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Na^+ currents generated by the purified ($Na^+ + K^+$)-ATPase on planar lipid membranes

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Purified ($Na^+ + K^+$)-ATPase from pig kidney was attached to black lipid membranes and ATP-induced electric currents were measured as described previously by Fendler et al. ((1985) EMBO J. 4, 3079–3085). An ATP concentration jump was produced by an ultraviolet-light flash converting non-hydrolysable caged ATP to ATP. In the presence of Na^+ and Mg^{2+} this resulted in a transient current signal. The pump current was not only ATP dependent, but also was influenced by the ATP/caged ATP ratio. It was concluded that caged ATP binds to the enzyme (and hence inhibits the signal) with a K_i of approx. 30 μM, which was confirmed by enzymatic activity studies. An ATP affinity of approx. 2 μM was determined. The addition of the protonophore 1799 and the Me^+/H^+ exchanger monensin made the bilayer conductive leading to a stationary pump current. The stationary current was strongly increased by the addition of K^+ with a $K_{0.5}$ of 700 μM. Even in the absence of K^+ a stationary current could be measured, which showed two Na^+-affinities: a high-affinity ($K_{0.5} \leq 1$ mM) and a low-affinity ($K_{0.5} \geq 0.2$ M). In order to explain the sustained electrogenic Na^+ transport during the Na^+-ATPase activity, it is proposed, that Na^+ can replace K^+ in dephosphorylating the enzyme, but binds about 1000-times weaker than K^+. The ATP requirement of the Na^+-ATPase was the same ($K_{0.5} = 2$ μM) with regard to the peak currents and the stationary currents. However, for the ($Na^+ + K^+$)-ATPase the stationary currents required more ATP. The results are discussed on the basis of the Albers-Post scheme.

Introduction

The ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) from mammalian cells is an electrogenic pump, which exchanges 3 Na^+ for 2 K^+ ions across the cell membrane. Pump currents have been observed in various intact cells [2,3]. Recently Gadsby et al. [4] and Lafaire and Schwarz [5] were able to measure the voltage dependency of the pump. In these studies the ouabain-sensitive current has been observed by a subtraction procedure. An alternative technique to study the pump current of the ($Na^+ + K^+$)-ATPase has been described by Fendler et al., 1985 [1]. The method consists of the adsorption of membrane fragments enriched with ($Na^+ + K^+$)-ATPase from the medulla of the kidney [6] to planar lipid membranes in a sandwich-like structure. Because of the capacitive coupling between the membranes in series the activity of the

Abbreviations: caged ADP, P^2-1-(2-nitro)phenylethyladenosine 5′-diphosphate; caged ATP, P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate; discs, ($Na^+ + K^+$)-ATPase-containing membrane fragments; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; TTFB, tetrachloro-2-(trifluoromethyl)benzimidazole; 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one.

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$(Na^+ + K^+)$-ATPase could be measured under short-circuit conditions. Pump currents were initiated by a light-induced concentration jump of ATP via the conversion of a photolabile inactive derivative, the caged ATP, into its active form.

Caged ATP was applied to study transport properties of the $(Na^+ + K^+)$-ATPase on erythrocyte ghosts [7], on proteoliposomes [8] and on black lipid membranes [1]. The lipid bilayer system allows the investigation of pump currents, generated by the purified $(Na^+ + K^+)$-ATPase with high sensitivity (0.5 pA) and 0.5 ms time resolution under well defined parameters such as electrolyte, substrates etc. With this most direct method for the determination of electrogenic pump activity a transient Na^+ pump current in the absence of K^+ has been shown by Fendler et al. 1985 [1], Similar results, measured with heart cells, have been obtained by Nakao and Gadsby, 1986 [9]. The electrogenicity of Na^+ transport in absence of K^+ is controversially discussed. Transport studies on reconstituted proteoliposomes showed K^+-independent, electrogenic Na^+ net transport [10,11]. Dissing and Hoffman [12], however, demonstrated that the Na^+ net transport of erythrocytes is electroneutral, because of cotransported anions. Further investigation of the electrogenicity of the Na^+ current (transient and stationary) on the planar lipid membrane system should clarify this point and allow the assignment of pump currents to different steps in the Albers-Post scheme.

Materials and Methods

(a) Caged ATP, caged ADP, $(Na^+ + K^+)$-ATPase

Caged ATP and caged ADP were prepared as reported previously for caged ATP [7,1]. Caged ADP and caged ATP were used in the form of triethylammonium salts. In a control experiment the 2-amino-2-hydroxymethylpropane-1,3-diol salt (Tris-caged ATP) was used, which resulted in the usual signal. The addition of triethylammoniumchloride to a concentration of 2 mM did not change the signal. $(Na^+ + K^+)$-ATPase was purified as described by Jørgensen, 1974 [6]. The enzyme activity at a concentration of 1 μg protein/ml was measured by spectrophotometric determination of inorganic phosphate, P_i, in the presence of 3 mM Tris-ATP, 3 mM $MgCl_2$, 120 mM NaCl, 20 mM KCl and 25 mM histidine-HCl (pH 7.5) [13]. The specific activities ranged between 20 and 30 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ at 37°C and 10–15 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ at 25°C. 2,4-Dinitrophenylphosphatase activity was measured at 37°C in 40 mM imidazole-HCl (pH 7.5) containing 5 mM $MgCl_2$ with the Tris-salt of caged ATP as inhibitor. For further details see Ref. 1.

