Electrogenic Transport of Sodium Ions in Cytoplasmic and Extracellular Ion Access Channels of Na⁺,K⁺-ATPase Probed by Admittance Measurement Technique¹

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Abstract—Electrogenic movements of sodium ions in cytoplasmic and extracellular access channel of the Na⁺,K⁺-ATPase have been studied by the admittance measurement technique which allows the detection of small changes of the membrane capacitance and conductance induced by phosphorylation of the ion pump. The measurements were carried out on a model system consisting of a bilayer lipid membrane, to which membrane fragments with ion pumps were adsorbed that contain the ion pumps in high density. Small changes of the membrane capacitance and conductance were induced by a fast release of ATP from caged ATP. The effect was measured at various frequencies and in solutions with different Na⁺ concentrations. The experimentally observed frequency dependences were explained using a theoretical model assuming that Na⁺ movement through the cytoplasmic access channel occurs in one step and through the extracellular access channel, in two steps. The phosphorylation of the protein by ATP leads to a block of the cytoplasmic access channel and an opening the extracellular access channel. The disappearance of electrogenic Na⁺ movements on the cytoplasmic side produces a negative change of capacitance and conductance, while the emergence of extracellular Na⁺ movements generates a positive change. Fitting the experimental dependences of capacitance and conductance by theoretical curves allowed the determination equilibrium and kinetic parameters of sodium transport in the access channels.

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The Na⁺,K⁺-ATPase is one of the key membrane proteins of eukaryotic cells that participates in maintaining the asymmetric distribution of Na⁺ and K⁺ ions across the cytoplasmic membrane. In the course of its physiological transport cycle the Na⁺,K⁺-ATPase transports three sodium ions from the cytoplasm to the extracellular medium and two potassium ions in the opposite direction (Fig. 1). A key step of the pump cycle is enzyme phosphorylation induced by ATP hydrolysis and occlusion of the ion-binding sites. The existence of an occluded state prevents simultaneous access to the binding sites from both sides of the membrane, a condition that would correspond to a continuous ion channel. Subsequently, a transition occurs between the two principal conformations, E₁ and E₂. These conformations differ by accessibility of the binding sites from the solutions on both sides of the membrane and by their affinity for sodium and potassium ions. Exchange of ions between the aqueous solutions and the binding sites inside the membrane domain of the protein occurs through so-called “access channels.” The conformational transition from E₁ to E₂ results in closing the

pathway to binding sites through the access channel from the cytoplasmic side and opening the pathway to the extracellular side. The structure of the access channels is not clear so far. The Na⁺,K⁺-ATPase is known to have three ion binding sites for sodium ions. The structure of Na⁺,K⁺-ATPase with two rubidium ions (which substitute the potassium ions) in conformation E₂ was obtained recently [1]. Presumably, the same two sites participate in the sodium ion binding. The structure of the Na⁺,K⁺-ATPase is similar to the Ca²⁺-ATPase; this allowed to identify two binding sites for sodium ions corresponding to the two binding sites for calcium ions in the Ca²⁺-ATPase [1–5]. These binding sites include negatively charged amino-acid side chains and are placed approximately in the middle of the membrane. The location of the third sodium binding site, which is unique for the Na⁺,K⁺-ATPase, is not clear so far. The responsible amino acids are proposed either by molecular modeling or by studying the effect of amino-acid substitution on transport function [6–10].

Essential information on the access channels of the Na⁺,K⁺-ATPase was obtained from the studies of electrogenic ion transport performed with the aid of electro-

¹ The text was submitted by the authors in English.

Fig. 1. The Albers–Post cycle and the electrogenic transport of sodium ions in the Na^+, K^+ -ATPase. The boxes show the steps, which are assumed to give contribution to the admittance increments. The left side of the figure designates the transitions at the cytoplasmic side of protein the right one, at the extracellular side.

chromic fluorescent probes or by measurements of electric signals [6, 11–13]. These experiments allow the evaluation the dielectric coefficients of distinct electrogenic transport steps. These coefficients characterize the relative depths of charge movements inside the protein. It has been demonstrated by various experimental techniques that the major contribution to the electric current through the membrane is the ion movement associated with the third Na^+ released (as first ion) through the extracellular access channel [6, 12, 14–16]. The large value of the dielectric coefficient of this process was explained by the assumption that the extracellular access channel for the third sodium ion is deep and narrow and its binding site is placed closer to the cytoplasmic side of the protein [8]. Non-steady-state transport of sodium ions through the Na^+, K^+ -ATPase was investigated by measuring electric currents in the absence of potassium ions. Under these conditions the sodium pump does not perform the whole cycle but can only relax between confined states. Such relaxation processes are triggered either by steps of the electric potential or by concentration jumps of ATP or other substrates of the cycle. Current transients induced by voltage steps in the Na^+, K^+ -ATPase were first reported by Nakao and Gadsby in cardiac myocytes using whole-cell patch-clamp technique [17]. Later similar currents were detected in other cellular systems [11, 14, 17–19]. Initially, only a monoexponential current decay was detected. This was explained by voltage-driven

redistribution of sodium ions in the extracellular access channel coupled with the conformational transition, which controls the observed slow kinetics. Improved experimental techniques allow the detection of additional, faster components in the relaxation currents [11, 19, 20]. The most detailed investigation on squid giant axons revealed three current components: a slow one, assigned to the conformation transition E_1/E_2 ; an intermediate one, the rate of which is controlled by binding/release of the third sodium ion, and an ultra fast step, attributed to the movement of two other sodium ions in wide access channel [11].

The asymmetric location of the binding site of the third sodium ion means that the cytoplasmic access channel for this ion should be rather shallow with a dielectric coefficient of 0.25 [24]. Therefore, electric signals associated with the transport in the cytoplasmic access channel evaded detection for a long time. Electrogenicity of the sodium transport in the cytoplasmic access channel was demonstrated by fluorescence techniques [21, 22]. Binding of sodium ions in the cytoplasmic access channel triggers the ATP-induced conformation transition and the opening of the extracellular access channel. This is manifested in the dependence on the cytoplasmic sodium concentration of the electrogenic movement of these ions in the extracellular access channel [23]. These experiments allowed the determination of the binding constants of sodium ions

on the cytoplasmic side of the Na^+, K^+ -ATPase (about 5 mM) and confirmed the dielectric coefficient for the movement of the third ion in the access channel. However, the kinetic parameters of this partial reaction could not be determined.

Electrogenic ion transport in the Na^+, K^+ -ATPase can be studied in a system consisting of a lipid bilayer (BLM) and small membrane fragments that contain ion-pumps and adsorb to the bilayer surface in an oriented manner [15, 24–28]. The BLM serves as capacitive electrode transmitting the electrical signals that arise due to electrogenic transport in the membrane fragments to an external detection circuit. Fast photo-activated release of ATP from (inactive) caged ATP triggers the transfer of sodium ions through the Na^+, K^+ -ATPase from the bulk solution into the water-filled gap between BLM and membrane fragments (Fig. 2). This ion movement can be detected under short-circuit conditions as a transient current. A modification of this technique by application of an alternating voltage between both aqueous solutions allows the study of the effect of the electric field on the ion transport. The shape of the alternating voltage can be either rectangular [15], or sinusoidal. In the latter case the influence of voltage on the movement of ions inside the Na^+, K^+ -ATPase can be detected as small increments of the membrane admittance (capacitance and conductance) [13, 16, 29–34]. It was shown that this technique is equivalent to the usual voltage-clamp technique allowing the determination of the same set of kinetic parameters of the transport process [30, 34]. The advantage of the admittance measurements is higher sensitivity and the possibility to work with purified protein.

The application of this technique allowed us to show the existence of several steps of electrogenic movements of sodium ions in the extracellular access channel of the Na^+, K^+ -ATPase in the conditions of high concentration of these ions [16]. In a medium containing a low concentration of sodium ions, negative ATP-driven increments of the membrane capacitance were found. They were explained by electrogenic translocations of sodium ions through the cytoplasmic access channel that disappears after phosphorylation of the Na^+, K^+ -ATPase [34]. A more detailed investigation showed that these negative increments depend on frequency [35]. However, this explanation was only qualitative. To prove that this effect is related to the sodium ion transport in the cytoplasmic access channel and to evaluate its parameters, a more detailed study of the frequency dependence of the admittance at different sodium concentrations is necessary, as well as an analysis of the results in the framework of a theoretical model of such a transport mechanism. The theoretical models describing the electrogenic non steady-state sodium transport available at present are based either on the kinetic equations describing a modified Post–Albers reaction cycle [15, 27, 36] or on a simplified description of the ion movement relaxation in the access channel [14, 23, 34, 37]. These

Fig. 2. The bilayer lipid membrane with adsorbed membrane fragment containing Na^+, K^+ -ATPase (a) and its simplified equivalent circuit (b).

models can, however, describe neither the new experimental data indicating the existence of several steps of the sodium transport in the extracellular access channel nor the electrogenic sodium transport in the cytoplasmic access channel. The development of a more detailed model as well as a more detailed study of the frequency-dependent increments of capacitance and conductance at different concentration of sodium ions was the goal of this study.

EXPERIMENTAL

Purified membrane fragments containing exclusively Na^+, K^+ -ATPase were obtained from rabbit kidney according to the Jorgensen's procedure "C" [38]. No proteins with a molar mass different from that of the Na^+, K^+ -ATPase could be detected in the membrane fragments by gel-electrophoresis. The membrane fragments are planar disks 0.2–1 μm in diameter, containing about 0.8 mg of phospholipids and 0.2 mg of cholesterol per 1 mg of protein [39]. The ATPase activity at 37°C was 1800–2000 μM of inorganic phosphate released per hour per 1 mg of protein. Suspensions of the fragments in water with a protein content of about 3 mg/ml can be stored in a freezer for several months without notable loss of the ATPase activity. For the experiments, an aliquot of the suspension was thawed and subsequently stored at +4°C for not more than 2 weeks.

