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Effect of chaotropic anions on the sodium transport by the Na,K-ATPase

Abstract The effect of choline iodide, bromide and chloride on the kinetics of the electrogenic sodium transport by the Na,K-ATPase was investigated in a model system of ATPase-containing membrane fragments adsorbed on the lipid bilayer membrane. The kinetic parameters of Na^+ transport were determined from short circuit currents after fast release of ATP from its caged precursor. The falling phase of the current transients could be fitted by a single exponential with the time constant, τ_2. Its temperature dependence allowed an estimation of the activation energy of the rate-limiting reaction step, the conformation transition E_1/E_2. Choline iodide and bromide caused a decrease of the activation energy as well as the overall rate of the process expressed as the pre-exponential factor A of the Arrhenius equation. If choline iodide or bromide were present on the cytoplasmic and extracellular sides of the protein, the temperature dependent changes were more pronounced than when present on the cytoplasmic side only. These results can be explained by an effect of the anions on water structure on the extracellular surface of the protein, where a deep access channel connects the ion-binding sites with the solution. Chloride ions also caused a deceleration of the electrogenic transport, however, in contrast to iodide or bromide, they did not affect the activation energy, and were more effective when added on the cytoplasmic side. This effect can be explained by asymmetric screening of the negative surface charges which leads to a transmembrane electric potential that modifies the ion transfer.

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Introduction

The Na,K-ATPase is one of the most important and widespread proteins in the animal kingdom, which performs active transport of Na and K ions across the cytoplasmic membrane of the cells. Intensive studies were conducted during recent years and resulted in a significant progress in elucidating pump function. Conformational rearrangements of the Na,K-ATPase play a key role in the transport process. While the pump runs through its cycle, alternating access to the ion binding sites through channel-like structures on both sides of the membrane domain enables in the E_1 conformation an exchange of three Na^+ against two K^+ ions on the cytoplasmic side and vice versa on the extracellular side in the $P-E_2$ conformation (Apell 2003; Pavlov and Sokolov 2000).

It has been known for more than 15 years that flat membrane fragments with a high density of Na,K-ATPase molecules (about 5,000 per μm^2), isolated and purified from rabbit kidneys, can be adsorbed to bilayer lipid membranes (BLM). This system is convenient to investigate the ion-transport mechanism of the Na,K-ATPase (Borlinghaus et al. 1987; Fendler et al. 1985; Wuddel and Apell 1995). In such a setup, in the absence of potassium ions, short circuit current can be generated by the transfer of sodium ions through the membrane domain of the Na,K-ATPase towards the extracellular side of the membrane, which is facing the BLM. Such currents can be recorded as synchronized response to a fast ATP-concentration jump, which is produced by the release of ATP from its inactive precursor, caged ATP, upon an UV light flash. The triggered current transients have three characteristic phases: rise and decay of current in response to the flash, as well as third phase of a negative current component which is associated with the slow passive discharge of the electric capacity of the

adsorbed fragments and of the BLM (Sokolov et al. 2001; Wuddel and Apell 1995). The transfer of Na^+ ions consists of several sequential steps, including binding of three Na^+ ions from the cytoplasmic side of the membrane, binding and hydrolysis of ATP, phosphorylation, $P\text{-}E_1/P\text{-}E_2$ conformation transition, and release of the ions on the extracellular side. Determination of the time constants of the three current phases does not provide sufficient information to obtain the rates of all the reaction steps, however, the rate of the slowest step controlling the time constant of the respective partial reaction can be identified. The conformation transition $P\text{-}E_1/P\text{-}E_2$ is supposed to be the slowest step of the ion transfer (Sokolov et al. 1998b). Although this interpretation is most widely adopted (Holmgren et al. 2000; Sokolov et al. 1998a; Wuddel and Apell 1995), there is an alternative point of view that the conformation transition controls the rising phase of the current signal (Fendler et al. 1993).

One method to solve this issue is to vary the rate of the conformational transition by the so-called "salt effect". It is well known that the conformation of macromolecules dissolved in water depends upon the ionic composition of the solution. The most likely explanation is that the energy of water molecules bound to the macromolecule-water interface depends on the presence of other substances in the solution. Some substances, known as chaotropes, increase the binding energy for water whereas other substances, referred to as cosmotropes, decrease it (Cacace et al. 1997). The effect of the chaotropic ions on the equilibrium between the two principal conformations of the Na,K-ATPase, E_1 and $P\text{-}E_2$, was demonstrated earlier by investigation of the effect of different salts on binding of inorganic phosphate to the Na-K-ATPase (Post and Suzuki 1991).

