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Inhibitory Effect of Pb^{2+} on the Transport Cycle of the Na^+,K^+-ATPase

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The effect of Pb^{2+} on the transport cycle of the Na^+,K^+-ATPase was characterized in detail at a molecular level by combining electrical and biochemical measurements. Electrical measurements were performed by adsorbing purified membrane fragments containing Na^+,K^+-ATPase on a solid-supported membrane. Upon adsorption, the Na^+,K^+-ATPase was activated by carrying out concentration jumps of different activating substrates, for example, Na^+ and ATP. Charge movements following Na^+,K^+-ATPase activation were measured in the presence of various Pb^{2+} concentrations to investigate the effect of Pb^{2+} on different ion translocating steps of the pump cycle. These charge measurements were then compared to biochemical measurements of ATPase activity in the presence of increasing Pb^{2+} concentration. Our results indicate that Pb^{2+} inhibits cycling of the enzyme, but it does not affect cytoplasmic Na^+ binding and release of Na^+ ions at the extracellular side at concentrations below 10 μM. To explain the inhibitory effect of Pb^{2+} on the Na^+,K^+-ATPase, we propose that Pb^{2+} may interfere with the hydrolytic cleavage of the phosphorylated intermediate E_2P, which occurs in the K^+-related branch of the pump cycle.

Introduction

Lead (Pb) is an ubiquitous heavy metal, used by humans since prehistoric times (*1*). Anthropogenic lead pollution occurs through a variety of activities, including mining, metal processing, battery manufacturing and disposal, burning of leaded fuels, or release from wearing of tires, just to mention some (*2*). Noxious effects of this contaminant have been described for all ecosystem components, including humans, and environmental lead exposure has been recognized as a public health problem of global dimensions (*3*). At the macroscopic level, the exposure to high levels of this metal may cause severe damage to, for example, the nervous system, kidneys, and blood, which may eventually culminate with death (*4*). Among the effects occurring at the cellular and molecular level, it is known that lead has the potential to inducing oxidative stress and cause direct damage to the structure and function of biological membranes (for example, see ref *5*). It is known that several heavy metals, including lead, affect the function of biological membranes inhibiting the activity of P type ATPases. For instance, toxic heavy metal ions (Cd^{2+}, Hg^{2+}, Pb^{2+}, Cu^{2+}, and Zn^{2+}) have been shown to affect the activity of Ca^{2+}-ATPase in different types of membranes [erythrocyte plasma membrane, sarcoplasmic reticulum, and liver microsomes (*6–9*)]. Inhibitory effects have also been reported on the activity of Mg^{2+}-ATPase and Na^+,K^+-ATPase (*10–14*). Despite such evidence, however, the molecular mechanism by which heavy metals cause ATPase inhibition is still largely unknown.

The Na^+,K^+-ATPase is an enzyme present in the plasma membrane of almost all animal cells. This enzyme utilizes the energy provided by the hydrolysis of ATP to actively transport

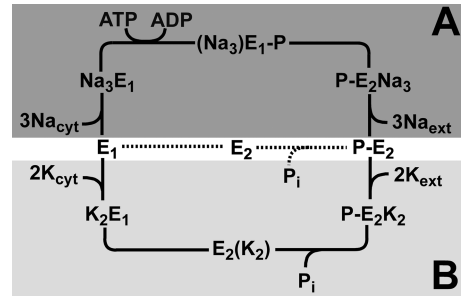

Figure 1. Reaction cycle of the Na^+,K^+-ATPase according to the Albers−Post model. Panels A and B indicate the Na^+-related branch and K^+-related branch of the pump cycle, respectively.

Na^+ out of and K^+ into the cell against their electrochemical potential gradients. The Na^+ and K^+ electrochemical gradients are required for basic cellular functions such as maintenance of membrane potential, regulation of cell volume, secondary transport of other solutes, and signal transduction. In the brain, about one-half of the ATP provided by oxidative metabolism is used to power the Na^+,K^+-ATPase (for a review, see, for example, refs *15–18*). The functioning of this ion pump is generally described by the Albers−Post model (*19, 20*), which involves the existence of two main conformations, E_1 and E_2, characterized by having ion binding sites exposed to the cytoplasm and the extracellular medium, respectively (Figure 1). The E_1 conformation binds Na^+ ions and, in the presence of ATP, is converted into the phosphorylated intermediate E_2P. In the E_2P state, Na^+ is released to the extracellular medium, and K^+ ions are bound. Binding of K^+ results in accelerated dephosphorylation. A subsequent conformational change to the E_1 state leads to translocation of K^+ ions to the cytoplasmic side of the membrane and their release there (Figure 1).

