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Short-term exposure to waterborne free silver has acute effects on membrane current of *Xenopus* oocytes

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Abstract

Waterborne free silver can cause osmo- and ionoregulatory disturbances in freshwater organisms. The effects of a short-term exposure to extracellular Ag^+ ions on membrane currents were investigated in voltage-clamped defolliculated *Xenopus* oocytes. At a holding potential of −60 mV, ionic silver (1 μM Ag^+) increased inward currents (=I_{Ag}) from −8±2 nA to −665±41 nA (n=74; N=27). I_{Ag} activated within 2 min of silver exposure and then rose impetuously. This current was largely reversible by washout and repeatable. I_{Ag} reversed around −30 mV and rectified slightly at more positive potentials. Na^+-free bath conditions reduced the silver-induced current to a smaller but sustained current. The response to silver was abolished by the Cl^- channel blockers DIDS and SITS, whereas niflumic acid strongly potentiated I_{Ag}. Intraoocyte injection of $AgNO_3$ to about 1 mM $[Ag]_i$ strongly potentiated I_{Ag}. Extracellular application of either dithiothreitol (DTT), a compound known to reduce disulfide bridges, or L-cysteine abolished Ag^+-activated increase of membrane current. In contrast, n-ethylmaleimide (NEM) which oxidizes SH-groups potentiated I_{Ag}. Hypoosmotic bath solution significantly increased I_{Ag} whereas hyperosmolar conditions attenuated I_{Ag}. The activation of I_{Ag} was largely preserved after chelation of cytosolic Ca^{2+} ions with BAPTA/AM. Taken together, these data suggest that *Xenopus* oocytes are sensitive to short-term exposure to waterborne Ag^+ ions and that the elicited membrane currents result from extra- and intracellular action of Ag^+ ions on peptide moieties at the oocyte membrane but may also affect conductances after internalization.

Keywords: Silver; *Xenopus* oocyte; Gadolinium; Metal toxicity; Freshwater organism

1. Introduction

All freshwater life-forms are osmoregulators that have to actively absorb ions to maintain their ion homeostasis. Therefore, they are particularly susceptible to certain ionic forms of metals. The predominant mode-of-action for metal toxicity in aquatic organisms is via perturbations of ion regulation [1]. Previous studies have shown that different metal ions can produce a variety of rather specific effects on cells and any generalization about the physiological mechanisms of metal-induced toxicity is unlikely to be correct [2].

Silver, a transition metal, is most common in the +I (uc ai) oxidation state that in the following will be referred to as Ag(I). Although silver is most often complexed to organic or inorganic ligands aquatic organisms that live in ion poor water can be exposed to free ionic silver (Ag^+) [3]. Various studies addressed physiological impacts of exposures to waterborne Ag^+. At nanomolar concentrations, Ag^+ perturbs Na^+ and Cl^- homeostasis of freshwater fish and invertebrates [3,4] due to the inhibition of branchial Na^+/K^+-ATPase and carbonic anhydrase activity [5]. To exert its ionoregulatory disturbances silver has to be taken up by the organisms and enter cells. Freshwater fish, for instance, have been found to accumulate silver in a concentration dependent manner via a sodium uptake pathway [6]. Increasing the external Na^+ concentration reduced Ag^+

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uptake via the fish gill, whereas increasing other cations (K^+ and Ca^{2+}) had no effect. Phenamil, an analogue of the epithelial Na^+ channel blocker amiloride, and bafilomycin A_1, an inhibitor of V-type ATPases, both reduce silver uptake in freshwater fish [6]. In consequence of impaired absorption of ions, a depletion of extracellular ions is the primary cause of death of silver exposed organisms in freshwater. At higher concentrations Ag^+ has been shown to stimulate short circuit current (SCC) of various epithelial preparations [7–11]. In toad or frog skin the rise in SCC is attributed to either an increase in the shunt permeability or an altered cation selectivity of ion conductances [7]. Some of the physiological mechanisms of silver toxicity are believed to be due to Ag^+ binding to extracellular sulphydryl moieties of membrane proteins such as ionic channels or transporters [8,9].

While the chronic toxicity of silver due to long-term exposures has been subject to studies on freshwater organisms the present study investigated the impact of acute short-term exposure to Ag^+ ions on membrane conductances in native oocytes of *Xenopus laevis*. *Xenopus* oocytes are routinely used as a tool for heterologous expression and functional characterization of various ion transporters and ion channels. Therefore, the endogenous conductances and transport systems in native oocytes are well characterized and even sensitivities to various divalent metal cations have been described previously [12]. These amphibian oocytes are large cells with a large surface area to volume ratio. They are spawned into freshwater and after fertilization a delicate developmental program sets in. Endogenous membrane transport systems play a significant role in fertilisation and development and findings obtained in these oocytes can help to evaluate if silver toxicity poses a potential threat to reproduction of freshwater life-forms.

2. Materials and methods

2.1. Oocyte preparation

Female *Xenopus laevis* were obtained from Kaehler (Hamburg, Germany). Oocytes were isolated by partial ovariectomy. The incision was immediately adhered and after 2 days of isolation the frogs returned to aquaria. Oocytes were treated with 1.5 mg (1.3 IU) ml^{-1} collagenase (Serva) for 90 min in storage solution (in mM: 90 NaCl; 1 KCl; 2 $CaCl_2$; 5 HEPES, 2.5 pyruvate, 0.06 penicillin, 0.03 streptomycin, adjusted to pH 7.4 with NaOH). The follicular layers were removed by treatment with Ca^{2+}-free solution (in mM: 90 NaCl; 1 KCl, 0.5 EDTA, 5 HEPES, adjusted to pH 7.4 with NaOH) for 10 min. Oocytes were then stored at 14 °C in storage solution. Only healthy stage V or VI oocytes [13] were used for experiments.

