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ARTICLE

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Assignment of charge movements to electrogenic reaction steps of Na,K-ATPase by analysis of salt effects on the kinetics of charge movements

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Abstract Na,K-ATPase-enriched membrane fragments adsorbed to lipid bilayers were used to study electrogenic Na^+ movements induced by enzyme phosphorylation when ATP was photo-released from inactive caged ATP, and simultaneously by externally applied alternating voltages which allowed the measurement of small ATP-induced membrane admittance changes. A detailed analysis of frequency dependence of the capacitance and conductance increments showed that the observed process consists of more than one electrogenic step. The frequency dependence could be described by the sum of two Lorentzian functions and a constant term. The faster process (~2000 s^{-1}) was assigned to the release of the first extracellular Na^+ ion. The corner frequency of the slower Lorentzian (about 30 s^{-1}) coincided with the reciprocal exponential time constant of the falling phase of the transient current, which can be assigned to the conformational transition. Preferentially, the slower process showed a dependence on the ion concentration of choline salts with different anions. The effectiveness of the used chaotropic anions to decelerate the kinetics decreased in agreement with the Hofmeister series, $I^- > Br^- > Cl^-$. This observation matches their effect on the partition between two phosphoenzyme states of the Na,K-ATPase, as established previously.

Keywords Na,K-ATPase · Ion movement · Electrogenicity · Capacitance · Transport kinetics

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Introduction

Na,K-ATPase is an important housekeeping protein in most animal cells and it maintains the intracellular high K^+ and low Na^+ concentrations by active ion transport. Recent extensive studies have led to substantial progress in the understanding of the transport mechanism of the ion pump (Apell and Karlish 2001; Jørgensen 1992). Detailed information on the kinetics of the active transport mechanism was obtained by electrical and optical studies, which allowed the identification of the electrogenic reaction steps of the transport cycle and the determination of their characteristic parameters (Clarke et al. 1998; Cornelius 1999; Heyse et al. 1994; Holmgren et al. 2000; Sokolov et al. 1998a; Wuddel and Apell 1995). Electric measurements can be performed in stationary and non-stationary modes. The non-stationary electrogenic transport was investigated mainly in the absence of K^+ ions when the Na,K-ATPase is confined to that part of the pump cycle which is associated with Na^+ transfer. Investigation of non-stationary electrogenic transport allows separation and analysis of the individual electrogenic steps of Na^+ transport (Fig. 1).

Two methods are widely used to trigger partial reactions of Na,K-ATPase and to induce transient electric currents: voltage-jump experiments and fast concentration steps of ATP. Lipid bilayer membranes (BLMs) with absorbed Na,K-ATPase-containing membrane fragments proved to be a convenient experimental system (Borlinghaus et al. 1987; Fendler et al. 1985). In such a set-up the ion pumps are capacitively coupled to the BLM, and it is possible to detect simultaneously transient currents generated by a fast release of ATP from caged ATP and by an externally applied alternating voltage (Sokolov et al. 1992, 1994, 1998a, 1998b). The interpretation of transient currents from ATP-jump experiments was based on a mathematical model of the Albers-Post cycle (Apell et al. 1987; Heyse et al. 1994; Sokolov et al. 1998a; Wuddel and Apell 1995), whereas

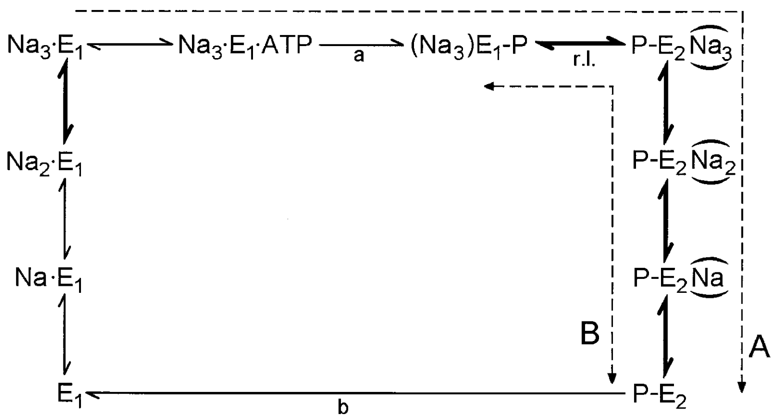

Fig. 1 Simplified Post-Albers reaction scheme of the Na-only transport mode of Na,K-ATPase on the basis of a scheme from Wuddel and Apell (1995). The left side of the diagram represents reactions occurring on the cytoplasmic side of membrane, the right side reactions at the extracellular side. Symbols E_1 and E_2 denote the two basic conformations of the protein, the *bold arrows* indicate transitions which are electrogenic and which contribute to a change of the apparent membrane capacitance. The *dashed arrows* indicate the transitions which may be included in two types of kinetic experiments investigated: (*A*) transient current after an ATP concentration jump induced by a photo-release from caged ATP and (*B*) reversible charge movement induced by an alternating voltage applied during admittance measurements in the presence of Na^+ and ATP. Owing to the nominal absence of ADP and of inorganic phosphate, the reaction steps *a* and *b* are irreversible. Also the reaction $P\text{-}E_2 \rightarrow E_1$ is negligible in the time frame of the experiments, owing to its low rate constant. The reaction $(Na_3)E_1-P \rightleftharpoons P-E_2(Na_3)$ is the rate-limiting step in the observed partial reactions

the interpretation of the voltage-jump experiments was based on a phenomenological "access channel" model (Rakowski 1993). Both treatments imply different assumptions and describe the transport process in terms of different parameters, so that a direct comparison of the results meets certain difficulties. However, in both types of non-stationary transport experiments the ions move through the same protein structures.

As can be seen from Fig. 1, the ATPase proceeds through a sequence of states to transfer Na^+ ions from the cytoplasm to the extracellular medium, and at least the electrogenic reaction steps are the same for both non-stationary techniques. If the rate-limiting step is part of both types of experiments, then it has to be manifest in the kinetic parameters measured by both techniques. Therefore, if the rate-limiting step is affected by appropriate experimental conditions, a comparison of the results from both types of experiments can be used to identify the crucial observable(s). A potential candidate for such experiments is the "salt effect". It was established that solutions of different salts affect the stability of protein conformations differently. All salts can be classified by their influence on protein conformations in two groups, as stabilizing (cosmotropic) and as destabilizing (chaotropic) agents. Following this classification, the ions can be ordered in a Hofmeister series according to their ability to affect proteins (Cacace et al. 1997; Collins and Washabaugh 1985). The influence of different anions on the conformational stability of Na,K-ATPase was clearly demonstrated, and the effectiveness of the anions was in agreement with the Hofmeister series (Post and Suzuki 1991). Recently we demonstrated that in a concentrated NaCl solution the kinetics of the electrogenic transport was notably slowed and the effect was comparable when Na^+ ions were replaced by choline$^+$ (Sokolov et al. 1998b). The salt effect on the investigated electrogenic transport correlates with the effect on the fluorescence changes of the dye RH421, which can be used to observe the action of Na,K-ATPase (Ganea et al. 1999).

However, it is also uncertain whether the salt effect is possibly related to other phenomena rather than to the influence of the chaotropic ions on protein conformation. Proposed phenomena are anion-induced changes of lipids surrounding the ion pump (Klodos et al. 1994), unspecific screening of charged amino acids of the protein, or competition at the ATP-binding site (Nørby and Esmann 1997). In order to test the effect of chaotropic ions on the protein conformation as the origin of the observed altered kinetics, more profound studies of the electrogenic ion transport in salt solutions with different anions are needed. To contribute to this discussion, with an advanced experimental technique and analysis, was the major purpose of this study.

Materials and methods

The measurements of electric signals related to the Na,K-ATPase activity were based on a method described previously (Borlinghaus et al. 1987). A BLM was formed on the orifice in the partition between two compartments of a Teflon cell. The cell had two optically transparent windows, one for visual observation of the BLM and the other, made from quartz, to illuminate the BLM with UV flashes. The cuvette was thermostated by a Peltier element mounted on the cuvette holder. The temperature was stabilized by control electronics with an integrated-circuit thermosensor in the cuvette holder. All experiments were carried out at a temperature of 24 °C. After formation of the BLM, Na,K-ATPase-containing membrane fragments were added (at a concentration of ~20 µg/mL), together with 1 mM DTT and 100 µM caged ATP, to the back compartment of the cuvette, which was not penetrated by the UV flashes. Adsorption of the membrane fragments to the BLM took about 60 min and resulted in capacitively coupled membranes. This process could be monitored by a decreasing system capacitance.

