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ARTICLES

Binding of Potassium Ions Inside the Access Channel at the Cytoplasmic Side of Na^+,K^+-ATPase

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Abstract—Binding of potassium ions through an access channel from the cytoplasmic side of Na^+,K^+-ATPase and the effect of pH and magnesium ions on this process have been studied. The studies were carried out by a previously developed method of measuring small increments of the admittance (capacitance and conductivity) of a compound membrane consisting of a bilayer lipid membrane with adsorbed membranes fragments containing Na^+,K^+-ATPase. The capacitance change of the membrane with the Na^+,K^+-ATPase was induced abruptly by release of protons from a bound form (caged H^+) upon a UV-light flash in the absence of magnesium ions. The change of admittance consisted of an initial fast jump and a slow relaxation to a stationary value within a time of about 1–2 s. The kinetics of the capacitance relaxation depended on pH and the concentration of magnesium and potassium ions. The dependence of the rapid capacitance jump on the potassium concentration corresponded to the predictions of the model developed earlier that describes binding of sodium or potassium ions in competition with protons. The effect of magnesium ions can be explained by the assuming that they bind to the Na^+,K^+-ATPase and affect binding of potassium ions because of either changes in protein conformation or the creation of an electrostatic field in the access channel on the cytoplasmic side.

Keywords: Na^+,K^+-ATPase, sodium pump, electrogenic ion transport, caged proton, admittance measurement, K^+ binding by the Na^+,K^+-ATPase

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INTRODUCTION

The Na^+,K^+-ATPase transfers sodium ions from the cell and potassium ions into the cell consuming the energy of ATP hydrolysis. The transport mechanism is based on the Post–Albers cycle [1, 2], consisting of successive conformational rearrangements, during which the access for the ions to binding sites inside the protein is open alternately from both solutions. Ion transfer between the binding sites and solutions occurs passively through "access channels". Their structures resemble ion channels that are closed on one side. The energy for the active transport is provided by ATP hydrolysis, and the terminal phosphate of the ATP molecule is transferred to the protein. The Na^+,K^+-ATPase belongs to a family of proteins, P-type ATPases, which function by a similar mechanism. They are similar in structure and are able to transfer various metal ions and even phospholipids through the membrane [3–5].

Studies of Na^+,K^+-ATPase for more than 50 years allowed the establishment of its structure and mechanism of function, however, some details of this mechanism remained obscure. One unresolved issue is the role of protons in ion transport. It is known that the Na^+,K^+-ATPase is able to transfer protons through the membrane in the absence of sodium and potassium ions [6–9], or to carry out the transport of protons in exchange for potassium ions (like the closely related H^+,K^+-ATPase) in the absence of sodium ions [10, 11]. Binding of sodium and potassium ions in the protein occurs with the participation of deprotonated amino acids [12–18], and their binding occurs in exchange for protons [6, 19–22].

The movement of sodium and potassium ions in exchange for protons in the access channels becomes electrogenic under certain conditions, and the electric currents arising from this process can be studied using electrical measurements. Previously, the electrogenic ion transport by the Na^+,K^+-ATPase in membrane fragments adsorbed on a bilayer lipid membrane was studied by recording short-circuit currents and changes of the admittance (membrane capacitance and conductivity) induced by rapid photo-activated release of ATP from an inactive form (caged ATP) [23–25]. The same method allowed it to study the transport of sodium and potassium ions in exchange for protons by recording changes of the membrane

capacitance after a rapid decrease of pH caused by the photo-activated release of protons (caged H^+) [26, 27]. This method opens up new possibilities to study binding and transport of potassium ions, which was previously inaccessible by the use of caged ATP. In this paper, binding of potassium ions has been studied in more detail, in particular, the effect of ATP, magnesium ions and pH on this process has been investigated. Complex processes have been discovered: binding of potassium or magnesium ions, the competitive binding of protons occurs at different conformations of the protein, and the replacement of one type of ions against another is accompanied by a conformational transition of the Na^+,K^+-ATPase.

