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Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel

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Bury, Nicolas R., and Chris M. Wood. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. *Am. J. Physiol.* 277 (*Regulatory Integrative Comp. Physiol.* 46): R1385–R1391, 1999.—The branchial uptake mechanism of the nonessential heavy metal silver from very dilute media by the gills of freshwater rainbow trout was investigated. At concentrations >36 nM $AgNO_3$, silver rapidly entered the gills, reaching a peak at 1 h, after which time there was a steady decline in gill silver concentration and a resulting increase in body silver accumulation. Below 36 nM $AgNO_3$, there was only a very gradual increase in gill and body silver concentration over the 48-h exposure period. Increasing water sodium concentration ($[Na^+]$; 0.05 to 21 mM) significantly reduced silver uptake, although, in contrast, increasing ambient $[Ca^{2+}]$ or $[K^+]$ up to 10 mM did not reduce silver uptake. Kinetic analysis of silver uptake at varying $[Na^+]$ showed a significant decrease in maximal silver transport capacity (173 ± 34 $pmol \cdot g^{-1} \cdot h^{-1}$ at 0.1 mM $[Na^+]$ compared with 35 ± 9 at 13 mM $[Na^+]$) and only a slight decrease in the affinity for silver transport (K_m; 55 ± 27 nM at 0.1 mM $[Na^+]$ compared with 91 ± 47 nM at 13 mM $[Na^+]$). Phenamil (a specific blocker of Na^+ channels), at a concentration of 100 μM, blocked Na^+ uptake by 78% of control values (58% after washout), and bafilomycin A_1 (a specific blocker of V-type ATPase), at a concentration of 2 μM, inhibited Na^+ uptake by 57% of control values, demonstrating the presence of a proton-coupled Na^+ channel in the apical membrane of the gills. Phenamil (after washout) and bafilomycin A_1 also blocked silver uptake by 62 and 79% of control values, respectively, indicating that Ag^+ is able to enter the apical membrane via the proton-coupled Na^+ channel.

silver transport; teleost; sodium channel; phenamil; bafilomycin A_1

SILVER, WHEN PRESENT as silver nitrate, is one of the most toxic metals to freshwater fish, with a 96-h half-maximal lethal concentration of between 5 and 70 μg total Ag/l (45–650 nM) (11). However, water chemistry influences toxicity (11) and, in the case of freshwater rainbow trout, silver toxicity can be correlated to the "free" water ionic silver (Ag^+) concentration (2, 3, 8, 19). In freshwater rainbow trout, the toxic mechanism for $AgNO_3$ involves the inhibition of Na^+ and Cl^- uptake at the gills (20), resulting in a decline in plasma ion levels, which in turn results in perturbed internal fluid-volume regulation and hemoconcentration, with death occurring due to cardiovascular collapse (11, 30). The perturbation to ionoregulation is a result of inhibition of the branchial enzymes involved in ion transport (3, 19, 20). The principal enzyme affected by silver is the Na^+/K^+-ATPase, which is situated on the basolateral membrane of gill cells (13). Consequently, silver must cross the apical membrane and traverse the cytoplasm before exerting its toxic action.

Silver does not affect Ca^{2+} transport (30), but divalent heavy metals, such as cadmium, zinc, and cobalt, have been shown to perturb branchial Ca^{2+} uptake in freshwater fish (4, 10, 25). Inhibition of Ca^{2+} uptake by these metals is primarily due to interference with basolateral membrane Ca^{2+} extrusion, although there is also competition with Ca^{2+} at the lanthanum-sensitive Ca^{2+} channel present on the mitochondria-rich chloride cells of the gills (22). There is now clear evidence that Zn, Cd, and Co enter the gills of freshwater fish via the apically situated Ca^{2+} channel. In the presence of increased ambient Ca^{2+} concentration, the affinity of the transport system for Zn, Cd, and Co decreases [i.e., affinity constant (K_m) increases], illustrating competition between Ca^{2+} and the respective metal ions. Treatments that reduce branchial Ca^{2+} entry, such as inducing hypercalcemia by injecting $CaCl_2$ or blockade of the Ca^{2+} channel by lanthanum (13) or diltiazem (5), also reduce Cd, Zn, and Co uptake.

By analogy to the premise that metals that inhibit Ca^{2+} transport share the same branchial Ca^{2+} uptake process, then it is feasible that silver, which inhibits Na^+ transport, may share the apical Na^+ uptake pathway of freshwater fish gills (2, 3, 19, 20, 30). There are two hypotheses for apical Na^+ uptake by freshwater fish (24). The first is that Na^+ is exchanged for protons by an antiport carrier. However, a second system, first proposed by Avella and Bornancin (1), is now favored and is comparable to the model for Na^+ uptake in the frog skin (7). Protons are transported across the apical membrane by an H^+-ATPase, creating an electrochemical gradient for the transport of Na^+ into the cell via an amiloride-sensitive channel (1, 17, 18, 21, 23).