After the indicated period of time, the membrane was exposed to UV light flashes to produce ATP-concentration jumps by a fast release of ATP from caged ATP, which caused transient currents associated with the transfer of positive charge by the Na,K-ATPase from the bulk phase towards the BLM. A modification of the detection electronics allowed the determination of minor variations of the capacitance and conductance of the compound membrane system (Sokolov et al. 1992, 1998b). Electrical signals were recorded after the UV flash. A number of measurements could be collected from the same membrane; however, it was necessary to wait long enough between two UV flashes until all the ATP was hydrolyzed so that the ion pumps had returned to their initial state, Na_3E_1. A period of 10 min proved to be sufficient. In a subset of the experiments, apyrase was added to the compartment containing caged ATP to obtain a reduction of the necessary time interval between two flashes to 2–3 min, owing to the ATPase activity of the apyrase (Borlinghaus et al. 1987). Data collection was started only after the amplitude of the current transients was constant between successive UV flashes.

Planar membrane fragments containing Na,K-ATPase were isolated from rabbit kidneys according to Jørgensen (1974). They had diameters in the order of 0.2–1 µm and contained about 0.8 mg phospholipids and 0.2 mg cholesterol per mg protein (Bühler et al. 1991). The ATPase activity was 1800–2000 µmol P_i per h per 1 mg protein at 37 °C. Suspensions of the membrane fragments with a protein concentration of about 3 mg/mL were stored at –70 °C for several months without notable loss of ATPase activity. To perform measurements, small aliquots were thawed and kept at +4 °C for up to 2 weeks.

The BLM were formed from diphytanoylphosphatidylcholine (Avanti Polar Lipids, USA) in *n*-decane (15 mg/mL). Buffers contained NaCl, $MgCl_2$ (Merck, Germany), choline chloride, choline bromide, choline iodide, and apyrase (type VI, Sigma, USA), DTT (Fluka, Switzerland), imidazole, and P^3-1-(2-nitro)phenylethyladenosine-5′-triphosphate ("caged ATP", Calbiochem, USA).

The standard buffer contained 150 mM NaCl, 30 mM imidazole, 10 mM $MgCl_2$, and 1 mM EDTA. Various choline salts with different anions were added to this standard buffer. The pH was adjusted to 6.5 by addition of NaOH.

A xenon flash lamp with a sapphire window, FJ-249U (EG&G, USA), was used as the source of the UV flashes. Currents were measured with Ag/AgCl electrodes connected to the compartments of the cell via salt bridges. These bridges were filled with the same electrolyte as the cell. The resistance of the electrodes with the salt bridges was in the range 10–100 kΩ, which corresponded to a charging time constant of the BLM (capacitance of 1–3 nF) of less than 0.1 ms. This time constant allowed determination of the capacitance and conductance in the frequency range up to at least 300 Hz. In control experiments without salt bridges, the time constant was decreased to 0.01 ms, and proved that in the analyzed frequency range the electrodes did not distort the results of the admittance measurements.

Short-circuit currents were measured by a Keithley 427 (Keithley, Cleveland, Ohio, USA) current amplifier. Its voltage output was fed into the analog-to-digital converter (ADC) of either a digital oscilloscope, KDS-102, or of a transient recorder, TRM-120 (Kawasaki, Japan). The digitized voltage signals were transferred to a computer. The oscilloscope was triggered simultaneously with the UV flash. The current transients could be fitted by the sum of three exponentials.

In the case of admittance measurements, a sine wave voltage with an amplitude of 40–60 mV was applied to the (compound) bilayer and the current response was measured by a Keithley 427 current amplifier. Input and output voltages were collected simultaneously in two ADC channels. The background current, which was generated by capacitance and conductance of the compound membranes but not by the ion pumps, could be reduced by a compensation circuit as described before (Sokolov et al. 1992). With software written by the authors, the effect of the background current component was minimized numerically while the membrane admittance increments were calculated. This digital procedure allowed the determination of admittance increments after the UV flash virtually from a zero base level. The admittance was calculated by approximation of the current signal, I_{meas}, with a linear combination of sine and cosine components of the frequency of the voltage applied. The linear coefficients obtained from this approximation were used to calculate the conductance increment, G_{meas}, and the capacitance increment, C_{meas}, of the BLM with the adsorbed membrane fragments according to:

$$I_{\mathrm{meas}} = G_{\mathrm{meas}} V \cos(\omega t) - C_{\mathrm{meas}} \omega V \sin(\omega t) \qquad (1)$$

Figure 2 shows the typical record of a short-circuit current, *I*, its integral, *Q* (which was used to normalize the admittance measurements), as well as the capacitance and conductivity increments, ΔC and ΔG, respectively. The admittance increment was defined as

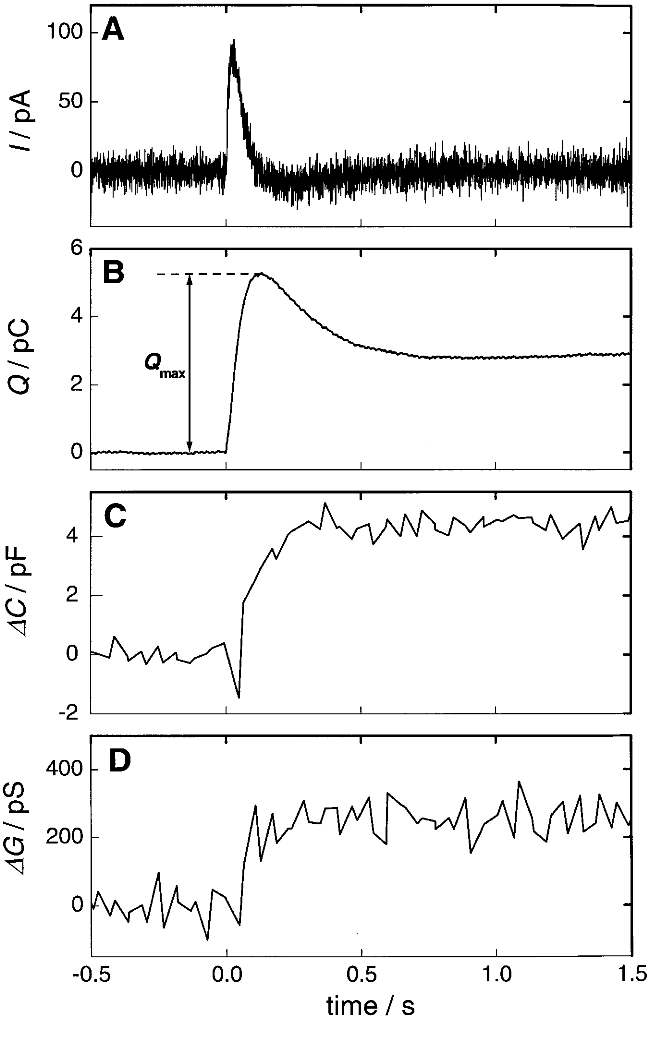

Fig. 2 A Typical record of a Na,K-ATPase-induced transient current in the presence of a superimposed sine wave. From this trace, three parameters may be determined: the amount of charge transferred (**B**), changes of the membrane capacitance (**C**), and the conductance (**D**), determined from the response of an externally applied sine-wave voltage of 20 Hz. Ion pump activity was triggered by a concentration jump of ATP released from caged ATP by a UV-light flash at time 0. The lipid bilayer (initial capacitance about 0.9 nF, corresponding to a membrane area of about 0.2 mm^2) was formed from diphytanoyl-PC in *n*-decane (15 mg/mL) in standard buffer containing 150 mM NaCl, 30 mM imidazole, 10 mM $MgCl_2$, 1 mM EDTA, pH 6.5

the difference between the initial level and the new quasi-stationary value that was reached within 0.5 s after the flash (Fig. 2). The calculated capacitance and conductance values were corrected to account for a systematic error caused by a phase shift due to the filter function of the current amplifier according to the following relation, which can be easily derived (see Appendix):

$$C = C_{\text{meas}} + \tau G_{\text{meas}}, \qquad G = G_{meas} - \tau\omega^2 C_{\text{meas}} \tag{2}$$

where τ is the time constant of the filter of the Keithley 427 current amplifier (in most of measurements its value was set to 1 ms), and C and G are the corrected values of the capacitance and conductance, correspondingly.

