Charge Translocation by the Na^+/K^+-ATPase Investigated on Solid Supported Membranes: Cytoplasmic Cation Binding and Release

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ABSTRACT In the preceding publication (Pintschovius and Fendler, 1999. *Biophys. J.* 76:000–000) a new technique was described that was able to produce concentration jumps of arbitrary ion species at the surface of a solid supported membrane (SSM). This technique can be used to investigate the kinetics of ion translocating proteins adsorbed to the SSM. Charge translocation of the Na^+/K^+-ATPase in the presence of ATP was investigated. Here we describe experiments carried out with membrane fragments containing Na^+/K^+-ATPase from pig kidney and in the absence of ATP. Electrical currents are measured after rapid addition of Na^+. We demonstrate that these currents can be explained only by a cation binding process on the cytoplasmic side, most probably to the cytoplasmic cation binding site of the Na^+/K^+-ATPase. An electrogenic reaction of the protein was observed only with Na^+, but not with other monovalent cations (K^+, Li^+, Rb^+, Cs^+). Using Na^+ activation of the enzyme after preincubation with K^+ we also investigated the K^+-dependent half-cycle of the Na^+/K^+-ATPase. A rate constant for K^+ translocation in the absence of ATP of 0.2–0.3 s^{-1} was determined. In addition, these experiments show that K^+ deocclusion, and cytoplasmic K^+ release are electroneutral.

INTRODUCTION

Electrogenicity of partial reactions is an important topic in view of the mechanisms of active ion transport. The knowledge of certain reaction steps being electrogenic yields information about the dielectric barriers crossed by the moving ions. By measuring the current generated by the transport proteins during the pump process, ion movement across the membranes can be monitored. In a pre-steady-state experiment—e.g., after a concentration jump—time constants of the reaction steps involved in the pump cycle can also be determined.

The Na^+/K^+-ATPase has important physiological functions in the plasma membrane of almost all animal cells. In the physiological pump mode, hydrolysis of one molecule of ATP results in the transport of three Na^+ ions out of the cell, and of two K^+ ions into the cytoplasm. During this cycle, one positive elementary charge is translocated from the cytoplasmic to the extracellular side of the membrane. It has been postulated and experimentally verified that during its transport cycle the enzyme assumes two different conformational states, E_1 and E_2 (Fahn et al., 1966; Post et al., 1969; Jørgensen and Andersen, 1988). In the E_1 state the enzyme has a high affinity for Na^+ at the cytoplasmic surface so that Na^+ can be bound at the cytoplasmic cation binding site. After binding and hydrolysis of ATP, the Na^+/K^+-ATPase is converted into the phosphorylated E_2P state, where the cation binding region is accessible to the extracellular side. In the E_2P conformational state Na^+ is released, and because of the high affinity for K^+ ions on the extracellular surface, K^+ is bound. Extracellular K^+ uptake results in accelerated dephosphorylation and a subsequent conformational change to the E_1 state, thus translocating K^+ to the cytoplasmic side of the membrane.

Time-resolved electrical current measurements on the Na^+/K^+-ATPase have been performed using UV-cleavable caged ATP (Kaplan et al., 1978; Fendler et al., 1993; Friedrich et al., 1996) to trigger ion pumping. Alternatively, rapid ATP-concentration jumps can be generated using a rapid solution exchange technique combined with a solid supported lipid membrane. As is described in the preceding publication (Pintschovius and Fendler, 1999) comparable results are obtained.

In view of the mechanism of ion transport, electrogenicity of cation binding is an important issue. Therefore, in this publication we focus on concentration jump experiments performed in nontransport modes, i.e., in the absence of ATP.

MATERIALS AND METHODS

Chemicals

Na^+ concentration jumps were generated using a rapid solution exchange technique at the surface of a solid supported membrane (SSM). The SSM carried membrane fragments from pig kidney containing purified Na^+/K^+-ATPase. Standard solutions contained 25 mM imidazole, 3 mM $MgCl_2$, and 0.2 mM dithiothreitol (DTT; 99.5%, Roth, Karlsruhe, Germany) at pH 7.0. The experiments were carried out at room temperature (23°C) and ionic strength was kept constant using choline chloride. For details see Pintschovius and Fendler (1999).

$MgCl_2$ was present in most experiments to allow comparison with the experiments, where ATP was involved. Mg^{2+} is a cofactor for phosphorylation from ATP in the Na^+/K^+-ATPase, but it is not necessary for cation binding to the unphosphorylated protein. In contrast, it acts as an antagonist to Na^+ binding (Robinson and Pratap, 1991a). Indeed, electrical signals of Na^+ binding in the absence of ATP were also observed when Mg^{2+}-free solutions were used. In Mg^{2+}-free solutions, 125 μM EDTA were added to avoid Mg^{2+} impurities.

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To exclude effects of the imidazole buffer (Schuurmanns Stekhoven et al., 1984) we repeated the experiments with TRIS buffer. No difference was observed.

Current measurements

Transient pump currents were generated via concentration jumps at the SSM as described in the preceding paper (Pintschovius and Fendler, 1999). The risetime of the concentration jump on the membrane surface is ~90 ms, but under certain conditions an effective time resolution of up to 10 ms can be obtained (Pintschovius and Fendler, 1999).

When electrogenic events with relaxation times shorter than the effective time resolution are considered (Na^+ jumps at 0 mM ATP), the time course of the current signal is mainly determined by hydrodynamic properties of the apparatus. Under these conditions, turbulent flow patterns may lead to irregularly shaped signals (see Fig. 2, *inset*). Therefore, the electrical signal was characterized as the time integral of the measured current. This quantity is independent of the time course of charge translocation and therefore insensitive to rapid variations of Na^+ concentration at the surface of the SSM because of turbulent flow. On-currents after addition of Na^+ and off-currents upon removal of Na^+ were integrated separately.