MATERIALS AND METHODS

Bilayer lipid membranes (BLMs) were formed by the method of Mueller–Rudin [28] in a teflon cell with two compartments across a hole with a diameter of 1 mm from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, USA) dissolved in *n*-decane. KCl, $MgCl_2$ (Sigma, USA), EDTA (Sigma, USA) and dithiotreitol (Sigma, USA) were used to prepare the solutions. All solutions were prepared in distilled water, additionally purified with Pure PACK-1 filter of MILI-Q50 C (Thermo Scientific). The composition of the solutions is given in the captions to the figures. To create a photoinduced pH jump, 2-methoxy-5-sodium nitrophenyl sulfate (MNPS, or caged H^+) synthesized at the N.D. Zelinsky Institute of organic chemistry, Russian Academy of Sciences, was used. Its photochemical properties in solution and on BLM were studied earlier [26, 29].

Membrane fragments containing purified Na^+,K^+-ATPase were isolated at a concentration of about 2 mg/mL from the kidney of rabbit according to the procedure described in [30]. The activity of Na^+,K^+-ATPase at 37°C was 1300–1700 μmol of inorganic phosphate released per hour per mg of protein. The suspension of fragments with Na^+,K^+-ATPase was stored at –60°C for several months without significant loss of activity. During the experiments, aliquots were thawed and stored at 4°C for no longer than two weeks.

The increments of the capacitance and conductivity of the compound membrane of BLM and adsorbed membrane fragments containing Na^+,K^+-ATPase caused by a stepwise decrease of pH were measured using the technique described in [26]. Abrupt pH decreases were induced by photolysis of caged H^+ under illumination of the BLM by a UV flash of a Xenon lamp FJ-249U with a sapphire window (EG&G, USA). In all experiments, the suspension of membrane fragments with Na^+,K^+-ATPase (at a concentration of 20 μg/mL) and caged H^+ (300 μM) were added to the far (relative to the light source) compartment. In the course of the measurements, all the chemicals (KCl, $MgCl_2$, and ATP) were added into the same compartment. Adsorption of the membrane fragments occurred for about 1 h before the first experiment and was monitored by a reduction of the total capacitance of the compound membrane.

Silver chloride electrodes with agar bridges filled with the same solution that was in the cell were used for the electrical measurements. A sinusoidal voltage with an amplitude of 50 mV and a frequency of 64 Hz from the output of the DAC of the L-780 LCard board (Russia) was applied to the BLM to measure the capacitance increments. This voltage was applied to one of the electrodes. The second electrode was connected to a Keithley-427 amplifier (USA), the output of which was connected to the ADC input of the L-780 board. The kinetics of the changes of electric current and voltage during and after a UV flash was recorded in the computer using this board. Further numerical analysis of capacitance increments were carried out using a program developed by the authors. The kinetics of the capacitance change after the UV flash was approximated by an exponential function; the capacitance increment was the difference between the mean capacitance calculated before the light flash and the value at the time when the flash transient had vanished. Relative increments of capacitance were averaged from experiments under the same condition taken from several (at least three) BLMs.

RESULTS

Figure 1 shows the dependence of the membrane capacitance change initiated by a UV flash on the KCl concentration in the electrolyte. The induced pH shift led to a small increase of the BLM capacitance in the absence of Na^+,K^+-ATPase containing membrane fragments similarly to that observed earlier [26, 27]. The addition of KCl up to 10 mM did not affect this increase. After membrane fragments with Na^+,K^+-ATPase were adsorbed to the BLM, the change of capacitance was larger, and the addition of KCl to the solution reduced this capacitance change, again similarly to that observed earlier [27].

The effect of potassium ions on the capacitance change was suppressed in the presence of ATP. Figure 2 shows the dependence of the relative capacitance change as function of the KCl concentration at different ATP concentrations. The effect of potassium ions disappeared at ATP concentration of 0.5 mM, and it was reduced to about half at 0.125 mM ATP. The effect of ATP was the same, independent of the sequence of ATP and KCl additions. Upon the initial addition of ATP, addition of potassium ions did not change the capacitance, and if in the absence of ATP the capacitance change was reduced by the addition of potassium ions, subsequent addition of ATP restored the capacitance change that was observed before the addition of potassium ions.

