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Biochimica et Biophysica Acta 1190 (1994) 402–408

BBA Biochimica et Biophysica Acta

# Potent and reversible interaction of silver with pure Na,K-ATPase and Na,K-ATPase-liposomes

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(Received 11 August 1993)

**Abstract**

The Na,K-ATPase (EC 3.6.1.37) is the receptor for cardioactive steroids, the only specific inhibitors known at the present time for this unique membrane bound transport system. We report here that silver is the most rapid and potent inhibitor of isolated Na,K-ATPase ever described. Inhibition of Na,K-ATPase activity by silver is immediate and strikingly distinct from other inhibitors: addition of 1 mM of cysteine or DMPS reactivates the silver blocked-enzyme immediately. The results reveal that silver interacts with Na,K-ATPase and inhibits differently by an on-off mechanism involving most likely a few critical sulfhydryl groups. Inhibition of Na-K transport by silver has been demonstrated also in an artificial membrane, e.g., in liposomes reconstituted with pure Na,K-ATPase performing active transport. Silver inhibits the active $^{86}Rb$ transport mediated by the pure Na,K-ATPase molecule. The Na,K-ATPase contained in the liposomes was labeled specifically with $^{110m}Ag$ and appeared to bind two silver ions. Taken together, the results show that the mechanism of silver interaction with Na,K-ATPase might be different from other metals, for instance, mercury. The unique action mechanism of silver suggests a fundamental role of a few critical sulfhydryl groups for Na,K-transport.

*Key words:* ATPase, $Na^+/K^+$-; Silver; Liposome; Inhibition, potent; Reversible interaction

## 1. Introduction

The cell surface Na,K-ATPase, an enzymatic expression of the sodium and potassium transport system, is a most versatile and fundamental membrane receptor composed of a catalytic $\alpha$-subunit ($\sim$ 110 kDa) and a $\beta$-subunit glycoprotein ($\sim$ 50–60 kDa) [1–3]. This membrane embedded enzyme performs a vital function as an electrical battery and creates an inside-out negative membrane potential [4]. The electrogenic effect results from the molecular mechanisms of the pump which extrudes more intracellular Na ions as compared to the number of absorbed K ions. The membrane potential and the transmembrane Na-gradient resulting from the Na,K-ATPase activity furnish the primary driving force for other membrane associated transport systems or channels (Na, K and Ca channels, Na/Ca and Na/H antiporters and Na-dependent transporters for phosphate, glucose and amino acids). All these different linked functions confer on the Na,K-ATPase a role of a primordial communication system between the extra and the intracellular medium [5]. In view of the functional importance of Na,K-ATPase, the regulation of this system by exo- and endogenous compounds is crucial for understanding the control of electrolyte balance under various physiological or pathological conditions.

Heavy metals such as copper and zinc perform vital structural and functional roles in cell metabolism and macromolecule interactions [6] and are important components in several gene regulatory proteins [7,8]. In excess, however, these as well as non-essential metals, e.g., cadmium, mercury and lead, can be detrimental to cellular processes. The adverse effects of IB and IIB metals are due to their remarkable ability to interact with sulfhydryl groups of proteins [9,10].

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*Abbreviations:* Na,K-ATPase, sodium-potassium-activated adenosine triphosphatase; DMPS, 2,3-dimercapto-1-propanesulfonic acid.


*SSDI* 0005-2736(93)E0371-G

Metals might affect primarily membrane transport system while crossing the plasma membrane. Na,K-ATPase is a principal member of the ATPase family [11] and contains numerous sulfhydryl groups which could be the target for metal interaction. There are few reports available on effect of metals on Na,K-ATPase activity. Inhibition of Na,K-ATPase by lead in rat tissues has been reported earlier [12,13]. Rajanna et al. [14] reported effect of lead on Na,K-ATPase of rat brain tissues and its protection by thiol groups. Other experiments have shown that cadmium is a potent Na,K-ATPase inhibitor [15]. Nechay and Saunders [16] have confirmed these findings with human Na,K-ATPase. Ballatori et [17] showed that relatively low mercury concentrations (10 $\mu$M) inhibit the active $^{86}$Rb uptake within 5 min in suspension cultures of skate hepatocytes indicating interaction with Na,K-ATPase. All these studies were previously shown with Na,K-ATPase in animal tissues.

