed. The results are in agreement with previously published data of reconstituted Na,K-ATPase [33] and can be fitted by the empirical equation

$$\dot{U} = \dot{U}_1 \frac{C_T}{K_{M,1} + C_T} + \dot{U}_2 \frac{C_T}{K_{M,2} + C_T} \tag{3}$$

with $\dot{U}_1 = 0.3$ mV/s, $K_{M,1} = 60$ nM, $\dot{U}_2 = 5.5$ mV/s, and $K_{M,2} = 13$ μM. In Fig. 6 the values of $\dot{U}_1$ and $\dot{U}_2$ are scaled down by factor of 10 to allow a direct comparison of both pumping modes. The K_M value of the Na-only mode and that of the high-affinity binding in the Na,K-mode are in good agreement.

In Fig. 7 the initial slope of the membrane potential, dU_{AV}/dt, is plotted as a function of ADP concentration, C_D, for an ATP concentration of 250 μM. The experimental values of $dU_{AV}/dt = \dot{U}$ in the Na-only mode can be approximately fitted by the empirical equation

$$\dot{U} = (\dot{U}_0 - \dot{U}_1) \frac{K_{I,1}}{K_{I,1} + C_D} + \dot{U}_1 \frac{K_{I,2}}{K_{I,2} + C_D} \tag{4}$$

with $K_{I,1} = 6.0$ μM, $K_{I,2} = 4$ mM and $\dot{U}_0 = 0.72$ mV/s, $\dot{U}_1 = 0.17$ mV/s. Trials with a single inhibition do not lead to an acceptable fit. In Fig. 7 the fit of the ADP-concentration dependence of the Na,K-mode are plotted with a dashed line, normalized to $\dot{U}_0$ of the Na-only mode for better comparison. The K_I of 140 μM in the Na,K-mode is in agreement with previous data and similar conditions [34]. These data are in good agreement with a single inhibition process (i.e., $\dot{U}_1 \approx 0$ in Eqn. 4). Varying of ATP concentration (25 μM–500 μM) has no influence on the inhibitor binding constants, $K_{I,1}$ and $K_{I,2}$. This finding for both pumping modes indicates that the inhibition process is noncompetitive between ATP and ADP. This was already shown previously for the Na,K-mode [34]. The data points at [ADP] = 0 were measured in the absence of externally added ADP. The contamination of ATP with ADP was determined to be $\leq 10^{-8}$ M at [ATP] = 50 μM.

(d) Activation energy

The time course of the fluorescence signal after ATP addition strongly depends on temperature [23,26]. For the determination of the activation energy, E_a, values of the initial slope of membrane potential, $\dot{U}_{AV}$, obtained at different temperatures T are plotted in Fig. 8 on a logarithmic scale as a function of $1/T$, according to the Arrhenius equation

$$\dot{U} = \dot{U}_0 \cdot \exp(-E_a/RT) \tag{5}$$

$\dot{U}_0$ is a temperature-independent constant. From the slopes of the straight lines in Fig. 8 activation energies are obtained for both pumping modes to be E_a(Na-only) = 110 kJ/mol and E_a(Na,K) = 160 kJ/mol. The data are obtained from four preparations of vesicles