(b) Lipid bilayer setup

Optically black lipid membranes with an area of 10^{-2} cm^2 have been formed in a thermostated teflon cell with 1.5 ml of an appropriate electrolyte solution in each compartment. The temperature was kept at 22°C. The membrane forming solution contained 1.5% w/v diphytanoylphosphatidylcholine (Avanti Biochemicals, Birmingham, AL) and 0.025% w/v octadecylamine (Riedel de Haen, Hannover, F.R.G.) in *n*-decane. Octadecylamine was used to introduce a positive surface charge on the lipid bilayer, which leads to a higher adsorption of the negatively charged $(Na^+ + K^+)$-ATPase membrane discs to the supporting planar film. A similar procedure has been used for purple membrane from *Halobacterium halobium* [14]. The membrane was connected to an external measuring circuit via Ag/AgCl electrodes. To avoid artificial photoeffects, the electrodes were separated from the aqueous compartments of the teflon cell by agar-agar salt bridges. To prevent light pipe effects the salt bridges were made from polyethylene tubes and the agar-agar was prepared in black ink. For further details see Ref. 15.

Caged ATP or caged ADP and if necessary different salts from stock solutions were added under stirring to the cuvette.

To photolyse the caged ATP, light pulses with a duration of 125 ms were applied. The light beam was focused on the lipid bilayer membrane. As light source a mercury-high-pressure lamp with an maximal light intensity of 3.7 W/cm^2 was used. If not otherwise indicated, the system was kept in the dark after each ultraviolet-light flash for 10 min to allow relaxation to the conditions before the ultraviolet-light pulse. By this means the liberated ATP is diluted and hydrolysed by the enzyme in the cuvette and the original concentration of caged ATP is recovered on the membrane

surface. The irradiated volume in the protein containing compartment is 1% of total volume of the aqueous phase. That means, that each flash with a maximal conversion rate of 34% reduces the concentration of caged ATP by 0.3%.

The conversion rate of caged ATP was determined in two ways. (a) ATP was liberated within the light beam in the cuvette. After stirring a sample of the cuvette solution was analyzed for ATP concentration with the luciferin-luciferase assay. The concentration of released ATP in the light beam was calculated according to the geometry of the beam. (b) A droplet of 2 μl of a 20 μM caged ATP solution was placed in the hole of the empty cuvette instead of the membrane. After flashing the droplet was analyzed with the luciferin-luciferase assay. Both procedures gave a conversion rate of maximal 34% at 125 ms pulse duration and 3.7 W/cm^2 light intensity. By improvement of the procedure described above, the conversion rate could be determined more precisely, than was possible in our previous paper [1], where a $K_{0.5}$ value for ATP of 6.5 μM was found, compared to 2 μM shown in this paper.

The ultraviolet-light was attenuated by neutral density filters (Melles-Griot, The Netherlands). Luciferin luciferase (Boehringer, Mannheim, FRG) was used to determine ATP concentration. A schematic representation of the membrane set up is given in Fig. 1.

(c) Chemicals

The protonophore 1799, the 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one, was kindly provided by Dr. P. Heytler, Dupont Nemours. Monensin was a gift from Dr. G. Szabo, Galveston, TX. Valinomycin was purchased from Serva, Heidelberg, F.R.G. All other reagents were analytical or suprapure grade from Merck, Darmstadt, F.R.G.

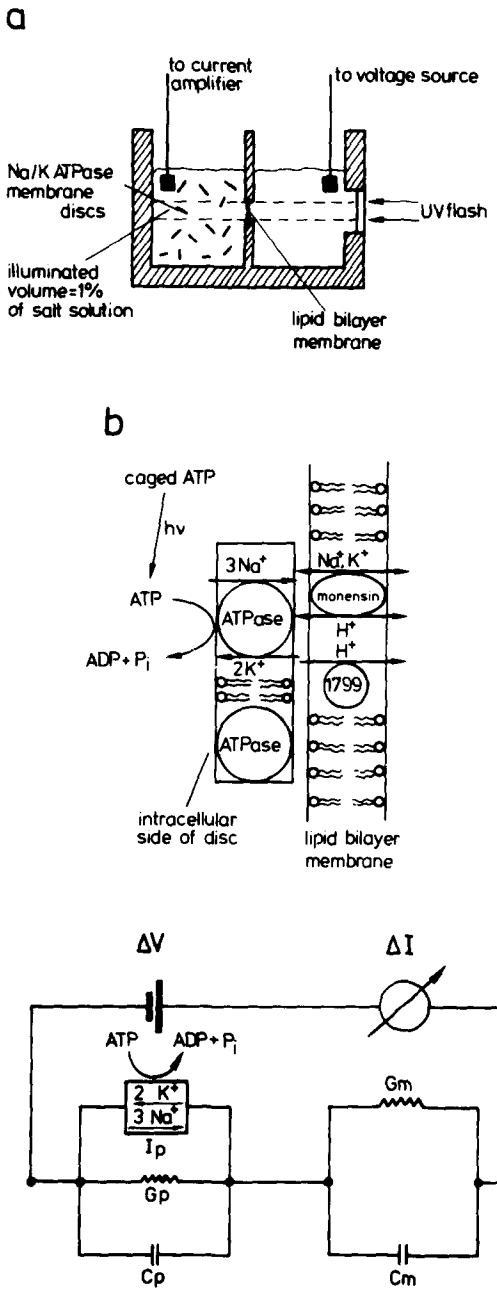

Fig. 1. Schematic representation of the bilayer setup. (a) Teflon cuvette with black lipid membrane (BLM) and adsorbed ($Na^+ + K^+$)-ATPase discs. (b) Proposed sandwich-like arrangement of discs and underlying lipid membrane. The two membranes are capacitively coupled. It has to be noted, that in experiments with ionophores, incorporation of ionophores into the disc membrane cannot be excluded (not shown in the figure). (c) Equivalent circuit diagram of the two membranes in series. G_m, G_p refer to the conductance of the planar bilayer (BLM) and the disc membrane, respectively; C_m and C_p to the capacitance of BLM and discs, respectively. I_p designates the pump current generator. For small values of G_m only transient currents are observable, whereas for large values of G_m in the presence of 1799 and monensin stationary pump currents are obtained.

