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Inhibition by mercuric chloride of Na-K-2Cl cotransport activity in rectal gland plasma membrane vesicles isolated from *Squalus acanthias*

Evamaria Kinne-Saffran [a,b], Rolf K.H. Kinne [a,b,*]

[a] *Max-Planck-Institut für Molekulare Physiologie, Abteilung Epithelphysiologie, Postfach 50 02 47, 44227 Dortmund, Germany*
[b] *Mount Desert Island Biological Laboratory, Salsbury Cove, ME 04672, USA*

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Abstract

The rectal gland of the dogfish shark is a model system for active transepithelial transport of chloride. It has been shown previously that mercuric chloride, one of the toxic environmental pollutants, inhibits chloride secretion in this organ. In order to investigate the mechanism of action of $HgCl_2$ at a membrane-molecular level, plasma membrane vesicles were isolated from the rectal gland and the effect of mercury on the activity of the Na-K-2Cl cotransporter was investigated in isotope flux studies. During a 30 s exposure $HgCl_2$ inhibited cotransport activity in a dose-dependent manner with an apparent K_i of approx. 50 μM. The inhibition was complete after 15 s, partly reversible by dilution of the incubation medium and completely attenuated upon addition of reduced glutathione. The extent of inhibition by mercury depended on the ionic composition of the medium. The sensitivity of the cotransporter was highest when only the high affinity binding sites for sodium and chloride were saturated. Organic mercurials such as *p*-chloromercuribenzoic acid and *p*-chloromercuriphenylsulfonic acid at 100 μM did not inhibit the cotransporter, similarly exposure of the vesicles to 10 mM H_2O_2 or 1 mM dithiothreitol for 30 min at 15°C did not change cotransport activity. Transport activity was, however, reduced by 45.9 ± 2.5% after an incubation with 3 mM *N*-ethylmaleimide for 20 min. Blocking free amino groups by *N*-hydroxysuccinimide or biotinamidocapronate-*N*-hydroxysulfosuccinimide had no effect. Investigations on the sidedness of the plasma membrane vesicles, employing the asymmetry of the (Na+K)-ATPase, demonstrated a right-side-out orientation in which the former extracellular face of the membrane is exposed to the incubation medium. In addition, extracellular mercury (5×10^{-5} M) inhibited bumetanide-sensitive rubidium uptake into T84 cells by 48.5 ± 7.1% after a 2 min incubation period. This inhibition was reversible in a manner similar to that observed in the plasma membrane vesicles. These studies suggest that in isolated rectal gland plasma membrane vesicles the Na-K-2Cl cotransporter (sNKCC1) exposes functionally relevant mercury binding sites at its external surface. These sites represent probably cysteines, the accessibility and/or sensitivity of which depends on the functional state of the transporter.

Keywords: Mercuric chloride; Shark rectal gland; Na-K-2Cl cotransport; sNKCC1; Plasma membrane vesicle; Active chloride transport; *Squalus acanthias*

1. Introduction

One of the main model systems used to unravel the cellular and molecular basis of active transepithelial chloride transport is the rectal gland of the shark *Squalus acanthias* [1]. The rectal gland is an organ

* Corresponding author. Fax: +49-231-133-2299; E-mail: rolf.kinne@mpi-dortmund.mpg.de

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that contains a homogenous population of cells which can be stimulated to secrete sodium chloride in extremely high rates. Compared to mammalian sources it is also the organ that contains the highest amount of (Na+K)-ATPase and Na-K-2Cl cotransporter and has served as starting material to isolate and clone these transporters [2–4].

In perfusion studies it could be demonstrated by Silva et al. [5] that mercuric chloride but not *p*-chloromercuriphenylsulfonic acid (PCMBS) inhibits chloride secretion; this inhibition was only partly reversible. Mutational and functional studies on the shark rectal gland Na-K-2Cl cotransporter (sNKCC1) activity also showed an inhibitory action of mercury when the transporter and mutants were stably expressed in human embryonic kidney (HEK) cells. In those investigations mainly cysteines located in transmembrane segments and intracellular domains of the transporter were identified as potential target sites [6].

Studies in whole organs and intact cells are always complicated by the fact that not only one member in the chain of events comprising transepithelial transport might be affected or that intracellular regulatory processes might be altered and not the cotransporter itself. In addition, interesting mutants can sometimes not be expressed in heterologous cells and therefore important regions of the molecule are difficult to be investigated. We, therefore, chose to examine the effect of mercury on the Na-K-2Cl cotransporter of the rectal gland by employing functional studies in isolated plasma membrane vesicles where some of the above-mentioned interdependences are no longer present. Our results suggest that the Na-K-2Cl cotransporter possesses binding sites for mercury also at the external face, the reactivity of which seems to be dependent on the functional state of the transporter.