Here we report that silver is the most potent inhibitor of isolated Na,K-ATPase ever described despite the presence of 1 mM EDTA:. Silver interacts with Na,K-ATPase by an on-off mechanism in the condition used for activity measurement. To analyse further the effects of silver on the active transport of Na,K-ATPase, purified Na,K-ATPase molecules were incorporated in a functional state into the membranes of artificially formed phospholipid vesicles (liposomes) and the silver effect on the active $^{86}$Rb transport measured. Silver induced K-transport inhibition was demonstrated.

The results described in the present study show that silver inhibits Na,K-ATPase by a unique pathway and in addition delineate the role of cysteine and *N*-acetylcysteine as possible physiological chelators for preventing the interaction of silver with this vital transport system.

## 2. Materials and methods

*Materials*

$AgNO_3$ and EDTA were from Fluka. NADH (grade I), $Na_2ATP$ and pyruvatekinase/lactate dehydrogenase were purchased from Boehringer, phospho-*enol*pyruvate-cyclohexane-Tris from Sigma. Lithium dodecyl sulfate (LDS) solution (25%wt/vol) was obtained from Serva. Cholic acid were purchased from Merck. Phosphatidylcholine (grade IIa) and phosphatidylserine were from Lipid Products, Nutfield, UK. $^{86}$RbCl and $^{110m}AgNO_3$ were purchased from New England Nuclear and Amersham. DMPS, cysteine, *N*-acetylcysteine were from Merck. All chemicals and salts used were of the highest purity available; only bidistilled water was used. $AgNO_3$, *N*-acetylcysteine and cysteine solutions were freshly prepared. The Pierce bicinchonic acid assay kit (Rockford, IL) for protein determination was obtained from Sigma.

*Purification of Na,K-ATPase*

The enzyme was purified from the outer medulla of lamb kidney by a dodecyl sulfate extraction microprocedure adapted from Dzhandzhugazyan and Jørgensen [18] as follows: 11.2 mg of microsomal protein were incubated in 7 ml of a solution containing (in mM) 3-disodium ATP, 25 imidazole, 1 tris(hydroxymethyl)aminomethane (Tris)-EDTA, and 2.5 lithium dodecyl sulfate (LDS) for 20 min at 25°C. The enzyme was then recovered as a pellet after a centrifugation for 110 min at 250 000 $\times g$ in a fixed-angle rotor through a 20 ml gradient (4 ml 15% sucrose, 16 ml 15% sucrose, 25 mM imidazole, 1 mM Tris-EDTA (pH 7.5), 0°C), suspended in 0.5 ml 1% sucrose, 25 mM imidazole and 1 mM Tris-EDTA (pH 7.2), and stored at −70°C. The Na,K-ATPase activity was determined by the linked-enzyme assay as described below. The purity of the enzyme was assessed by gel electrophoresis followed by laser densitometric scan of the Commassie blue-stained subunits as shown previously [19]. The lamb preparation was used for the present study. Rat and rabbit Na,K-ATPase interacted similarly with silver.

*Na,K-ATPase activity*

The Na,K-ATPase activity was measured at 37°C by the enzyme-linked assay as follows: 1–2 mg of enzyme protein was added to the cuvette containing 1 ml of enzyme solution (0.3 mM NADH, 2.5 mM phospho-*enol*pyruvate-cyclohexamine-Tris, 8 $\mu$l pyruvate kinase/lactate dehydrogenase suspension, 30 mM imidazole, 1 mM Tris-EDTA, 2.5 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl (pH 7.2)). The oxidation rate of NADH was recorded at 340 nm wavelength in the automated enzyme kinetic accessory of a Philips Unicam SP1800 or Varian Cary 210 spectrophotometer. Calculation was done by considering that one molecule of oxidised NADH is equivalent to the production of 1 ADP molecule. The original recording was copied on transparancies then reversed to recopy on a paper to yield a time scale going from left to right. The copied recording were then redrawn to enlarge experimental points designed by the pen recorder at 1 min if the recorder has been synchronized with the cuvette changer or to add the 1 min values manually when the recording has been continuously made without cuvette changer.

