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# Investigation of $\text{Na}^+, \text{K}^+$ -ATPase on a solid supported membrane: the role of acylphosphatase on the ion transport mechanism

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## Abstract

Charge translocation by  $\text{Na}^+, \text{K}^+$ -ATPase was investigated by adsorbing membrane fragments containing  $\text{Na}^+, \text{K}^+$ -ATPase from pig kidney on a solid supported membrane (SSM). Upon adsorption, the ion pumps were activated by performing ATP concentration jumps at the surface of the SSM, and the capacitive current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase were measured under potentiostatic conditions. To study the behavior of the ion pump under multiple turnover conditions, ATP concentration jump experiments were carried out in the presence of  $\text{Na}^+$  and  $\text{K}^+$  ions. Current transients induced by ATP concentration jumps were also recorded in the presence of the enzyme  $\alpha$ -chymotrypsin. The effect of acylphosphatase (AcP), a cytosolic enzyme that may affect the functioning of  $\text{Na}^+, \text{K}^+$ -ATPase by hydrolyzing its acylphosphorylated intermediate, was investigated by performing ATP concentration jumps both in the presence and in the absence of AcP. In the presence of  $\text{Na}^+$  but not of  $\text{K}^+$ , the addition of AcP causes the charge translocated as a consequence of ATP concentration jumps to decrease by about 50% over the pH range from 6 to 7, and to increase by about 20% at pH 8. Conversely, no appreciable effect of pH upon the translocated charge is observed in the absence of AcP. The above behavior suggests that protons are involved in the AcP-catalyzed dephosphorylation of the acylphosphorylated intermediate of  $\text{Na}^+, \text{K}^+$ -ATPase.

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**Keywords:**  $\text{Na}^+, \text{K}^+$ -ATPase; Acylphosphatase; Lipid membrane; Ion transport

## 1. Introduction

$\text{Na}^+, \text{K}^+$ -ATPase is an ion translocating membrane protein that is found in the plasma membrane of virtually all animal cells. Under physiological conditions  $\text{Na}^+, \text{K}^+$ -ATPase transports three  $\text{Na}^+$  ions out of and two  $\text{K}^+$  ions into the cell against their electrochemical potential gradients at the expense of the chemical energy provided by the hydrolysis of one molecule of ATP. Due to its stoichiometry (3  $\text{Na}^+$  vs. 2  $\text{K}^+$ ) the reaction cycle of this ion pump is electrogenic, that is, the transport process results in a charge separation across the membrane. The mechanism of this enzyme is generally described by the Albers–Post model [1,2]. During its transport cycle  $\text{Na}^+, \text{K}^+$ -ATPase can assume two main

conformations,  $E_1$  and  $E_2$ , with ion binding sites exposed to the cytoplasm and the extracellular medium, respectively. The  $E_1$  conformation binds  $\text{Na}^+$  ions and, in the presence of ATP, is converted into the phosphoenzyme  $E_2\text{P}$ . In the  $E_2\text{P}$  state or during  $E_2\text{P}$  formation  $\text{Na}^+$  is released to the extracellular medium, and  $\text{K}^+$  ions are bound. Binding of  $\text{K}^+$  ions results in accelerated dephosphorylation. A subsequent conformational change to the  $E_1$  state leads to translocation of  $\text{K}^+$  ions to the cytoplasmic side of the membrane and their release there.

Acylphosphatase (AcP) is a widespread cytosolic enzyme of about 11 kDa that catalyzes the hydrolysis of the carboxyl phosphate bond of acylphosphates, such as carbamoyl phosphate [3] and succinoyl phosphate [4]. AcP is a highly basic protein, with an isoelectric point (pI) of approximately 11. In mammalian tissues, AcP is present in two isoforms: one is prevalent in skeletal and cardiac muscles, the other in red blood cells. Although the two isoenzymes have a similar molecular weight, they exhibit

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remarkable differences in catalytic potency, affinity towards substrates and sensitivity to inhibitors [5]. More recently, Nassi and coworkers reported that AcP can act not only on the abovementioned low molecular weight substrates, but also on the acylphosphorylated intermediates of various transport ATPases, including  $\text{Na}^+, \text{K}^+$ -ATPase [6,7]. Although this effect was observed with a denatured phosphoenzyme [6], the same authors demonstrated the occurrence of a molecular interaction between AcP and native, undenatured  $\text{Na}^+, \text{K}^+$ -ATPase by dot immunobinding analysis [7]; they also found that AcP addition modifies the functional properties of this active transport system by increasing the rate of ATP hydrolysis and the  $\text{Na}^+/\text{K}^+$  exchange ratio with respect to the well-documented 3/2 value. Based on these results, AcP has been proposed as a potential modulator of the sodium pump [7].

In order to gain a deeper insight into the ion transport mechanism of  $\text{Na}^+, \text{K}^+$ -ATPase and the effect of AcP on this particular ion pump, the present research makes use of a novel experimental method, which was recently developed to perform concentration jumps of an arbitrary substrate at the surface of a solid supported membrane (SSM) [8]. The SSM consists of an alkanethiol monolayer firmly anchored to the gold surface via the sulfhydryl group, with a second phospholipid monolayer on top of it. This technique combines the high mechanical stability of the SSM with a rapid solution exchange procedure. Especially for those substrates that are not available in a caged form, such as  $\text{Na}^+$  and  $\text{K}^+$  ions, the SSM technique was shown to be capable of providing additional information on the transport mechanism of ion translocating membrane proteins [8,9]. In particular, this method was successfully used to investigate the electrogenic partial reactions in the enzymatic cycle of  $\text{Na}^+, \text{K}^+$ -ATPase [8,9], yielding results similar to those obtained by other techniques. In the present study, membrane fragments containing  $\text{Na}^+, \text{K}^+$ -ATPase from pig kidney were adsorbed on the SSM. Upon adsorption, the ion pumps were activated by performing concentration jumps of ATP at the surface of the SSM, and the electric currents generated by  $\text{Na}^+, \text{K}^+$ -ATPase were measured under potentiostatic conditions. To study the behavior of the sodium pump under multiple turnover conditions, ATP concentration jump experiments were carried out in the presence of  $\text{Na}^+$  and  $\text{K}^+$  ions. Current transients induced by ATP concentration jumps were also recorded in the presence of  $\alpha$ -chymotrypsin. Moreover, to investigate the effect of AcP on the ion transport by  $\text{Na}^+, \text{K}^+$ -ATPase, the SSM technique was employed to perform ATP concentration jumps both in the presence and absence of AcP. The present results indicate that AcP modifies significantly the function of  $\text{Na}^+, \text{K}^+$ -ATPase in terms of  $\text{Na}^+$  and  $\text{K}^+$  transport. This effect of AcP is tentatively explained taking into account the catalytic activity of this enzyme on the acylphosphorylated intermediate of  $\text{Na}^+, \text{K}^+$ -ATPase.

## 2. Materials and methods

### 2.1. Chemicals

Sodium, potassium and magnesium chlorides, orthophosphoric acid and imidazole were obtained from Merck at analytical grade. Adenosine-5'-triphosphate disodium salt (ATP, ~97%) and dithiothreitol (DTT, ≥99%) were purchased from Fluka. Octadecyl-mercaptan (98%) from Aldrich was used without further purification.  $\alpha$ -Chymotrypsin (type II, from bovine pancreas) was obtained from Sigma.