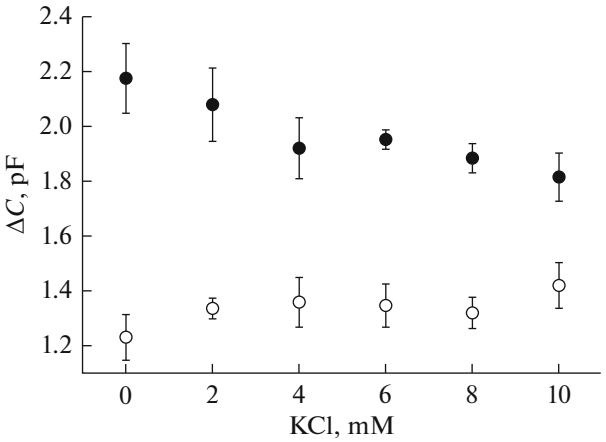

Fig. 1. The influence of KCl concentration on the BLM capacitance change induced by a pH jump upon the photolysis of MNPS by a flash of UV light. *Open circles*: BLM only, made of diphytanoyl PC. *Solid circles*: BLM of the same composition with adsorbed membrane fragments containing Na^+,K^+-ATPase. The initial solution contained 12 mM NMG and 0.1 mM EDTA, pH 7.2.

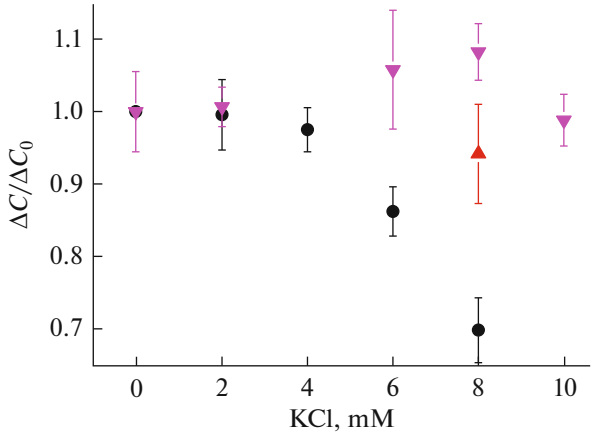

Fig. 2. Effect of ATP on the dependence of the capacitance change on the KCl concentration induced by a pH jump applied on a BLM with adsorbed membrane fragments containing Na^+,K^+-ATPase. The initial solution contained 12 mM NMG and 0.1 mM EDTA, pH 7.5. *Circles*: measurements without ATP; *triangle apex up*: measurements after the addition of 0.125 mM ATP; *triangles apex down*: measurements with ATP at a final concentration of 0.5 mM.

The addition of magnesium ions significantly affected the kinetics of the membrane capacitance change upon a pH jump. This is illustrated in Fig. 3a, which shows the kinetics of the change of the capacitance of the membrane with Na^+,K^+-ATPase in the absence of the metal ions, as well as after the addition of magnesium and potassium ions. The change of the capacitance was stepwise-like in the absence of magnesium ions, but in their presence a capacitance relaxation from the initial increase back to approximately the initial value was observed. The relaxation kinetics was influenced by a subsequent addition of potassium ions. It is interesting to note that magnesium ions affected not only the kinetics of the capacitance change, but also the kinetics of the short-circuit current. The integral of this current is proportional to the amount of charge transferred by the external circuit under voltage clump conditions, and this charge divided by the membrane capacitance is equal to the potential change across the membrane [25]. The kinetics of membrane potential change is shown in Fig. 3b. In the absence of magnesium ions, the release of protons from the caged H^+ induced a step-like potential increase corresponding to an addition of positive charge on the surface of the membrane on the side to which H^+ was released. In the presence of magnesium ions, the potential decreased again rapidly, corresponding to transfer of positive charges from the membrane surface to the solution. The effect of magnesium ions depended on pH, the capacitance relaxation in the presence of magnesium ions was observed only at high pH. As it will be discussed below, even if assumed that binding of potassium ions is accompanied by a conformational transition of the protein, the rate of it is high enough to ensure a completion of this process during the time interval in which the initial, membrane capacitance increase was observed. The mechanism of the capacitance relaxation (and the corresponding change of membrane potential) in the presence of magnesium ions is not clear so far. Probably, this process is caused by a slow release of magnesium ions bound to the protein (and possibly to the head groups of phospholipids in the membrane) due to protonation of the membrane surface upon the pH jump.