2.2. Electrophysiological measurements with two-microelectrode voltage-clamp technique

Single oocytes were placed in plexiglass chambers of 1 ml volume and superfused with a normal bath solution (NBS) (in mM: 90 $NaNO_3$; 1 KNO_3; 2 $Ca(NO_3)_2$, 5 HEPES, adjusted to pH 7.4 with NaOH) at a constant rate (~2 ml/min). Cl^--ions were omitted from the solutions to ensure ionic silver (Ag^+) was the predominant silver species in the perfusate and not $AgCl_n^{n-}$ complexes [14]. Silver was added at a concentration of 1 μM $AgNO_3$. Isoosmolar Na^+-free bath solution comprised of (in mM:) 180 mannitol, 1 KNO_3; 2 $Ca(NO_3)_2$, 5 HEPES, adjusted to pH 7.4 with KOH. Transmembraneous currents were measured by the two-electrode voltage-clamp technique (OC-725B Oocyte clamp, Warner Inst. Corp., USA) interfaced to a computer via a CED 1401 (CED, Cambridge, UK). Microelectrodes for transmembrane current analysis were made from borosilicate glass capillaries. The voltage and current electrode were filled with 1 M or 3 M KCl, respectively, and had a resistance of 1–5 MΩ. Replacement of the Cl^- ions in the bathing solutions with nitrate and shielding bath electrodes with 2% Agar in 300 mM $NaNO_3$ solution left the Ag(I) almost exclusively as Ag^+ and not as AgCl complexes. Similar precautions were taken by other groups [14]. Oocytes were clamped at a holding potential of −60 mV and the holding current recorded on computer or on strip chart recorder, all experiments commenced once the current was stable. Inward currents are denoted by a downward deflection. I_{Ag} was determined after oocytes had been exposed to 1 μM Ag^+ for 5 min. Unless otherwise stated all experiments were performed in NBS. Results are reported as means ± SEM and represent the mean of n independent experiments with oocytes originating from N different donors. The results were analyzed with the Student's *t*-test or paired *t*-test and values which are significantly different are indicated.

2.3. Chemicals and experimental procedure

Chemicals for oocyte preparation, oocyte storage and experimental bath solutions were obtained from Fluka. The following compounds and blockers were purchased from Sigma-Aldrich: $AgNO_3$, 9AC (anthracene-9-carboxylic acid), EDTA (ethylenediaminetetraacetic acid), DIDS (diisothiocyanatostilbene-2,2-disulfonic acid), DPC (diphenylamine-2-carboxylic acid), DTT (dithiothreitol), $Gd(NO_3)_3$, L-cysteine, NEM (n-ethylmaleimide), NFA (niflumic acid), ouabain, penicillin, pyruvate, SITS (4-Acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid), streptomycin and trypsin. BAPTA/AM was purchased from Calbiochem Corp,. NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) was purchased from Tocris.

To avoid chemical complexation of Ag^+ ions in experiments using either SITS, DIDS, NEM, DTT or L-cysteine, these compounds were washed out for 2 min before $AgNO_3$ was added to the bath. Control experiments were conducted with oocytes from the same batch, respectively.

3. Results

3.1. Ag^+-ions stimulated clamp current in native oocytes

Defolliculated oocytes were superfused with normal bath solution (NBS) and clamped to a holding potential of −60 mV. After the membrane current had stabilized 1 μM $AgNO_3$ in NBS was superfused for 5 min. Such treatment increased the transmembrane current from −8 ± 2 nA to −665 ± 41 nA (n=74; N=27). The activation of this inward current (I_{Ag}) started within 2 min of exposure to silver (Fig. 1A). The range of I_{Ag} varied between oocyte batches and the clamp current in 7% of the batches did not respond to 1 μM Ag^+ (data not shown). A washout of Ag^+ ions resulted in a rapid decrease of I_{Ag}. However, I_{Ag} was not fully reversible (Fig. 1A) and the current stabilized at −131 ± 9 nA within 25 min (n=74, N=27). A following removal of extracellular Na^+ ions further reduced the current to −14 ± 4 nA (n=74, N=27). Extracellular Na^+-free conditions did not affect the baseline current (2 ± 4 nA) but eliminated the inward Na^+ current and thereby significantly reduced I_{Ag} to only 39% of the current obtained in NBS (from −617 ± 51 nA to −240 ± 45 nA (p=0.001, n=12, N=5)). The subsequent washout of Ag^+ ions reduced I_{Ag} under Na^+-free conditions by 154 ± 52 nA compared to 464 ± 64 nA in NBS.

As shown in Fig. 1B, the induction of I_{Ag} by Ag^+ ions was repeatable and a second application of silver increased I_{Ag} significantly from −703 ± 68 nA to −1755 ± 297 nA (p=0.005,

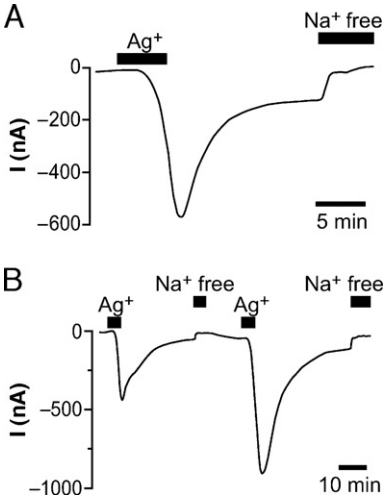

Fig. 1. (A) Time course of membrane current (I_{Ag}) elicited by 1 μM $AgNO_3$ in a *Xenopus* oocyte at a holding potential of −60 mV. The oocyte was continuously superfused with NBS and the exposure to $AgNO_3$ (1 μM for 5 min) is indicated by a bar. Note the latency of I_{Ag}. Washout of Ag^+ was followed by a superfusion with Na^+-free solution. (B) An example of the repeatable nature of I_{Ag}. The oocyte was clamped at a holding potential of −60 mV and repeatedly exposed to Ag^+. Note that after the second exposure I_{Ag} was markedly larger.