The lipid solution contained diphytanoylphosphatidylcholine (Avanti Polar Lipids) and octadecylamine (puriss., Fluka) [60:1] and was prepared at a concentration of 1.5% (w/v) in *n*-decane (Merck) as described by Bamberg et al. [10].

Membrane fragments containing  $\text{Na}^+, \text{K}^+$ -ATPase were prepared and purified from the outer medulla of pig kidney according to the procedure described in Ref. [11]. Proteoliposomes containing  $\text{Na}^+, \text{K}^+$ -ATPase from shark rectal glands were prepared as described in Ref. [12].

Human muscle AcP recombinant protein was obtained in *E. coli* as a fusion protein with glutathione *S*-transferase and purified by affinity chromatography [13]. The acylphosphatase Asn-41 mutant to Ser, Asn41Ser, which is virtually devoid of catalytic activity, was obtained by site-directed mutagenesis according to Taddei et al. [14]. AcP activity was measured by a continuous optical test at 283 nm, using benzoyl phosphate as substrate [15]. Benzoyl phosphate was synthesized according to Camici et al. [16].

Inhibition experiments were carried out using vanadate (sodium orthovanadate, Sigma) and ouabain (ouabain octahydrate, Fluka). A 30 mM stock solution of vanadate was added to the buffer solution at a final concentration of 1 mM, as described in Ref. [8]. A 15 mM stock solution of ouabain was added to the buffer solution at a final concentration of 1 mM. In both cases, the protein adsorbed on the SSM was incubated in the inhibitor-containing solution for approximately 20 min.

### 2.2. The solid supported membrane (SSM)

The SSM consisted of an alkanethiol monolayer covalently bound to a gold surface via the sulfur atom, with a phospholipid monolayer on top of it [17,18]. To prepare the SSM the procedure described by Pintschovius and Fendler [8] was followed. Briefly, the mixed bilayer was formed in two sequential self-assembly steps. A self-assembled octadecanethiol monolayer was first formed on a gold electrode by incubating a freshly deposited gold film in an ethanolic solution of 1 mM octadecyl-mercaptan for 6 h at room temperature. The bilayer was then formed by spreading a drop of lipid solution (usually 5  $\mu\text{l}$ ) on the surface of the thiol-coated gold electrode. Typically, the effective membrane area ranged from 2 to 3  $\text{mm}^2$ .

### 2.3. Setup

For the rapid concentration jumps, a plexiglass cuvette with an inner volume of 20  $\mu\text{l}$  was used. The SSM and an O-ring, which contained the actual reaction volume, were sandwiched between the upper and the lower part of the cuvette. The SSM acted as the working electrode, while a Ag/AgCl(0.1 M KCl) electrode was employed as a counter-electrode. The counter-electrode was separated from the streaming solution by an agar/agar gel bridge. For details see Pintschovius and Fendler [8].

Two different 100-ml glass containers were used for the nonactivating and the activating solution. When performing a concentration jump experiment, the solution flow was kept constant at approximately 60 ml/min by applying a pressure of 0.4 bar to the system and by controlling the pressure with a precision digital manometer. The cuvette was connected to the outlet of a Teflon block on which two solenoid valves were mounted (Model 225T052, NResearch Inc., West Caldwell NJ, USA). The two valves, which were computer-controlled through a digital-to-analog converter (IOtech Inc. DAC 488/2), allowed a fast switching between the activating and the nonactivating solution. All parts of the setup conducting the electrolyte solutions were enclosed in a Faraday cage. The current generated by the ion pumps upon keeping the applied potential between the SSM and the counter-electrode equal to zero was amplified by a current amplifier (Keithley 428, gain:  $10^9$  V/A), filtered (low-pass, 3 ms), recorded (16-bit analog-to-digital converter, IOtech Inc. ADC 488/8SA), visualized (Oscilloscope, Tektronix TDS 340A) and stored (Power PC G3, Macintosh). Operation of the experimental setup and data acquisition were carried out under computer control (GPIB interface, National Instruments board) using a homemade acquisition program written in LabView environment.

### 2.4. Solution exchange technique

Two hours after forming the SSM and filling the cuvette, the capacitance and conductance of the SSM remained constant at  $C_m = 0.3\text{--}0.4$   $\mu\text{F}/\text{cm}^2$  and  $G_m = 100\text{--}300$  nS/ $\text{cm}^2$ . At this stage of the procedure, control experiments were usually performed with the protein-free SSM in order to exclude any artifacts generated by the solution exchange [8]. The membrane fragments containing  $\text{Na}^+, \text{K}^+$ -ATPase were then added by injecting 20  $\mu\text{l}$  of their suspension into the cuvette through the outlet opening. The suspension was vigorously mixed using a pipette. The membrane fragments were adsorbed on the SSM for 15 min. The usual procedure for a concentration-jump experiment consisted of three steps: (i) washing the cuvette with the nonactivating solution for 1 s; (ii) injecting the activating solution into the cuvette for 1 s; (iii) removing the activating substrate from the cuvette with the nonactivating solution for 1 s.

In order to verify the reproducibility of the current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase within the same

set of measurements, each single measurement of the set was repeated five to six times and then averaged to improve the signal-to-noise ratio. Average standard deviations were always found to be no greater than  $\pm 5\%$ . At the beginning of each set of measurements, 100  $\mu\text{M}$  ATP jumps were carried out to test the activity of the ion pumps previously adsorbed on the gold-supported alkanethiol/phospholipid mixed bilayer. The same ATP jump was performed at the end of the experiment, and the initial and final ATP-induced current transients were then compared to rule out any loss of activity during the time of the experiment. If differences between the two transients were greater than  $\pm 5\%$ , the set was discarded.

## 3. Results

### 3.1. ATP concentration jumps

Electrical currents generated by  $\text{Na}^+, \text{K}^+$ -ATPase were measured by adsorbing membrane fragments containing  $\text{Na}^+, \text{K}^+$ -ATPase from pig kidney on the SSM. The ion pumps were then activated by ATP concentration jumps. In the presence of  $\text{Na}^+$  and ATP but in the absence of  $\text{K}^+$ , only the Na-transport limb of the cycle is operative. In this case the phosphoenzyme  $\text{E}_2\text{P}$  undergoes a very slow dephosphorylation [19] and is reconverted to the  $\text{E}_1$  conformation. Therefore, under these experimental conditions, only the  $\text{Na}^+$ -dependent steps of the  $\text{Na}^+, \text{K}^+$ -ATPase cycle can be investigated.