Chaotropic ions are known to slow down the electrogenic transport of sodium ions by the Na,K-ATPase in the model system consisting of a BLM with adsorbed Na,K-ATPase containing membrane fragments. Specifically, in the presence of different anions a deceleration of the kinetics of the short circuit current, and a shift of the frequency dependence of the admittance towards lower frequencies were observed (Ganea et al. 1999; Sokolov et al. 2001; Sokolov et al. 1998b). The magnitude of the effect depended on the anions present: the effectiveness of choline salts with different halogenoids increased in the sequence $Cl^- < Br^- < I^-$ corresponding to their position in the Hofmeister series (Sokolov et al. 2001). This finding can be explained by the effect of chaotropic anions on the rate of the conformation transition in the protein. Other explanations, however, could not be excluded so far, e.g. salt-induced changes of the aggregation state of the lipids surrounding the protein (Klodos et al. 1994), as well as non-specific screening of the charged groups of the protein, or competition of anions for the ATP binding site (Nørby and Esmann 1997).

Investigations of temperature dependencies of the salt effects can be used to prove that the effects are associated with the conformation transition of the Na,K-ATPase. This rate is known to be highly sensitive to temperature, as indicated by the high-activation energy, which was estimated to be about 80 kJ/mol, based on the electric measurements with cardiomyocytes (Gadsby et al. 1992). If the choline salts affect the rate of the conformational transition, they may also affect the corresponding activation energy. Besides the membrane domain with 10 transmembrane helices, the Na,K-ATPase has a large cytoplasmic part that consists of three different domains that represent the enzymatic machinery, and a small extracellular part that is formed mostly by the β subunit of the protein. Therefore, it may be expected that the chaotropic salts produce different effects on the two sides of the membrane. The Na,K-ATPase-containing membrane fragments are known to adsorb on the BLM with a preferred orientation, the extracellular membrane side facing the BLM. This property allows comparison of the effects of choline salts with various anions on the Na,K-ATPase on both sides of the membrane, when the salts are added to the solution either prior to the adsorption on BLM or after the tight adsorption has occurred. In the first case the salts can affect the ATPase on both sides of the membrane fragment, whereas in the second case only the cytoplasmic side is exposed to the bulk solution. (During the time period of the experiments the ions or ouabain do not penetrate into the narrow space of the contact area between membrane fragment and BLM (Borlinghaus et al. 1987; Fendler et al. 1985).) In the following presentation we will refer to a 'bilateral' or 'unilateral' addition of ions when the choline salts were added before or after adsorption of the membrane fragments, respectively.

In the present study, we have proven by measurements of the temperature dependency that the choline salts of chaotropic anions affect the activation energy of the slowest step of electrogenic transport, being much more effective when present on the extracellular side of the protein. This implies that the salts do affect the $P\text{-}E_1/P\text{-}E_2$ conformation transition rather than ATP hydrolysis, since hydrolysis occurs at the cytoplasmic side of the protein and has a different temperature dependence.

Materials and methods

Membrane fragments containing purified Na,K-ATPase were obtained from rabbit kidney according to Jorgensen procedure "C" (Jørgensen 1974). The membrane fragments are planar patches with the diameter of 0.2–1 μm, containing about 0.8 mg of phospholipids and 0.2 mg of cholesterol per 1 mg of protein (Deguchi et al. 1977). The enzyme activity was 1,800–2,000 μmol of inorganic phosphate released per 1 mg of protein per hour at 37°C. The aqueous suspension of the fragments with a protein concentration of about 3 mg/ml can be stored in a deep freezer for several months without notable loss of ATPase activity. For the experiments, the

suspension was thawed and subsequently stored at +4°C for less than 2 weeks.

BLMs were formed in a Teflon cuvette from a 15 mg/ml solution of diphytanoyl lecithin (Avanti Polar Lipids, USA) in n-decane (Aldrich, USA). The electrolyte contained NaCl, $MgCl_2$ (Merck, Germany), imidazole (Serva, Germany), dithiothreitol (DTT, Sigma, USA), caged ATP (Calbiochem, USA). The basic buffer solution (which will be referred to as "standard") contained 150 mM NaCl, 30 mM imidazole, 10 mM $MgCl_2$, and 1mM EDTA at pH 6.5 (HCl).

The electric current signals generated by the Na,K-ATPase were measured by the method described earlier (Borlinghaus et al. 1987). BLM were formed in a thermostated Teflon cuvette with two optically transparent windows for visual control of the membrane and for exposure to the UV light flash. After the BLM was formed in standard buffer, DTT was added to both compartments of the cuvette, then membrane fragments and caged ATP were added to the compartment opposite to the source of ultra-violet light (concentrations of the caged ATP and protein in the cuvette were 100 μM and about 20 μg/ml, respectively). The adsorption of the membrane fragments on the BLM was monitored by a decrease of the measured capacitance.