In experiments where ATP was involved, the electrical signal is followed by a slow negative current component which has been assigned to the discharge of the membrane fragments containing Na^+/K^+-ATPase, the system time constant (Borlinghaus et al., 1987; Fendler et al., 1987; Seifert et al., 1993). In this case only the first part of the signal corresponding to a positive current was used for integration. No negative component was observed in Na^+ jump experiments without ATP. Here, the integration limits were set such that the current amplitudes exceeding noise level were contained within the integration range. The reason for the absence of the negative phase in these experiments is unclear. It has been speculated that the negative component is in fact a protein-mediated rather than a passive backflow of charge (Fendler et al., 1993). This could explain a different behavior in the presence and absence of ATP.

RESULTS

In a preceding publication we presented experiments where the Na^+ transport of the Na^+/K^+-ATPase was triggered either by a Na^+ concentration jump in the presence of ATP or an ATP concentration jump in the presence of Na^+. The latter experiment is analogous to investigations using black lipid membranes and caged ATP (Borlinghaus et al., 1987; Fendler et al., 1985, 1987). Under these conditions active ion transport by the Na^+/K^+-ATPase takes place and an electrogenic Na^+ translocation step was identified.

Here we address the question of whether ion binding and release steps in the Na^+/K^+-ATPase reaction cycle represent electrogenic events. We probed Na^+ binding to the Na^+/K^+-ATPase by Na^+ concentration jumps in the absence of ATP. Under these conditions the protein is able to bind Na^+ ions, but it should be unable to transport them across the membrane. Therefore, the binding process can be studied independently of other steps.

Na^+ concentration jumps in the absence of ATP

In Fig. 1 a comparison between Na^+ concentration jumps and subsequent Na^+ removal under different conditions is shown. Trace *A* corresponds to a Na^+ jump ($0\ s < t < 1\ s$) in the presence of ATP. Note that ATP was present in all buffers used for this experiment. The sign of the peak current indicates transport of positive charge toward the SSM (or negative charge in the opposite direction). It is known that under those conditions the enzyme is activated by Na^+ and transports Na^+ ions to the extracellular surface. Here the reaction is virtually stopped because in the absence of K^+, the reactions following the transport of Na^+ are too slow to be observed. No signal occurs when Na^+ is removed from the solution ($t > 1$ s).

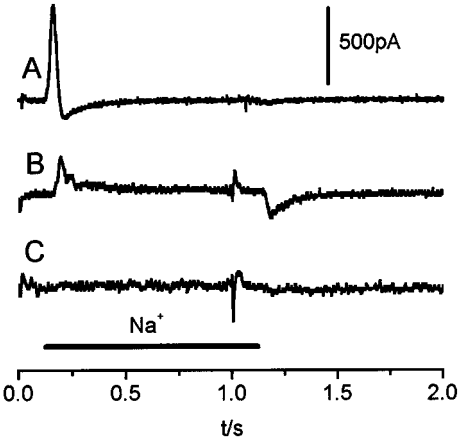

FIGURE 1 Na^+ concentration jump (10 mM, addition at $t = 0$ s and removal at $t = 1$ s) under different conditions. (*A*) In the presence of 100 μM ATP electrogenic Na^+ transport by the Na^+/K^+-ATPase is activated. The sign of the current signal is compatible with transport of positive charge to the gold electrode. When Na^+ is removed, no current response is observed. (*B*) The same procedure as in (*A*), but in the absence of ATP. After addition of Na^+ charge translocation takes place. The signal has the same sign as in (*A*) and is smaller. In contrast to (*A*) there is a current response upon removal of Na^+, indicating a fast backward reaction. (*C*) In order to ensure that (*B*) is dependent on the Na^+/K^+-ATPase, the same experiment was done after incubation with the specific inhibitor of P-type ATPases, orthovanadate. This procedure results in complete inhibition of the electrogenic process.

Trace *B* shows an Na^+ concentration jump in the absence of ATP. Again, positive charge is moved toward the SSM after addition of Na^+. In contrast to the previous trace, a peak into the opposite direction occurs when Na^+ is removed again. To exclude artefacts, the same sample was incubated in 1 mM orthovanadate, a specific inhibitor of the P-type ATPases. Na^+ concentration jumps after vanadate treatment (trace *C*) show no electrogenic effect.

In Fig. 2 the current response after a 100-mM Na^+ concentration jump is shown at greater time resolution. Signal A was recorded at 100 μM ATP present in both solutions, signal B without ATP. Both measurements were carried out on the same membrane. It should be noted that the transient currents generated by the Na^+ jumps without ATP vary widely in shape, depending on the fluid mechanical properties of the system (position, size of the SSM, etc.). For example, an SSM positioned right under the fluid inlet resulted in irregular, burstlike signals (Fig. 2, *inset*) reflecting the turbulent behavior of the solution flow in this part of the reaction volume. It also shows that both the

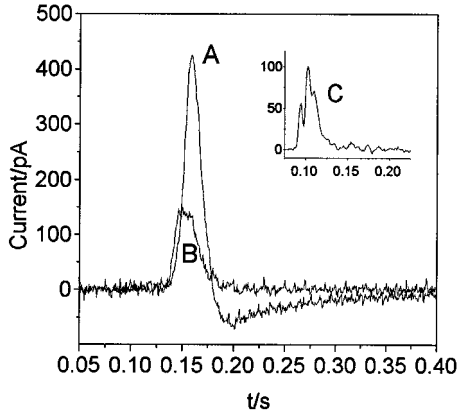

FIGURE 2 Na^+ concentration jump (100 mM) in the presence (A) and absence (B) of ATP. The electrolyte solution contained 300 mM choline chloride, 25 mM imidazole, 3 mM $MgCl_2$, and 0.2 mM DTT at pH 7.0 (HCl). The activating solution contained 200 mM choline chloride plus 100 mM NaCl instead of 300 mM choline chloride. The experiments were performed at 25°C. *Inset*: depending on the fluid mechanical behavior at the SSM surface, irregular signals were observed in the ATP-free case. These are explained by concentration inhomogeneities in the solution stream, which cause fast forward *and* backward reactions.

forward *and* backward reaction must be fast. In order to obtain quantitative results, the amount of charge translocated instead of peak currents or relaxation times was evaluated (see Materials and Methods).

