ANION EFFECTS ON CURRENTS GENERATED BY THE SODIUM PUMP

A. BABEŞ, FLORENTINA PLUTEANU

Department of Animal Physiology and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independenţei 91 – 95, 76201 Bucharest, Romania

Abstract. This paper describes some effects of lyotropic anions on currents generated by the Na^+,K^+-ATPase recorded in excised inside-out patches from guinea pig cardiac myocytes. We show that these effects are reversible, that ionic strength is a major factor in influencing the pumping turnover and that various anions have effects on the pump current in the presence and in the absence of potassium at the extracellular side. We also report that anions affect the ATP affinity at the catalytic high affinity site, but do not change the dissociation constant for ATP at the low affinity binding site. It is proposed that these effects are related to a change in the local electric field due to anion binding to the lipid membrane or to the protein itself.

Key words: Na^+,K^+ - ATPase, electrogenic, anions, lyotropic, membrane dipole moment.

INTRODUCTION

It has already been known for many years that various inorganic anions are able to influence certain physiological processes. It has been shown that in the presence of nitrate and other anions there is an increase in the force of contraction during twitches of isolated muscle fibres [8]. The voltage dependence of activation of several ion channels is shifted as an effect of thiocyanate or perchlorate [5, 20].

It was also relatively early discovered that the order of effectiveness of various anions in influencing these processes is in good agreement with the so-called Hofmeister series, or lyotropic series, described for the first time by Hofmeister [9] and related to the efficiency of various salts in precipitating egg globulin. The amplitude of the effect is increased in the following order: $Cl^- < Br^- < NO_3^- < I^- < SCN^- < ClO_4^-$. Small differences have been found later in this order of effectiveness, depending on the process. It was then noted that this order corresponds to the order of efficiency in which these anions are adsorbed to the plasma membrane, suggesting that these effects can be related to their membrane adsorption which would modify the local electric field, and thus influence voltage-dependent processes.

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The Na^+,K^+-ATPase, also known as the sodium pump, is a membrane protein, which can be found in all animal cells and is able to extrude three sodium ions and to import two potassium ions against their electrochemical gradient. The energy required for ion translocation is provided by the hydrolysis of ATP to ADP and inorganic phosphate [16]. As it exports three monovalent cations against only two imported, the sodium pump generates electric current through the membrane resistance thereby contributing to the resting membrane potential of the cell. The membrane potential has a pronounced effect on the pump activity, which is enhanced at depolarized potentials and inhibited upon hyperpolarization. Due to these properties, the sodium pump is called electrogenic. By extension, each reaction step that is associated with the charge movement within the membrane dielectric is called electrogenic. Several studies have shown that there is at least one major electrogenic step within the sodium transport branch of the reaction cycle of the Na^+,K^+-ATPase [16].

In this study we intend to describe some effects of various common anions on the kinetics of the sodium pump. Post and Suzuki [19] have shown for the first time that anions modify the conformational equilibrium between the two major phosphorylated states of the sodium pump: E_1P and E_2P. Other authors have reached the same conclusion, using various techniques [12, 13, 14]. There is no agreement yet upon the mechanism by which anions are modifying the activity of this enzyme. It has been suggested by Post and Suzuki [19] that the anions can act by changing the structure of water molecules that surround the phosphate group attached to the protein. Klodos and colleagues [14] have attributed this effect to a change in protein conformation induced by anions. Other authors [18] have investigated the effects of various salts on the ADP affinity of the sodium pump and they have interpreted these as pure ionic strength effects: the anions and cations in the electrolyte solution are screening the surface charges on the protein. However, the same authors have observed more pronounced effects of certain salts, especially $NaNO_3$, NaSCN and $NaClO_4$, which could not be explained solely by the Debye-Hückel theory. They have proposed that there is a specific interaction between the anions of these salts and the protein. Moreover, NO_3^- acts as if it competes with ADP for its binding site on the protein. It can be therefore assumed that there is indeed a specific effect of anions on the conformational equilibrium of the phosphoenzyme.