$n=10$, $N=5$). The sustained current after washout of Ag^+ did not change between repeated applications of Ag^+ (from −105±16 nA to −156±33 nA ($n=10$, $N=5$)). By clamping the membrane potential in 10 mV steps from −90 mV up to +30 mV, we determined the reversal potential (E_{rev}) to be −49 mV for oocytes in NBS. A 5 min treatment of oocytes with 1 μM Ag^+ shifted E_{rev} of I_{Ag} to −32 mV ($n=14$, $N=3$, Fig. 2A). This finding suggests an activation of rather unselective conductances and that an increased Na^+ conductance contributes to I_{Ag}.

3.2. Do intracellular Cl^- ions contribute to I_{Ag}?

The usage of Cl^--free bath solutions in our experiments may suggest that at least a fraction of I_{Ag} is due to an increased loss of Cl^- ions by the oocytes. A comparison of the current–voltage relationships after maximal activation of I_{Ag} either obtained under Cl^--free conditions or in presence of 90 mM extracellular Cl^- revealed only a minor shift in the reversal potential (Fig. 2B). However, we also tested several blockers of Cl^- channels. In the presence of the tested inhibitors $AgNO_3$ was not maintained in the bathing solution due to concerns regarding Ag^+ forming complexes with the blockers, and thereby reducing its bioavailability. Thus, 5 min prior to application of 1 μM Ag^+ the oocytes were pre-treated with the compounds. We found DPC, 9AC and NPPB (1 mM each) to have no effect on I_{Ag} ($n=5$, $N=3$) whereas niflumic acid (NFA, 1 mM) significantly potentiated I_{Ag} (327±34%; $n=5$, $N=3$; $p<0.001$) (Fig. 3). Interestingly, a pre-treatment of oocytes with either one of the two disulfonic Cl^- channel blockers (1 mM) DIDS or SITS abolished I_{Ag} to 1±2 nA ($n=11$; $N=2$; $p<0.001$) and 1±1 nA ($n=9$; $N=2$; $p<0.001$, Fig. 3), respectively. A substitution of NO_3^- for SO_4^{2-} did not affect the effect of silver ions (data not shown).

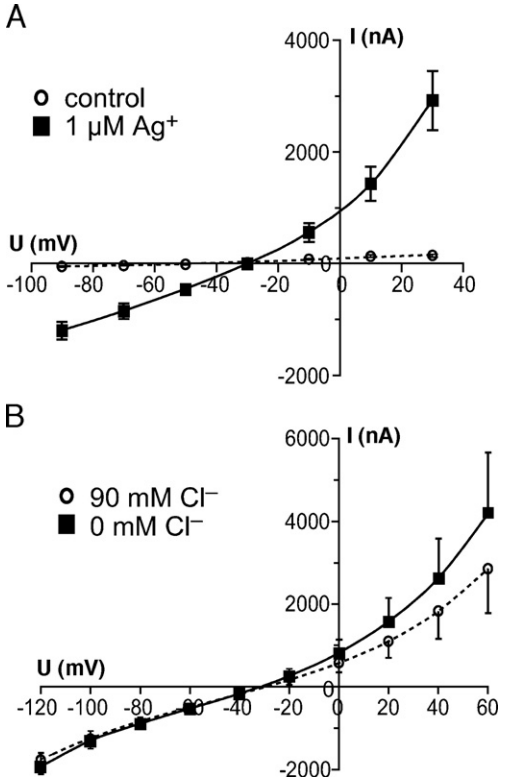

Fig. 2. (A) Current–voltage relationship of I_{Ag} induced in a *Xenopus* oocyte by a 5 min exposure to 1 μM Ag^+ in NBS. The filled squares show current evoked by 1 μM Ag^+ ($n=14$; $N=3$, open circles: control). (B) Current–voltage relationship of I_{Ag} induced by 1 μM Ag^+ in the presence (open circles) and absence of Cl^- ions (filled squares) in the bath solution ($n=4$; $N=2$). Voltage-step protocols were run when I_{Ag} reached steady-state.

3.3. Ag^+-induced mechanisms: restricted to the cell surface?

The steep rise of the activated I_{Ag} and its fast reversibility indicates an interaction of extracellular Ag^+ with surface expressed membrane proteins. Putative mechanisms for metal ions to affect gating of ion channels are interactions with sulfhydryl residues of extracellular amino acids. Free Ag^+ ions are very likely to bind these groups of extracellularly exposed amino acids. However, covalent modifications of the sulfhydryl

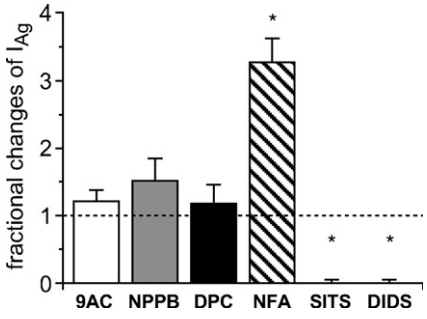

Fig. 3. Effect of chloride channel blockers on I_{Ag} in *Xenopus* oocytes. Two minutes prior to application of 1 μM Ag^+ the oocytes had been treated for 5 min with one of the compounds. The concentrations used were 1 mM of 9AC, NPPB, DPC or NFA ($n=5$, $N=3$;*$p<0.001$). Concentrations of DIDS ($n=11$; $N=2$; *$p<0.001$) and SITS ($n=9$; $N=2$; *$p<0.001$) were 1 mM. Shown are the fractional changes I_{Ag} in comparison to control.

