Eur Biophys J (1998) 27: 605–617 © Springer-Verlag 1998

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Influence of sodium concentration on changes of membrane capacitance associated with the electrogenic ion transport by the Na,K-ATPase

Received: 3 November 1997 / Revised version: 19 February 1998 / Accepted: 21 February 1998

Abstract Electrogenic ion transport by the Na,K-ATPase was investigated in a model system of protein-containing membrane fragments adsorbed to a lipid bilayer. Transient Na^+ currents were induced by photorelease of ATP from inactive caged ATP. This process was accompanied by a capacitance change of the membrane system. Two methods were applied to measure capacitances in the frequency range 1 to 6000 Hz. The frequency dependent capacitance increment, ΔC, was of sigmoidal shape and decreased at high frequencies. The midpoint frequency, f_0, depended on the ionic strength of the buffer. At 150 mM NaCl f_0 was about 200 Hz and decreased to 12 Hz at high ionic strength (1 M). At low frequencies ($f \ll f_0$) the capacitance increment became frequency independent. It was, however, dependent on Na^+ concentration and on the membrane potential which was generated by the charge transferred. A simple model is presented to analyze the experimental data quantitatively as a function of two parameters, the capacitance of the adsorbed membrane fragments, C_P, and the potential of maximum capacitance increment, ψ_0. Below 5 mM Na^+ a negative capacitance change was detected which may be assigned to electrogenic Na^+ binding to cytoplasmic sites. It could be shown that the results obtained by experiments with the presented alternating current method contain the information which is determined by current-relaxation experiments with cell membranes.

Key words Na,K-ATPase · Membrane capacitance · Dielectric properties · Ion movement · Electrogenicity

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Introduction

Active ion transport across membranes is vital for the maintenance of the electrochemical gradients of ions in cells, and a variety of proteins are known which facilitate this process. Many of them utilize ATP as energy source for ion pumping. To study active transport transmembrane fluxes has been investigated. However, there are experimental difficulties in detecting fluxes with a time resolution which is necessary to analyze the kinetics of the transport process. This task becomes much simpler if the transport is electrogenic, that is, if it is accompanied by a net charge movement perpendicular to the plane of the membrane. In such a case the transport across a membrane can be studied by electric measurements. The Na,K-ATPase is one of the proteins mediating electrogenic ion transport (Glynn 1985; Läuger 1991). Investigation of the electrogenic ion transport provided considerable progress in the understanding of its mechanism (Nakao and Gadsby 1986; Borlinghaus et al. 1987; Gadsby et al. 1993; Hilgemann 1994; Wuddel and Apell 1995) and in determination of the structural features of the protein domain which is directly responsible for ion transport (Vasilets and Schwarz 1993, 1994). Studies of electrogenic sodium translocation by the Na,K-ATPase in the absence of potassium reduces the number of reaction steps in the pump cycle (Fig. 1) and was therefore a preferred target of investigations. Under such conditions the kinetics of the ion transfer were studied by recording transient currents in ATP concentration-jump experiments (Fendler et al. 1985; Borlinghaus et al. 1987; Sokolov et al. 1998), or voltage-jump experiments (Nakao and Gadsby 1986; Rakowski 1993; Hilgemann 1994). The results of these experiments provided strong evidence for an "access channel" hypothesis (Läuger and Apell 1988) which implies that ions have to move through a narrow well between the binding sites inside the protein and the extracellular solution (Läuger 1991; Stürmer et al. 1991; Vasilets et al. 1991; Gadsby et al. 1993; Hilgemann 1994; Wuddel and Apell 1995). Transient currents observed in voltage-jump experiments with phosphorylated

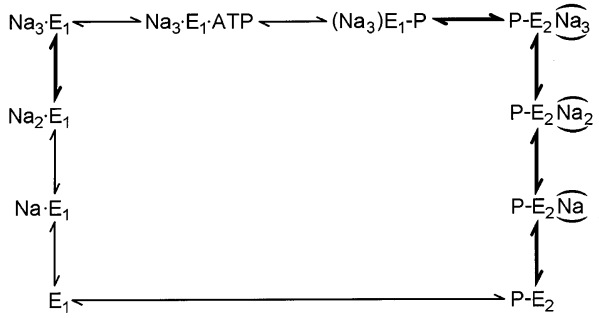

Fig. 1 Simplified kinetic diagram of sodium transport by the Na,K-ATPase on the basis of a modified Post-Albers reaction scheme from Wuddel and Apell (1995). The left side of the diagram represents reactions occurring at the cytoplasmic side of membrane, the right side reactions at the extracellular side. Symbols E_1 and E_2 denote the two basic conformations of the protein, the bold arrows indicate transitions which are electrogenic and which may produce a change of the apparent membrane capacitance. The main contribution to the capacitance increment is assigned to the dissociation/association of the first Na^+ ion on the extracellular side of protein which has a dielectric coefficient of 0.65 (Wuddel and Apell, 1995). The simplified model applied in this paper restricts the electrogenic process to this step and neglects all other electrogenic steps. The transition $E_1 \leftrightarrow P\text{-}E_2$ is extremely slow compared to all other reaction steps of the scheme and was not taken into account

Na,K-ATPase are described in the framework of this hypothesis by a redistribution of Na^+ ions within the access channel towards the electrochemical equilibrium (Rakowski 1993; Hilgemann 1994). This process is similar to the voltage-dependent movement of hydrophopic ions in black lipid membranes (BLM) which was studied by current relaxations after a voltage jump (Ketterer et al. 1971; Andersen and Fuchs 1975). An alternative method to study such transport process is the measurement of the impedance or admittance of transport systems in a membrane when an alternating voltage is applied. These methods provide results analogous to the current relaxation or charge relaxation methods (Markin and Chizmadzhev 1974; Pickar and Amos 1976; Hladky 1979; Rangarajan et al. 1979; Wang et al. 1994). The simplest version of the admittance method is the measurement of the "pseudo capacitance," an increase of the apparent membrane capacitance caused by ion transport. This apparent capacitance increase is frequency dependent. Initially this effect was observed with hydrophobic anions (Grigoriev and Ermishkin 1972); however, it was observed also with Na,K-ATPase containing membranes upon enzyme phosphorylation with ATP (Sokolov et al. 1992). The capacitance increment depended on the frequency of the applied voltage (Sokolov et al. 1994; Lu et al. 1995). However, a quantitative study of the electrogenic transport by the Na,K-ATPase with capacitance-increment measurements in the compound-membrane system was impeded by a poor reproducibility of the electric signals in these experiments, a disadvantage which was already reported in early research with this system (Borlinghaus et al. 1987). In this paper we present a calibration procedure and a theory which allows a quantitative and consistent analysis of experiments performed with different membranes. The procedure enabled us to measure capacitance increments at various sodium concentrations and to compare the results quantitatively with theoretical predictions.

Materials and methods

Chemicals. L-1,2-diphytanoyl-phosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL), n-decane from Aldrich (Steinheim, Germany, sodium dodecylsulfate (SDS) from Pierce Chemical (Rockford, IL). Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH and ATP (disodium salt, special quality) were from Boehringer (Mannheim). Apyrase VI from Sigma (Deisenhofen, Germany) and 3-O-(1-(2-nitrophenyl)-ethyl)ester ATP ("caged ATP") from Molecular Probes (Eugene, OR). NaCl (suprapure quality) and all other reagents (at least analytical grade) were from Merck (Darmstadt). All solutions were prepared with quartz distilled water.

Protein preparation. Na,K-ATPase was prepared from outer medulla of rabbit kidneys using procedure C of Jørgensen (1974). This method yielded purified enzyme in the form of membrane fragments, which consisted of flat sheets with a diameter of 0.2–1 μm and contained about 0.8 mg phospholipid and 0.2 mg cholesterol per mg protein (Bühler et al. 1991). Specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al. 1971). The protein concentration was determined by the Lowry method (Lowry et al. 1951), using bovine serum albumin as a standard. For all preparations the specific activity was in the range between 1,800 and 2,200 μmol P_i per hr and mg protein at 37 °C. The suspension of Na,K-ATPase-rich membrane fragments (about 3 mg protein per ml) in buffer (25 mM imidazole sulfate, pH 7.5, 1 mM EDTA, 10 mg/ml sucrose) was frozen in samples of 100 μl; in this form the preparation could be stored for several months at –70 °C without significant loss of activity. Storage of thawed preparations at +4 °C did not affect the activity over a period of 4 weeks.