Results

Figure 3 shows typical records of short-circuit currents after photo-release of ATP from caged ATP in standard buffer in the absence and presence of 300 mM choline chloride. The presence of choline salts in high concentrations caused a deceleration of the transient current kinetics and a decrease of its amplitude. In order to obtain quantitative parameters, the short-circuit currents were fitted by a sum of three exponential functions. The dependence of the exponential time constants of the rise (τ_1) and decay (τ_2) of the transients on the concentration choline halides is presented in Fig. 4. The decelerating effect of the anions on the kinetics decreased according to the series $\text{I}^- > \text{Br}^- > \text{Cl}^-$.

Figure 5A and B shows the normalized capacitance and conductance increments as functions of the frequency of the applied voltage in standard buffer (solid circles). Both quantities were normalized by the maximal transferred charge, Q_{max}, as defined in Fig. 2B. This was a prerequisite to compare results from different experiments. As can be seen from Fig. 5A, in standard buffer the capacitance increments decreased with frequency in the frequency range up to 10 Hz; at higher frequencies the capacitance increments were constant. The conductance increments increased over the range of frequencies, but had a reduced dependence in the frequency range between about 10 and 50 Hz (Fig. 5B).

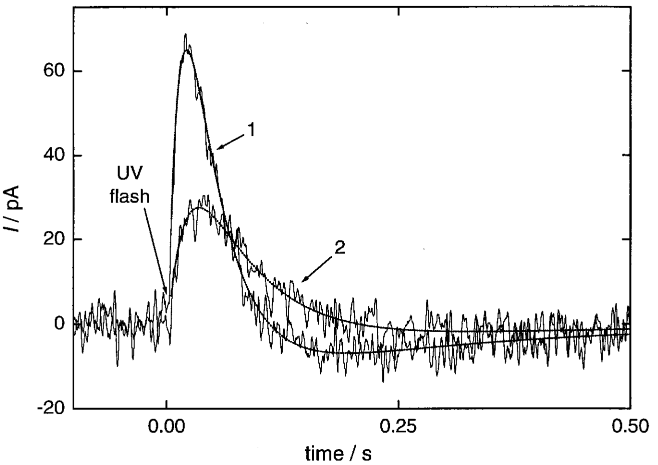

Fig. 3 Dependence of the shape of transient currents on the ionic strength of the electrolyte. Time course of the current as detected in standard buffer (*curve 1*) and after addition of 300 mM choline chloride (*curve 2*). The *lines* show the results of fits to the data with a sum of three exponential functions. The high ion concentrations led to a significant deceleration of the kinetics

As demonstrated recently (Sokolov et al. 1998b), the frequency dependencies of the capacitance and conductance increments may be described by a Lorentzian function in the case when the alternating current affects the charge translocation of a single step of the transport process:

$$\Delta C = C_0 \frac{\omega_0^2}{\omega^2 + \omega_0^2} \quad \text{and}$$

$$\Delta G = C_0\omega_0 \frac{\omega^2}{\omega^2 + \omega_0^2} \tag{3}$$

On the basis of this model, the capacitance increment, ΔC, must be frequency independent at low frequencies and approach zero at high frequencies. According to

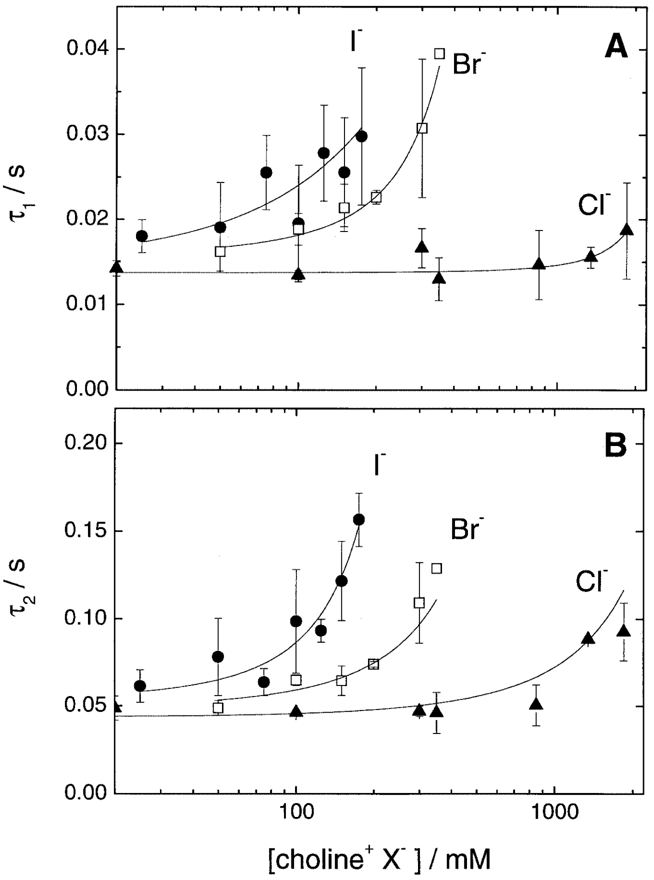

Fig. 4 Dependence of the time constants of the exponential function which fitted the rising phase (**A**) and falling phase (**B**) of the measured transient currents on the concentration of choline salts added to the standard solution: choline chloride (*solid triangles*), choline bromide (*open squares*), choline iodide (*solid circles*). Each point was obtained by averaging the time constants obtained from fits of 3–15 current transients (as shown in Fig. 3). The *lines* through the data points are fits with an exponential function, corresponding to a regression line when plotted on a linear concentration axis