A typical capacitive current transient following an ATP concentration jump at a constant  $\text{Na}^+$  concentration is shown in the inset of Fig. 1. Integration of the current peak gives the value of the translocated charge, which usually ranges from 40 to 50 pC. The electrical signal, whose amplitude is equal to 1.5 nA, rises with a time constant of 8 ms and decays with a time constant of 12 ms. A positive component with a time constant of approximately 115 ms is also observed. As expected, the time dependence of the current is similar to that obtained in analogous experiments [8]. The sign of the current peak is negative and corresponds to the transport of positive charge from the aqueous solution toward the SSM [20]. The direction of the current indicates that the  $\text{Na}^+, \text{K}^+$ -ATPase membrane fragments contributing to the electrical signal are adsorbed with the cytoplasmic side facing the aqueous solution. The same conclusion was drawn from inhibition experiments with orthovanadate and ouabain. In fact, the ATP signal was inhibited by 1 mM orthovanadate. For this purpose, the SSM with the adsorbed membrane fragments was incubated with a vanadate-containing solution for approximately 20 min. Following this treatment, ATP concentration jumps in the presence of vanadate did not yield an electrical signal. On the other hand, if the SSM with the adsorbed membrane fragments was incubated with a 1 mM ouabain solution for approximately 20 min, no inhibition occurred, and a current

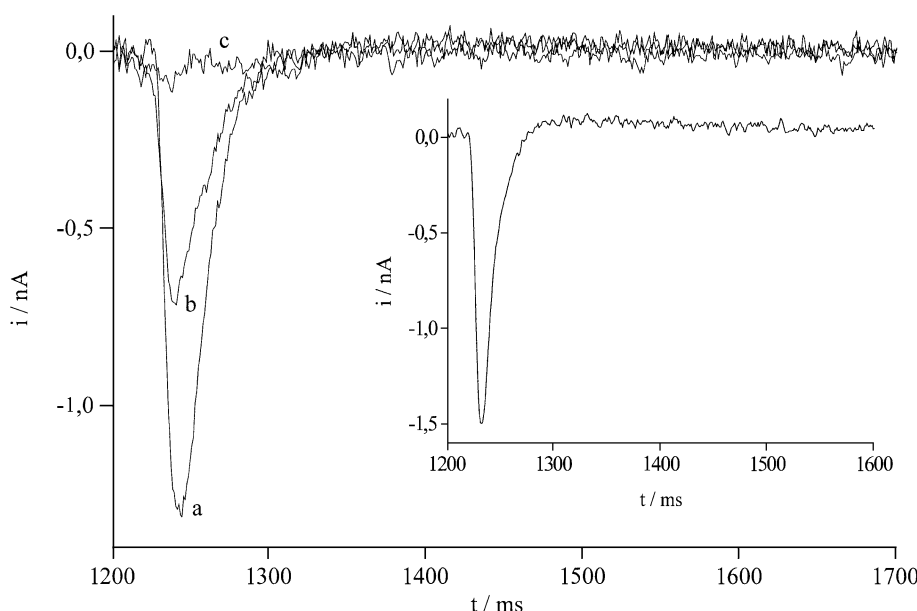


Fig. 1. Current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase following ATP concentration jumps in the presence of 5 mg/ml  $\alpha$ -chymotrypsin at different time intervals: (a)  $t=0$ , (b)  $t=1$  min and (c)  $t=4$  min. The nonactivating solution contained 10 mM NaCl, 50 mM TrisCl, 3 mM  $\text{MgCl}_2$ , 0.2 mM DTT at pH 7.0 (HCl). The activating solution had the same composition as the nonactivating one plus 100  $\mu\text{M}$  ATP. The inset shows a capacitive current transient following an ATP concentration jump obtained with a nonactivating solution containing 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$  and 0.2 mM DTT at pH 7.0 (HCl); the activating solution had the same composition as the nonactivating one plus ATP at a saturating concentration equal to 100  $\mu\text{M}$ .

transient following an ATP jump in the presence of ouabain could still be measured. Because vanadate inhibits the pump from the cytoplasmic side, whereas ouabain binds to the extracellular side of the protein, the inhibition experiments demonstrate that the enzyme population with the cytoplasmic side facing the aqueous solution is responsible for the ATP-induced current transient.

In a second series of experiments  $\alpha$ -chymotrypsin was used to block a specific transition in the Na-transport limb of the  $\text{Na}^+, \text{K}^+$ -ATPase enzymatic cycle. In fact, it is known that the treatment of  $\text{Na}^+, \text{K}^+$ -ATPase with  $\alpha$ -chymotrypsin in the presence of  $\text{Na}^+$  ions at low ionic strength leads to cleavage of a single peptide bond in the  $\alpha$ -subunit of the ion pump; the split is located in the cytoplasmic portion of the protein between Leu-266 and Ala-267 [21]. Under appropriate experimental conditions secondary cleavage is negligible [22]. In the chymotrypsin-treated enzyme, phosphorylation by ATP, occlusion of  $\text{Na}^+$  ions and ATP/ADP exchange are maintained, while  $\text{Na}^+, \text{K}^+$  pumping and  $\text{Na}^+, \text{Na}^+$  exchange are abolished [22,23]. According to these findings, modification of  $\text{Na}^+, \text{K}^+$ -ATPase by  $\alpha$ -chymotrypsin blocks the  $(\text{Na}_3)\text{E}_1\text{-P} \rightarrow \text{E}_2\cdot\text{Na}_3$  transition and leaves phosphorylation and occlusion of  $\text{Na}^+$  intact [24]. Fig. 1 shows the current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase following ATP concentration jumps in the presence of  $\alpha$ -chymotrypsin and of 10 mM  $\text{Na}^+$  and in the absence of  $\text{K}^+$ . Under the chosen experimental conditions the proteolytic reaction is expected to be selective, consisting in the cleavage of a single peptide bond, as discussed above. After checking the current transient generated by  $\text{Na}^+, \text{K}^+$ -ATPase in the absence of  $\alpha$ -chymotrypsin, a high concentration of  $\alpha$ -chymotrypsin (5

mg/ml) was added both to the nonactivating and the activating solution, and the electrical signal was recorded at different time intervals. As appears from Fig. 1, the amplitude of the current transient progressively decreases in a few minutes. It is important to point out that, before adding  $\alpha$ -chymotrypsin, the amplitude of the current transient was found to remain constant in a time period of 30 min. The observation that  $\alpha$ -chymotrypsin abolishes the current transient that is normally detected following the ATP concentration jump indicates that phosphorylation by ATP and occlusion of  $\text{Na}^+$  ions are electrically silent steps, and that charge translocation must occur in the deocclusion step and/or during the subsequent release of  $\text{Na}^+$  ions to the extracellular side. This conclusion agrees with findings from BLMs experiments with open membrane fragments [24].

If ATP concentration jumps are carried out on membrane fragments containing  $\text{Na}^+, \text{K}^+$ -ATPase in the presence not only of  $\text{Na}^+$  but also of  $\text{K}^+$  ions,  $\text{K}^+$  ions have a minor effect on the current transient, due to their difficulty in moving within the cleft between the membrane fragments and the supporting bilayer. In fact, the cleft is hardly accessible to ions, with the remarkable exception of protons, which are known to move rapidly along the polar heads of phospholipid monolayers. However, if we add to the solution monensin, an ionophore that exchanges  $\text{Na}^+$  and  $\text{K}^+$  ions against protons in an electroneutral way, the current decays slowly in time, denoting the occurrence of a number of pump turnovers. Fig. 2 shows the current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase following ATP concentration jumps in the presence of  $\text{Na}^+$ , monensin and increasing concentrations of  $\text{K}^+$ . As discussed above, only the  $\text{Na}^+, \text{K}^+$ -