Membrane experiments. Planar BLM were formed from 15 mg/ml solution of diphytanoyl-phosphatidylcholine in n-decane on a 1 mm orifice in a Teflon cell containing electrolyte of 30 mM imidazole, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7 and the indicated concentrations of NaCl at room temperature. After the bilayer was formed 100 μM caged ATP, 0.2 units apyrase and membrane fragments with a final concentration of ~15 μg/ml were added to one compartment of the cell whilst being smoothly mixed with a small magnetic stirrer (Sokolov et al. 1998). Membrane fragments adsorb to the BLM and generate a compound system of two capacitively coupled membranes (Fig. 2) with an RC time constant of several minutes.

Electric measurements. The electric signals were recorded by Ag/AgCl electrodes connected to the compartments of

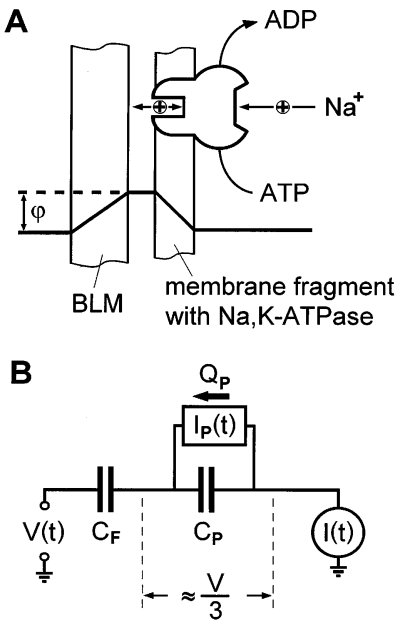

Fig. 2 **A** Schematic representation of the compound membrane consisting of a lipid bilayer (BLM) and a Na,K-ATPase containing membrane fragment. At the edge of the fragment both membrane are sealed together to isolate the compartment between both membranes against the bulk aqueous phase (not shown). When Na^+ ions are transported upon ATP hydrolysis through the pump a positive potential, φ, is generated in the enclosed compartment. An externally applied AC voltage is able to move ions in the ion well of the protein after phosphorylation of the protein by ATP. **B** Equivalent electric circuit of the compound membrane system. According the actual values of the capacitance of the BLM (C_F) and the membrane fragments (C_P) one third of the externally applied voltage $V(t)$ drops across the membrane fragments. C_P can be charged up by the pump current I_P

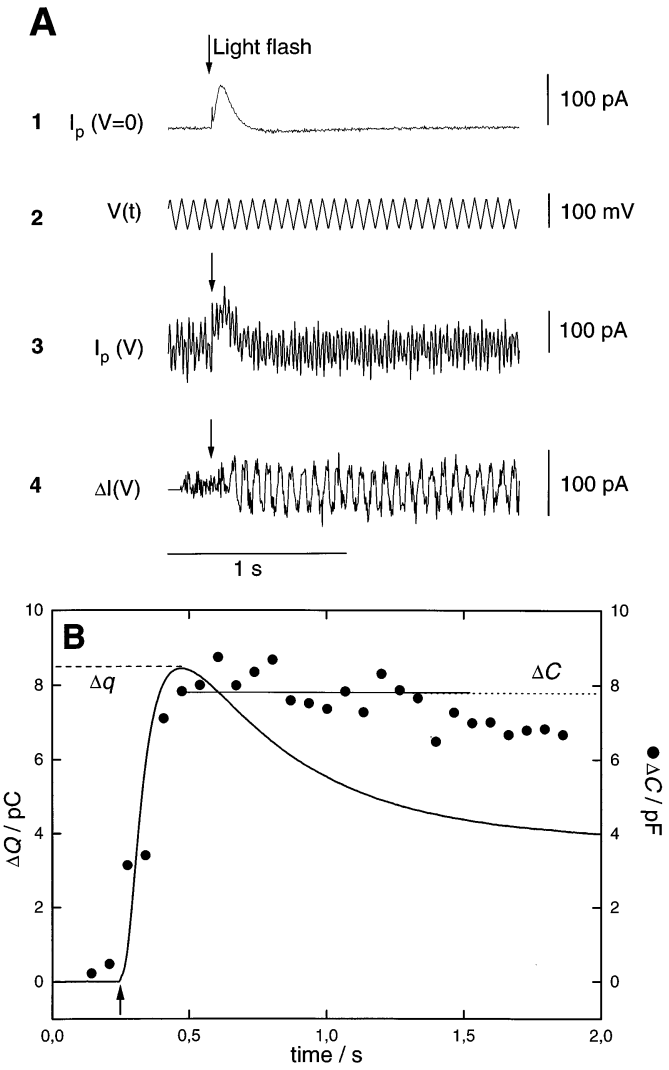

Fig. 3A, B Measurement of pump-induced charge transfers and capacitance changes. **A** Typical current transient triggered by a concentration jump of ATP photoreleased from caged ATP at the light flash (arrow) in the absence of an externally applied voltage (*trace 1*). The charge ΔQ was obtained as the integral of the current starting at the arrow. Then a triangular voltage of 15 Hz was applied to membrane (*trace 2*), and a second current transient was triggered (*trace 3*). The result of two subtractions from trace 3 resulted in the current components produced by the pump induced capacitance changes (*trace 4*): trace 1 was subtracted from trace 3 to account for the pump action, and the first period of alternating current of trace 3 (prior release of ATP from caged ATP) from all following periods to account for the uncompensated capacitive effects of the compound membrane system before pump activation. **B** Time courses of membrane capacitance ΔC (*circles*) and of the charge transferred across the membrane ΔQ, (*line*) induced by Na,K-ATPase activity after an ATP concentration jump induced by an UV-light flash (*arrow*). The data were obtained by analysis of the experiment presented in panel A. The buffer contained 150 mM NaCl, 10 mM $MgCl_2$, 30 mM imidazole, 1 mM dithiothreitol, pH 7. The concentration of membrane fragments added initially was approximately 50 μg/ml, the concentration of caged ATP was 100 μM. The experiment was performed at room temperature

the Teflon cell via salt or agar bridges. The solution in the salt bridges was identical to the electrolyte in the cell. One of the electrodes was either grounded or connected to an alternating voltage source. The other electrode was connected to the input of a Keithley 427 current amplifier (Keithley, Cleveland, OH). The setup is described in detail in a recent paper (Sokolov et al. 1998). the 'background' current induced by AC voltage due to conductance and capacitance of the compound membrane system, was minimized by an analogue compensation circuit before enzyme phosphorylation by an ATP-concentration jump as described before (Sokolov et al. 1992).

Two different techniques were used for capacitance measurements. The first is described by Sokolov et al. (1992). Since the pump induced charge transfer and the changes of capacitance cannot be measured at the same time, two consecutive experiments are performed within a short time interval, the first without and the second with an alternating voltage of triangular shape with the amplitude of 30–100 mV and a frequency of 15 Hz applied to the compound membrane (Fig. 3A, traces 1–3). Transferred charge and capacitance increment on the same membrane and at the same flash energy do not change significantly in subsequent repetitions, as has been checked (data not shown). The output of the current-voltage amplifier is recorded in one channel of a digital oscilloscope KDS-102 (Kawasaki, Japan) or Nicolet 4094A (Nicolet, Madison, WI), the correspondent membrane voltage in the second channel of the oscilloscope. After data transfer to a com-