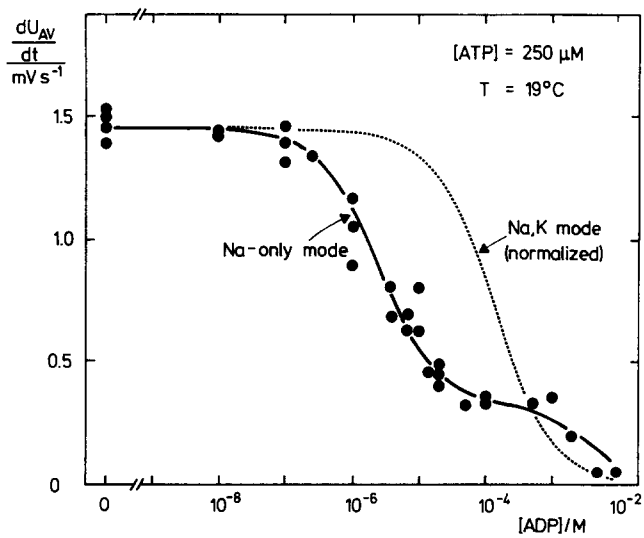

Fig. 7. Initial increase of membrane potential dU_{AV}/dt as a function of ADP concentration. The ATP concentration was 250 μM. The filled circles show the concentration dependence of the pump in the Na-only mode. The line follows a kinetic behaviour according to Eqn. 3 with $K_{I,1} = 2.2$ μM and $K_{i,2} = 4$ mM. The dashed line shows the concentration dependence of the pump working in the Na,K-mode and can be described by a single inhibition constant $K_I = 140$ μM as published previously [34]. Data points representing the Na,K-mode are removed for sake of clarity.

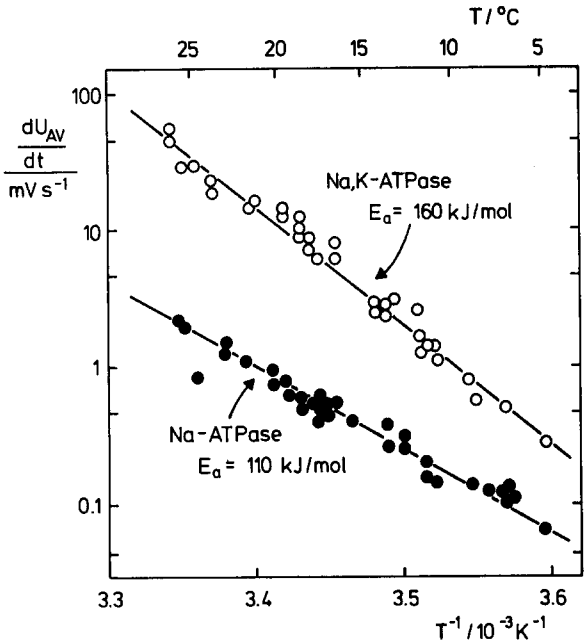

Fig. 8. Initial increase of membrane potential, dU_{AV}/dt, as function of reciprocal temperature. Open circles: Na,K-mode, vesicles containing 140 mM K^+ + 10 mM Na^+; activation energy $E_a = 160$ kJ/mol. Filled circles: Na-only mode, vesicles containing 150 mM Na^+; activation energy $E_a = 110$ kJ/mol. The external buffer contained 150 mM Na^+ in both sets of experiments.

with comparable enzymatic activity. Each preparation was prepared in the presence of 150 mM Na^+ and split into half and dialysed against 140 mM K + 10 mM Na^+ or 150 mM Na^+. The activation energy for Na,K-mode, E_a(Na,K), is in agreement with previously published experiments [26]. For the determination of the values of $\dot{U}$ at high temperature it has to be assumed that the redistribution of the dye across the vesicle membrane is not rate limiting. This is proved by recent experiments investigating the kinetics of the voltage-detecting fluorescence dye Oxonol VI [35].

Discussion

Effect of the limited internal vesicle volume

In this paper we have investigated the influence of the presence and absence of (extracellular) potassium ions on Na,K-ATPase isolated from rabbit kidney and reconstituted in lipid vesicles. Since in experiments with reconstituted Na,K-ATPase the inside out oriented pump molecules contribute to transmembranal transport activity, the intravesicular (= extracellular) potassium concentration is a crucial parameter. At an average vesicle diameter of 100 nm and an intravesicular concentration of 140 mM, the vesicle contains approximately 40000 potassium ions. Assuming a pumping rate of 25 s^{-1} and four transport active pumps in the vesicle membrane which is an average value for the used vesicle preparation, the vesicle is depleted of potassium within 400 s. A comparable finding to this rough estimate is demonstrated by the measurements of the production of inorganic phosphate (Fig. 2), where the rate of ATP hydrolysis for times longer than 10 min are equal for vesicles with or without internal potassium in the beginning. A similar conclusion follows from the measurements of membrane potentials with Oxonol VI (Fig. 3) where the traces converge for times longer than 1000 s. Also the difference between the ^{22}Na uptake in the presence and absence of internal potassium (Fig. 4) can be approximated by a saturation curve with a saturation time in the range of 2000 s (not shown).

