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ARTICLES

Electrogenic Binding of Ions at the Cytoplasmic Side of the Na^+,K^+-ATPase

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Abstract—Electrogenic binding of ions from the cytoplasmic side of the Na^+,K^+-ATPase has been studied by measurements of changes of the membrane capacitance and conductance triggered by a jump of pH or of the sodium-ion concentration in the absence of ATP. The pH jumps were performed in experiments with membrane fragments containing purified Na^+,K^+-ATPase adsorbed to a bilayer lipid membrane (BLM). Protons were released in a sub-millisecond time range from a photosensitive compound (caged H^+) triggered by a UV light flash. The sodium concentration jumps were carried out by a fast solution exchange in experiments with membrane fragments attached to a solid-supported membrane deposited on a gold electrode. The change of the membrane capacitance triggered by the pH jump depended on the sodium-ion concentration. Potassium ions had a similar effect on the capacitance change triggered by a pH jump. The effects of these ions are explained by the their competition with protons in the binding sites on cytoplasmic side of the Na^+,K^+-ATPase. The approximation of the experimental data by a theoretical model yields the dissociation constants, K, and the cooperativity coefficients, n, of the binding sites for sodium ions ($K = 2.7$ mM, $n = 2$) and potassium ions ($K = 1.7$ mM, $n = 2$). In the presence of magnesium ions the apparent dissociation constants of sodium increased. A possible reason of the inhibition of sodium-ion binding by magnesium ions can be an electrostatic or conformational effect of magnesium ions bound to a separate site of the Na^+,K^+-ATPase close to the entrance to the sodium-ion binding sites.

Keywords: sodium pump, electrogenic ion transport, Na^+,K^+-ATPase, caged H^+, solid-supported membranes

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INTRODUCTION

The Na^+,K^+-ATPase is a widely distributed membrane protein which transports sodium ions out of the cell, and potassium ions into the cytoplasm while consuming the energy of ATP hydrolysis. The mechanism of this transport is based on the conformational changes of the protein, due to which the access of ions to the binding sites inside the protein is accessible alternatively either from the cytoplasmic or the extracellular side, these conformations are designated as E1 or E2, respectively [1, 2]. An essential insight into the functional mechanism of the Na^+,K^+-ATPase was achieved when the structure of the protein in two basic conformations was obtained with atomic resolution [3–6]. However, the determination of the detailed mechanism of the active transport requires quantitative measurements of their kinetic and equilibrium characteristics and the influence of external conditions. Despite of intensive investigations during decades, many questions concerning the mechanism of Na^+,K^+-ATPase function remain unresolved. Among them is the structure of the access channel connecting the binding sites with the solution on the cytoplasmic side of the protein. Another question deals with the possibility that the Na^+,K^+-ATPase transports protons in addition to sodium and potassium ions which has been demonstrated recently in numerous publications, but the mechanisms and the role of such transport are not understood in full [7–11].

It has been shown by many researchers that ion binding from the cytoplasmic side of the Na^+,K^+-ATPase is electrogenic. It is affected by the voltage applied to the membrane, and the ion movement from the aqueous phase to the binding site creates an electric current [12–14]. Using fluorescent dyes it has been demonstrated that the Na^+,K^+-ATPase can bind not only sodium and potassium ions from the cytoplasmic side but also protons, and there is a competi-

tion at the binding sites of sodium and potassium ions with protons [7, 15, 16]. It has been shown also that the binding constant of Na^+ on the cytoplasmic site depends on the concentration of Mg^{2+} [15–18]. This indicates that magnesium ions are not only a cofactor of the ATP hydrolysis but can also influence the ion binding, but the mechanism of it remains unknown so far.

Earlier we studied the competitive binding of protons and sodium ions on the model system, in which the membrane fragments containing Na^+,K^+-ATPase were adsorbed on the surface of a bilayer lipid membrane (BLM). The observed small increments of the capacitance of the membrane with Na^+,K^+-ATPase were triggered by a fast decrease of pH due to photolysis of caged H^+ [19]. The dependence of this signal on pH at various sodium concentrations allowed the determination of the pK values of the binding sites. However, the accuracy of the measurements was not good enough to determine binding constants for sodium ions as well as to study the competition between potassium ions and protons at the binding sites.