The initial capacitance increase, measured immediately after the flash of light, was used for quantitative analysis. Figure 4 shows the dependence of the initial capacitance change as function of the potassium concentration in the presence and absence of magnesium ions (in the latter case, no subsequent relaxation was observed, and this value was measured as the average value after the stepwise shift similarly to that determined earlier [27]). The previously developed theoretical model of competition between proton and sodium or potassium binding was used for quantitative analysis. It allowed the estimation of the binding constants of these ions in the binding site on the cytoplasmic side of Na^+,K^+-ATPase [26, 27]. According to this model, the dependence of the capacitance change on pH and the concentration of potassium ions is accounted for by Eq. (1):

$$\frac{\Delta C\left([K^+]\right)}{\Delta C(0)} = \frac{\dfrac{\left(1-\bar{K}^n-\bar{H}\right)}{\left(1+\bar{K}^n+\bar{H}\right)^3}+C_0}{\dfrac{(1-\bar{H})}{(1+\bar{H})^3}+C_0}. \qquad (1)$$

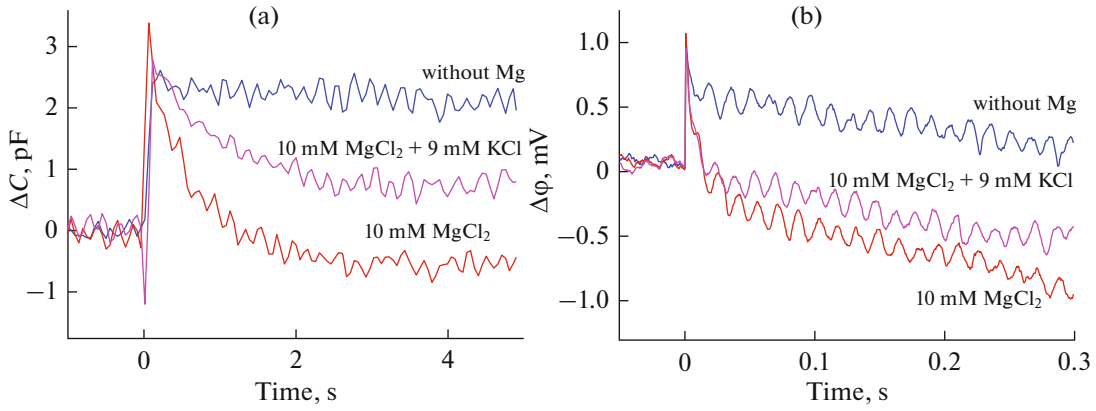

Fig. 3. Kinetics of the capacitance (a) and membrane potential (b) changes of the BLM with adsorbed membrane fragments containing Na^+,K^+-ATPase induced by a pH jump. The UV flash caused instant MNPS photolysis, which produced a pH jump at time zero on the horizontal axis. The initial solution contained 12 mM NMG and 0.1 mM EDTA, pH 7.5. Three curves correspond to measurements in the initial solution and after the addition of 10 mM $MgCl_2$ and 9 mM KCl. The time course of the membrane potential change was obtained by integrating the short-circuit current and divided by the membrane capacitance.

where $\Delta C([K^+])$ is the capacitance change caused by the pH jump in the presence and $\Delta C(0)$ in the absence of potassium ions. $\bar{K} = [K^+]/K_K$, and $\bar{H} = [H^+]/K_H$, $[K^+]$ and $[H^+]$ are the concentrations of potassium ions and protons in the solution, K_K and K_H are the corresponding dissociation constants of these ions in the binding site, n is the Hill coefficient that is needed to account for the cooperativity of potassium binding [20, 21], C_0 is the capacitance increment, which is independent of the potassium concentration, caused apparently by the changes of the surface charge or dielectric constant of the BLM due to the protonation of the phospholipids that was observed also in the absence of Na^+,K^+-ATPase (Fig. 1). Figure 4 shows the results of an approximation of the experimental capacitance change on the concentration of potassium ions in the absence and presence of magnesium ions. As follows from the parameter values given in the figure legend, the presence of magnesium ions led to a decrease of the dissociation constant and the Hill coefficient of the potassium binding sites.

The effect of potassium ions on the capacitance change depended on pH. Figure 5a shows the relative capacitance change on the potassium concentration at different pH. Similar experiments have been carried out in the presence of magnesium ions, but only the initial rapid capacitance increase has been determined and analyzed. The effect of potassium ions on the capacitance change at different pH was obtained by determination of the ratio of the capacitance changes in the presence of 6 mM KCl and in the absence of KCl, as in [26]. This dependence, shown in Fig. 5b, indicates that the effect of potassium ions increased with increasing pH in both the solutions without and with magnesium ions. The only effect of magnesium ions was a slight increase of the effect of potassium ions and in a shift of its pH dependence.