(d) Ultraviolet-light artifacts

Before pump currents were measured possible artifacts induced by caged ATP, the ionophores monensin and 1799 were tested. As shown previously [1], in the presence of monensin and valinomycin at highest light intensities (3.7 W/cm^2) only a small ultraviolet-light artifact occurred. In order to investigate the electrogenicity of the pump in presence of Na^+ without any K^+ the ionophore system monensin together with 1799 as described in Fig. 2b was used. The advantage of the protonophore 1799 compared to the usual H^+ carriers like FCCP or TTFB is that there is no absorbance in the range of wavelengths, which have been applied. Therefore no ultraviolet-light artifact occurs on the membrane system if 1799 is added in contrast to the protonophores FCCP and TTFB.

The ultraviolet-light artifact of the caged substrates, caged ATP and caged ADP (up to 500 μM), does not originate from the liberated hydrophobic nitrobenzyl residue but rather from the proton, which is also released during photolysis [16]. The proton presumably binds to the planar lipid bilayer. This was demonstrated by variation of the buffer concentration in the medium. At low concentration (1 mM) or absence of buffer a large capacitive current response occurred, which was completely abolished at buffer concentrations of 20 to 100 mM (Christensen, B., unpublished data).

Results

Purified ($Na^+ + K^+$)-ATPase membrane discs were added together with caged ATP under stirring to one side of the lipid bilayer. After 15 min a transient current could be induced by an ultraviolet-light pulse.

Fig. 2a shows the activation of the pump after a 125 ms ultraviolet-light flash under normal conditions, i.e. 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.5). In the absence of ionophores the current is transient, whereas the permeabilization of the underlying lipid bilayer (Fig. 2b) yields a sustained current which decays slowly to zero due to the consumption and the dilution of the liberated ATP into the not irradiated volume.

Previously it has been shown already, that the

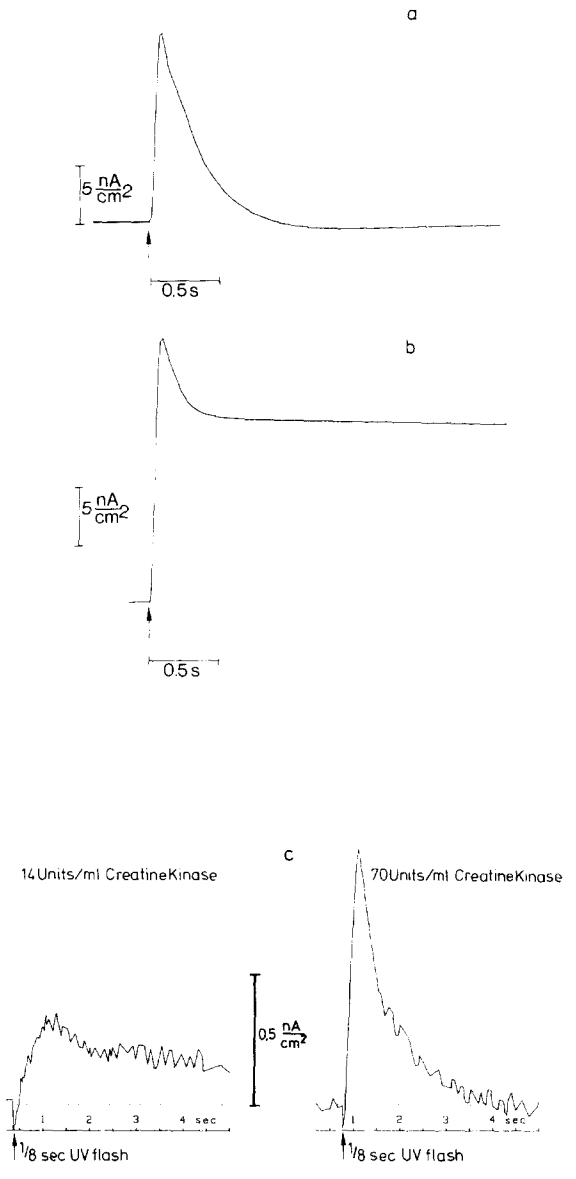

Fig. 2. Short-circuit currents on the lipid bilayer system under different experimental conditions. The membrane bathing solution contained 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.5). The arrows indicate opening of the shutter, which was opened for 125 ms. (a) 100 μM caged ATP, no addition of ionophores. (b) Same membrane system after addition of monensin and 1799 to a concentration of 10 μM and 0.6 μM, respectively. (c) Control experiment without ionophores, no caged ATP but 5 μM caged ADP, 1 mM creatine phosphate and 14 units/ml creatine kinase (left side) or with 70 units/ml creatine kinase (right side). Notice different current-scaling.