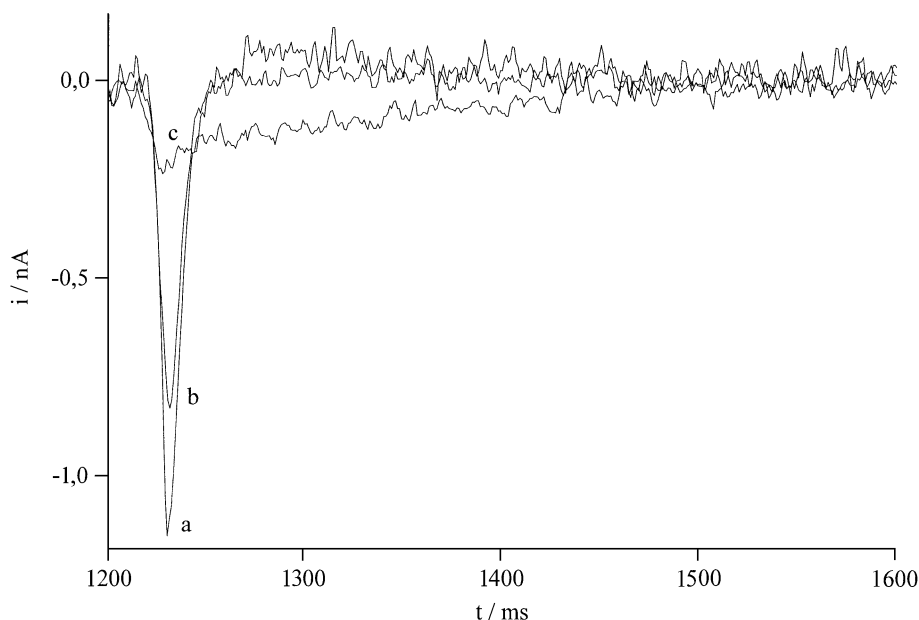


Fig. 2. Current transients induced by ATP concentration jumps in the presence of  $\text{Na}^+$ , monensin and increasing concentrations of  $\text{K}^+$ . The nonactivating solution contained 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$ , 0.2 mM DTT, 25  $\mu\text{M}$  monensin and 0 mM (a), 2 mM (b), 15 mM (c) KCl at pH 7.0 (HCl). The activating solution had the same composition as the nonactivating one plus 100  $\mu\text{M}$  ATP.

pumps with their cytoplasmic side turned toward the aqueous solution are affected by an ATP concentration jump. In the presence of  $\text{K}^+$  ions within the cleft, these ATP-activated pumps can pump  $\text{Na}^+$  ions into and  $\text{K}^+$  ions out of the cleft. Therefore, the resulting current transient is due to a  $\text{Na}^+/\text{K}^+$  exchange, which represents the physiological transport mode of  $\text{Na}^+/\text{K}^+$ -ATPase. Naturally, under the present conditions the capacitive current ultimately vanishes, when a steady state is attained in which the number of ions pumped into the cleft in a given time becomes equal to those leaving it in the same time either by leakage through the membrane fragment or after reaching the rim of the fragment. In particular, a small addition of  $\text{K}^+$  ions has the only effect of eliminating the typical overshoot of the current transient, which denotes the complete absence of a pump stationary current [20], and therefore no turnover (see curve b in Fig. 2). It should be noted that  $\text{K}^+$  ions can be translocated to the cleft by monensin only thanks to the high mobility of protons within the cleft.

The results discussed above confirm the electrogenicity of  $\text{Na}^+$  translocation during the conformational transition  $\text{E}_1\text{P} \rightarrow \text{E}_2\text{P}$ , in agreement with results obtained by different techniques on various enzyme preparations [25,26]. In a previous work, the SSM technique was also used to investigate  $\text{K}^+$  transport by  $\text{Na}^+/\text{K}^+$ -ATPase [12]. In this study, proteoliposomes incorporating  $\text{Na}^+/\text{K}^+$ -ATPase were adsorbed on the SSM and activated by  $\text{K}^+$  concentration jumps both in the presence and absence of inorganic phosphate ( $\text{P}_i$ ). In the presence of  $\text{P}_i$ , a  $\text{K}^+$  concentration jump causes an on-current peak ascribable to a translocation of positive charge by the inside-out oriented ion pumps and an off-current peak whose associated charge is practically equal

and opposite to that of the on-current [12]. This confirms an electrogenic release of  $\text{K}^+$  to the extracellular side, in agreement with results obtained by other authors [27–29].

However, some caution must be used as to the estimate of the amount of  $\text{K}^+$  ions actually pumped by  $\text{Na}^+/\text{K}^+$ -ATPase. Fig. 3 shows the current transients generated by

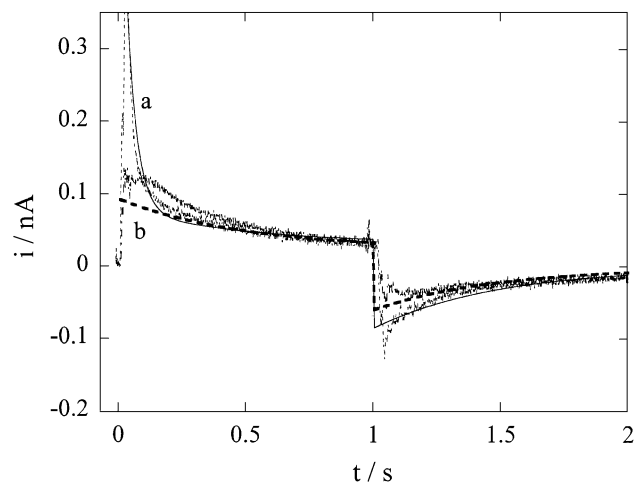


Fig. 3. Current transients following a 100 mM  $\text{K}^+$ -jump in the presence of inorganic phosphate both before (gray fuzzy curve) and after (black fuzzy curve) the addition of 0.1 mM digitoxigenin. The nonactivating solution contained 300 mM choline chloride, 25 mM imidazole, 3 mM  $\text{MgCl}_2$ , 2 mM  $\text{H}_3\text{PO}_4$  and 0.2 mM DTT at pH 7.0 (HCl). The activating solution contained 100 mM KCl, 200 mM choline chloride, 25 mM imidazole, 3 mM  $\text{MgCl}_2$ , 2 mM  $\text{H}_3\text{PO}_4$  and 0.2 mM DTT at pH 7.0 (HCl). The solid and dashed curves are the best fits of the experimental currents to the equivalent circuit described in the text (see the appendix of Ref. [20] for the expressions of the on- and off-currents).