DISCUSSION

Previously, this method has been used to study the electrogenic transport of sodium ions in exchange for protons in the access channel of the cytoplasmic side of Na^+,K^+-ATPase [26, 27]. The studies presented in this paper with potassium ions are to some extent similar, therefore, it makes sense to compare the results. The effect of sodium ions on the capacitance change caused by a jump of pH was explained by a simple model in which the cytoplasmic ion access channel to the binding sites in Na^+,K^+-ATPase is considered to be always open, and sodium ions compete with protons in the binding sites. Potassium ions, like sodium ions, suppressed the increase of the capacitance caused by the pH jump. The way how potassium ions affect the capacity changes as function of their concentration and pH resembles the dependencies in the case of sodium ions [27].

The difference between the binding of potassium and sodium ions to the Na^+,K^+-ATPase in the E_1 conformation is that the enzyme undergoes a conformation transition into the occluded E_2 state upon potassium binding . The transition to an occluded state is fast with a rate constant of about hundreds per second [31, 32]. The rate of back transition to E_1 is about three orders of magnitude lower [33, 34]. Thus, binding of potassium ions should be accompanied by an essential change in the protein conformation to the occluded state and lead, most likely, to the closure of the cytoplasmic access channel. This proposal can explain the observed suppression of the effect of potassium ions by ATP (Fig. 2). The presence of ATP has the opposite effect on the binding of sodium and potassium ions. In the case of sodium ions, ATP (which is hydrolyzed with a magnesium ion as cofactor) leads to occlusion of sodium in Na^+,K^+-ATPase and closure of the intracellular ion access channel. In the case of potassium

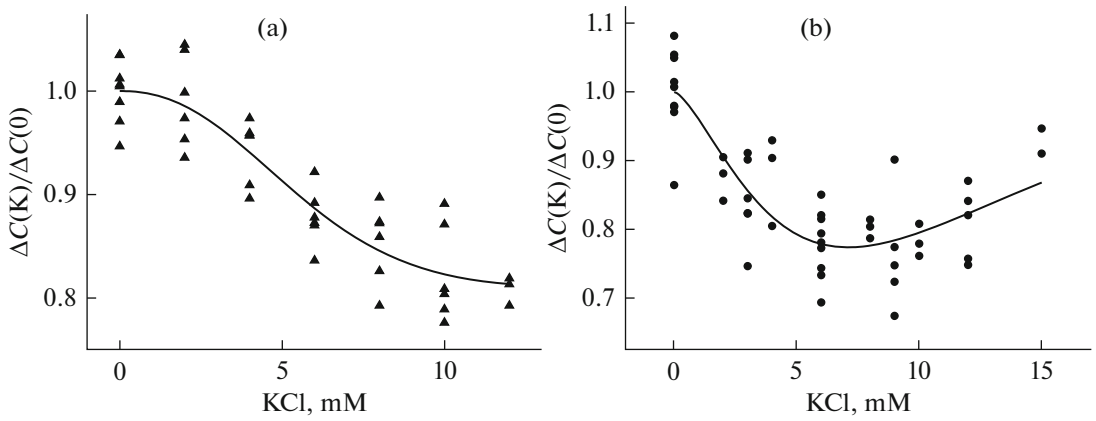

Fig. 4. Dependence of the relative capacitance change of the BLM with adsorbed membrane fragments containing Na^+,K^+-ATPase, ΔC(K)/ΔC(0), induced by a pH jump on the KCl concentration in the cell in the absence (a) and in the presence (b) of 10 mM $MgCl_2$. The initial solution contained 12 mM NMG and 0.1 mM EDTA, pH 7.5. The capacitance changes were normalized to the mean values of the capacitance changes measured before the addition of KCl in each experiment. Each graph presents data from three experiments. *Curves* were calculated from Eq. (1) using the following parameters: in the absence of magnesium, pK = 6.8, K_K = 12 mM, n = 2, C_0 = 2; in the presence of 10 mM $MgCl_2$, pK = 6.8, K_K = 5 mM, n = 1.5, C_0 = 2.