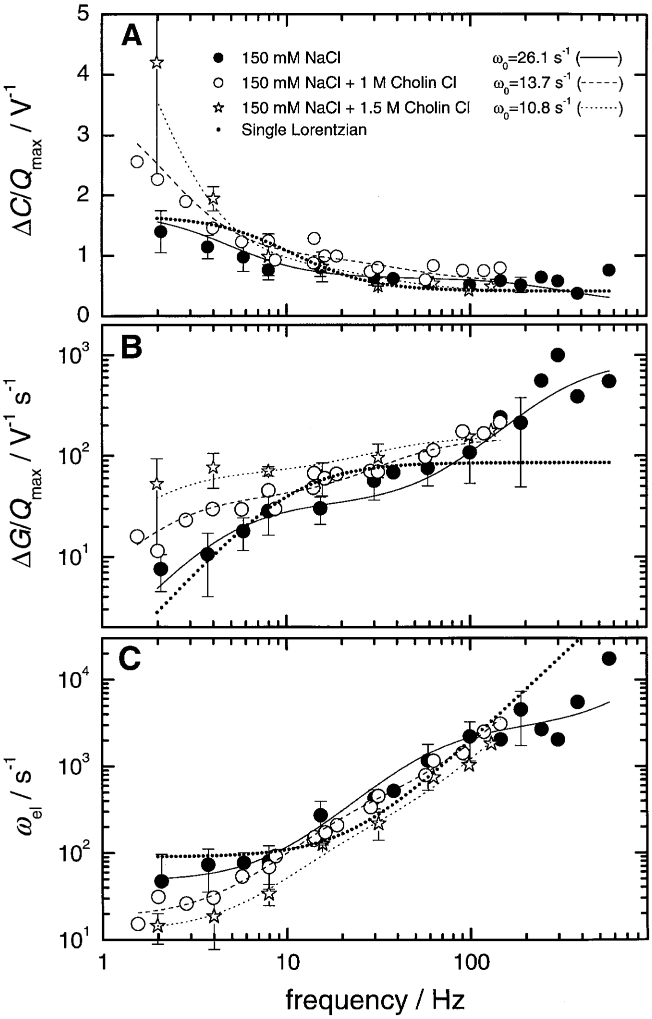

Fig. 5 Frequency dependence of Na,K-ATPase-induced changes of the membrane capacitance, $\Delta C/Q_{\max}$ (**A**), of the conductance, $\Delta G/Q_{\max}$ (**B**), and of the derived characteristic quantity, $\Omega_{el} \equiv \Omega^2 \delta C/\delta G$ (**C**). The experimental conditions were the same as in Fig. 2. The experimental data were normalized by $Q_{\max}$ (see text) and averaged over 3–5 measurements. In all three panels, *solid circles* show the result of eight experiments in standard solution and the *solid lines* are fits to the data by a sum of two Lorentzians with a bias capacitance, C_i (cf. Eq. 7), with the parameters: $\omega_0 = 26.1\ \mathrm{s}^{-1}$, $\omega_1 = 1081.8\ \mathrm{s}^{-1}$, $C_0/Q_{\max} = 1.16\ \mathrm{V}^{-1}$, $C_1/Q_{\max} = 0.331\ \mathrm{V}^{-1}$, $C_i/Q_{\max} = 0.301\ \mathrm{V}^{-1}$. The *dotted lines* show an attempt to fit the same data with a single Lorentzian with a bias capacitance ($\omega_0 = 67.9\ \mathrm{s}^{-1}$, $C_0/Q_{\max} = 1.25\ \mathrm{V}^{-1}$, $C_i/Q_{\max} = 0.408\ \mathrm{V}^{-1}$). *Open circles* represent the data of two experiments in standard solution + 850 mM choline-Cl (the total concentration of Cl^- is 1 M). The *dashed lines* were obtained from Eq. (7) with the following parameters: $\omega_0 = 13{,}7\ \mathrm{s}^{-1}$, $\omega_1 = 274{,}5\ \mathrm{s}^{-1}$, $C_0/Q_{\max} = 2.84\ \mathrm{V}^{-1}$, $C_1/Q_{\max} = 0.409\ \mathrm{V}^{-1}$, $C_i/Q_{\max} = 0.562\ \mathrm{V}^{-1}$. *Open stars* show the data of two experiments in standard solution + 1350 mM choline-Cl (total concentration of Cl^- is 1.5 M). The *dotted lines* were obtained from Eq. (7) with the parameters: $\omega_0 = 10.8\ \mathrm{s}^{-1}$, $\omega_1 = 279.5\ \mathrm{s}^{-1}$, $C_0/Q_{\max} = 6.49\ \mathrm{V}^{-1}$, $C_1/Q_{\max} = 0.336\ \mathrm{V}^{-1}$, $C_i/Q_{\max} = 0.392\ \mathrm{V}^{-1}$

these considerations, in the earlier studies the frequency dependence of the capacitance increment was fitted by a Lorentzian function to determine the characteristic parameter ω_0. However, in the present work we measured the capacitance and conductance increments in the lower frequency range in more detail than previously (Sokolov et al. 1998b). These measurements indicated, however, that the capacitance increment cannot be described satisfactorily by the simple Lorentzian of Eq. (3). The first step towards a more sophisticated model would be to account for capacitance changes due either to unspecific effects (such as electrostriction of the membrane) or to a very fast electrogenic charge displacement process (Babes and Fendler 2000; Lu et al. 1995; Sokolov et al. 1998b), which can be modeled by addition of a frequency-independent term to the capacitance:

$$\Delta C = C_0 \frac{\omega_0^2}{\omega^2 + \omega_0^2} + C_\mathrm{i} \quad \text{and}$$

$$\Delta G = C_0 \omega_0 \frac{\omega^2}{\omega^2 + \omega_0^2} \tag{4}$$

The dotted lines in Fig. 5 show the fit of Eq. (4) to the experimental data. It is obvious that this function is able to reproduce changes of the capacitance and conductance at low frequencies, but it cannot reproduce the conductance changes at high frequencies. A possible explanation for this finding is that the measured alternating current is caused by more than one electrogenic reaction step. In this case the increments of capacitance and conductance have to be described by a sum of Lorentzian functions (Eq. 3) with different characteristic frequencies, ω_0. Obviously, fitting the data by a more complicated function would not be unique if the data analysis is restricted to the capacitance increments only, as published recently (Sokolov et al. 1998b), since even for a definite fit with a single Lorentzian the frequency dependence of the data would have to cover a wider range of frequencies. In the experiments presented here, in addition to capacitance increments, also conductance increments were measured, which correspond to the imaginary and real components of the complex membrane admittance, respectively. These quantities allowed the evaluation of the characteristic parameters of a multi-step process. A technical difficulty was the variation of the current amplitude between different membranes and even during the lifetime of the same membrane. Therefore, we chose a different approach for the analysis, which was based on measurements of the phase shift of the alternating current as a function of frequency. Information about the phase shift can be obtained from the square and the in-phase components of the alternating current, which are proportional to ΔC and ΔG. In this case the tangent of the phase shift can be represented as:

$$\tan(\varphi) = -\omega \frac{\Delta C}{\Delta G} \tag{5}$$

A more convenient parameter is the product of $\tan(\varphi)$ and frequency, ω:

$$\omega^2 \frac{\Delta C}{\Delta G} \equiv \omega_{\mathrm{el}} \tag{6}$$

It can be easily checked that in the simple case, when the capacitance and conductance increments comply with Eq. (3), this parameter is equal to the characteristic frequency of the Lorentzian function. Consequently, in the simplest case when the process consists of one elementary step only, and the capacitance increment is described by a single Lorentzian function, ω_{el} will be a constant and equal to the characteristic frequency of the Lorentzian function, ω_0. If the analyzed process includes two electrogenic steps, each of which is described by a function of the type of Eq. (3), then the frequency dependence of ω_{el} derived from the data will form an S-shaped curve with two horizontal asymptotes at low and high frequencies. In the low-frequency limit it will approach the characteristic frequency of the Lorentzian representing the slower reaction step, and the high-frequency limit will represent the characteristic frequency of the faster process. Hence, the dependence of the parameter $\Omega_{el} \equiv \Omega^2 \delta C/\delta G$ on frequency directly allows the discrimination of the number of contributing reaction steps. This parameter is independent of the amplitudes of the capacitance and conductance and, therefore, is a good measure to compare experimental results from different membranes.

To test this approach, experiments with electronic replacement circuits were performed which show the behavior of charge-translocating systems. A resistor and a capacitor in series allowed the modeling of the dependence of the membrane capacitance, C, and conductance, $G = 1/R$, on frequency with a Lorentzian function. It is known that the characteristic frequency of a Lorentzian is equal to the product of the reciprocal capacitance and conductance in an electronic circuit, $1/(RC)$. Therefore, simulation experiments were performed in which R and C were selected to obtain characteristic frequencies close to those expected in the experiments with Na,K-ATPase-containing membranes. In Fig. 6 the corresponding measured frequency characteristics of apparent capacitance, C, conductance, G, and of parameter ω_{el} are shown. It can be seen that C and G are not really suitable to discriminate between systems consisting of one or two RC elements (Fig. 6A, B), in contrast to the frequency characteristics of ω_{el}. In the case of a single RC circuit, ω_{el} was nearly frequency independent, corresponding to the respective values of ω_0 (Fig. 6C). At the edges of the experimental frequency range, deviations could be observed, which were caused at low frequencies by the uncertainty of the capacitance measurements and at high frequencies by an inadequate compensation of the intrinsic time constant of the current amplifier used. Based on these measurements, the frequency range analyzed in the experiments with the ion-pump-containing membranes was restricted to avoid notable distortions. If two RC elements were connected in parallel, processes with two charge-translocating steps can be simulated. In this case the dependence of ω_{el} on frequency was indeed S-shaped with asymptotic values close to the characteristic frequencies of the RC circuits (Fig. 6C). These findings indicated that the suggested approach allows discrimination between one- and multiple-step processes and an estimation of the characteristic frequencies of the Lorentzian functions.