Bilayer lipid membranes (BLM) were formed in a Teflon cell from 15 mg/ml solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, USA) in *n*-decane (Aldrich, USA). To prepare experimental solutions, the following substances were used: NaCl, MgCl₂ (Merck, Germany), choline chloride (Sigma, USA), imidazole (Calbiochem, USA), dithiotreitol (Sigma, USA), caged ATP (Calbiochem, USA). The basic buffer solution (which will be referred to as "standard") contained 30 mM imidazole, 10 mM MgCl₂, and 1 mM EDTA, pH 6.5; concentrations of NaCl varied from 3 to 150 mM; pH was adjusted by addition of HCl.

Temperature was maintained with the aid of a Peltier element mounted under the metallic jacket, in which the Teflon cell was placed. The current through the Peltier element was controlled by a thermo-element built into the jacket and an electronic module designed by the authors.

Measurements of the electric signals produced by the Na⁺,K⁺-ATPase were based on the method described in [28] and adapted for measurements of small admittance increments [31]. BLM was formed across a hole in the septum between both compartments of the Teflon cell. The cell had two optically transparent windows for visual control of the BLM and its exposure to UV flashes. After the BLM was formed, dithiotreitol was added to both compartments of the cell. Then the suspension of membrane fragments and caged ATP were added to the compartment, opposite to the source of UV light (concentrations of the caged ATP and protein in the cell were 100 μM and about 20 μg/ml, respectively). Adsorption of the membrane fragments on the BLM was monitored by the decrease of the membrane capacitance and lasted about 60 min. Electrical currents were recorded after a UV flash which caused a rapid release of ATP from its inactive precursor. A Xenon pulse flash lamp with a sapphire window FJ-249 (EG&G, USA) was used as a light source. Release of ATP caused transient currents generated by the transfer of sodium ions in the Na⁺,K⁺-ATPase from the bulk solution to the contact area between BLM and the membrane fragments [28]. Transfer of sodium ions in the potassium-free environment generate a long-lived quasi-equilibrium state. The next UV flash was applied at least 10 min later to ensure a return of the ion pumps to the initial state. When current transients were recorded before the adsorption of membrane fragments was complete, their amplitude slowly increased with successive UV flashes. Constant amplitude was interpreted as evidence for complete adsorption. This occurred usually about two hours after the addition of the membrane fragments to one compartment of the cell.

Electrical signals were picked up by Ag/AgCl electrodes with agar bridges. The bridges were made of micropipette tips, which were sealed with agar at the ends. The tips were filled with electrolyte, in which the electrodes were immersed. The solution was the same

as in the experimental cell. The electric resistance of the electrodes with salt bridges did not exceed 10–20 kΩ, BLM capacity was 1–3 nF; so, the characteristic time constant of the BLM charging was about 50 μs and reliable measurements could be performed at frequencies up to 2000 Hz.

Electric signals were recorded using L-783 or L-780 ADC boards (LCard, Russia). To record short-circuit currents, one of the electrodes was grounded and the other connected to the input of an operational amplifier Keithley 427; the output signal of the amplifier was fed to an inlet of the ADC board. To record admittance increments, sine voltage with an amplitude of 50 mV was applied to one of the electrodes from the analog output of the ADC board. This voltage was also supplied to one of the input channels of the ADC. The other electrode was connected to the input of the operational amplifier, and the output of the amplifier was connected to another input channel of the ADC. To compensate for the response of the membrane to the initial alternating current applied before the UV flash, which is associated to capacitance and conductance of the BLM with adsorbed membrane fragments, an analog electronic circuit was used. This circuit added an inverted current generated by the electric equivalent of the experimental setup with adjustable parameters which allowed the compensation of the capacitive background current of the BLM, and thus significantly decreased the initial alternating current (by more than two orders of magnitude) and enabled recording of small current increments generated by the Na⁺,K⁺-ATPase activation after a UV flash [31]. Precise subtraction of the initial alternating current and calculation of admittance changes were executed digitally using software developed by the authors. The software calculated the admittance changes by means of approximation of the light flash-induced increments of alternating current i by a linear combination of cosine and sine functions with the frequency f of the applied voltage. Amplitudes of these two functions representing in-phase components (with respect to the applied voltage) and quadratic components (90° phase shift) of the alternating current were used to determine the increments of capacitance C_{meas} and conductance g_{meas} of the BLM with adsorbed membrane fragments according to the equation

$$i = g_{\text{meas}} V \cos(\omega t) - C_{\text{meas}} \omega V \sin(\omega t), \quad (1)$$

where V is the amplitude of the alternating voltage applied to the membrane and $\omega = 2\pi f$ is its circular frequency. An example of the calculated time-dependence of capacitance and conductance after a light flash is presented in Fig. 3. Changes of the admittance associated with transition of the ATPase into a new state were calculated as the difference between the capacitance and conductance averaged before the UV flash and over a time interval starting 0.5 s after the flash. After this period, new steady-state values of the parameters are accomplished (Fig. 3). To account for the phase shift introduced by the filter function of the amplifier, capac-

Fig. 3. The kinetics of change of the current (*I*), its integral (2), capacitance (3), and conductance (4) of the BLM with adsorbed membrane fragments containing Na⁺,K⁺-ATPase and the charge transferred across the membrane after the release of ATP from caged ATP initiated by a UV light flash at *t* = 0.9 s. The aqueous solution contained 10 mM MgCl₂, 1 mM EDTA, 30 mM imidazole, pH 6.5, 100 μM caged ATP, 50 μg/ml Na⁺,K⁺-ATPase, and either 150 mM NaCl (a) or 3 mM NaCl plus 150 mM choline chloride (b). The measurements were performed under application of alternating voltage with an amplitude of 50 mV and a frequency of 15 Hz (a) or 25 Hz (b).

itance and conductance values were corrected according to the equations derived in [16]:

$$C = C_{\text{meas}} + \tau g_{\text{meas}}, \quad G = g_{\text{meas}} - \tau \omega^2 C_{\text{meas}}, \quad (2)$$

where τ is the time constant of the Keithley 427 amplifier filter (usually set to 0.01–1 ms), C_{meas} and g_{meas} are the measured increments of capacitance and conductance; C and g , the respective corrected values.

Approximation of the experimental data by theoretical curves was performed by software written by the authors on the base of known algorithm of minimization of the function (the sum of squares of deviations) of several variables. The linear parameters C_0 , C_1 , C_2 , and C_{lim} of Lorentz functions (Eqs. (3)) were calculated by the method of linear regression.

RESULTS

Kinetics of Capacitance and Conductance

Figure 3 shows time course of the currents (panel 1) and the integral of the currents, which represent the charge transferred by the pumps (panel 2), as well as the change of the capacitances (panel 3) and conductances (panel 4) of the compound membrane at 3 or

150 mM NaCl. At high (150 mM) concentration of NaCl (Fig. 3a), the capacitance initially increased after the light flash. The kinetics of conductance and capacitance gradually changed with decreasing Na⁺ concentrations and at concentrations below 10 mM, the change of capacitance and conductance induced by the ATP concentration jump became negative in a definite region of frequencies. The experiment presented in Fig. 3b was performed in the presence of 3 mM NaCl. The ATP-induced current produced a charge signal (panel 2) with a time course similar to that at 150 mM NaCl; however, the change of capacitance (panel 3) was negative, while the conductance change (panel 4) was still positive.

The negative capacitance change, $\Delta C < 0$, which can be observed after the release of ATP at low Na⁺ concentrations, has to be related to an electrogenic process that disappears (or is significantly decreased) after enzyme phosphorylation and the concomitant Na⁺ occlusion. A viable candidate for such an electrogenic process is the movement of the third Na⁺ in the cytoplasmic access channel of the protein. Ion movements in a narrow access channel reduce the apparent thickness of the membrane, and therefore, increase the capacitance of the membrane. When this process is blocked by occlu-

sion of the binding sites and the transition into the P-E₂ conformation, the apparent thickness of the membrane increases and the capacitance decreases [29]. As will be shown theoretically (see below), the maximal effect of the electrogenic transport on the change of capacitance and conductance is expected when Na⁺ concentration is in the range of half saturation of the binding sites. The cytoplasmic sodium-binding constant of non-phosphorylated Na⁺,K⁺-ATPase is known to be 4 mM in the presence of 5 mM Mg²⁺, pH 7.2 [22] and 8 mM in the presence of 10 mM Mg²⁺, pH 7.2 [40]. In the P-E₂ conformation the extracellular sodium-binding constant is about 500 mM [41]. Therefore, after the transition to P-E₂ at low Na⁺ concentrations, only minor charge movements are expected through the extracellular access channel of the Na⁺,K⁺-ATPase, and only an insignificant apparent increase of the membrane capacitance occurs, which, in consequence, is overcompensated by the abolished negative capacitance change on the cytoplasmic side (Fig. 3b).

In the presence of higher Na⁺ concentrations (≥100 mM NaCl), due to the saturation of the (cytoplasmic) binding sites, no significant charge movements occur in the corresponding access channel prior phosphorylation and amplitude of the negative component of the capacitance change after enzyme phosphorylation is much smaller than the increase of capacitance produced by the now possible charge movement in the extracellular access channel after the transition into the P-E₂ conformation. Therefore, a positive capacitance change, ΔC, can be observed at 150 mM NaCl (Fig. 3a).

In summary, the fact that the capacitance change has a (negative) minimum around the cytoplasmic half-saturation Na⁺ concentration and increases with enhanced Na⁺ concentrations confirms the assumption that the capacitance changes are caused by the electrogenic transport of Na⁺ in the cytoplasmic channel in conformation E₁, followed by the electrogenic ion movements in the P-E₂ conformation after phosphorylation of the Na⁺,K⁺-ATPase. From the experimentally obtained amplitudes it can be concluded that the cytoplasmic access channel is less deep than the extracellular one, which is in agreement with other findings reported earlier [15, 21, 29].