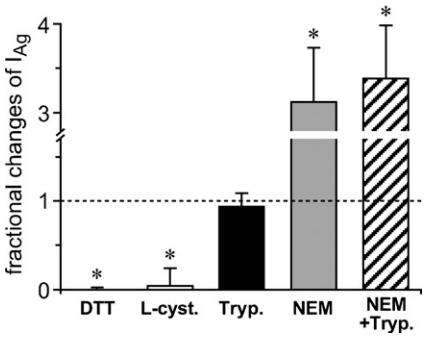

Fig. 4. Sulfhydryl-reactive compounds affect I_{Ag}. Interaction with extracellular SH-groups is one mechanism by which Ag^+ affects function of membrane proteins. *Xenopus* oocytes were pretreated for 5 min with either 1 mM DTT, 300 μM L-cysteine or 300 μM NEM. The compounds were washed out before 1 μM Ag^+ was added to the bath. DTT ($n=10$, $N=5$; *$p<0.001$) and L-cysteine ($n=11$; $N=4$; *$p<0.001$) significantly reduced I_{Ag} whereas NEM potentiated the effect of Ag^+ ($n=7$, $N=2$). Further, I_{Ag} is not sensitive to extracellular pretreatment of oocytes with trypsin. Oocytes were exposed for 5 min to trypsin (20 μg ml^{-1}) at RT. I_{Ag} in response to 1 μM $AgNO_3$ was determined at a holding potential of −60 mV ($n=7$, $N=2$).

groups are expected to be reversed after washout with Ag^+-free solution and in presence of O_2. In order to determine whether the activation of I_{Ag} is due to such an interaction, we tested the effect of dithiothreitol (DTT), a compound known to reduce disulfide bridges to sulfhydryl groups and thus maintaining additional SH-groups for Ag^+ to bind. Oocytes were pre-treated for 5 min with 1 mM DTT, which did not affect baseline current (0±0 nA; $n=10$; $N=5$). Two minutes prior to administration of Ag^+ ions, the DTT was removed. I_{Ag} was diminished in oocytes treated with DTT (Fig. 4). This inhibition of I_{Ag} by DTT was reversible within several minutes after washout of the agent (not shown). Further, we tested the effect of extracellular L-cysteine, a mildly reducing amino acid that is expected to have similar effects on disulfide bridges as DTT. A preincubation of oocytes for 5 min with 300 μM of L-cysteine was sufficient to prevent the activation of I_{Ag} (Fig. 4).

Interestingly, oxidation of thiol groups had the opposite effect. In a series of experiments using NEM, a cell-permeable sulfhydryl-reactive agent which oxidizes sulfhydryl groups, we obtained a significant increase of I_{Ag} from 1362±125 nA to 4171±459 nA ($n=7$, $N=2$). The exposure of cells to the protease trypsin has been shown to cleave extracellular portions of membrane proteins including ion channels and thereby affecting their gating. However, in our experiments a pre-treatment of oocytes with trypsin (20 μg ml^{-1}) for 5 (and up to 30) min did not significantly affect I_{Ag} in response to extracellular silver. Further, a preceding application of trypsin did not reduce the potentiation of I_{Ag} by NEM (Fig. 4).

3.4. I_{Ag}-inducing mechanisms: a cytosolic action?

Previous studies suggest that cells internalize Ag(I) when they are exposed to them and this uptake is linked to transcellular Na^+ transport [6]. The delayed onset of I_{Ag} after the oocytes were exposed to silver may indicate an internalization of Ag(I) which activates I_{Ag} via direct interactions with ion channels and associated proteins or by interference with downstream signaling mechanisms. Further, our results obtained with cell-permeable DTT or NEM may indicate cytosolic residues to be targeted by silver. To assess whether I_{Ag} was influenced by intracellular accumulation of silver, a small volume (50.6 nl) of 10 mM $AgNO_3$ was injected into oocytes (approx. 1 mM silver in the cytosol). Initially, the intracellular application of Ag^+ did not affect the baseline current at the holding potential of −60 mV. Nevertheless, I_{Ag} in response to a subsequent exposure to extracellular 1 μM Ag^+ was approximately doubled in oocytes after $AgNO_3$ was administered to their cytosol (Fig. 5). In addition the residual current after washout of Ag^+ was significantly increased (not shown).

The inhibition of branchial Na^+/K^+-ATPases is one mechanism by which silver exerts its toxicity in freshwater animals. We treated oocytes with 100 μM ouabain for 10 min prior to Ag^+ exposure. However, the I_{Ag} generated in oocytes treated with ouabain did not differ significantly from control oocytes (Fig. 5). In order to determine the importance of Ca^{2+} signalling mechanisms or the influx of Ca^{2+} ions for the induction of I_{Ag}, oocytes were treated with a calcium chelating agent. Oocytes had been either preincubated for 30 min under Ca^{2+}-free conditions with membrane permeable BAPTA/AM (500 μM) or injected with BAPTA/AM to a final 1 mM intracellular concentration. However, I_{Ag} in response to 1 μM Ag^+ of these BAPTA-loaded oocytes did not differ from the control (Fig. 5).