These findings lead to the conclusion that information about the physiological Na,K-mode of the sodium pump with reconstituted vesicle can be obtained only within the period of time when the internal potassium concentration is not significantly reduced. This period of time is dependent on the initial concentration of potassium as revealed by Fig. 3. For investigating the electrogenic action of the pump by monitoring the fluorescence of Oxonol VI, the initial slope of the fluorescence signal after addition of ATP, dF/dt, turned out to be an easily accessible and reliable parameter. Using an appropriate calibration curve [26], the fluorescence can be transformed into a voltage signal, $dU_{AV}/dt \equiv \dot{U}$, representing the mean voltage change averaged over all vesicles which are inhomogeneous in size.

Comparison of the results of different experimental methods

To investigate transport activities, the tracer flux method is widely used (see, for example, Refs. 12, 36–41). This method can be applied to determine by the use of ^{22}Na the total amount of sodium ions transported across the membrane. Comparing under otherwise identical conditions the tracer flux of vesicles with and without 140 mM K^+ in the interior volume, a ratio of ^{22}Na uptake, $R_T = I_p(140\ K)/I_p\ (0\ K) = 4.1 \pm 1.5$, is found. Garay and Garrahan have determined $R_T = 5$ for red blood cells [54].

The ATP-hydrolysing activity of the Na,K-ATPase is often measured using [γ-^{32}P]-ATP and counting the liberated [γ-^{32}P]P_i [40,42–44]. A different approach is the determination of P_i by a color reaction [29,32]. Since it is known that 1 ATP molecule is hydrolysed per 1 turnover this method was used to obtain the ratio of ATP hydrolysis in the presence and absence of intravesicular potassium. A ratio of hydrolysing activity R_H = activity (140 K)/activity (0 K) of 6.0 ± 2.0 was found at 150 mM Na^+. At 20 mM Na^+ R_H was measured to be 6.6 ± 2.0, i.e., not significantly different. In the literature different values for R_H are reported. For bovine brain enzyme $R_H = 10$ [55] to 25 [56] is published, for pig kidney enzyme $R_H = 5.3$ was found [57].

The use of potential-sensitive dyes to investigate pumping activity of the Na,K-ATPase is now also an

established method [17,23,26,45]. The change of membrane potential is determined by the amount of charge transferred per cycle and number of turnovers (when the number of pumps/area is assumed to be constant). The initial rise of membrane potential was determined by experiments in the presence and absence of intravesicular potassium and used to calculate the ratio of electrogenic potential increase $R_E = \dot{U}$ (140 K)/$\dot{U}$ (0 K) = 18.5. Assuming a vesicle radius of 48 nm and a specific membrane capacity of 1 $\mu F/cm^2$, a capacity of $2.9 \cdot 10^{-16}$ F is obtained. Since the net current through the membrane can be calculated by $I = C \cdot \dot{U}$ one finds $I(140\ K^+) \approx 20\ e_0/s$ and $I(0\ K^+) \approx 1\ e_0/s$ per vesicle (e is the elementary charge).

The comparison of the three ratios R_T, R_H and R_E allows some conclusions on the basis of the generally accepted stoichiometric relations of 3 Na/2 K/1 ATP for Na,K-ATPase under physiological conditions and of 3 Na/1 ATP for the (first) half cycle of the sodium-only mode. The ratio R_H gives directly the ratio of the hydrolysis rates (which equals the turnover rate) is 6.0 ± 2.0. This number should equal the ratio R_T since for short time-periods the intravesicular ^{22}Na concentration is negligible. The actual numbers show that $R_T \leq R_H$. This finding suggests that all extravesicularly bound Na ions are released to the intravesicular medium. It does not exclude, however, that intravesicular Na ions are transported in a K^+-like manner. If extravesicularly bound Na^+ ions would not be released into the vesicle, $\phi_{Na}(0\ K)$ would become smaller than $\phi_{Na}(140$ K) and the relation $R_T > R_H$ would be valid. This was not found.