Besides the admittance increments associated with charge translocations inside the Na⁺,K⁺-ATPase other processes may affect the admittance of the compound membrane. In the absence of other charge transporters, the only known process capable of producing measurable effects is electrostriction, which is the compression of the membrane by the electric field leading to an increase of membrane capacitance. Na⁺ ions, which are pumped by the ATPase into the gap between the membrane fragments and the BLM, change the electric potential gradient across both membranes (Fig. 2). The initial electrical potential in the gap may be negative because of the negative surface charge of the membrane fragments which are placed very close to the BLM [42].

Pumping of Na⁺ by Na⁺,K⁺-ATPase into the gap should shift the potential to more positive values. This shift decreases the absolute value of the electric field inside the lipid bilayer that can decrease the value of its capacitance due to electrostriction. If the negative capacitance change observed at low Na⁺ concentrations is caused by electrostatic charging in the gap between BLM and membrane fragments, this effect should correlate with the change of potential in the gap, which is known to be proportional to the charge transferred by the ATPase through the membrane fragments. This transferred charge and its time course can be determined by integrating the short circuit current [28]. The time course of the currents and their integrals are shown in Fig. 3 (traces 1 and 2). After the flash-induced ATP release, the transferred charge (i.e., the integral of the current) increases, reaches maximum, and then relaxes to a steady-state value, which corresponds to the steady-state potential in the gap established in the conditions of the Na–Na exchange [15, 28]. The steady-state value typically was about 50% of its maximum at 150 mM NaCl (Fig. 3a) and less than 20%, at NaCl concentrations below 10 mM (Fig. 3b). Comparison of the time course of the transferred charge (traces 2) and of the admittance increments (traces 3) demonstrates that at low Na⁺ concentrations no correlation exists between both signals. In other words, almost instantaneous increment of capacitance and conductance is not a function of the electric potential in the gap. This is quite a compelling reason to claim that the negative changes of capacitance and conductance observed under such conditions are not caused by electrostriction, but rather by a process directly related to the charge transfer by the protein, presumably, by ion movements in the cytoplasmic access channel.

Frequency Dependencies of the Changes of Capacitance and Conductance

The frequency dependence of the capacitance and conductance increments was studied at various NaCl concentrations between 3 mM and 1 M. To take into account a different number of active pumps in various experiments, these increments were normalized to the charge transferred across the membrane after the UV-flash-induced release of ATP, as introduced in [16]. This amount of charge was defined by the maximal value of the integrated current recorded after actuation of the ATPase (Fig. 3). Figure 4 shows typical frequency dependences at three Na⁺ concentrations, 3, 7, and 150 mM. The dependence obtained at 150 mM (curve 1) is similar to that obtained earlier [16]. At low sodium concentrations, 7 mM (curve 2) and 3 mM (curve 3), the capacitance and conductance increments became negative within a defined frequency range (Fig. 4). While in the frequency window 10–100 Hz the capacitance change was negative, the conductance change was positive. Above 100 Hz the behavior was inverted.

To verify that the observed effects on capacitance and conductance increments are associated with the electrogenic Na^+ transport, control experiments were performed to assess the sensitivity of the effects to ionic strength and the chloride concentration in the electrolyte. If it is an effect of the low Na^+ concentration, no change should be detected after addition of choline chloride. The frequency dependences measured in solutions containing 3 mM Na^+ in the presence or absence of 150 mM choline chloride are presented in Fig. 4 (4). It can be seen that the presence of choline chloride in the electrolyte did not notably affect the frequency dependence. This result justifies to the conclusion that the observed negative changes of the capacitance are associated with low Na^+ concentration and not with the presence of Cl^- . The absence of the choline chloride effect also confirms the conclusion that the negative changes of capacitance and conductance observed at low concentrations of sodium ions cannot be explained by electrostriction, because this effect should depend on ionic strength, which can be excluded by the presented experiments.

Recently we have demonstrated that frequency dependencies at high Na^+ concentrations can be well approximated by a sum of two Lorentz functions with positive amplitudes and a frequency-independent component [16]. The frequency dependence of the negative capacitance changes, which was observed at low Na^+ concentrations, can be approximated by introduction of an additional Lorentz term with a negative amplitude [35]. The fitting curves in Fig. 4 were plotted using the following equations:

$$C_p = C_0 \frac{\omega_0^2}{\omega^2 + \omega_0^2} + C_1 \frac{\omega_1^2}{\omega^2 + \omega_1^2} - C_2 \frac{\omega_2^2}{\omega^2 + \omega_2^2} + C_{\text{lim}}, \quad (3)$$

$$G_p = C_0 \omega_0 \frac{\omega^2}{\omega^2 + \omega_0^2} + C_1 \omega_1 \frac{\omega^2}{\omega^2 + \omega_1^2} - C_2 \omega_2 \frac{\omega^2}{\omega^2 + \omega_2^2}.$$

To approximate the experimental points at 150 mM NaCl , it was sufficient to omit the negative component (i.e., $C_2 = 0$). The characteristic frequencies of the “slow” ($\omega_0 = 58 \text{ s}^{-1}$) and “fast” ($\omega_1 = 2570 \text{ s}^{-1}$) process were close to our estimates published previously [16]. The frequency dependence at 3 mM NaCl could be fitted by restriction to the “slow” and “negative” Lorentz functions, neglecting the “fast” component (i.e., $C_1 = 0$). At Na^+ concentrations between 3 and 10 mM (the data presented in Fig. 4 were taken at 7 mM Na^+), the data points could be fitted only if all three amplitudes (C_0 , C_1 , and C_2) had non-zero values.

The frequency dependencies of the capacitance and conductance changes in the concentration range between 3 mM and 1 M NaCl were measured and fitted by the respective equations (Eqs. (3)). The Na^+ -concentration dependences of the amplitudes, C_0 , C_1 , and C_2 , and corner frequencies, ω_0 , ω_1 , and ω_2 , of the corresponding Lorentz functions are shown in Fig. 5. As can

Fig. 4. The dependences of the capacitance (a) and conductance (b) increments on the frequency of the applied alternating voltage. The aqueous solution contained: 10 mM MgCl_2 , 1 mM EDTA, 30 mM imidazole, pH 6.5, and various concentrations of NaCl : 150 mM (1), 7 mM (2), 3 mM (3 and 4). In (4), the solution also contained 150 mM choline chloride. Each point corresponds to the average of at least 3 measurements. The solid lines show the fitting of these dependences by the sum of three Lorentzians according to Eq. (3) with the following parameters: $C_0 = 1.17 \text{ V}^{-1}$, $C_1 = 0.485 \text{ V}^{-1}$, $C_2 = 0$, $C_{\text{lim}} = 0.169 \text{ V}^{-1}$, $\omega_0 = 58 \text{ s}^{-1}$, $\omega_1 = 2570 \text{ s}^{-1}$ (solid line); $C_0 = 3.7 \text{ V}^{-1}$, $C_1 = 0$, $C_2 = -0.43 \text{ V}^{-1}$, $C_{\text{lim}} = 0.06 \text{ V}^{-1}$, $\omega_0 = 17 \text{ s}^{-1}$, $\omega_2 = 377 \text{ s}^{-1}$ (dashed line); $C_0 = 2.84 \text{ V}^{-1}$, $C_1 = 0.128 \text{ V}^{-1}$, $C_2 = -0.245 \text{ V}^{-1}$, $C_{\text{lim}} = -0.006 \text{ V}^{-1}$, $\omega_0 = 19.5 \text{ s}^{-1}$, $\omega_1 = 1240 \text{ s}^{-1}$, $\omega_2 = 569 \text{ s}^{-1}$ (dotted line).

be seen, the amplitude of the “negative” component, C_2 , decreases with increasing Na^+ concentrations and disappears at $>10 \text{ mM}$. The corresponding corner frequency ω_2 increases with concentration (at $>10 \text{ mM}$ NaCl it could not be resolved). The value of the “slow,” positive amplitude, C_0 , decreases, while the value of the “fast” amplitude, C_1 , increases with concentration. The

Fig. 5. The dependences of amplitudes (a) and corner frequencies (b) of the Lorentzian fitting the frequency dependences of capacitance and conductance increments on the concentration of sodium ions in the solution. The points were obtained by fitting of frequency dependences of capacitance and conductance increments and following averaging of at least 3 experiments. The solid lines are the theoretical curves plotted by Eqs. (A27)–(A29) and (A31)–(A33) with the following values of the parameters: $A = 30 \text{ mM}$; $\alpha_e = 0.89$; $\alpha_c = 0.21$; $\alpha = 0.06$; $K = 200$; $1/K_e = 0.74 \text{ M}$; $1/K_i = 0.0015 \text{ M}$; $k_{12} = 2.4 \text{ s}^{-1}$; $k_{23}^0 = 1500 \text{ s}^{-1}$; $k_{01}^0 = 160 \text{ s}^{-1} \text{ M}^{-1}$.

corner frequency of “slow” component, ω_0 , increases with concentration, while ω_1 does not change significantly. The solid lines are the calculated curves according to the theoretical model described below.

THEORETICAL MODEL

The model describing the dependence of the capacitance and conductance changes on the frequency of the applied AC voltage is based on the extended Albers–Post cycle of the Na^+, K^+ -ATPase (Fig. 1). The ion pump presents its ion-binding sites alternately either to the cytoplasmic phase of the membrane (conformation E_1) or to the extracellular side (conformation $P-E_2$). Both principal conformations are linked by the occluded state ($PE_1(3\text{Na})$), in which none of both access channels

is functional. Since the experiments were performed in the absence of ADP, the presence of ATP produces an irreversible transition from E_1 to $P-E_2$ conformation. Therefore, either cytoplasmic or extracellular electrogenic effects will occur in each pump and the release of ATP by the UV-light flash induces a synchronized transition from the cytoplasmic to the extracellular contributions.

The major contribution to the electric current through the membrane is related to the transfer of only one of the three sodium ions [11, 14, 15, 23, 27, 36, 41], and therefore, only this transfer is considered in our model. Na^+ transfer consists of several steps depicted in Fig. 1 by boxes. Let us consider these steps in more details.

