

Involvement of Protons in the Ion Transport Cycle of Na⁺,K⁺-ATPase¹

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Abstract—The effect of pH on electrogenic sodium transport by the Na⁺,K⁺-ATPase has been studied. Experiments were carried out by admittance recording in a model system consisting of a bilayer lipid membrane with adsorbed membrane fragments containing purified Na⁺,K⁺-ATPase. Changes in the membrane admittance (capacitance and conductance increments in response to photo-induced release of ATP from caged ATP) were measured as function of AC voltage frequency, sodium ion concentration, and pH. In solutions containing 150 mM Na⁺, the frequency dependence of capacitance increments was not significantly dependent on pH in the range between 6 and 8. At a low NaCl concentration (3 mM), the capacitance increments at low frequencies decreased with the increasing pH. In the absence of NaCl, the frequency-dependent capacitance increment at low frequencies was similar to that measured in the presence of 3 mM NaCl. These results may be explained by involvement of protons in the Na⁺,K⁺-ATPase pump cycle, i.e., electroneutral exchange of sodium ions for protons under physiological conditions, electrogenic transport of sodium ions at high pH, and electrogenic transport of protons at low concentrations (and in the absence) of sodium ions.

Keywords: pH, protons, Na⁺,K⁺-ATPase, electrogenicity, electroneutrality.

The Na⁺,K⁺-ATPase pump transports three sodium ions out of a cell and two potassium ions into a cell using the energy of ATP hydrolysis. This membrane protein is a member of the family of P-type ATPases characterized by similar structures and pump mechanisms [1, 2]. The mechanism of such active transport is described by the Albers–Post cycle (Fig. 1). It provides for the two major protein conformations, E₁ and E₂, with an open access channel for ions to the binding centers from the cytoplasmic or extracellular side of the membrane, respectively. The conformation transition from E₁ to E₂ coupled with ATP hydrolysis and subsequent protein phosphorylation is the key step of active transport. Reversible transfer of the ions between the binding centers inside the protein and the solutions occurs passively through the so-called “access channels”. As a result of the conformation transition, the access channels on the cytoplasmic side of the protein are closed and those on the opposite side opened; the gating is arranged in such a way that simultaneous opening of the channels on both sides of the membrane is prevented. A significant achievement during recent years is the establishment of the 3D structure of the Ca²⁺-ATPase in several conformations. This allows the identification of the movement

of protein segments during conformation transitions and the structure of the ATP and ion binding centers [3, 4]. Recently, the first 3D structure of Na⁺,K⁺-ATPase was established [5, 6], and the structure of the α -subunit proved to be similar to the structure of the Ca²⁺-ATPase. Moreover, the transmembrane fragments of these two proteins have practically the same amino acid sequences and three-dimensional structures. The Na⁺,K⁺-ATPase has three binding centers for sodium ions. Two of them are approximately in the middle of the membrane, similarly to the Ca²⁺ binding centers of Ca²⁺-ATPase. These same two sodium binding centers of Na⁺,K⁺-ATPase are assumed to be responsible for the binding of potassium ions [2, 7, 8]. In contrast to other P-type ATPases, Na⁺,K⁺-ATPase can additionally bind a third sodium ion. The position of this binding center in the protein has not yet been established.

The information about the structure of the Na⁺,K⁺-ATPase channels can be obtained from the study of electrogenic transport by measurements either of fluorescence with electrochromic dyes or directly electrical signals (see [7, 9, 10]). It has been shown that the main contribution to electric currents in the Na⁺,K⁺-ATPase is due to the movement of the third sodium ion in the access channel on the extracellular side of the protein [11, 12]. This fact indicates

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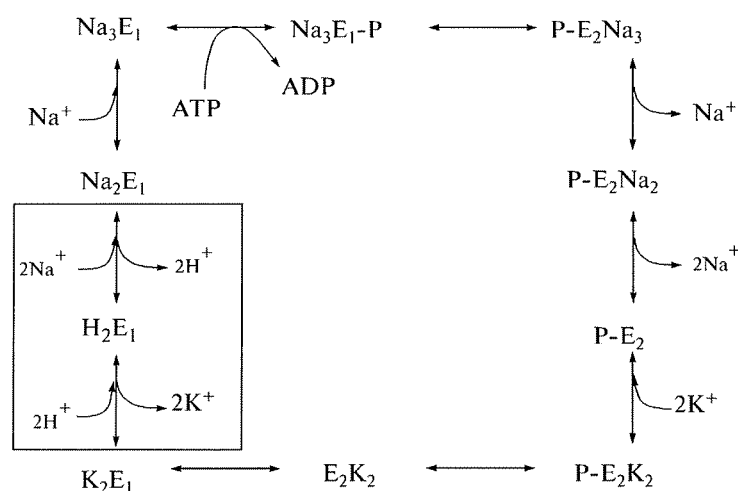