proteoliposomes incorporating  $\text{Na}^+, \text{K}^+$ -ATPase following a 100 mM  $\text{K}^+$ -jump in the presence of 2 mM  $\text{P}_i$  both before and after the addition of the inhibitor digitoxigenin, a membrane permeable ouabain analogue. In this experiment, the proteoliposomes adsorbed on the SSM were incubated in a 0.1 mM digitoxigenin solution for approximately 30 min in order to allow digitoxigenin to diffuse into the proteoliposomes and to bind to the ouabain site, which is accessible from the extracellular side, i.e. from the inside of the proteoliposomes. The inhibition of the pump activity by digitoxigenin suppresses only a part of the on- and off-currents and does not affect the quasi-stationary current component, as appears from Fig. 3. A similar result was obtained by performing a 100 mM  $\text{K}^+$ -jump on an orthovanadate-inhibited sodium pump (data not shown). Moreover, a 100 mM  $\text{K}^+$ -jump in the presence of  $\text{P}_i$  was also carried out with liposomes of the same lipid composition but containing no protein. In this case neither a current peak nor a quasi-stationary current was observed. These results indicate that the inhibitor-insensitive current, which increases with an increase in  $\text{K}^+$  concentration, requires the presence of  $\text{Na}^+, \text{K}^+$ -ATPase but is not related to its specific activity. This current can be ascribed to a leakage pathway promoting downhill movement of  $\text{K}^+$  ions, possibly along the edge of sodium pumps not perfectly sealed into the lipid bilayer. The solid and dashed curves in Fig. 3 were calculated from an equivalent circuit in which  $\text{Na}^+, \text{K}^+$ -ATPase is represented as a current source, in parallel with the capacity  $C_p$  and resistance  $R_p$  of the pump, while the supporting mixed thiol/diphytanoylPC bilayer is represented

as a  $R_m C_m$  mesh in series with the circuit elements representing the pump [20].  $R_m$  and  $C_m$  were ascribed the experimental values,  $3.9 \times 10^6 \Omega \text{ cm}^2$  and  $0.5 \mu\text{F cm}^{-2}$ , of the mixed bilayer obtained from independent impedance spectroscopy measurements, while  $R_p$  and  $C_p$  were given the reasonable values of  $3.6 \times 10^5 \Omega \text{ cm}^2$  and  $1 \mu\text{F cm}^{-2}$ . The pump current was set equal to the sum of an exponentially decaying contribution,  $a \times \exp(-t/\tau)$ , and of a stationary contribution  $b$ . In the presence of digitoxigenin the exponential contribution was omitted, and the leakage current was expressed by a stationary contribution  $b'$ . The best fit was obtained for  $\tau = 0.04 \text{ s}$ ,  $a = 2 \text{ nA}$ ,  $b = 0.37 \text{ nA}$  and  $b' = 0.20 \text{ nA}$ . Therefore, the actual pump current is equal to  $b - b' = 0.17 \text{ nA}$ . It is evident that the current due to leakage is far from negligible at this relatively high  $\text{K}^+$  concentration. However, the off-current in the absence of digitoxigenin being appreciably higher than in its presence must necessarily be related to a continuous release of  $\text{K}^+$  ions to the extracellular side due to the activity of  $\text{Na}^+, \text{K}^+$ -ATPase.

### 3.2. ATP concentration jumps in the presence of acylphosphatase

To investigate whether AcP can affect the electrical currents that are directly related to the transport of  $\text{Na}^+$  ions by  $\text{Na}^+, \text{K}^+$ -ATPase, ATP concentration jumps were carried out both in the absence and presence of this enzyme.

Fig. 4 shows the capacitive current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase following a 100  $\mu\text{M}$  ATP concentration

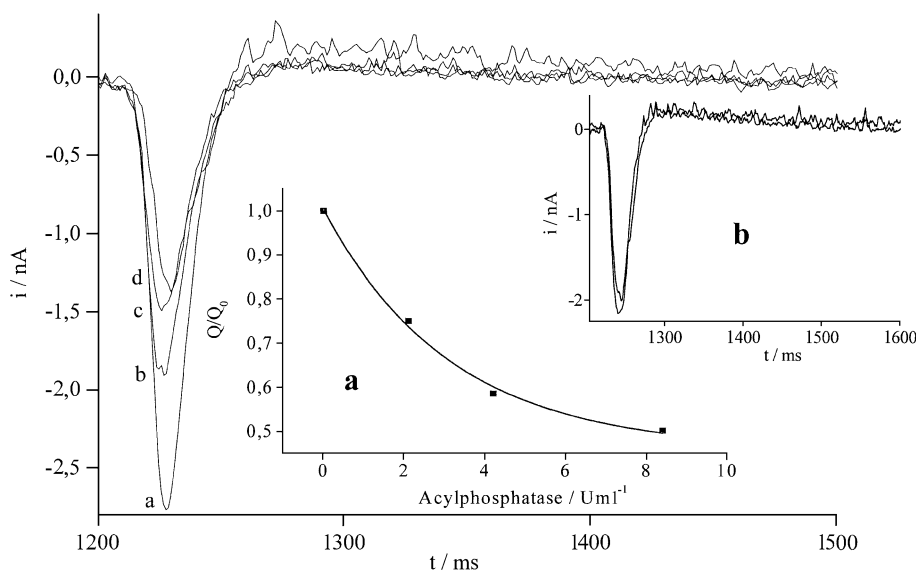


Fig. 4. Current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase following 100  $\mu\text{M}$  ATP concentration jumps in the presence of varying amounts of AcP: 0 (a), 2.1 (b), 4.2 (c) and 8.4 U/ml (d), corresponding to 0, 2.3, 4.6 and 9.2  $\mu\text{g/ml}$  of AcP. The AcP concentration was the same in both the nonactivating and the activating solution. Both solutions also contained 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$  and 0.2 mM DTT at pH 7.0 (HCl). Inset (a) shows the ratio of the charge  $Q$  translocated in the presence of AcP to that,  $Q_0$ , translocated in its absence against the AcP concentration. Inset (b) shows the current transients following 100  $\mu\text{M}$  ATP concentration jumps in the presence and absence of the AcP mutant N41S. The concentration of the mutant in both the activating and nonactivating solutions was equal to 9.2  $\mu\text{g/ml}$  and equivalent to 8.4 U/ml of active AcP. Both solutions also contained 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$  and 0.2 mM DTT at pH 7.0 (HCl).

jump at pH 7 in the presence of  $\text{Na}^+$  and of varying amounts of AcP. The AcP concentration was the same both in the nonactivating and activating solutions. It is apparent that the current amplitude decreases when increasing the concentration of AcP in the buffer solution, and becomes approximately one half of the initial value in the presence of 8.4 U/ml AcP. The ratio of the charge  $Q$  translocated by the sodium pump in the presence of a concentration,  $[\text{AcP}]$ , of AcP to the charge,  $Q_0$ , translocated in its absence shows an analogous trend when plotted against  $[\text{AcP}]$  (inset (a) of Fig. 4). By carrying out the above ATP concentration jumps with AcP in the activating solution but not in the nonactivating one, under otherwise identical conditions, no decrease in the current transient was observed with respect to that obtained in the absence of AcP in both the activating and the nonactivating solution.

To verify that the AcP-induced modification of the ATP signal is actually related to the catalytic activity of this enzyme, a 100  $\mu\text{M}$  ATP concentration jump was performed in the presence of the AcP mutant N41S, a catalytically inactive form of the enzyme (inset (b) of Fig. 4). The N41 residue is in close spatial proximity to R23, which was indicated as the main phosphate binding site in the muscular AcP [30]. Several pieces of experimental evidence strongly support the existence, in the N41 mutants, of a native-like structure even at the active site level [31]. The dramatic loss of activity of N41 mutants, whose ability to bind the substrate appears unchanged, was therefore specifically ascribed to the absence of the N41 side chain. Inset (b) of Fig. 4 shows that the presence of the inactive mutant N41S

causes the current amplitude to decrease only slightly (7%) with respect to the value measured in the absence of the mutant. In these experiments, the concentration of the mutant in both the activating and nonactivating solutions was equal to 9.2  $\mu\text{g/ml}$  and equivalent to 8.4 U/ml of active AcP.