In the absence of ATP, pump function is confined to the reaction steps in the conformation denoted by E_1 . First, the third sodium ion can exchange between solution and its binding site through the cytoplasmic access channel and this electrogenic process contributes to the electric current:

The next step, phosphorylation of the sodium pump by ATP, is associated with occlusion of the three Na^+ and closure of the cytoplasmic access channel. This transition can be considered as irreversible under the condition of our experiment, since the ADP concentration in the aqueous phase is virtually zero. During this step, no charge movement could be detected [28]:

The following transitions are reversible. The first one is a slow and rate-limiting conformational transition in which there is no (or negligible) charge transfer across the membrane [11, 15]:

The second one consists of two steps: release/binding of one Na^+ from/to the binding site at the inner end of the access channel, the so-called “medium-speed component” with the rate faster than the conformation transition, but slower than the movement of sodium ion in the access channel [11]:

followed by the ion movement in the access channel which is probably diffusion controlled. Release of this Na^+ produces the most significant contribution to the electric current [11, 15]:

The following transition consists also of two steps, consecutive release/binding of the subsequent 2Na^+ and their movement through a significantly widened access channel. These steps produce only minor current com-

ponents [11, 15] and therefore, may be neglected in our first-order theoretical description.

We will consider the physical model of sodium ion transport as a series of several steps/transitions between five states of protein (Fig. 6). The electrogenic transport of the third sodium ion on the cytoplasmic side occurs during the transition between the first state, $E_1(2Na)$ (labeled “c0”) and the second state, $E_1(3Na)$ (labeled “c1”). After phosphorylation by ATP, the Na^+, K^+ -ATPase performs the transition into the occluded state (labeled “1”), which closes the cytoplasmic access channel. The occluded state is not stable but relaxes with a sequence of reaction steps, shown in the next two transitions: first, the conformation transition into conformation $P-E_2$, in which the extracellular access channel is open but the three Na^+ still occupy the binding site inside the protein, $P-E_2(3Na)$ (labeled “2”); after that, Na^+ is released to the aqueous phase, $P-E_2(2Na)$ (labeled “3”). The energetic conditions of the Na ion are very different in these states: in the occluded state 1 the ion is bound in a potential well between two high-energy barriers. In the other states a high barrier closes only one of both access channels either on the extracellular side (in c0 and c1) or on the cytoplasmic side of protein (in states 2, 3). As mentioned above, only movements of the sodium ion in the narrow cytoplasmic and extracellular access channels are considered electrogenic (transitions between the states c0 and c1 and between 2 and 3). These transitions comprise diffusion or migration of the Na^+ in the access channels without any essential conformational change of the protein. Na^+ ions in the access channels respond to the externally applied electric field by a respective movement, and thus contribute to the electric current measured in the external circuit. If the electric field is small, it is possible to describe the transport process as jumps over an energy barrier. This approach is discussed in [36, 43].

The number of Na^+, K^+ -ATPase molecules in the membrane will be defined as the surface density of proteins (i.e., the number of the molecules per unit of membrane surface). The application of an alternative voltage to the membrane in the absence of ATP initiates the movement of sodium ions inside the cytoplasmic access channel. The kinetic equation describing this process can be written in the following form:

$$\frac{dn_{c1}}{dt} = -k_{10}n_{c1} + k_{01}[Na_c]n_{c0}, \quad (9)$$

where n_{c0} is the surface density of the Na^+, K^+ -ATPase in state c0; n_{c1} , the surface density in state c1; $[Na_c]$ is the concentration of sodium ions in the cytoplasmic aqueous solution; k_{01} and k_{10} are the rate constants of forward and backward transitions between the states c0 and c1, respectively.

Ion movements in the cytoplasmic access channel occur in all (active) protein molecules present in the membrane. However, with the experimental method applied only the ATP-dependent changes of charge

Fig. 6. The model of electrogenic sodium-ion transport in the intracellular and extracellular access channels of Na^+, K^+ -ATPase. The cartoons show the energy profiles in access channels for sodium ion in various states of the Na^+, K^+ -ATPase. The dotted lines show hypothetical energetic profile in closed access channels, the energy of ion in which should be very high.

movements are detected, which are confined to the fraction N of all protein molecules, which have been phosphorylated. In the phosphorylated molecules ion move-

ment is blocked in the cytoplasmic access channel and begins instead in the extracellular access channel. The total number of ion pumps in states c_0 and c_1 , the transport in which is blocked by the enzyme phosphorylation, is

$$n_{c1} + n_{c0} = N. \quad (10)$$

The kinetic equations describing Na^+ transport on the extracellular side include the transitions between states 1 and 2 (conformational transition and deocclusion of the sodium ion) and between states 2 and 3 (movement of sodium ion in the access channel connecting binding site and the aqueous solution):

$$\frac{dn_1}{dt} = -k_{12}n_1 + k_{21}n_2, \quad (11)$$

$$\frac{dn_2}{dt} = k_{12}n_1 - (k_{21} + k_{23})n_2 + k_{32}[\text{Na}_e]n_3, \quad (12)$$

$$\frac{dn_3}{dt} = k_{23}n_2 - k_{32}[\text{Na}_e]n_3, \quad (13)$$

where n_1 , n_2 , and n_3 are the surface densities of the protein in state 1, 2, and 3, respectively; $[\text{Na}_e]$ is the sodium concentration in the extracellular aqueous solution; k_{12} and k_{21} are the rate constants of the forward and backward transition between states 1 and 2; k_{23} and k_{32} , the rate constants between states 2 and 3, respectively. The number of pump molecules per unit area participating in these reaction steps on extracellular sodium ions transport is equal to the number of pumps phosphorylated by ATP,

$$n_1 + n_2 + n_3 = N. \quad (14)$$

In the absence of an alternating voltage applied to the membrane, there is an equilibrium between different states of the Na^+, K^+ -ATPase. The long-lived phosphorylated state of Na^+, K^+ -ATPase is also close to equilibrium. Equilibrium densities of the Na^+, K^+ -ATPase in the different states can be determined from Eqs. (9) and (11)–(13), when the left sides of these equations are set to zero. Solving this equation system and taking into account the normalization equations (10) and (14), leads to

$$n_{c1}^0 = N \frac{K_c[\text{Na}_c]}{1 + K_c[\text{Na}_c]}, \quad n_{c0}^0 = N \frac{1}{1 + K_c[\text{Na}_c]}, \quad (15)$$

where

$$K_c = \frac{k_{01}}{k_{10}} \quad (16)$$

and

$$n_1^0 = N \frac{[\text{Na}_e]K_eK_1}{1 + [\text{Na}_e]K_e(K_1 + 1)}, \quad (17)$$

$$n_2^0 = N \frac{[\text{Na}_e]K_e}{1 + [\text{Na}_e]K_e(K_1 + 1)}, \quad (18)$$

$$n_3^0 = N \frac{1}{1 + [\text{Na}_e]K_e(K_1 + 1)} \quad (19)$$

with

$$K_e = \frac{k_{32}}{k_{23}}, \quad K_1 = \frac{k_{21}}{k_{12}}. \quad (20)$$

The application of an alternating voltage to the membrane leads to movements of sodium ions either in the cytoplasmic or extracellular access channels. In the model these movements are considered to be jumps over (low) energetic barriers. Due to the fact that the access channels are narrow, ion movements in them may be treated as in a medium with a low dielectric constant [36]. In this case the effect of the electric field on the rate constants of the corresponding reaction steps can be accounted for by simple correction factors [44]:

$$k_{01} = k_{01}^* \exp(\beta\phi_c/2), \quad (21)$$

$$k_{10} = k_{10}^* \exp(-\beta\phi_c/2)$$

in the cytoplasmic access channel and

$$k_{23} = k_{23}^* \exp(\beta\phi_e/2), \quad (22)$$

$$k_{32} = k_{32}^* \exp(-\beta\phi_e/2)$$

in extracellular access channel. Here $\beta = e/kT$; k is the Boltzmann constant; T , absolute temperature; e , the elementary charge; ϕ_c , the potential drop in cytoplasmic access channel, and ϕ_e , the potential drop in extracellular access channel; the upper index “*” denotes the corresponding rate constants without electric field.

To determine the voltage drop inside the access channels and the relation of the ion movements inside the protein to the current measured in the experiment, it is necessary to analyze the equivalent circuit of the complex membrane consisting of a bilayer lipid membrane with adsorbed membrane fragments containing the sodium pump (Fig. 2). We will use a simplified equivalent circuit of this system representing the BLM as a capacitor connected in series to the membrane fragments with Na^+, K^+ -ATPase (Fig. 2b). It was shown that the voltage effect on the pump as well as the current detected in such a system are attenuated by factor α depending on the BLM to membrane fragment capacitance ratio (C_B and C_F , respectively) [15, 16, 27, 34]:

$$\alpha = \frac{C_B}{C_B + C_F}. \quad (23)$$

Inside the Na^+, K^+ -ATPase the effect of the applied voltage is attenuated by so-called dielectric coefficients. They are defined by the relative depth of the access channels in the membrane [14, 27, 36]. With respect to the membrane fragment capacity, it can be represented as two capacitors in series (Fig. 2b) [13]. The capacitance C_c corresponds to the layer of the membrane in which the charge moves in the cytoplasmic access channel of the protein. C_e corresponds to the

respective capacitance of the extracellular access channel. These considerations allow determining the voltage drop inside the protein, ϕ_c and ϕ_e , with respect to the voltage U applied between both aqueous solutions:

$$\phi_c = \alpha \alpha_c U, \quad \phi_e = \alpha \alpha_e U, \quad (24)$$

where

$$\alpha_e = \frac{C_c}{C_e + C_c}, \quad \alpha_c = \frac{C_e}{C_e + C_c}. \quad (25)$$

The same coefficients define the relation of the ion movements in the access channels to the current measured in the external circuit, I . The charge movements inside the protein correspond to recharging of one of both internal capacitors, C_c and C_e . The current I is defined as

$$I_c = \alpha \alpha_c e \frac{dn_{c1}}{dt}, \quad I_e = \alpha \alpha_e e \frac{dn_3}{dt}. \quad (26)$$