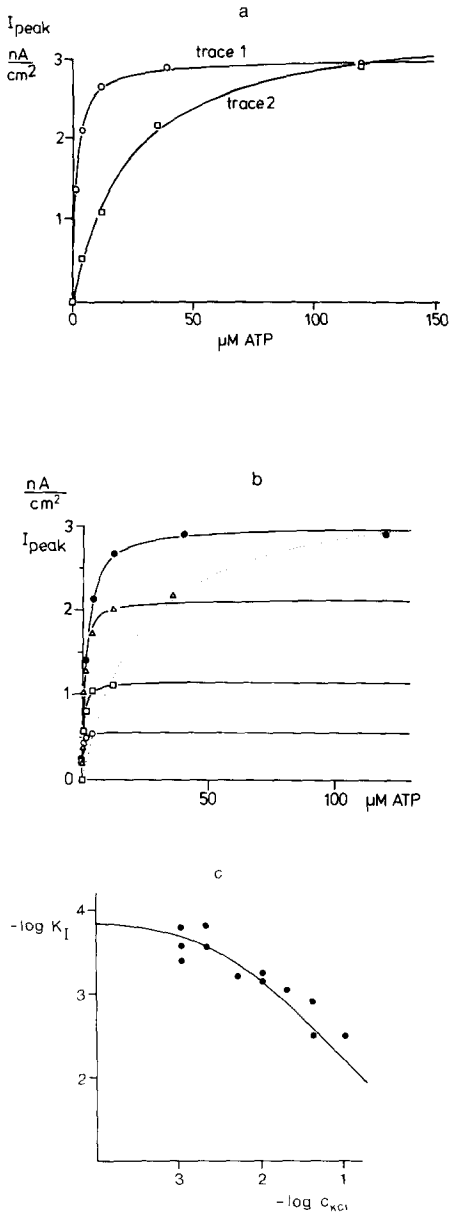

Fig. 3. (a) ATP dependency of the peak current, measured by two different procedures. Electrolyte composition as in Fig. 2. Omission of K^+ did not change the result. Trace 1 (○), conversion rate was always 34%, ATP concentration was varied by addition of caged ATP. Trace 2 (□): initial caged ATP concentration was kept constant at 355 μM, ATP concentration was varied by varying the conversion rate. The solid lines are fit curves, calculated as described for Fig. 3b, yielding $I_{peak\,max} = 3$ nA/cm², $K_{0.5} = 1.7$ μM for trace 1 and $I_{peak\,max} = 3.5$ nA/cm², $K_{0.5} = 23$ μM for trace 2.
(b) ATP dependency of the peak current at different conversion rates. The solid lines are fit curves, which correspond to signals presented in Figs. 2a and 2b are due to the enzymatic activity and the related charge translocation [1]. The sign of the current was always the same and corresponded to a positive charge movement toward the protein-free side. The discs were shown to be preferentially oriented with the extracellular side facing the planar bilayer [1], see Fig. 1b.

In addition the following control experiments have been performed:

(a) The addition of caged ATP to the protein-free compartment of the membrane system had no effect on the pump current. Caged ATP, added only to the protein-free side did not result in any signal, indicating the negligible membrane permeability to the molecule.

(b) The signal was blocked by vanadate with an halfmaximal effect below 1 μM.

(c) Caged ADP could not substitute for caged ATP, but in the presence of phosphocreatine and creatine kinase, which converts ADP to ATP, a pump current could be measured depending on the concentration of the kinase (Fig. 2c).

Mg^{2+} was necessary for the appearance of pump currents [1]. If not otherwise indicated, in all experiments a concentration of free Mg^{2+} of 3 mM was used.

The inhibitory effect of caged ATP on the transient pump current (I_{peak})

The ATP dependence of the pump current was obtained in two ways: Caged ATP was added successively in increasing concentrations, where the light-induced conversion rate was constant

different conversion rates, and were calculated according to the equation

$$I_{peak} = I_{peak\,max} \cdot c_{ATP}/(K_{0.5} + c_{ATP})$$

Conversion rate, $I_{peak\,max}$ and $K_{0.5}$: (●), 34%, 3 nA/cm², 1.7 μM; (△), 12%, 2.1 nA/cm², 0.66 μM; (□), 4%, 1.15 nA/cm², 0.46 μM; (○), 1.3%, 0.56 nA/cm², 0.15 μM.
(c) Inhibition of dinitrophenylphosphatase-activity by the Tris salt of caged ATP at 37 °C. Dependence of the inhibition constant (K_I) on KCl concentration. The solid line is calculated according to $K_I = K_I^0 \cdot (1 + c_{KCl}/K_{KCl})$, where K_I^0 represents the KCl-independent caged ATP binding constant and K_{KCl} represents the K^+ binding constant, assuming a 1:1 complex with the enzyme. The corresponding value for $-\log K_I^0$ is 3.85 ± 0.3 and for $-\log K_{KCl}$ is 2.7 ± 0.2.

(34%). Alternatively the ATP concentration was changed by the variation of the light intensity at a fixed concentration of caged ATP. (The saturation curves for the two different methods are shown in Fig. 3a). Depending on the procedure different saturation curves were obtained. The dependency of peak currents on the concentration of liberated ATP is shown in Fig. 3a. Curve 1 was obtained by successive addition of caged ATP up to 355 μM. As the conversion rate was kept constant at 34% it corresponds to a maximal ATP concentration of 121 μM. Curve 2 was obtained on the same membrane at 355 μM caged ATP by attenuation of the light intensity yielding the corresponding liberation of ATP. In contrast to the Ca^{2+}-ATPase from sarcoplasmic reticulum, where the shape of the two curves is the same [17], the two different procedures gave different results at the same concentration of released ATP. This phenomenon suggests that caged ATP inhibits the enzyme. A more detailed representation and analysis of this effect is shown in Fig. 3b. Caged ATP was added at different concentrations. At each concentration the conversion rate was varied by changing the intensity of the incident ultraviolet light. A set of saturation curves was obtained, each of which could be fitted by a simple Michaelis-Menten formalism. The dotted line represents the pump current at the same concentration of caged ATP and different conversion rates. This corresponds to trace 2 in Fig. 3a.