2. Materials and methods

2.1. Materials

Male and female dogfish (*S. acanthias*) were caught in Frenchman's Bay, ME, USA. The fish were kept in live cars and were used within 2 days–1 week of capture. ^{86}RbCl (0.06–4.2 Ci/mmol), ^{22}Na (carrier free), ^{36}Cl (0.18 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA, USA). All other chemicals employed were of the highest grade of purity commercially available.

2.2. Isolation of plasma membranes and determination of (Na+K)-ATPase activity

Isolation of plasma membranes was carried out at 0–4°C by a modification of the method described by Hokin et al. [3]. The procedure consists of a series of differential centrifugation steps in which plasma membranes are separated from nuclei, mitochondria, and endoplasmic reticulum. The marker enzyme (Na+K)-ATPase was enriched about 4-fold in the final membrane fraction. (Na+K)-ATPase activity was determined in an assay medium containing 50 mM imidazole (pH 7.4), 100 mM NaCl, 20 mM KCl, 6 mM $MgCl_2$, 3 mM Tris-ATP, in the absence or presence of 2 mM ouabain. After 30 min of incubation at 25°C ice-cold 10% trichloroacetic acid was added to the samples to stop the reaction. After centrifugation of the samples the amount of liberated phosphate was determined according to Fiske and Subbarow [7]. Sidedness of the plasma membrane fraction was determined by comparing ATPase activity in the presence of magnesium alone (Mg-ATPase), in the presence of magnesium, sodium and potassium (Mg-ATPase and (Na+K)-ATPase) and in the presence of magnesium, sodium, potassium, and 2 mM ouabain, before and after shell-freezing and freeze-drying of the vesicle preparation.

2.3. Determination of protein

Protein was determined by a modification of the method of Lowry et al. [8] after precipitation of the samples in ice-cold 10% trichloroacetic acid, using bovine serum albumin as standard.

2.4. Transport studies

Isotope uptake into the plasma membrane vesicles suspended in 200 mM mannitol, 1.5 mM $MgNO_3$, 20 mM Tris-HEPES, pH 7.4, was followed at 15°C using a rapid filtration technique through mixed cellulose nitrate filter (pore size 0.45 μm; Schleicher und Schuell, Dassel, Germany) [9]. Radioactivity associ-

ated with the filters was determined by standard liquid scintillation techniques using Ultima Gold (Packard Instruments) as scintillation fluid. Values were corrected for the amount of radioactivity bound to the filters in the absence of plasma membranes. Isotope uptake was measured by incubating the vesicles in transport buffer of the following composition (in mM): 200 mannitol, 20 HEPES (pH adjusted to 7.6 with Tris-base), 1 $Mg(NO_3)_2$, and various salt gradients and isotopes. To determine chloride-dependent rubidium uptake the medium contained in addition 0.1 $RbNO_3$, 10 μCi ^{86}Rb, and either 170 NaCl or 170 $NaNO_3$ in each assay. The difference in uptake between the chloride and the nitrate containing medium was defined as chloride-dependent rubidium uptake. Potassium-dependent chloride uptake was determined in medium containing 4 mM chloride, contributed by 10 μCi ^{36}Cl used as isotope, 100 Na_2SO_4 or 50 Na_2SO_4 and 50 K_2SO_4. The difference in chloride uptake between the potassium containing medium and the potassium-free medium was defined as potassium-dependent chloride uptake. In order to measure the chloride-dependent sodium flux media containing 0.1 $NaNO_3$, 10 μCi ^{22}Na, and either 170 KCl or 170 KNO_3 were used. The difference in sodium uptake between the chloride and the nitrate containing medium constituted the chloride-dependent sodium flux. In previous studies it has been shown that all of these fluxes can be completely inhibited by bumetanide [9]. As 'stop solution' a medium containing (in mM) 200 mannitol, 200 KNO_3, 20 HEPES-Tris (pH 7.6), and 1 $Mg(NO_3)_2$ was used. For further details see Hannafin et al. [9].

2.5. Uptake studies in T84 cells

T84 cells were purchased from ATCC (Manassas, VA, USA) and grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium supplemented with 15 mM HEPES buffer, pH 7.5, 1.2 g $NaHCO_3$, 40 mg penicillin, 8 mg ampicillin, and 90 mg streptomycin/l, and 6% newborn calf serum (Gibco BRL-Life Technologies, Rockville, MD, USA). For uptake experiments cells were seeded in 6-well plates (Falcon No. 3046). Cells from passages 45–55 were used. Rubidium uptake was determined in duplicate as described by Jacoby et al. [6].

2.6. Modification of amino groups

To study the effect of amine-specific reagents membrane vesicles were initially prepared as described above. The final membrane pellet was resuspended in 35 ml sodium-phosphate buffer, pH 7.4. After centrifugation the resulting pellet was suspended in vesicle buffer containing 10 mM sodium-phosphate buffer instead of Tris or HEPES as buffer substances. These membranes were subsequently incubated in a freshly prepared *N*-hydroxysuccinimide (NHS) solution or a freshly prepared biotinamidocapronate-*N*-hydroxysulfosuccinimide (BAC-SulfoNHS) solution. This represents a 10 times higher concentration than recommended for biotinylation of proteins by the manufacturer (Sigma, St. Louis, MO, USA).