3.5. I_{Ag} and osmotic stress

In all experiments I_{Ag} activated with a latency of minutes after the oocytes were exposed to Ag^+ in the bath. These latencies and time courses of activation may indicate that

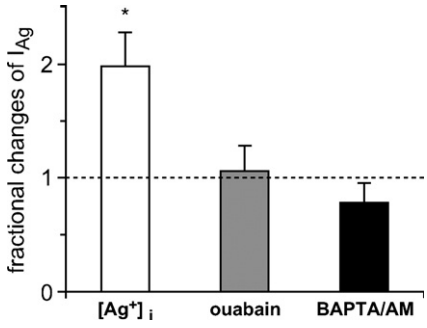

Fig. 5. Effect of intracellular $AgNO_3$, ouabain or BAPTA/AM on I_{Ag} in *Xenopus* oocytes. Intraoocyte injection of $AgNO_3$ potentiates I_{Ag}. Comparison of I_{Ag} in *Xenopus* oocytes injected with 10 mM $AgNO_3$ (50.6 nl) and water injected control. After the injection the oocytes was allowed 10 min for healing of the membranes. The effect of 1 μM Ag^+ was determined at a holding potential of −60 mV ($n=11$; $N=4$; *$p<0.05$). Neither inhibition of Na^+/K^+-ATPase activity nor chelation of intracellular calcium affects I_{Ag} in *Xenopus* oocytes. The inhibition of Na^+/K^+-ATPases is one mechanism by which silver exerts its toxicity. Na^+/K^+-ATPases of oocytes were inhibited with 100 μM ouabain for 10 min prior to application of 1 μM $AgNO_3$. I_{Ag} generated in oocytes treated with ouabain did not differ significantly from control oocytes ($n=7$, $N=3$). *Xenopus* oocytes had been loaded for 30 min under Ca^{2+}-free conditions with membrane permeable BAPTA/AM (500 μM in Ca^{2+}-free solution). I_{Ag} evoked by 1 μM $AgNO_3$ did not differ significantly from control oocytes ($n=8$, $N=5$).

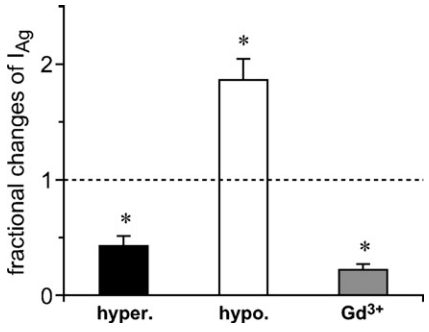

Fig. 6. Volume changes of *Xenopus* oocytes affect I_{Ag}. Oocytes were subjected for 10 min to either hypoosmotic (96.5 mosM, n=11, N=3) or hyperosmotic (293 mosM, n=11, N=3) conditions. Shrinkage of oocytes reduced I_{Ag} in response to 1 μM $AgNO_3$ (5 min exposure) whereas swelling potentiated I_{Ag}. I_{Ag} is markedly decreased by the lanthanoid Gd^{3+}. Oocytes had been exposed to 300 μM Gd^{3+} before I_{Ag} was induced (n=11, N=2).

cellular volume changes trigger at least a portion of the activation of I_{Ag}. In order to address the question whether the Ag^+ ions interfere with volume regulation, we osmotically challenged oocytes before I_{Ag} was induced. Swelling of oocytes was achieved by a hypoosmotic bath solution. A 50% reduction in osmolarity significantly increased I_{Ag}. In contrast, hyperosmolar conditions that result in the shrinkage of oocytes (the bath media contained additional 100 mM mannitol) attenuated I_{Ag} (Fig. 6).

Gadolinium (Gd^{3+}) and other lanthanides have been shown to block the nonselective stretch-activated cation and anion channels that are endogenously expressed in *Xenopus* oocytes [15–18]. Under isoosmolar conditions I_{Ag} proved to be sensitive to 300 μM Gd^{3+} which reduced I_{Ag} to 22±2% (n=11; N=2) of the initial control value (Fig. 6). The reversible portion of I_{Ag} after washout was significantly smaller with gadolinium than without. Further, under Na^+-free conditions I_{Ag} was drastically reduced by Gd^{3+} from −100±13 nA to −8±10 nA (n=10; N=3). Thus, the remaining non-Na^+ current was sensitive to Gd^{3+}.

4. Discussion

Various studies have shown that metal ions are toxic to osmoregulating organisms because they interfere with ion channel and transporter function. One toxicant is nanomolar waterborne free silver of anthropogenic origin that causes osmo- and ionoregulatory disturbances in freshwater fish and crustacea [5,19,20] via the inhibition of apical sodium entry [21] and/or the branchial Na^+/K^+ ATPases and carbonic anhydrases [5,20,22,23].

Silver, and other metals, have been shown to effect transepithelial ion transport of amphibian skin preparations. A silver-induced stimulation of short circuit current across amphibian epithelia has been attributed to both Ag^+ and the circumneutral AgCl [9,23]. Curran [7], utilising a sulphate based medium, showed that Ag(I) can stimulate frog skin SSC, and this was probably due to increased K^+ permeability rather than Na^+ permeability. Gerencser et al. [8] demonstrated that Ag (I) only elicited a response in toad skin in the presence of NaCl in the perfusate, and the increased inward movement of Na^+ could be induced by the addition of Ag^+ to the basolateral bathing medium. Interestingly, the increased permeability of the frog skin induced by silver resulted not just in an increase in Na^+ and K^+ movement, but also in an increase in uptake of Ag (I) by the skin [7].