As can be seen in Fig. 3b, the apparent I_{max} and $K_{0.5}$ values are lower at lower conversion rates, indicating the inhibition of the pump by caged ATP. The real value for the $K_{0.5}$ of the pump-current dependency on the concentration of ATP can therefore only be experimentally determined at a conversion rate of 100% or by extrapolation of the $K_{0.5}$ values to 100%. This procedure gave a K_m value for ATP of 2 ± 1 μM, as revealed by further analysis given in the Discussion.

In order to determine the degree of binding of caged ATP to the $(Na^+ + K^+)$-ATPase under the conditions used here, inhibition constants K_I were determined on the basis of enzymatic activity (dinitrophenylphosphatase-activity) studies at different concentrations of KCl. As shown in Fig. 3c the $-\log K_I$ value decreased with increasing K^+ concentration. Assuming that caged ATP binds only to the Mg^{2+} complex of the enzyme and not to the K^+, Mg^{2+} complex (E_2 form), a binding constant of about 140 μM at 37°C is found for vanishing K^+ concentrations.

Stationary pump currents in the absence of K^+

In the absence of K^+ the $(Na^+ + K^+)$-ATPase induces a transient pump current on the reconstituted lipid bilayer system. This demonstrates directly that at least parts of the Na^+ cycle of the pump are electrogenic. Only the appearance of a stationary Na^+ pump current, however, can prove that the enzyme acts as an electrogenic Na^+ pump.

Stationary currents on the sandwich-like structure (Fig. 1b) can be detected by increasing the conductivity of the supporting lipid bilayer with an appropriate carrier system. Since no electrogenic Na^+ carrier is available, ultraviolet light-insensitive monensin as an alkali metal ion/H^+ exchanging agent was applied together with the ultraviolet light-insensitive protonophore 1799 (see Materials and Methods). As shown below only such experiments on the stationary pump currents were evaluated, where the ionophore induced conductance was stable during the measurements. (Maximal variation was 30%).

The purely capacitively (AC) coupled membrane system is changed by the application of the ionophores to a DC coupled system (Fig. 2b). The $(Na^+ + K^+)$-ATPase creates an electrochemical

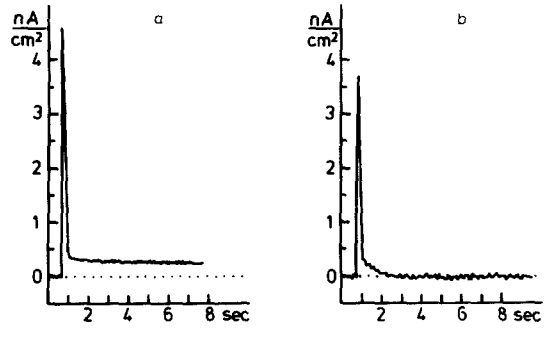

Fig. 4. (a) Stationary Na^+ pump activity in the absence of K^+, other conditions as in Fig. 2b. (b) Same membrane as in (a) after addition of 1 mM glucose and 50 units/ml hexokinase for rapid consumption of released ATP.

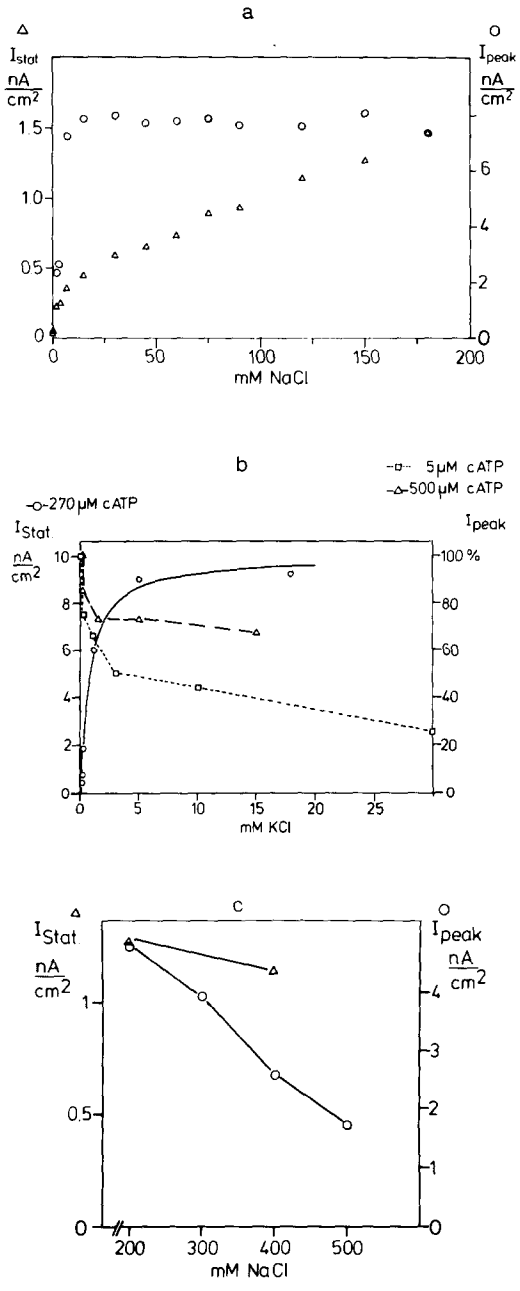

Fig. 5. Dependency of peak and stationary currents on Na^+ and K^+. (a) Na^+ dependency of peak (○) and stationary (△) currents in the presence of monensin and the protonophore 1799. Electrolyte: 25 mM imidazole-HCl, 3 mM $MgCl_2$ pH 7.5, residual K^+ concentration = 6 µM. The 1 M Na^+ stock solution for titration contained 10 µM K^+. Three membranes were measured showing the same affinities within an error limit of ±25%.