2.7. Statistics

All transport studies were performed in duplicate. '*n*' indicates the number of membrane preparations derived from separate animals. For statistical analysis Student's *t*-test for unpaired data was employed. A difference with a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of mercuric chloride on Na-K-2Cl cotransport activity in isolated rectal gland plasma membrane vesicles

3.1.1. Concentration dependence

In order to elucidate whether the Na-K-2Cl cotransporter is affected by mercury in the isolated plasma membranes we tested the effect of various mercuric chloride concentrations on the chloride-dependent rubidium uptake into the membrane vesicles. Control rubidium uptake in the presence of chloride amounted in this experimental series to 34.6 ± 10.1 pmoles/mg protein $\times 30$ s ($n = 5$), uptake in the absence of chloride (chloride replaced by nitrate) to 6.9 ± 0.7 pmoles/mg protein $\times 30$ s. As shown in Fig. 1, a significant inhibition of chloride-dependent rubidium uptake was observed at a concentration higher than 10 μM mercuric chloride. The inhibition was complete at 100 μM. At the same time

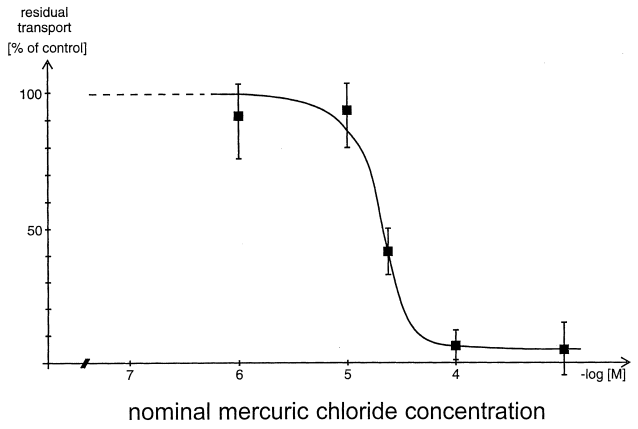

Fig. 1. Effect of mercuric chloride on chloride-dependent rubidium uptake into isolated rectal gland plasma membrane vesicles. Uptake was measured for 30 s at 15°C in the presence of mercuric chloride concentrations indicated in the figure. Mean values ± S.E.M. derived from five experiments are given.

the uptake of rubidium into the membrane vesicles in the presence of nitrate was not impaired (95.0 ± 8.9% of control ($n = 5$)). These results suggest that the inhibition observed for the Na-K-2Cl cotransport was not due to unspecific changes in the permeability of the plasma membranes. Furthermore, mannitol uptake into the vesicles was also not altered, indicating that membrane integrity and membrane vesiculation were preserved (data not shown).

3.1.2. Divalent cation specificity

Next, the specificity of the inhibitory effect with regard to other heavy metals was investigated. The results of these experiments are compiled in Table 1. When mercury was replaced by cadmium, zinc or copper no inhibition of the cotransport activity was observed.

Table 1
Effect of various divalent cations (concentration 5×10^{-5} M) on Na-K-2Cl cotransport in isolated rectal gland plasma membrane vesicles

	% control	n	P
Hg^{2+}	21.4 ± 8.6	4	< 0.01
Cd^{2+}	109.6 ± 13.8	3	n.s.
Zn^{2+}	98.6 ± 10.2	3	n.s.
Cu^{2+}	93.5 ± 5.5	6	n.s.

Na-K-2Cl cotransport activity was determined by measuring chloride-dependent rubidium flux at 15°C. The divalent cations added as chloride salts were present during the 30 s uptake period. Mean values ± S.E.M. derived from n experiments are given. n.s., not significantly different from control.

3.1.3. Effect of other mercurials

In order to study whether also other mercurials affected the cotransport activity the action of *p*-chloromercuribenzoic acid (PCMB) and PCMBS was investigated. As shown in Table 2 neither PCMB nor PCMBS in concentrations as high as 100 μM altered the cotransport activity. The same concentration of mercuric chloride abolished transport completely.