The ratio R_E of electrogenic transport activity is defined as $R_E = R_H \cdot R_Q$ where $R_Q = q(140\ K)/q(0\ K)$ is the amount of charge q transported per pumping cycle in the presence and absence of intravesicular potassium. Since $R_E = 18.5$ and $R_H = 6$ the value of R_Q is calculated to be 3. Under physiological conditions the transfer of one net charge per pumping cycle, $q(140$ K) = 1, is generally accepted which leads to the surprising result of $q(0\ K) = 1/3$ in the Na-only mode. In case of an uncoupled Na pumping $q(0\ K) = 3$ would be observed, assuming a potassium-like effect for sodium on the intravesicular side $q(0\ K) = q(140\ K) = 1$ would hold, and if no electrogenic transport occurs $q(0\ K) = 0$. On this basis one has to assume that on the average only every third turnover transfers one charge. Fig. 5 reveals that at high extravesicular (cytoplasmic) Na concentrations the initial pump current is reduced. Since the electrogenic transport ratio is determined at 20 mM Na^+ to be $R_E(20\ Na) = 6.4$, R_Q then is calculated to be approximately 1 since $R_H(20\ Na) = 6.6$ is measured. This result can be understood as a potassium-like effect of sodium. As Fig. 5 shows, the increase of R_E at high extravesicular sodium concentrations is due to a decrease of $\dot{U}$ in the sodium-only mode. If the ratio of the concentration independent values, $\dot{U}_\infty$, in the presence and absence of intravesicular potassium are taken (cf. Eqns. 1 and 2), $R_E(\infty) = 7$ is calculated which is close to R_H.

Summarizing these findings one has to claim that the Na,K-ATPase from rabbit kidney reconstituted in lipid vesicles exhibits under Na-only conditions a transport mode where the extracellular Na ions are bound and transported in a potassium like manner. This means that under Na-only conditions, the contribution of the middle limb of the pumping cycle of Fig. 1B is negligible.

Substrate interactions

As shown in Fig. 1 several reaction steps are associated with binding or release of substrate molecules. ATP, ADP and inorganic phosphate are involved in the enzymatic processes and ions like Na^+ and K^+ are transported across the membrane.

ATP. The binding of ATP is well investigated (for review, see Refs. 2 and 5). Under physiological conditions the Na,K-pump binds ATP in the state $(K_2)E_2$, accelerating the transition to state $K_2E_1 \cdot ATP$ with an equilibrium dissociation constant above 10 μM ('low-affinity binding site') [2,34,46]. Under conditions of low ATP concentrations ATP binds to the state E_1 to form E_1ATP, presumably with or without Na ions bound, with an equilibrium dissociation constant in the range of 50–500 nM ATP ('high-affinity binding constant') [5,33,46]. The high-affinity binding was observed in the absence of potassium [47] and it is also seen with an equilibrium dissociation constant of ≈ 50 nM in the sodium-only mode experiments of this paper. In the Na,K-mode the ATP concentration dependence is controlled by low-affinity binding with an equilibrium dissociation constant of 13 μM (more than 90% of the effect) although a low (but significantly above background) transport activity is observed at ATP concentrations below 500 nM which would fit into high-affinity activated ion-pumping.

The comparison of the ATP concentration dependence for both transport modes reveals that ATP binding as an accelerating step in the 'K-limb' of the Na,K-mode has no counterpart in the Na-only mode. The reason could be that the rate-limiting step is a reaction before ATP binding (e.g., $P\text{-}E_2Na_2 \rightarrow (Na_2)E_2$) and is even rate limiting compared to the transition $(Na_2)E_2 \rightarrow Na_2E_1$. An other possible explanation would be that the assumed analogy of the lower limbs in Fig. 1A and B describing the $E_2 \rightarrow E_1$ transition of the reaction cycles does not hold. Although less likely, this argument cannot be excluded with the available experimental findings.

ADP. The ADP concentration dependence in the Na,K-mode was found to show a binding behaviour not competitive with ATP binding using reconstituted

Na,K-ATPase from rabbit kidneys [34]. These results could be confirmed and compared to experiments in the Na-only mode. Essentially also an inhibition kinetics was found with a half maximal inhibitor concentration of 6 μM. This is by a factor 20 lower than the half-maximal inhibitor concentration in the Na,K-mode.