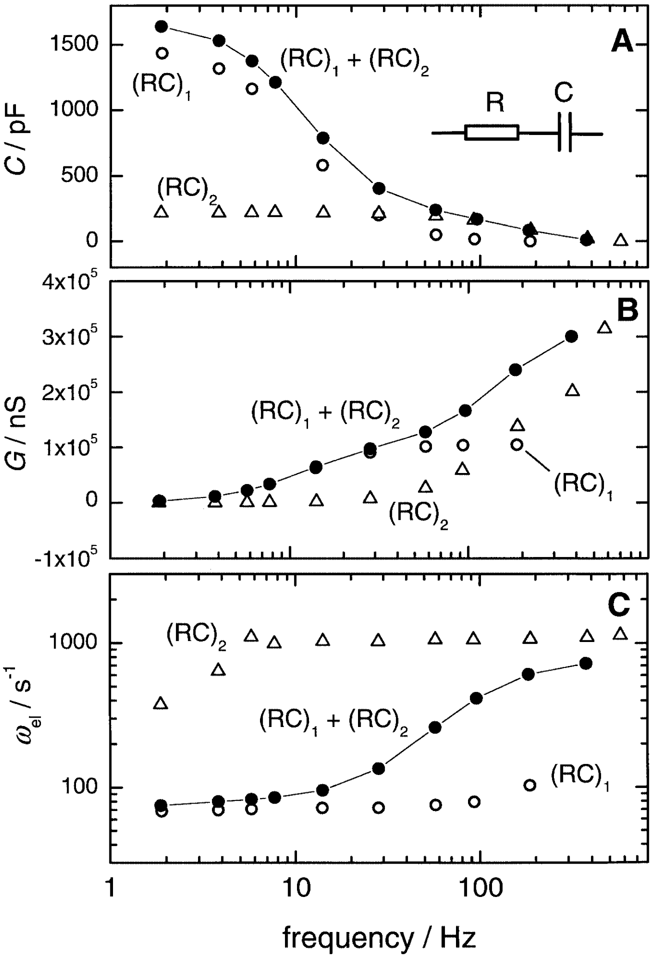

Fig. 6A–C Control experiments with RC equivalents to simulate the effect of one and two relaxation processes. The frequency dependence of (**A**) capacitance, C, of (**B**) conductance, G, and of (**C**) the characteristic quantity, $\Omega_{el} \equiv \Omega^2 \delta C/\delta G$, are shown for RC circuits simulating slow relaxation (*open triangles*, $1/(RC)_1 = 75\ \mathrm{s}^{-1}$), fast relaxation (*open circles*, $1/(RC)_2 = 1150\ \mathrm{s}^{-1}$), and for the combination of both circuits simulating the two relaxation process (*solid circles*, connected by *lines* to guide the eye)

Figure 5C shows the frequency dependence of ω_{el} for the Na,K-ATPase obtained from the results of measurements of the capacitance and conductance increments as functions of the frequency. From this figure it appears that in the case of Na,K-ATPase the parameter ω_{el} increased monotonically with frequency, and the experimental points could be fitted by an S-shaped curve similar to the one obtained in the replacement measurements with two parallel RC circuits. The lower limit of ω_{el} was in the order of $100\ \mathrm{s}^{-1}$, whereas the upper limit was about $2000\ \mathrm{s}^{-1}$. This result suggests that there are at least two electrogenic steps in the investigated partial reaction of Na,K-ATPase. To fit the characteristic frequencies of both steps, the frequency dependence of the capacitance and conductance increments, ΔC and ΔG, was described by a sum of two Lorentzians and a constant term:

$$\Delta C = C_0 \frac{\omega_0^2}{\omega^2 + \omega_0^2} + C_1 \frac{\omega_1^2}{\omega^2 + \omega_1^2} + C_i \quad \text{and} \quad \Delta G = C_0 \omega_0 \frac{\omega^2}{\omega^2 + \omega_0^2} + C_1 \omega_1 \frac{\omega^2}{\omega^2 + \omega_1^2} \tag{7}$$

The constant term, C_i, can be related either to another, still faster, electrogenic process or to artifacts, such as charge movements by the ATPase into the space between the BLM and the membrane fragments, which, in turn, could alter the geometric sizes and capacitances of the membranes owing to electrostriction phenomena (Sokolov et al. 1992, 1998b).

Figure 5 shows that the theoretical approach introduced above is able to reproduce the experimental results adequately, at least under standard conditions of 150 mM NaCl (full circles). The fit to the data yielded two characteristic frequencies, corresponding to the two processes. The characteristic frequency of the fast process was about 1100 Hz. This value is close to our earlier data, when the frequency dependency was approximated by a single Lorentzian (Sokolov et al. 1998b). The characteristic frequency of the slow process was found to be about 30 Hz. This value is close to the rate constant of the exponential decay of the transient current (Fig. 3A).

When the ionic composition of the buffer was varied to determine the salt effects on the frequency dependence of the capacitance and conductance increments, and of ω_{el}, different characteristic frequencies of the Lorentzian functions were obtained for the applied ion concentrations. Figure 5 also shows results from experiments in which choline chloride was added to the standard buffer to obtain a concentration of 850 mM (open circles) and 1350 mM (stars). A comparison of the data shows that significant effects were found preferentially in the low-frequency region. Another effect of the increased salt concentration was the magnitudes of the signals: when normalized to the total charge transferred by the Na,K-ATPase, at low frequencies the normalized quantities, $\Delta C/Q_{max}$ and $\Delta G/Q_{max}$, were larger in the presence of high choline chloride concentrations, while they remained unaffected at frequencies above 20 Hz (Fig. 5A, B). In the case of ω_{el}, the data points were shifted to lower values when the anion concentration was increased. This indicates a decrease of the characteristic frequency of the slower of the observed processes (Fig. 5C). A fit with the sum of two Lorentzian functions and a constant component was used to determine the concentration dependence of the characteristic frequency of the slow process. The results of such an analysis of experiments with various choline chloride and bromide concentrations are shown in Table 1. The ability of the anions to reduce the characteristic frequency increased in agreement with the Hofmeister series in the order $Cl^- < Br^-$. For comparison, the dependence of the time constants of the exponential decay of the transient pump currents after ATP release (cf. Fig. 4) is also shown in Table 1. Both parameters are apparently close to each other, within the confidence range of the data, and they decease with similar concentration dependence. Table 1 illustrates another salt effect: an increasing choline salt concentration decreased the relative amplitude of the fast components when compared with the slow one. Owing to this effect, the characteristic frequencies and amplitudes of fast electrogenic processes could not be determined at high salt concentrations.

Discussion

When the Na,K-ATPase is activated by ATP in buffer containing Na^+ ions, a partial reaction is induced in which three Na^+ ions are transported from the cytoplasmic side of the membrane to the extracellular side per ATP hydrolyzed, according to the reaction sequence $(Na_3)E_1{-}P \rightarrow P - E_2(Na_3) \rightarrow P - E_2Na_2 \rightarrow P - E_2Na \rightarrow P - E_2$. In the absence of K^+ ions the reaction back to the E_1 conformations is extremely slow (Heyse et al. 1994), and in the nominal absence of ADP the dephosphorylation of state $(Na_3)E_1$-P can be neglected during the time period of the experiments, so that the

Table 1 Concentration dependence of the kinetic parameters obtained from the analysis of short-circuit currents and from the frequency dependence of admittance increments. τ_1 and τ_2 are the time constants of the two exponentials which fitted the transient currents as shown in Fig. 3. Their values were averaged from the data shown in Fig. 4. The errors given are standard errors of the mean. ω_0 is the characteristic frequency of the Lorentzian which describes the slower process. C_0, C_1, and C_i are the capacitive contributions as defined in Eq. (7)

Buffer	τ_1 (s)	τ_2 (s)	$1/\tau_2$ (s^{-1})	ω_0 (s^{-1})	$(C_1+C_i)/C_0$
Standard	0.0120 ± 0.005	0.035 ± 0.005	28.4 ± 4.0	26.1	0.54
Choline-Cl					
350 mM	0.013 ± 0.003	0.046 ± 0.012	21.6 ± 5.6	25.3	0.56
850 mM	0.015 ± 0.004	0.051 ± 0.012	19.7 ± 4.7	13.7	0.37
1350 mM	0.016 ± 0.002	0.088 ± 0.016	11.3 ± 2.0	10.8	0.11
1850 mM	0.019 ± 0.006	0.093 ± 0.016	10.8 ± 1.9	16.5	–
Choline-Br					
150 mM	0.021 ± 0.002	0.064 ± 0.008	15.5 ± 1.9	13.7	0.35
350 mM	0.031 ± 0.008	0.109 ± 0.023	9.2 ± 1.9	12.3	–

enzyme is confined virtually to a steady state in the sequence $(Na_3)E_1-P \rightleftharpoons P-E_2(Na_3) \rightleftharpoons P-E_2Na_2 \rightleftharpoons P-E_2Na \rightleftharpoons P-E_2$ (Fig. 1) after phosphorylation has taken place.