Current transients were recorded after the ATP release from caged ATP, triggered by the UV flash. A xenon flash lamp with sapphire window (FJ-249, EG&G, USA) was used as UV-light source. The release of ATP caused a transient current generated by transfer of the Na^+ ions through the Na,K-ATPase from the (cytoplasmic) bulk solution to the space between BLM and membrane fragments (Borlinghaus et al. 1987). Multiple records were made with the same membrane under identical conditions and averaged in order to increase the signal/noise ratio. Typical time intervals between two light flashes were longer than 10 min to ensure recovery of the initial state of the Na,K-ATPase. Some experiments were made in the presence of apyrase, a soluble enzyme that hydrolyses the free ATP in the buffer and accelerates restoration of the initial state of the Na,K-ATPase (Borlinghaus et al. 1987). In these cases the interval between the flashes could be decreased to 3 min. The amplitude of the transient currents increased between the sequential flashes, as long as the adsorption of the fragments continued. The electrical signals generated by the Na,K-ATPase were recorded only after the current amplitude and the membrane capacitance became stable, which indicated that the adsorption process was completed (usually more than 2 h after addition of the membrane-fragment suspension).

The electric currents were measured with Ag/AgCl electrodes, connected to the electrolyte in the membrane cuvette via salt bridges. The bridges were filled with the same solution as the cuvette. The resistance of the electrodes with the bridges did not exceed 30 kΩ. In order to record the short circuit currents, one of the electrodes was grounded, and the other connected to the input of the operational amplifier Keithley 427 (USA). The output signal of the amplifier was recorded by a digital oscilloscope KDS-102 (Kawasaki, Japan), or L780 ADC/DAC board (Lcard, Russia), and transferred to a PC. The current trace was fitted by a sum of exponential functions with a computer program designed by the authors. The temperature in the cuvette was maintained by an electronic thermostat based on a Peltier element.

Results

A typical record of a short circuit current is shown in Fig. 1. The time dependence of the current was fitted by a sum of three exponentials to obtain the three time constants: τ_1, τ_2 and τ_3. Only the time constants τ_1 and τ_2 were of interest for subsequent analysis, since the constant τ_3 characterizes the slow, passive discharge of the membrane capacitances, and is not directly related to the function of the ion pump (Borlinghaus et al. 1987). The measurements were conducted at different temperatures in the range between 15 and 30°C. The determined time constants at each temperature were plotted as Arrhenius plot, $\ln(1/\tau)$ versus $1/T$. Such a dependence of τ_2 is shown in Fig. 2. The activation energy, E_a, was obtained from the slope of the linear fit of the data points in the Arrhenius plot. The pre-exponential, temperature-independent factor A characterizes the vertical position of the data points in the Arrhenius equation:

$$k = A \cdot \exp\left(-\frac{E_a}{R} \cdot \frac{1}{T}\right),$$

where the rate, $k = 1/\tau$, is the reciprocal time constant of the corresponding exponential function. The activation energy of τ_1, E_a, which represents the rising phase of the current transient, was 98 kJ/mol in the standard solution, whereas an E_a of 65 kJ/mol was obtained for τ_2 characterizing the falling phase. These values are in good agreement with previously reported results of measurements performed in the same model system under similar conditions (Fendler et al. 1993), where the activation energies were reported to be 101 kJ/mol and 68 kJ/mol, respectively.