Fig. 1. Modified model of ion transport in Na^+, K^+ -ATPase based on the Albers–Post cycle and the hypothetical proton involvement [13]. The left side of the Figure corresponds to the conformation with an open cytoplasmic access channel (E_1); the right side corresponds to the conformation with an open extracellular channel (P-E_2). The phosphorylated states of the protein are marked by P. Protons are supposed to be bound in the same sites where potassium ions and two of the three sodium ions can be bound. The box marks the hypothetical steps, in which competition of Na^+ and H^+ occurs in the ion binding sites.

that the channel is considerably long and the binding center of third sodium ion is located closer to the cytoplasmic side of the protein [7]. The transport of two other sodium ions on the cytoplasmic side has been considered to be almost electroneutral, because it provides only a low contribution to the electric current. A possible mechanism of such a transport was suggested, according to the fact that binding of two sodium ions is coupled with protonation/deprotonation of the binding center, i.e., it is an electroneutral exchange for protons [13]. This assumption is confirmed by the above mentioned structural homology of Na^+, K^+ -ATPase with other ATPases of this family, which perform the exchange transport of various metal ions for protons. This mechanism has been experimentally confirmed by fluorescence measurements with the fluorescent dyes showing that ion binding depends on pH and is accompanied by charge transfer in a protein molecule at high pH [13]. Consequently, it may be expected that under certain conditions the electroneutral exchange of sodium for protons is converted into electrogenic transport.

The effect of pH on electrogenic transport can be studied by electric measurements. To study non-stationary electrogenic transport in a model system, we have previously developed the method using a bilayer lipid membrane (BLM) with adsorbed membrane fragments containing Na^+, K^+ -ATPase, to measure membrane admittance increments caused by a rapid release of ATP from caged ATP [14]. It allowed us to study the movement of sodium ions in the access channels of the Na^+, K^+ -ATPase. The proposed theoretical model considers the transport of one of the three sodium ions in the cytoplasmic and extracellular access channels. The model can explain satisfactorily

the frequency dependences of capacitance and conductance increments in a wide range of Na^+ concentrations, with an exception of low concentrations, where a significant discrepancy between theory and experiments was observed at low frequencies. Probably, electrogenic transport under these conditions has an additional step comprising the transport of not only sodium ions but also protons. To check this assumption, in the present investigation we have studied the effect of pH on the frequency characteristics of admittance increments at different sodium concentrations.

MATERIALS AND METHODS

Planar BLMs were formed from diphytanoyl phosphatidyl choline (Avanti Polar Lipids, USA) dissolved in *n*-decane (Aldrich, USA), 15 mg/ml, according to the Mueller–Rudin technique in a Teflon cell on a 1-mm hole. The solutions were prepared from the following reagents: NaCl, MgCl_2 (Merck, Germany), imidazole (Serva, Germany), dithiothreitol (Sigma, USA), and caged ATP (Calbiochem, USA). All solutions were prepared in distilled water additionally purified in MILLI-Q50 with a Pure PACK-1 filter (Thermo Scientific, USA). The buffer solution used for the study contained different concentrations of NaCl (see Figure legend), 10 mM MgCl_2 , and 1 mM EDTA (Sigma). In the case of low concentrations or in the absence of NaCl, 150 mM chloride N-methyl-D-glutamine (Sigma, USA) were added to the solution to maintain the concentration of chloride ions in the medium. In addition, the solution contained as a buffer 30 mM MES at pH below 6; 30 mM imidazole, at pH 6 to 7.5, and 30 mM EPPS (4-(2-hydroxyethyl)-1-piperazinepropanesulfoacid, Sigma), at pH 8 and

higher. Membrane fragments containing the purified Na^+, K^+ -ATPase were prepared from rabbit kidneys according to the procedure described in [15]. The activity of Na^+, K^+ -ATPase at 37°C was 1300–1700 μM of inorganic phosphate per h per 1 mg protein. Suspensions of the fragments with Na^+, K^+ -ATPase were stored at -60°C for several months without notable loss of activity. For measurements, the suspension was thawed and stored at 4°C for no longer than 2 weeks.