The translocated charge depends on the total salt concentration in the presence of ATP (Q_{ATP}) but not in the absence of ATP (Q_0, Fig. 3). High concentrations of anions, Cl^- in this case, are known to affect the phosphorylated Na^+/K^+-ATPase. This observation has earlier been identified as a

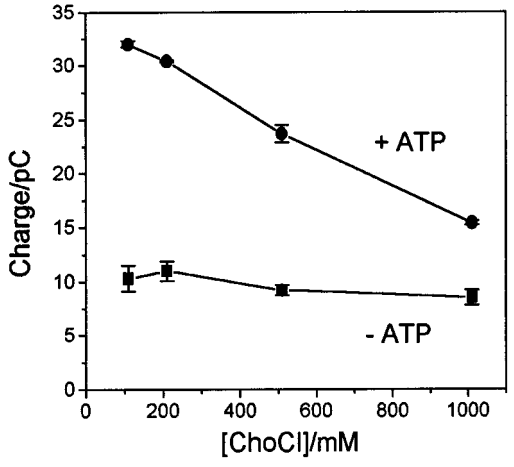

FIGURE 3 The dependence of the integrated peak-currents on choline chloride concentration in the presence (*circle*) and absence (*square*) of ATP. The detected charge drops considerably with increasing salt concentration, when 100 μM ATP is present, whereas it remains almost constant if only the electrogenic effect of cytoplasmic Na^+ binding with no ATP present is measured. Electrolyte composition: The indicated amount of [ChoCl] plus 10 mM ChoCl (nonactivating solution) or NaCl (activating solution), 3 mM $MgCl_2$, 0.2 mM DTT, and 25 mM TRIS (HCl) at pH 7.0 and 22°C.

Hofmeister effect (see, e.g., Post and Suzuki, 1991). Other authors have ascribed this decrease of pump activity to an effect of the choline cation (Robinson and Pratap, 1991b). Evaluation of the integrated signal currents, i.e., the charge translocated, yields a ratio of $q_r = Q_0/Q_{ATP} \sim 30\%$ at sufficiently low ionic strength. If it is assumed that in the presence of ATP exactly one elementary charge is translocated over the total thickness of the membrane, the ratio q_r corresponds to the dielectric coefficient α of the partial reaction (Läuger, 1991). Note that the dielectric coefficient is a measure for the effective distance of charge transport within the membrane dielectric compared to the transport of one charge unit over the entire membrane.

The knowledge of the amount of charge Q_{ATP} translocated after an Na^+ jump in the presence of saturating ATP concentrations allows one to estimate the density of active Na^+/K^+-ATPase molecules on the SSM surface. We therefore assume that only one half-cycle of the Na^+/K^+-ATPase is observed, during which one elementary charge per pump molecule is translocated over the membrane. With a membrane area of 1 mm^2 and a translocated charge of ~30 pC (Fig. 3) a pump density of $\sim 2 \cdot 10^{10}$ active molecules per cm^2 is obtained. This has to be compared with the density of Na^+/K^+-ATPase molecules within the membrane fragments of $\sim 10^{12}$ cm^{-2} (Maunsbach et al., 1988). We conclude that only ~2% of the surface of the SSM is covered with membrane fragments. To be precise, the value calculated in this way must be interpreted as a lower limit: The current generated by the Na^+/K^+-ATPase is coupled to the amplifier via the capacitances of the SSM and the membrane fragments that act as a capacitive current divider. Therefore, the measured current is only ~50% of the current generated by the membrane fragments. This means that at least 2% of the SSM area is covered with active pumps, the remaining area being uncovered or covered with inactive enzyme; also, SSM surface regions, where no thiol/PC double layer has formed (but multilayers or decane lenses), do not contribute (Seifert et al., 1993).

It is one of the advantages of the fast flow technique that it enables substances not only to be added but also to be removed again. The removal of Na^+ in the ATP-free experiment leads to an oppositely directed current with about the same absolute value of translocated charge (integrated current signal $Q_0^{on} = -Q_0^{off}$) as in the forward direction, indicating that the backward reaction is also a fast process. As already mentioned, their rates are beyond the limit for the time resolution of the apparatus. From the steepness of the signal slope rates of at least 100–200 s^{-1} can be estimated.

The measurement of charge translocation with variable Na^+ concentrations is shown in Fig. 4. The concentration dependence shown in the figure was fitted using a Michaelis-Menten model equation:

$$Q(c_0) = Q_{max} \frac{c_0}{c_0 + K_M}. \quad (1)$$

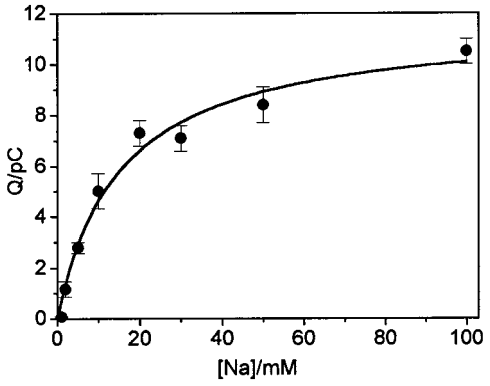

FIGURE 4 Na^+ dependence of the Na^+ concentration jump experiments in the absence of ATP. The electrolyte solution contained 25 mM imidazole, 3 mM $MgCl_2$, and 0.2 mM DTT at pH 7.0 (HCl). The nonactivating solution contained 300 mM choline chloride, whereas the activating solution contained varying Na^+ concentrations plus the amount of choline chloride required to yield 300 mM. The experiments were performed at 22°C. On-peaks (Na^+ jump) and off-peaks (Na^+ removal) of five current traces per concentration value were integrated to give the charge translocated. The resulting on-peak values were averaged and plotted versus the Na^+ concentration. The absolute values of the on-peak and off-peak curves are equal within the error bars. The best fit is obtained using a Michaelis-Menten function with $K_M = 15 \pm 3$ mM.