The function of the Na^+, K^+ -ATPase in such a system can be affected by way of the contact of the membrane fragments with the BLM. The first problem is that ion movements inside the Na^+, K^+ -ATPase may modify the electric field within the protein and the voltage across the whole membrane. For the sake of simplicity this effect of Na^+, K^+ -ATPase on the electric field will be neglected. In addition, one cannot exclude that the Na^+ concentration changes in the gap between membrane fragments and BLM. This gap is a water-filled cavity of a very small volume. Evaluations of the size of this gap [42] were based on the experimental investigation of the adsorption of Na^+, K^+ -ATPase-containing membrane fragments on the BLM surface. The Na^+, K^+ -ATPase exposes its extracellular hydrophilic parts to the gap. This volume can be regarded as a water-filled compartment containing fixed anions belonging to the ectodomains of the Na^+, K^+ -ATPase. The ions in this compartment are in a Donnan equilibrium with the external solution. According to the Donnan equation, Na^+ concentration in the gap $[\text{Na}_e]$ is related to the concentration of these ions in the external solution $[\text{Na}_c]$ and the concentration of the fixed anions A as

$$[\text{Na}_e] = \frac{1}{2}[\text{Na}_c] + \frac{1}{2}\sqrt{[\text{Na}_c]^2 + 4A^2}. \quad (27)$$

This formula shows that at high sodium ion concentration, the value of $[\text{Na}_e]$ does not significantly differ from $[\text{Na}_c]$. The correction of $[\text{Na}_e]$ becomes important only at low concentration, when the value of $[\text{Na}_e]$ reaches a limit, which is determined by the concentration of fixed anions in the gap between the membranes (Fig. 7).

In the experiments, the voltage applied to the membrane changes with time as

$$U = V \cos(\omega t). \quad (28)$$

The applied periodic voltage U evokes an alternating current that changes in time as a sum of cosine and

Fig. 7. Dependence of the concentration of sodium ions in the gap between BLM and membrane fragment with Na^+, K^+ -ATPase on the concentration of these ions in the bulk solution plotted according to Eq. (27) with $A = 30$ mM.

sine functions. The ATP-induced contribution of the electrogenic ion transport by the Na^+, K^+ -ATPase to the current is determined as the difference between the currents recorded before and after the ATP release (Fig. 3). This contribution can be either negative or positive. When ATP “switches on” the charge movements generated by the AC voltage, then this contribution to the electric current has a positive sign. Such a current contribution, I_e , is produced by electrogenic ion movements in the extracellular access channel which is opened by the conformation transition after the enzyme phosphorylation. In the case of electrogenic ion movements in cytoplasmic access channel, phosphorylation of the protein by ATP causes closure or blocking of the access channel and disables the electrogenic transport. Therefore, the current I_c evoked by the alternating voltage vanishes and the admittance change has a negative sign. This difference of the currents is detected in the experiment as ATP-induced increments of the membrane capacitance C_p and conductance G_p :

$$\Delta I|_{\text{ATP}} = I_e - I_c = G_p U + C_p \frac{dU}{dt}. \quad (29)$$

To retrieve this contribution, the kinetic equations describing Na^+ transfer in intracellular and extracellular access channels and the main conformation transition were solved. The admittance changes associated with this solution were calculated (see Appendix). It was shown that the ATP-driven changes of capacitance and conductance could be presented by a sum of Lorentz functions:

$$C_p = C_0 \frac{\omega_0^2}{\omega^2 + \omega_0^2} + C_1 \frac{\omega_1^2}{\omega^2 + \omega_1^2} - C_2 \frac{\omega_2^2}{\omega^2 + \omega_2^2}, \quad (30)$$

$$G_p = C_0 \omega_0 \frac{\omega^2}{\omega^2 + \omega_0^2} + C_1 \omega_1 \frac{\omega^2}{\omega^2 + \omega_1^2} - C_2 \omega_2 \frac{\omega^2}{\omega^2 + \omega_2^2} \quad (31)$$

The amplitudes C_i and corner frequencies ω_i ($i = 0, 1, 2$) are derived from the kinetic constants of the corresponding electrogenic transport steps. The first two terms in Eqs. (30) and (31) correspond to the ion movements in the extracellular access channel. The exact solutions for C_0 , C_1 , ω_0 , and ω_1 are derived in the Appendix. The general formulas, however, can be simplified considerably if the rate of Na^+ transport in the access channel is assumed to be much higher than the rate of the conformation transition, i.e., $k_{23} \gg k_{12}$. In this case the following approximated derivations are obtained:

$$C_0 \approx eN\beta(\alpha\alpha_e)^2 \frac{[\text{Na}_e]K_e K_1}{(1 + [\text{Na}_e]K_e)(1 + [\text{Na}_e]K_e(K_1 + 1))^2}, \quad (32)$$

$$\omega_0 \approx k_{12} + k_{21} \frac{[\text{Na}_e]K_e}{(1 + [\text{Na}_e]K_e)},$$

$$C_1 \approx eN\beta(\alpha\alpha_e)^2 \frac{[\text{Na}_e]K_e}{(1 + [\text{Na}_e]K_e)(1 + [\text{Na}_e]K_e(K_1 + 1))}, \quad (33)$$

$$\omega_1 \approx k_{23}^0(1 + [\text{Na}_e]K_e).$$

The last term in Eqs. (30) and (31), which has a negative sign, represents the ion movements in the intracellular access channel. It was obtained as an exact solution of kinetic equation (9), as shown in the Appendix:

$$C_2 = eN\beta(\alpha\alpha_e)^2 \frac{[\text{Na}_e]K_e}{(1 + [\text{Na}_e]K_e)^2}, \quad (34)$$

$$\omega_2 = k_{10}^0(1 + [\text{Na}_e]K_e).$$

The dependence of C_p and G_p on frequency is controlled by the parameters ω_0 , ω_1 , and ω_2 . Each of these parameters is the corner frequency of the correspondent Lorentz function; their values are defined by the sum of forward and back rates of transitions between the correspondent states of protein. (For the extracellular ion movements this is valid only in the approximation accepted above, i.e., when the rates of the ion movements in the access channel are much higher than the rates of conformation transition.) In the limit of an infinite frequency ω , the value of C_p approaches zero, whereas G_p reaches a plateau. In the limit of low frequency, G_p tends to zero, whereas C_p reaches a plateau. The plateau values and their dependences on Na^+ concentrations are defined by the amplitudes C_0 , C_1 , and C_2 of the Lorentz functions. They depend non-monotonically on the Na^+ concentration. It should be mentioned that these amplitudes could be obtained in a simpler way, without solving the kinetic equations, but by differentiation of the charge bound to the ion sites in the equilibrium, which is a function of the applied alternating voltage. In the case of charge movements in the

cytoplasmic access channel it can be shown immediately that

$$C_2 = e\alpha\alpha_e \frac{dn_{e1}^0}{dU}. \quad (35)$$

The extracellular transport consists of two steps represented by two Lorentz functions. The charge bound inside the protein from the extracellular side is the sum of the charge bound in two states, n_1 and n_2 . Differentiation of this sum leads to the sum of amplitudes of the corresponding Lorentz functions:

$$C_0 + C_1 = e\alpha\alpha_e \frac{dn_3^0}{dU}$$

$$= eN(\alpha\alpha_e)^2 \beta \frac{[\text{Na}_e]K_e(1 + K_1)}{(1 + [\text{Na}_e]K_e(K_1 + 1))^2}. \quad (36)$$

The contribution of C_1 to this sum can be found by a similar differentiation procedure, if it is taken into account that C_1 defines the capacitance due to the fast Na^+ movement in the extracellular access channel measured at a frequency low enough to allow the ions inside the access channels, n_2 , to be in equilibrium at the applied voltage (i.e., in equilibrium between n_2 and n_3), but nevertheless too high to change the amount of n_1 . Therefore,

$$C_1 = e\alpha\alpha_e \frac{dn_2^0}{dU} \Big|_{n_1^0 = \text{const}}. \quad (37)$$

Note that the derivations for C_2 and for $C_0 + C_1$ are always valid, even for the exact solution of the kinetic equations (see Appendix). They are defined only by the equilibrium distribution between different states of the Na^+ , K^+ -ATPase, and so they do not depend on a detailed mechanism of charge movements in the access channels and their kinetic parameters. Therefore, the bell-shaped dependence of the values of C_0 , C_1 , and C_2 on the Na^+ concentration in solution is a general feature, which is obvious in the simplified equations (32)–(34), dependent only on equilibrium binding of the sodium ions inside the protein. The amount of ions bound depends on their concentration in accordance with equations (15)–(20) with two plateaus. It is zero at low concentration and attains a maximum, which corresponds to the total occupancy of the binding sites at high concentration. Obviously, at Na^+ concentration, at which the binding sites are half-saturated, an externally applied voltage can modify the occupation of the binding sites most effectively. Such a concentration corresponds to the maximum amplitude of the corresponding Lorentz function that determines the value of the capacitance and conductance change. The value of C_2 (Eq. (34)) should be maximal at the concentration corresponding to half-maximal cytoplasmic Na^+ binding in the E_1 conformation (according to Eq. (15), this concentration corresponds to $n_{1c}^0 = N/2$). The maximum of the amplitudes, C_0 and C_1 , will be found at Na^+ concen-

trations that correspond to half-maximal binding at the extracellular side. The latter is obvious for $C_0 + C_1$, which is maximal at the concentration corresponding to half-maximal binding at all sites accessible from the extracellular side (i.e., $n_1^0 + n_2^0 = N/2$).

It is known that Na^+ binding on the cytoplasmic side occurs with a significantly higher affinity than that on the extracellular side [41]. Therefore, the contribution of electrogenic ion movements in the cytoplasmic access channel should be maximal at low concentrations and disappear at higher concentrations, at which the contribution of ion movements should be maximal in the extracellular access channels. This gives a possibility to discriminate these contributions by measuring the dependence of admittance change on the concentration of sodium ions.