The amplitude of short circuit current decreased and its kinetics was slowed down in the presence of choline iodide (Fig. 1). This effect was reflected in an increase of the time constants, τ_1 and τ_2 as reported recently by the authors (Sokolov et al. 2001). It has been shown previously that this effect is caused by the anions and not by choline (Sokolov et al. 1998, 2001). In order to reveal the underlying mechanism, the effect of anions on the temperature dependence of the kinetical parameter was studied. Addition of iodide ions changed only insignificantly the process which controls the rising phase of the current transient with its time constant, τ_1, (data not shown), whereas the effect on the falling phase, represented by τ_2, was much more pronounced. Fig. 2a
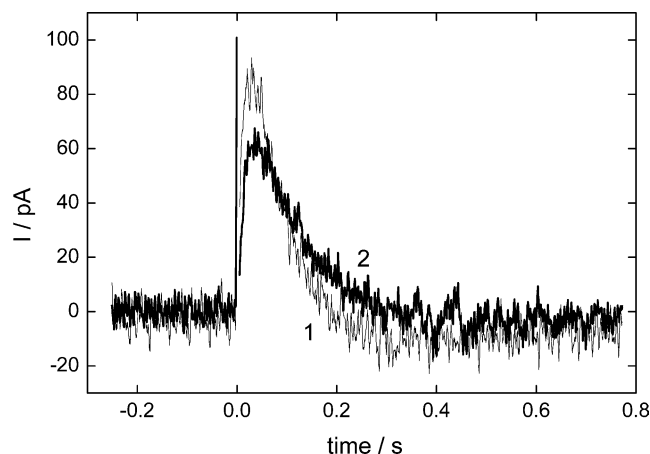
Fig. 1 Kinetics of the short circuit current caused by UV flash induced ATP-concentration jump before (1) and after (2) unilateral addition of 50 mM choline iodide to the electrolyte. The curve was fitted by a sum of three exponentials that yields the following values of the time constants (ms): $\tau_1 = 17$, $\tau_2 = 34$, $\tau_3 = 309$ (1); $\tau_1 = 19$, $\tau_2 = 76$, $\tau_3 = 331$ (2). The BLM was formed from diphytanoyl phosphatidylcholine (15 mg/ml in n-decane) in standard buffer at 24°C and had an area of about 0.3 mm^2 (capacitance of 1,3 nF). After formation of the BLM, dithiothreitol was added as reducing agent to both compartments of the cuvette, and the suspension of the membrane fragments containing Na,K-ATPase (protein concentration about 30 μg/ml) and caged ATP (100 μM) were added to the compartment opposite of the light source

illustrates the temperature dependency of $1/\tau_2$ at different choline-iodide concentrations (0–60 mM) when the ions were added prior to the adsorption of the membrane fragments (bilateral addition). Choline iodide caused a decrease of the rate parameter A (downwards shift of the data points) as well as a decrease of the activation energy E_a. The parameters A and E_a were found to change in a correlated manner as function of the choline iodide concentration. In Fig. 2b each point corresponds to the values of $\ln(A)$ and E_a, obtained from the fit of the temperature dependence of the Na$^+$ transport by the Na,K-ATPase at the iodide concentration shown in Fig. 2a. As can be seen from Fig. 2b, $\ln(A)$ is linearly related to E_a, which means that the temperature dependencies obtained at different choline iodide concentrations intersect in one point. The coordinates of this point, which can be easily deduced from the slope of the line on Fig. 2b, correspond to a temperature of 270 K, which is close to freezing temperature of water.

If the bromide or iodide salts were added to the solution after complete adsorption of Na,K-ATPase-containing fragments (unilateral addition), the changes of A and E_a were much smaller than in the case of bilateral addition (Fig. 3). Hence, these salts affect the protein more effectively when present on both the cytoplasmic and the extracellular sides. This observation indicates that the extracellular side is more sensitive to the effects of iodide.

One possible explanation of these salt effects is—as it was discussed in the literature—that the anions affect
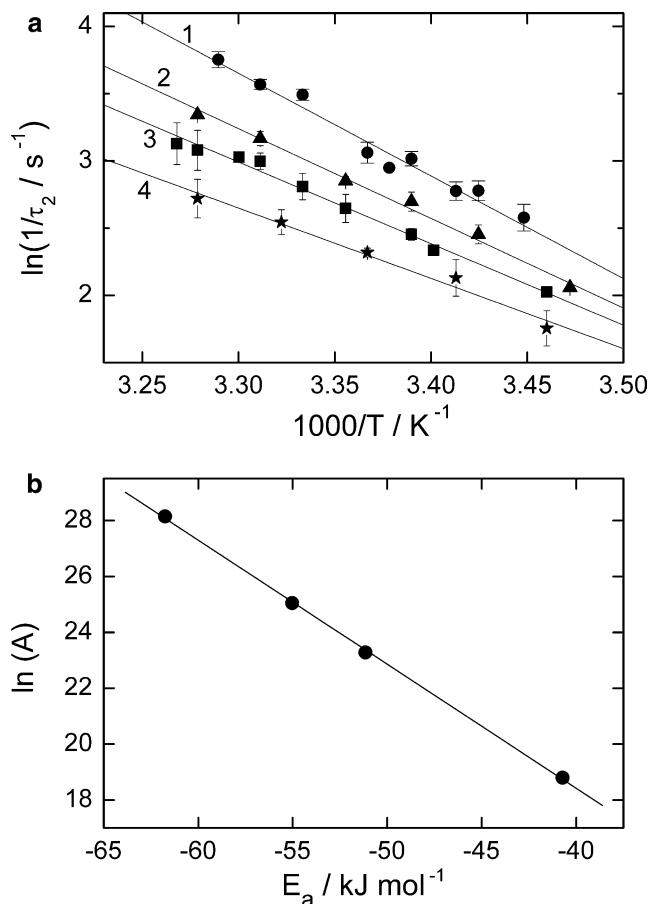