The main objectives of the present study were to describe the time course and kinetics of silver uptake (presumably as the free ion, Ag^+) and to determine whether Ag^+ can share the apical Na^+ uptake pathway. For the latter objective, three experimental approaches were taken. First, we tested whether increasing water cation concentration (Ca^{2+}, K^+, Na^+) influences whole body silver uptake. Second, having established the potency of Na^+ in this regard, we evaluated the nature of the competition via classical Michaelis-Menten analy-

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sis. Finally, we tested whether the drugs phenamil, a specific blocker of the sodium channel (9), or bafilomycin A_1, a specific inhibitor of V-type ATPases (28), inhibits Ag^+ entry via a proton-coupled sodium channel.

MATERIALS AND METHODS

Fish husbandry. Rainbow trout fingerlings (3–10 g) were obtained from Humber Springs Hatchery, Orangeville, ON, Canada, and maintained in flowing dechlorinated Hamilton tap water (approximate ionic composition in mM: 0.5 [Na^+], 0.7 [Cl^-], 1 [Ca^{2+}], 0.2 [Mg^{2+}], and 0.05 [K^+], pH 7.8–8.0). Fish were fed ~1% of their body weight daily (Martin Mills, Tavistock, ON, Canada). All fish were acclimated to synthetic soft water (dechlorinated Hamilton tap water treated by reverse osmosis) over a 2-wk period and were maintained in soft water (approximate ionic composition in mM: 0.05 [Na^+], 0.05 [Cl^-], 0.05 [Ca^{2+}], 0.02 [Mg^{2+}], and 0.02 [K^+], pH 6.8 and temperature 10–15°C) for a further 2 wk before experiments, during which time the fish were fed a normal ration.

Time course study. Silver was added to darkened Plexiglas boxes containing 10 liters of synthetic soft water (composition as above), with a combination of $^{110m}AgNO_3$ (Amersham International, 94.3 Mbq/ml, 2.5 mCi/ml) and unlabeled $AgNO_3$ at least 16 h before addition of the fish, a procedure designed to ensure saturation of Ag binding sites on the box walls so that silver levels would remain constant during the subsequent experiment. Each box was treated with a different total silver concentration. Water pH was adjusted to 6.0–7.0 by addition of KOH. A total of 64 fish was placed into each container, and 8 fish were sampled at 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h. Throughout the experiment, the water received continuous aeration. All fish in this experiment and all subsequent experiments received a wash procedure to remove loosely bound $AgNO_3$. Initially, fish were anesthetized with MS-222 (200 mg/l), washed for 1 min in 150 mg/l NaS_2O_3 and then 250 μg Ag/l, and finally killed by an overdose of MS-222. Gills were excised, and then the body and gills were counted separately on a gamma counter (MINAXI autogamma 5000 series, Canberra Packard). At each time point, a 10-ml water sample was taken for radioactivity counting and ion and silver analysis (see below). The average measured total Ag concentrations ($n = 7$) used in the exposure were 13 ± 1, 21 ± 4, 36 ± 3, and 128 ± 22 nM.

Uptake kinetics study. Ten total silver concentrations, in synthetic soft water (composition as above), were tested in separate containers (11, 19, 37, 46, 77, 84, 88, 118, 228, and 289 nM $AgNO_3$). Eight fish were placed in 400-ml black Plexiglas containers 16 h before the experiment. The containers received silver-free soft water at a flow rate of 40 ml/min throughout this period and were continuously aerated. At the start of the experiment, the water flow was stopped and a combination of $^{110m}AgNO_3$ and unlabeled $AgNO_3$ to yield the desired total silver concentrations was added. In this and all subsequent experiments, a 10-ml water sample was taken 15 min after starting to allow thorough mixing of the radiolabel- and a further 10-ml sample was taken at the end of the experiment (2 h) for radioactivity counts and water ion and total silver measurements (see below). Gills were counted separately from the body.

Competition studies. All subsequent experiments were performed in deionized water (Barnstead Nanopure system), rather than synthetic soft water, to eliminate the effects of complexation on the free silver ion activity. Consequently, we predominately studied the uptake of ionic silver, Ag^+, and not the Ag complexes (such as $AgCl_0$) that we have shown accumulate in rainbow trout (3, 11, 19). Black Plexiglas containers were filled with 400 ml of deionized water, pH was adjusted to 6.0–7.0 with KOH, and seven or eight fish were added. These containers received continuous aeration. A combination of ~50 nM $^{110m}AgNO_3$ and the increasing concentrations of $Ca(NO_3)_2$, KNO_3, or $NaNO_3$ was added. In addition to the competition studies described above, the nature of Na^+ interaction with silver uptake was studied more thoroughly by performing a sequence of experiments in which the effects of three Na^+ concentrations (0.1, 2.3, and 13 mM) on silver uptake at various total silver concentrations (6, 25, 40, 95, and 175 nM) were investigated. Water samples were taken, and fish were sampled (after 2 h exposure) as described above.