(b) △, □: Measurement of peak currents with 5 or 500 µM caged ATP, corresponding to 1.5 and 150 µM ATP after ultraviolet-light flash, respectively. Values are normalized to 100%. ○: K^+ dependency of stationary currents in the presence of monensin and the protonophore 1799 with 270 µM caged ATP, corresponding to 81 µM released ATP. Electrolyte: 130 mM NaCl, 25 mM imidazole-HCl, 3 mM $MgCl_2$ (pH 7.5). The solid line is a fit curve according to a Michaelis-Menten equation, resulting in a $K_{0.5}$ of 0.7 mM.

(c) Decline of peak currents (○) and stationary currents (△) at concentrations higher than 200 mM NaCl. Conditions as in Fig. 5a, except that peak currents were determined without ionophores.

potential, which is positive within the interfacial space between the adsorbed discs and the planar lipid membrane (Fig. 1b). Monensin abolishes the Na^+ and K^+ gradient electroneutrally by exchanging Na^+ or K^+ with protons. Then, the protons will be transported electrogenically by the protonophore 1799 across the planar lipid membrane, yielding an electric current. Assuming a similar ionophore induced permeability of the discs and the lipid bilayer the expected pump current is reduced by a factor of 2.

A stationary Na^+ pump current was obtained indeed after the addition of monensin and the protonophore 1799 to the membrane system (Fig. 4a). Comparing Fig. 2b and Fig. 4 shows that the stationary pump current without K^+ is smaller than the Na^+K^+ pump current. The stationary Na^+ current disappeared after the addition of hexokinase and glucose, which depletes the free ATP in solution (Fig. 4b). In all experiments concerning the Na^+ current the K^+ concentration was determined by atomic absorption spectroscopy. A maximal concentration of 6 µM K^+ was obtained.

The addition of 100 µM K^+ to the K^+ free electrolyte ($c_{K^+} \leqslant 6$ µM) gave only an increase of the pump current by a factor of 2. By comparison with the K^+ dependency (Fig. 5b) it can be excluded that the residual K^+ is responsible for the Na^+ stimulated stationary pump current. For the same reasons residual NH_4^+ ions ($C_{NH_4^+} \leqslant 2$ µM) cannot account for the stationary pump current.

Fig. 5a shows the dependency of the peak and the stationary current on the Na^+ concentration. For the peak current only a high affinity ($K_{0.5}$ = approx. 3 mM) was obtained, whereas the stationary current shows a high (1 mM) and a low

affinity to Na^+ (0.2 M). Additional potassium added to the same membrane stimulates the pump current with an $K_{0.5}$ of 700 μM, according to a simple Michaelis-Menten formalism (Fig. 5b, solid line). Fig. 5b dashed and broken line demonstrate the reduction of the peak current depending on the K^+ concentration at different concentrations of ATP 1.5 μM and 150 μM, respectively, see also Discussion. Further addition of Na^+ up to 500 mM depressed drastically the peak current (Fig. 5c). The stationary current declines less at Na^+ concentrations above 200 mM (Fig. 5c). Choline chloride in high concentrations could not imitate the effect of NaCl at concentrations higher than 200 mM.

ATP dependency of stationary pump currents

Under the experimental conditions in Fig. 6 each flash converts 34% of the caged ATP to ATP within the incident light beam. Therefore at a starting concentration of 50 μM in the cuvette 15 μM ATP are released with the first flash and with the second flash additional 10 μM. This means, the concentration of ATP can be increased significantly by a second ultraviolet-light flash of the same intensity and duration.

Under K^+-free conditions and at low K^+ concentrations (100 μM) the stationary current is not increased by a second ultraviolet-light flash (Fig. 6, trace a). Measurements of the ATP dependency of the stationary Na^+ current (data not shown) showed the same affinity for ATP as the peak current (Fig. 3). In presence of K^+ (10 mM and 100 mM Na^+), however, the stationary current increases remarkably with a second concentration jump of ATP (Fig. 6, trace b), indicating a second lower affinity binding site. This is in agreement with early ($Na^+ + K^+$)-ATPase measurements and the current conception of the enzyme [18–20]. Therefore K^+ titration of stationary currents was carried out with a high (270 μM) caged ATP concentration, but this could even be too low for saturating the low-affinity ATP binding site.

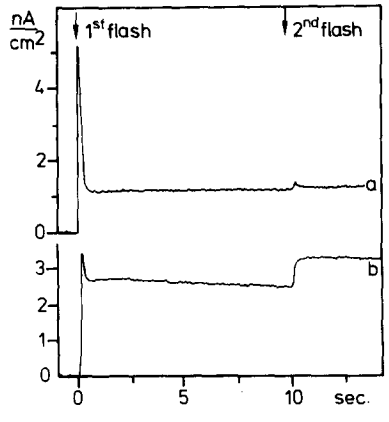

Fig. 6. Demonstration of ATP stimulation of pump currents in the presence and absence of K^+. Caged ATP concentration = 50 μM corresponding to 15 μM released ATP with the first flash and additional 10 μM ATP with the second flash. (a) 100 mM NaCl, 3 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.5). (b) Same membrane and electrolyte as in (a) after addition of 10 mM KCl.