Measurements of the electric signals caused by a fast change of the sodium ion concentration can be carried out by methods based on a fast exchange of solutions. Experiments with the solution exchange require a system that is more stable than BLM, such as a gold electrode covered by monolayers of thiols and phospholipids [20]. In this system transient currents initiated by changes in sodium ion concentration were detected [14, 21]. These currents were observed both in the presence and in the absence of ATP. In the latter case these signals were associated with movements of sodium ions in the cytoplasmic access channel of the Na^+,K^+-ATPase.

These approaches were used in the present investigation for detailed measurements of the admittance increments initiated by a fast decrease of pH, as well as by a fast change of sodium ion concentration that allows determination of binding constants of sodium and potassium ions at the cytoplasmic side of the H^+-ATPase.

MATERIALS AND METHODS

BLMs were formed by the Mueller–Rudin technique from diphytanoyl phosphatidylcholine dissolved in *n*-decane on the hole with 1 mm diameter of the septum dividing two compartments of the Teflon cell. The aqueous solution contained $MgCl_2$ and NaCl (Sigma, USA) at various concentrations (see legends to the figures), 0.1 mM EDTA (acid), and 12 mM *N*-methyl-*D*-glucamine chloride (NMG) (Sigma, USA), to maintain the chloride concentration as high as necessary for the silver-chloride electrodes. All solutions were prepared in distilled water that was additionally purified by MILI-Q50 with filter Pure PACK-1 (Thermo Scientific). In the experiments with the caged H^+, sodium 2-methoxy-5-nitrophenyl sulfate (MNPS-Na) was synthesized in Zelinsky Institute of Organic Chemistry RAS (Moscow, Russia).

Membrane fragments containing the purified Na^+,K^+-ATPase were prepared from rabbit kidney according to the procedure described in [22]. The activity of Na^+,K^+-ATPase at 37°C was 1300–1700 μM of inorganic phosphate per 1 h per 1 mg protein. Suspensions of the membrane 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.

The capacitance and conductance increments induced by the photolysis of caged H^+ were measured by the technique described in [19]. The suspension of the membrane fragments with Na^+,K^+-ATPase (in a concentration of 20 μg/mL) and caged H^+ (300 μM) were added into the distal (with respect to the light source) compartment of the cell. The BLM was illuminated by a xenon flash lamp with sapphire window FJ-249 (EG&G, USA). The adsorption of the membrane fragments took about 1 h and was controlled by a decrease of the BLM capacitance. Relative increments of the capacitance were averaged over various BLMs from at least 3 experiments.

The shifts of the capacitance and conductance induced by fast changes of the sodium ion concentration were measured on a gold electrode covered by monolayers of covalently bound hexadecanethiols and phospholipids (Fig. 1a). The gold electrode was prepared from the wire of rectangle profile 0.05 × 0.127 mm (Biomund Cohn Corp. Mt Mt Veanon, USA, purity 99.99%). The piece of wire was first cleaned with ethanol in an ultrasonic bath for 10 min, then in a mixture of sulfuric acid with hydrogen peroxide (25% H_2O_2, 75% H_2SO_4) for 20 min, and finally washed with distilled water. The rest of water was removed by washing in acetone, followed by drying in a flow of argon. The purified wire was immersed into a 10-mM solution of hexadecanethiol (Sigma–Aldrich, USA) in hexane for incubation overnight at room temperature. Immediately before the experiment the wire was washed for 2 min with hexane. To fix the area of the working surface during the experiment, all remaining surface was coated with a dope. After measuring the capacitance of the wire's thiole cover, the electrode was immersed into the solution of 15 mg/mL diphytanoylphosphatidylcholine in decane (Avanti Polar Lipids, USA) for 3 min and then transferred into the cell filled with the working solution. Spreading of the lipid and formation of its monolayer on the thiol layer on the electrode was monitored by the capacitance change (it decreased approximately twofold). After the monolayer of phospholipids was formed, the suspension of the membrane fragments with Na^+,K^+-ATPase was added into the cell. Their adsorption took about 1 h, and measurements began after the electrode capacitance reached a steady-state value. The solution in the cell was changed by the addition with a pipette of 200 μL of a new solution that displaced the previous solution.