Pharmacological studies. The basic structure of amiloride and its analogs consists of a pyrazine ring with an acylguanidine group attached (15). Pharmacological studies are complicated by the fact that these compounds will avidly bind heavy metals such as Cu and Ag (M. Grosell, N. R. Bury, and C. M. Wood, personal observation). This binding, rather than competition, may inhibit metal uptake when the drug remains present in solution. Phenamil is an analog of amiloride, which specifically inhibits the Na^+ channel and, in vitro, has been shown to be a nonreversible inhibitor of Na^+ influx (9, 15). The nonreversible nature of phenamil means that after a preincubation period, the compound can be removed from the bathing solution and still be pharmacologically active. This approach avoids the problem of the drug complexing silver in solution and inadvertently affecting silver influx by this nonspecific mechanism. Studies using phenamil to investigate Na^+ transport have primarily been conducted in vitro (15). Consequently, preliminary studies were performed to find the effective dose range and to confirm that this drug also acts as a nonreversible inhibitor of Na^+ influx in vivo. For each trial studying the effects of phenamil on Na^+ influx, eight fish were transferred to 400 ml of deionized water containing 50 μM $NaNO_3$ and 50 μM $Ca(NO_3)_2$ adjusted to pH 6.0–7.0 with KOH; containers were continuously aerated. Preliminary studies showed that the Na^+ influx was strongly inhibited (78%) by the presence of 100 μM phenamil (RBI, Natick, MA) compared with controls, whereas a concentration of 1 μM phenamil was without effect. The Na^+ influx rates of fish that received a 3-h incubation in 100 μM phenamil, followed by a washout procedure to remove the phenamil, were inhibited by 58% compared with controls (receiving the same washout procedure in the absence of phenamil), thereby demonstrating the nonreversible inhibitory nature of the drug phenamil on sodium uptake.

To determine if phenamil blocked silver entry in a comparable fashion, fish were placed in the same media containing 100 μM phenamil for 3 h, after which the fish were removed and placed in drug-free media. The medium was then discarded, the containers were rinsed, 400 ml of the aforementioned water was added containing either 50 nM $^{110m}AgNO_3$ or 50 μM Na^+ plus 2 μCi/l $^{22}Na^+$ (NEN-DuPont), and the fish were replaced. Fish treated in the same way but without phenamil acted as controls. Ag and Na^+ uptake were determined by accumulation of radiolabel over a 1-h period. Fish exposed to $^{22}Na^+$ were then washed in excess $NaNO_3$ and killed by the addition of an excess of MS-222; whole body radioactivity was counted. Fish exposed to $^{110m}AgNO_3$ were washed and killed as described above.

Because of the high cost and limited availability of bafilomycin A_1, experiments with this drug were performed using very small rainbow trout alevins to economize on volumes. Tests were performed on 21-day posthatch rainbow trout alevins (approximate weight 100 mg) obtained from Rainbow Springs

Trout Farm (Thamesford, ON, Canada). At this stage of development the yolk sac was 90% absorbed, but exogenous feeding had not begun, and the alevins were starting to swim up. Acclimation to soft water followed the protocol described above for the rainbow trout fingerlings.

Four groups of 10 alevins each were placed in 30 ml of deionized water containing 50 μM $NaNO_3$ and 50 μM $Ca(NO_3)_2$ adjusted to pH 6.0–7.0 with KOH 1 h before starting the experiment. During this time, as well as throughout the experiment, the solution was continuously aerated and kept at 15°C. Bafilomycin A_1 was obtained from Prof. K. Altendorf, Universität Osnabrück, Osnabrück, Germany, and its concentration was determined using the methods of Werner et al. (28). A 2.6 mM bafilomycin A_1 stock solution was prepared in dimethyl sulfoxide (DMSO). One hour before measurement of flux rates, two containers received 0.08% DMSO (vehicle control) and the remaining two containers received 2 μM bafilomycin A_1 (+0.08% DMSO). One control and one experimental container then received a spike of 50 nM $^{110m}AgNO_3$, whereas the other control and experimental containers received a spike of 50 μM $(^{24}Na)_2CO_3$ (0.05Ci/ml, made at McMaster University Nuclear Reactor). Water samples were taken 5 min after spiking and then 2 h later, at which point the experiment was terminated. Fish were killed and washed, and whole body radioactivity was counted as described above. Note that with this procedure, the total time during which the fish were subjected to a closed system was the same in the phenamil and bafilomycin A_1 experiments, and the flux measurements were also made at a comparable time.

Water total silver, ammonia, and ion measurements, calculation, and statistics. Water ion concentrations were measured by an atomic absorption spectrophotometer (Varian 1275-AA, Mississauga, ON, Canada), and silver concentrations were measured by graphite furnace atomic absorption spectrophometry (Varian 1275-AA fitted with a GTA-95 atomizer). Water ammonia levels were determined by the methods of Verdouw et al. (26). Water ammonia did not exceed 10.3 μM NH_4^+/l in any experiment. Water containing $^{110m}AgNO_3$ could not be analyzed for dissolved organic carbon (DOC) content, because it would irreversibly contaminate the DOC analyzer. Consequently, it was not possible to accurately determine silver speciation. The inward Ag and Na^+ influxes were calculated from the formula $J_{in} = CT/(SA \cdot wt \cdot t)$, where CT is the counts present in the tissue (cpm), SA is the measured specific activity of the water [cpm/(min·nmol)], wt is wet weight of the tissue (g), and t is the time of exposure (h). For time course experiments, t was omitted from the calculation. Kinetic parameters [maximal flux (J_{max}) and K_m] were calculated, fitting the data to the Michaelis-Menten equation using nonlinear regression analysis. Differences in values of silver uptake in the ion competition studies, at different time points, and Na^+ uptake in the presence of phenamil were assessed by ANOVA in combination with least significant difference (LSD) test. All other comparisons were performed using a one-tailed unpaired Student's t-test (STATISTICA, Statsoft, Tulsa, OK).