3.2. Accessibility of mercury-sensitive sites

3.2.1. Time dependence of inhibition by mercuric chloride

In Fig. 2 an experiment is depicted from which the time dependence of the inhibition of the cotransport activity by mercuric chloride can be deduced. In the absence of mercuric chloride the typical rubidium uptake in the presence of a sodium chloride gradient is observed. Furthermore, rubidium uptake in the presence of a sodium nitrate gradient is markedly reduced indicating the presence of an active Na-K-2Cl cotransporter in these membrane vesicles. Mercuric chloride, at a concentration of 5×10^{-4} M, inhibited the cotransporter completely within 15 s, i.e., rubidium uptake in the presence of sodium

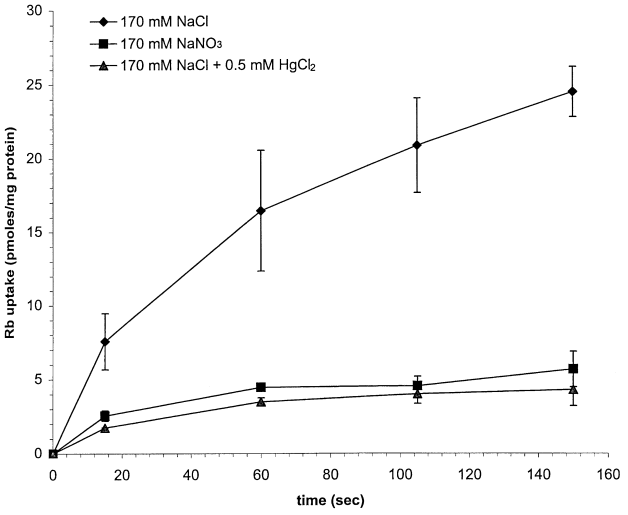

Fig. 2. Time course of rubidium uptake into isolated rectal gland plasma membrane vesicles: effect of mercuric chloride. One representative experiment performed in duplicate is given.

Table 2
Inhibition of Na-K-2Cl cotransport activity in isolated rectal gland plasma membrane vesicles by various mercurials

	% control	*n*	*P*
10^{-4} M $HgCl_2$	4.6 ± 0.9	4	< 0.01
10^{-4} M PCMB	110.5 ± 7.3	3	n.s.
10^{-4} M PCMBS	95.1 ± 6.1	3	n.s.

PCMB, *p*-chloromercuribenzoic acid; PCMBS, *p*-chloromercuriphenylsulfonic acid. Na-K-2Cl cotransport activity was determined at 15°C as chloride-dependent rubidium uptake for 30 s in the presence of the compounds indicated above. Mean values ± S.E.M. derived from *n* experiments are given. n.s., not significantly different from control.

chloride was identical to the rubidium uptake in the presence of sodium nitrate. In further experiments, even after 5 s already a reduction of the transport activity to 58.1 ± 10.2% ($n = 4$) was observed.

3.2.2. Reversibility of inhibition by mercuric chloride

The rapid onset of inhibition suggests that the mercury-sensitive sites are located at the surface of the isolated plasma membrane vesicles. In order to test this hypothesis further the reversibility of inhibition was investigated. For this purpose three different experimental protocols were chosen. The first constituted a simple dilution step. The second employed reduced glutathione (GSH) as a scavenger with supposedly low membrane permeability and dithiothreitol (DTT) as a scavenger with a supposedly high membrane permeability. The results of these experiments are shown in Table 3. In this series membrane vesicles were preexposed for 30 s to medium containing mercuric chloride or medium free of mercuric chloride. The membrane vesicles were then transferred into various transport media and subsequently the cotransport activity was measured for 30 s. In control vesicles preincubated in mercury-free medium and then exposed to 50 μM mercury during the transport cotransport activity was inhibited by 84.5% compared to vesicles which had not been exposed to mercury at all. When vesicles were preexposed to mercury and then transferred to mercury-free transport medium the inhibition of their cotransport activity decreased to 21.6%. This degree of inhibition is almost identical to the one to be expected from the mere dilution of the mercury present in the membrane suspensions (50 μM) to 14 μM (line 3 of Table 3). If, in addition, reduced glutathione was present in the transport medium, the activity of the cotransporter recovered completely and was no longer statistically different from that of non-exposed membranes (line 5 in Table 3). Similarly, the presence of DTT completely reversed the inhibition by mercuric chloride. Both the rapid reversibility upon dilution into mercury-free medium and the additional effect of GSH strongly support the notion that the sites of the transporter which are affected by mercury are located at the external face of the membrane vesicles. Surprisingly, even after a prolonged exposure of the membrane vesicles to mercury for up to 6.5 min at 15°C a complete reversal of the inhibition was achieved by GSH (105.6 ± 2.3% of control uptake ($n = 3$)).

3.3. Identification of target site(s) for mercury

3.3.1. Chemical identification

Classically mercury is assumed to form mercaptide

Table 3
Reversibility of inhibition of Na-K-2Cl cotransport in isolated rectal gland plasma membrane vesicles

Preincubation medium	Transport medium	Activity in % control	*n*	*P*
Vesicle buffer	no $HgCl_2$	100	6	
Vesicle buffer	5×10^{-5} M $HgCl_2$	15.5 ± 6.1	3	< 0.01
Vesicle buffer	1.4×10^{-5} M $HgCl_2$	83.2 ± 6.2	3	< 0.05
5×10^{-5} M $HgCl_2$	no $HgCl_2$	78.4 ± 3.8	3	< 0.05
5×10^{-5} M $HgCl_2$	1 mM GSH	96.6 ± 4.1	6	n.s.
5×10^{-5} M $HgCl_2$	1 mM DTT	115.9 ± 9.2	3	n.s.