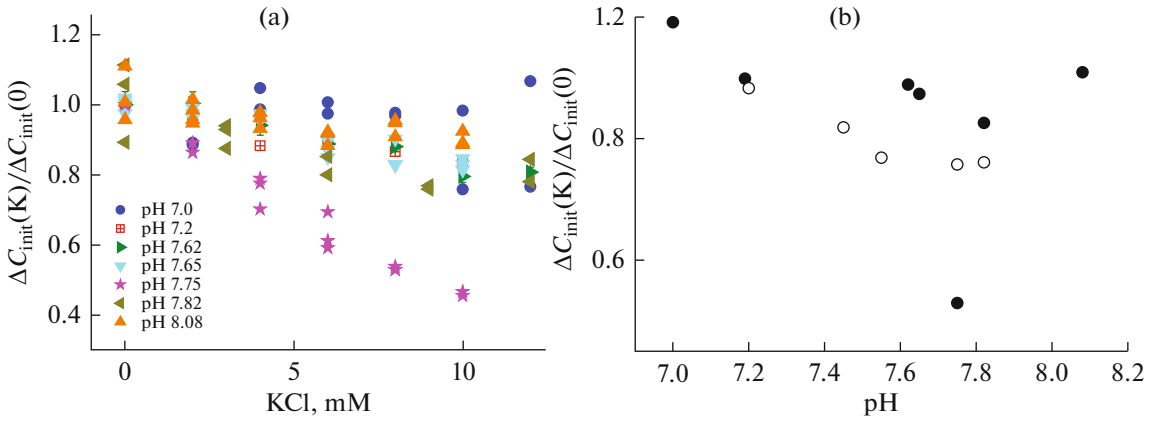

Fig. 5. Dependence of the relative capacitance change of the BLM with adsorbed membrane fragments containing Na^+,K^+-ATPase induced by a pH jump on the KCl concentration in the cell at different pH (as indicated) (a). Effect of KCl on the initial increase of the relative capacitance change at different pH in the absence (*solid circles*) and in the presence of 10 mM $MgCl_2$ (*open circles*) (b). The relative capacitance changes were determined as ratio of the capacitance change in the presence of 6 mM KCl and in the absence of KCl. The data points in the absence of magnesium ions were taken from Fig. 5a. Data from measurements in the presence of magnesium ions are the results of corresponding experiments. The initial solution contained 12 mM NMG and 0.1 mM EDTA.

ions, ATP causes the opposite effect. ATP binds to a "low-affinity binding site", which promotes the transition to the E_1 conformation, and causes deocclusion of the binding sites to the cytoplasm, opening of the channel, and the release of potassium ions [34, 35]. In the presence of potassium ions and absence of ATP the capacitance increment caused by pH jumps was decreased due to binding and occlusion of potassium ions in the protein, and a closed access channel. On the other hand, the presence of ATP induced deocclusion of the binding sites and opening of the access channel, thus making way to the binding sites, which in turn disabled the suppressing effect of potassium on the capacitance change. Therefore, the simple model we considered earlier for sodium ions is inapplicable in the case of potassium ions. To explain the effect of ATP on the capacitance changes, two aspects have to be considered. First, ATP leads to a shift of the conformation equilibrium from the occluded P-E_2 state preferentially towards E_1 with the open access channel, which enables permanent exchange of ions in the binding sites of the Na^+,K^+-ATPase. Second, in the E_1 conformation exchange of potassium ions and protons at the binding sites takes place, and the average occupation by one or the other or none ion depends on their actual concentrations [19]. Such a competition, since each site may be occupied only by one ion at a time, has to be described by a mechanism similar to

that in the case of sodium ions, which explains the increased effect of potassium ions with increasing pH (Fig. 5). Thus, in summary, from the formal point of view, binding of potassium ions in competition to protons and their subsequent occlusion are comparable with binding of sodium ions where binding of the third sodium to the sodium-specific site prevents also exchange with protons. Therefore, the dependence of the capacitance change on the concentration of potassium ions may be explained by the equation obtained in the framework of the introduced simple model [27], although the obtained potassium dissociation constants probably are only apparent values.

Ion transfer into the Na^+,K^+-ATPase is a complex multistep process consisting of ion binding to their corresponding sites and the thereby associated conformational relaxation. For this reason, the experimentally obtained ion binding constants may be significantly different from the true binding constants. The experimental methods for the determination of the binding constants of sodium and potassium ions to the cytoplasmic binding sites may be divided into three groups. The first group consists of methods to measure unidirectional or bidirectional ion fluxes primarily depending on the concentration of the ions. In this case, the binding constants for ions to their sites in the protein are not measured directly, but corresponding to the effect of ion concentration on the rate of their transfer [36]. In the investigation presented above, the values of dissociation constants to the cytoplasmic side of the protein for sodium and potassium ions were $K_{Na} = 0.19$ mM and $K_K = 9$ mM, respectively, by the effect of the ions on capacitance changes, also a rather indirect method. In a second approach direct measurements used the radioactive isotope ^{42}K [37, 38]. This method yielded very low values, about 0.1 mM. The third, widely applied technique to determine the dissociation constants used fluorescent probes. One of the most popular probes is FITC, a molecule of which covalently binds to the protein at the ATP binding site. The fluorescence of FITC is sensitive to a conformational transition [32]. Since binding of K^+ leads to a conformational transition with the formation of an occluded state, this label allows to determine the effective (see experiments with ^{42}K) K_K. The K_K values determined by this method were also very low: 0.23 [31], 0.1 [21], and 0.07 mM [39].