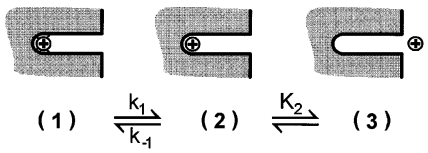

Fig. 4 Schematic representation of the simplified reaction scheme. In the absence of K^+ ions and ADP the action of the ion pump has been reduced to a three state model in which in state (1) the ion is bound to the binding site at the inner terminus of the access channel, in state (2) the ion is released from its site but still inside the protein, and in state (3) the ion is outside the protein. Only the latter reaction step contributes to the measured charge movement. Compared to the binding/release step the electrogenic diffusion through the access channel is so fast that it can be treated as a quasi equilibrium between states (2) and (3) with the equilibrium constant K_2

puter the current component induced by capacitive changes in the membrane fragments is determined by subtraction of the pump current in the absence of the alternating voltage and the uncompensated system capacitance immediately before pump activation (Fig. 3A, trace 4). Conductance and capacitance changes are calculated from a linear approximation of the current-voltage dependence (Sokolov et al. 1992). This technique has limitations at high frequencies due to limited conversion rate of the A/D converter available. In the second technique a sine voltage with an amplitude of 50 mV is applied to the compound membrane system and the current-voltage amplifier output is transmitted to a lock-in analyzer PAR 5204 (Princeton Applied Research, Princeton, NJ). The change of capacitance is determined by the amplitude of the current component with a phase shifted by 90° with respect to the phase of the sine voltage input to the BLM. According to principles of the lock-in technique this current component is generated by capacitive effects of the analyzed system, i.e. the compound membrane system. The influence of the current amplifiers used on the phase shift is tested and taken into account. The second technique is used in the high frequency range, since its sensitivity is decreased at low frequencies. To obtain the frequency dependence in a wide range, both techniques were combined: the first method was used at lower frequencies (1–300 Hz), the second at higher frequencies (30–5000 Hz). In the overlapping range of frequency both methods led to capacitance changes which coincided within the scatter of data of each method.

Since the model system was composed of two membranes connected in series, the BLM and the adsorbed membrane fragments (Fig. 2), it is impossible to measure directly both the current through the Na,K-ATPase (or correspondingly the net charge transferred, ΔQ) and the contribution of the transport to the capacitance increment, ΔC. However, the 'true' current through Na,K-ATPase can be calculated on the basis of equivalent circuit analysis (Borlinghaus et al. 1987). A similar, simplified procedure was used in the present study. It is described in Appendix II of this paper. Using the simplified equivalent circuit of a BLM with adsorbed membrane fragments restricts the analysis of measurements: the time interval of a single capacitance measurement should not exceed 0.5 s and in consequence the frequency of the measurement has to be higher then 2 Hz. Typically a frequency of 15 Hz was used. The capacitance increment, ΔC, reached the maximum value within 0.5 s, and decayed slowly with time (Fig. 3B). To decrease errors due to the scatter of data, ΔC was determined as average value in a time interval of one second starting 0.5 s after the light flash when ΔC had reached a plateau. During this time interval the decay of ΔC was negligible.

To determine the charge transferred after release of ATP from caged ATP the short circuit current after the triggering light flash was recorded in digitized form, transferred to a computer and subsequently integrated numerically to obtain the charge transferred by Na^+ translocation. The charge increased after the light flash, reached its maximum within 200–300 ms and decreased slowly thereafter. The maximum value, Δq, was taken as a measure of the total charge transferred by the pump (Fig. 3). The subsequent decrease of the charge was caused by discharging of the compartment between the two membranes through leak conductance pathways (Wuddel and Apell 1995). In order to decrease discrepancies caused preferentially by variations of the membrane bilayer area in different experiments, the 'background' capacitance of each BLM, which can be assumed to be proportional to the area, was measured after adsorption of membrane fragments. The charge transferred after the ATP-concentration jump as well as the capacitance increment were measured and normalized to a 'background' capacitance of 2 nF which corresponds to a 'standard' BLM area.

Theory: Calculation of the Na,K-ATPase admittance within the 'access channel' model. Let us consider a model suggesting that Na^+ exchanges between binding sites in the states of P-E_2 and the extracellular solution through an 'ion well' or 'access channel' (Lauger 1991). According to recent data with Na,K-ATPase containing membrane fragments from rabbit kidney the electric current is carried mainly by release of the first sodium ion to the aqueous bulk, P-$E_2(Na_3) \rightarrow$ P-$E_2(Na_2)$, with a dielectric coefficient of ~0.65 (Heyse et al. 1994; Wuddel and Apell 1995). Data of membrane patches from cardiac cell led to similar results (Hilgemann 1994). Therefore, we will consider for the sake of simplicity the translocations of only a single Na^+ ion. The ion is supposed to be in one of three states: (1) bound in the protein ('occluded' state), (2) inside the channel close to the binding site, and (3) outside of the protein in the solution (Fig. 4). To transfer the ion from the bound state (1) into the solution, state (3), it must pass through state (2). It is assumed also that ions in different protein molecules are independent of each other (Nieto-Frausto et al. 1992). Let N be the total number of protein molecules participating in the process, and n_1 the number of proteins with ions bound, state (1), n_2 the number of proteins with ions in the channel, state (2), and n_3 the number of 'empty' proteins in state (3). We designate

$$\theta_1 = n_1/N; \quad \theta_2 = n_2/N; \quad \theta_3 = n_3/N; \quad \theta_1 + \theta_2 + \theta_3 = 1 \qquad (1)$$

Binding of an ion to its site in the channel is characterized by rate constant k_1, the dissociation process by k_{-1}. In agreement with Rakowski (1993) we assume binding of the ion to and release from the site as electroneutral. Then we can write the following balance equation for state θ_1

$$\frac{d\theta_1}{dt} = -k_1 \cdot \theta_1 + k_{-1} \cdot \theta_2 \quad (2)$$

In contrast to this process the transfer of ions from inside the channel to the external solution is electrogenic. According to Hilgemann (1994) we assume that the translocation within the channel is so fast that the channel is in a quasi equilibrium with the solution at the frequencies which we applied in the presented experiments. Instead of the trans-membrane voltage φ (Fig. 2) we use in the following the reduced, dimensionless voltage $\psi = e_0\varphi/kT$ expressed in units of $kT/e_0 \approx 25$ mV; k is the Boltzmann constant, T the absolute temperature, and e_0 the elementary charge. The equilibrium between state (2) and state (3) can be described by the Boltzmann distribution between both states

$$\theta_2 = K_2 \cdot [\mathrm{Na}] \cdot \theta_3 \exp(\alpha\psi) \quad (3)$$

where K_2 is equilibrium constant of the reaction, $\theta_2 \rightleftharpoons \theta_2 + \mathrm{Na}^+$. [Na] is the sodium concentration in the solution and α the dielectric coefficient, determined by the fraction of the trans-membrane potential, ψ, that drops in the access channel (Läuger 1991). A voltage-induced shift of the equilibrium between θ_2 and θ_3 affects also the population of the state θ_1 according to Eq. (2). The maximum charge inside the protein, $Q_{\max}$, (i.e. $\theta_1 + \theta_2 = 1$), is given by

$$Q_{\max} = N e_0 \quad (4)$$

When the Na,K-ATPase is supplied with ATP in the presence of Na^+ ions only and when the concentration of ADP is negligible, the pumps are confined almost quantitatively to their posphorylated states of E_2 since the back reaction to E_1 may be neglected due to the extremely slow back reaction $P\text{-}E_2 \rightarrow E_1$ (Fig. 1). Under this condition the electric currents, $I(t)$, which are detected upon application of a sine-wave voltage, result from ion movements through the access channel between external solution and ion binding site inside the protein. Therefore $I(t)$ can be defined by the derivative of states (1) and (2) which contains ions inside the protein:

$$I = \alpha Q_{\max} \, d/dt(\theta_1 + \theta_2) \quad (5)$$

The dielectric coefficient α represents also a dielectric distance (normalized to the thickness of the protein) across which the ion is moving. If the protein is not a homogeneous dielectric, the topologic and dielectric distance are not necessarily the same. Equations (1)–(5) allow the calculation of the system admittance. The voltage across the membrane is composed of a constant and an alternating component

$$\varphi = U + V\cos(2\pi ft) \quad \text{or} \quad \psi = u + v\cos(2\pi ft) \quad (6)$$

with $u = e_0 U/kT$ and $v = e_0 V/kT$. For the sake of simplicity the amplitude of the alternating voltage is assumed to be small:

$$v \ll 1 \quad (7)$$

This constraint allows the linearization of Eq. (3) and the finding of an approximate solution of Eq. (2) when nonlinear terms are neglected.