As proposed initially by Hodgkin and Horowicz [8], these anion effects can also be seen as due to a change in the local electric field in the plasma membrane, without having to take into consideration changes in the protein or water structure. By modifying the electric field, the anions can stabilize or destabilize certain charged conformations of the protein. We can expect that the electrogenic reactions of the pump cycle in particular are affected in the presence of various anions. Some authors already used such a model based on the local electric field in order to explain the effect of the hydrophobic anions tetraphenylborate (TPB^-) and

tetraphenylsulphonate (TPP^+) on partial reactions of the sodium pump [4, 15, 21]. However, it can also be argued that these ions can bind to hydrophobic pockets of the protein, and thus modify the local electric field.

In a recent paper, Clarke and Lüpfert [2] have shown using voltage dependent fluorescent probes that anions bind to lipid vesicles and reduce in this manner the membrane electric dipole moment. The order of effectiveness in decreasing the membrane dipole moment was $ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^- > SO_4^{2-}$. This order is in good agreement with the Hofmeister series. Using the same technique and a stationary electrical perturbation technique, the same group has reported pronounced anion effects on the kinetics of some partial reactions of the sodium pump: conformational transition $E_1P \rightarrow E_2P$, sodium and ATP binding, dephosphorylation [7]. In this study we have used a complementary technique, patch-clamp, in order to extend these observations to other steps in the reaction cycle of the sodium pump.

MATERIALS AND METHODS

Most of the results reported in this study have been obtained using the giant patch-clamp technique [6], which allows the recording of ionic currents generated by electrogenic ion pumps and transporters.

As opposed to ion channels which pass a large number of ions per unit time (10^7 ions/s), thus generating relatively large single-channel currents which can be recorded with conventional patch-clamp electrodes, the current generated by ion pumps and transporters is much smaller. The turnover frequency in this case is limited by the rate constant of the major conformational transition to values of less than 10^2 ions/s. As a result, it is impossible to record 'single-pump currents' and, even to record macroscopic currents, it is not effective to use conventional patch pipettes with tip diameters of 1 – 5 μm. Pipettes with much larger tip openings (18 – 40 μm diameter) must be used to record electric currents generated by a large number of sodium pumps in excised inside-out patches from guinea pig cardiac myocytes.

Cardiac myocytes have been isolated from guinea pig ventricles according to the procedure of Collins and collaborators [3]. In brief, cells were obtained by retrograde perfusion of the heart through the aorta using a Langendorf device through which a constant flow rate of solution was maintained using a peristaltic pump. A Ca^{2+}- free Tyrode solution containing 1 mg/ml collagenase (Worthington) was used for cell isolation. The procedure continued with the fragmentation of the heart and cell dispersion using scissors, followed by filtration of the cells through a nylon sieve. Cells were then kept for no longer than three days in a K^+- rich, Ca^{2+}-free solution (storage solution, see below) at 4 °C. During this time the cells developed plasma membrane blebs (or vesicles) [3], which were then used to obtain inside-out excised patches.

Pipettes were obtained from borosilicate glass capillaries (1.6 mm inner diameter, 2.2 mm outer diameter, type N-51, Drummond Scientific Co., Broomall, PA), using a conventional two stage pipette puller (PP-83, Narishige, Tokyo, Japan). Tip openings varied between 18 and 24 μm. The tips were then heat polished and immersed in D, L-tocopherol-acetate immediately before use, in order to increase the probability of obtaining giga-seals [6].

The Tyrode solution for cell isolation contained (in mM): NaCl 130, KCl 4.5, $NaH_2.2H_2O$ 0.3, $MgCl_2$ 1, taurine 20, creatine 5, HEPES 10, glucose 11, $CaCl_2$ 0.036. The pH was 7.35 (NaOH).

The storage solution contained (in mM): KCl 134, EGTA 10, HEPES 10, glucose 10, $MgCl_2$ 2. The pH was 7.4 (KOH).

In order to study the anion effects at the intracellular side of the sodium pump, several bath solutions have been used. B-NaCl (or B-NaX) contained (in mM): NaCl 145 (or NaCl 45 + NaX 100, where X = ClO_4, NO_3, CH_3COO), HEPES 10, TEA-Cl 20, $MgCl_2$ 2. The pH was 7.4 (HCl).

Two different pipette solutions have been used. P-NaK contained (in mM): NaCl 145, KCl 5, $BaCl_2$ 2, $MgCl_2$ 2, $CdCl_2$ 0.5. The pH was 7.4 (NaOH). P-Na contained (in mM): same as P-NaK, without 5 mM KCl.