RESULTS

An increase in gill accumulation of silver was observed in fish exposed to 36 and 128 nM $AgNO_3$ in synthetic soft water, peaking after 1 h of exposure at ~800 pmol/g^{-1} gill wet wt. A gradual decline in gill silver levels followed, reaching a plateau after 24 h of exposure at a level of 100 and 200 pmol/g for fish exposed to 128 and 36 nM $AgNO_3$, respectively (Fig. 1*A*). There was no rapid accumulation of silver in the gills of fish exposed to 13 and 21 nM $AgNO_3$, but all values were significantly greater than zero (t-test, $P < 0.05$). The gill silver concentration of fish exposed to 13 nM $AgNO_3$ remained at 6 pmol/g gill wet weight throughout the exposure, whereas the gill silver concentration of fish exposed to 21 nM $AgNO_3$ gradually increased over the exposure period, reaching a stable concentration at 24 h of ~160 pmol/g gill wet wt.

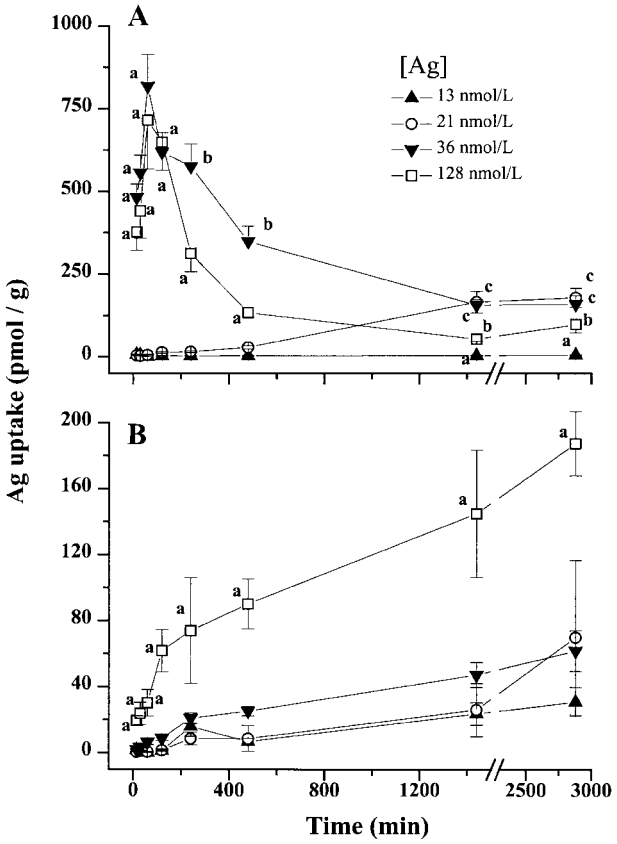

Fig. 1. Time course of silver accumulation in gills (*A*) and body (*B*) of rainbow trout exposed to 13 (▲), 21 (○), 36 (▼), or 128 nmol (□) $AgNO_3$/l in synthetic soft water. At each time point, values that are unlabeled or labeled with same letter are not significantly different from each other [$P < 0.05$, ANOVA followed by least significant difference (LSD) test]. All values are significantly different from zero (t-test, $P < 0.05$) and significantly increase relative to starting levels for all groups ($P < 0.05$, ANOVA followed by a LSD test), except for gill silver concentrations of fish exposed to 13 nmol $AgNO_3$/l, where values remained constant over exposure period. Values are means ± SE; $n = 6–8$.

Fish exposed to 128 nM $AgNO_3$ showed a much greater rise in body silver accumulation, which was significantly greater than all other treatments at each time point. There appear to be two components to the rate of accumulation in the body of fish exposed to 128 nM $AgNO_3$. Initially there was a rapid accumulation of body silver over the first 2 h of exposure, reaching a concentration of 60 pmol/g, and then a slower accumulation rate, reaching 180 pmol/g after 48 h of exposure (Fig. 1*B*). Fish exposed to 36 nM $AgNO_3$ exhibited a similar biphasic trend. Fish exposed to 13, 21, and 36 nM $AgNO_3$ showed no significant differences in their body silver levels at each time point, although for all groups the increases over time were significant.

Whole body silver uptake by rainbow trout on exposure to various $AgNO_3$ concentrations in synthetic soft water over a 2-h period increased exponentially at low concentrations. At silver concentrations >88 nM, the uptake rate increased more gradually but did not saturate over the range tested (highest 280 nM $AgNO_3$; Fig. 2).