*Inhibition by silver*

$AgNO_3$ stock solution (100 mM) was prepared freshly in water and added to the cuvette containing 1 ml of above enzyme-solution plus enzyme to the final various concentrations mentioned in results. Activity was recorded as described above. Control experiments

were run by adding only ADP to the enzyme solution in presence of $AgNO_3$ in order to make sure that the linked enzyme system was not affected by the metal at the concentrations used in the present study.

*Treatment with DMPS, cysteine and N-acetylcysteine*

Stock solutions (100 mM) of cysteine, *N*-acetylcysteine and DMPS were prepared and added to the enzyme in the cuvette to yield 1 mM final concentrations. Reversal of silver inhibition by these thiol compounds was assesed by measuring Na,K-ATPase activity continuously by the linked enzyme assay as described above.

*$^{86}Rb$ uptake and $^{110m}Ag$ binding to Na,K-ATPase reconstituted in liposomes*

Functional Na,K-ATPase reconstituted in ATP-filled liposomes were prepared as previously described [20]. Briefly, 300 $\mu$g purified Na,K-ATPase was suspended in 60 $\mu$l of a solution containing (in mM): 30 histidine, 1 Tris-EDTA, 50 $Na_2ATP$, 5 $MgCl_2$, and 23 cholic acid (pH 7.2), 0°C, was centrifuged for 10 min at $100000 \times g$ in an Airfuge Beckman at 0°C and the resulting supernatant was added to 50 $\mu$l lipid solution (5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA, 23 mM cholic acid, 0.8 mg phosphatidylcholine, 0.2 mg phosphatidylserine (pH 7.2)) at 0°C. Na,K-ATPase containing liposomes were formed by a 15-h dialysis at 0°C in 10 ml solution (50 mM $Na_2ATP$, 5 mM $MgCl_2$, 1 mM Tris-EDTA, 30 mM histidine (pH 7.2)) and cholestyramine resine to bind cholate. External ATP was removed by two centrifugations at $100000 \times g$ at 0°C in a Beckman airfuge. The washed ATP-filled liposomes were suspended in 100 $\mu$l of 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 30 mM histidine (pH 7.2), 0°C

5 $\mu$l of solution containing 20 $\mu$M $^{110m}$AgN03, in 100 mM NaCl, 5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA (pH 7.2), were added to aliquots of liposomes which were then incubated from 10 min at 25°C; 100 $\mu$l of stop solution (100 mM NaCl, 30 mM imidazole, 1 mM Tris-EDTA (pH 7.2), 0°C) were added to the liposome suspension and the free isotope was removed in a Sephadex G-50 medium column ($1 \times 20$ cm) in stop-solution at 0°C. The washed liposomes containing the $^{110m}$Ag-labeled Na,K-ATPase were collected within the first 10 min at a flow rate of 0.8 ml/min and their $^{110m}$Ag content was determined by beta counting in scintillation fluid.

It was previously demonstrated that $^{86}$Rb can be used as a precise analogue for K fluxes in this system [21]. ATP-filled Na,K-ATPase liposomes were prepared [26] and $^{86}$Rb uptake measured by the addition of external $^{86}$Rb and by determination of the intraliposomal $^{86}$Rb by the procedure described for $^{110m}$Ag-liposomes by gel filteration on $1 \times 20$-cm Sephadex medium columns at 0°C described [22].