Possible reasons for the higher sensitivity of the pump for ADP in the Na-only mode can be on the one hand an enhanced ratio of the backward over the forward directed transition step of the reaction $Na_3E_1 \cdot ATP \rightleftharpoons (Na_3)E_1\text{-}P + ADP$ or on the other hand an enhanced binding of ADP to the state $E_2(Na_2)$ in competition to binding of ATP. An argument against the latter possibility is that in the range of 25–500 μM ATP the same inhibition kinetics is observed, in contradiction to a competitive binding of ATP and ADP. The shift of the half-maximal inhibitor concentration is caused by an appropriate increase of the reaction rate $[(Na_3)E_1\text{-}P] \cdot [ADP] \cdot p_b$ in the Na-only mode (cf. Fig. 1B). This increase must be due to a higher population of the state $(Na_3)E_1$-P and/or a lower of $Na_3E_1 \cdot ATP$, if one refuses to assume that p_f and p_b are regulated by the presence/absence of potassium. A discrimination between the two possibilities by numerical simulation of the reaction scheme according to Fig. 1B as described in [34] is not possible at present since no reliable information on states and rate constants for the lower limb in Fig. 1B is available.

Sodium. The comparison of the sodium concentration dependence of both pumping modes is striking (Fig. 5). The increase of the pumping activity with (cytoplasmic) Na^+ concentration is in agreement with previous measurements and it is understood by a Michaelis-Menten type binding kinetics [32,48,49]. In the Na-only mode up to a concentration of 10 mM Na^+ also a Michaelis-Menten type binding kinetics is found with a K_M value of 8 mM which is smaller by a factor of 2 compared to the K_M value of the Na,K-mode. The higher K_M value in the Na,K-mode may be caused by the presence of K^+ ions which bind competitively to the E_1 conformation of the pump and lead to an apparent K_m value of 15 mM.

Above 10 mM Na^+ the pump becomes increasingly inhibited by Na^+ in the Na-only mode. A reduction of pumping activity in the Na-only mode was found also by Forgac and Chin with reconstituted canine enzyme [50]. They could also observe at high sodium concentrations a stoichiometry of < 0.3 Na^+/ATP. A possible mechanism on the basis of the modified Post-Albers cycle could be due to the following effects: At high concentrations of (cytoplasmic) sodium the population of the state $Na_2E_1 \cdot ATP$ is enhanced (Fig. 1B) and in the absence of K^+ ions the reaction equilibrium $Na_2E_1 \cdot ATP \rightleftharpoons ATP \cdot E_2(Na_2)$ is shifted towards the right side, thus reducing the overall turnover of the pump. But this argument is not sufficient to explain the reduced stoichiometry. A proposal for this observation is given by Forgac and Chin [50], who refer to the finding that at high concentrations of Na^+ the $(Na_3)E_1$-P state is highly populated [51]. This state can also hydrolyze in a K^+-independent process [52,53]. This suggestion is supported by the high sensitivity of the Na-only mode for ADP which needs an increased population of the state $(Na_3)E_1$-P, as shown above.

Comparison of activation energies

The activation energy of the electrogenic ion transport should reflect the activation energy of the rate limiting step of the pumping cycle. The great difference of the measured energies of the Na,K-ATPase (160 kJ/mol) and the Na-only ATPase (110 kJ/mol) reveal two distinct rate-limiting steps for both modes. An interesting finding is the fact that the slower process has the lower activation energy; an explanation cannot be offered so far.

For the Na,K-ATPase the reaction step $E_2(K_2) + ATP \rightarrow K_2E_1 \cdot ATP$ is rate-limiting as shown by Steinberg and Karlish [48] and Stürmer et al. [10]. They found under nonlimiting ATP, Na^+ and K^+ concentrations 60%–80% of the enzyme in state $E_2(K_2)$. In the Na-only mode good evidence is found that $E_2(Na_2) \rightarrow Na_2E_1$ cannot be accelerated by ATP, a possible indication of a different rate-limiting step. At 18°C the rate constants of the limiting steps in both transport modes are different by a factor of 10, being 15 s^{-1} [10] in the Na,K-mode and in consequence 1.5 s^{-1} in the Na-only mode. This is in agreement with previous findings [9,10]. A candidate for such a slow reaction could be the occlusion step $P\text{-}E_2Na_2 \rightarrow E_2(Na_2) + P_i$ which is most strongly influenced by the 'misfitting' substrate Na^+. Additional experiments in preparation shall elucidate states and reaction steps of this part of the reaction cycle.

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