In experiments where silver uptake was assessed in deionized water, fish lost ions due to the osmotic gradient from the body tissues to the water. On the basis of a total fish weight of 25 g/400 ml, the net loss rates were ~400 μM $Na^+ \cdot kg^{-1} \cdot h^{-1}$, 160 μM $K^+ \cdot kg^{-1} \cdot h^{-1}$, and 80 μM $Ca^{2+} \cdot kg^{-1} \cdot h^{-1}$. Whole body silver uptake was significantly lower in deionized water compared with the uptake rate after the addition of 0.1 mM $Ca(NO_3)_2$, KNO_3, or $NaNO_3$ (Fig. 3). Increasing water [Ca^{2+}] or [K^+] to 10 mM did not prevent the accumulation of silver in the body (Fig. 3, *A* and *B*), except at 1 mM KNO_3, where there was a significant decrease in whole body silver uptake (Fig. 3*B*). However, even small increases in [Na^+] resulted in reduced whole body silver uptake; the inhibition increased progressively with increasing [Na^+] (Fig. 3*C*). Interestingly, elevating water [Ca^{2+}] actually appeared to stimulate whole body silver uptake (Fig. 3*A*).

Whole body Ag uptake in deionized water containing 0.1 mM Na^+ followed Michaelis-Menten kinetics, with a J_{max} of 173 ± 34 $pmol \cdot g^{-1} \cdot h^{-1}$ and K_m of 55 ± 27 pmol. As water [Na^+] was increased to 13 mM, there was a significant fivefold decrease (to 35 $pmol \cdot g^{-1} \cdot h^{-1}$, $P < 0.05$, *t*-test) in the maximal transport capacity of silver. In contrast, there was only a nonsignificant increase in K_m, with increasing [Na^+] (Fig. 4).

Whole body silver uptake rates in fish that received a 3-h pretreatment with 100 μM phenamil followed by a washout procedure to remove the phenamil were significantly reduced by 62% compared with control whole body silver influx rates [from 295 ± 9.5 to 110 ± 11 (SE) $pmol \cdot g^{-1} \cdot h^{-1}$]. Similarly, whole body Na^+ influx was reduced by 40% compared with control fish Na^+ influx rates (from 685 ± 40 to 411 ± 47 $nmol \cdot g^{-1} \cdot h^{-1}$; Fig. 5).

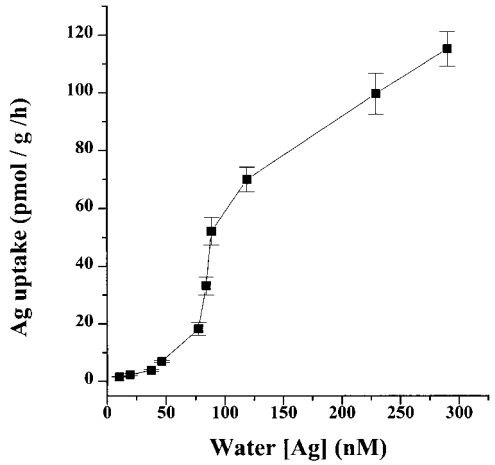

Fig. 2. Whole body silver uptake rate of rainbow trout exposed to a variety of $AgNO_3$ concentrations in synthetic soft water. Values are means ± SE; $n = 8$.

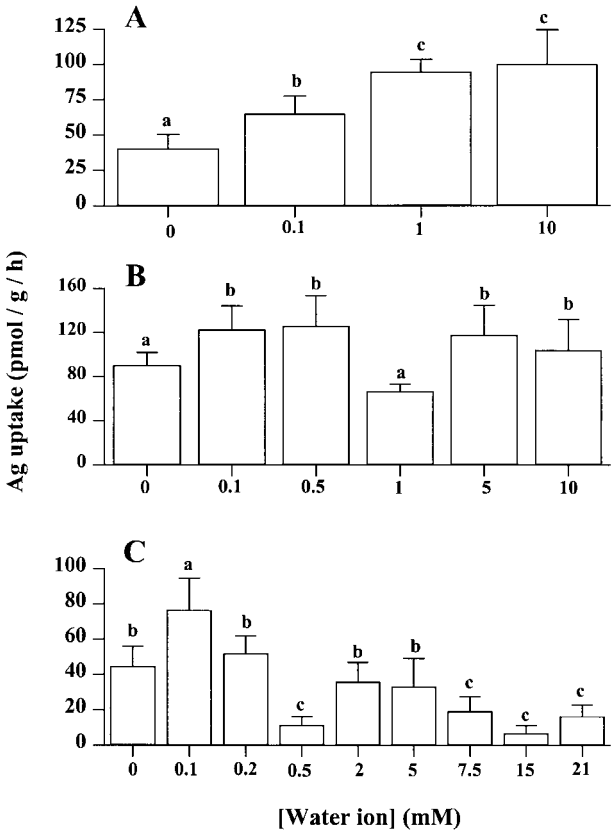

Fig. 3. Whole body silver uptake rate of rainbow trout over a 2-h exposure period to 50 nmol $AgNO_3$/l in deionized water (0) and with increasing concentrations of $Ca(NO_3)_2$ (*A*), KNO_3 (*B*), or $NaNO_3$ (*C*). Values are means ± SE; $n = 6–8$. Columns marked with same letter were not significantly different from each other ($P < 0.05$, ANOVA followed by LSD test)

Silver and sodium whole body influx rates of swim-up rainbow trout alevins treated with 2 μM bafilomycin A_1 were significantly reduced by 79 and 57%, respectively, compared with control flux rates. The decrease was from 20.8 ± 2.2 to 4.3 ± 0.7 $pmol \cdot g^{-1} \cdot h^{-1}$ for the silver influx rate and 510 ± 28 to 219 ± 11 $nmol \cdot g^{-1} \cdot h^{-1}$ for Na^+ influx rate (Fig. 6).