Mg^{2+} dependency of the pump currents

The presence of Mg^{2+} is necessary for the occurrence of the pump currents. Fig. 7 shows the dependency of the peak current on the concentration of free Mg^{2+} at saturating concentrations of ATP for the high-affinity binding site. The pump current is half maximal at 2 μM Mg^{2+}. Measurements of the ATP dependency at 10 and 100 μM Mg^{2+}, compared to the usual 3 mM, resulted in a 2-fold increase of $K_{0.5}$ for ATP, indicating that MgATP binds with a slightly higher affinity than ATP to the enzyme, as also reported by Robinson and Flashner [21].

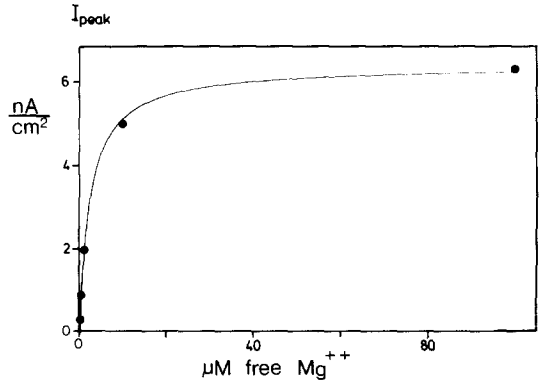

Fig. 7. Mg^{2+} dependency of the peak currents. Conditions: 50 μM caged ATP, corresponding to 17 μM released ATP. Electrolyte: 130 mM NaCl, 25 mM imidazole-HCl, 2 mM EDTA (pH 7.5). The solid line is a fit curve according to a Michaelis-Menten equation, yielding $K_{0.5} = 2$ μM free Mg^{2+}.

Discussion

ATP hydrolysing Na^+/Na^+ exchange of pig kidney enzyme is electrogenic

Transport activity related to the electrogenicity of the $(Na^+ + K^+)$-ATPase in the absence of K^+ has been investigated previously by different methods: Tracer measurements on reconstituted proteoliposomes [10,11] demonstrating an electrogenic effect and on erythrocytes, showing an electroneutral transport in the absence of extracellular Na^+ [12], as well as measurements of the Na^+/K^+ pump, reconstituted on a planar lipid bilayer [1], showing a transient electric pump current. In this paper continuous electrogenic Na^+ pumping activity in the absence of K^+ is demonstrated by direct measurement of the electric current.

The reconstituted lipid bilayer system allows us to investigate a highly purified enzyme under well defined electrolyte composition. Therefore interference of other transport proteins and contamination of the electrolyte with K^+ and its congeners can be excluded almost completely.

Although the whole system is clamped to zero voltage, an electrochemical potential can be built up across the discs due to the ion transport across the disc membrane. This potential can be reduced by the application of the ionophores. The agreement of the substrate dependencies obtained in this and the previous paper [1] with data from enzymatic experiments supplies strong evidence, that a possible effect of the electrochemical potential can be neglected.

The Na^+ current decays after the initial transient phase to a small stationary component. The fast decay to the stationary value is not due to the fast consumption of ATP because a second flash showed only a negligible effect on the current, indicating the saturation of the system with ATP (Fig. 6a) (15 μM ATP released after the first flash compared to a K_m value for ATP of 2 μM).

The ATP and Na^+ dependencies for the peak and the stationary currents suggest, that the transient component of the signal reflects the phosphorylation step with concomitant Na^+ transport. The values of the ATP ($K_{0.5} = 2$ μM) and Na^+ ($K_{0.5} = 3$ mM) affinities agree well with data from earlier work on the enzymatic activity [22,23]. The stationary current represents the continuous function of the enzyme, which is stimulated by higher Na^+ concentrations (Fig. 5a). Stimulation of ATP-hydrolysis with high (up to 200 mM) Na^+ concentrations has been reported previously [11,23,24].

The different properties of the transient and the stationary current are discussed on the model based on the Albers-Post scheme [25,26], as modified by Karlish et al. [20] (Fig. 8).

Loop 1 in Fig. 8 is the reaction cycle in the absence of K^+, i.e. of the Na^+-ATPase, phosphorylation with and without K^+ both involve the high-affinity ATP binding step. Therefore the ATP affinity of the peak currents are the same under both conditions. The reduction of the peak current in the presence of K^+ (Fig. 5b) is presumably due to the shift of the E_1Na-E_2K equilibrium to the E_2K form. Thereby the number of molecules in the E_1Na state, which can be activated by the released ATP, is reduced. The reduction of the peak current by K^+ is less pronounced at high ATP concentrations (Fig. 5b). At a high ATP level the E_2K intermediate is not accumulated, but decays to the E_1 form (loop 3 in Fig. 8), which can then be phosphorylated.