The number of liposomes was calculated on the basis of extensive ultrastructural data [27] yielding an average diameter of 100 nm for a single liposome which corresponds to a volume of $382\ 10^{-21}$ l or 382 zl; from the fraction of $^{86}$Rb entrapped by all liposomes (10 l per ml on the average), a liposome number of $2.62 \cdot 10^{13}$ per ml can be calculated [20,22].

## 3. Results

*Effect of silver on Na,K-ATPase activity*

The effect of silver on Na,K-ATPase activity was studied to elucidate the potential interaction of the transport system with a metal other than mercury in the presence of 1 mM EDTA using a system for measuring continuously the activity of pure isolated enzyme Na,K-ATPase in a cuvette before and after addition of an inhibitor at 37°C in an automated spectrophotometer. Fig. 1 shows the spectrophotometric recording of absorbance change measured in 3 cuvettes each containing 1–2 $\mu$g of Na,K-ATPase in 1 ml of enzyme solution. The activity of Na,K-ATPase and its inhibition by silver can be distinguished on each line in a biphasic manner. The first phase of the line represents the normal activity of Na,K-ATPase followed by a second phase which shows the immediate inhibition of activity. A sudden break in the line reveals a rapid inhibition without latency period. Whereas at 0.1 $\mu$M silver there is some residual activity, inhibition at 1 and 2.5 $\mu$M is total and immediate. Conversely, in our previous study mercury inhibited the enzyme with a latency period [23]. The data described here demonstrate that silver is the most rapid Na,K-ATPase inhibitor described so far.

*Potency of silver inhibition*

To determine the potency of silver inhibition, the effect of increasing concentrations on the Na,K-ATPase

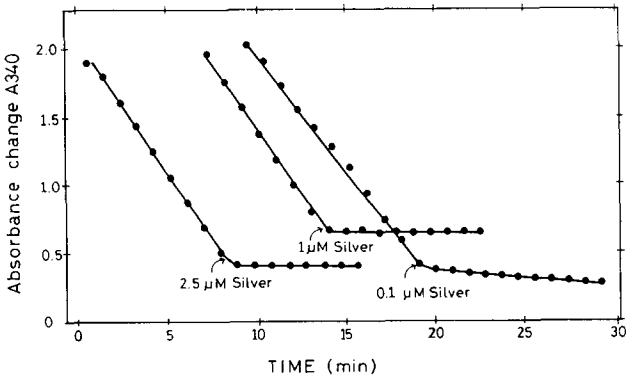


Fig. 1. Rapid inhibition of Na,K-ATPase activity by silver. Experimental points show absorbance change measured at 1-min intervals in an automated enzyme kinetics analyzer as described in Section 2. Typical experiments are shown.

activity was determined (Fig. 2A). A dose dependent inhibition of Na,K-ATPase by silver was observed with an $IC_{50}$ of 9 nM; at 500 nM silver, the activity was below 2%.

A Hill plot was done to determine nature and putative binding sites of silver interaction (Fig. 2B). The slope of the line shows a Hill coefficient of about two in the beginning of the reaction. However, with increasing concentrations, the Hill coefficient was found below 1 indicating negative cooperativity.

*Reversal of silver-inhibition*

Since silver was found to be a potent inhibitor of Na,K-ATPase, we intended to find out whether DMPS, a known metal chelator, reactivated enzyme activity. DMPS was added to the enzyme at 1 mM final concentration and the activity of Na,K-ATPase was measured (Fig. 3A); the two lines represent the duplicate values of Na,K-ATPase activity. The first phase of the line shows a normal activity of Na,K-ATPase, the second phase indicates the immediate inhibition (90%) of Na,K-ATPase at 100 nM silver and the third phase represents the full reversal of the Na,K-ATPase activity by 1 mM of the dithiol DMPS, a potent metal antidote.