The voltage-induced current consists of components controlled by membrane conductance G and capacitance C:

$$I(t) = G\varphi + C\,d\varphi/dt \quad (8)$$

from which we derived the following equations to describe the influence of voltage induced ion transport on conductance and capacitance:

$$C = C_0 \frac{f_0^2}{f^2 + f_0^2} + C_1, \qquad G = C_0 \cdot 2\pi f_0 \frac{f_0^2}{f^2 + f_0^2}, \quad (9)$$

$$f_0 = \frac{1}{2\pi}\left[k_1 + \frac{k_{-1}}{1 + \exp(\psi_1 - \alpha u)}\right],$$

$$C_0 = \frac{\alpha^2 Q_{\max} \cdot e_0 / kT}{4\left[\cosh\left(\frac{\alpha u - \psi_0}{2}\right)\right]^2}, \qquad C_1 = \frac{\alpha^2 Q_{\max} \cdot e_0 / kT}{4\left[\cosh\left(\frac{\alpha u - \psi_1}{2}\right)\right]^2},$$

$$\psi_0 = \ln\left(\frac{k_1}{k_1 + k_{-1}} \cdot K_2 \cdot [\mathrm{Na}]\right), \quad \psi_1 = \ln(K_2 \cdot [\mathrm{Na}])$$

If no alternating component of the membrane voltage is present, an equilibrium state will be reached. The charge, Q_b, inside the access channel of the protein in the equilibrium state is described by the well known Fermi function (Nakao and Gadsby 1986; Rakowski 1993), which can be easily obtained from Eqs. (2) and (3)

$$\theta_1 + \theta_2 = \frac{Q_b(u)}{Q_{\max}} = \frac{1}{1 + \exp(\psi_0 - \alpha u)}. \quad (10)$$

Results

After membrane fragments were adsorbed to a BLM a 40 µs UV-light flash was applied which released ATP from 100 µM caged ATP with a yield of approximately 13%, and the Na,K-ATPase underwent the reaction sequence from state Na_3E_1 to state $P\text{-}E_2$ (Fig. 1) in a synchronized way and produced a current transient as shown in trace 1 of Fig. 3A. In this partial reaction charge was transferred (Borlinghaus et al. 1987, Wuddel and Apell 1995) and produced a capacitance increment. In Fig. 3B the amount of charge transferred, ΔQ, upon the release of ATP (solid line) and the corresponding capacitance increment, ΔC, (points) are plotted as a function of time. These experiments were repeated at various frequencies of the externally applied alternating voltage between 15 Hz and 6000 Hz. The dependence of the capacitance increment, ΔC, on the frequency f of the voltage in the presence of

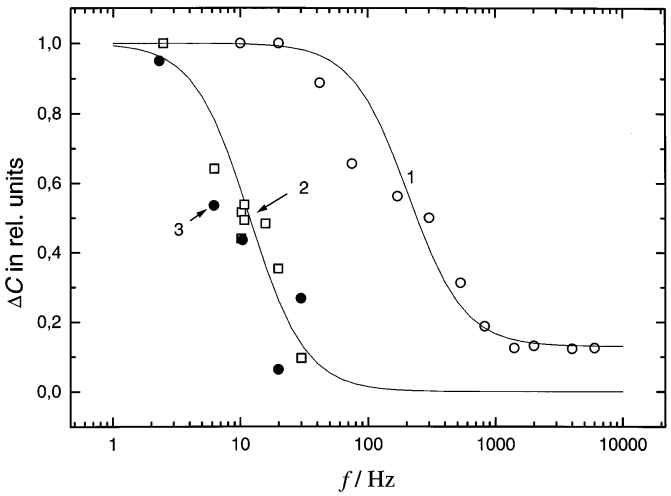

Fig. 5 Dependence of capacitance increment (in relative units) on frequency of alternating voltage with frequency f and an amplitude of 50 mV. The aqueous buffer contained 10 mM $MgCl_2$, 30 mM imidazole, 1 mM dithiothreitol, pH 7 and varying salt concentrations: (1) 150 mM NaCl, (2) 1 M NaCl, (3) 150 mM NaCl+850 mM choline chloride. The values of ΔC at the lowest frequency measured (2.48 Hz) were used normalize the relative changes of capacitances. The *solid lines* are the theoretical curves calculated using Eq. (9) with the following values of parameter f_0: 207 Hz (1), 12 Hz (2 and 3)

150 mM CaCl is shown in Fig. 5 (open circles). Below 20 Hz the capacitance increment was frequency independent. As shown in the Theory section, the low-frequency plateau allows the determination of the capacitance increment corresponding to an equilibrium distribution of ions in state P-E_2 in the protein. At high frequencies ($f > 2000$ Hz) the capacitance increment decreased to a constant finite value of approximately 15% of the low-frequency plateau. Equation (9) describes the frequency dependence of the capacitance increment induced by redistribution of mobile charge between two states. As can be seen from this equation, the dependence has a 'characteristic' frequency, f_0, at which the capacitance increment is half maximal. The experimental data were fitted to obtain f_0. For this purpose we neglected the constant high frequency component of the capacitance increment and used a slightly modified version of Eq. (9) which describes respective capacitance changes as:

$$\frac{C}{C_0} = \frac{f_0^2}{f^2 + f_0^2} + \Delta \qquad (11)$$

where Δ is a fitting parameter to the data which corresponds to C_1/C_0. At high ionic strength Δ was set to zero since no capacitance changes could be determined at high frequencies. At 150 mM NaCl f_0 was about 200 Hz (Fig. 5). At a sodium concentration of 1 M f_0 was shifted to a lower value in the range of 12 Hz (Fig. 5). It was impossible to observe explicitly the low frequency plateau, since the lowest applicable frequency in our experiments was 2 Hz. However, a similar shift of frequency dependence could be obtained when the experiment was performed in buffer containing 150 mM NaCl and an ionic strength elevated to 1 M by addition of 850 mM choline chloride (Fig. 5). Choline ions do not bind to the ion sites of the Na,K-ATPase. This observation indicated that the shift of f_0 is caused by ionic strength rather than by sodium concentration.

In contrast to f_0 the low-frequency capacitance increment, $\Delta C(f \to 0)$, was dependent on Na^+ concentration but independent of the ionic strength (data not shown). For further investigations we therefore measured the maximum capacitance increment at low frequencies only. Measurements of capacitance increments conducted at 15 Hz produced values of the low-frequency plateau of the frequency dependence when the Na^+ concentrations was restricted to the range $\leqslant 400$ mM (data not shown).

In order to obtain detailed information on electrogenic properties of the Na,K-ATPase we studied the dependence of the capacitance increment, ΔC, on the amount of charge transferred in ATP-concentration jump experiments. The experiments were performed with different membranes, and the time course of the current transients and capacitance increments were measured at various light energies and in the presence of Na^+ concentrations between 0.5 and 400 mM. The amount of ATP released from caged ATP was proportional to the intensity of the light flash. Transferred charge and capacitance increment were determined as described above. The capacitance increment per amount of charge transferred by the ion pumps was fairly constant when measured with different membranes as long as the Na^+ concentration was kept constant. This observation allowed a quantitative comparison of data from different membranes with buffer of the same Na^+ concentration.