The ATP-driven capacitance and conductance changes measured in experiments were normalized to the (total) charge, Q_m , transported by the Na^+, K^+ -ATPase after phosphorylation by ATP.

$$\Delta C = C_p/Q_m, \quad \Delta G = G_p/Q_m. \quad (38)$$

This charge can be calculated as the charge moved across the membrane between both equilibrium states of the Na^+, K^+ -ATPase before and after release of ATP from caged ATP:

$$Q_m = \alpha \alpha_e n_3^0. \quad (39)$$

By this normalization method all measured values became independent of the unknown total amount of the protein in the membrane. However, the normalization affects the dependences of ΔC and ΔG on Na^+ concentration, because the charge Q_m itself depends also on the Na^+ concentration. This dependence is non-monotonic. The increase of the Na^+ concentration leads to a decrease of Q_m due to the decrease of n_3^0 , according to Eq. (19). In principle, Q_m can also decrease with Na^+ at low sodium ions concentration due to the decreased number of phosphorylated molecules, N , but it does not affect ΔC and ΔG , because after normalization the effect of N is cancelled.

The dependency of C_p and G_p on Na^+ concentration and frequency of the alternating voltage is similar to that derived earlier [34], with the modification that we consider now a more complex model with three transport steps, which results in more complex expressions for C_p and G_p , each being the sum of three Lorentz functions. The main properties of these derivations are similar to those discussed earlier. For example, it was shown that the capacitance increments depend on the DC voltage bias as the bell-shaped, non-monotonic function with a maximum corresponding to half saturating of binding sites [30, 34]. This is similar to the bell-shaped dependences of C_0 , C_1 , and C_2 on Na^+ concentration obtained in the present model, the maxima of

which also correspond to the half saturation of binding sites.

The experimental dependence of the parameters of the Lorentz functions on the Na^+ concentration (Fig. 5) was fitted by a theoretically derived dependence as presented in Eqs. (A27)–(A29) and (A31)–(A33) of the Appendix. These equations represent the exact solution of the kinetic equations and are close to the approximated equations discussed above. The theoretical curves are shown by solid lines in Fig. 5.

DISCUSSION

The experimental data on the frequency dependence of capacitance and conductance increments induced by phosphorylation of the Na^+, K^+ -ATPase are adequately described by a theoretical model that includes the main conformation transitions of the protein and movements of sodium ions in cytoplasmic and extracellular access channels. It allows the determination of the rate constants and equilibrium dissociation constants of the corresponding processes. At high sodium concentration, the frequency dependencies are similar to that measured earlier and explained qualitatively if the rate of sodium ion transport in extracellular access channel is assumed to be controlled by at least two distinct steps, the conformation transition and binding/release of a sodium ion in the binding sites exposed to the extracellular access channel [16]. Earlier we suggested a model in which transport of the sodium ions in the extracellular access channel consists of these two steps. The experimental frequency dependence of the capacitance and conductance increments was measured at various sodium ions concentrations; however, it could be fitted by theoretical curves in an acceptable manner only in a very limited concentration range [45]. In the present model the structure of the contact between membrane fragments and bilayer lipid membrane was accounted for by the assumption that the Na^+ concentration in the small volume between both membranes can differ from that in the bulk solution. This effect is negligible at high Na^+ concentrations but becomes significant at low concentrations. When the Na^+ concentration in the gap is calculated as a function of the bulk Na^+ concentration, it can be shown that pumping of ions into the gap results in a significant effect in the low concentration range, where the Na^+ concentration in the gap approaches a limiting value (Fig. 7). This means that at low Na^+ concentrations, Na^+, K^+ -ATPase performs ion transport under the conditions of a Na^+ concentration gradient, when the concentration at the extracellular side of protein becomes significantly higher than at the cytoplasmic side. This effect becomes significant when the aqueous volume in the gap is small. Consideration of this effect allows fitting the experimental data over a wide range of Na^+ concentrations. The experimental support of this approach was obtained in our recent study of the adsorption of membrane fragments on lipid bilayer [42].

The corner frequencies of the fast and slow steps of ion transport in the extracellular access channel are similar to that found earlier [16] except that we determined these values now in a wide range of Na^+ concentrations. Fitting the experimental data by the theoretical model allowed determining the equilibrium and kinetic parameters of the transport in the extracellular access channel. The dielectric coefficient defining the relative depth of the access channel α_e is about 0.89; equilibrium dissociation constant $1/K_e$ is 0.74 M; equilibrium constant K_1 , which determines the distribution of Na^+, K^+ -ATPase molecules between states 1 (Na^+ occluded) and 2 (Na^+ deoccluded), is about 200; rate constant k_{12} of the transition from state 1 to state 2 is 2.4 s^{-1} , while the rate of the back reaction is much higher (about 500 s^{-1}). The rate constant of the transition between states 2 and 3 (Na release through the access channel) is about 1500 s^{-1} .

The most interesting result obtained in the presented experiments is the decrease of the membrane capacitance and conductance at low sodium concentrations. This effect is explained by an electrogenic sodium transport in the cytoplasmic ion access channel. The negative sign is a result of the experimental technique, in which the capacitance and conductance increments are caused by the ATP-induced enzyme phosphorylation. Electrogenic sodium transport in the cytoplasmic access channel is possible in the absence of ATP, when the protein is in E_1 conformation. But this process is blocked after phosphorylation of Na^+, K^+ -ATPase by ATP, and this effect produces a negative capacitance and conductance change. The ion transport in the cytoplasmic access channel is theoretically described correspondingly to the transport in extracellular access channel, with the only modification, that is, the sign of the Lorentz function is negative. The characteristic frequency of this Lorentz function was found to be $500\text{--}800 \text{ s}^{-1}$, and it increased with Na^+ concentration, while the amplitude had a maximum at 3 mM Na^+ and became negligible at concentrations above 10 mM (Fig. 5). The fit of the experimental data by the theoretical model yields the transport parameters: the rate constant of Na^+ binding to the binding site, the equilibrium dissociation constant ($1/K_e = 1.5 \text{ mM}$), and the dielectric coefficient characterizing the relative depth of the cytoplasmic channel (0.21). The value of $1/K_e$ is about 2 times smaller than the constants measured by other methods. In [21–23], $1/K_e$ was found to be in the range between 3 and 10 mM, the dielectric coefficient was in the range of 0.2–0.25. This deviation can be explained by the environment of the Na^+, K^+ -ATPase, because it has been shown that the apparent binding constants strongly depend on pH and other (competitive) cations present in the aqueous phase [46]. The dielectric coefficient of the ion movement in cytoplasmic access channel was much lower than the corresponding coefficient for the extracellular side, which indicates that the third Na^+ binding site may be located closer to the cytoplas-

mic surface of the membrane. The model allowed also the determination of the binding rate constant k_{01}^0 of the third Na^+ to the Na^+, K^+ -ATPase from the cytoplasmic side. It was found to be about $160 \text{ s}^{-1} \text{ M}^{-1}$. This value is obtained for the first time, because the methods employed earlier allowed determining only equilibrium constants for Na^+ binding from the cytoplasmic side of the protein.

The best fit of the experiments was achieved at the attenuation coefficient α of 0.06. This value is five times smaller than usually accepted value of 0.33, which was obtained on the assumption that the ratio of the capacitance of the lipid bilayer (containing decane as a solvent) to the capacitance of the membrane fragment (free of solvent) is 0.5. The divergent value of α is a consequence of the fact that the magnitude of admittance increments measured in the experiments became much lower than predicted theoretically. For example, the theoretically expected change of capacitance in the limit of high sodium ions concentration and low frequency can be evaluated by Eq. (36) as $C_0 + C_1$. After normalization to the transferred charge Q_m (Eq. (39)), it can be expressed by a very simple formula:

$$\frac{C_1 + C_0}{Q_m} = \alpha \alpha_e \beta. \quad (40)$$

This value does not contain any parameters of the model, except the dielectric coefficient α_e and the capacitances ratio α . The value of β is $e/kT \approx 40 \text{ V}^{-1}$. If the usually accepted values, $\alpha = 0.33$ and $\alpha_e = 0.8$, are taken into account, then the value of $(C_1 + C_0)/Q_m$ is approximately 10 V^{-1} , which is much higher than the maximum values measured in the experiments (about 2 V^{-1}). One possible reason of this discrepancy may be an incorrect normalization with the transferred charge, Q_m . The experimental value of Q_m was measured as the maximum of the integrated short circuit current after the light flash, although the time course of this value was complex and its steady-state value was much lower (Fig. 3). We used such a way of normalization because it was shown that the integral of the actual current through the membrane fragments approaches the limiting value which is close to this maximum [28]. If the normalization is correct, the only possible way to explain the low values of capacitance increments is that the actual value of α is less than the usually assumed value of 0.33. A smaller value indicates that the capacitance C_b in the equivalent circuit on the Fig. 2 is considerably smaller than the capacitance of a bilayer lipid membrane with solvent, i.e., the lipid membrane in the contact region with a membrane fragment has a much higher thickness. Arguments in favor of this proposal are discussed in our recent study of adsorption of membrane fragments on lipid bilayer. It was shown that the total capacitance of the membrane decreased about 30% of its original value due to the adsorption of the fragments [42]. Provided that the fragments do not

cover more than 50% of the lipid bilayer surface, this result means that the thickness of the “sandwich” consisting of lipid bilayer and membrane fragment is much higher than the sum of the thicknesses of membranes itself. This explanation is strongly supported by optical and electrical surveillance of the lipid bilayer membrane after addition of membrane fragments, which then developed a “silvery” structure on the black membrane. This indicates an increased thickness. At the same time, the membrane capacitance decreased up to a factor of 2–3 (I. Wuddel, personal communication). Another possible explanation of the apparent thickness increase is that the compartment between membrane fragment and lipid bilayer contains the extracellular domains of the densely packed membrane proteins and therefore can be regarded as a dielectric rather than an aqueous solution. In this case capacitance C_B is the capacitance of a complex structure including lipid bilayer and the ectodomains of the protein. The increase of the thickness may be also a result of the redistribution of the solvent that increases the thickness of the lipid membrane, specifically in the region covered by fragments.