To verify whether an AcP concentration jump may cause a rapid hydrolysis of the preformed phosphoenzyme  $\text{E}_2\text{P}$  of  $\text{Na}^+, \text{K}^+$ -ATPase, AcP concentration jumps were carried out in the presence of both  $\text{Na}^+$  ions and ATP, which induce the  $\text{E}_2\text{P}$  conformational state. In these experiments the non-activating solution contained 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$ , 0.2 mM DTT and ATP ranging from 0.2 to 1 mM at pH=7.0 (HCl). The activating solution had the same composition as the nonactivating one plus AcP of concentration ranging from 50 to 125 U/ml. These AcP concentration jumps did not yield detectable current transients.

Further experiments were carried out to investigate the pH dependence of the effect exerted by AcP on the current transient induced by an ATP concentration jump. First of all, 100  $\mu\text{M}$  ATP jumps were performed in the absence of AcP at three different pH values ranging from 6 to 8. The resulting current transients are shown in the inset of Fig. 5. The charge under the current peak is almost identical at all pH values, but the peak current is higher at pH 7; this denotes a maximum phosphorylation rate at the physiological pH. In a further experiment, 100  $\mu\text{M}$  ATP jumps were carried out at pH 6.05 first in the absence, and then in the presence of increasing amounts of AcP. The AcP concen-

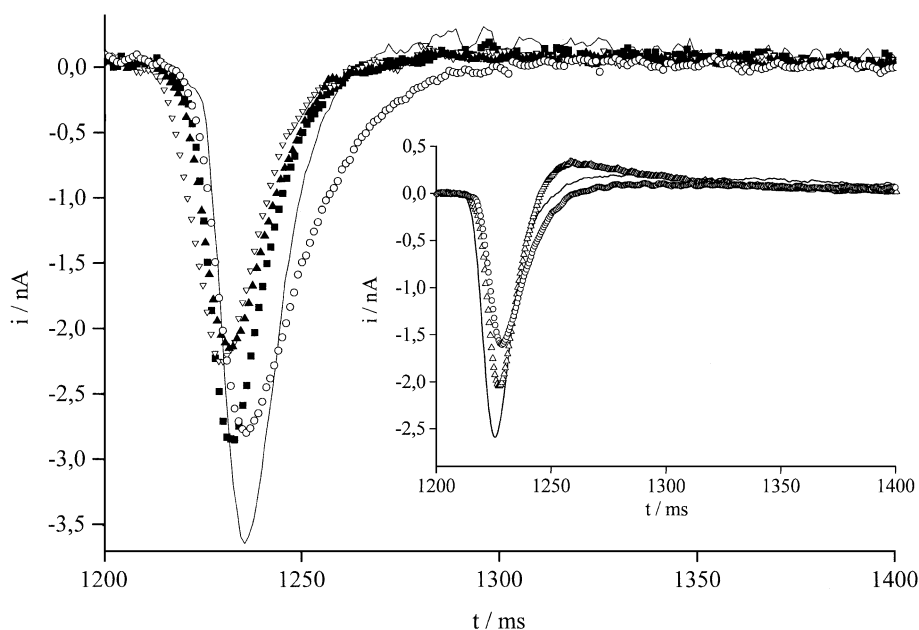


Fig. 5. Current transients following 100  $\mu\text{M}$  ATP jumps performed at pH 6.05 first in the absence (solid line), and then in the presence of 1 (solid squares), 2 (open down-triangles) and 5 U/ml (solid up-triangles) AcP. The solution pH was then increased to 8.08, and a further 100  $\mu\text{M}$  ATP jump was carried out in the presence of 5 U/ml AcP (open circles). Both the nonactivating and the activating solution also contained 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$  and 0.2 mM DTT. The inset shows the current transients following 100  $\mu\text{M}$  ATP jumps in the absence of AcP at different pH values: 6 (up-triangles), 7 (solid line) and 8 (circles). Both the nonactivating and the activating solution contained 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$  and 0.2 mM DTT.



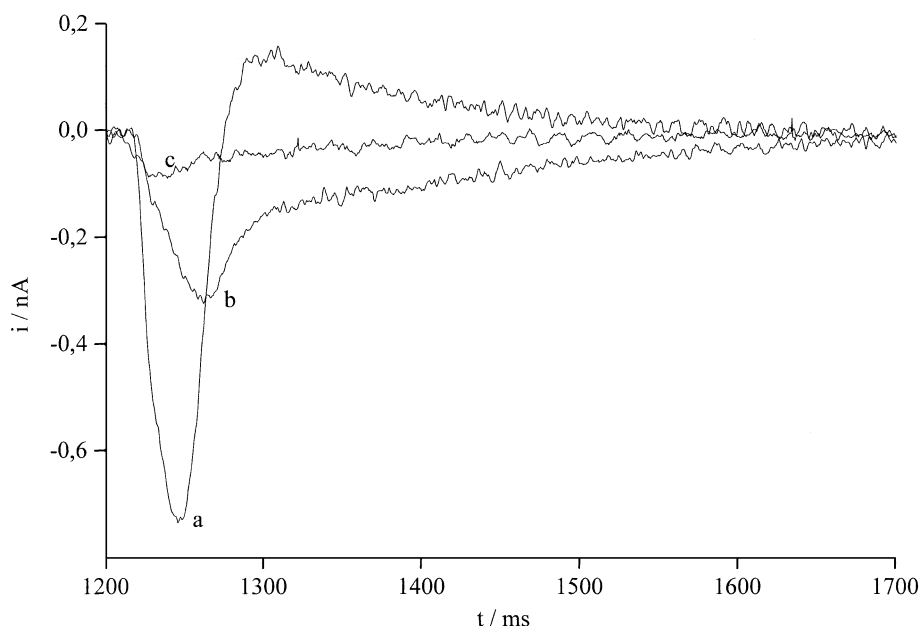


Fig. 6. Current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase following 100  $\mu\text{M}$  ATP jumps in the absence of KCl (a), in the presence of 10 mM KCl and 25  $\mu\text{M}$  monensin (b), and in the presence of 10 mM KCl, 25  $\mu\text{M}$  monensin and 20 U/ml AcP (c). The activating and nonactivating solutions also contained 130 mM NaCl, 25 mM imidazole, 3 mM  $\text{MgCl}_2$  and 0.2 mM DTT at pH 6.9 (HCl).

tration was the same in both the nonactivating and the activating solution. As shown in Fig. 5, progressive additions of AcP cause a decrease in the charge under the current peak down to a minimum value of about 50%, in analogy with what observed at pH 7. The solution pH was then increased to 8.08, and a further 100  $\mu\text{M}$  ATP jump was carried out under otherwise identical conditions. The resulting peak current is higher than that at pH 6.05 in the presence of the same amount of AcP, albeit lower than that in its absence. However, the charge under the current peak is about 20% greater than that measured in the absence of AcP over the pH range from 6 to 8, where this charge is practically constant (see inset of Fig. 5).