Figure 6 represents the dependency of the capacitance increment, ΔC, on the amount of charge transferred, ΔQ, measured at variable intensities of the light flash in solutions of standard buffer with the indicated concentrations

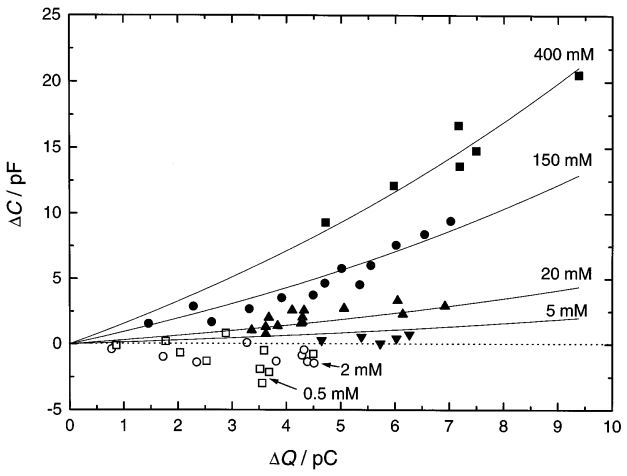

Fig. 6 Dependence of capacitance increment, ΔC, on charge transferred across the membrane, ΔQ, after an ATP concentration jump at various NaCl concentrations. Other buffer conditions as given in Fig. 4. The theoretical curves (solid lines) were calculated according to Eq. (13) with the adjustable parameters C_m=447 pF for all curves, and Ψ_0 (dependent on the Na^+ concentration): 53 mV (400 mM), 67 mV (150 mM), 96 mV (20 mM), 116 mV (5 mM). The data obtained at Na^+ concentrations below 5 mM could not be fitted by the model presented

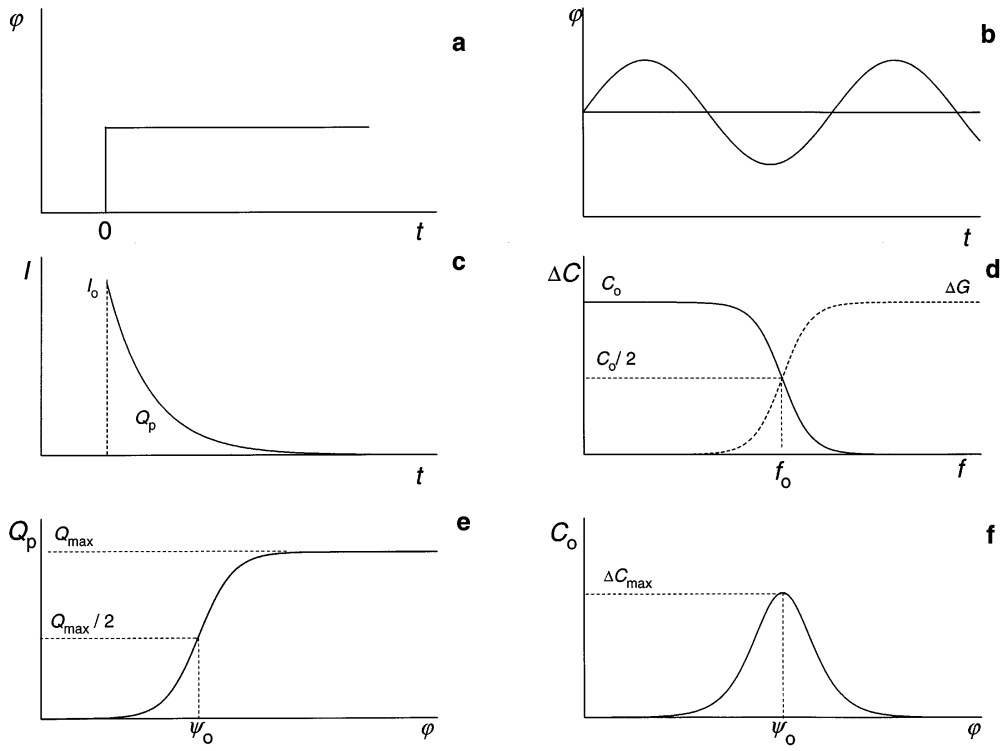

Fig. 7 a–f Comparison of features of current relaxation experiments (*left column*) and admittance experiments (*right column*) performed with electrogenic transport systems and the information obtained there from. Panels **a** and **b** represent the potential pattern applied to the membrane. **c** Current transient induced by the voltage jump. The integral of the current, Q_P, corresponds to the amount of charge transferred across the membrane by the relaxation process. **d** Dependence of capacitance increment, ΔC, and conductance increment, ΔG, on frequency f of the externally applied AC voltage. A half maximum change of the capacitance increment is observed at the frequency f_0. **e** Dependence of Q, on the applied membrane potential φ; Q_{max} is the maximum amount of charge which can be moved through the protein. Half of the maximum charge is moved at the midpoint potential, Ψ_0. **f** Dependence of the capacitance increment at the low frequency plateau, C_0, as function of the transmembrane potential, φ. $C_0(\varphi)$ is the derivative of $Q_P(\varphi)$ (see text)

of NaCl. At a given ΔQ the capacitance increment was dependent on the Na^+ concentration. The higher the Na^+ concentration the greater was ΔC at the low-frequency plateau. The theoretical curves in Fig. 6 are drawn according to calculations from the model presented in the Discussion section. In solutions with Na^+ concentrations of 0.2 mM and 2 mM the capacitance increment became negative.

Discussion

Dependence of capacitance upon frequency

In a previous paper we detected a capacitance increment associated with pumping activity of Na,K-ATPase and proposed several possible explanations for the effect (Sokolov et al. 1992). The most interesting proposal suggested that the capacitance increment is associated with ion translocations inside the protein. In earlier investigations the Na,K-ATPase was studied by a current relaxation method (Nakao and Gadsby 1986; Rakowski 1993; Hilgemann 1994). However, it can also be studied by the admittance method, i.e. by measurement of membrane capacitance and conductance. In the Appendix we illustrate the correspondence of the results from analysis of transient currents by the two methods with a simple model. A comparison of both methods is shown in Fig. 7. Kinetic information is obtained in current relaxation experiments from the rate constant of the exponential decay (Fig. 7c). In the admittance method the frequency dependent capacitance increment can be described by a Lorentzian function with a characteristic or midpoint frequency, f_0 (Fig. 7d). As has been shown in the Appendix, the rate constant of the relaxation method and the midpoint frequency of the admittance method contain identical information. This finding allows the correlation of our results with the data obtained in earlier experiments.

The frequency dependency in our experiments was of Lorentzian type (Fig. 5) with the midpoint frequency f_0 equal to 200 Hz. With, Na,K-ATPase purified from a different source (pig kidney) we obtained the same frequency dependence and a comparable value of f_0 (Sokolov et al. 1994). A similar dependence was obtained on giant patch clamp of cardiac myocytes with f_0 equal to 140 Hz (Lu et al. 1995). However, a comparison of these numbers with rate constants of the exponential decay of currents obtained by voltage jump experiments with Na,K-ATPase in cell membranes indicates that different processes may be monitored. As follows from Eq. (A1), the frequency $f_0 = 200$ Hz corresponds to a relaxation with an exponential-decay time

constant of $2\pi f_0 \approx 1250\ s^{-1}$. The time constant measured in voltage-jump relaxations is the rate-limiting conformational transition, $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3)$, which was $200\ s^{-1}$ at 0 mV and 37 °C in cardiac cells using whole-cell patch clamp (Nakao and Gadsby 1986). In oocytes the same value was obtained at room temperature (Rakowski 1993; Holmgren and Rakowski 1994). A higher time constant ($600\ s^{-1}$) was observed in the cardiac myocytes using the giant patch clamp technique (Hilgemann 1993). A recent study of relaxation processes in the Na,K-ATPase, in which a so-called charge-pulse technique was applied to the same compound membranes, resulted in the finding that the conformational change has only a minor contribution to the electrogenicity of the Na^+ transport, and that the release of the first Na^+ produces the major component of the observed current which had a comparable time constant of about $1000\ s^{-1}$ (Wuddel and Apell 1995). A similar finding has been published recently for the Na,K-ATPase of the squid axon (Wagg et al. 1997). In the light of these observations the experiments presented in Fig. 5 detect predominantly the release of the first Na^+ ion on the extracellular side of the ATPase, $P\text{-}E_2(Na_3) \rightleftharpoons P\text{-}E_2(Na_2)$.