The best fit was obtained for $K_M = 15$ mM; the maximal amount of charge transported, Q_{max}, was 12 pC/mm^2.

Fig. 5 shows the process of electrogenic binding of monovalent cations has a high specificity for Na^+. For each cation species, control measurements were performed before the protein was added (gray bars). In this experiment, integrated currents (on peak) generated by 10-mM concentration jumps of the cation species indicated were evaluated.

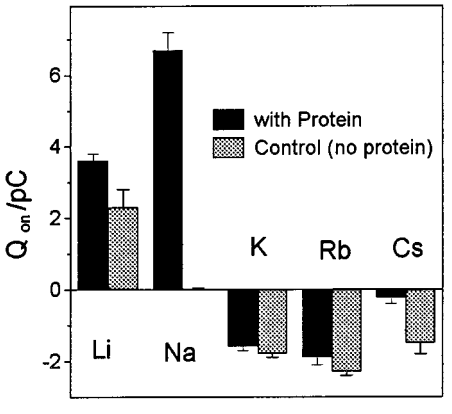

FIGURE 5 Charge movement in the Na^+/K^+-ATPase elicited by different cations. The electrolyte solution contained 210 mM choline chloride, 25 mM imidazole, 3 mM $MgCl_2$, and 0.2 mM DTT at pH 7.0 (HCl). The activating solution contained 200 mM choline chloride plus 10 mM of the cation indicated instead of 210 mM choline chloride. The experiments were performed at 22°C. On-peak currents (cation jump) and off-peak currents (removal) were integrated to obtain the charge moved. As a control measurement each concentration jump experiment was also performed before addition of protein. Electrogenic cation binding turns out to be specific for Na^+. Possibly Li^+ binding is an electrogenic process, too.

For the Na^+ jump, the control experiment shows a negligible artefact, whereas relatively large artefactual response signals are observed for the other cations. A signal size significantly larger than the control was measured only for Na^+. There is possibly also an effect of Li^+, but no detectable effect of K^+, Rb^+, or Cs^+. In the case of Cs^+, a remarkable difference between the control and protein experiment is observed. The reason for this behavior is unclear.

Of course, the experiment shown in Fig. 5 does not rule out that ions other than Na^+ bind electrogenically with an affinity significantly lower than that of Na^+. However, experiments with concentrations higher than 10 mM are difficult to perform with ions other than Na^+ because of the increase in artefactual amplitudes. Also, the assessment of ion specificity requires a comparison of all ions at the same concentration that should not be saturating. This requirement is met by the choice of 10 mM as the concentration for the comparison of the ions.

The origin of such artefactual currents after concentration jumps in the absence of protein could be explained by one of the following effects: 1) a difference in diffusion velocities across the unstirred boundary layer ($\sim$1 μm above the SSM surface, see Pintschovius and Fendler, 1999). For example, an inward stream of K^+ ions and an outward stream of less mobile choline ions during a K^+ jump would lead to an artefactual charge translocation of positive charge toward the SSM. 2) Unspecific binding of cations to the lipid surface of the SSM. Fig. 5 illustrates that the ion with the lowest mobility (Li^+) shows a positive artefact, whereas the ion with the highest mobility (Cs^+) has the most negative artefact. On one hand, hypothesis 1 predicts the contrary and has therefore to be rejected. On the other hand, hypothesis 2 gives the correct sign and order of magnitude of the artefacts, if we assume unspecific binding to a "strong field" cation binding site (Hille, 1992). Here we have to assume that choline binds with about the same affinity as Na^+, thus yielding a negligible artefact. Li^+ binding more tightly than choline shows a positive artefact, whereas Cs^+ binding less tightly than choline yields negative artefacts.

Inhibition experiments

In the previous article (Pintschovius and Fendler, 1999) we demonstrated that transient currents can be induced by ATP concentration jumps. From the sign of the pump currents and the finding that these currents could not be inhibited by the addition of ouabain, it was concluded that only those membrane fragments oriented with their extracellular side to the SSM contribute to the electrical signal. The same effect has been observed using Na^+/K^+-ATPase membrane fragments adsorbed to a black lipid membrane (Fendler et al., 1985). This shows that ouabain cannot be used to inhibit Na^+/K^+-ATPase in membrane fragments adsorbed with their extracellular side to a lipid bilayer (Fendler et al., 1985) or an SSM (Pintschovius and Fendler, 1999). In contrast, their cytoplasmic binding sites—as those for ATP

or the phosphate analog orthovanadate—are accessible from the bulk solution, so that orthovanadate was able to inhibit ATP-activated Na^+ currents. However, after these experiments, the question still remains whether two populations of membrane fragments exist at the SSM: one that is activated by ATP and is adsorbed with the extracellular side to the SSM, and a second one adsorbed with the intracellular side to the SSM and which is not activated by ATP because the ATP binding site is not accessible.

In Na^+ binding experiments, the possible existence of a second population of membrane fragments with inverse orientation poses a problem because binding and dissociation of Na^+ (and K^+) can occur on both surfaces of the Na^+/K^+-ATPase. Fig. 1 *C* shows that orthovanadate inhibits electrogenic Na^+ binding and thus must be able to access the cytoplasmic surface. Since we cannot rule out that orthovanadate can also access its binding site, even if the membrane fragment is adsorbed with the cytoplasmic side to the SSM, this experiment does not directly prove the sidedness of the Na^+/K^+-ATPase.

To ascertain the orientation of the membrane fragments responsible for electrogenic Na^+ binding, we again made use of the fact that ouabain cannot inhibit Na^+/K^+-ATPase in membrane fragments adsorbed with their extracellular side to the SSM. The addition of 1 mM ouabain to both solutions (Na^+-free and 20 mM Na^+) after the membrane fragments had been attached to the SSM did not affect the signal response. This shows that the membrane fragments responsible for electrogenic Na^+ binding are oriented with their intracellular side to the bulk solution. As a control, membrane fragments were used which had been incubated with 1 mM ouabain prior to protein adsorption to the SSM. They show no activity, demonstrating that the electrogenic effect can be blocked by ouabain under our conditions, provided it has access to the extracellular side of the membrane fragments. The corresponding experiments are shown in Fig. 6.