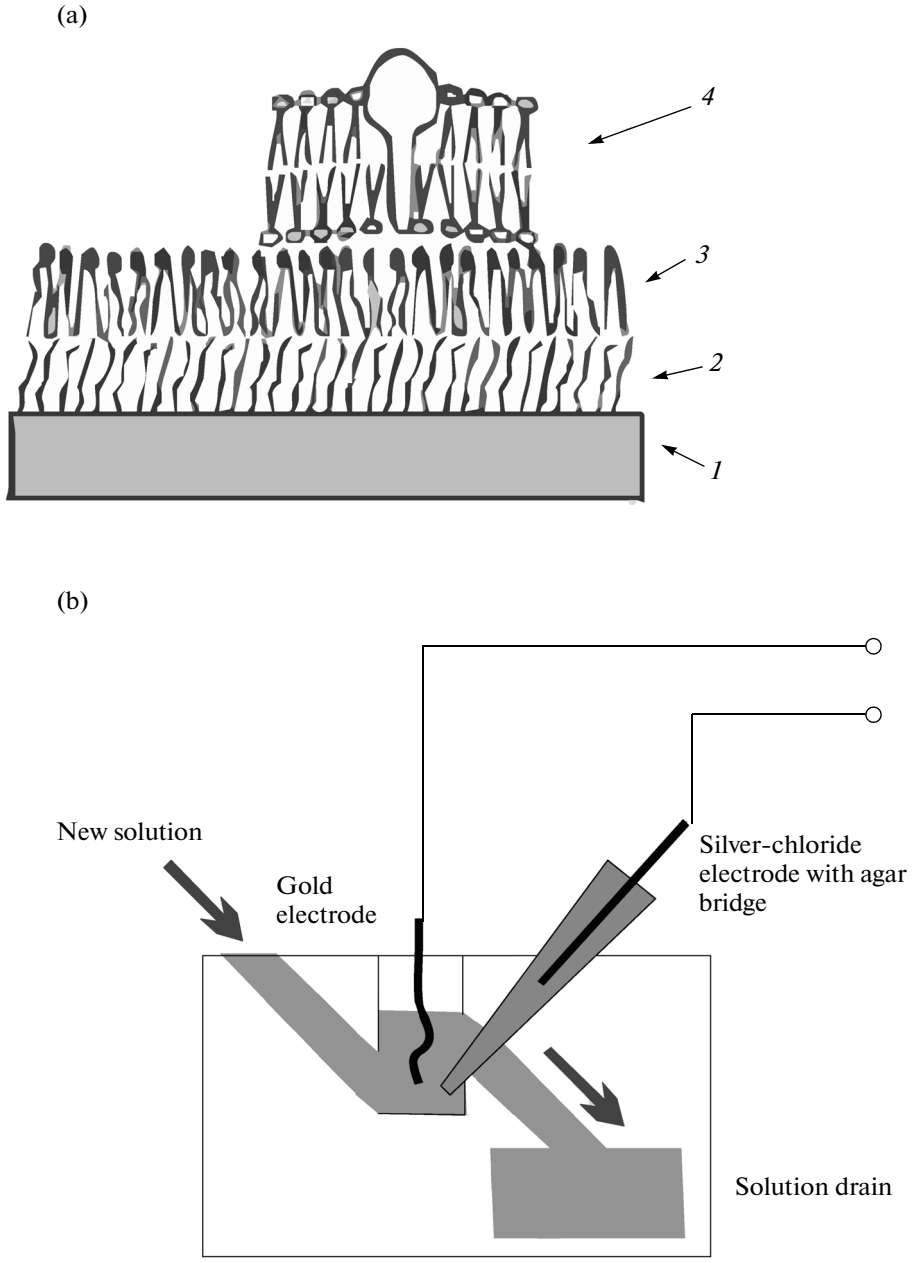

Fig. 1. The structure of the supported membrane (a) and a scheme of the experimental cell for a fast exchange of the solution (b). Figures in (a) designate: *1*, gold electrode; *2*, monolayer of hexadecanethiole; *3*, monolayer of lipids; *4*, membrane fragment with Na^+,K^+-ATPase.