From recent work it is known that at least three of the four reaction steps are electrogenic and their dielectric coefficients and rate constants have been determined. It was found that the first Na^+ ion moves through ~65% of the protein dielectric (Holmgren et al. 2000; Wuddel and Apell 1995). This may be explained by a narrow and deep access channel or "ion well" between the binding site in the protein and the aqueous outside of the protein (Läuger 1991; Läuger and Apell 1989). The release of the first Na^+ is followed by another conformational relaxation which brings the remaining two Na^+ ions closer to the extracellular aqueous phase, because they have to cross only 10–20% of the membrane dielectric when they are released in the next reaction steps (Holmgren et al. 2000; Wuddel and Apell 1995). The conformational transition $(Na_3)E_1-P \rightleftharpoons P-E_2(Na_3)$ was found to be only of minor electrogenicity (<10%; Wuddel and Apell 1995) with rate constants in the order of 30 s^{-1} to 400 s^{-1}, depending on the experimental conditions (Clarke et al. 1998; Ganea et al. 1999; Heyse et al. 1994; Pratap and Robinson 1993; Sokolov et al. 1998a). The kinetics of the deocclusion/release reactions have been analyzed recently in great detail by electrophysiological methods in squid axon, and it was found to occur with increasing rate constants from the first ion ($\leq 1000\ s^{-1}$) to the third ($\sim 10^6\ s^{-1}$) (Holmgren et al. 2000). With charge-pulse experiments on a coupled BLM system, about 25 s^{-1} were derived for the conformational transition step, 1400 s^{-1} for the release of the first ion, and above 700 s^{-1} and 4000 s^{-1} for the second and third Na^+ ions (Wuddel and Apell 1995). These findings have to be compared with the results obtained by the method applied in this study.

Frequency dependence of the capacitance and conductance increments

Recently we demonstrated that a correlation exists between two experimental methods to investigate nonstationary ion transport: the analysis of the current relaxation kinetics after a voltage step and the analysis of the frequency dependence of the admittance of the membrane system (Sokolov et al. 1998b). When measurements were performed on the same membranes by both methods it was found that, in the simplest case of mono-exponential current relaxation, the relaxation time constant is equal to the characteristic frequency of the Lorentzian function which fits the dependence of the capacity increments on frequency. Therefore, it is interesting to compare the results obtained from frequency-dependent admittance measurements with published results of current relaxation experiments. When the frequency dependence of charge movements in the reaction sequence $(Na_3)E_1-P \rightleftharpoons P-E_2(Na_3) \rightleftharpoons P-E_2Na_2 \rightleftharpoons P-E_2Na \rightleftharpoons P-E_2$ is fitted by a single Lorentzian function, we found a value of the characteristic frequency, f_0, of about 200 Hz, which corresponds to a circular frequency $\omega_0 = 2\pi \times f_0 = 1200\ s^{-1}$ (Sokolov et al. 1998b). This value is much higher than the reciprocal of the time constant of the current relaxation which was detected on cardiac cells or oocytes (Hilgemann 1994; Nakao and Gadsby 1986; Rakowski 1993) or of the lowest time constant observed with squid axons (Holmgren et al. 2000). However, fitting of the frequency dependence of the measured admittance with a single Lorentzian showed, on the one hand, at low frequencies a systematic deviation from the data, which can be seen in published data (Babes and Fendler 2000; Ganea et al. 1999; Sokolov et al. 1998b), and, on the other hand, it was impossible to obtain reliable data for the low-frequency plateau of the frequency-dependent capacitance (Babes and Fendler 2000; Ganea et al. 1999; Lu et al. 1995; Sokolov et al. 1998b). Owing to latter problem, the characteristic frequency, f_0, could be determined only with large uncertainties.

To overcome this problem we used an alternative method to analyze the frequency dependence of the admittance increments, based on measurements of the phase shift of the current with respect to the applied sine voltage. The advantage of this approach is that the results are independent of the signal amplitude, which is poorly reproducible between different membranes. In addition, the measurements could be restricted to a narrower frequency range since the value of the signal amplitude at the low-frequency plateau is irrelevant to the accuracy of the results. A possible difficulty of this approach is that the experimental determination of the phase shift may be affected by sources other than the charge movements in the ion pump. First of all, the contribution of the current amplifier to the total phase shift due to its built-in frequency filter function had to be considered. This systematic error can be accounted for accurately by a phase offset (see Appendix), and this was included in our analysis (see Materials and methods). An additional phase shift could cut into the signal at high frequencies when the membrane is no longer fully charged/discharged during a voltage semi-cycle. The time constant of this process is equal to the product of the membrane capacitance and total resistance of the electrodes, salt bridges, and solution. In our experiments this time constant was about 10^{-4} s, which corresponds to a circular frequency of about 2×10^3 Hz. In order to avoid errors from this artifact, the measurements were analyzed only in the frequency range below 1 kHz. Concerning unspecific phase shifts, the most difficult problem to analyze (and possibly account for) was the effect of the complex structure of the system consisting of a BLM and adsorbed membrane fragments. If we make the reasonable assumption that the membrane fragments adsorbed to the BLM are described by the equivalent circuit presented in Fig. 7B, a simple equation can be derived to quantify the phase shift (see Appendix):

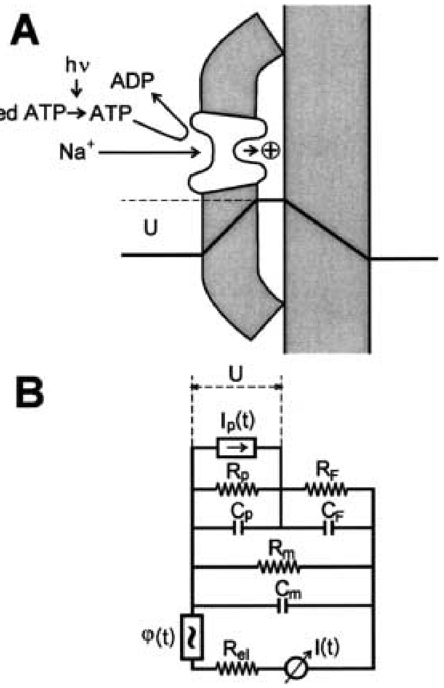

Fig. 7 A Schematic representation of a lipid bilayer with an adsorbed membrane fragment containing Na,K-ATPase. The *bold line* indicates the profile of the electric potential generated after the compartment enclosed by a membrane fragment and the BLM has been charged up by active Na^+ transport; U is the voltage across the membranes. **B** An electric equivalent circuit of the experimental set-up [adapted from (Borlinghaus et al. 1987)]. C_p and R_p are the total capacitance and resistance of adsorbed membrane fragments which contain Na,K-ATPase; C_F and R_F are capacitance and resistance of the supporting BLM region in contact with the fragments; C_m and R_m are capacitance and resistance of the BLM not covered by membrane fragments; R_{el} is the resistance of the electrodes and the aqueous solution; $I_p(t)$ is the ATPase-generated electric current, $I(t)$ the measured current, and $\varphi(t)$ the externally applied voltage

$$\varphi_{meas} = \varphi_p - 2\varphi_t + 2\varphi_F \tag{8}$$

where φ_{meas} is the measured phase shift and φ_p the phase shift of the current through the membrane fragment generated by the ion pumps; φ_t is the phase shift controlled by the time constant of the passive discharge of the total capacitance of the compound system, i.e. BLM plus membrane fragments, according to:

$$\tan(\varphi_t) = -\frac{\omega(C_F + C_p)}{G_F + G_p} \tag{9}$$

and φ_F is the phase shift controlled by the time constant of the BLM discharge:

$$\tan(\varphi_F) = -\frac{\omega C_F}{G_F} \tag{10}$$

As we have shown recently (Sokolov et al. 1998b), on the basis of the known capacitance and conductance of the BLM and membrane fragments, and with frequencies exceeding 10 Hz, the relation $\varphi_t \approx \varphi_F \approx -\pi/2$ holds, so that the error of determination of the phase shift caused by the BLM connected in series with the membrane fragments can be neglected:

$$\varphi_{meas} \approx \varphi_p \tag{11}$$

At lower frequencies the effect of the compound membrane system on the phase shift measurement becomes substantial, when the conductance of the membrane fragments has to be taken into account. The conductance of the pure BLM can be neglected as shown before (Borlinghaus et al. 1987), and, as a consequence, $\tan(\varphi_F)$ becomes infinite according to Eq. (10). In other words, the BLM behaves as an ideal capacitor and introduces a phase shift $\varphi_F \approx -\pi/2$. In Eq. (9), $\tan(\varphi_t)$ is a product of the circular frequency and time constant of the passive discharge of the contact area of both membranes. Values of the corresponding time constant were estimated earlier: the first estimate was about 300 ms (Borlinghaus et al. 1987), but later this phenomenon was revisited and the time constant was estimated to be about 4 s (Wuddel and Apell 1995). However, even with the less favorable assumption of 300 ms, a phase shift φ_t which differs notably from $-\pi/2$ will be observed only at circular frequencies below 3 s^{-1} (corresponding to a frequency of the alternating voltage of about 0.5 Hz). In our measurements the lowest frequency applied was 2 Hz, which was well above the crucial limit. In summary, errors induced by possible phase shift problems were not significant in the selected experimental frequency range and the major contribution to the detected phase shift was generated by the alternating charge movements in the Na,K-ATPase.