Fig. 6 shows the capacitive current transients generated by  $\text{Na}^+, \text{K}^+$ -ATPase following 100  $\mu\text{M}$  ATP jumps in the presence of  $\text{Na}^+$  (a), in the presence of  $\text{Na}^+$ ,  $\text{K}^+$  and the ionophore monensin (b), and in the presence of  $\text{Na}^+$ ,  $\text{K}^+$ , monensin and AcP (c). It is apparent that the presence of AcP suppresses almost completely the current generated by an ATP concentration jump when in the presence of  $\text{Na}^+$ ,  $\text{K}^+$  and monensin. This behavior is, therefore, different from that observed in the absence of  $\text{K}^+$  ions under otherwise identical conditions, where the presence of AcP decreases the current transient induced by the ATP concentration jump down to a minimum limiting value, but does not suppress it.

#### 4. Discussion

The above results indicate that AcP affects considerably the capacitive current transients directly related to the transport of  $\text{Na}^+$  and  $\text{K}^+$  ions by  $\text{Na}^+, \text{K}^+$ -ATPase. The effect of

AcP on the current transients induced by an ATP concentration jump in the presence of  $\text{Na}^+$  ions will first be discussed. As shown in Figs. 4 and 5, over the pH range from 6 to 7 the presence of increasing concentrations of AcP causes an asymptotic decrease in the peak current, until ultimately a limiting value about one half of the initial value is attained. On the other hand, at pH 8 the presence of AcP causes an increase in the charge translocated by  $\text{Na}^+, \text{K}^+$ -ATPase by about 20%. These results should be compared with those of steady-state measurements of ATP hydrolysis,  $\text{Na}^+$  influx and  $\text{K}^+$  efflux in  $\text{K}^+$ -preloaded vesicles immersed in a solution containing  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and ATP [7]. According to the latter results, the presence of increasing amounts of AcP increases the strophantidine-sensitive  $\text{Na}^+, \text{K}^+$ -ATPase activity by enhancing the rates of ATP hydrolysis and of the ATP-dependent  $\text{Na}^+$  influx, while strongly decreasing the rate of  $\text{K}^+$  efflux.

Taken together, these results suggest an association of the cytosolic enzyme AcP to the cytoplasmic side of the sodium pump, which does not prevent the access of ATP to its binding site but allows AcP to rapidly hydrolyze the acylphosphate bond after its formation. This association is a relatively slow process, since it does not occur to a detectable extent during AcP concentration jumps. The nature of this association is unknown and hardly predictable, also in view of the narrow and highly preserved NPA domain in the P-E<sub>2</sub> state of  $\text{Na}^+, \text{K}^+$ -ATPase on the basis of the meanwhile known 3D structure of the closely related SR  $\text{Ca}^{2+}$ -ATPase [32]. A mere steric block of ATP binding to the Na pump by AcP, as might be suggested by the decrease in the translocated charge at pH 7, is to be excluded for the following reasons: (1) such a decrease tends asymptotically

to a well-defined and appreciable limiting value with an increase in AcP concentration; (2) an increase in the translocated charge in the presence of AcP, rather than a decrease, is observed at pH 8 under otherwise identical condition; (3) the effect of the AcP mutant N41S is almost negligible at pH 7; at most, only the 7% decrease in the translocated charge induced by this mutant might be ascribed to some steric hindrance; (4) steady-state measurements at the physiological pH reveal that the presence of AcP increases the  $\text{Na}^+, \text{K}^+$ -ATPase activity and the rate of ATP hydrolysis; (5) under the latter steady-state conditions, the effect of heat-inactivated AcP was found to be practically negligible, as opposed to that of the wild type [6].

We will postulate that the association of AcP with  $\text{Na}^+, \text{K}^+$ -ATPase leads to a complex  $\text{P}^*\text{AcP}$  characterized by a faster hydrolysis of the phosphoenzyme than the free sodium pump P. Denoting the equilibrium constant for the formation of  $\text{P}^*\text{AcP}$  from P and AcP by  $K_a$ , in view of the slow attainment of such an equilibrium, it is reasonable to assume that it remains practically frozen during the time of the capacitive current transient. In this case the free pump P and the  $\text{P}^*\text{AcP}$  complex will pump  $\text{Na}^+$  ions independently. Upon denoting by  $q_1$  and  $q_2$  the charges translocated by the free sodium pump and by the  $\text{P}^*\text{AcP}$  complex, the  $Q/Q_0$  ratio can be obtained from the self-explanatory relationships:

$$Q = q_1[P] + q_2[\text{P}^*\text{AcP}]; \quad Q_0 = q_1[P_t];$$

$$[P_t] = [P] + [\text{P}^*\text{AcP}], \quad K_a = \frac{[\text{P}^*\text{AcP}]}{[P][\text{AcP}]}$$

whose combination yields:

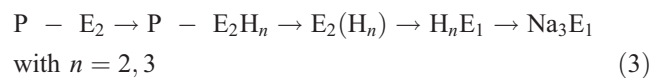
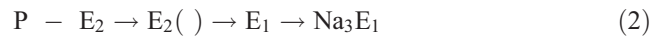
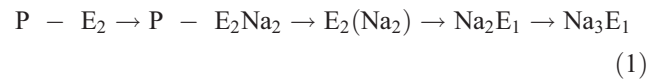
$$\frac{Q}{Q_0} = \frac{q_1 + q_2 K_a [\text{AcP}]}{q_1 (1 + K_a [\text{AcP}])}$$

Here  $[P]$ ,  $[P_t]$  and  $[\text{P}^*\text{AcP}]$  are the concentration of the free pump, its value in the absence of AcP, and the concentration of the  $\text{P}^*\text{AcP}$  complex. The best fit of the  $Q/Q_0$  versus  $[\text{AcP}]$  plot is obtained by setting  $K_a = 0.55 \text{ ml U}^{-1}$  and  $q_2/q_1 = 0.4$ , and is shown by the solid curve in inset (a) of Fig. 4.

This behavior can be reasonably explained by considering the dielectric coefficients relative to the different steps of the pump cycle, as reported by Apell and Karlish [29, and references therein]. In the absence of AcP, the ATP concentration jump in the presence of  $\text{Na}^+$  causes the pump to pass from the  $\text{Na}_3\text{E}_1$  state to the  $\text{P}-\text{E}_2$  state. In the  $\text{Na}_3\text{E}_1$  state two  $\text{Na}^+$  ions are considered to be bound to two negatively charged cytoplasmic sites, while a third  $\text{Na}^+$  ion is bound to an exclusive neutral cytoplasmic site, with a high affinity for  $\text{Na}^+$ . Phosphorylation by ATP causes the three  $\text{Na}^+$  ions to be occluded in an electroneutral step. Following an almost electroneutral conformational transition from the  $\text{E}_1$  to the  $\text{E}_2$  state, one  $\text{Na}^+$  ion is first released to the extracellular side of the pump through a narrow access channel [33]; this release is highly electrogenic, with a dielectric coefficient of

0.65–0.70 [29,34]. This is followed by a further conformational relaxation that brings the remaining two occluded  $\text{Na}^+$  ions closer to the extracellular side of the pump, such that the release of each of them is characterized by a low dielectric coefficient of about 0.1–0.2 [29,34]. The latter conformational transition can be regarded as a widening of the access channel, which is then filled by water or else, more simply, as a penetration of water molecules into the access channel concomitant to the release of the last two  $\text{Na}^+$  ions. In both cases the “physical distance” between the two deoccluding ions and the aqueous phase is characterized by a higher dielectric constant and, hence, the corresponding “dielectric distance” decreases. The sum of the dielectric coefficients of the steps interposed between the  $\text{Na}_3\text{E}_1$  and  $\text{P}-\text{E}_2$  states is, therefore, close to unity.