DISCUSSION

The major conclusion drawn from this study is that silver, probably in the ionic form (Ag^+), enters freshwater-adapted rainbow trout via a proton-coupled Na^+ channel situated on the apical membrane of the branchial epithelium.

Analogs of amiloride that are more specific to either the Na^+ channel or Na^+/H^+ exchanger have seldom been employed in the study of teleost branchial Na^+ transport. Phenamil is the most potent amiloride derivative that blocks the Na^+ channel (9, 15). A concentration of 100 μM phenamil caused a 78% reduction in branchial Na^+ transport (see MATERIALS AND METHODS), which is very similar to the values we and others have obtained for amiloride (29). On the basis of the reported potency of phenamil (15) for the two Na^+ transport mechanisms, a concentration of 100 μM phenamil would not influence Na^+ uptake via the Na^+/H^+ ex-

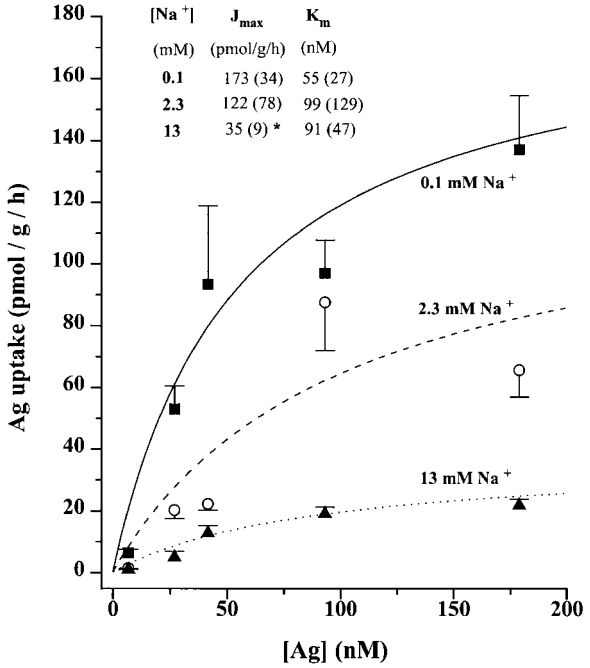

Fig. 4. Effects of increasing concentrations of $NaNO_3$ in deionized water (■, 0.1 mM Na^+; ○, 2.3 mM Na^+; ▲, 13 mM Na^+) on silver uptake kinetics in rainbow trout. Values are means ± SE; $n = 6$. Curves were generated using the Michaelis-Menten equation (solid line, 0.1 mM Na^+; dashed line, 2.3 mM Na^+; dotted line, 13 mM Na^+) using maximal transport capacity (J_{max}) and affinity (K_m) values for silver uptake shown in *inset*. *Significant difference from value at 0.1 mM Na^+ ($P < 0.05$, *t*-test).

changer. Consequently, the 78% reduction in Na^+ uptake by rainbow trout in the presence of phenamil supports the idea that Na^+ uptake in freshwater fish is via an Na^+ channel (1, 24). Even after washout of

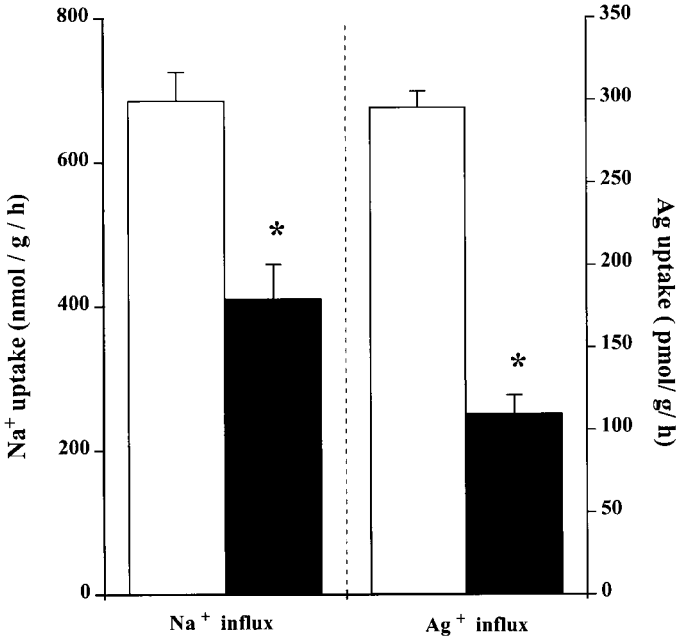

Fig. 5. Whole body Na^+ and silver influx rates in control rainbow trout (open bars) and trout after an incubation with 100 μM phenamil for 3 h followed by washout (filled bars). Values are means ± SE; $n = 8$. *Significant difference from control values ($P < 0.05$, *t*-test).