First, the inhibition of the Na^+ jump signals by specific inhibitors shows that the electric currents observed after a Na^+ jump are not caused by unspecific binding processes of Na^+ to the surface of the SSM or of the membrane fragments. Second, the signal results from binding of Na^+ to membrane fragments that are adsorbed with their extracellular surface to the SSM. These experiments do not completely rule out extracellular Na^+ binding to the enzyme via rapid diffusion of Na^+ into the cleft between membrane fragment and SSM. However, in the following section (Discussion) we will provide further evidence that the electrical signal generated by a Na^+ jump is due to binding of Na^+ to the intracellular side of the protein.

Cytoplasmic K^+ binding and occlusion

We have reported that the electrogenic response following the addition of monovalent cations has a high specificity for Na^+ (Fig. 5). This also means that no such effect appears with K^+ (or is beyond the detection limit), though K^+ could have served as a candidate for an electrogenic binding process, too.

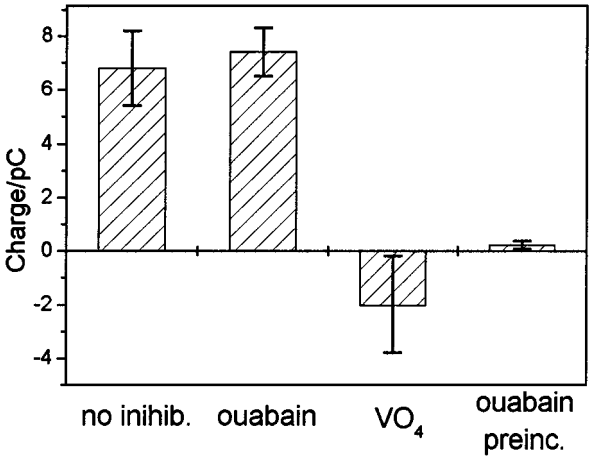

FIGURE 6 Inhibition of the currents generated by a 20-mM Na^+ jump in the absence of ATP. The bars show the translocated charge in the absence of inhibitors (1st bar) and after addition of 1 mM ouabain (2nd bar) to the adsorbed membrane fragments. Inhibition by ouabain could be demonstrated by the adsorption of membrane fragments preincubated in 1 mM ouabain (4th bar). Also, 1 mM orthovanadate (3rd bar) added to the adsorbed membrane fragments was able to inhibit the currents.

Nevertheless, K^+ affects ATP-free binding of Na^+ by driving the enzyme into the E_2 state that cannot bind Na^+ (see Fig. 7). In Fig. 8 we show the amount of translocated charge after Na^+ concentration jumps ([ATP] = 0) in the presence of variable K^+ concentrations in both solutions. The antagonistic effect of K^+ is very clear and obeys a relation for the inhibition

$$Q = Q_{\max}\frac{[K^+]}{[K^+] + K_I} \tag{2}$$

with $K_I = 0.6 \pm 0.12$ mM.

An experiment that can be performed only with a flow-through method and an immobilized protein sample is the reversible exchange of substrates. Fig. 9 shows an experi-

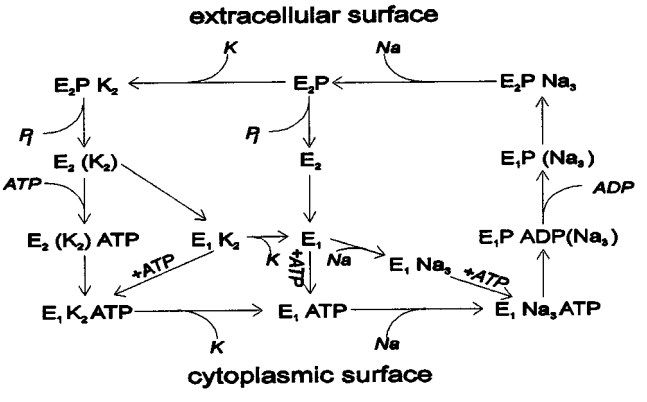

FIGURE 7 The Albers-Post reaction cycle of the Na^+/K^+-ATPase according to Glynn, 1985.

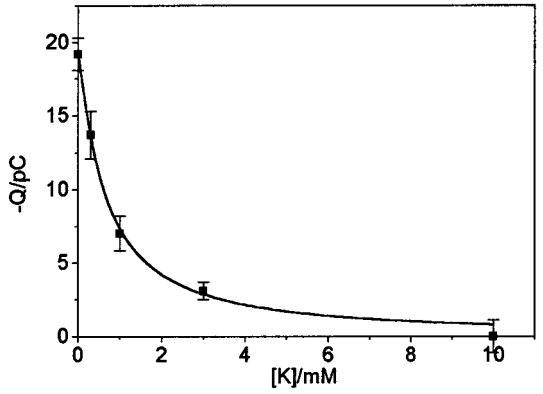

FIGURE 8 Influence of K^+ on Na^+ concentration jumps in the absence of ATP. The electrolyte solution contained 25 mM imidazole, 3 mM $MgCl_2$, 0.2 mM DTT, and varying amounts of K^+ at pH 7.0 (HCl). The nonactivating solution contained 300 mM choline chloride, whereas the activating solution contained 20 mM Na^+ plus 280 mM choline chloride. The experiments were performed at 22°C. On-peaks (Na^+ jump) and off-peaks (Na^+ removal) were integrated to obtain the charge translocated. The resulting absolute charges were averaged and plotted versus the K^+ concentration. The presence of K^+ has an inhibiting effect on the Na^+ jump signals without ATP. The charge moved is half-maximal at $[K^+]$ = 0.6 mM ± 0.12 mM.