The cardiac myocytes were suspended in a 35 mm plastic Petri dish mounted on an inverted microscope (Zeiss Axiovert, 35 M, Carl Zeiss, Oberkochen, Germany). Giga-ohm seals were obtained on membrane blebs by applying small negative pressure (suction) in the pipette, and often just by abolishing the positive pressure. Upon patch excision, the pipette was taken to the temperature controlled perfusion chamber. The extremely small volume of the chamber (10-20 μl) allows a very fast exchange of the extracellular solution (80% of the volume in one second). This can be seen in Fig. 1 by observing the decrease in the pump current following the replacement of the activating ATP solution with a solution without ATP. The perfusion chamber consists in a small metal cube (dimensions 1x1x1 cm) covered with an insulating layer. A channel was created inside the cube to allow water flow for temperature control. Electric valves (General Valves, Fairfield, NJ) controlled the intracellular solution flow. Up to eight different solutions could be simultaneously temperature controlled before entering the perfusion chamber.

Pump generated electric currents through inside-out excised patches were measured at 0 mV, using a EPC-7 patch-clamp amplifier (List Instruments, Darmstadt, Germany). The currents were continuously recorded at low resolution (10 Hz) on a paper chart and at 50 Hz sampling rate on the hard-disk of a PC using the KAN 1 acquisition software (MFK, Niederhausen, Germany).

RESULTS

We have monitored the current generated by the Na^+,K^+-ATPase in the presence of 40 mM $NaClO_4$ compared to 40 mM NaCl. The sodium pump was

activated by 0.5 mM ATP, at first in 40 mM NaCl, then in 40 mM $NaClO_4$ and, finally, again in 40 mM NaCl (Fig. 1). It can be seen that there is a substantial decrease (ca. 30%) in the pump current in the presence of $NaClO_4$, which is entirely reversible.

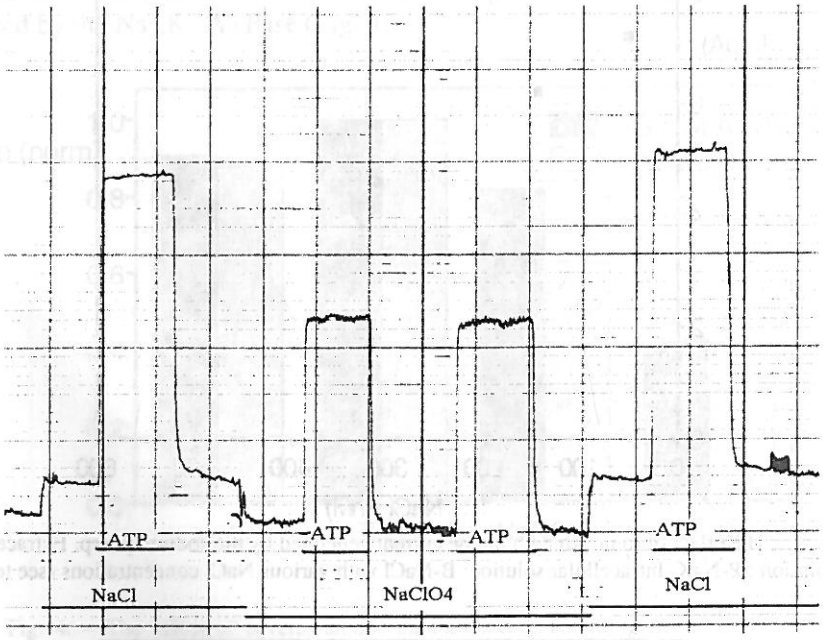

Fig. 1. – The effect of ClO_4^- on the pump current of the Na^+,K^+-ATPase recorded in excised inside-out patches from cardiac myocytes. Intracellular solution: B-NaCl, B-$NaClO_4$. Extracellular solution: P-NaK. Sodium pumps were activated with 0.5 mM ATP.

In order to monitor the effect of the ionic strength on the current generated by the Na^+,K^+-ATPase, the pumps were activated by 0.5 mM ATP at four different concentrations of NaCl: 50, 145, 300 and 525 mM (Fig. 2). A monotonous decline of the current was observed with increasing salt concentration. At 525 mM NaCl the current activated by 0.5 mM ATP was only 35% of the current at 50 mM NaCl.