In the presence of AcP, this is expected to catalyze a rapid dephosphorylation of the phosphoenzyme  $\text{P}-\text{E}_2$ , and the resulting rapid conformational transition to the  $\text{E}_1$  state. In principle, three dephosphorylation pathways are conceivable, which are related to the noncanonical ATP-driven  $\text{Na}^+, \text{Na}^+$ -exchange and to the ATP-driven  $\text{Na}^+$ -efflux:



Of these three pathways, the third one, involving a  $3\text{Na}_{\text{cyt}}/n\text{H}_{\text{exc}}$  exchange, is by far the most probable, also in view of the appreciable pH dependence of the charge translocated by  $\text{Na}^+, \text{K}^+$ -ATPase in the presence of AcP. In fact, the first pathway is unlikely to make an appreciable contribution, in view of the low mobility of  $\text{Na}^+$  ions in the cleft and of their low concentration, which according to Wuddel and Apell [34] should be only about 55–60% of that in the bulk phase. Moreover, the  $\text{Na}^+$  affinity for the  $\text{P}-\text{E}_2$  is very low. The second pathway is also to be discarded on the basis of the experimental behavior over the pH range from 6 to 7. According to this pathway, the conformational transition from  $\text{E}_2$  to  $\text{E}_1$  would be accompanied by a movement of the two negatively charged ion-binding sites from the extracellular to the cytoplasmic side, which is electrostatically equivalent to a movement of positive charges in the opposite direction. If this were the case, then the presence of AcP would cause an increase in the charge under the current peak induced by the ATP concentration jump, contrary to the experimental behavior at pH values from 6 to 7. Incidentally, pathway (2) was claimed by Cornelius [35] at physiological pH on the basis of transmembrane potential measurements in liposomes with recon-

stituted shark  $\text{Na}^+, \text{K}^+$ -ATPase. Different conclusions were drawn by Polvani and Blostein [36] using membrane vesicles derived from human red cells and by Goldshleger et al. [37] from transmembrane potential measurements in liposomes with reconstituted renal  $\text{Na}^+, \text{K}^+$ -ATPase. In particular, Goldshleger found that the ATP-driven  $\text{Na}^+$  efflux is electroneutral at pH 6.5–7 and becomes progressively electrogenic as the pH is raised to 8.5. This behavior was interpreted by a  $3\text{Na}_{\text{cyt}}/3\text{H}_{\text{exc}}$  exchange at pH 6.5–7.0 and by a progressive decrease in proton involvement with an increase in pH, until the ATP-driven  $\text{Na}^+$  efflux becomes completely uncoupled at pH 8.5. Subsequently, Apell et al. [38] observed a similar pH dependence of Na pump phosphorylation by inorganic phosphate (back-door phosphorylation) in the absence of  $\text{Na}^+$  and  $\text{K}^+$  ions. This behavior was explained by pathway (3) proceeding in the backward direction from  $\text{H}_n\text{E}_1$  to  $\text{P-E}_2$ , with  $n=2$ , at pH 7, with protons acting as congeners of  $\text{K}^+$ .

The pH dependence of the effect exerted by AcP upon the charge translocation induced by an ATP concentration jump (see Fig. 5) suggests that protons are involved in the AcP-catalyzed dephosphorylation pathway. The charge translocated by the sodium pump in the presence of AcP tending asymptotically to about 40% of that translocated in its absence over the pH range from 6 to 7 can be tentatively explained by a  $3\text{Na}^+/2\text{H}^+$  exchange. If we ascribe a maximum dielectric coefficient of 0.2 to the binding of each proton to the extracellular sites of the  $\text{P-E}_2$  state and we consider that the subsequent steps leading to the  $\text{E}_1$  state are electroneutral, such a  $3\text{Na}^+/2\text{H}^+$  exchange induced by AcP would only involve a decrease in the translocated charge down to about 60% of its value in the absence of AcP. However, a modest steric hindrance of AcP similar to that exhibited by the N41S mutant might account for the further residual decrease in charge.

The 20% increase in the translocated charge at pH 8.08 following the addition of AcP corresponds to about a 70–80% increase in the translocated charge in the presence of AcP following an increase in pH from 6.05 to 8.08, as appears from Fig. 5. This increase in charge is relatively close to that expected for the lack of translocation of one proton from the extracellular to the cytoplasmic side of the sodium pump following the rapid dephosphorylation induced by AcP. We may, therefore, postulate that such an increase in pH tends to decrease the number of translocated protons from 2 to 1. However, we cannot exclude that some contribution to the translocated charge due to the increase in pH may stem from a modest alteration of the interaction between the sodium pump and AcP. What we can state with certainty is that such an increase in pH does not affect the amount of charge translocated by the sodium pump in the absence of AcP.

The almost complete suppression of the current transient following an ATP concentration jump by AcP on membrane fragments containing  $\text{Na}^+, \text{K}^+$ -ATPase in the presence of  $\text{Na}^+$ ,  $\text{K}^+$  and the ionophore monensin, as shown in Fig. 6,

is difficult to explain. The steady-state measurements of ATP hydrolysis,  $\text{Na}^+$  influx and  $\text{K}^+$  efflux in  $\text{K}^+$ -preloaded vesicles [7] exclude a steric block, even leaving apart the absence of such a block in the present measurements carried out in the absence of  $\text{K}^+$  ions. This behavior can be tentatively explained by assuming that, after ATP phosphorylates the pump and AcP hydrolyzes the acylphosphate bond along the sodium limb of the enzymatic cycle, AcP slows down the low-affinity binding of ATP along the subsequent potassium limb. This would delay the conformational change from the  $\text{E}_2(\text{K}_2)$  to the  $\text{E}_1$  state and the resulting turnover of the sodium pump.

The present concentration-jump experiments may account for the results of steady-state measurements of ATP hydrolysis,  $\text{Na}^+$  influx and  $\text{K}^+$  efflux in  $\text{K}^+$ -preloaded vesicles immersed in a solution containing  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and ATP [7]. According to the latter results, the presence of increasing amounts of AcP increases the strophanthidine-sensitive  $\text{Na}^+, \text{K}^+$ -ATPase activity as well as the  $\text{Na}^+/\text{K}^+$  ratio from the well documented 1.5 value up to 6.7, while the  $\text{Na}^+/\text{ATP}$  ratio remains constantly close to 3. The present experiments suggest that the notable increase in the  $\text{Na}^+/\text{K}^+$  ratio and the acceleration in the  $\text{Na}^+, \text{K}^+$ -ATPase turnover may be ascribed to the involvement in the  $\text{E}_2$  to  $\text{E}_1$  conformational transition of the only monovalent cation present in the solution other than  $\text{Na}^+$  and  $\text{K}^+$ , i.e. the proton; in particular, they support the active role of protons as congeners of potassium ions. They also confirm that AcP, owing to its catalytic activity on the acylphosphorylated intermediate, can alter the functioning of  $\text{Na}^+, \text{K}^+$ -ATPase in terms of sodium and potassium transport.

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