ment where the initial solution contained 10 mM K^+. Then the solution in the cuvette was exchanged such that K^+ was replaced by 10 mM Na^+. No signal could be obtained after this solution exchange. Again replacing the Na^+ solution by the initial K^+ solution yielded a current (positive charge translocated from the membrane to the solution) that was dependent on the time Δt Na^+ had been present in the reaction volume. The dependence of the integral of these currents on the duration Δt of Na^+ incubation (Fig. 10) was recorded. For small times almost no response was observed. Half-maximal response could be obtained after $t_{0.5} \approx 2$–3 s of Na^+ incubation. An exponential fit using the model

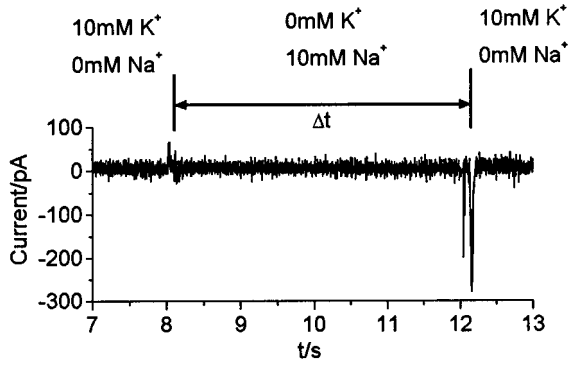

FIGURE 9 Exchange between solutions containing 10 mM K^+ and 10 mM Na^+: I. The solution in the cuvette contained 10 mM K^+. II. K^+ was removed and replaced by 10 mM Na^+. No current signal was observed at this point. III. The enzyme was allowed to incubate in the Na^+-solution for the duration Δt. IV. Na^+ was removed again; at this point a current peak was detected showing translocation of positive charge away from the gold electrode. Conditions: 300 mM choline chloride, 25 mM TRIS (pH 6.9, HCl), 0.2 mM DTT, 125 μM EDTA, and 10 mM Na^+ or K^+. Temperature 23°C.

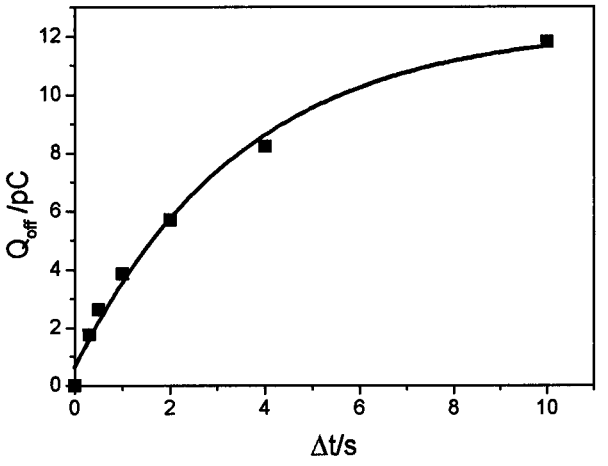

FIGURE 10 Exchange between solutions containing 10 mM K^+ and 10 mM Na^+ as shown in Fig. 9. The charge translocated after removal of Na^+ is shown to be dependent on the time interval Δt. The solid curve represents a numerical fit using Eq. 3. As a result the rate $k_f = 0.29 \pm 0.04\ s^{-1}$ is obtained.

function

$$Q(\Delta t) = Q_{max} - \Delta Q \cdot \exp(-k_f \Delta t) \quad (3)$$

yields $Q_{max} = 12.3 \pm 0.7$ pC, $\Delta Q = 11.7 \pm 0.7$ pC, and $k_f = 0.29 \pm 0.04\ s^{-1}$.

Since for $\Delta t = 0$ one expects zero amplitude ($Q(0) = 0$), the relation $Q_{max} = \Delta Q$ should hold. This condition is met within the error range of ±0.7 pC. Deviations of the data points from the given model (Eq. 3) are due to the relatively slow time dependence of the solution exchange.

DISCUSSION

Active ion transport by the Na^+/K^+-ATPase is an electrogenic process. Therefore, at least one of the partial reactions of the Na^+/K^+-ATPase has to be electrogenic, but several possibly exist. Assignment of the electrogenic steps to the partial reactions of the biochemically defined reaction cycle is important with respect to the clarification of the transport mechanism of the ATPase. Equally important is the identification of the underlying charge translocation mechanisms of the electrogenic steps.

The kinetic model of the Na^+/K^+-ATPase, the Albers-Post scheme (Fahn et al., 1966; Post et al., 1969), is shown in Fig. 7. The electrogenicity of many of the partial reactions of the Albers-Post cycle has been investigated in the past. However, the conclusions drawn in the numerous studies were not always in agreement with one another (for a review see Rakowski et al., 1997).

In the present study, the electrical charge movement associated with the Na^+ binding reaction was directly measured for the first time. By using the rapid solution exchange technique, the current response after Na^+ concentration jumps could be studied. In the absence of phosphorylating agents the investigation was restricted to the reactions of the

unphosphorylated pump, where cations are bound to the cytoplasmic surface.

Binding of Na^+ to the cytoplasmic surface of the Na^+/K^+-ATPase is electrogenic

Our experiments demonstrate that in the absence of ATP an electrogenic event is initiated by both the addition of Na^+ to the Na^+/K^+-ATPase and the removal of Na^+ from the Na^+/K^+-ATPase. Contamination of the preparation with ATP as an origin of the currents can be ruled out, since before and during the experiment the SSM is in contact only with ATP-free buffers. From inhibition experiments with orthovanadate and ouabain, we were able to show that an enzyme specific reaction is responsible for the observed signals. Making use of the side specificity of these inhibitors, we concluded that only MF adsorbed with their extracellular side to the SSM contribute to the signal (see Results). Although this does not rigorously rule out electrogenic Na^+ binding at the extracellular surface of the protein, there are two results that strongly argue in favor of a cytoplasmic Na^+ binding reaction: 1) since the protein population contributing to the electrical signal is oriented with its extracellular side toward the SSM, binding of a cation to this side would yield a negative transient current, but a positive current is observed. 2) The accessibility of the extracellular side is greatly restricted by the strong adsorption of the MF to the SSM. Indeed, current leakage out of the interstitial space between the MF and the SSM takes place on a time range of several hundred milliseconds (cf. the system time constant $\tau_0 \sim 200$ ms, Pintschovius and Fendler, 1999).