This study aims at investigating the effect of Pb^{2+} on the transport cycle of the Na^+,K^+-ATPase using an electrical method, which makes use of a solid-supported membrane

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(SSM). The SSM represents a model system for a bilayer lipid membrane with the advantage of being mechanically so stable that solutions may be rapidly exchanged at the surface. Membrane fragments or vesicles containing the transport protein are adsorbed on a SSM and are activated using a fast solution exchange technique (*21, 22*). Thus, the rapid change from a solution containing no substrate for the protein to one that contains a substrate, herein referred to as substrate concentration jump, allows protein activation by any substrate, for example, ionic species or organic molecule. The electrical currents generated by the transport protein following its chemical activation can provide useful information about protein function and its modulation by drugs, peptides, and small soluble proteins. The SSM technique has been successfully used in the study of electrogenic transport by several membrane transporters (for a review, see refs *21* and *22*). More recently, this method has been employed to identify and analyze the effect of various inhibitors on the Na^+,K^+-ATPase (*23*) and Ca-ATPase (*24–26*).

The experiments reported in this work were performed to investigate the effect of Pb^{2+} on different partial reactions of the transport cycle of the Na^+,K^+-ATPase. In particular, electrical currents generated by the Na^+,K^+-ATPase were measured by adsorbing purified membrane fragments containing the ion pump on the SSM. The Na^+,K^+-ATPase was then activated by performing concentration jumps of different activating substrates, for example, Na^+ and ATP, in the absence and presence of Pb^{2+}. Electrical measurements were compared to biochemical measurements of ATPase activity in the presence of increasing Pb^{2+} concentration. Our results show that Pb^{2+} interferes with the ion transport cycle of the Na^+,K^+-ATPase. A possible mechanism of enzyme inhibition by Pb^{2+} is proposed.

Materials and Methods

Chemicals. Magnesium, sodium and potassium chloride, lead(II)nitrate, and imidazole were obtained from Merck (Whitehouse Station, NJ) at analytical grade. ATP (~97%) and ouabain were purchased from Fluka (Buchs, Switzerland). Phosphoenolpyruvate (PEP), pyruvate kinase/lactate dehydrogenase suspension (PK/LDH), b-nicotinamide adenine dinucleotide (reduced disodium salt hydrate, NADH), and choline chloride were purchased from Sigma-Aldrich (St. Louis, MO) at the highest quality available. Octadecanethiol (98%) from Aldrich was used without further purification. Diphytanoylphosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL) and then solubilized (7.5 mg/mL) in *n*-decane (Merck).

Protein Preparation and Biochemical Measurements. Membrane fragments containing Na^+,K^+-ATPase were obtained by extraction and purification from the outer medulla of New Zealand White Rabbit's kidney, following method C of Jørgensen (*27*).

The protein concentration was determined by the Lowry method using bovine serum albumin as a standard (*28*). The total protein content of membrane fragments was usually comprised between 1.5 and 2.5 mg/mL.

The ATP hydrolytic activity of the ion pump was measured by the PK/LDH method (*29*). The PK/LDH assay is a colorimetric method by which the Na^+,K^+-ATPase activity is determined following P_i production. The reaction mixture contained 100 mM NaCl and 10 mM KCl (or 100 mM NaCl only), 5 mM $MgCl_2$, 25 mM imidazole (pH 7.2), 2 mM PEP, 100 μM Na_2ATP, 290 μg of Na^+,K^+-ATPase/mL (or ≈1 mg/mL in the presence of Na^+ only), 740U/mL PK, and 931U/mL LDH. The reaction was initiated by the addition of 100 μM NADH. The specific ATPase activity at 37 °C was ≈1000 μmol Pi/(h mg protein) in the presence of Na^+ and K^+, and ≈350 μmol Pi/(h mg protein) in the presence of Na^+ only. The ion pump activity was fully blocked by 1 μM ouabain. In Pb^{2+} titrations, $Pb(NO_3)_2$ was added to the solution at the required concentration.