Chloride-dependent rubidium uptake was measured for 30 s at 15°C in the transport medium indicated above. Plasma membranes were preincubated in vesicle buffer either in the presence or the absence of mercuric chloride for 30 s at 15°C. Mean values ± S.E.M. derived from *n* experiments are given. GSH, reduced glutathione; DTT, dithiothreitol. n.s., not significantly different from control.

complexes with SH groups in proteins when exerting its inhibitory effect [10]. In several other instances the action of mercury could be mimicked by oxidation of the mercury-sensitive SH groups [11,12]. We, therefore, investigated the action of the oxidizing reagent H_2O_2 on the cotransporter. No changes in cotransport activity in isolated membrane vesicles were observed when 1 mM H_2O_2 was present during the 30 s uptake period or 10 mM H_2O_2 during preincubation for 30 min (96.0 ± 4.5% of control ($n = 4$)). Another possibility to modify SH groups is to induce thiol-disulfide exchange. To this end membranes were pretreated with 10 mM dithiothreitol for 30 min or the cotransport activity was measured in the presence of 1 mM dithiothreitol. No change in activity was found under either condition.

On the other hand, incubation of the membrane vesicles with 3 mM *N*-ethylmaleimide (NEM) for 20 min at 15°C reduced the transport activity to 55.1 ± 2.4% ($n = 4$) of control activity. Although NEM is assumed to interact predominantly with SH groups it is also known to modify free amino groups [10]. We therefore tested whether amine-specific reagents, such as *N*-hydroxysuccinimide [13] or biotinamidocapronate-*N*-hydroxysulfosuccinimide [14], had an effect on transport activity. In appropriately amine-free buffer solutions, pH 7.4, incubation with 1 mM NHS at 15°C for up to 30 min did not significantly modify transport activity. The same holds for 4.5 mM BAC-SulfoNHS.

3.3.2. Functional identification

It is now generally accepted that the Na-K-2Cl cotransporter possesses four binding sites at the extracellular face which have to be occupied in an ordered sequence before translocation of the ions can occur [15]. First, sodium binds to a high affinity sodium binding site, then chloride associates with a high affinity chloride binding site. Potassium binding follows and, finally, the low affinity chloride binding site is engaged. In studies using membrane vesicles it is possible to alter the incubation conditions in such a way that on the average one particular ion/cotransporter complex predominates [9]. We, therefore, tested whether in these various functional states the mercury-sensitive sites on the protein might differ in their accessibility and, therefore, the cotransport would exhibit different sensitivities to mercury. The

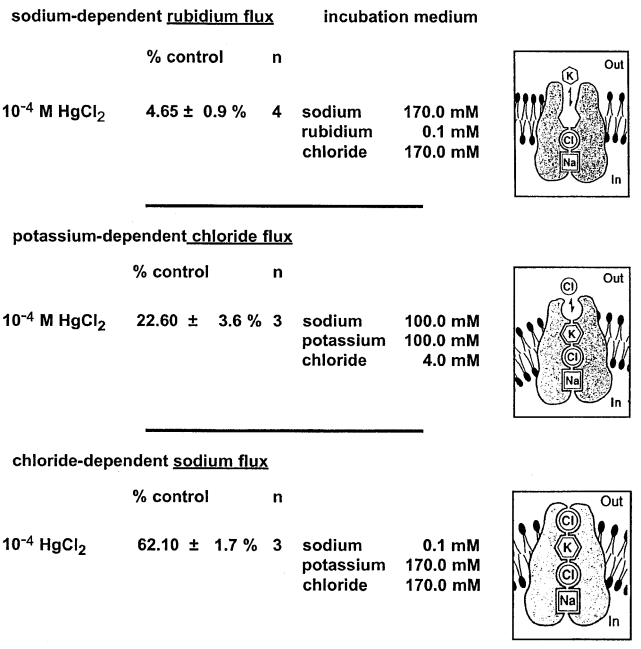

Fig. 3. Inhibition of Na-K-2Cl cotransport activity by mercuric chloride in isolated rectal gland plasma membrane vesicles: dependence on the ionic composition of the uptake medium. The schematic drawings on the right side of the figure depict the predominant states of the Na-K-2Cl cotransporter with regard to the occupancy of ion binding sites. Uptake was measured for 30 s at 15°C. Chloride-dependent rubidium flux under control conditions amounted to 0.034 nmoles/mg protein, potassium-dependent chloride flux amounted to 0.8 nmoles/mg protein, and chloride-dependent sodium flux amounted to about 0.18 nmoles/mg protein, respectively. The differences in transport rates are due to different affinities of the transporter for the isotopes used to measure transport activity and due to the differences in driving forces. The mercury-induced inhibition of chloride and sodium flux are significantly different from the inhibition observed in rubidium flux (P at least < 0.05).

results of these studies are compiled in Fig. 3 which also depicts the various functional states assumed to predominate under the experimental conditions employed. During the rubidium flux experiments used thus far to quantify the cotransport activity (composition of transport medium: 0.1 mM rubidium, 170 mM sodium, and 170 mM chloride), the sodium binding site and high affinity chloride binding site can be assumed to be occupied. Since the rubidium concentration is, however, very low the potassium (rubidium) site and consequently the second chloride site are rarely occupied. Under these conditions, as noted above, 100 μM mercuric chloride almost completely inhibited the cotransporter.