In *Xenopus* oocytes the presence of extracellular divalent cations has been shown to evoke cation-specific electrophysiological responses. For example, Co^{2+} elicited slow oscillatory Cl^- currents, whereas Zn^{2+} induced a smooth K^+ current [12]. In our experiments silver, added as $AgNO_3$ to a chloride-free media to ensure the majority of the metal being present as Ag^+, induced I_{Ag} with a short latency and the activated inward current reached steady state within minutes. I_{Ag} was partially reversible by washout but a small fraction of I_{Ag} persisted after removal of Ag^+. The reversible portion of I_{Ag} suggests that Ag^+ interacts with extracellular moieties of membrane proteins. However, the lag time before onset of I_{Ag} and the washout-resistant current might be due to intracellular mechanisms that are activated by cytosolic silver or covalent intracellular modifications of membrane proteins. This may explain our observation that I_{Ag} got larger with repeated exposures of oocytes to Ag^+ (Fig. 1B) and thus accumulation of cytosolic silver. This notion is supported by an increased I_{Ag} observed after intraoocyte injection of $AgNO_3$ (Fig. 5).

4.1. Non-selective cation channels

Xenopus oocytes express a plethora of endogenous ion channels in their plasma membrane including non-selective channels with low preference for specific cations [24,25]. Under our experimental conditions I_{Ag} was predominantly carried by sodium ions because Na^+-free conditions decreased I_{Ag} to 39% compared to control conditions. E_{rev} of I_{Ag} was shifted by +17 mV to −32 mV due to additional Na^+- and possibly Ca^{2+} conductances. Further, I_{Ag} could be activated in Na^+-free but either K^+- or Cs^+-containing salines (Bury and Schnizler, personal observations) suggesting activation of non-selective cationic conductances.

Xenopus oocytes have a low permeability for Cl^- ions under resting conditions and the minor shift in the I/V relationship obtained under Cl^--free conditions (Fig. 2B) did not indicate a significant contribution of Cl^- currents to I_{Ag}. However, DIDS and SITS, two to a certain degree membrane permeable blockers of chloride channels abolished I_{Ag}. These compounds are known to react with ε-NH_2 groups of lysines to yield covalent adducts. Both stilbenedisulfonates are not only rather unspecific blockers of various anion channels but they also affect cation conductances. This and the fact that DIDS and SITS can react with sulfhydryl groups and thereby occupy putative Ag-binding sites make it difficult to speculate about the ion channels that are responsible for I_{Ag}.

The fluorine atoms of niflumic acid readily form hydrogen bonds with amino acid residues. NFA largely potentiated I_{Ag} but a variety of endogenous ion channels in the oocytes are sensitive to this agent [24,25]. In particular, NFA was reported to reversibly block Ca^{2+}-activated Cl^- channels in *Xenopus*

oocytes [26]. However, I_{Ag} is unlikely to be generated by Ca^{2+}-activated conductances since the injection of Ca^{2+} chelators as BAPTA (Fig. 5) or EGTA (not shown) had no effect on I_{Ag}. Presumably, Ag^+ does not interact with the signalling system which involves mobilization of intracellular Ca^{2+}-ions. DIDS, SITS and NFA may either competitively occupy binding sites for Ag(I) or their interactions have allosteric effects on the activation of I_{Ag} due to conformational changes of membrane proteins that are involved.

The common action of extracellular Ag^+ appears to be an interaction with surface expressed membrane peptide moieties that influence ion conductances. This may be either due to binding to specific structures that regulate gating of channels or unspecific binding that distorts the structural integrity of the channel protein [14]. The strong covalent bonds that Ag^+ ions form with SH-groups would suggest an interaction with cysteines. Further, Ag^+ interacts by forming weaker bonds to other amino acid terminal moieties such as imidazole or carboxyl groups. In previous studies, the addition of sulphydryl-containing compounds (i.e. glutathione or cysteine) to the medium inhibited silver-induced SCC in amphibian skin epithelia and suggested that the action of Ag^+ or AgCl is via binding to membrane bound sulphydryl groups [7,9].

In our experiments oxidizing agents sensitized Ag-activated current whereas reducing agents abolished I_{Ag}. An external action of Ag(I) is likely because in our study I_{Ag} was abolished after oocytes were treated with either DTT or L-cysteine (Fig. 4). Both compounds are expected to reduce disulfide bridges and thus provide more SH-groups to interact with Ag^+ ions. Presumably, L-cysteine not only opened disulfide bridges but also occupied some of the sulphydryl groups before Ag(I) could bind them. Surprisingly, DTT and L-cysteine diminished I_{Ag} but this was reversible after several minutes of recovery without the compound. DTT is a cell-permeable dithiol-reducing agent and thus it is not clear whether certain extra- or intracellular disulfide bonds of ion channels or transporters must not be reduced in order to confer the silver effect.

In order to substantiate the notion that I_{Ag} was activated by modulation of redox-sensitive sites we used NEM, a cell-permeable sulfhydryl alkylating agent. NEM covalently modifies cysteine residues in proteins and possibly prevents further chemical oxidation or reduction of these sites. Modifications by these redox-active substances affect tertiary and quarternary structures of membrane proteins. The susceptibility of I_{Ag} to these redox-active compounds may result from allosteric and/or conformational changes in channel protein complexes. Further, alterations of inter- or intramolecular disulfide crosslinks affect protein–protein interactions or the activity of proteins associated with Ag-sensitive ion channels. The proteolysis of membrane structures by extracellular trypsin did not change I_{Ag} but this does not necessarily argue against an extracellular action of silver. The relevant membrane proteins may simply lack accessible cleavage sites or cleavages do not interfere e.g. with Ag-induced protein–protein interactions. Interestingly, in *Xenopus* oocytes trypsin has been shown to specifically activate endogenous Ca^{2+}-mobilizing G protein-coupled receptors [27,28]. Therefore, our data provide indirect evidence for intracellular calcium not being crucial for mediating the activation of I_{Ag}. The potentiating effect of the cell-permeable NEM on I_{Ag} and its resistance to trypsin (Fig. 4) may suggest that the activation of I_{Ag} is at least partially attributable to modifications of intracellular protein thiol groups. However, not all of the batches of oocytes responded to Ag^+ treatment and the extent of stimulation was variable indicating that I_{Ag} was not a biophysical artefact, reasoned by Ag^+ in the bath solutions (not shown).