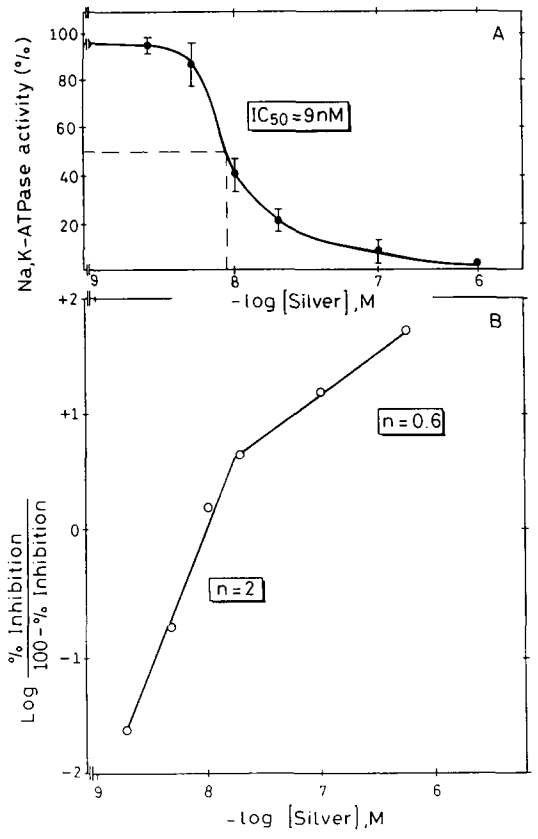


Fig. 2. (A) Dose response curve of Na,K-ATPase inhibition by silver. The enzyme activity before and after addition of silver was measured as described in Section 2. Vertical bars denote S.E. of the mean for three separate determinations. (B) Hill transformation of the curve and determination of the interaction coefficient ($n$) of silver effect on Na,K-ATPase.

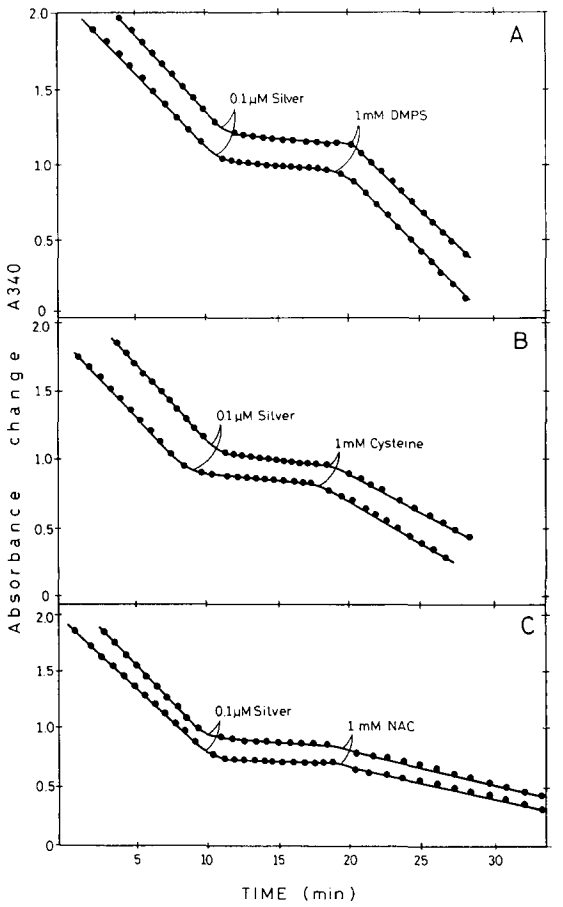


Fig. 3. (A) Reversal of silver inhibition by DMPS. (B) Reversal of silver inhibition by cysteine. (C) Reversal of silver inhibition by *N*-acetylcysteine. Na,K-ATPase was measured as described in Section 2.

Cysteine is known to interact with metals with its free -SH group [33]. In our previous experiments, cysteine was able to protect Na,K-ATPase from mercury inhibition [24] but unable to extract it once it had been bound to Na,K-ATPase [23]. The normal activity of Na,K-ATPase, its inhibition by silver and an approximately 80% reversal by cysteine is seen in Fig. 3B illustrating that the monothiol cysteine is able to extract silver that has been bound to Na,K-ATPase.