We found that the value of f_0 decreased considerably at high ionic strengths. This means that the kinetics of charge redistribution in the electric field are slowed down with increasing ionic strength. A likely explanation for the influence of ionic strength on the kinetics of charge translocation may be that ion translocation involves conformational rearrangements in the protein structure which become slow enough to affect the observable rates at high ionic strength. In this case a deceleration of the transition should slow down the charge relaxation process. Sodium transport includes a major conformational transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3)$ which is the rate-limiting step of this partial reaction (Sokolov et al. 1998), and which occurs prior to release of the first sodium ion (Fig. 1). It is reasonable to assume that the kinetics of sodium transfer are controlled by the E_1/E_2 transition which may be affected by the ionic strength of the solution. In addition, Wuddel and Apell (1995) provided evidence that the release of the first Na^+ ion is also accompanied by a minor conformational relaxation as proposed by Hilgemann (1994) and Wagg et al. (1997). It seems to be likely that the conformational transitions are affected by anions rather than by ionic strength according to the so-called Hoffmeister effect. It was shown by Post and Suzuki (1991) that high salt concentration influences the distribution of Na,K-ATPase between conformations E_1/E_2 and that this effect was specific to anions in the Hoffmeister series. Later it was demonstrated that high salt concentrations decelerate the E_1/E_2 conformational transition (Klodos et al. 1994). The mechanism of the influence of high salt concentrations on conformational transitions is unknown so far. According to a recent hypothesis the high salt concentration will influence the conformational state of the protein via a phase transition of the surrounding lipids (Post and Klodos 1996).

Recently the concept of an 'access channel' in the Na,K-ATPase became widely accepted. This concept implies that sodium ions are transported passively through a narrow well connecting ion binding sites and the extracellular aqueous phase of the membrane and which crosses part of the transmembrane potential (Läuger 1991; Stürmer et al. 1991; Rakowski 1993; Gadsby et al. 1993; Hilgemann 1994). When the membrane voltage is changed the Na^+ ions redistribute accordingly between the states of the sequence $(Na_3)E_1\text{-}P, \ldots, P\text{-}E_2$. In our simplified model we reduce this process to its two end states: bound to the phosphorylated protein in the E_1 conformation, $(Na_3)E_1\text{-}P$, and outside the pump molecule, $P\text{-}E_2$ (Fig. 4). These two states can be assigned to θ_1 and θ_3 of the model, respectively. The observation that the rate constant of the current relaxation after a membrane voltage step is small and that it exhibits a strong temperature dependence (Gadsby et al. 1991) is another indication that the kinetics of redistribution are affected by the rate-limiting conformational transition E_1/E_2 or a subsequent conformational rearrangement related to the release of the first Na^+ ion. The diffusion of ions in the channel-like well is expected to be much faster, and such fast processes were observed recently in patch clamp experiments (Hilgemann 1994; Rettinger et al. 1994) and charge-pulse experiments (Wuddel and Apell 1995). According to the 'access channel' concept a model was proposed to explain the kinetics of the slow current relaxation as well as the sodium concentration dependence (Rakowski 1993). Since this model was applied so far to voltage-clamp experiments only, in the Appendix we analyzed a similar model for application in admittance measurements. If we take into account only the slow charge relaxation, to be in agreement with Rakowski (1993), the capacitance according to Eq. (A2) should drop to zero at high frequencies. However, this is in contradiction to the experimental observation. From Fig. 5 it is obvious that the frequency dependence has two plateaus, both at low and high frequencies. According to the model discussed in the Theory section, the plateau at high frequencies (substantially above the characteristic frequency f_0) corresponds to a condition when the slow relaxation process, which is associated with binding/release of the ions, no longer contributes and the capacitance increment may be caused by fast charge relaxation within the access channel. At the moment we cannot rule out other possible explanations for the high frequency plateau, for instance, a change of the system capacitance due to electrostriction in the membrane (Sokolov et al. 1994). However, the existence of the high frequency plateau is supported by results obtained in a different experimental system (Lu et al. 1995), therefore the latter explanation seems to be less probable. From the experimental results presented we can derive an important conclusion: since the capacitance increment at high frequencies is much smaller than at low frequencies, the contribution of fast processes (together with possible artifacts such as electrostriction) are negligible at the low frequency plateau.

Low frequency capacitance measurements

In measurements at low frequencies the ion distribution is in equilibrium with respect to the applied voltage. As

shown in the Appendix, the dependence of the capacitance of the low frequency plateau on voltage, $C_0(u)$, can be obtained as the derivative of the steady state charge distribution with respect to voltage, dQ_b/du. Dependencies of the steady state charge distribution on voltage were studied with cells by the voltage clamp technique (Nakao and Gadsby 1986; Rakowski 1993; Hilgemann 1994), and two characteristic parameters could be derived, the maximum amount of transferred charge, Q_{max}, and the potential, ψ_0, at which the mobile charge was distributed equally between two states. This potential is equal to the constant voltage component at which the maximum capacitance increment can be observed (Fig. 7e, f). The existence of such a maximum capacitance in the voltage dependence of the capacitance increment was reported by Lu et al. (1995); however, the measurements were conducted at high frequencies where the increment is due to fast ion migration processes. In order to determine the equilibrium parameters of the slow process the measurements have to be performed at the low frequency plateau. This was not possible, however, in our experimental system, which consists of two coupled membranes (Fig. 2) and which thus did not allow direct control and maintenance of a (constant) voltage, φ, across the ATPase-containing membrane. Therefore, the dependence of the capacitance on constant transmembrane voltage and its maximum, ΔC_{max}, could not be determined directly as plotted in Fig. 7f.

It is possible, however, to estimate the location of the maximum, $\Delta\psi_0$, even in the compound membrane system. The Na,K-ATPase transports ions from the bulk solution into the enclosed compartment between the two membranes, thus creating a virtually constant voltage across the membrane for several seconds, as long as ATP hydrolysis occurs. The amplitude of this voltage depends on the amount of charge transferred per area. The amount of charge per area can be controlled by the concentration of ATP which is released by an UV-flash of a given intensity in the presence of a chosen concentration of caged ATP (Sokolov et al. 1998). The dependence of the capacitance increment, ΔC, on the charge transferred, ΔQ, is shown in Fig. 6. The transferred charge can be used as an independent parameter and, despite a variation in amplitude of the short-circuit current in different experiments and membranes, the same ΔQ produced quantitatively the same ΔC when the Na^+ concentration was kept constant. The charge transferred is proportional to the number of ATP-activated pump molecules since upon ATP release each pump performs only one turnover in the observation period of 1–2 s (Wuddel and Apell 1995). Therefore the capacitance increment should be a function of the charge under the assumption that the increment is generated from a redistribution of sodium ions sites in the protein and the aqueous phase. A functional relation between ΔC and ΔQ could be derived (see Appendix) and was applied to the experiments presented in Fig. 6. First of all, the capacitance increment depended not only on the charge transferred, ΔQ, but also on the sodium concentration. In addition, the dependence of ΔC on ΔQ was non linear (Fig. 6). The deviation from linearity indicates that the capacitance increment depends on at least one more parameter. In the following we discuss the voltage across the membrane fragments, which is generated by the charge transferred, as the origin of the observed non-linearity in the framework of the presented model.

An expression for the capacitance increment across the membrane fragments, ΔC_P, at low frequencies can be derived from the Theory section of this paper. If we disregard fast processes at low frequencies (i.e. C_1=0), we can simplify Eq. (9) to be:

$$\Delta C_P\big|_{f\to 0} \cong C_0 = \frac{\alpha^2 Q_{max}\cdot e_0/kT}{4\left[\cosh\left(\frac{\alpha u-\psi_0}{2}\right)\right]^2}, \qquad (12)$$

where Q_{max} and ψ_0 are defined as above. The dielectric coefficient, α, determines the fraction of the external voltage that influences the charge relaxation process (Läuger 1991). In the case of our simplified model it was considered as 'depth' of the access channel, but generally it may also include the valence of ions and a Hill coefficient to characterize the cooperativity of ion binding in the sites when more than one ion binds per ATPase (Rakowski 1993).