Our results from injecting oocytes with $AgNO_3$ showed that intracellular Ag^+ potentiated I_{Ag}. This suggests that, besides extracellular Ag^+-mediated modulation, specific intracellular mechanisms, perhaps secondary messenger pathways or direct modifications of channels or transporters, are involved in generation of I_{Ag}. Several authors claim an inhibition of the Na^+/K^+ ATPase to be one key mechanism for cytotoxicity of silver. Ag^+ is known to inhibit Na^+/K^+ ATPase activity by interaction with the intracellular Mg^{2+} binding site of the subunit of this enzyme [29–31]. However, our studies did not determine the inhibition of pump activity to be involved in generating I_{Ag} because ouabain had no effect (Fig. 5).

4.2. Mechanosensitive cation channels

Xenopus oocytes lack intrinsic volume-regulatory mechanisms but express mechanosensitive non-selective cation channels (MSCC) [18,32,33]. MSCC are normally quiescent and are activated upon an increase in cell volume or physical stretch. These endogenous stretch-activated cation channels proved to be sensitive to gadolinium [16,17] and may belong to the canonical transient receptor potential channels (TRPC) [34]. I_{Ag} was largely mediated by a net uptake of Na^+ that was accompanied by osmotically obliged water and thereby swelling of the oocytes. The fact that cell swelling due to hypoosmotic conditions (96.5 mosM) increased I_{Ag} whereas hyperosmotic bath solution (293 mosM) and thus shrinkage of the oocytes reduced I_{Ag} indicates volume-sensitive mechanisms to be involved in generating I_{Ag}. Further, the partial sensitivity of I_{Ag} to micromolar concentrations of Gd^{3+} may indeed suggest an involvement of MSCC in Ag^+ induced upregulation of cation currents [18]. Other osmo-sensitive transport systems in oocytes have been reported not only to be sensitive to Gd^{3+} but also to be prone to the unspecific actions of DIDS and SITS [15,35]. Considering the inhibition of I_{Ag} by DIDS or SITS it is conceivable that these transports systems contribute to I_{Ag}. Moreover, *Xenopus* oocytes express endogenous hypertonicity-activated Na^+/H^+ exchangers (NHE) that are sensitive to DIDS [32]. Remarkably, branchial apical silver uptake in the fish gill has been linked to proton-coupled Na^+ currents [6] and perhaps analogue mechanisms of silver actions are to be found in amphibian oocytes.

In this study, we have shown that waterborne free silver affects the electrophysiological properties of *Xenopus laevis* oocytes. Ag^+ activated I_{Ag} via an interaction with extracellular sites of membrane proteins and increasing the intraoocyte concentration of silver potentiated the effects of extracellular Ag^+. This suggests that acute short-term exposure to Ag^+ ions is

potentially toxic to *Xenopus* oocytes by interfering with ion homeostasis. However, water chemistry greatly influences silver speciation [36] and thus, it will be of interest to test whether other forms of Ag (e.g. AgCl) elicit similar electrophysiological responses in amphibian oocytes and may impair reproduction.