Fig. 2 a Temperature dependence of the reciprocal time constant of the exponential function approximating the decay phase of the short circuit current, ($1/\tau_2$), represented as Arrhenius plot. The measurements were performed in the standard buffer (trace 1), or after bilateral addition of several choline–iodide concentrations: 15 mM (trace 2), 35 mM (trace 3) and 60 mM (trace 4). The lines fitted through the data correspond to the following values of the parameters of the Arrhenius equation, E_a (kJ/mol) and $\ln(A)$ (s^{-1}), respectively: 61.7 and 28.2 (0 iodide); 55.0 and 25.0 (15 mM); 51.1 and 23.3 (35 mM); 40.7 and 18.8 (60 mM). **b** Correlation between E_a and $\ln(A)$, as obtained from the fit of the data shown on panel (**a**). The linear correlation could be fitted by: $\ln(A) = 0.67 - 0.44 \cdot E_a$

ATP binding to the Na,K-ATPase or compete with ATP for the nucleotide-binding site (Ganea et al. 1999; Nørby and Esmann 1997). An alternative explanation would be that the anions screen the electrostatic field produced by the charged groups of the protein in the vicinity of the ATP-binding site (Fedosova et al. 2003; Nørby and Esmann 1997). In order to verify whether the effect of choline salts is caused by these reasons, we investigated the temperature dependency of $1/\tau_2$ at variable intensities of the UV flash, thus varying the amount of ATP released from caged ATP. The results are presented in Fig. 4. A decrease of the flash intensity (or ATP concentration) affected the temperature dependence of $1/\tau_2$ in a different manner when compared to addition of iodide. When the light intensity was reduced to 50%, the slope of temperature dependence of $1/\tau_2$ increased, and correspondingly, the activation energy grew from
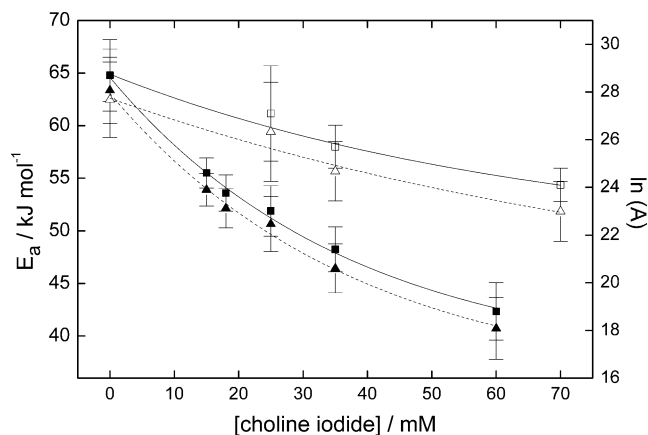
Fig. 3 Dependence of the activation energy, E_a, (*triangles*) and pre-exponential factor (*squares*) on the concentration of choline iodide when added either bilateral, i.e. before addition of the membrane fragments (*solid symbols*), or unilateral, i.e. after completion of their adsorption on BLM (*open symbols*)

Fig. 5 Dependence of the time constant, τ_2, of the fit to the decay of the short circuit current (cf. Fig. 1) on the concentration of choline chloride added to the cuvette either (1) before addition of the membrane fragments ("bilateral addition"), or (2) after completion of their adsorption on BLM ("unilateral addition"). T = 24°C

62.3 kJ/mol to 119.7 kJ/mol in the temperature range between 21°C and 31°C. Addition of the iodide caused the slope to decrease (Fig. 2), and the activation energy to be reduce from 61.2 kJ/mol (0 I⁻) to 18.8 kJ/mol (60 mM I⁻). Moreover, the intersection point of the fitting lines in the Arrhenius plot was about 28°C when the amount of ATP released was reduced by about a factor of two, while it was close to 0°C for all iodide concentrations between 0 and 60 mM.

When the temperature dependence of $1/\tau_2$ was investigated in the presence of various choline-chloride concentration the slope of the Arrhenius plot (and accordingly the activation energy) was not significantly affected (not shown), and hence the (temperature independent) decrease of $1/\tau_2$ at elevated chloride
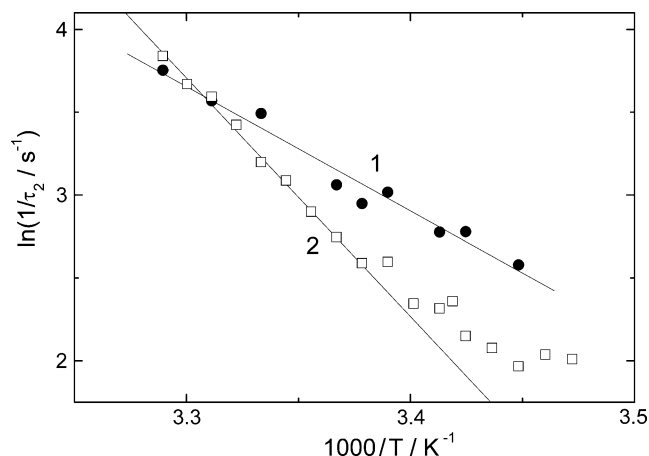
concentrations has to be attributed to a decrease of the pre-exponential factor A.