The rate of the electrogenic Na^+ binding reaction is still unclear, as we were unable to resolve the time course of either the forward or the backward reaction. We suggest that upon addition or removal of 10–100 mM Na^+ the relaxation times are shorter than the time resolution of the technique and the highest rates given in Pintschovius and Fendler (1999) ($>100\ s^{-1}$). This is in agreement with the current concept of cation binding steps, which are generally believed to be very rapid.

Rather than Na^+ binding itself ($E_1 + Na \rightarrow E_1Na$), a preceding electrogenic reaction could also result in an electrical signal measured after the Na^+ concentration jump. A possible candidate for this is the conformational transition $E_2 \rightarrow E_1$. However, based on results from literature as well as our experiments with K^+, we conclude that a contribution of $E_2 \rightarrow E_1$ can be ruled out because 1) the rate of the conformational transition in the absence of transported cations (Na^+, K^+) is very slow (Apell et al., 1996; $0.023\ s^{-1}$), and thus should not yield a detectable signal. 2) If the initial state of the enzyme in the absence of the cations Na^+ and K^+ was E_2, then a K^+ jump as well as the Na^+ jump should involve the transition $E_2 \rightarrow E_1$ (see Fig. 7). Instead, with K^+, no electrical signal is obtained (Fig. 5). 3) In the experiment, where a solution containing K^+ was exchanged for one, which contained Na^+ (Fig. 9, cf. Fig. 7), an electrical signal was obtained when Na^+ was removed. As in this case the conformational transition $E_2 \rightleftharpoons E_1$ is not involved in the reaction cycle, this transition cannot be identified with the electrogenic step observed. As will be shown later, in this experiment only cytoplasmic Na^+ dissociation can be responsible for the observed charge translocation.

In conclusion, the electrogenic process observed after an Na^+ jump in the absence of ATP has to be attributed to the binding of Na^+ to the intracellular side of the protein, $E_1 + Na \rightarrow E_1Na$. In particular, the possibility that these currents can be explained by an unspecific surface binding processes can be ruled out, since they require the presence of active Na^+/K^+-ATPase. At the same time, the fact that an active protein is required for the effect strongly supports the concept that these binding sites are the specific transport sites of the Na^+/K^+-ATPase, in which the Na^+ ions are translocated and released to the extracellular medium.

Properties of electrogenic Na^+ binding

The dependence of the amount of electrical charge translocated on Na^+ concentration could be fitted using a hyperbolic relation with a $K_{0.5}^{Na}$ of 15 mM. This is comparable to the value of 8 mM determined by Heyse et al. (1994) who evaluated the $[Na^+]$-dependence of the relative RH421 fluorescence change. Also, using Na^+/K^+-ATPase from electric eel a binding constant of 10 mM was determined with a bilayer technique (Fendler et al., 1994). With Na^+/K^+-ATPase from pig kidney somewhat lower values were obtained (Nagel et al., 1987: 3 mM; Pintschovius and Fendler, 1999: 2.6–3.7 mM, but Kane et al., 1997: 8–10 mM). However, the latter experiments were performed under turnover conditions, i.e., in the presence of ATP. It is therefore not surprising that an increased apparent affinity is measured under conditions where the Na^+ binding equilibrium is followed by an additional reaction (ATP binding and phosphorylation).

Interestingly, the $[Na^+]$-dependent experiments presented in our previous publication (Pintschovius and Fendler, 1999) could not be fitted with a simple hyperbolic relationship, but required a Hill-exponent of at least $n = 2$. This indicates that in the presence of ATP positive cooperativity of at least two cation binding sites can be observed. This is not observed in the experiments performed under ATP-free conditions (Fig. 4). It can be speculated that although three Na^+ ions are necessary for the phosphorylation of the ATPase, only one Na^+ ion is responsible for the electrogenic binding process.

The amount of charge translocated during Na^+ binding (in the absence of ATP) can be compared to charge translocation during Na^+ transport (in the presence of ATP) to give the relative charge q_r, determined by the experiments shown in Fig. 3 at sufficiently low ionic strength. The value obtained in the presence of ATP represents the charge

translocated during both processes, namely binding and subsequent transport to the extracellular side. Binding, therefore, accounts for ~30% of charge displacement, indicating that cytoplasmic Na^+ binding represents a minor electrogenic event in the reaction cycle.

Fig. 3 shows that with increasing choline chloride concentrations (and at 10 mM NaCl) the transported charge in the presence of ATP decreases. This has been explained as a Hofmeister effect of the anions acting on the $E_1P \rightleftharpoons E_2P$ equilibrium (Post and Suzuki, 1991). High Cl^- concentrations destabilize E_2P in favor of E_1P thereby leading to a reduced charge translocation. This effect is not observed when ATP is absent, consistent with the notion that it is the $E_1P \rightarrow E_2P$ transition that is responsible for the Hofmeister effect.

We probed the specificity of the binding process (Fig. 5) for different cations. A charge displacement significantly larger than that of the control experiments was found for Na^+ only. This lends further support to the notion that the observed effect is due to specific binding of Na^+ to the transport sides of the Na^+/K^+-ATPase. It has to be noted that for ionic species other than Na^+ a relatively high control artefact occurs. These artefacts represent up to one-third of the charge translocated during electrogenic Na^+ binding (or 10%, if expressed in terms of the relative charge translocation q_r).

Various techniques have been employed to probe cytoplasmic Na^+ binding. From the voltage-sensitivity of kinetic data evidence was provided for electrogenic intracellular Na^+ binding (Goldshleger et al., 1987; Or et al., 1996). These arguments are based on flux measurements into vesicles in the absence and presence of a diffusion potential. Electrogenicity of both intracellular and extracellular Na^+ binding and release has been postulated from experiments making use of the electrochromic fluorescence dye RH421 (Heyse et al., 1994). As in our experiments, the effect was specific for Na^+ ions and yielded an Na^+ affinity of 8 mM. The relative contribution of intracellular Na^+ binding to the total electrogenicity of the ion pump was estimated to be 16% (Or et al., 1996) and 25% (Heyse et al., 1994) which is comparable to the value found in our investigation (30%).