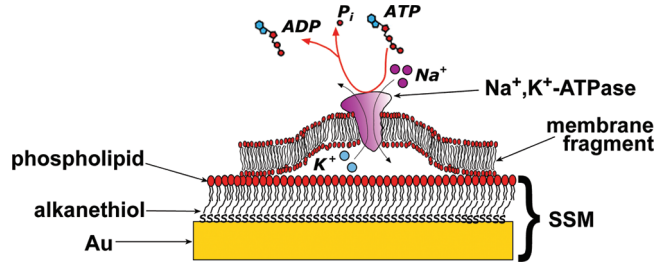

Figure 2. Cartoon of a membrane fragment containing Na^+,K^+-ATPase adsorbed on a SSM and subjected to ATP activation (not drawn to scale).

Enzyme Phosphorylation. Enzyme phosphorylation with [γ-^{32}P]ATP was estimated following equilibration (60 s at 0 °C) of Na^+,K^+-ATPase containing membrane fragments (25 μg/mL) with 50 μM [γ-^{32}P]ATP, in a medium containing 50 mM MOPS (pH 7.0), 150 mM NaCl, and 3 mM $MgCl_2$, in the presence or in the absence of different concentrations of free Pb^{2+}. The reaction was quenched with 5 mL of 5% trichloroacetic acid, and the quenched reaction mixture was filtered through 0.45 μm Millipore filters. The protein collected on the filters was washed three times with 0.125 M perchloric acid and once with water. Filters were finally dissolved with dimethylformamide and processed for scintillation counting. Control experiments were performed by quenching samples with trichloroacetic acid before the addition of the radioactive substrate.

SSM Measurements. The SSM is a hybrid bilayer supported by a gold electrode, and it consists of an octadecanethiol monolayer covalently linked to a gold surface via the sulfur atom, with a diphytanoylphosphatidylcholine monolayer on top of it (*30*).

Membrane fragments containing Na^+,K^+-ATPase, following a brief sonication in the absence of detergent, were allowed to adsorb on the SSM electrode during 90 min of waiting time at 4 °C. After adsorption, the ion pump was activated by a rapid concentration jump of a suitable substrate, for example, Na^+ or ATP (Figure 2).

If at least one electrogenic step is involved in the relaxation process following protein activation, a current transient can be recorded by the SSM method (*21, 22*). In particular, the electrical response of the ion pump can be monitored under potentiostatic conditions. In this case, movement of a net charge across the activated protein is compensated by a flow of electrons along the external circuit to keep the applied voltage ΔV constant across the whole metal/solution interphase. The resulting current transient is recorded as a function of time. Usually, experiments are carried out under short circuit conditions, that is, at zero applied voltage relative to the reference electrode. It should be pointed out that the SSM technique detects presteady state current transients within the first transport cycle and is not sensitive to stationary currents following the first cycle.

Useful information is gained from current transients. In fact, numerical integration of each transient is related to a net charge movement, which depends upon the particular electrogenic event (i.e., after Na^+ or ATP jumps). In addition, kinetic information can be obtained by fitting a sum of exponentially decaying terms to the current versus time curves. Further details can be found in ref *22*. In the concentration jump experiments, two buffered solutions were used, that is, the "nonactivating" and the "activating" solution. The composition of the two solutions was as follows.

In Na^+ concentration jump experiments, the nonactivating solution contained 150 mM choline chloride, 25 mM imidazole (pH 7), and 3 mM $MgCl_2$; the activating solution contained 20 mM choline chloride, 25 mM imidazole (pH 7), 3 mM $MgCl_2$, and 130 mM NaCl. In ATP concentration jump experiments, the nonactivating solution contained 130 mM NaCl (or 80 mM NaCl and 50 mM KCl), 3 mM $MgCl_2$, and 25 mM imidazole (pH 7); the activating solution contained, in addition, 100 μM ATP.