ΔC_P, Q_{max} and $u = e_0 U/kT$ would be directly accessible in experiments with cell membranes; however, they cannot be measured directly in the case of compound membranes. As shown in the Appendix it is possible to relate these parameters to the measured quantities, $\Delta C\big|_{f\to 0}$ and ΔQ, which leads to the following relation

$$\Delta C\big|_{f\to 0} \cong \alpha\gamma\ \frac{e_0}{kT}\cdot\frac{\Delta Q}{1+\exp(\psi_0-\alpha u)} \qquad (13)$$

$$\text{with}\quad u=\frac{e_0}{kT}\cdot\frac{\Delta Q}{C_m}$$

which provides the functional description of the relation between measured capacitance increment, ΔC, and charge, ΔQ, as shown in Fig. 6. The value of dielectric coefficient α was taken to be 0.65, the coefficient γ depends on the relation between the specific capacitances of BLM and protein containing membrane fragment (see Appendix) and was adjusted at 1/3. Thus, to fit experimental data by the function (Eq. (13)) we used only two free parameters, the capacitance of the active region of membrane covered by the adsorbed membrane fragments, C_m, and the potential of maximum capacitance increment, ψ_0.

It seems reasonable to suggest that C_m did not vary considerably in the experiments since they were conducted only after adsorption of membrane fragments was saturated and the results were normalized to standard BLM area. Therefore, we used the same value of C_m for all experiments in Fig. 6. All theoretical curves fit satisfactorily the experimental results at all Na^+ concentrations above 5 mM with C_m = 447 pF. (At lower Na^+ concentrations negative capacitance increments were observed, which could not be fitted by the presented theory, see below). The value of C_m was approximately 1/5 of the total BLM capacitance, which was normalized to a 'background' capacitance of

2 nF. Taking into account that the specific capacitance of the fragments is about twice that of the lipid bilayers (Eq. (18)) it can be estimated that the fragments occupy about 15% of the bilayer in our experiments. This value is in the range of the estimates made by other authors (Borlinghaus et al. 1987).

The values of ψ_0 depended on Na^+ concentration. A similar result obtained by Rakowski (1993), where the steepest slope in the charge-voltage curve, the so-called midpoint potential, was shifted with Na^+ concentration. This shift could be adequately described by the Nernst equation. In our experiments ψ_0, which corresponds to the midpoint potential (Fig. 7e, f), exhibited a less pronounced dependence upon Na^+ concentration than expected by the theory. According to Eq. (9) the dependence of the capacitance maximum point upon sodium concentration should be described by the Nernst equation

$$\frac{\alpha V_0}{kT/e_0} \equiv \psi_0 = -\ln\left(\frac{k_1}{k_1 + k_{-1}} \cdot K_2 \cdot [\mathrm{Na}]\right) \qquad (14)$$

where V_0 is the midpoint potential, all other parameters are as given in the Theory section. The ψ_0 obtained for different Na^+ concentrations from Fig. 6, however, could not be fitted with Eq. (14). It should be noted, however, that we made measurements in a very wide range of concentrations of NaCl (5–400 mM). This range includes the conditions of high ionic strength, which are known to influence the kinetics of pumping (Wuddel and Apell 1995; Sokolov et al. 1998, present paper) as well as the conformational state of the Na,K-ATPase (Post and Suzuki 1991). Therefore, the disagreement of the dependence of ψ_0 on the concentration of NaCl determined experimentally and predicted by Eq. (14) may be explained by an influence of high ionic strength on the Na,K-ATPase. Another possible explanation may be that the presented model simplified the voltage-dependent process to the movement of only one Na^+ ion. Wuddel and Apell (1995) showed that the most electrogenic partial reaction, to which we reduced our model (viz. P-$E_2(Na_3) \rightleftharpoons$ P-$E_2(Na_2)$) is characterized by a dielectric coefficient of 0.65, while the complete reaction sequence $(Na_3)E_1P \rightleftharpoons \ldots \rightleftharpoons$ P-E_2 has a total dielectric coefficient of $\leqslant 1.05$ for the release of 3 Na^+ ions. If we assume that the reaction $\theta_2 \rightleftharpoons \theta_3$ in reality includes the electrogenic effect of all three Na^+ ions, the simplified model (Eq. (14)) would underestimate the observed electrogenic process by $1.05/0.65 \approx 1.6$. This indication of an imperfection of the applied model led us to the decision not to derive binding constants from the data at the present state of the investigations.

At low Na^+ concentrations negative capacitance increments, ΔC, were detected (Fig. 6). It has to be stated that, due to basic principles, the contribution of a charge movement within the membrane protein dielectric cannot produce negative capacitance change. Therefore, a likely explanation of the negative capacitance increment is that at low Na^+ concentrations another charge relaxation process exists prior to ATP release and its contribution to the apparent membrane capacitance disappears upon enzyme phosphorylation. In the absence of ATP the pump molecules are confined to the states $E_1 \rightleftharpoons \ldots \rightleftharpoons Na_3E_1$ (Fig. 1). It has been shown that binding of one Na^+ ion in the E_1 conformation is electrogenic (Heyse et al. 1994; Schulz and Apell 1995; Or et al. 1996), Na^+ has an (apparent) equilibrium dissociation constant in the range of 3–6 mM in state E_1 (and a dielectric coefficient in the range of 0.25 according to Wuddel and Apell 1995). Therefore, at low Na^+ concentrations a capacitance increment may be expected due to Na^+ binding and release to the cytoplasmic sites in the E_1 conformation of the Na,K-ATPase. Upon enzyme phosphorylation after the ATP concentration jump the E_1/E_2 conformational change takes place and the ion-binding sites disappear from the cytoplasmic side of the protein and are presented to the extracellular face of the protein. Because of the low sodium affinity of the P-E_2 states it has to be expected that only one state, P-E_2, will be populated at Na^+ concentrations below 5 mM (Heyse et al. 1994; Wuddel and Apell 1995). With a K_M of about 100 mM and a dielectric coefficient of <0.2 for the reaction step P-E_2 + $Na^+ \rightleftharpoons$ P-E_2(Na) the applied sine-wave voltages (with amplitudes in the range of $kT/e_0 \approx 25$ mV) will not be effective enough to lead to a considerable population of state P-E_2(Na) by the provided electric driving force and virtually no capacitance increment can be detected. In consequence phosphorylation of the Na,K-ATPase in buffers of low Na^+ concentration will produce an apparent negative capacitance change as observed (Fig. 6). Negative capacitance increments were also observed in experiments with the GAT1 cotransporter, another type of electrogenic transport system (Lu et al. 1995).

It seems possible that investigations of the electrogenic transport by the Na,K-ATPase at extremely high and extremely low sodium concentrations will allow the study of so far unresolved states of the transport cycle. This will be the focus of subsequent research.

Appendix

I Equivalence between current relaxation and admittance method

The discussed model is similar in many respects to the 'two state model' described by Rakowski (1993). Hence, it seems useful to illustrate the equivalence of the two methods used for ion transport studies, current relaxation and admittance methods, on the basis of the simple model presented, as shown in Fig. 7. It should be noted that without any sophisticated setup the current relaxation method allows only a detection of slow charge translocations with time constants >1 ms, which correspond to the ion binding process inside the protein in the case of the Na,K-ATPase rather than diffusion of Na^+ ions in the access channel. Only recently have higher time resolutions been reported (Hilgemann 1994; Wagg et al. 1997). In the current relaxation method the membrane voltage is changed stepwise from one constant value to another (Fig. 7a). In the

case of the simple model, as shown in Fig. 4, the dependence of current on time is exponential (Fig. 7c)

$$I(t) = I_0 \exp(-2\pi f_0 \cdot t), \qquad (A1)$$

with f_0 as given in Eq. (9). Thus, the dependence of current on time allows the determination of the kinetic parameter f_0. The net charge translocated, Q_P, can be determined as the integral of the time course of the current, $I(t)$, after the voltage jump of amplitude $\Delta\varphi = u$. Measuring the dependence of Q_P on the increment of voltage we can obtain the dependence described by Eq. (10). The dependence of the charge on the trans-membrane voltage, φ, reflects the equilibrium distribution of ions (Fig. 7e) and allows the determination of the parameters $\alpha \cdot \Psi_0$ and Q_{max}. These parameters were determined for the Na,K-ATPase by Na-exchange experiments (Nakao and Gadsby 1986; Rakowski 1993).