The rate-limiting transport step and the effect of concentrated salt solutions

On the basis that the experimentally recorded phase shifts were produced by the action of Na,K-ATPase, the analysis of the frequency dependence (Fig. 5) allowed the conclusion that the charge relaxation process in the protein includes at least two charge translocating reaction steps with rate constants of about 30 s^{-1} and 1500 s^{-1}. The value of the slow rate constant is in agreement with that of the decaying phase of the current transients (Table 1) and with the rate constant derived from the slow phase of the current relaxation found previously in cardiac cells (Nakao and Gadsby 1986) when scaled down from 36 °C to 20 °C with an activation energy of 90 kJ/mol. This indicates that the slow process in both types of experiments represents the same rate-limiting reaction step.

The existence of salt effects on the charge transfer kinetics of Na,K-ATPase was shown previously (Ganea et al. 1999; Heyse et al. 1994; Sokolov et al. 1998a, 1998b). We observed a significant shift in the frequency dependence of the capacitance increments when high concentrated solutions of NaCl or choline-Cl were added (Sokolov et al. 1998b). However, the more detailed measurements presented in this paper reveal that, in previous experiments, different steps of electrogenic transport were compared, the fast step in the standard solution with the slow one in concentrated salt solutions.

The detailed data analysis above shows that the characteristic frequency of the slow step decreased in concentrated solutions of choline salts, and this effect correlated with the decrease of the rate constant of the exponential decay of transient currents measured under the same conditions (Table 1). The effectiveness of the deceleration increased in the series $Cl^- < Br^- < I^-$ (Fig. 4). The agreement with the order in the Hofmeister series (Cacace et al. 1997) suggests that the deceleration was related to the chaotropic effect of the anions on the protein conformation, similar to their effect on the partition between the two phosphoenzyme states, P-E_1 and P-E_2, which was observed before (Post and Suzuki 1991). These findings support the concept that the slow (and rate-limiting) reaction step in the non-stationary ion transport sequence in our model system is the conformation transition between P-E_1 and P-E_2.

In the literature, an alternative assignment of the rate-limiting step has been discussed, in which the conformation transition was proposed to be a fast process in the partial reaction, and it was assumed that it controls the kinetics of the rising phase of the transient current after photolysis of the caged ATP. The falling phase was assigned to the exchange of the inactive caged ATP against the ATP photo-released in the nearby buffer (Babes and Fendler 2000; Fendler et al. 1993; Friedrich and Nagel 1997). However, as shown above, the addition of choline salts caused a stronger effect on the time constant of the falling phase of the current than on the rising one (Fig. 4 and Table 1). In the admittance experiments the reaction steps before the conformation change are not present (owing to the nominal absence of ADP; Fig. 1); therefore, the step affected by high salt concentrations can be only the conformational change, since it is well accepted that the ion binding and release steps are fast (Apell and Karlish 2001; Holmgren et al. 2000; Wuddel and Apell 1995). These observations support the proposal that the rising time is controlled by caged-ATP photolysis and phosphorylation of the Na,K-ATPase and the falling one by the conformation transition, as proposed previously (Heyse et al. 1994; Sokolov et al. 1998a; Wuddel and Apell 1995).

Assignment of the fast process detected by admittance analysis

The results of the frequency dependence of capacitance and conductance increments and, more importantly, of the parameter ω_{el} revealed the existence of a second Lorentzian function with a characteristic frequency with a rate constant of about 2000 s^{-1}, indicating the presence of a second electrogenic process (Fig. 5). This process is, in contrast to the slow one, not significantly affected by high concentrations of choline salts. In Fig. 5 a salt effect can be seen only in the low-frequency range. The high concentrations of choline salts reduced the amplitude of the fast processes relative to the slow one, as can be concluded from Table 1 (last column). This observation can be explained again by a shift in the equilibrium distribution between the two protein conformations. The rate constants of the fast process could not be obtained with sufficient accuracy in the highly concentrated choline salt solutions (data not shown). According to the reaction scheme (Fig. 1), the sequence of (electrogenic) ion release and binding steps, $P-E_2(Na_3) \rightleftharpoons P-E_2Na_2 \rightleftharpoons P-E_2Na \rightleftharpoons P-E_2$, are the candidates for this process. The enzyme phosphorylation and dephosphorylation reactions can be excluded since, in these experiments, ADP was not present in significant amounts. It was found earlier that the most pronounced charge-moving step of the whole pump cycle is the release of the first Na^+ ion, $P-E_2(Na_3) \rightleftharpoons P-E_2Na_2$, with a dielectric coefficient of 0.65 and a rate constant of $\leq 1000\ s^{-1}$ (Holmgren et al. 2000) or 1400 s^{-1} (Wuddel and Apell 1995). The rate constants of the subsequently released Na^+ ions are much higher (Holmgren et al. 2000). Therefore, we propose that the second process detected in our experiments reflects release/binding of the first Na^+ ion to the extracellular side. The constant term, C_i, in Eq. (7), which is necessary to fit the data at high frequencies, may be interpreted as the beginning of another Lorentzian function caused by the still faster release of the second Na^+ ion to the extracellular aqueous phase. Further improvements of the set-up to reduce the *RC* time constant generated by the membrane capacitance and total resistance of the electrodes, solution, and salt bridges will possibly enable us to resolve and analyze also this frequency domain.

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Appendix

Correction of distortions due to the electronic filter of the current amplifier

For this treatment the simple filter is considered, which usually is installed in standard current amplifiers (e.g. in the Keithley 427 used here). It consists of a capacitance C connected in parallel with feedback resistance R of the operational amplifier (Fig. 8A). The ideal current-voltage converter should give an output voltage U proportional to the input current I:

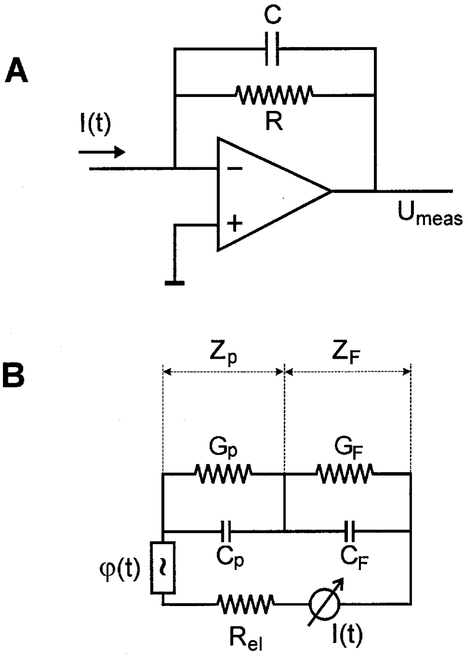

Fig. 8 **A** Schematic representation of an operation amplifier with a filter function. **B** Simplified equivalent circuit of the membrane fragment with capacitance C_P and conductance G_P contacting with the lipid bilayer with capacitance C_F and conductance G_F and resistance of the electrodes and aqueous solution, R_{el}, used to evaluate the errors of the phase shift in the admittance measurements

$$U = IR \tag{A1}$$

Owing to the presence of the filter function, a current converter has a more complex relation between the input circuit current I and the output voltage U_{meas}:

$$I = \frac{U_{meas}}{R} + C\frac{d}{dt}U_{meas} \tag{A2}$$

Equations (A1) and (A2) give a simple relation between ideal (U) and filtered (U_{meas}) output voltages:

$$U = U_{meas} + \tau\frac{d}{dt}U_{meas} \tag{A3}$$

where $\tau = RC$ is the time constant of the filter. In the case of admittance measurements, the amplifier is used for measurement of alternating currents due to the sine voltage applied to the membrane:

$$V(t) = V\cos(\omega t) \tag{A4}$$

The resulting current consists of real and imaginary components which are proportional to cosine and sine functions of time. The capacitance, C, and conductance, G, are calculated according to:

$$I = \frac{U}{R} = GV\cos(\omega t) - C\omega V\sin(\omega t) \tag{A5}$$

for an ideal amplifier, or:

$$I_{meas} = \frac{U_{meas}}{R} = G_{meas}V\cos(\omega t) - C_{meas}\omega V\sin(\omega t) \tag{A6}$$

for a real amplifier with a filter function as introduced above. Combining Eqs. (A5) and (A6) with (A3) leads to Eq. (2).