In conclusion, we measured the frequency dependences of the capacitance and conductance increments in a wide range of concentrations of sodium ions, developed a theoretical model of the Na^+ transport in both access channels, and evaluated their parameters. It was confirmed that the transport of single sodium ion in extracellular access channel consists of two steps. We have studied in detail the electrogenic transport of sodium ions in the cytoplasmic access channel and determined its kinetic constants.

APPENDIX

Calculation of the Contribution of the Electrogenic Na^+ Movements in the Na^+, K^+ -ATPase to the Detected Capacity and Conductance Changes

Extracellular access channel. The coupled kinetic differential equations (11)–(14) are solved in the case of sine voltages applied to the membrane, and the contribution of ion movements to the membrane capacitance and conductance is determined after phosphorylation of the ion pumps by ATP. For the sake of convenience, normalized surface densities of the three states will be used as follows: $\theta_1 = n_1/N$, $\theta_2 = n_2/N$, and $\theta_3 = n_3/N$. Due to the conservation condition of Eq. (14), the surface density of state 2, n_2 , can be eliminated. Thus, two coupled equations have to be solved:

$$\frac{d\theta_1}{dt} = -(k_{12} + k_{21})\theta_1 - k_{21}\theta_3 + k_{21}, \quad (\text{A1})$$

$$\frac{d\theta_3}{dt} = -k_{23}\theta_1 - (k_{23} + k_{32}[\text{Na}_e])\theta_3 + k_{23}. \quad (\text{A2})$$

The electric potential difference will be expressed as dimensionless potential $\psi = \beta U$, with $\beta = F/RT$. Let the

potential difference between both aqueous solutions be a periodic harmonic function of time with a small amplitude:

$$\psi(t) = \psi^0 + \delta\psi(t). \quad (\text{A3})$$

This perturbation results in small variations of the normalized surface densities:

$$\theta_j = \theta_j^0 + \delta\theta_j, \quad j = 1, 2, 3.$$

The voltage dependence of the rate constants k_{23} and k_{32} is defined by Eq. (22). In the case of small $\delta\psi$ they have the form:

$$k_{23} = k_{23}^0(1 + 1/2\delta\psi), \quad (\text{A4})$$

$$k_{32} = k_{32}^0(1 - 1/2\delta\psi) \quad (\text{A5})$$

with $k_{23}^0 = k_{23}^* e^{\psi^0/2}$, $k_{32}^0 = k_{32}^* e^{-\psi^0/2}$. Introducing (A3)–(A5) into (A2), we obtain

$$\begin{aligned} \frac{d\delta\theta_3}{dt} = & -k_{23}^0 \left(1 + \frac{\delta\psi}{2}\right) (\theta_1^0 + \delta\theta_1) \\ & - \left[k_{23}^0 \left(1 + \frac{\delta\psi}{2}\right) + k_{32}^0 \left(1 - \frac{\delta\psi}{2}\right) [\text{Na}_e] \right] \\ & \times (\theta_3^0 + \delta\theta_3) + k_{23}^0 \left(1 + \frac{\delta\psi}{2}\right). \end{aligned} \quad (\text{A6})$$

In equilibrium, the normalized surface densities can be determined by

$$0 = -(k_{12} + k_{21})\theta_1^0 - k_{21}\theta_3^0 + k_{21}, \quad (\text{A7})$$

$$0 = -k_{23}\theta_1^0 - (k_{23}^0 + k_{32}^0[\text{Na}_e])\theta_3^0 + k_{23}^0. \quad (\text{A8})$$

If the variations of the normalized surface densities are small, the equation for θ_3 can be rewritten as

$$\begin{aligned} \frac{d\delta\theta_3}{dt} = & -k_{23}^0\delta\theta_1 - k_{23}^0\frac{\delta\psi}{2}\theta_1^0 \\ & - \left(k_{23}^0\frac{\delta\psi}{2} - k_{32}^0\frac{\delta\psi}{2}[\text{Na}_e] \right) \theta_3^0 \\ & - (k_{23}^0 + k_{32}^0[\text{Na}_e])\delta\theta_3 + k_{23}^0\frac{\delta\psi}{2}. \end{aligned} \quad (\text{A9})$$

This expression can be further simplified, using the relations $1 - \theta_1^0 - \theta_3^0 = \theta_2^0$ and $k_{32}^0\theta_3^0[\text{Na}_e] = k_{23}^0\theta_2^0$:

$$\begin{aligned} \frac{d\delta\theta_3}{dt} = & -k_{23}^0\delta\theta_1 + k_{23}^0\delta\psi\theta_2^0 \\ & - (k_{23}^0 + k_{32}^0[\text{Na}_e])\delta\theta_3. \end{aligned} \quad (\text{A10})$$

Introducing the expressions

$$\delta\psi = \alpha_e \alpha \beta \delta U$$

into Eq. (A9) leads to

$$\frac{d\delta\theta_3}{dt} = -k_{23}^0\delta\theta_1 + k_{23}^0\left(\alpha_e\alpha\beta\delta U - \frac{\beta Ne\delta\theta_3}{C_m}\right)\theta_2^0 - (k_{23}^0 + k_{32}^0[\text{Na}_e])\delta\theta_3. \quad (\text{A11})$$

For further calculations we rewrite the kinetic equations describing the charge transfer in extracellular access channel in the following form:

$$\frac{d\delta\theta_1}{dt} = -k_{12}(\rho\delta\theta_1 + K_1\delta\theta_3), \quad (\text{A12})$$

$$\frac{d\delta\theta_3}{dt} = -k_{23}^0(\delta\theta_1 + \chi\delta\theta_3 + \gamma\delta U) \quad (\text{A13})$$

with

$$\begin{aligned} \rho &= 1 + K_1, \\ K_1 &= \frac{k_{21}}{k_{12}}, \\ \gamma &= \beta\alpha_e\alpha\theta_2^0, \quad \text{and} \end{aligned} \quad (\text{A14})$$

$$\chi = 1 + \frac{k_{32}^0}{k_{23}^0}[\text{Na}_e].$$

If a sine-wave voltage is applied in the external circuit

$$U = V\cos(\omega t), \quad (\text{A15})$$

the solution of the kinetic equations should be a combination of sine and cosine functions of time:

$$\begin{aligned} \delta\theta_1 &= V[A\sin(\omega t) + B\cos(\omega t)], \\ \delta\theta_3 &= V[L\sin(\omega t) + M\cos(\omega t)]. \end{aligned}$$

For the values of A, B, L, M , a system of linear equations is obtained:

$$\begin{aligned} -k_{12}\rho A + \omega B - k_{21}L &= 0, \\ \omega A + k_{12}\rho B + k_{21}M &= 0, \\ -k_{23}^0A - k_{23}^0\chi L + \omega M &= 0, \\ k_{23}^0B + \omega L + k_{23}^0\chi M &= -k_{23}^0\gamma. \end{aligned} \quad (\text{A16})$$

Solving this system we obtain:

$$A = \frac{k_{23}^0\gamma k_{21}(k_{12}\rho + k_{23}^0\chi)\omega}{\omega^4 + [(k_{23}^0)^2\chi^2 + 2k_{23}^0k_{21} + (k_{12}\rho)^2]\omega^2 + (k_{23}^0)^2(k_{12}\chi\rho - k_{21})^2}, \quad (\text{A17})$$

$$B = \frac{k_{23}^0\gamma k_{21}[-\omega^2 + k_{23}^0(\chi k_{12}\rho - k_{21})]}{\omega^4 + [(k_{23}^0)^2\chi^2 + 2k_{23}^0k_{21} + (k_{12}\rho)^2]\omega^2 + (k_{23}^0)^2(k_{12}\chi\rho - k_{21})^2}, \quad (\text{A18})$$

$$L = \frac{k_{23}^0\gamma[\omega^2 + (k_{12}\rho)^2 + k_{23}^0k_{21}]\omega}{\omega^4 + [(k_{23}^0)^2\chi^2 + 2k_{23}^0k_{21} + (k_{12}\rho)^2]\omega^2 + (k_{23}^0)^2(k_{12}\chi\rho - k_{21})^2}, \quad (\text{A19})$$

$$M = -\frac{k_{23}^0\gamma[\chi\omega^2 + k_{12}\rho(k_{12}\chi\rho - k_{21})]}{\omega^4 + [(k_{23}^0)^2\chi^2 + 2k_{23}^0k_{21} + (k_{12}\rho)^2]\omega^2 + (k_{23}^0)^2(k_{12}\chi\rho - k_{21})^2}. \quad (\text{A20})$$

As determined earlier, the contribution of the Na^+, K^+ -ATPase to the current in external circuit can be presented in the form

$$\begin{aligned} \Delta I_{\text{out}} &= \alpha_e\alpha e \frac{dn_3}{dt} \\ &= \alpha_e\alpha e NV(L\omega\cos(\omega t) - M\sin(\omega t)). \end{aligned} \quad (\text{A21})$$