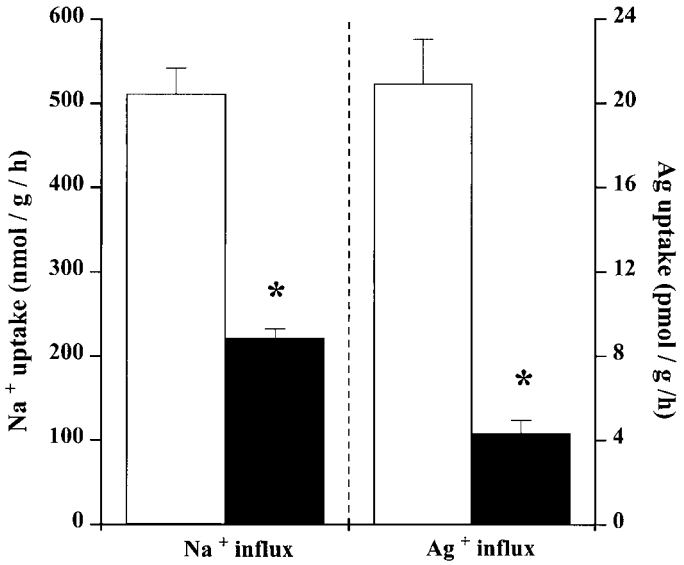

Fig. 6. Whole body Na^+ and silver influx rates in 21-day-posthatch rainbow trout control alevins (open bars) and alevins in presence of 2 μM bafilomycin A_1 (filled bars). Values are means ± SE; $n = 10$. *Significant difference from control values ($P < 0.05$, *t*-test).

phenamil, substantial inhibition of Na^+ influx persisted (58% in the preliminary experiments, 40% in the experiment of Fig. 5). Bafilomycin A_1 also inhibited Na^+ influx in rainbow trout alevins by 57% (Fig. 6). This drug is a specific inhibitor of V-type ATPases (6) and has been shown to significantly inhibit rainbow trout gill homogenate H^+-ATPase activity (18). Consequently, these two results indicate that at least part of the apical Na^+ entry is via a proton-coupled Na^+ channel. Further evidence for this mechanism of Na^+ apical entry comes from Nelson et al. (21), who demonstrated the presence of a proton-coupled Na^+ channel in the gills of brown trout (*Salmo trutta*) by the use of different concentrations of amiloride to uncouple the branchial Na^+ and proton transport, and from immunohistochemical studies that have localized vacuolar type H^+-ATPase at the branchial apical membrane (reviewed by Perry and Fryer in Ref. 23).

The evidence that silver (probably as Ag^+) enters the fish via a proton-coupled Na^+ channel comes from four separate experiments: the measurement of whole body silver uptake while altering the ambient cation concentration (Na^+, K^+, Ca^{2+}), the measurement of silver uptake kinetics in the presence of various Na^+ concentrations, the measurement of silver influx after phenamil treatment, and the measurement of silver influx after treatment with bafilomycin A_1.

Na^+ prevented the whole body accumulation of silver, whereas the other cations tested, Ca^{2+} and K^+, had no inhibitory effect. (However, it is unclear why increasing ambient calcium concentration appeared to increase whole body silver uptake. Possibly it may involve the effect that calcium has on membrane stability.) Janes and Playle (12) found that an increase of approximately five orders of magnitude in the concentration of Na^+ was necessary to reduce gill silver accumulation (16 mM Na^+ vs. 110 nM total Ag); in the present study, a

four orders of magnitude increase in [Na^+] was sufficient to reduce whole body silver accumulation (0.5 mM Na^+ vs. 50 nM total Ag). Both studies illustrate that the Na^+ uptake pathway has a very high affinity for silver. Interestingly, other heavy metals, such as Zn and Co, have a higher affinity for the Ca^{2+} channel than Ca^{2+} itself, when based on in vitro competition studies between Ca^{2+} and the respective ion (4, 10).