There was also a difference between the different anions in the experiments with bilateral and unilateral addition of the salts. Unilateral addition of choline chloride caused a more pronounced deceleration of the short circuit current kinetics than a bilateral addition. The value of τ_2, increased only insignificantly, and τ_1 remained constant in the case of a bilateral addition of choline chloride at concentrations up to 850 mM (Sokolov et al. 2001). In the case of unilateral addition, τ_2 increased significantly at concentrations above 50 mM (Fig. 5). This observation is in contrast to the results obtained in the presence of iodide. When iodide

Fig. 4 Effect of the variation of the amount of ATP released on the temperature dependence of $1/\tau_2$. Dataset 1 was obtained under standard conditions (as trace 1 in Fig. 2). Dataset 2 was obtained at a flash energy reduced by a factor of two, corresponding to a reduction of the amount of ATP released by about the same factor
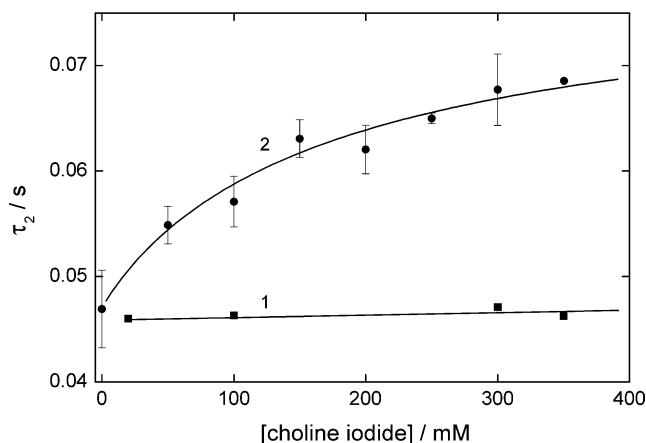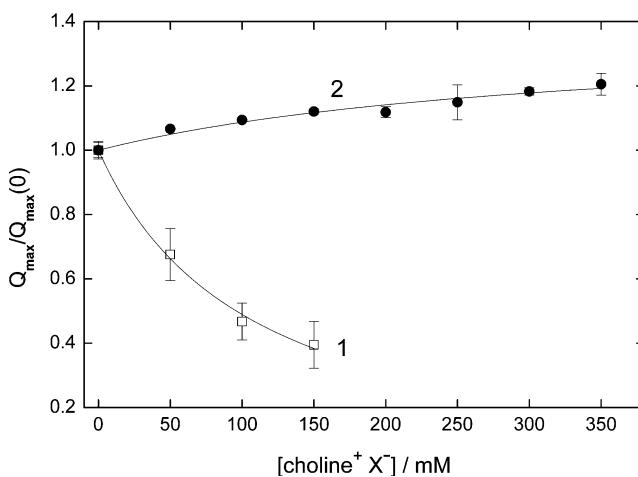

Fig. 6 Dependence of the charge transferred by the Na,K-ATPase on the concentration of choline iodide (1) or chloride (2) added to the cuvette after adsorption of the membrane fragments to the BLM ("unilateral addition"). The charge transfer was triggered by an ATP-concentration jump. The condition of the experiments is described in Fig. 1. The transported charge was defined as maximum of the integral of short circuit current, the values are normalized to the charge obtained before addition of choline salts

was added unilaterally, τ_2 developed as function of concentration similarly to the findings with chloride, i.e. $\tau_2 = 0.057 \pm 0.002$ s at 100 mM, while τ_2 increased to about 0.1 s at 100 mM when iodide was added bilaterally (Sokolov et al. 2001). In contrast, chloride had no significant effect on $\tau_2 = 0.046 \pm 0.001$ s when added bilaterally in concentrations between 50 mM and 350 mM (Fig. 5). This behavior, as it will be discussed below, can be explained rather by screening of surface charge than by a direct interaction of choline with the Na,K-ATPase.

The experiments repeated with choline bromide instead of choline iodide showed that bromide gives the same effect as iodide (data not shown).