The addition of K^+ speeds up dephosphoryla-

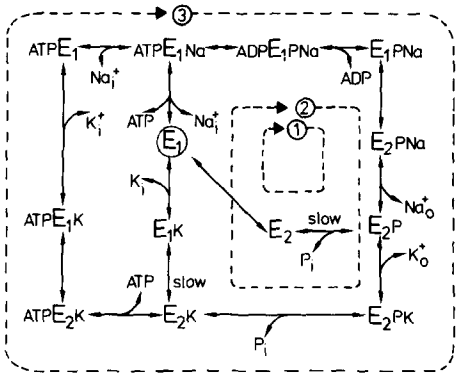

Fig. 8. Mechanism of the $(Na^+ + K^+)$-ATPase. E_1 and E_2 refer to different conformations, EP to the phosphorylated form of the enzyme. Na_i and K_i designate intracellular concentrations of Na^+ and K^+, respectively. Na_o and K_o refer to the concentrations outside the cell. Loop 1 shows the Na^+-ATPase cycle; loop 2 the $(Na^+ + K^+)$-ATPase cycle at low ATP concentrations, loop 3 the physiological case, where the low-affinity ATP binding site is saturated. (Scheme modified from Karlish et al., 1978 [20]).

tion of the enzyme and therefore increases the stationary current (Fig. 5b). In this case the reaction cycle is loop 2 or loop 3 depending on the ATP concentrations. The fact, that in the presence of K^+ the reaction cycle involves a second, low-affinity ATP binding site (loop 3) is clearly demonstrated by the increase of the stationary current after a second ATP-releasing flash (Fig. 6). The affinity for K^+ stimulation of the stationary current has been measured at high ATP concentrations in order to avoid limitation of the signal by the slow E_2K-E_1K step (loop 2). A value of 700 μM for the K^+ stimulation is found, which is in good agreement with data reported in the literature for K^+ binding at the extracellular side of the pump [27–29] and with the K_{KCl} of 2 mM at 37 °C, found by measurements of dinitrophenylphosphatase activity of the $(Na^+ + K^+)$-ATPase (Fig. 3c).

The model in Fig. 8 includes only one step with Na^+ binding (and another with Na^+ release). Experiments, however, show an additional increase of the stationary Na^+ current at high Na^+ concentrations (Fig. 5a). The stationary Na^+ current implies that during dephosphorylation less Na^+ ions are transported (inward) than during phosphorylation (outward), that means one or two. Two is most reasonable, because then this function of the ATPase can be attributed to replacement of K^+ by Na^+, allowing dephosphorylation to take place via E_2Na. This function of the ATPase is represented by loop 2 or 3 in the scheme depending on ATP concentration. In this case K^+ has to be replaced by Na^+ in the scheme shown in Fig. 8.

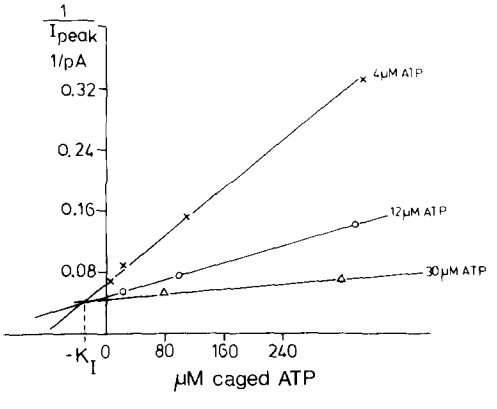

Fig. 9. Dixon-plot of data of Fig. 3b, yielding an inhibition constant of approx. 30 μM caged ATP.

At high Na^+ concentrations ($c_{Na^+} \geqslant 200$ mM) both the peak current and the stationary current decline. On the basis of the results with high choline chloride concentrations a pure salt effect can be excluded, therefore the reduction of the peak current has to be explained by the assumption, that the E_1PNa-E_2PNa transition is slowed down by the accumulation of the intermediate E_2PNa at high Na^+ concentrations. The declined stationary current implies that under high Na^+ conditions the E_1PNa-E_2PNa transition becomes rate limiting. Similar conclusions based on kinetic measurements of the conformational changes of the enzyme have been drawn by Taniguchi et al., 1985 [30].

The effect of caged ATP

As shown before, the presence of caged ATP reduces the pump current. This can be explained by competition of ATP and caged ATP for the ATP binding site. As caged ATP cannot phosphorylate the enzyme, it acts as an inhibitor of phosphorylation, when it can bind to the ATP binding site. Replotting the data of Fig. 3b in a Dixon plot (Fig. 9) under assumption of caged ATP as an inhibitor led to an intersection point, which indicates a competitive inhibition with a K_I of approx. 30 μM.

For competitive inhibition the following equation holds

$$v = \frac{v_{max} \cdot c_S}{c_S + K_m \cdot (1 + c_I/K_I)}$$

With a constant inhibitor concentration an apparent half saturation constant $K_{0.5} = K_m \cdot (1 + c_I/K_I)$ is obtained, which is significantly higher than K_m for inhibitor concentrations c_I in the range of K_I or higher (see Fig. 3a trace 2, where the caged ATP concentration is nearly the same at different concentrations of ATP). If, however, the ATP concentration is varied at a constant conversion ratio the caged ATP concentration has to be changed. These conditions yield a different saturation curve, where both $K_{0.5}$ and the apparent

maximal velocity v'_{max} change to reduced values

$$K_{0.5} = K_M/(1 + r \cdot K_m/K_I)$$

$$v'_{max} = v_{max}/(1 + r \cdot K_m/K_I)$$

where r designates the caged ATP/ATP ratio.

As can be seen in Fig. 3b, the initial slope of the saturation curves, $v'_{max}/K_{0.5}$, remains constant, but the v'_{max} and $K_{0.5}$ values are reduced with decreasing conversion ratios. Taking this into account, a K_m/K_I ratio of approx. 1/15 is obtained. Together with the inhibition constant from Fig. 9 (30 μM) the binding constant for ATP is calculated to $K_m = 2 \pm 1$ μM.

An inhibition constant of 30 μM caged ATP is in reasonable agreement with data obtained from enzymatic activity studies ($K_I = 140$ μM at 37 °C, Fig. 3c) as well as with data from binding studies [8], where a binding constant of 43 μM was reported for caged ATP in the absence of Mg^{2+}.

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