If the cotransport activity is determined by measuring the potassium-dependent chloride flux (4 mM chloride, 100 mM sodium, and 100 mM potassium) the sodium binding site, the high affinity chloride binding site, and the potassium site can be assumed to be saturated [9]. In this state mercury was less effective. An inhibition of only 77.4% was observed.

A third possibility to monitor cotransport activity is to determine chloride-dependent sodium flux [9]. These experimental conditions (0.1 mM sodium, 170 mM potassium, 170 mM chloride) induce a functional state in which all four cotransported ions, sodium, potassium, and the two chloride ions, interact with the carrier. In this state the sensitivity of the cotransporter to mercury was further reduced as indicated by the even lower inhibition.

According to these experiments the site(s) sensitive to mercury are most accessible when only the sodium and the high affinity chloride binding site are saturated: these were the conditions when the effects of other mercurials, H_2O_2, GSH, dithiothreitol, and the *N*-hydroxysuccinimides on the cotransport were investigated.

3.3.3. Topological identification

In order to define whether the mercury-sensitive sites of the membrane vesicles are located at the intracellular or extracellular face of the cotransporter, we investigated first the orientation of the plasma membrane vesicles. For this purpose the functional asymmetry of the membrane bound (Na+K)-ATPase with regard to its extracellularly oriented ouabain binding site and its intracellularly oriented site of ATP hydrolysis was employed [16–18]. As shown in Table 4, the increase of ATPase activity in freshly isolated plasma membranes induced by the presence of sodium and potassium ((Na+K)-ATPase) was completely inhibited by ouabain, indicating that all (Na+K)-ATPase molecules which under these experimental conditions hydrolyze ATP are also accessible to ouabain. When the plasma membrane fraction was shell-frozen and subsequently freeze-dried prior to analyzing the enzymatic activity, the (Na+K)-ATPase activity increased about 3-fold and again all activity was inhibited by ouabain. Thus, the membrane preparation employed in these studies contains open membrane sheets – which do not exhibit any activity in the transport studies – and right-side-out vesicles exposing the former extracellular face of the cell to the mercury containing transport medium. These findings suggest that the mercury-sensitive sites are located within the extracellular loops of the transport protein. In order to provide further support for this assumption, which was based on vesicle data where the orientation of the membrane is always debatable, rubidium uptake studies on intact T84 cells were performed, where the orientation of the transporter is much better defined. These cells have been shown to contain a member of the secretory type cotransporter family NKCC1 [19]. Control rubidium uptake (in the presence of 1 mM ouabain) amounted to 3.2 ± 0.5 nmoles rubidium/mg protein $\times$ 2 min, the bumetanide-sensitive uptake (determined in the presence of ouabain and 0.2 mM bumetanide) averaged 2.5 ± 0.5 nmoles/mg protein $\times$ 2 min). Exposure of these cells for 2 min to an extracellular medium containing 5×10^{-5} M mercuric chloride inhibited bumetanide-sensitive rubidium uptake by $48.5 \pm 7.1\%$ ($n = 3$). Adopting the same strategy as described above for rectal gland membrane vesicles it could be shown that rubidium uptake into cells, first exposed to $HgCl_2$ for 2 min, and then transferred for 2 min to a mercury-free transport medium was only inhibited by $24.6 \pm 5.0\%$ ($n = 3$). The inhibition could be completely reversed in the presence of 1 mM GSH ($115.0 \pm 8.2\%$ of control uptake ($n = 3$)) [20].

Table 4
(Na+K)-ATPase activity in isolated rectal gland plasma membranes

Compounds present in the incubation medium	MgATP	MgATP+Na+K	MgATP+Na+K+ouabain (2 mM)
Freshly prepared membranes	0.140 ± 0.01	0.315 ± 0.02	0.094 ± 0.01
Flash-frozen membranes	0.129 ± 0.01	0.682 ± 0.06	0.095 ± 0.01

Enzyme activities are given in μmoles phosphate released/h $\times$ mg protein. Mean values $\pm$ S.E.M. derived from three experiments are given. For details see text and Section 3.