The volume of the cell with electrode was 60 μL. A schematic design of the cell is presented in Fig. 1b.

Electrical measurements were performed using silver-chloride electrodes with agar bridges filled with the same solution as in the cell. To measure the capacitance increments of the BLM or gold electrode with adsorbed membrane fragments containing the Na^+,K^+-ATPase, a sine wave voltage of an amplitude of 50 mV and a frequency of 64 Hz from the DAC output of the ADC/DAC board L-780 (Lcard, Russia) was applied to one of the electrodes of the cell with the BLM or gold electrode. The other silver-chloride electrode (in the case of BLM) or the gold electrode (if the measurements were carried out with gold electrode) was connected to the input of the current amplifier Keithley-427 (USA). The output signal was connected to the ADC input of the same board. Both current and applied voltage were recorded and transmitted into the computer, the capacitance and conductance increments were calculated by the software written by the authors.

RESULTS

Studies of the competitive binding of sodium ions and protons. Recently, we studied the increments of the capacitance triggered by fast pH shift due to photolysis of caged H^+ as a function of frequency of the applied voltage. The effect of sodium ions on these increments was determined as a difference of the increments measured at a fixed concentration of 10 mM and zero NaCl [19]. In the present study, to determine the binding constant of Na^+ and K^+, experiments were carried out at a constant applied frequency (64 Hz) along with a gradual increase of the ion concentration in the solution. Measurements of the capacitance increments at alternating voltage frequencies between 2 and 200 Hz showed that the capacitance changes did not depend on the frequency (data not shown). This fact allows us to suppose that the exchange of ions between the solution and the binding sites at the cytoplasmic side of the Na^+,K^+-ATPase is sufficiently fast (in contrast to the transport of sodium ions at the extracellular side, studied earlier [19]). Taking this finding into account, we further performed measurements at 64 Hz and assumed that at this frequency the ion distribution between solution and the binding sites is in equilibrium.

Figure 2 shows the dependence of the capacitance increments triggered by the caged H^+ photolysis on the NaCl concentration in the cell. The capacitance increments were normalized to the increment measured in the absence of sodium ions. The capacitance decreases with the NaCl concentration reaching the minimal value at the concentration of about 4 mM. In the control measurements without membrane fragments containing Na^+,K^+-ATPase, the capacitance changes did not depend on the concentration of sodium ions in the range from 0 to 10 mM; a notable effect was detected only at considerably higher concentrations exceeding 100 mM (data not shown).

The results were analyzed quantitatively with the help of the model described in [19]. This model is a generalization of the model of electrogenic transport of ions in the cytoplasmic access channel of the Na^+,K^+-ATPase developed earlier [13]. The model is based on the assumption that the sites of the Na^+,K^+-ATPase accessible from cytoplasmic side can bind not only sodium ions but also protons. A competition between both ion species indicates that simultaneous binding of sodium ions and protons in the same site is prohibited. The capacitive current is assigned to ion movements in the access channel when an alternating voltage is applied. The quantitative description of this process can be considerably simplified when the frequency of alternating voltage is low enough to assume that the ion distribution between binding sites and solution is at or close to equilibrium. As was supposed in [19], the sites are charged negatively, so that the net charge Q of the sites at equilibrium is defined by the amount of free sites which are occupied neither by sodium nor by protons, according to the following equation:

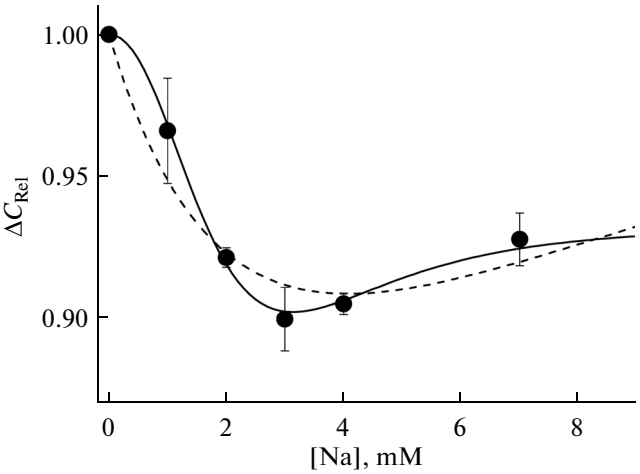

Fig. 2. Dependence of normalized capacitance increments of the BLM with adsorbed membrane fragments on the concentration of NaCl added to the cell. Capacitance changes were triggered by photolysis of caged H^+. The initial aqueous solution contained 12 mM NMG, 0.14 mM EDTA, pH = 7.3. *Symbols* show experimental values; *dashed line* is a plot constructed according to Eq. (3) with the following parameters: K_{Na} = 4.6 mM, pK = 7.35, $dC_0 = 0.27$; *solid line* represents a fit using Eq. (4) with the parameters: K_{Na} = 2.7 mM, n = 2.1, pK = 7.1, $dC_0 = 1.1$.

$$Q = \frac{Ne}{1 + \overline{Na}\exp(-\alpha\beta\varphi) + \overline{H}\exp(-\alpha\beta\varphi)},$$

where N is the number of proteins per unit of membrane area, $\overline{Na} = [Na^+]/K_{Na}$, and $\overline{H} = [H^+]/K_H$, $[Na^+]$ and $[H^+]$ are the sodium and proton concentrations in the solution, K_{Na} and K_H are the sodium and proton dissociation constants, respectively, α is the fraction of the applied ac voltage, φ, that affects the distribution of ions in the channel (it is proportional to so called "dielectric coefficient", which is determined by the relative length of the channel [13]); $\beta = e/kT$, e is the elementary charge, k is the Boltzmann's constant, and T, absolute temperature.

The contribution of the current caused by the movement of ions in the channel to the electrical capacitance of the membrane can be found as a derivative of the charge Q with respect to voltage φ:

$$C = \alpha\left.\frac{dQ}{d\varphi}\right|_{\varphi=0} = \frac{N\alpha^2e^2\left(\overline{Na} + \overline{H}\right)}{kT\left(1 + \overline{Na} + \overline{H}\right)^2}. \quad (1)$$

To find the capacitance changes initiated by a small pH shift, Eq. (1) should be differentiated with respect to $\overline{H}$ (such a function was used for the data fitting in [19]):

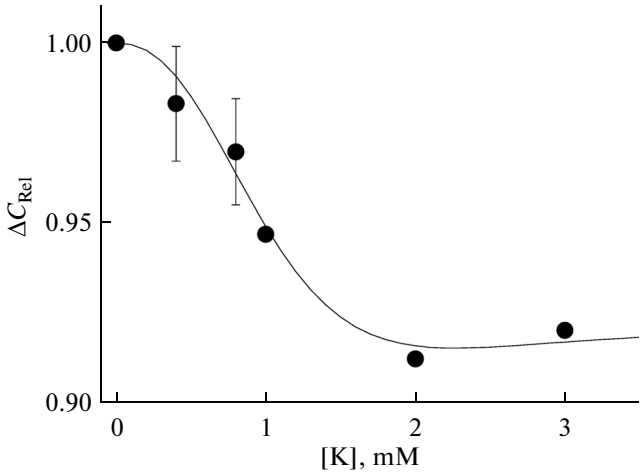

Fig. 3. The dependence of normalized capacitance increments of BLM with adsorbed membrane fragments on the concentration of KCl added to the cell. Capacitance changes were triggered by photolysis of caged H^+. The initial aqueous solution contained 12 mM NMG, 0.14 mM EDTA, pH = 7.2. *Symbols* show experimental values, *solid line* is a plot constructed according to Eq. (5) with the parameters: $K_K = 1.7$ mM, $n = 2.3$, pK = 6.5, $dC_0 = 5.7$.

$$\frac{dC}{d\bar{H}} = \frac{N\alpha^2 e^2 \left(1 - \overline{Na} - \bar{H}\right)}{kT\left(1 + \overline{Na} + \bar{H}\right)^3}. \quad (2)$$