The pump current was then recorded at several concentrations of the anion ClO_4^-: 0, 10, 40, 100 mM (Fig. 3). In order to account for the ionic strength effect the total salt concentration in the bath was maintained constant at 145 mM, using NaCl. We have observed a strong decrease in the pump current with increasing concentrations of ClO_4^-. In the presence of 100 mM $NaClO_4$ (+45 mM NaCl), the current is ca. 50% of the current recorded in 145 mM NaCl. It is clearly a specific effect of ClO_4^-, independent of the ionic strength.

Previous studies have reported an effect of anions on the kinetics of sodium transport by the Na^+,K^+-ATPase [7, 18]. In the presence of ClO_4^- the rate constant of the conformational transition is reduced and the affinity for nucleotides is decreased [18]. We have recorded sodium pump currents activated by 0.5 mM ATP

in 100 mM Cl^-, CH_3COO^-, NO_3^-, ClO_4^-. It is obvious that these four anions have a strong effect on the current generated by the Na^+,K^+-ATPase, and this effect can be used to arrange the anions in a Hofmeister type series: $ClO_4^- > NO_3^- > Cl^- > CH_3COO^-$ (Fig. 4).

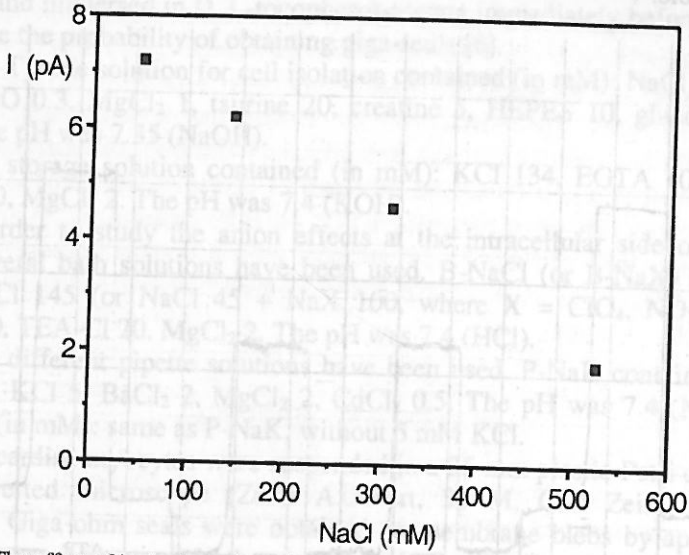

Fig. 2. – The effect of ionic strength on the current generated by the sodium pump. Extracellular solution : P-NaK. Intracellular solution: B-NaCl with various NaCl concentrations (see text).

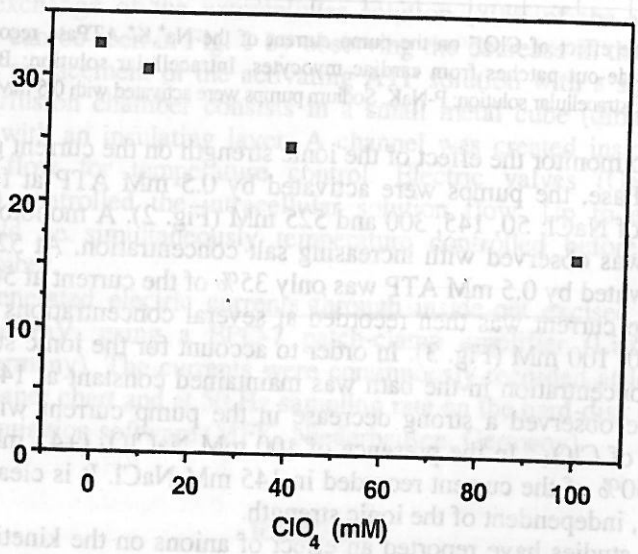

Fig. 3. – The effect of ClO_4^- on the current generated by the Na^+,K^+-ATPase. Extracellular solution: P-NaK. Intracellular solution B-$NaClO_4$.

It can be assumed that the reduction of the sodium pump current is due to a decrease in the affinity for ATP at the low affinity binding site, so that 0.5 mM ATP is not a saturating concentration in the presence of anions such as ClO_4^- or NO_3^-. In order to test this hypothesis we have recorded pump currents in the presence of 4 mM ATP and there was no significant change in the currents generated by the Na^+,K^+-ATPase (Fig. 4).