In Pb^{2+} titrations, $Pb(NO_3)_2$ was added at the same concentration to both the nonactivating and the activating solutions. Free Pb^{2+} concentration was calculated with the computer program Win-

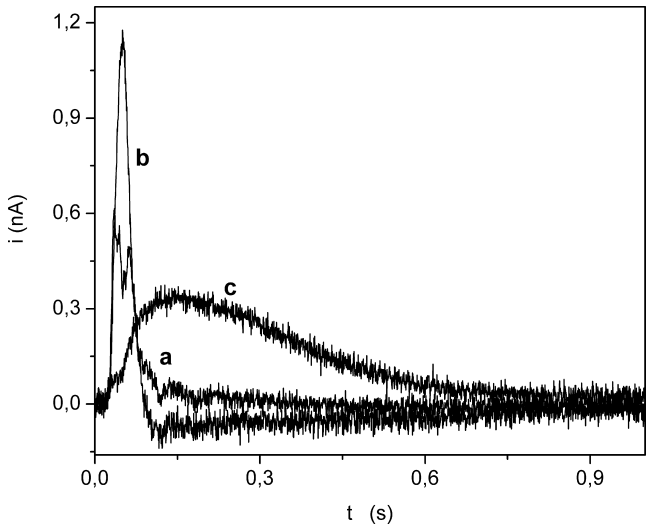

Figure 3. Current transients induced by a 100 mM Na^+ concentration jump in the absence of ATP (curve a) and a 100 μM ATP concentration jump in the presence of 130 mM Na^+ (curve b) and in the presence of 80 mM Na^+ and 50 mM K^+ (curve c).

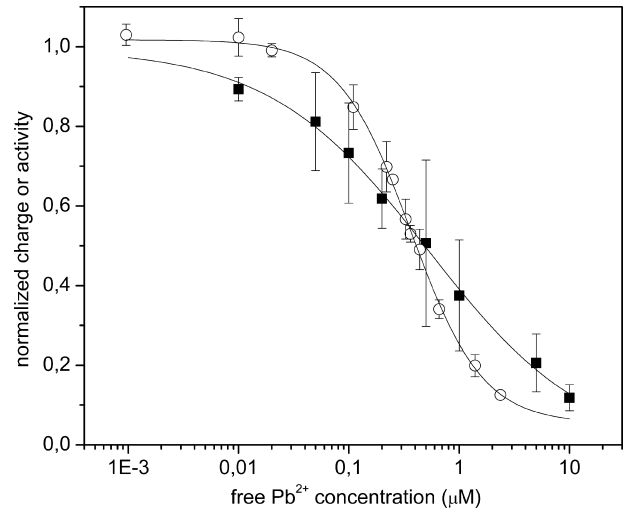

Figure 4. ATPase activity in the Na^+,K^+-mode (empty circles) and charge after a 100 μM ATP jump in the presence of 80 mM Na^+ and 50 mM K^+ (filled squares) as a function of Pb^{2+} concentration. Both the ATPase activity and the charge were normalized with respect to the corresponding maximum value measured in the absence of Pb^{2+}. The solid lines represent fits of the Hill function to the experimental data: $K_{0.5} = 0.35 \pm 0.01$ μM and $n = 1.39 \pm 0.07$ (ATPase activity) and $K_{0.5} = 0.5 \pm 0.1$ μM and $n = 0.60 \pm 0.05$ (charge).

MAXC (*31*). The concentration jump experiments were carried out at room temperature, that is, 22−23 °C.

All concentration jump experiments were carried out by using the $SURFE^2R^{One}$ device (Ion-Gate Biosciences, Frankfurt, Germany). The SSM sensor, the experimental setup, and the solution exchange technique are described in ref *32*.

To verify the reproducibility of the current transients generated within the same set of measurements on the same SSM, each single measurement of the set was repeated 4−5 times and then averaged to improve the signal-to-noise ratio. Standard deviations were usually found to be no greater than ±5%. Moreover, each set of measurements was usually reproduced using three different gold sensors; therefore, the data points reported in the figures represent the mean of three independent normalized values. Standard deviations are given by individual error bars in each figure.