Lu et al. (1994) studied capacitance changes of the cell membrane of cardiac myocytes using the giant patch-clamp technique. In these experiments, cytoplasmic Na^+ binding seemed to be voltage-insensitive, whereas a voltage-sensitive process was observed when both Na^+ and ATP were added to the solution. The discrepancy between the first two studies including ours and that of Lu et al. (1994) could be related to the fact that the former were performed using purified enzyme while in the latter the enzyme was in the plasma membrane of whole cells. Also, the origin of the Na^+/K^+-ATPase was different: kidney as opposed to heart.

K^+ translocation in the absence of ATP proceeds with a rate of 0.2–0.3 s^{-1}

The initial state of the Na^+/K^+-ATPase before the cation concentration jump is E_1. This was concluded from the analysis of the Na^+ jump experiments given above. The Na^+/K^+-ATPase is known to occlude K^+ in the $E_2(K)$ state. K^+ therefore acts by affecting the $E_1 \rightleftharpoons E_2$ equilibrium. This has been tested in the experiment shown in Fig. 8. The figure illustrates that K^+ is bound, although K^+ concentration jumps (Fig. 5) do not yield an electrogenic response of the protein.

For the explanation of the experiment of Fig. 9 (exchange of Na^+ versus K^+ solution) a slow rate of the transition $E_2(K) \rightarrow E_1$ has to be assumed: After Na^+ addition (and K^+ removal) the low transition rate k_f limits the rate of electrogenic Na^+ binding via the sequence

$$E_2(K) + Na \overset{k_f}{\rightarrow} E_1K + Na \rightarrow E_1Na + K \qquad (4)$$

Due to the small value of k_f the corresponding current response is below the detection limit. The number of pumps in the Na^+-bound state increases in time (Δt) with the rate k_f. Upon Na^+ removal (K^+ jump) the electrogenic Na^+ dissociation from those pump molecules already in the E_1Na state gives a contribution to the current signal, so that the amplitude of the Na^+ removal peak depends on the duration Δt, during which Na^+ was present in the reaction volume (Fig. 10).

From this experiment a value of $0.29 \pm 0.04\ s^{-1}$ was obtained for the rate k_f. This value can be directly compared to the literature results obtained with FITC-labeled Na^+/K^+-ATPase using a stopped-flow technique. In these experiments a K^+ concentration jump ("K^+ quench") had been carried out in the absence of both ATP and Na^+. For the backward transition $E_1 + K \rightarrow E_2(K)$ a rate of $k_b = 150\ s^{-1}$ was found (Smirnova et al., 1995). The forward transition was probed by Na^+ jumps performed with the $E_2(K)$ enzyme ("Na^+ reversal") yielding the rate constant k_f of the forward reaction Eq. 4 (Steinberg and Karlish, 1989: $0.2\ s^{-1}$ (20°C); Smirnova et al., 1995: $0.13\ s^{-1}$ (15°C)). These values agree well with our $k_f = 0.29 \pm 0.04\ s^{-1}$ (23°C).

K^+ deocclusion, and cytoplasmic K^+ release are electroneutral

The K^+ concentration jumps of Fig. 5 do not yield signals larger than the corresponding control experiments. Taking the size of the control signals as an error limit, a K^+ jump in the absence of ATP and Na^+ is electroneutral within an error of 10% compared to the charge translocation in the presence of ATP.

As discussed in the preceding section, the reaction sequence following a K^+ jump in the absence of Na^+ and ATP is

$$E_1 + K \rightarrow E_1K \rightarrow E_2(K). \qquad (5)$$

In this reaction sequence (which is backward in terms of the physiological operation mode of the ion pump) K^+ is generally believed to be taken up from the cytoplasmic side of

the protein. Our experiments, therefore, probe cytoplasmic K^+ binding and subsequent translocation. The "K^+-quench" experiments of Smirnova et al. (1995) show that the corresponding transition rate is 150 s^{-1}. This rate would be sufficiently fast to yield a detectable current response if at least one of the steps was electrogenic. As no electrogenic signal is observed, the sequence shown above must be electroneutral.

Electroneutrality of K^+ transport has been a matter of controversial literature discussion. Bahinski et al. (1988) report voltage-insensitive K^+ transport using voltage-clamp measurements of guinea pig myocytes. The same conclusion was drawn from BLM experiments using proteoliposomes containing purified Na^+/K^+-ATPase from shark rectal gland (Gropp and Fendler, 1998).

Other authors have proposed electrogenic K^+ translocation or electrogenic K^+ binding via a high-resistance access channel (Schwarz and Vasilets, 1991; Stürmer et al., 1991; Peluffo and Berlin, 1997). In these publications extracellular K^+ binding was suggested to be the electrogenic step. This is not in contradiction to our results because our experiments were carried out in the absence of any phosphorylating agents, restricting the range of possible partial reactions to the reactions of the unphosphorylated enzyme (sequence Eq. 5).

CONCLUSIONS

By using a rapid solution exchange technique combined with a lipid membrane on a solid support, we have investigated the binding reactions of Na^+ and K^+ to the Na^+/K^+-ATPase. Cytoplasmic Na^+ binding (or release) was found to be electrogenic, accounting for ~30% of the total charge translocated during Na^+ transport. In contrast, cytoplasmic K^+ binding (or release) is electroneutral. The same holds for the other partial reactions on the K^+ deocclusion pathway $E_2(K) \rightarrow E_1K \rightarrow E_1 + K$. For future studies, extracellular cation binding would be an attractive subject. For such investigations the situation is more complicated because the MF are adsorbed with their extracellular side facing the solid supported membrane. This limits direct access to the extracellular side of the protein. Further developments are underway to overcome this limit of the technique.

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