A difference between chloride on the one hand and iodide or bromide on the other hand was also found for the amount of charge moved across the membrane after an ATP-concentration jump. The amount of charge transported by the Na-K-ATPase was determined as described before as a maximum value of the integrated short circuit current (Sokolov et al. 2001). When bromide or iodide were added, the amount of charge was significantly reduced to about 50% at 100 mM, whereas addition of even 350 mM chloride led only to a slight increase of about 20% (Fig. 6).

Discussion

We measured the temperature dependency of the characteristic time constants of the ATP-induced current transients, τ_1 and τ_2, in solutions containing choline salts of three different anions, chloride, bromide and iodide. The latter two anions affected the temperature dependence of $1/\tau_2$ by significantly decreasing the activation energy of the rate-limiting reaction step. The effect of iodide was stronger than that of bromide. From the literature it is known that bromide and iodide anions are strong chaotropic agents, with iodide being notably more effective than bromide according to the Hofmeister series (Post and Suzuki 1991). Therefore, the observed effect of these ions may be explained by their chaotropic influence on the "basic" free energies (Hill 1977; Läuger 1991) of the different conformational states of the protein. It has been shown in a number of studies that the conformation transition, P-E_1/P-E_2, is the rate limiting step of the electrogenic Na^+ transport, and that its rate constant controls the kinetics of the decay of the short circuit current (Holmgren et al. 2000; Sokolov et al. 1998a; Wuddel and Apell 1995). The observation that these anions modify the temperature dependence of $1/\tau_2$, while the dependence of $1/\tau_1$ is practically unaffected, supports this conclusion, since the rising phase with its time constant represents the (faster) preceding partial reactions, which includes ATP release from its caged form, ATP binding, and enzyme phosphorylation (Sokolov et al. 1998a). The effects of iodide were much stronger when it was added bilaterally, than when iodide was added after adsorption of the membrane fragments

on the BLM, where it could affect only the cytoplasmic side of the protein. These experiments imply that the effect of iodide on the temperature dependence of τ_2 is primarily due to its interaction with the extracellular parts of the protein.

The conformation transition, P-E_1/P-E_2, is known to open the access to the ion-binding sites from the extracellular aqueous phase through a so-called ion well. Therefore, it is possible that iodide (or bromide) affects the conformation transition by changing the free energy of the open-channel formation. The chaotropic effect of iodide and bromide can be related to changes of the energy necessary to bind water to the extracellular surface of the protein or to allow ions to enter the access channel. The fact that all the temperature dependences of the reciprocal time constants of τ_2 intersect in the Arrhenius plot for the different iodide concentrations at the same temperature, the freezing point of water (Fig. 2a), implies that the effects of the anions on the time constant, τ_2, vanished at this temperature. This observation supports the proposal of an effect on water binding to the protein surface. An alternative explanation of the observed effects, however, cannot be ruled out, such as specific binding of iodide (and bromide) to extracellular sites of the protein.

In the literature other possible explanations of the salt effects have been introduced. One proposal was an interaction of anions with the ATP-binding site—either by competitive binding or by shielding of electrostatic protein charges located in the vicinity of the binding center (Fedosova et al. 2003; Nørby and Esmann 1997). Neither mechanisms, however, can explain the iodide effects shown above: (1) significant effects occur already at low-iodide concentrations when the ionic strength of the solution remains practically unchanged, (2) the relaxation time τ_2 is controlled by the conformation transition and not by enzyme phosphorylation, and (3) the ATP-binding site is situated on the cytoplasmic side of the membrane and is accessible for the chaotropic ions in the same way when added unilaterally or bilaterally, in contrast to the observation that iodide (or bromide) acted more efficiently when present at the extracellular side.

Alternative mechanisms stand to reason, however, in the case of the less chaotropic chloride ions. Significant chloride effects were observed only at higher concentrations when the ionic strength of the solution was enhanced considerably. Further support for an alternative mechanism is that chloride ions were more effective upon unilateral addition than upon bilateral addition (Fig. 5). Chloride ions did not measurably change the slope of the temperature dependence of $1/\tau_2$, and therefore, the activation energy was not significantly modified (not shown). Finally, there is another important difference between the effects produced by chloride on the one hand and bromide or iodide on the other: Addition of the latter two anions to the buffer caused a decrease of the amount of electric charge transferred by the protein upon phosphorylation, whereas addition of
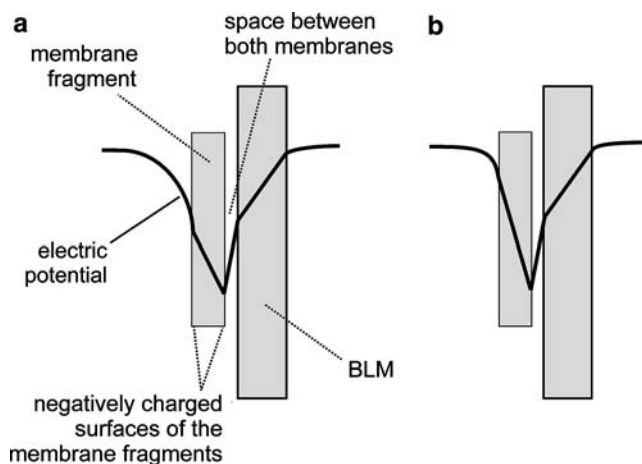
Fig. 7 Schematic representation of the electrostatic energy profile for cations across the BLM and an adsorbed membrane fragment in a solution of low-ionic strength (**a**) and after unilateral increase of ionic strength (**b**). The changes of the electrostatic potential have their origin in a shielding of the negative surface charges on the Na,K-ATPase-containing membrane fragments by the increased ionic strength in the solution with a high-choline chloride concentration