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References

[1] C.M. Wood, Toxic response of the gill, in: D. Schlenk, W.H. Benson (Eds.), Target organ toxicity of marine and freshwater teleosts, Taylor & Francis, New York, USA, 2001, pp. 1–89.
[2] H. Passow, A. Rothstein, T.W. Clarkson, The general pharmacology of the heavy metals, Pharmacol. Rev. 13 (1961) 185–224.
[3] C. Hogstrand, C.M. Wood, Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: implications for water quality criteria, Environ. Toxicol. Chem. 17 (1998) 547–561.
[4] N.R. Bury, J. Shaw, C. Glover, C. Hogstrand, Derivation of a toxicity based model to predict how water chemistry influences silver toxicity to invertebrates, Comp. Biochem. Physiol., C Toxicol. Pharmacol. 133 (2002) 259–270.
[5] I.J. Morgan, R.P. Henry, C.M. Wood, The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (Oncorhynchus mykiss) is inhibition of Na^+ and Cl^- transport, Aquat. Toxicol. 38 (1997) 145–163.
[6] N.R. Bury, C.M. Wood, Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel, Am. J. Physiol. 277 (1999) R1385–R1391.
[7] P.F. Curran, Effect of silver ion on permeability properties of frog skin, Biochim. Biophys. Acta 288 (1972) 90–97.
[8] G.A. Gerenscer, K.M. Cornette, S.Y. Loo, S.K. Hong, Effect of silver chloride on the short-circuit current across the isolated toad skin, Life Sci. 20 (1977) 1883–1889.
[9] G.A. Gerencser, S.Y. Loo, Glutathione inhibition of silver-enhanced sodium transport across toad skin, Comp. Biochem. Physiol., C Pharmacol Toxicol. Endocrinol. 114 (1996) 67–70.
[10] S.D. Klyce, W.S. Marshall, Effects of Ag^+ on ion transport by the corneal epithelium of the rabbit, J. Memb. Biol. 66 (1982) 133–144.
[11] J.H. Li, R.C. de Sousa, Effects of Ag^+ on frog skin: interactions with oxytocin, amiloride and ouabain, Experientia 33 (1977) 433–436.
[12] R. Miledi, J. Parker, R.M. Woodward, Membrane currents elicited by divalent cations in *Xenopus* oocytes, J. Physiol. 417 (1989) 173–195.
[13] N. Dascal, The use of *Xenopus* oocytes for the study of ion channels, CRC Crit. Rev. Biochem. 22 (1987) 317–387.
[14] C. Dart, M.L. Leyland, R. Barrett-Jolley, P.A. Shelton, P.J. Spencer, E.C. Conley, M.J. Sutcliffe, P.R. Stanfield, The dependence of Ag^+ block of a potassium channel, murine Kir 2.1, on a cysteine residue in the selectivity filter, J. Physiol. 511 (1998) 15–24.
[15] M.J. Ackerman, K.D. Wickman, D.E. Clapham, Hypotonicity activates a native chloride current in *Xenopus* oocytes, J. Gen. Physiol. 103 (1994) 153–179.
[16] R.A. Caldwell, H.F. Clemo, C.M. Baumgarten, Using gadolinium to identify stretch-activated channels: technical considerations, Am. J. Physiol. 275 (1998) C619–C621.
[17] O.P. Hamill, D.W. McBride Jr., The pharmacology of mechanogated membrane ion channels, Pharmacol. Rev. 48 (1996) 231–252.
[18] X.C. Yang, F. Sachs, Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions, Science 243 (1989) 1068–1071.
[19] A. Bianchini, M. Grosell, S.M. Gregory, C.M. Wood, Acute silver toxicity in aquatic animals is a function of sodium uptake rate, Environ. Sci. Technol. 36 (2002) 1763–1766.
[20] C.M. Wood, C. Hogstrand, F. Galvez, R.S. Munger, The physiology of waterborne silver toxicity In freshwater rainbow trout (Oncorhynchus mykiss) 1. The effects of ionic Ag^+ speciation, Aquat. Toxicol. 35 (1996) 93–109.
[21] T.P. Morgan, M. Grosell, R.C. Playle, C.M. Wood, The time course of silver accumulation in rainbow trout during static exposure to silver nitrate: physiological regulation or an artifact of the exposure conditions? Aquat. Toxicol. 66 (2004) 55–72.
[22] J.C. McGeer, C.M. Wood, Protective effects of water Cl^- on physiological responses to waterborne silver in rainbow trout, Can. J. Fish Aquat. Sci. 55 (1998) 2447–2454.
[23] M. Walser, Calcium transport in the toad bladder: permeability to calcium ions, Am. J. Physiol. 218 (1970) 582–589.
[24] W.M. Weber, Endogenous ion channels in oocytes of *Xenopus laevis*: recent developments, J. Membr. Biol. 170 (1999) 1–12.
[25] W.M. Weber, Ion currents of *Xenopus laevis* oocytes: state of the art, Biochim. Biophys. Acta 1421 (1999) 213–233.
[26] M.M. White, M. Aylwin, Niflumic and flufenamic acids are potent reversible blockers of $Ca2^+$-activated Cl^- channels in *Xenopus* oocytes, Mol. Pharmacol. 37 (1990) 720–724.
[27] M.E. Durieux, M.N. Salafranca, K.R. Lynch, Trypsin Induces $Ca2^+$-activated Cl^- currents in *X. laevis* oocytes, FEBS Lett. 337 (1994) 235–240.
[28] H. Shapira, I. Amit, M. Revach, Y. Oron, J.F. Battey, Gα14 and Gαq mediate the response to trypsin in *Xenopus* oocytes, J. Biol. Chem. 273 (31) (1998) 179–185.
[29] N.R. Bury, J.C. McGeer, C.M. Wood, Effects of altering freshwater chemistry on physiological responses of rainbow trout to silver exposure, Environ. Toxicol. Chem. 18 (1999) 49–55.
[30] E.A. Ferguson, D.A. Leach, C. Hogstrand, Metallothionein protects against silver blockage of Na^+/K^+-ATPase, Proceedings, 4th International Conference on Transport, Fate, and Effects of Silver in the Environment, Madison, WI, USA, 1997, pp. 191–197.
[31] S. Hussain, E. Meneghini, M. Moosmayer, D. Lacotte, B.M. Anner, Potent and reversible interaction of silver with pure Na,K-ATPase and Na,K-ATPase-liposomes, Biochim. Biophys. Acta 1190 (1994) 402–408.
[32] L. Jiang, M.N. Chernova, S.L. Alper, Secondary regulatory volume increase conferred on *Xenopus* oocytes by expression of AE2 anion exchanger, Am. J. Physiol. 272 (1997) C191–C202.
[33] C. Methfessel, V. Witzemann, T. Takahashi, M. Mishina, S. Numa, B. Sakmann, Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels, Pflügers Arch. 407 (1986) 577–588.
[34] R. Maroto, A. Raso, T.G. Wood, A. Kurosky, B. Martinac, O.P. Hamill, TRPC1 forms the stretch-activated cation channel in vertebrate cells, Nat. Cell. Biol. 7 (2) (2005) 179–185.
[35] A. Diakov, J.P. Koch, O. Ducoudret, S. Müller-Berger, E. Frömter, The disulfonic stilbene DIDS and the marine poison maitotoxin activate the same two types of endogenous cation conductance in the cell membrane of *Xenopus laevis* oocytes, Pflügers Arch. 442 (2001) 700–708.
[36] J.R. Kramer, G. Benoit, K.C. Bowles, D.M. Di Toro, R.T. Herrin, G.W. Luther III, H. Manolopoulos, K.A. Robillard, M.M. Shafer, J.R. Shaw, in: A.W. Andren, T.W. Bober (Eds.), Environmental Chemistry of Silver in the Environment: Transport, Fate and Effects, Setac Press, Pensacola, FL, USA, 2002, pp. 1–25.