Results

The SSM method has been employed to investigate charge translocating steps in the reaction cycle of the Na^+,K^+-ATPase (*30, 33, 34*). In the present research, the SSM technique was used to study the effect of Pb^{2+} on the ion transport mechanism of the sodium pump. In our experiments, electrical currents generated by the Na^+,K^+-ATPase were measured by adsorbing membrane fragments containing the ion pump on the SSM. The Na^+,K^+-ATPase was then activated by performing concentration jumps of different activating substrates, for example, Na^+ and ATP. Figure 3 shows the current transients obtained following activation of the enzyme by a Na^+ concentration jump in the absence of ATP and ATP concentration jumps in the presence of Na^+ only and in the presence of Na^+ and K^+ ions.

In particular, the current transient induced by a Na^+ jump in the absence of ATP (Figure 3, curve a) can be attributed to electrogenic binding of Na^+ ions to the cytoplasmic side of the Na^+,K^+-ATPase, as previously demonstrated (*33*). As shown in Figure 3, the current transients obtained after an ATP jump in the presence of Na^+ only (Figure 3, curve b) and in the presence of both Na^+ and K^+ (Figure 3, curve c) have different shapes, which were investigated thoroughly in the past (*35, 36*). It was shown that the current transient generated by an ATP jump in the presence of Na^+ ions only corresponds to the translocation and release of bound Na^+ to the extracellular side upon utilization of ATP (*30, 34–36*). In the absence of K^+, the reactions following the transport of Na^+ cannot be observed by the SSM method, because the enzyme enters a long-lived phosphorylated state, that is, the E_2P state (see Figure 1), from which return to the initial E_1 state is rather slow (*37–41*). On the other hand, when K^+ is present in the solution in addition to Na^+, the peak amplitude of the current transient is reduced and the decay of the signal is prolonged (Figure 3, curve c). The prolonged decay of the current signal was assigned to a quasi-stationary pump current, which is maintained by the Na^+,K^+-ATPase in the presence of K^+ ions (*34–36*). In particular, the current transient generated by the Na^+,K^+-ATPase upon addition of ATP in the presence of both Na^+ and K^+ can be attributed to Na^+/K^+ exchange within the first transport cycle (*34*). Following the first cycle, when steady state conditions are attained, the experimental current measured along the external circuit vanishes, and no further signal is detected.

Charge movements following Na^+,K^+-ATPase activation were measured in the presence of various Pb^{2+} concentrations to investigate the effect of Pb^{2+} on different ion translocating steps of the pump cycle. These charge measurements were then compared to biochemical measurements of ATPase hydrolytic activity in the presence of increasing Pb^{2+} concentration.

Figures 4 and 5 were obtained by plotting the moved charge, obtained by numerical integration of the current transient, and the ATPase activity as a function of Pb^{2+} concentration. The experimental data were normalized with respect to the corresponding maximum value measured in the absence of Pb^{2+} and can be satisfactorily fitted by the Hill equation, which is widely used for biochemical characterization:

$$Q_{norm} = (Q_{norm})_0 + (Q_{norm})_{max}\left[\frac{c^n}{c^n + K_{0.5}^n}\right] \qquad (1)$$

$(Q_{norm})_0$ is the initial normalized charge measured at 0 Pb^{2+}, $(Q_{norm})_{max}$ is the maximum variation of normalized charge at highest Pb^{2+} concentration, c represents the Pb^{2+} concentration, and n is the Hill coefficient, related to the cooperativity of the substrate binding process.