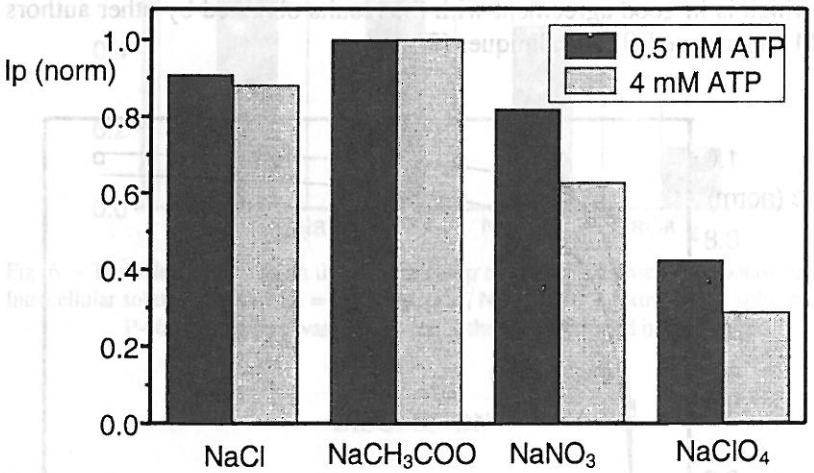

Fig. 4. – The effect of several anions on the electric current generated by the Na^+,K^+-ATPase in excised inside-out patches from cardiac myocytes. Extracellular solution P-NaK. The current was normalized to the value obtained in CH_3COO^-.

In the presence of 100 mM $NaClO_4$ (+ 45 mM NaCl), the sodium pump current is decreased by more than 50% compared to 145 mM NaCl (same ionic strength, Figs. 3 and 4). We have recorded the ATP dependence of the pump current in both ionic conditions and found that the affinity for ATP at the low affinity binding site in 100 mM $NaClO_4$ (+ 45 mM NaCl) is very similar to the affinity in 145 mM NaCl (Kd = 75 μM in $NaClO_4$ compared to Kd = 90 μM in NaCl) (data not shown). Therefore, the anion effect on the pump current is not due to a decrease in ATP affinity and must reflect an influence on the rate limiting step of the reaction cycle, probably the $E_2(K) \rightarrow E_1(K)$ transition.

When potassium ions are present at the extracellular side, the Na^+,K^+-ATPase is exchanging three sodium ions for two potassium ions for each molecule of ATP hydrolysed to ADP and inorganic phosphate. In these conditions, the ATP dependence of the pump current reflects the allosteric action of ATP at the low affinity binding site [16], whereby ATP accelerates the $E_2(K) \rightarrow E_1(K)$ transition. In order to monitor the catalytic action of ATP at the high affinity binding site (Kd = 1 μM) we have measured pump currents in the absence of potassium. In

these conditions, the Na^+,K^+-ATPase is carrying out sodium-sodium exchange [16] and the rate limiting step becomes the dephosphorylation reaction, which is ATP independent. Thus, the ATP dependence of the pump current in the absence of potassium will reflect the catalytic action of ATP (binding and hydrolysis). We have determined the ATP affinity at the catalytic site in 145 mM NaCl and in 100 mM $NaClO_4$ + 45 mM NaCl (Fig. 5). It can be seen that there is a pronounced decrease of the ATP affinity in ClO_4^- (Kd = 35.17 μM compared to 16.65 μM in NaCl), which is in good agreement with the results obtained by other authors using the BLM and stopped-flow techniques [7].