Electrical signals associated with the Na^+, K^+ -ATPase activity were measured by the method described in [16, 17] and improved for the measurement of minor changes in capacitance and conductance [18]. The release of ATP from caged ATP was initiated by UV light flash using xenon flash lamp with sapphire window (FJ-249, EG&G, USA). The cell had two optically transparent windows: one for visual observation of BLM and the other for illumination with UV light flash. After BLM formation, dithiothreitol was added into the cell on both sides of the membrane, up to 1 mM. The suspension of membrane fragments containing the purified Na^+, K^+ -ATPase was added into the compartment opposite to the UV source. Protein concentration in the cell was 20 $\mu\text{g}/\text{ml}$. Adsorption of the fragments took about 2 h and was monitored as a decrease in the membrane capacitance. After the adsorption was complete (the capacitance had ceased to decrease), the caged ATP solution was added to the cell compartment with the Na^+, K^+ -ATPase up to 100 μM and the recording of electrical signals associated with the function of the Na^+, K^+ -ATPase was started. Changes in membrane capacitance and conductance induced by the ATP release were measured as described in [14, 19]. To combine the results of measurements with different BLMs, the capacitance changes were normalized with respect to the total charge ΔQ transferred across the membrane after the photolysis of caged ATP, defined as the maximum value of integral of short-circuit current.

RESULTS

The effects of pH on frequency-dependent capacitance variations were studied at the two concentrations of sodium ions, 150 and 3 mM, and in the absence of these ions. These concentrations of Na^+ were selected on the basis of our previous investigations [14] and other published works [17], in which the constants of Na binding in the cytoplasmic and extracellular channels were determined. The experiment was directed to detect the ATP-induced change of the membrane capacitance and conductance caused by the transport of sodium ions only in the extracellular channel at high concentrations and, in contrast, at low concentrations in the cytoplasmic channel only.

The frequency dependences of membrane capacitance changes at 150 mM NaCl and different pH val-

ues are shown in Fig. 2 a. The data obtained at pH 6.5 were similar to those obtained previously [14] (not shown). In the pH range between 5 and 8 [HJA1], no significant variation of frequency dependence was found. At $\text{pH} > 8.5$, the capacitance increments became close to zero at all frequencies (data not shown). In the presence of 3 mM NaCl, the effect of pH was more significant (Fig. 2 b). From pH 6 to 8 a systematic downward shift of frequency dependences was observed in the low-frequency range. At low pH values, the whole dependence was above zero; at pH 6–7, the capacitance variation was positive in the low-frequency range, negative at higher-frequencies, and tended to zero when the frequency increased to the highest values. At pH 8.0, the whole dependence in the low-frequency range was below zero.

In the absence of sodium ions (Fig. 2 c), the changes in capacitance at low frequencies proved to be positive at all pH values [HJA2]. At the same time, the shape of frequency dependence was close to that obtained at pH 6–7 and low Na^+ concentration (Fig. 2 b). No short-circuit current was present under these conditions. To compare these curves with those obtained in the presence of sodium ions, 3 mM NaCl was added to the cell after frequency dependence measurement, the short-circuit current was measured, the maximum value of its integral was determined, and capacitance increments were normalized with respect to this value. In the control experiments without Na^+, K^+ -ATPase, changes of the membrane capacitance were absent upon the photo-induced release of ATP (data not shown).

DISCUSSION

Previously we have studied in detail the electrogenic transport in Na^+, K^+ -ATPase by the admittance method [14]. The frequency dependences of changes of the membrane capacitance and conductance initiated by the ATP release were measured at different Na^+ concentrations and pH 6.5. The model developed to interpret the results considers the electrogenic movement of one of the sodium ions in the cytoplasmic and extracellular access channels. Both channels were supposed to contribute with opposite sign to the changes in capacitance and conductance. The contribution of the cytoplasmic channel (state E_1 in Fig. 1) has the negative sign, because this channel is closed after ATP hydrolysis, followed by protein phosphorylation, and the electrogenic transport here is stopped. The extracellular channel (state $P-E_2$ in Fig. 1) accounts for a positive contribution, because the channel opens and the transport begins. In addition, the contributions of Na^+ transport in these channels to capacitance and conductance variations depend on the concentration of these ions in the solution. The contribution must be highest at a concentration corresponding to half-saturation of ion binding sites in the Na^+, K^+ -ATPase. The binding constants for sodium