*N*-Acetylcysteine is an endogenous product of cysteine metabolism which might be playing a role in metal detoxification owing to the presence of -SH groups. *N*-Acetylcysteine has been shown to protect mercury-induced nephrotoxicity in vivo although it is not known by what mechanism [25]. Thus, it is crucial to know whether *N*-acetylcysteine has any role in sequestering metals in in vitro systems. *N*-Acetylcysteine (1 mM) was added to the enzyme inhibited by silver but only about 20–30% recovery was seen (Fig. 3C). Although *N*-acetylcysteine has a free -SH group, its

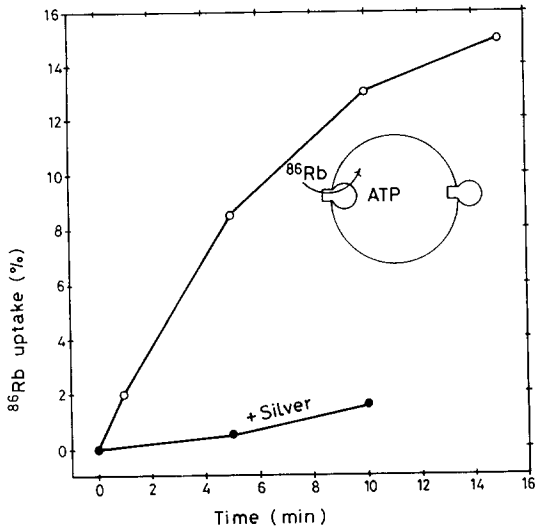


Fig. 4. Inhibition of Rb uptake in liposomes reconstituted with Na,K-ATPase; 10 $\mu$M $^{86}$Rb was added to both control (○) and silver-treated liposomes (●), $^{86}$Rb uptake was measured in parallel and expressed in percentage ot total $^{86}$Rb added. Techniques used for liposome preparation and radioflux measurements are described in Section 2. A typical experiment is shown.

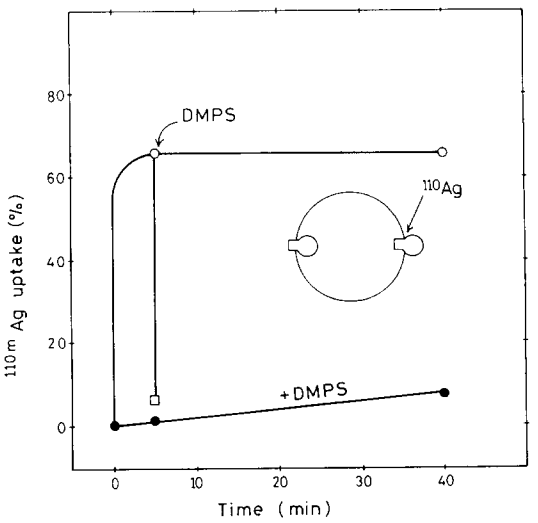


Fig. 5. $^{110m}$Ag-uptake by Na,K-ATPase reconstituted in liposomes. Purified lamb kidney Na,K-ATPase reconstituted into liposomes was incubated in the presence of 20 $\mu$M $^{110m}AgNO_3$ for 10 min at 25°C in 10 $\mu$l solution containing 100 mM NaCl, 1 mM NaCl, 5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA (pH 7.2). The $^{110m}$Ag uptake (○) was measured as described in Section 2; 10 mM DMPS was added 5 min after $^{110m}$Ag addition to extract silver from the enzyme (□). $^{110m}$Ag binding to Na,K-ATPase-liposomes was prevented by the presence of 10 mM DMPS (●). A typical experiment is shown.

affinity for silver seems poor in our system compared to cysteine and DMPS. Therefore, our results suggest that *N*-acetylcysteine has less ability than its analogue cysteine to extract silver from the enzyme.