4. Discussion

The results presented above clearly demonstrate an inhibitory action of mercuric chloride on the Na-K-2Cl cotransporter in isolated plasma membrane vesicles of the rectal gland. They confirm and extend observations made on the in vitro perfused rectal gland by Silva et al. [5]. In the perfusion experiments the concentration of mercuric chloride needed for half-maximal inhibition of chloride secretion was 25 μM, a K_i quite similar to the one observed in this study. In the perfusion studies PCMBS also did not inhibit chloride secretion. It could, however, not be distinguished whether the Na-K-2Cl cotransporter itself was inhibited by mercury or whether mercury affected other transport systems involved in chloride secretion such as (Na+K)-ATPase [21,22], potassium channels or the chloride channel in the luminal membrane of the rectal gland. The partly irreversible effect of mercuric chloride in the intact gland versus the reversibility in the vesicle studies already suggests that mercury might interact with multiple sites in the intact cells.

At the molecular level sequencing the transporter revealed cysteines in the putative transmembrane domain tm1 and tm11 as well as in the putative extracellular loop between tm7 and tm8, in the intracellular loop between tm8 and tm9 and in the large intracellular C-terminal loop [4]. In an extensive study involving mutagenesis and expression of the transporter in a mammalian expression system, using HEK-293 cells, Jacoby et al. [6] observed that mercuric chloride inhibits Na-K-2Cl cotransport mediated by the shark carrier sNKCC1 with an inhibitory constant of 25 μM. This apparent K_i is in good agreement with the results obtained in the present experiments. Furthermore, the authors identified one of the cysteine residues within tm11 as a binding site for mercury. From the rather slow onset of inhibition and the lack of reversibility by GSH the authors concluded that additional mercury-SH interactions are occurring, probably within the lipid bilayer or at intracellular domains [6]. The rapid onset of inhibition and the complete reversibility observed in our studies indicate that mercury-sensitive sites are located on the surface of the membranes at either the intracellular or the extracellular side of the transport protein. If one takes into account in addition the evidence obtained about the orientation of the plasma membrane vesicles and the transport studies on intact T84 cells, the most probable sites are those located in the extracellular loop between tm7 and tm8. Unfortunately, no direct information about the role of these cysteines is available since Jacoby et al. [6] as well as the authors of this study were unable to express mutants in these amino acids in a functionally active state. Western blot analysis, performed in our laboratory, revealed that any mutation in this loop of cysteine to serine led to a drastic reduction of the expression of the protein. Moreover, the protein appeared not to be incorporated into the plasma membrane (E. Kinne-Saffran et al., unpublished data). The studies by Jacoby et al., in contrast to the experiments presented above, provide strong evidence for a role of intracellular SH groups in mercury sensitivity of the cotransporter. They do, however, not exclude the presence of extracellularly oriented mercury-sensitive sites, since MTSET, a membrane-impermeant SH reagent inhibited rubidium uptake in HEK-293 cells, be it only partly and to a lesser extent than its membrane-permeant congener MTSEA [6].

In the current study the highest sensitivity of the transporter to mercury was observed when only the sodium binding site and the high affinity chloride binding site were occupied. In this state the accessibility and/or reactivity of mercury-sensitive sites seem to be most pronounced. The same has been shown for the Na-K-2Cl cotransporter and its relative affinity for loop diuretics which are dependent on the occupation of the binding sites by the respective transported ions [9,23]. This result also provides evidence that mercury acts directly on the transport protein and probably not on regulatory proteins that might be still active in the membrane preparation [24].

Therefore it could be envisaged that the conformation of the loop between tm7 and tm8 is affected by the ionic composition of the medium, the incubation temperature and/or the membrane lipids the transporter is embedded in. Differences of these parameters exist in the two studies and thus might explain the discrepancies in the results. It can, however, also not be excluded that in the isolated rectal gland plasma membranes the cysteines in tm11 might be more exposed to the external medium than when the co-

transporter is expressed in HEK-293 cells. Thus, the reactivity to mercuric chloride could be higher and could account for the divergent results.

Another interesting point is the apparently very low permeability of the rectal gland plasma membranes to mercury. Even after 6.5 min none of the other mercury-sensitive sites of the cotransporter present in the membrane bilayer or at the cytoplasmic face appear to have been reached, whereas at this time point in HEK-293 cells almost maximal inhibition of rubidium influx was observed. A different lipid composition and the low temperature (which reflects the temperature of the water the shark lives in) used in the current study might be the cause for this phenomenon.

In summary, studies on the role of cysteines in the sNKCC1 transporter using plasma membrane vesicles isolated from shark rectal gland have provided evidence that also cysteines located at the extracellular surface of the protein can play a significant role in sodium, potassium, and chloride cotransport activity. It remains to be established whether this reactivity can also be demonstrated in other members of the NKCC family in which these cysteines are conserved [25].