On the basis of Michaelis-Menten kinetics, the significant decrease in J_{max} indicates that there is noncompetitive inhibition of silver uptake by Na^+, whereas the nonsignificant increase in K_m for silver transport in the presence of increasing Na^+ concentrations suggests weak competition between Na^+ and Ag^+ for a common uptake site (Fig. 4). In the converse experiments, Morgan et al. (20) and Bury et al. (3) found that silver noncompetitively inhibits Na^+ influx and concluded that this was due to binding of Ag to the Na^+/K^+ ATPase at a different site to Na^+, which results in a conformational change to the enzyme and a concomitant reduction in transport capacity. However, because sodium (in contrast to silver) is an essential element, it is difficult to imagine that sodium would cause nonspecific conformational changes to an enzyme. A possible explanation for the decrease in J_{max} of silver uptake caused by high [Na^+] is the branchial handling of Na^+. There is an abundance of Na^+/K^+-ATPase on the basolateral membrane of freshwater fish gills (13, 22), which enables the rapid extrusion of cellular sodium, thus maintaining stable cytoplasmic Na^+ concentrations (22). However, the addition of 13 mM Na^+ to the external medium may exceed the internal branchial capacity for the regulation of cytoplasmic [Na^+]. Consequently, to maintain cytoplasmic ion concentrations, the fish would have to reduce the apical entry of that ion. If Na^+ and Ag^+ share a similar uptake pathway, then a reduction in apical Na^+ entry will also result in a decrease in the maximal Ag^+ uptake. Indeed, pharmacological studies confirmed that an important uptake route of silver is via the apical Na^+ channel (Figs. 5 and 6).

Phenamil, unlike amiloride, is a nonreversible blocker of the sodium channel, and thus its inhibitory action persists after removal from the bathing solution (9). Branchial Na^+ transport was reduced by 40–58% after preincubation with phenamil (and washout), a value that is comparable to the 38–75% irreversible inhibition by phenamil of toad urinary bladder short-circuit current reported by Garvin et al. (9). The 62 and 78% inhibitions of whole body silver uptake rate by phenamil and bafilomycin A_1, respectively, show that a substantial fraction of branchial Ag^+ entry is via the proton-coupled Na^+ channel, although it is possible that other apical entry routes also exist.

Once the fish are exposed to silver, there is a favorable inward concentration gradient, which may result in a passive uptake of Ag^+. However, branchial silver uptake saturates in deionized water, suggesting that part of the uptake process is carrier mediated (Fig. 4). The pattern of silver uptake differs in soft water (Fig. 2). There is an initial exponential relationship with Ag concentration, which is followed by a slower uptake rate at [$AgNO_3$] above 88 nM, yielding an overall sigmoidal relationship. It is unclear why the silver uptake patterns in the two water types differ. One explanation may be that in soft water the chloride present will form $AgCl_0$ complexes, so that both Ag^+ and $AgCl_0$ are present (Fig. 2), whereas in deionized water only ionic silver, Ag^+, will exist (Fig. 4). The $AgCl_0$ complex is small and neutral and will readily cross the branchial membranes and thus will have a different uptake pattern to the charged Ag^+ ion.

The carrier-mediated component of silver uptake may be either the apical entry step, the transfer of the ion across the cytoplasm, or the extrusion of the ion across the basolateral membrane, or may involve a combination of these processes. Potts (24) demonstrated that Na^+ influx via the proton-coupled Na^+ channel in freshwater animals exhibits saturation kinetics, and this may partially explain the pattern of silver uptake. However, the temporal pattern of silver uptake suggests that at least a portion of the rate-limiting step is in fact an extrusion process across the basolateral membrane. There are several possible explanations for the similar gill silver concentrations observed over the first 2 h of exposure to either 36 or 128 nM $AgNO_3$ (Fig. 1). One possibility is that there is saturation of the gill silver binding sites, including the mucus, or there is saturation of the apical membrane uptake process. However, the significantly greater accumulation of silver in the body of trout exposed to 128 nM would suggest that the gill silver turnover rate of these fish is greater than that of fish exposed to 36 nM. The mechanism behind this difference may include an increase in basolateral membrane silver transport.

In conclusion, the inhibition of whole body Na^+ influx by the drug phenamil, which specifically blocks the Na^+ channel, and bafilomycin A_1, which specifically inhibits V-type ATPases, demonstrates that Na^+ enters freshwater rainbow trout predominantly via an apical proton-coupled Na^+ channel. Blockade of silver entry by these drugs, as well as competition between Na^+ and Ag^+ uptake processes, suggests that the nonessential heavy metal silver can also enter via this proton-coupled Na^+ channel. However, it is not clear how these heavy metals traverse the cytoplasm, which is rich in heavy metal binding proteins, to exert their toxic action on the Na^+/K^+-ATPase at the basolateral membrane (19, 20). This is an area of research that warrants further studies.

Perspectives

Heavy metals are notorious for their deleterious effects on ion uptake processes. In freshwater fish, the main site for heavy metal toxicity is the gills, where these heavy metals primarily inhibit transport enzymes on the basolateral membrane. To exert this toxic action the metal has to enter the gill and traverse the cytoplasm. There is growing evidence that in freshwater fish the branchial entry route for a heavy metal is linked to the cation uptake process, which that metal perturbs. There is now clear evidence that heavy

metals that inhibit freshwater teleost Ca^{2+} uptake, such as Co^{2+}, Zn^{2+}, and Cd^{2+}, can enter freshwater fish via an apical Ca^{2+} channel (5, 10, 25). Silver appears to follow a similar pattern: it inhibits Na^+ influx in freshwater rainbow trout (11, 20, 27, 30) and mimics Na^+ in the entry step across the apical membrane via an H^+-coupled Na^+ channel. It awaits to be seen if other metals that inhibit teleost Na^+ uptake, such as copper, also follow this trend.

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