In ATP concentration jumps in the presence of Na^+ and K^+ (Figure 4, filled squares), an increasing concentration of Pb^{2+}

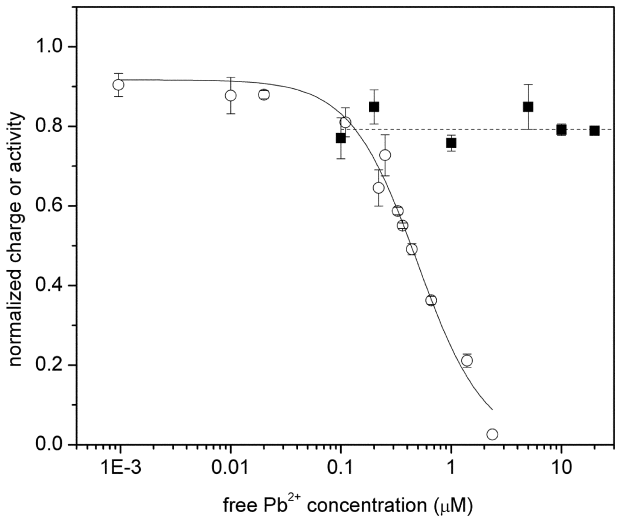

Figure 5. ATPase activity in the Na^+-only mode (empty circles) and charge after a 100 μM ATP jump in the presence of 130 mM Na^+ and in the absence of K^+ (filled squares) as a function of Pb^{2+} concentration. Both the ATPase activity and the charge were normalized with respect to the corresponding maximum value obtained in the absence of Pb^{2+}. The solid line represents the best fitting by the Hill function: $K_{0.5} = 0.47 \pm 0.04$ μM and $n = 1.4 \pm 0.2$.

causes a decrease of the moved charge by the Na^+,K^+-ATPase. A similar behavior was observed in biochemical measurements of ATPase activity under the same experimental conditions (Figure 4, empty circles). The decrease of the moved charge and protein activity occurs in the 0.01−10 μM Pb^{2+} concentration range. Thus, similar values of $K_{0.5}$ (concentration of Pb^{2+} producing half maximal inhibition), obtained with the Hill function (eq 1), were determined. From charge measurements, the $K_{0.5}$ was 0.5 ± 0.1 μM ($n = 0.60 \pm 0.05$), whereas the $K_{0.5}$ obtained from biochemical measurements was found to be 0.35 ± 0.01 μM ($n = 1.39 \pm 0.07$).

Figure 5 (filled squares) shows the dependence of the normalized charge after ATP jumps in the presence of Na^+ only on Pb^{2+} concentration. In the presence of increasing Pb^{2+} concentration, the moved charge remains constant at ∼80% of the maximum value measured in the absence of Pb^{2+}. On the contrary, the enzyme activity in the Na^+ only mode (Figure 5, empty circles) decreases by increasing the concentration of Pb^{2+}, as also observed for the ATPase activity measured in the presence of Na^+ and K^+ ions (Figure 4, empty circles). By fitting the Hill function (eq 1) to the biochemical data of Figure 5, a $K_{0.5}$ of 0.47 ± 0.04 μM and a Hill coefficient $n = 1.4 \pm 0.2$ were found. Thus, biochemical measurements of ATPase activity in the Na^+-only mode and in the Na^+,K^+-mode yield similar values of $K_{0.5}$ and of n for Pb^{2+}.

The effect of Pb^{2+} on cytoplasmic Na^+ binding to the Na^+,K^+-ATPase was also examined. In Na^+ concentration jumps in the absence of ATP (Figure 6), we observed that the moved charge was practically unaffected by Pb^{2+} in the concentration range up to 10 μM and then decreased with an increase of Pb^{2+} concentration. In this case, a higher value of $K_{0.5}$ was found, that is, 30 ± 3 μM, while the Hill coefficient, n, indicate a noncooperative binding of Pb^{2+} ($n = 1.0 \pm 0.1$).

To examine whether Pb^{2+} can affect the phosphorylation step of the Na^+,K^+-ATPase, we determined the amount of the phosphorylated intermediate (E_2P) obtained by [γ-^{32}P]ATP in the absence of K^+ ions and in the presence of various concentrations of free Pb^{2+} (Figure 7). Our results indicate that E_2P formation is not affected by Pb^{2+} in the concentration range 0−1 μM, and it is reduced to ∼80% of the initial value in the