### *$^{86}$Rb uptake and inhibition by silver in liposome reconstituted Na,K-ATPase*

Liposomes were incubated for different times with or without silver to look for inhibition of $^{86}$Rb transport. It was previously demonstrated that in the presence of external $^{86}$Rb, accumulation of $^{86}$Rb reflects K-transport by Na,K-ATPase [21,26]. $^{86}$Rb accumulation by ATP containing liposomes augmented with increasing time as shown in Fig. 4. At 10 min of incubation, 13% of the externally added 10 $\mu$M $^{86}$Rb were incorporated by the liposomes whereas in presence of silver the uptake was less than 2% indicating that silver blocked the ion-transport activity of Na,K-ATPase.

### *Silver ($^{110m}$Ag) uptake by liposomes reconstituted with Na,K-ATPase*

Liposomes reconstituted with pure Na,K-ATPase were incubated with 20 $\mu$M radiolabeled silver ($^{110m}$Ag) for 5–40 min. The results showed that 65% of $^{110m}$Ag was bound to the Na,K-ATPase within 5 min and the binding remained constant up to 40 min (Fig. 5, Table 1). When 10 mM DMPS were added to the liposomes 5 min after binding of $^{110m}$Ag, the bound silver was reduced from 65% to 8% (Fig. 5). This observation is

Table 1
Silver ($^{110m}$Ag) binding to pure Na,K-ATPase (NKA) reconstituted in liposomes

| No. of expts. ($n$) | Time (min) | No. of liposomes added | NKA | DMPS | $^{110m}$Ag in liposomes (pmol) | $^{110m}$Ag in liposomes (% total) | $^{110m}$Ag ions bound per liposome (No.) | $^{110m}$Ag ions bound per NKA (No.) |
|---|---|---|---|---|---|---|---|---|
| 2 | 15 | $3.12 \cdot 10^{12}$ | + | – | 1440 | 60 | 8 | 2 |
| 7 | 30 | $3.20 \cdot 10^{12}$ | + | – | 1580 ± 152 | 62.0 ± 5 | 8 ± 1.2 | 2 ± 0.3 |
| 2 | 30 | $4.70 \cdot 10^{12}$ | – | – | 11.75 | 0.63 | 0.4 | 0.1 |
| 1 | 30 | $1.00 \cdot 10^{12}$ | + | + | 142 | 18 | 2 | 0.5 |
| 1 | 40 | $3.12 \cdot 10^{12}$ | + | – | 1400 | 58 | 8 | 2 |

Purified lamb kidney ATPase reconstituted into liposomes was incubated in the presence of 20 $\mu$M of silver ($^{110m}$Ag) for 15, 30 and 40 min at 25°C in a solution containing 50 mM RbCl, 1 mM NaCl, 5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA (pH 7.2). The $^{110m}$Ag uptake was measured as described in Section 2. Liposome without Na,K-ATPase (NKA) was incubated as above to measure silver uptake. In parallel silver uptake was measured in liposomes incubated with DMPS. The number of liposomes, the amount of silver bound per liposome and per NKA molecule was calculated as described in Section 2. Data represent means ± S.E.

in analogy with the reversal of silver-inhibited Na,K-ATPase activity by DMPS measured in the spectrophotometer (Fig. 3A). Liposomes incubated with 10 mM DMPS showed no silver uptake; at 5 min less than 1% silver was bound when compared to 65% binding without DMPS. These results show that silver binds to the Na,K-ATPase and that the binding is prevented or reversed by thiols. However, the precise mechanism of inhibition and reversal is not yet known.

*Quantification of silver ($^{110m}$Ag) binding to Na,K-ATPase-liposomes*

Table 1 shows the determination of $^{110m}$Ag binding sites per liposome and per Na,K-ATPase molecule. Liposomes reconstituted with Na,K-ATPase were treated with 20 $\mu$M silver ($^{110m}$Ag) for different times and the binding calculated per liposome and reconstituted pump molecule as described previously [20,22] as in Materials and methods. Eight silver molecules were bound per liposome and 2 per Na,K-ATPase molecule respectively, considering 4 Na,K-ATPase molecules per liposome on the average at the protein/lipid ratio used for reconstitution [27]. $^{110m}$Ag binding was less than 0.6% in liposomes without Na,K-ATPase compared to 60% uptake with Na,K-ATPase liposomes demonstrating that binding took place to the enzyme and not the lipids and that silver directly binds to Na,K-ATPase molecules.