As mentioned, the determination of the dissociation constant of K^+ is complicated because binding of potassium ions is accompanied by the protein transition into the occluded state, $E_1 + 2K^+ \rightarrow E_2(2K^+)$. The rate of the reaction $E_1 + 2K^+ \rightarrow E_2(2K^+)$ measured by stopped-flow fluorometry [31] was approximately of 300 s^{-1}. In the absence of ATP the rate of the back reaction, $E_2(2K^+) \rightarrow E_1 + 2K^+$, which was measured in deocclusion experiments with ^{86}Rb [33, 40], was approximately only 0.1–0.3 s^{-1}. Such a large difference in the rates of forward and back reaction leads to a very significant shift of the equilibrium to the right, so the dissociation constant of K^+ obtained in equilibrium conditions (see above) should be underestimated. The dissociation constant of K^+, determined by stopped-flow fluorometry, appeared to be 74 mM when separated from the subsequent conformation transition [31]. Since this value is about three orders of magnitude higher than the values obtained at overall equilibrium conditions, it is possible that it is close to the true dissociation constant of K^+ for E_1 conformation.

It is known that magnesium is not only a crucial cofactor of ATP hydrolysis, but also affects the activity of Na^+,K^+-ATPase. It has been suggested that this ion has a specific binding site in the protein, different from the enzymatic site, and its presence leads to an apparent decrease of the sodium and potassium affinity of binding sites. Magnesium ions reduce binding of sodium ions, which can be caused by both allosteric and electrostatic actions on the Na^+,K^+-ATPase [41]. The quantitative estimates made earlier on the basis of competition of sodium and magnesium ions in the cytoplasmic access channel of Na^+,K^+-ATPase, showed that the dissociation constant of Na^+ in the binding site increased by approximately six times in the presence of magnesium ions [27]. However, in the case of potassium ions, the effect of magnesium ions was different. Magnesium ions affected the kinetics of the capacitance change after the pH jump significantly (Fig. 3). The influence of magnesium ions on the quick jump of capacitance was manifested rather as a change of effect of potassium ions (shift down of points in Fig. 5b). The approximation of experimental dependences of the capacitance jump on the potassium concentration by theoretical curves (Fig. 4) has shown that the dissociation constant of K^+ in the presence of magnesium ions changes. The effect of magnesium ions on the binding of potassium ions can, however, be explained by allosteric and electrostatic mechanisms, similarly to how it has been explained in the case of sodium ions. For this explanation, it is necessary to assume that Mg^{2+} ions can shift the conformational equilibrium of Na^+,K^+-ATPase to E_2 conformation (i.e., compared to ATP, Mg^{2+} has an opposite effect on potassium binding). The presence of Mg^{2+} in this case causes two oppositely directed effects: a decrease in the concentration of potassium ions within the channel due to an electrostatic field (which reduces K^+ binding similarly to the suppression of Na^+ binding), and a shift of the conformation equilibrium towards the potassium occluded E_2 conformation (which, in contrast, facilitates K^+ binding). These two effects can balance each other to some extent. It is also interesting to note a decrease in the coefficient of cooperability of binding potassium ions upon the addition of magnesium ions from 2 to 1.4. A similar effect has been observed for binding of

sodium ions [27]. The similarity of the effect of both ions species in sign and magnitude is in favor of the electrostatic hypothesis of the magnesium effect on sodium and potassium binding in the E_1 conformation. The presence of Mg^{2+} both in the double layer close to the membrane surface and bound to the protein near the entrance to the access channel [41] is expected to create a potential barrier for cations on the way from the solution to the binding sites. Then the decrease of cooperativity can be explained by a rate-limitation of the ion flux from the solution to the binding sites since ions have to jump over the barrier one by one.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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