In the experiments shown in Fig. 2, the capacitance changes were normalized to the value measured in the absence of Na^+. As was mentioned earlier [19], in the experiments without protein the capacitance changes were also observed, but the values of these changes did not depend on the sodium concentration. These changes were likely caused by the variation of the surface charge or dielectric permeability of the bilayer due to the binding of protons to the phospholipids. To take this fact into account, function (2) has to be modified by the addition of a constant parameter C_0. Thus, the equation used to fit the data is represented by the following expression:

$$\frac{\frac{dC}{d\bar{H}}(Na)}{\frac{dC}{d\bar{H}}(0)} = \frac{\frac{(1 - \overline{Na} - \bar{H})}{(1 + \overline{Na} + \bar{H})^3} + C_0}{\frac{(1 - \bar{H})}{(1 + \bar{H})^3} + C_0}. \quad (3)$$

When we attempted to fit the measured data by this function using the least-square deviation method, a systematic deviation of the theoretical curve from the measured data was found (Fig. 2, *dotted line*). To improve the agreement of the theoretical curve with the experimental data, cooperative binding of the ions on the binding sites was assumed. Similar assumptions on the cooperativity of ion binding at the cytoplasmic side of the Na^+,K^+-ATPase were introduced in the investigation using fluorescent probes [15, 16]. To account for the cooperativity, the Hill coefficients n were introduced into function (3), which yielded the following equation:

$$\frac{\frac{dC}{d\bar{H}}(Na)}{\frac{dC}{d\bar{H}}(0)} = \frac{\frac{\left(1 - \overline{Na}^n - \bar{H}\right)}{\left(1 + \overline{Na}^n + \bar{H}\right)^3} + C_0}{\frac{(1 - \bar{H})}{(1 + \bar{H})^3} + C_0}. \quad (4)$$

Fitting the data with function (4) is shown in Fig. 2 by *solid line*. It can be seen that function (4) fits the data adequately. The values of the parameters of the binding centers are given in the legend to the figure. The most important ones are the Hill coefficient, $n = 2$, the sodium dissociation constant, $K_{Na} = 2.7$ mM, and pK = 7.1.

The binding sites for sodium ions are assumed to be located almost at the same position in the protein as those for potassium ions, so the protons were expected to compete also with potassium ions. To verify this assumption, experiments similar to those described above were carried out with potassium ions. The results are presented in Fig. 3. The dependence of the capacitance increments on the potassium ion concentration was similar to that for sodium ions (Fig. 2), but the position of the minimum slightly differs, it was slightly below 2 mM K^+.

The data were fitted by function (5) that corresponds to the function used above for the sodium ions (4):

$$\frac{\frac{dC}{d\bar{H}}(K)}{\frac{dC}{d\bar{H}}(0)} = \frac{\frac{\left(1 - \bar{K}^n - \bar{H}\right)}{\left(1 + \bar{K}^n + \bar{H}\right)^3} + C_0}{\frac{(1 - \bar{H})}{(1 + \bar{H})^3} + C_0}. \quad (5)$$

The fitting procedure by the least-square deviation method yielded the Hill coefficient, $n = 2$, and the potassium dissociation constant, $K_K = 1.7$ mM.

To study the influence of magnesium ions on the binding of sodium ions, experiments were carried out, in which the dependence of the capacitance increments on sodium ion concentration was measured at three different magnesium concentrations in the solution (0, 5, and 10 mM). The results are presented in Fig. 4. All dependencies have a similar shape: the capacitance increments decrease with sodium ions concentration approaching to minimum at 4 mM (0 Mg^{2+}), 5 mM (5 mM Mg^{2+}), and 9 mM (10 mM Mg^{2+}). The amplitudes of the effects of sodium ions (i.e., the values of the relative capacitance increments at the point of minimum) were approximately the same at all concentrations of the magnesium ions. The dependencies were fitted by function (4) with different parameters K_{Na} and n for each magnesium concentration, and it was found that the values of K_{Na} increase with the Mg^{2+} concentration. Their values were 2.7,

7.3, and 18 mM at Mg^{2+} concentrations of 0, 5, and 10 mM, respectively. A similar relationship between the value of K_{Na} and the magnesium concentration has been found using the fluorescent probe RH421 [16], but the determined K_{Na} values were 0.4, 4.5, and 7 mM, respectively.