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References

[1] F.H. Epstein, P. Silva, Na-K-Cl cotransport in chloride-transporting epithelia, Ann. NY Acad. Sci. 456 (1985) 187–197.
[2] J.B. Riordan, B. Forbush, J.W. Hanrahan, The molecular basis of the chloride transport in shark rectal gland, J. Exp. Biol. 196 (1994) 405–418.
[3] L.E. Hokin, J.L. Dahl, J.D. Deupree, J.F. Dixon, J.F. Hackney, F. Perdue, Studies on the characterization of the sodium-potassium transport adenosine triphosphatase, J. Biol. Chem. 248 (1973) 2593–2605.
[4] J.-C. Xu, C. Lytle, T.T. Zhu, J.A. Payne, E. Benz, B. Forbush, Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter, Proc. Natl. Acad. Sci. USA 91 (1994) 2201–2205.
[5] P. Silva, F.H. Epstein, R.J. Solomon, The effect of mercury on chloride secretion in the shark (*Squalus acanthias*) rectal gland, Comp. Biochem. Physiol. C 103 (1992) 569–575.
[6] S.C. Jacoby, E. Gagnon, L. Caron, J. Chang, P. Isenring, Inhibition of Na^+-K^+-$2Cl^-$ cotransport by mercury, Am. J. Physiol. 277 (1999) C684–C692.
[7] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorous, J. Biol. Chem. 66 (1925) 375–388.
[8] O.H. Lowry, N.J. Roseborough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[9] J. Hannafin, E. Kinne-Saffran, D. Friedman, R. Kinne, Presence of a sodium-potassium chloride cotransport system in the rectal gland of *Squalus acanthias*, J. Membr. Biol. 75 (1983) 73–83.
[10] P.R. Iwaarden, A.J.M. Driessen, W.N. Konings, What we can learn from the effects of thiol reagents on transport proteins, Biochim. Biophys. Acta 1113 (1992) 161–170.
[11] A.E. Busch, S. Waldegger, T. Herzer, G. Raber, E. Gulbins, T. Takumi, K. Moriyoshi, S. Nakanishi, F. Lang, Molecular basis of I_{sK} protein regulation by oxidation or chelation, J. Biol. Chem. 270 (1995) 3638–3641.
[12] J.P. Ruppersberg, M. Stocker, O. Pongs, S.H. Heinemann, R. Frank, M. Koenen, Regulation of fast inactivation of cloned mammalian I_k(A) channels by cysteine oxidation, Nature 352 (1991) 711–714.
[13] R.L. Lundblad, The modification of lysine, in: Chemical Reagents for Protein Modification, CRC Press, Boca Raton, FL, 1991, pp. 129–172.
[14] E.A. Bayer, M. Wilchek, Protein biotinylation, Methods Enzymol. 184 (1990) 138–160.
[15] C. Lytle, T.J. McManus, M. Haas, A model of Na-K-2Cl cotransport based on ordered ion binding and glide symmetry, Am. J. Physiol. 274 (1998) C299–C309.
[16] B. Forbush, Characterization of right-side-out membrane vesicles rich in (Na,K)-ATPase and isolated from dog kidney outer medulla, J. Biol. Chem. 257 (1982) 12678–12684.
[17] R. Kinne, E. Kinne-Saffran, Membrane vesicles as tools to elucidate epithelial cell function, Eur. J. Cell Biol. 25 (1981) 346–352.
[18] O.J. Møller, Activation by freezing of (Na^++K^+)-ATPase in a microsomal fraction from ox kidney cortex, Exp. Cell Res. 68 (1971) 347–355.
[19] C. Lytle, J.-C. Xu, D. Biemesderfer, B. Forbush, Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies, Am. J. Physiol. 269 (1995) C1496–C1505.
[20] E. Kinne-Saffran, R.K.H. Kinne, Sidedness and reversibility of mercury inhibition of Na-K-Cl cotransport in intact T84 cells, a human colon cancer cell line, MDIBL Bull. 36 (1997) 19–20.
[21] B.M. Anner, M. Moosmayer, Mercury inhibits Na-K-ATP-

ase primarily at the cytoplasmic side, Am. J. Physiol. 262 (1992) F843–F848.
[22] X. Wang, J.-D. Horisberger, Mercury binding site on Na^+/K^+-ATPase: a cysteine in the first transmembrane segment, Mol. Pharmacol. 50 (1996) 687–691.
[23] B. Forbush, H.C. Palfrey, [^{3}H]Bumetanide binding to membranes isolated from dog kidney outer medulla. Relationship to the Na,K,Cl co-transport system, J. Biol. Chem. 258 (1983) 11787–11792.
[24] B. Forbush, M. Haas, C.Y. Lytle, Na^+-K^+-Cl^- cotransport in the shark rectal gland. I. Regulation in the intact perfused gland, Am. J. Physiol. 262 (1992) C1000–C1008.
[25] J.A. Payne, J.-C. Xu, M. Haas, C. Lytle, D. Ward, B. Forbush, Primary structure, functional expression, and chromosomal localization of the bumetanide-sensitive Na-K-Cl cotransporter in human colon, J. Biol. Chem. 270 (1995) 17977–17985.