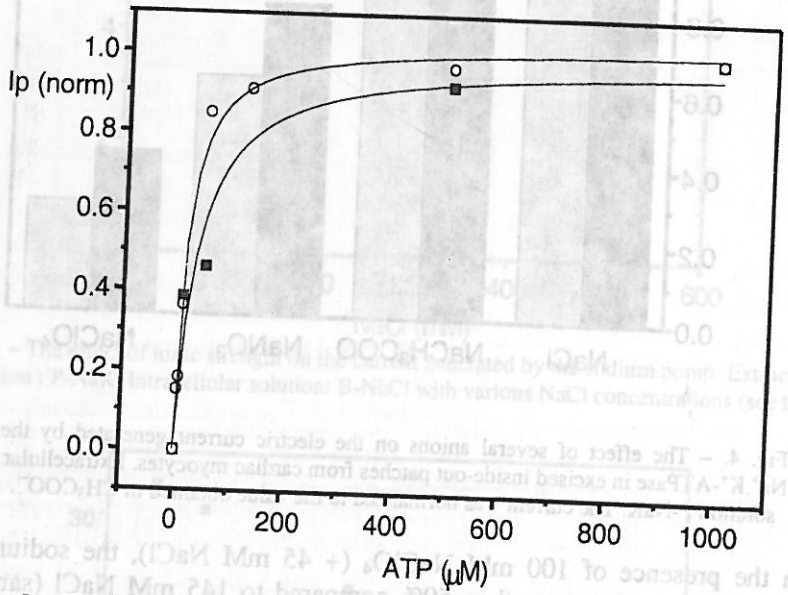

Fig. 5. – The ATP dependence of the sodium pump current in the absence of potassium. Intracellular solutions: B-NaCl (open symbols), B-$NaClO_4$ (filled symbols). Extracellular solution: P-Na. The current was normalized to the largest value in each ionic condition.

The sodium-sodium exchange current generated by the Na^+,K^+-ATPase in the absence of potassium has been recorded in the presence of 100 mM sodium salts of various anions (+ 45 mM NaCl): Cl^-, CH_3COO^-, NO_3^-, ClO_4^- (Fig. 6, recordings from two different patches). It can be seen that these anions have a pronounced effect on the pump current, and they can be arranged in a series in the decreasing order of the current: $Cl^- > CH_3COO^- > NO_3^- > ClO_4^-$. In comparison to the effect of the same anions on the pump current in the presence of potassium (Fig. 4), there is a change in the relative position of Cl^- and CH_3COO^- in the series. The current was activated by saturating concentrations of ATP (0.5 mM), which implies that we were monitoring the effect on anions on the rate constants of the reaction steps involved in sodium-sodium exchange.

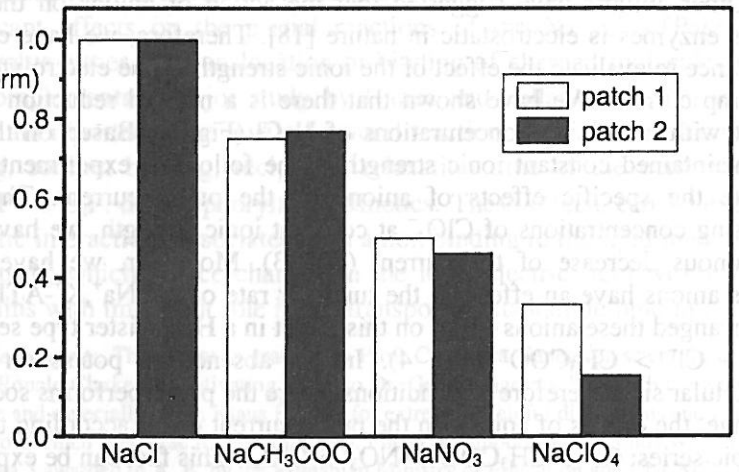

Fig. 6. – The effect of anions on the sodium pump current in the absence of potassium. Intracellular solution: B-NaX (X = Cl^-, CH_3COO^-, NO_3^-, ClO_4^-). Extracellular solution: P-Na. The current was normalized to the value recorded in Cl^-.

DISCUSSION

We have investigated the effect of anions, especially perchlorate (ClO_4^-), on the currents generated by the Na^+,K^+-ATPase in two different ionic conditions: with and without potassium at the extracellular side. We have used the giant patch-clamp technique, which allowed us to record macroscopic currents activated by ATP at the intracellular side of excised inside-out patches from guinea pig cardiac myocytes. These currents have been recorded in steady-state conditions, when the enzyme is performing ion transport activated by ATP hydrolysis and it alternates between two major conformations, E_1 and E_2. In E_1 the ion binding sites are oriented towards the cytoplasm, and in E_2 they are accessible from the extracellular side. We have activated the sodium pump by 0.5 mM ATP at the intracellular side and we have recorded the ion current generated in turnover conditions due to the stoichiometry of the transport: 3 sodium ions are extruded, and only 2 potassium ions are imported. This means that one single positive charge is continuously transported from the inside to the outside of the cell when the pump is active. We have used this pump current in order to evaluate the global effect of anions on the kinetics of ion transport by the enzyme.