Studies of sodium binding by the Na^+,K^+-ATPase on solid-supported membranes. In the experiments with caged H^+, the dissociation constants of sodium ions were determined by measuring the capacitance increments initiated by fast pH shift and assuming competition of sodium ions with protons in the binding sites. These constants can be determined by another, more direct method, detecting the capacitance increments initiated by sodium-ion concentration changes. Such measurements were carried out with a gold electrode covered with thiols and phospholipids instead with a BLM. In these experiments the capacitance changes were triggered by the fast substitution of the cell solution without sodium ions for another one that contained these ions in the desired concentration. The dependence of the capacitance changes on the sodium-ion concentration was bell-shaped with the maximum at about 2.5 mM. In the control experiments without Na^+,K^+-ATPase the capacitance changes were smaller and depended monotonically on the sodium concentration (Fig. 5). To fit this dependence, Eq. (1) was modified assuming cooperativity of sodium-ion binding with Hill coefficient n. The experimental data were normalized according to the value of the capacitance change at zero sodium,

$$\Delta C_{Rel} = \frac{(1+\bar{H})^2}{\bar{H}} \frac{(\overline{Na}^n + \bar{H})}{(1+\overline{Na}^n + \bar{H})^2}. \quad (6)$$

The fit of the experimental data by this equation yielded the sodium dissociation constant, $K_{Na} = 2.5$ mM, and the cooperativity coefficient, $n = 2$. These values are in agreement with those obtained in the experiments with caged H^+ ($K_{Na} = 2.7$ mM, $n = 2.1$).

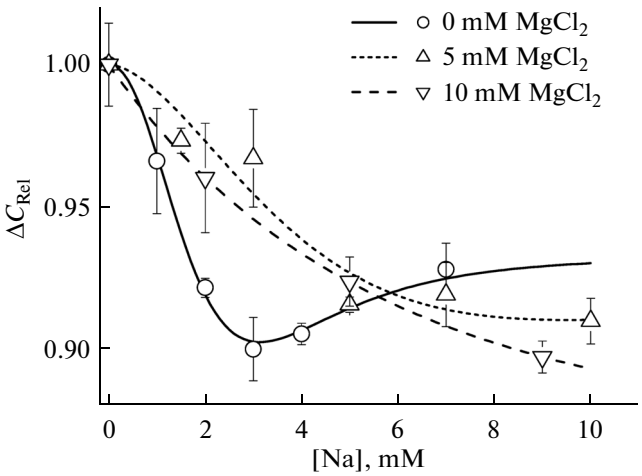

Fig. 4. The dependence of normalized capacitance increments of BLM with adsorbed membrane fragments on the concentration of NaCl at various concentrations of $MgCl_2$. Capacitance changes were triggered by photolysis of caged H^+. The initial aqueous solution contained 12 mM NMG, 0.14 mM EDTA, pH = 7.2. *Symbols* show experimental values, *lines* are plots constructed according to Eq. (4) with the following parameters: $K_{Na} = 2.7$ mM, $n = 2.1$, pK = 7.1, $dC_0 = 1.1$, (*solid line*); $K_{Na} = 7.3$ mM, $n = 1.7$, pK = 6.9, $dC_0 = 2.4$ (*dotted line*); $K_{Na} = 18$ mM, $n = 1$, pK = 6.5, $dC_0 = 3.2$ (*dashed line*).

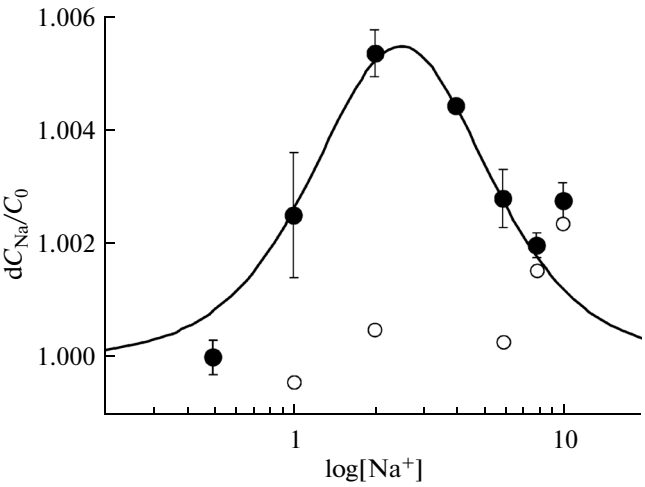

Fig. 5. The dependence of the normalized capacitance of support membrane on gold electrode on the concentration of NaCl in the presence (*black circles*) and absence (*open circles*) of Na^+,K^+-ATPase. The initial aqueous solution contained 50 mM NMG-Cl, 1 mM EDTA, 10 mM imidazole, pH 7. *Solid line* is a plot constructed according to Eq. (6) with the parameters: $K_{Na} = 2.5$ mM, $n = 2$.