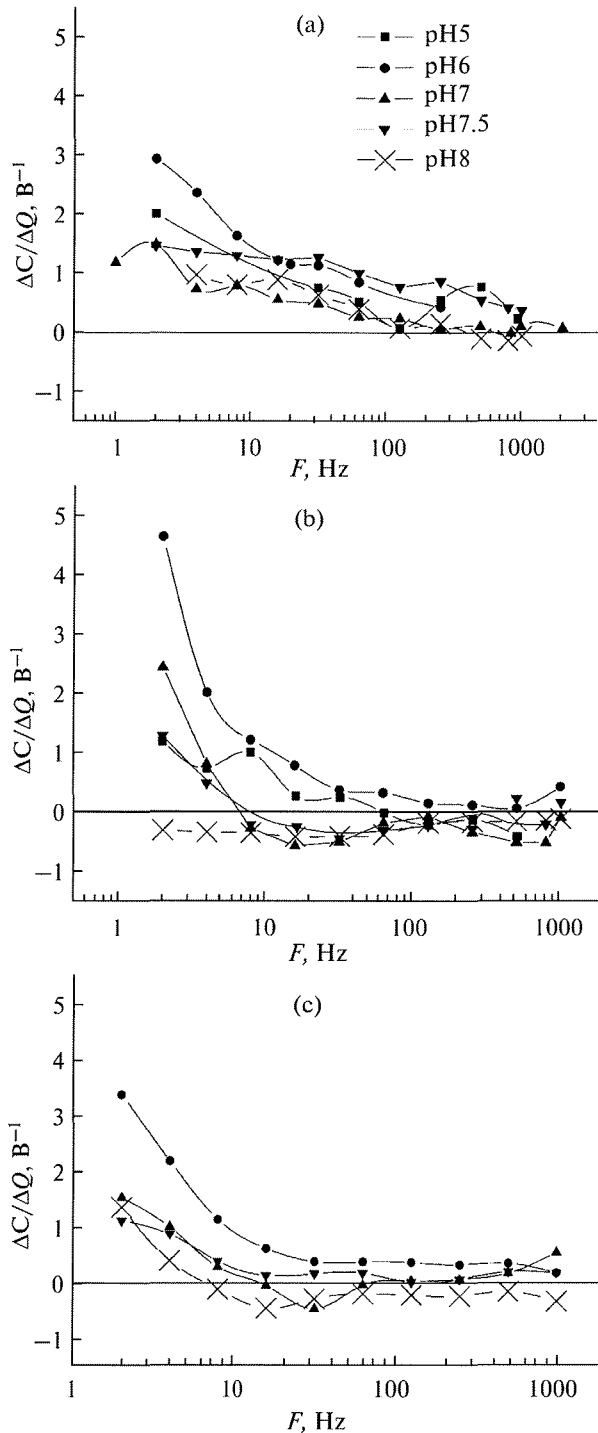


Fig. 2. Frequency dependences of the changes in membrane capacitance caused by the photo-induced release of ATP from caged ATP at NaCl 150 μ M (a), 3 mM (b), and in the nominal absence of NaCl (c). Data were measured in the pH range from 5 to 8 (see symbols in Fig. 2 a). Compositions of the solutions are given in the text. In order to normalize the capacitances the data were divided by ΔQ , the charge obtained as the maximum integral of the short-circuit current.

ions on both the cytoplasmic and extracellular sides of the protein are known [20]. According to these values, the contribution of the cytoplasmic channel to admittance variation must be highest at a sodium concentration of about 4 mM, and of 4[HJA3]00 mM for the extracellular channel. In the experiments published earlier [14] and in this work, negative changes in capacitance and conductance were recorded at low Na^+ concentrations, from 3 to 10 mM. At higher concentrations, they disappear and only positive changes were observed. However, positive capacitance and conductance changes (recognizable especially at low frequencies) were also observed at low Na concentrations and could not be explained theoretically.