On the other hand, the extracellular current component can be derived as capacitances and conductance changes

$$I_{\text{out}} = G_p U + C_p \frac{dU}{dt}. \quad (\text{A22})$$

A comparison of equations (A21) and (A22) provides a relation for the change of capacitance C_p and conductance G_p of the membrane due to ion transport by Na^+, K^+ -ATPase

$$G_p = -eN\alpha_e\alpha\omega L, \quad (\text{A23})$$

$$C_p = -eN\alpha_e\alpha M. \quad (\text{A24})$$

Introducing $[\text{Na}_1] = \frac{k_{32}^0}{k_{23}^0}[\text{Na}_e]$ as a dimensionless concentration of sodium ions, the contribution of Na^+ transport by the Na^+, K^+ -ATPase to the capacitance and the conductance can be written as

$$C_p = \frac{eN\alpha_e^2\alpha^2\beta[\text{Na}_1](k_{23}^0)^2[\omega^2(1 + [\text{Na}_1]) + k_{12}^2(1 + K_1)(1 + [\text{Na}_1] + [\text{Na}_1]K_1)]}{(1 + [\text{Na}_1] + [\text{Na}_1]K_1)[\omega^4 + \omega^2(2k_{23}^0k_{12}K_1 + (k_{23}^0)^2(1 + [\text{Na}_1])^2 + k_{12}^2(1 + K_1)^2) + k_{12}^2(k_{23}^0)^2(1 + [\text{Na}_1] + [\text{Na}_1]K_1)^2]}, \quad (\text{A25})$$

$$G_p = \frac{eN\alpha_e^2\alpha^2\beta[\text{Na}_1]k_{23}^0\omega^2[\omega^2 + k_{12}^2(1 + K_1)^2 + k_{12}k_{23}^0K_1]}{(1 + [\text{Na}_1] + [\text{Na}_1]K_1)[\omega^4 + \omega^2(2k_{23}^0k_{12}K_1 + (k_{23}^0)^2(1 + [\text{Na}_1])^2 + k_{12}^2(1 + K_1)^2) + k_{12}^2(k_{23}^0)^2(1 + [\text{Na}_1] + [\text{Na}_1]K_1)^2]}. \quad (\text{A26})$$

The expressions for the C_p and G_p have the same denominator, which is the polynomial in ω of the 4th power. They can be factorized as sums of simple fractions like Lorentz functions. The denominator can be decomposed on the factors like

$$(\omega^2 + \omega_1^2)(\omega^2 + \omega_0^2),$$

in which ω_1^2 and ω_0^2 can be found as roots of the denominator, which is a quadratic polynomial for ω^2 . These roots are real:

$$\omega_0^2 = \frac{\Omega_1^2 + \Omega_2^2 + 2k_{12}k_{23}^0K_1 - (\Omega_1 + \Omega_2)\sqrt{(\Omega_2 - \Omega_1)^2 + 4k_{12}k_{23}^0K_1}}{2}, \quad (\text{A27})$$

$$\omega_1^2 = \frac{\Omega_1^2 + \Omega_2^2 + 2k_{12}k_{23}^0K_1 + (\Omega_1 + \Omega_2)\sqrt{(\Omega_2 - \Omega_1)^2 + 4k_{12}k_{23}^0K_1}}{2} \quad (\text{A28})$$

with

$$\Omega_1 = k_{12}(K_1 + 1) \text{ and } \Omega_2 = k_{23}^0([\text{Na}]_1 + 1). \quad (\text{A29})$$

In the final solutions of the model, C_p and G_p can be presented in the form of the sums of Lorentz functions, which are convenient to fit the experimental data:

$$C_p = C_0 \frac{\omega_0^2}{\omega^2 + \omega_0^2} + C_1 \frac{\omega_1^2}{\omega^2 + \omega_1^2} \text{ and} \quad (\text{A30})$$

$$G_p = C_0 \omega_0 \frac{\omega^2}{\omega^2 + \omega_0^2} + C_1 \omega_1 \frac{\omega^2}{\omega^2 + \omega_1^2}$$

with

$$C_0 = \frac{(A_1 - \omega_0^2)[\text{Na}_1](1 + [\text{Na}_1])(k_{23}^0)^2}{(\omega_1^2 - \omega_0^2)(1 + [\text{Na}_1] + [\text{Na}_1]K_1)\omega_0^2}, \quad (\text{A31})$$

$$C_1 = \frac{(\omega_1^2 - A_1)[\text{Na}_1](1 + [\text{Na}_1])(k_{23}^0)^2}{(\omega_1^2 - \omega_0^2)(1 + [\text{Na}_1] + [\text{Na}_1]K_1)\omega_1^2}, \quad (\text{A32})$$

$$A_1 = \frac{k_{12}^2(1 + K_1)(1 + [\text{Na}_1] + [\text{Na}_1]K_1)}{(1 + [\text{Na}_1])}. \quad (\text{A33})$$

Further simplification can be achieved in the case when the rate constants of ion movements in the access channel are much higher than the rate constants of the conformational transitions, $k_{23} \gg k_{12}$. Then $\Omega_2 \gg \Omega_1$ is

valid and this condition allows a considerable simplification of all terms:

$$\begin{aligned} \omega_0^2 &= \frac{1}{2} \left[\Omega_1^2 + \Omega_2^2 + 2k_{12}k_{23}^0K_1 \right. \\ &\quad \left. - (\Omega_2^2 - \Omega_1^2) \sqrt{1 + \frac{4k_{12}k_{23}^0K_1}{(\Omega_2 - \Omega_1)^2}} \right] \\ &\approx \frac{1}{2} \left[\Omega_1^2 + \Omega_2^2 + 2k_{12}k_{23}^0K_1 - (\Omega_2^2 - \Omega_1^2) \right. \\ &\quad \left. \times \left(1 + \frac{2k_{12}k_{23}^0K_1}{(\Omega_2 - \Omega_1)^2} \right) \right] \approx \Omega_1^2. \end{aligned}$$

Similarly, it can be shown that $\omega_1^2 \approx \Omega_2^2$. Thus, in this limiting case the solution of the equations can be transformed into the simple form of Eqs. (32) and (33), similar to those used in [34].

Cytoplasmic access channel. The ion transport in the intracellular access channel is described by the kinetic equations (9)–(10). The calculation can be executed in the same way as in the case of extracellular transport. After eliminating n_{c0} by $n_{c0} = N - n_{c1}$ and introducing the normalized surface density of state c1, $\theta_{c1} = n_{c1}/N$, one obtains

$$\frac{d\theta_{c1}}{dt} = -(k_{10} + k_{01}[\text{Na}_c])\theta_{c1} + k_{01}[\text{Na}_c]. \quad (\text{A34})$$

The rate constants k_{01} and k_{10} depend on the electric potential in accordance with Eq. (21). We will use the dimensionless potential $\psi_c = \beta\phi_c$ that varies with time only slightly:

$$\psi_c = \psi_c^0 + \delta\psi_c, \quad (\text{A35})$$

where

$$\delta\psi_c = \alpha_c \alpha \beta \delta U. \quad (\text{A36})$$

The rate constants are in a linear approximation:

$$k_{01} = k_{01}^0 (1 + 1/2 \delta\psi_c), \quad (\text{A37})$$

$$k_{10} = k_{10}^0 (1 - 1/2 \delta\psi_c). \quad (\text{A38})$$

The potential change leads to a small variation of normalized surface densities of the state c1 around its equilibrium value:

$$\theta_{c1} = \theta_{c1}^0 + \delta\theta_{c1}, \quad (\text{A39})$$

in which the equilibrium surface density is defined by Eq. (12):

$$-(k_{10} + k_{01}[\text{Na}_c])\theta_{c1}^0 + k_{01}[\text{Na}_c] = 0. \quad (\text{A40})$$

Introducing (A37)–(A40) into (A34), and accounting for first-order variations, an equation for the variation of surface densities of the state c1 is obtained:

$$\begin{aligned} \frac{d\delta\theta_{c1}}{dt} = & -(k_{10}^0 + k_{01}^0[\text{Na}_c])\delta\theta_1 \\ & + [(k_{10}^0 + k_{01}^0[\text{Na}_c])\theta_{c1}^0 + k_{01}^0[\text{Na}_c]]\delta\psi_c/2. \end{aligned} \quad (\text{A41})$$

This equation can be simplified by using the identity $(k_{10}^0 - k_{01}^0[\text{Na}_c])\theta_{c1}^0 + k_{01}^0[\text{Na}_c] = 2k_{10}^0\theta_{c1}^0$ which follows from (A40):

$$\frac{d\delta\theta_{c1}}{dt} = -(k_{10}^0 + k_{01}^0[\text{Na}_c])\delta\theta_1 + k_{10}^0\theta_{c1}^0\delta\psi_c. \quad (\text{A42})$$

If the externally applied voltage is $U = V\cos(\omega t)$, then $\delta\psi_c = \alpha\alpha_c V\cos(\omega t)$ holds, and the solution of equation (A41) will have the form

$$\delta\theta_{c1} = A\cos(\omega t) + B\sin(\omega t). \quad (\text{A43})$$

The detected alternating current in the external circuit due to this process stops after phosphorylation of the Na^+, K^+ -ATPase and can be expressed as the change of total capacitance and conductance as

$$\begin{aligned} \Delta I_c = & -eN \frac{d\delta\theta_{c1}}{dt} = G_p U + C_p \frac{dU}{dt} \\ = & G_p V\cos(\omega t) - C_p V\omega\sin(\omega t). \end{aligned} \quad (\text{A44})$$

Solving Eq. (A42) and applying it to Eq. (A44) leads to

$$\begin{aligned} G_p = & -e\beta N \alpha^2 \alpha_c^2 \frac{K_c[\text{Na}_c]}{1 + K_c[\text{Na}_c]} \\ & \times \frac{k_{10}^0 \omega^2}{\omega^2 + (k_{10}^0)^2 (1 + K_c[\text{Na}_c])^2}, \end{aligned} \quad (\text{A45})$$

$$C_p = -e\beta N \alpha^2 \alpha_c^2 \frac{(k_{10}^0)^2 K_c[\text{Na}_c]}{\omega^2 + (k_{10}^0)^2 (1 + K_c[\text{Na}_c])^2}. \quad (\text{A46})$$

These expressions can be transformed into:

$$C_p = -C_2 \frac{\omega_2^2}{\omega^2 + \omega_2^2}, \quad G_p = -C_2 \omega_2 \frac{\omega^2}{\omega^2 + \omega_2^2}, \quad (\text{A47})$$

with

$$C_2 = -e\beta N \alpha^2 \alpha_c^2 \frac{[\text{Na}_c]}{(1 + K_c[\text{Na}_c])^2}, \quad (\text{A48})$$

$$\omega_2 = k_{10}^0 (1 + K_c[\text{Na}_c]).$$

Combining the solutions for capacitance and conductance changes occurring in the extracellular and cytoplasmic access channels results in the sum of the three Lorentz functions (30) and (31).

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