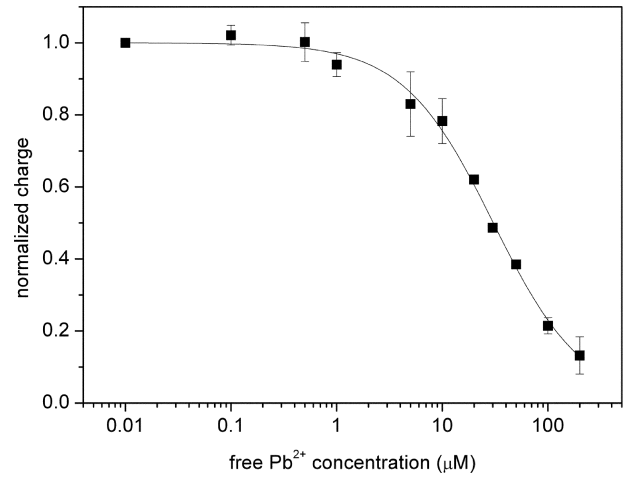

Figure 6. Charge after a 130 mM Na^+ jump in the absence of ATP as a function of Pb^{2+} concentration. The charge was normalized with respect to the maximum value measured in the absence of Pb^{2+}. The solid line is obtained by fitting the experimental data with the Hill function: $K_{0.5} = 30 \pm 3$ μM and $n = 1.0 \pm 0.1$.

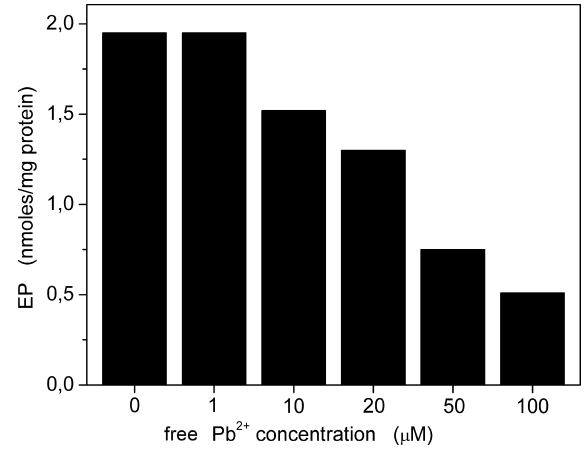

Figure 7. Phosphoenzyme intermediate obtained by [γ-^{32}P]ATP in the absence of K^+ ions and in the presence of increasing Pb^{2+} concentration.

presence of 10 μM free Pb^{2+}. Finally, E_2P formation is strongly inhibited by 100 μM free Pb^{2+}.

Discussion

The effect of Pb^{2+} on the ion transport mechanism of the Na^+,K^+-ATPase was characterized by combining biochemical measurements with detection of charge movements. Biochemical measurements of ATPase activity were performed in the Na^+,K^+-mode and in the Na^+-only mode. It should be mentioned that the sodium pump is able to complete many enzymatic cycles in the presence of Na^+ and K^+, but it is still able to complete the enzymatic cycle in the presence of Na^+ only, although with a slower turnover rate. In the latter case (i.e., absence of K^+), the rate-limiting step of the pump reaction cycle is the dephosphorylation of the intermediate P-E_2 (*37–41*). In particular, biochemical measurements of enzyme activity in the presence of increasing Pb^{2+} concentration show that Pb^{2+} has an inhibitory effect on the ATPase activity both in the Na^+-only mode and in the Na^+,K^+-mode, yielding similar values of $K_{0.5}$ and of n for Pb^{2+}.

The effect of Pb^{2+} on the Na^+,K^+-ATPase was previously investigated using biochemical methods (*11–14, 42*). It was found that Pb^{2+} inhibits the sodium pump in a wide range of

preparations, that is, microsomal preparation from beef cerebellar cortex (*11*), human erythrocyte membranes (*12*), rat brain synaptic plasma membranes (*13, 14*), and *Electrophorus* electroplax microsomes (*42*). In particular, the $K_{0.5}$ for Pb^{2+} determined in our measurements of ATPase activity agrees satisfactorily with values reported in the literature, for example, 2 (*14*) and 0.5 μM (*42*). Similar values of $K_{0.5}$ for divalent heavy metals were found in previous investigations on the Na^+,K^+-ATPase: for example, Cd^{2+} [1 (*13*) and 0.6 μM (*14*)] and Hg^{2+} [0.7 μM (*13*)].