This study has shown that positive capacitance increments in the accessible frequency range depend significantly on pH (Fig. 2). Moreover, they were also observed in experiments, where sodium ions were practically absent (Fig. 2 c). Note that it is impossible to completely remove sodium ions under such conditions, because caged ATP was used as a disodium salt, so the concentration of sodium ions in the solution was about 0.2 mM. These data obtained in the absence of Na^+ indicate that the capacitance increments in the low-frequency range are caused not by the transport of Na^+ but some other ions, most likely protons. Analysis of the results was complicated by the fact that protons participate not only in electrogenic transport. It is known that pH has a regulatory effect on the function of Na^+, K^+ -ATPase and that the maximum rate of ATP hydrolysis in the presence of sodium and potassium ions is observed at pH 7.5 [21]. It has also been noted that at low pH values (below 5), the Na^+, K^+ -ATPase loses its ability of active transport but is transformed into a passive, nonselective cation channel [22, 23]. These facts have to be taken into account to explain the effect of pH at high sodium concentrations as well.

However, the experiments at low concentrations and in the absence of sodium ions cannot be explained by the regulating pH effect alone. We have observed capacitance increments in the absence of sodium and potassium ions (Fig. 2 c). Positive increments of capacitance, which are observed at low frequencies, cannot be explained by the competition between sodium ions and protons in the binding centers on the cytoplasmic side of the protein (Fig. 1, the respective partial reaction is marked by a box). A positive sign of this increment implies that the release of ATP activates electrogenic transport. In the previous explanation of the experimental results in the presence of sodium ions, we have considered that the positive contribution to the capacitance and conductance increments is provided by the transport in the extracellular channel, which opens as a result of conformation transition from E_1 to E_2 . However, in the absence of sodium ions, according to the Albers-Post cycle (Fig. 1), this conformation transition may[HJA4] not happen. Now it is proposed that, contrary to the existing concepts, conformation transition still occurs in

the absence of sodium ions and the observed effect is an electrogenic proton transport in the extracellular channel of Na^+, K^+ -ATPase. This assumption is supported by the results obtained by independent methods on cell membranes. The Na^+, K^+ -ATPase has been shown to preserve its ability for ATP hydrolysis in the absence of sodium and potassium ions [24]. Besides, in the absence of these ions, steady-state electric currents were observed across the membranes of oocytes with Na^+, K^+ -ATPase, which are only possible if this enzyme can perform the complete cycle including E_1/E_2 [HJA5] transition [25, 26]. If the E_1/E_2 conformation transition is not performed in our conditions, it remains to assume that this effect is associated with some other process. It is known from the structural data that ATP binding is coupled with a significant conformation transition in the protein [4]. This transition may affect the cytoplasmic channel of Na^+, K^+ -ATPase by changing its properties, including the dissociation constant of amino acids present in this channel, which could alter the characteristics of electrogenic proton transport and lead not only to negative but also to positive contributions of capacitance and conductance.

A comparison of frequency dependences in the absence and at low concentrations of sodium ions (Fig. 2 b, c) shows that these ions cause a shift of the curves toward negative values, and the magnitude of this shift depends on pH. In some experiments, such shift could be detected with the same membrane, when the frequency dependent capacitance increments were measured first in the absence, and then again after the addition of sodium ions (data not shown). This shift can be due to the electrogenic transport of sodium ions in the cytoplasmic channel of Na^+, K^+ -ATPase and the competition between sodium ions and protons. In this case, the value of negative contribution to the capacitance and conductance changes depends not only on Na^+ concentration but also on pH. Since sodium ions and protons cause similar contributions to the capacitance increment, as can be expected by analogy to the model developed by us recently [14], both dependences must be non-monotonous. The maximum value will be reached either at a sodium ion concentration corresponding to the half-saturation of the binding sites, or at a pH value equal to the pK of the respective amino acids. As can be seen from Fig. 2 b, the effect of pH on negative membrane capacitance increments at low Na concentrations is most significant at highest used pH equal to 8. This result is in agreement with the data of fluorescence measurements [13] that allow the study of pH effect on the binding of sodium ions in the cytoplasmic channel of Na^+, K^+ -ATPase, where a pK of 7.3 has been obtained.

Thus, we have shown that ATP-induced increments in capacitance of the membrane with Na^+, K^+ -ATPase depend not only on the concentration of sodium ions but also on pH. Positive capacitance

increments in the low frequency range at low concentrations of sodium ions or in their absence may be caused by the electrogenic ion transport including protons. The detailed mechanism of this process is still unknown. These results explain the previously noted discrepancy between experimental results and the theoretical model considering the transport of sodium ions only [14]. The negative capacitance changes that are associated with the transition of sodium ions in the cytoplasmic channel are observed on the background of the changes generated by proton transport. This shift depends on both the concentration of sodium ions and pH, and can be explained by competition between sodium ions and protons for the binding sites in Na^+, K^+ -ATPase.

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