DISCUSSION

In the experiments described above binding of sodium and potassium ions on the cytoplasmic side of the Na^+,K^+-ATPase as well as the influence of magnesium ions have been studied using electrical measurements. These results allow us to draw some conclusions concerning the structure of the cytoplasmic binding sites.

We assume that the measured currents have to be assigned to ion binding to the cytoplasmic sites of the Na^+,K^+-ATPase only. There are two strong arguments in favor of this assumption. First, the experiments were carried out in the absence of ATP, when the protein is in the E1 conformation in which the access channel is open from cytoplasmic side, but closed from extracellular side. Second, according to previous investigations [23, 24] the membrane fragments adsorbed onto the surface of a BLM are predominantly oriented with the extracellular side facing the BLM. Therefore, the cytoplasmic side is in contact with the aqueous solution, in which the ion concentrations are controlled and the pH shift due to photolysis of caged H^+ is taking place.

The experimental data on ion binding could not be described on the assumption that the ions bind in each

site independently from each other; it was necessary therefore to propose a cooperative ion binding. This implies that the detected capacitance changes are the results of ion binding to two sites, and ion binding to one site affects the binding to the other. A similar conclusion has been made in a study using the fluorescent probe RH421 [16]. The values of the sodium dissociation constants derived from the experiments with a BLM and a gold electrode are approximately the same. But it is worth to note that in the experiments with caged H^+ the determination of the dissociation constants is based on the competition between sodium ions and protons, while the bell-shaped dependence of the capacitance changes on the sodium ions concentration, as measured in the other experiments, indicates that most of the binding sites are not occupied by protons. The fact that these results do not contradict each other may be attributed to the finding that the least-square fit of the experimental data with caged H^+ revealed that the pK value of the binding sites was lower than the pH of the experiments.

The model used to describe the experimental data assumed cooperativity of binding of sodium or potassium ions but not of protons. However, the possibility of the cooperative proton binding cannot be ruled out. The obtained data do not allow the verification of the hypothesis of cooperativity, as the pH value and pH shift were constant, and if the proton-binding cooperativity is assumed, it would affect only the pK value but not the dissociation constant and the cooperativity of sodium and potassium ions.

The capacitance increments triggered by caged H^+ photolysis were affected not only by sodium, but also by potassium ions, and the amplitudes of the sodium and potassium effect (i.e., the differences of the capacitance changes in the absence of ions and at its lowest value) are approximately equal (Figs. 2, 3). The Na^+,K^+-ATPase is known to have three binding sites for sodium and two for potassium. The similarity of the amplitude effects of the sodium and potassium ions indicates that the dielectric coefficients of the electrogenic binding of these ions are similar; hence, the positions of the binding sites of sodium and potassium ions are located not far one from another. From the 3D structures of Na^+,K^+-ATPase it can be seen that the binding sites I and II for sodium and potassium ions are located in close proximity [6]. Therefore, one can conclude that protons competing with Na^+ and K^+ bind also to the sites I and II of the protein.

Magnesium ions exert a significant impact on sodium binding. The apparent constant of sodium dissociation increased significantly in the presence of magnesium. Therefore, the magnesium ions are not only an essential co-factor of the ATP hydrolysis but are also able to suppress ion binding. A similar effect has been observed in an investigation using the fluorescent probe RH421 [15]. However, the ion-binding sites are specific to monovalent cations, and magnesium ions are unlikely to bind to them. In an earlier study it was suggested that magnesium has a specific binding site differing from the sodium and potassium sites [25]; therefore, the increase of the apparent sodium-binding constant appears to be caused either by a conformational alteration of the protein or by the appearance of a local electric field caused by a magnesium ion bound to a site close to the entrance to the sodium-ion binding sites, which affects binding of monovalent ions (Debye –Hückel effect).

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