It is also useful to derive an equation to correct the phase shift φ between the current and applied voltage. Now $\tan(\varphi)$, the ratio of the imaginary and real components of the current, can be written as:

$$\begin{aligned}\tan(\varphi) &= -\frac{\omega C}{G} = -\frac{\omega C_{meas} + \tau\omega G_{meas}}{G_{meas} - \tau\omega^2 C_{meas}}\\ &= \tan(\varphi_{meas} - \varphi_{filter})\end{aligned} \tag{A7}$$

where φ_{meas} is the phase shift of current, I, against the voltage measured by an amplifier with a filter function given as $\tan(\varphi_{filter}) = -\omega\tau$. The correction equation can be rewritten in a more simple form:

$$\varphi = \varphi_{meas} - \varphi_{filter} \tag{A8}$$

Correction of the current phase shift due to the complex equivalent circuit of membrane fragments capacitively coupled to a BLM

The current measured in a system consisting of membrane fragments adsorbed to a BLM is different from the current generated by charge movements in Na,K-ATPase. Borlinghaus et al. (1987) published a transformation equation to determine the current flowing through Na,K-ATPase in the membrane fragments from the current measured in the external circuit. In the case of admittance measurements, the derivation of a transformation equation needs another approach.

The equivalent circuit of membrane fragments adsorbed on the surface of a BLM can be represented by a combination of capacitors and resistors, as shown in Fig. 7B. The most convenient way to analyze the alternating current in the ion pumps generated by an externally applied sine voltage is to use complex arithmetic. The complex conductance (admittance) of a capacitor and resistor connected in parallel can be written as:

$$Z = G - i\omega C \tag{A9}$$

where G is conductance, C the capacitance, ω the circular frequency, and i the imagine unit. First, we analyze the change of admittance, Z, due to the action of Na,K-ATPase at low frequencies (less than hundreds of hertz), when the relation:

$$\omega = R_{el}C_{total} \ll 1 \tag{A10}$$

holds, where R_{el} is the resistance of the electrodes and aqueous solution (≤ 100 kΩ) and C_{total} is the total system capacitance of the BLM and adsorbed membrane fragments (≤ 3 nF). In this case we have to take into account only the equivalent circuit of that part of the membrane covered by the membrane fragments with Na,K-ATPase (Fig. 8B). Generally, the transition of Na,K-ATPase from one state to another by an electrogenic partial reaction leads to a change of the admittance of the membrane fragments from Z_{p}^{0} to Z_{p}:

$$\Delta Z_{\mathrm{p}} = Z_{\mathrm{p}} - Z_{\mathrm{p}}^{0} \tag{A11}$$

and to change of the admittance of the BLM from Z_{F}^{0} to Z_{F}:

$$\Delta Z_{\mathrm{F}} = Z_{\mathrm{F}} - Z_{\mathrm{F}}^{0} \tag{A12}$$

The total admittance Z of a system consisting of membrane fragments in series with a lipid bilayer (Fig. 8B) can be calculated as:

$$Z = \frac{Z_{\mathrm{p}} Z_{\mathrm{F}}}{Z_{\mathrm{p}} + Z_{\mathrm{F}}} \tag{A13}$$

The change of the total admittance ΔZ due to changes of Z_{F} and Z_{p} is derived to be:

$$\Delta Z = \frac{Z_{\mathrm{p}}}{Z_{\mathrm{p}} + Z_{\mathrm{F}}} \frac{Z_{\mathrm{p}}^{0}}{Z_{\mathrm{p}}^{0} + Z_{\mathrm{F}}^{0}} \Delta Z_{F} + \frac{Z_{\mathrm{F}}}{Z_{\mathrm{p}} + Z_{\mathrm{F}}} \frac{Z_{\mathrm{F}}^{0}}{Z_{\mathrm{p}}^{0} + Z_{\mathrm{F}}^{0}} \Delta Z_{\mathrm{p}} \tag{A14}$$

If the change of the BLM admittance (due to the electrostriction) can be neglected because of the small voltage changes, then we can assume $Z_{\mathrm{F}} \approx Z_{\mathrm{F}}^{0}$, or $\Delta Z_{\mathrm{F}} \approx 0$. It can be also assumed that the changes of the admittance are small, so that $Z_{\mathrm{p}} \approx Z_{\mathrm{p}}^{0}$. Then Eq. (A14) reduces to:

$$\Delta Z \approx \left(\frac{Z_{\mathrm{F}}}{Z_{\mathrm{p}} + Z_{\mathrm{F}}} \right)^{2} \Delta Z_{\mathrm{p}} \tag{A15}$$

An analysis of Eq. (A15) becomes more transparent in polar coordinates with modulus $|\Delta Z|$ and phase angle φ_{z}. According to the arithmetic of complex numbers, the product of two complex numbers has a modulus equal to the product of the modulus of both numbers, and a phase angle equal to the algebraic sum of the phase angles of both numbers. Thus, the value of ΔZ can be calculated to be:

$$|\Delta Z| = \left(\frac{G_{\mathrm{F}}}{G_{\mathrm{F}} + G_{\mathrm{p}}} \right)^{2} \frac{(1 + \omega^{4} \tau_{\mathrm{F}}^{2} \tau_{\mathrm{t}}^{2} + \omega^{2} \tau_{\mathrm{F}}^{2} + \omega^{2} \tau_{\mathrm{t}}^{2})}{(1 + \omega^{2} \tau_{\mathrm{t}}^{2})^{2}} |\Delta Z_{\mathrm{p}}| \tag{A16}$$

where $\tau_{\mathrm{F}} = C_{\mathrm{F}}/G_{\mathrm{F}}$ corresponds to the time constant of the BLM and $\tau_{t} = (C_{F} + C_{P})/(G_{F} + G_{P})$ to the time constant of the total composite membrane consisting of the BLM plus the adsorbed membrane fragments [a similar time constant was introduced previously (Borlinghaus et al. 1987)]. The phase angle is:

$$\Delta\varphi_{\mathrm{z}} = \Delta\varphi_{\mathrm{p}} - 2\varphi_{\mathrm{t}} + 2\varphi_{\mathrm{m}} \tag{A17}$$

where $\Delta\varphi_{\mathrm{p}}$ is the phase shift due to the admittance change of the membrane fragments (which is to be measured), φ_{F} is the phase angle induced by the BLM, which can be calculated according to $\tan(\varphi_{\mathrm{F}}) = -\omega\tau_{\mathrm{F}}$, and φ_{t} is the phase angle of the total admittance of the BLM and the membrane fragment, which can be calculated from $\tan(\varphi_{\mathrm{t}}) = -\omega\tau_{\mathrm{t}}$.

Equation (A17) is similar to Eq. (8). It can be used to determine the frequency interval in which one can neglect the effect of the resistors of the equivalent circuit (Fig. 7B) on the results of a measurement.

The ideal case can be achieved when $\omega\tau_{\mathrm{F}} \to \infty$ and $\omega\tau_{\mathrm{t}} \to \infty$. In this case, φ_{m}, as well as φ_{t}, will approach a value of $-\pi/2$, and the error of the phase shift can be neglected because of $\Delta\varphi_{z} = \Delta\varphi_{\mathrm{p}}$. In this case the magnitude of the admittance change, ΔZ, is determined only by the capacitance of the two contacting membranes:

$$\Delta Z \approx \left(\frac{C_{\mathrm{F}}}{C_{\mathrm{p}} + C_{\mathrm{F}}} \right)^{2} \Delta Z_{\mathrm{p}} \tag{A18}$$

which is an approximation that was used previously (Sokolov et al. 1998b).

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