An inhibitory effect of Pb^{2+} was also observed on charge movements generated by the Na^+,K^+-ATPase following ATP activation in presence of Na^+ and K^+ ions, that is, under turnover conditions (Figure 4, filled squares). In fact, if Na^+ and K^+ ions are present, the Na^+,K^+-ATPase may go through the normal transport cycle corresponding to the Na^+/K^+ exchange mode (Figure 1). As mentioned above, the current transient obtained after an ATP jump in the presence of both Na^+ and K^+ (Figure 3, curve c) can be attributed to Na^+/K^+ exchange within the first transport cycle. Therefore, our charge measurements indicate an inhibitory effect of Pb^{2+} on cycling of the enzyme and provide a $K_{0.5}$ for Pb^{2+} of 0.5 μM, which is in good agreement with the values determined from ATPase activity assays in the Na^+,K^+ mode and in the Na^+-only mode. On the other hand, the Hill coefficient obtained from charge measurements in the presence of both Na^+ and K^+ ($n = 0.60 \pm 0.05$) is lower that that determined from ATPase activity assay in the same conditions ($n = 1.39 \pm 0.07$): This difference may be related to a certain degree of irreproducibility of charge measurements performed in this case, as evident from the error bars reported in Figure 4 (filled squares).

Performing ATP concentration jumps in the presence of Na^+ and in the absence of K^+ (Figure 3, curve b) ensures that only the Na^+-related branch of the pump cycle (Figure 1A) will contribute to the detected current transients in the time course of the SSM experiments (*23, 30, 34*). In particular, the charge measured can be attributed to translocation and release of bound Na^+ to the extracellular side upon utilization of ATP (*23, 30, 34*). Charge measurements performed in the presence of different Pb^{2+} concentrations (Figure 5, filled squares) demonstrate that Pb^{2+} does not interfere significantly with Na^+ release at the extracellular side, that is, the $E_2PNa_3 \rightarrow E_2P$ step of the pump cycle (Figure 1).

Finally, performing Na^+ concentration jumps in the absence of ATP (Figure 3, curve a) allows us to investigate the $E_1 \rightarrow E_1Na_3$ step of the Na^+,K^+-ATPase cycle (Figure 1), corresponding to Na^+ binding to the cytoplasmic side of the Na^+,K^+-ATPase (*33*). Electrogenicity of cytoplasmic Na^+ binding was previously demonstrated by rapidly exchanging a choline chloride containing solution for a NaCl-containing one on a SSM in the absence of ATP (*33*). Thus, our charge measurements (Figure 6) clearly indicate that Pb^{2+} does not interfere significantly with Na^+ binding at the cytoplasmic side in the concentration range up to 10 μM, over which we observed a strong inhibitory effect on enzyme cycling (see above).

Our results indicate that Pb^{2+} inhibits cycling of the enzyme, but it does not affect cytoplasmic Na^+ binding and release of Na^+ ions at the extracellular side at concentrations below 10 μM. Moreover, it is shown in Figure 7 that Pb^{2+} does not affect the phosphorylation step within the concentration range that was found to be strongly inhibitory of steady-state activity, that is, in the 0−1 μM range (Figure 5). On the other hand, E_2P formation is reduced to ~80% of the initial value at 10 μM Pb^{2+}, a lead concentration that produces total loss of enzyme hydrolytic activity (Figure 5).

All of the experimental evidence may be explained by considering an inhibitory effect of Pb^{2+} on enzyme dephosphorylation. Previous studies reported that Pb^{2+} can inhibit the dephosphorylation reaction of the Na^+,K^+-ATPase in rat brain synaptic plasma membranes (*14*) and in *Electrophorus* electroplax microsomes (*42*). It was suggested (*42*) that Pb^{2+} may act at a single independent binding site to produce or stabilize an enzyme conformation that can be phosphorylated but that cannot catalyze hydrolysis of enzyme phosphate. In conclusion, taken together, our results lead us to propose that the inhibitory effect of Pb^{2+} on the Na^+,K^+-ATPase might be due to an interference with hydrolytic cleavage of the phosphorylated intermediate E_2P, which occurs in the K^+-related branch of the pump cycle (Figure 1B).

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