## 4. Discussion

The results shown in the present work indicate that silver is the most rapid and powerful inhibitor of Na,K-ATPase described so far since the enzyme activity is immediately blocked when silver is added to the transport system. Interestingly, the silver inhibition is immediate in contrast to the latency period taken for mercury [23] although mercury interacts also strongly with Na,K-ATPase. No other metal showed inhibition of Na,K-ATPase in our well established enzyme assay system in the presence of 1 mM EDTA [23]. $^{86}$Rb transport in liposome reconstituted by Na,K-ATPase is also inhibited totally by silver.

Binding of metals to sulfhydryl groups [19,28] is the most likely mechanism to explain the inhibitory action of IB and IIB metals on Na,K-ATPase. Indeed, sulfhydryl groups in the Na-K pumps are necessary for activity as suggested by the pump inhibition seen with sulfhydryl reagents such as N-ethylmaleimide are added [29–30]. Na,K-ATPase contains 11–14 sulfydryl groups/mol of $\alpha$ $\beta$, dimer; 8–9 SH groups reside on the $\alpha$-subunit and 1–2 on the $\beta$-subunit [31]. Further, titration experiments show that only 1–4 sulfhydryl groups per complex are freely accessible [32]. In in agreement with this report, quantitative binding of silver to Na,K-ATPase in liposomes showed that about two silver ions bind to a reconstituted Na,K-ATPase molecule and Hill plots of silver inhibition indicated also that two silver ions might bind to a functional Na,K-ATPase unit. The potent and rapid inhibition of Na,K-ATPase is most likely attributed to silver interaction with freely accessible sulfhydryl groups. The reactivation of silver inhibited Na,K-ATPase by thiols indicates that sulfhydryl groups are crucial for pump activity.

A possibility which could be deduced from the present study is that silver might replace K or Na ions and thereby block transport instantaneously. Since reactivation of Na,K-ATPase by thiols is also immediate silver can also be released rapidly from the transport system. It has been recently rewieved by Clarkson et al. [33] that metals stucturally mimic endogenous ions to such an extent that the transport process takes them as real ionic forms. Some metals such arsenate and vanadate mimic phosphates so closely that they can participate in a sequence of metabolic reactions until finally the mimicry fails and toxic consequences result [34]. According to Ormos and Manyal [35], chromium competes for sulfate in the human red blood cell membrane anion exchanger. Lead can mimic calcium in a number of specific transort-receptor, and enzyme-mediated processes [36]. However, metals can produce toxic effects by a variety of still unknown mechanisms.

The activity and life cycle of the eukaryotic cells are governed by the transmembrane distribution of alkaline and earth-alkaline metals. The concentrations of these metals whithin the various intracellular compartments and across the plasma cell membrane are regulated by a family of metal transporting proteins (ATPases). Thus, sensitivity of Na,K-ATPase to metals may have the impact on various cellular systems; for instance, isolated human lymphocytes were impaired by low concentrations (25 $\mu$M) of silver and were protected by cysteine in accordance with the protection of isolated Na,K-ATPase [37].

The unique silver inhibition and rapid reversibility by thiols suggest possible on-off mechanism of ion transport involving sulfhydryl groups. Silver interaction with Na,K-ATPase and reversal by thiols offers a new tool for studying the molecular mechanism of Na,K-transport.

## 5. Acknowledgements

The work was supported by the Swiss National Science Foundation (grant No. 31-25666.88), the Swiss Public Health Service (grant No. 31-30317.90), the Sandoz Research Foundation and the Ernest and Lucie Schmidheiny Foundation. We are grateful to Dr. Rolf Anner for useful discussions.

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