The admittance method describes the contribution of the slow process, i.e. transitions between state (1) and (2) of our model (Fig. 4), to frequency dependent capacitance and conductivity increments according to Eq. (9)

$$C = C_0 \frac{f_0^2}{f^2 + f_0^2} + C_1, \qquad G = C_0 \cdot 2\pi f_0 \frac{f_0^2}{f^2 + f_0^2} \qquad (A2)$$

The capacitance decreases at high frequencies to a frequency independent constant C_1. The characteristic frequency f_0 is defined as the frequency where the capacitance increment is half maximal, $\Delta C = C_0/2$ (Fig. 7d). f_0 is the same parameter obtained by the current relaxation method from the exponential decay of the current (Eq. A1). There is a simple relation between frequency dependent fraction of capacitance and conductance:

$$\frac{G}{C - C_1} = \frac{2\pi f^2}{f_0} \qquad (A3)$$

Equation (A3) implies that f_0 can be calculated without measuring the frequency dependence. It would be sufficient to measure capacitance and conductance at a given frequency f. In practical operation, however, the accuracy of conductance measurements is not sufficiently precise.

At decreasing frequencies the capacitance reaches a steady value of $C_0 + C_1$. The value of C_0, as can be seen from Eq. (9), depends solely on equilibrium parameters. It can be obtained from Eq. (10) by determination of dQ_b/du. C_0 corresponds to the amount of charge transferred through (parts of) the membrane per change of trans-membrane voltage. At low frequencies the mobility of ions in the protein is fast enough to reach an equilibrium distribution at any voltage amplitude. Therefore, the capacitance can be determined from the equilibrium parameters.

There is a simple relation between the charge transferred, Q_P, (or charge still bound to the protein, Q_b) after a voltage jump measured by current relaxation methods as shown in Fig. 7e and the 'equilibrium' capacitance increment, C_0, as a function of the constant component of voltage (Fig. 7f). C_0 is the derivative of Q_P with respect to voltage, dQ_P/du (or $-dQ_b/du$). This statement holds regardless of the ion transport mechanism, although we proved it on the basis of the presented model. As can be seen from Eq. (9), C_0 reaches its maximum at the voltage ψ_0. In the current relaxation method this value is determined by the steepest slope point in the dependence of the transferred charge on voltage (Fig. 7e). According to Eq. (9) the relation between Q_{max} and C_0 becomes simple at the voltage ψ_0/α.

$$\alpha^2 \cdot Q_{max} = \frac{4kT}{e_0} \cdot C_0 \qquad (A4)$$

Taking into account that the current relaxation method measures charge movements by electrodes placed in the aqueous phases and that the ions are moved through the fraction α of the membrane only, the determined apparent maximum charge, $Q_{max,app} = \alpha \cdot Q_{max}$, can be calculated from C_0 according to Eq. (A4). Thus the admittance method allows the determination of the same parameters as the current relaxation method.

II Derivation of the functional dependence between ΔC and ΔQ

Under the assumption that BLM and fragments enclose an electrically isolated compartment (Fig. 3) and that the membrane fragments adsorbed to the lipid bilayer contain several thousands of active Na,K-ATPase molecules per μm^2, which are activated statistically by ATP, the voltage generated across each membrane fragment by the ion pumps may be assumed to be approximately the same across all fragments. From the equivalent circuit of the BLM and adsorbed membrane fragments, as shown in Fig. 2, the (steady state) voltage component across the membrane fragments, U, which results from transfer of the charge through the Na,K-ATPase, ΔQ_P, is given by the relation

$$\Delta Q_P = C_P U, \qquad (A5)$$

where C_P is the capacitance of the adsorbed membrane fragments. In our experiments the charge was determined as the integral of the current transient after a flash-induced ATP release. It should be noted that the current measured represented the external current, $I(t)$, rather then the pump current, $I_P(t)$, across the membrane fragment (Fig. 2); however, an analysis of the equivalent circuit allows an appropriate correction to determine I_P from I (Borlinghaus et al. 1987). The same approach was used in this treatment. We started, however, with a simplified equivalent circuit of BLM with adsorbed membrane fragments, in which the conductances of BLM and of protein containing membranes were neglected and only the capacitances were taken into account (Fig. 2B). This is justified for calculation of the fast charge movements and of capacitance changes at relatively high frequencies of the applied voltage. A criterion of validity of the simplified equivalent circuit can be derived from the analysis of the decay of cur-

rent integral with time (The current integral is proportional to voltage in the gap between BLM and membrane fragment, Fig. 2). The time constant of the decay was about 0.5 s which determined the lower limit of the applied frequency to be 2 Hz.

The charge Q_P transferred by the sodium pump through the adsorbed membrane fragments can be calculated from ΔQ, the integral of the measured current transient $I(t)$, if we take into account (1) the fact that Q_P is moved over the fraction α of the dielectric of the membrane capacitor of the fragments,

$$\Delta Q_P = \alpha Q_P \tag{A6}$$

and (2) the capacitive coupling of the BLM (C_F) and the protein containing membrane (C_P)

$$\Delta Q = \frac{C_F}{C_P + C_F} \cdot \Delta Q_P \equiv \gamma \cdot \Delta Q_P \tag{A7}$$

In the experiments we could not control directly the capacitance of membrane fragments containing the Na,K-ATPase but only the total capacitance of compound membrane consisting of BLM and adsorbed lipid fragments (Fig. 2). Only part of the membrane area was covered by the adsorbed membrane fragments and changed its "compound" capacitance, C_m, due to the action of the Na,K-ATPase. The capacitance of this active region can be derived from the two capacitors, C_P and C_F, connected in series

$$C_m = \frac{C_P C_F}{C_P + C_F} = \gamma C_P \tag{A8}$$

Using Eqs. (A5)–(A8) the voltage U across the membrane fragments can be derived from the measured charge ΔQ and parameter C_m

$$U = \frac{Q_P}{C_P} = \frac{\Delta Q}{C_m} \tag{A9}$$

The relation between the measured capacitance increment, ΔC, and the capacitance increment of the membrane fragments, ΔC_P, can be derived from the formula of two capacitors in series under the realistic assumption for the system used that C_F is not affected by the action of the Na,K-ATPase and that changes of the fragment capacitance are small compared to the fragment capacitance, $\Delta C_P \ll C_P$:

$$\Delta C = \Delta \frac{C_P C_F}{C_P + C_F} \cong \left(\frac{C_F}{C_P + C_F} \right)^2 \cdot \Delta C_P = \gamma^2 \cdot \Delta C_P \tag{A10}$$

Equations (A7) and A10) show that the measured parameters are related to the 'real' parameters by a simple coefficient, γ, the ratio of the capacitances of the bilayer, C_F, and the membrane fragments, C_P. The specific capacitance of phosphatidylcholine bilayers containing decane as solvent is two times less than the capacitance of solvent-free membranes (Benz et al. 1975; Benz and Janko 1976). Since the BLM contains decane while membrane fragments do not, the ratio of capacitances, C_F/C_P, differs from unity and can be estimated with fair accuracy to be 0.5 (Borlinghaus et al. 1987), hence

$$\gamma = \frac{C_F}{C_P + C_F} \cong \frac{1}{3} \tag{A11}$$

Q_b can be determined from the theoretical model (Eq. (10)) and a relation between Q_P and Q_b developed by the following considerations. When the ATPase transports Na^+ ions after ATP release from the cytoplasmic side to the outside, they are all bound initially. Therefore we can suppose that the charge of ions bound initially equals the maximum charge, Q_{max}, which can be transferred by the protein (Eq. (4)). After equilibration a fraction of the ions is released to the compartment at the extracellular side of the membrane, which is Q_P as determined in the experiment. Therefore, the initial charge, Q_{max}, is distributed between Q_b and Q_P

$$Q_{max} = Q_b + Q_P \tag{A12}$$

Inserting Eq. (10) in Eq. (A12) leads to

$$Q_P = \frac{Q_{max}}{1 + \exp(\alpha u - \psi_0)} \tag{A13}$$

Combining Eqs. (A6)–(A10) and (A13) results in the final expression given in Eq. (13).

Acknowledgements The authors are grateful to Prof. Yu. A. Chizmadzhev for fruitful discussions, to Prof. R. Post for his interest and direct support of the work which considerably facilitated its execution, and to Milena Roudna for excellent technical assistance. The work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 156).

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