chloride led even to a small increase (Fig. 6). The amount of the charge transferred depends on three factors: (1) the number of sodium ions transferred per protein molecule in response to the ATP-concentration jump, (2) the number of active protein molecules, and (3) how far the Na^+ transport is completed. There is no indication that the anions alter stoichiometry and functional activity of the Na,K-ATPase. In the absence of chaotropic anions the steady-state conformation after the relaxation process is almost completely state P-E_2 in the experimental conditions chosen. In the presence of the strongly chaotropic iodide, however, the P-E_1/P-E_2 conformational equilibrium is shifted in favor of the E_1 conformation, this condition will prevent the (electrogenic) release of part of the Na ions, and in consequence, reduce the amount of charge transferred, as shown in Fig. 6. A mechanistic explanation for the observed shift towards the E_1 state may be that the chaotropic anions produce a stronger decrease of the forward rate constant ($E_1 \rightarrow E_2$) than of the backward rate constant.

The increase of the number of Na ions transferred by the protein in the presence of chloride is most likely to be associated with the increased ionic strength of the electrolyte. In the case of an unilateral addition this leads to a decrease of the surface potential on the cytoplasmic side of the membrane due to shielding of the negative charges of lipid molecules on the cytoplasmic surface of the ATPase-containing membrane fragments. A change of the surface potential on the cytoplasmic side of the membrane can affect both the amount of ATP bound to the protein, and especially, the (apparent) transmembrane potential. This is illustrated by Fig. 7, a schematic representation of the electric-potential profile across the membrane fragments adsorbed to the BLM. A

quantitative determination of the electric potentials occurring in such a system is hardly possible, and the profile depicted in Fig. 7 illustrates the two known facts only in principle. Firstly, both the data available from the literature and measurements of the electrophoretic mobility (unpublished results) indicate the presence of negatively charged lipids in the membrane fragments. In consequence, there is a potential change in the diffuse double layer at the interface between the membranes and the solution when the ionic strength is increased. Secondly, the membrane fragments and BLM are in close contact to each other. When the distance between the charged surface of fragments and BLM becomes comparable to the thickness of the diffuse double layer, a transmembrane-potential drop will occur to both membranes (Aityan et al. 1981). Figure 7a illustrates the potential profile at low-ionic strength, taking into account the surface charge of the fragment and the intramembrane potential drop in both membranes. An increase of ionic strength and the resulting decrease of the electrostatic surface potential on the electrolyte side of the membrane fragment modifies the potential distribution within it (Fig. 7b). Such an increase of the electric field inside the membrane is equivalent to an increase of the transmembrane potential, and causes an increase of the number of Na^+ ions transferred by the Na,K-ATPase by a short-circuit current.

Thus, we have demonstrated that choline iodide and bromide not only decrease the rate of the rate-limiting step of the electrogenic ion transport, but also affect the activation energy of this step. This observation cannot be explained by the effect of the chaotropic anions on the phosphorylation step of the Na,K-ATPase, or by an significantly increased ionic strength that shields surface charges of the membrane (since, as we demonstrated in the case of choline chloride, the ionic strength has a different effect). The difference between the effects of unilateral and bilateral additions of iodide suggests that the primarily sensitive parts of the protein are located on the extracellular side. These results support the conclusion that the effects of iodide (and, to lesser degree, of bromide) are associated with an influence of the anions on the conformational transition. Since the P-E_1/P-E_2 conformation transition is known to open the extracellular ion-access channel, and alter the extracellular protein surface (Geibel et al. 2003), the iodide and bromide ions can be assumed to affect such modifications. A reasonable explanation for the observed effect is that chaotropic anions modify the intrusion of water molecules into the access channel and alter their coordination with the surface of the protein.

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