In a first series of experiments, we have shown that the effect of anions is reversible. For this we have used ClO_4^-, which is the most potent anion used in this study (see Figs. 4 and 6). The pumps were activated by 0.5 mM ATP in the presence of NaCl, then in 40 mM $NaClO_4$ and then again in NaCl (Fig. 1). The current was the same during the first and third applications (in NaCl), and reduced by ca. 30% in $NaClO_4$.

Other authors have suggested that the action of anions on the kinetics of various enzymes is electrostatic in nature [18]. Therefore, we have considered of importance to evaluate the effect of the ionic strength of the electrolyte solution on the pump current. We have shown that there is a marked reduction in the pump current with increasing concentrations of NaCl (Fig. 2). Based on this result we have maintained constant ionic strength in the following experiments in order to separate the specific effects of anions on the pump current. Thus, applying increasing concentrations of ClO_4^- at constant ionic strength, we have recorded a monotonous decrease of the current (Fig. 3). Moreover, we have shown that various anions have an effect on the turnover rate of the Na^+,K^+-ATPase, and we have arranged these anions based on this effect in a Hofmeister type series: $ClO_4^- > NO_3^- > Cl^- > CH_3COO^-$ (Fig. 4). In the absence of potassium ions at the extracellular side, therefore in conditions where the pump performs sodium-sodium exchange, the effects of anions on the pump current occur according to a modified lyotropic series: $Cl^- > CH_3COO^- > NO_3^- > ClO_4^-$. This fact can be explained if one considers that the partial reactions which are rate limiting the pump turnover, and thus dictate the amplitude of the current, are different in the two conditions. When both physiological cations are present, the rate limiting step is the backward conformational transition, $E_2(K) \rightarrow E_1(K)$. In the absence of potassium, dephosphorylation is the slowest, with a rate constant of 5 s^{-1} [11]. It is very realistic to assume that the two reactions are differently affected by lyotropic anions, considering their very different nature. One involves a major conformational change of the protein, associated with important rearrangement of protein domains, while the other is related to the hydrolysis of a phosphate group and comprises only small shifts of the phosphorylation site.

We have also shown that, besides their effect on the turnover rate, ClO_4^- anions are decreasing the ATP affinity at the catalytic binding site (Fig. 5), without modifying the dissociation constant for ATP at the allosteric site (also known as the low affinity binding site). Using equilibrium titrations, Nørby and Esmann [18] have reported that the enzyme affinity for ADP is substantially decreased in the presence of 100 mM $NaClO_4$. Although our results seem to indicate that the two types of interactions between ATP and the sodium pump occur at two different sites, one cannot exclude the possibility that ATP binds at the same site, while the affinity changes with the conformational transition.

This result can be explained based on electrostatic considerations. It has already been shown that ClO_4^- anions bind to the surface of the phospholipid membrane and induce a decrease of the intrinsic positive electric dipole of the membrane [2] and a negative surface potential [17]. Moreover, these anions can interact with the surface of various proteins which leads to a change of the local electric field inside the protein matrix [1]. It can be suggested that the changes in ATP affinity are due to alterations of the local electric field induced by ClO_4^- binding to hydrophobic protein domains at the intracellular side, since the phosphorylation site of the Na^+,K^+-ATPase is located in one of the cytoplasmic loops of the protein [10].

As a conclusion, we have shown that lyotropic anions and ClO_4^- in particular, have significant effects on the partial reactions of the Na^+,K^+-ATPase. These reactions involve either ion translocation or binding of charged molecules (ATP). Our work complements a major study by Ganea and collaborators [7] in which anion effects are reported at the level of several reaction steps of the sodium pump: ATP binding, sodium binding, reciprocal relaxation time of the conformational transition $E_1P \rightarrow E_2P$, dephosphorylation kinetics. These effects can be explained by electrostatic interactions associated with anion binding to the lipid membrane or the protein itself, which induce changes in the local electric field within various protein domains with important role in ion transport or nucleotide binding.

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