Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport

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Hogstrand, Christer, Pieter M. Verbost, Sjoerd E. Wendelaar Bonga, and Chris M. Wood. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. *Am. J. Physiol.* 270 (*Regulatory Integrative Comp. Physiol.* 39): R1141–R1147, 1996.—The uptake mechanism of Zn^{2+} through the gill epithelium of freshwater rainbow trout was investigated both in intact animals and in isolated basolateral membranes. Involvement of the apical Ca^{2+} uptake sites in Zn^{2+} uptake was examined in vivo by pharmacological manipulation of the apical Ca^{2+} permeability. The apical entries of Ca^{2+} and Zn^{2+}, but not Na^+ and Cl^-, were inhibited by addition of La to the water. Addition of 1.0 µM La reduced the influxes of Ca^{2+} and Zn^{2+} to 22 ± 3 and 53 ± 7% (means ± SE) of the control value, respectively. Injection of $CaCl_2$ also reduced the branchial influxes of Ca^{2+} and Zn^{2+}. This treatment decreased the influx of Ca^{2+} to 45 ± 4% of the control level and the Zn^{2+} influx to 68 ± 5%. These results strongly imply that Zn^{2+} passes across the apical membrane of the chloride cells of the gills via the same pathway as Ca^{2+}. The presence of an active basolateral transporter for Zn^{2+} was investigated in vitro on isolated basolateral membranes. There was no ATP-dependent or Na^+-gradient driven transport of Zn^{2+} at physiological Zn^{2+} activities. The same system was used to study potential effects of Zn^{2+} on the basolateral Ca^{2+}-adenosinetriphosphatase. Zn^{2+} was found to be a potent blocker of this transporter, causing a mixed inhibitory effect on the ATP-driven Ca^{2+} transport at a free Zn^{2+} activity of 100 pM.

lanthanum; chloride cells; ionocytes; Ca^{2+}-adenosinetriphosphatase; Ca^{2+} channel; regulation; inhibition; stanniocalcin

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ZINC IS AN ESSENTIAL micronutrient with a well-regulated physiology (10, 26). In mammals and other higher vertebrates Zn^{2+} is taken up by the intestine, but in fish there is an additional important pathway for Zn^{2+} absorption, namely the gills (10, 23, 26). The branchial influx of Zn^{2+} in freshwater-adapted rainbow trout (*Oncorhynchus mykiss*) shows typical Michaelis-Menten characteristics, indicating a transcellular carrier-mediated uptake route (25). Waterborne Ca^{2+} competitively inhibits the Zn^{2+} influx through the gills, which would suggest that the uptake mechanism for Zn^{2+} is at least partly the same as for Ca^{2+} (25). This hypothesis is supported by recent findings that the effect of Zn^{2+} on the branchial Ca^{2+} influx was mostly of a competitive nature and that a decrease in affinity for Ca^{2+} (increase in K_M) was paralleled by a reduction in the branchial influx of Zn^{2+} (8, 9).

Uptake of waterborne Ca^{2+} in freshwater teleosts occurs through the chloride cells, which are primarily located in the gill epithelium (5, 18, 20). Branchial uptake of Ca^{2+} is thought to start by the passive movement of Ca^{2+} through a voltage-insensitive channel in the apical membrane of the chloride cells (5). The activity of Ca^{2+} in the chloride cell cytoplasm is kept low (10^{-7} M) by Ca^{2+}-binding proteins and by pumps that transport Ca^{2+} to the interior of the endoplasmic reticulum [Ca^{2+}-adenosinetriphosphatase (ATPase)] and the mitochondria (Ca^{2+} uniporter). The transfer of Ca^{2+} across the basolateral membrane is mediated by a high-affinity Ca^{2+}-ATPase and a low-affinity Na^+/Ca^{2+} exchange system (32). The competitive interactions between the influxes of Ca^{2+} and Zn^{2+} suggest that one or several steps in this pathway are common for the two elements.

Although Zn^{2+} is required for a number of physiological processes, relatively low concentrations of waterborne Zn^{2+} are toxic to freshwater fish [reported 96-h half-maximal lethal concentrations (LC_{50}) values range from 1.4 to 610 µM, largely as a function of water hardness; Ref. 10]. At sublethal concentrations, Zn^{2+} affects fish by impairing the influx of Ca^{2+} with hypocalcemia as a consequence (24). The potent inhibition seems to be due to a more than 10-fold higher affinity of the common Ca^{2+}/Zn^{2+}-binding sites for Zn^{2+} than for Ca^{2+} (8, 9, 25).

The nonessential element Cd is considerably more toxic to fish than Zn^{2+}, but otherwise these two metals show physical and biological similarities (7). Like Zn^{2+}, the toxicity of waterborne Cd lies mainly in a strong perturbation of the Ca^{2+} metabolism (12, 21). More recent studies provide strong evidence that the Ca^{2+}-ATPases in the chloride cells are extremely sensitive to inhibition by Cd, and these may be the first targets during Cd poisoning in fish (29, 30). Although there is no evidence for a capacity of the branchial basolateral Ca^{2+}-ATPase to actually transport Cd, the apical entry of Cd probably takes place via the Ca^{2+} uptake route (33). Indeed, waterborne Cd inhibits the branchial influx of Zn^{2+} (1), which is indirect evidence that Zn^{2+} enters the chloride cells through the same Ca^{2+} pathway. Thus physicochemical and toxicological similarities between Zn^{2+} and Cd point toward the putative apical Ca^{2+}-channels and the Ca^{2+}-transporting ATPases in the chloride cells as possible sites of Zn^{2+} interaction with the Ca^{2+} uptake.

The objectives of the present study were to compare the uptake pathways of Ca^{2+} and Zn^{2+} via the chloride cells of the gill and to identify the site(s) where Zn^{2+} interferes with Ca^{2+} uptake. The possibility that Zn^{2+}

competes with Ca^{2+} for a common apical ion channel was investigated by La blockade of the Ca^{2+} channels (18, 33) and by stimulating endogenous stanniocalcin release by $CaCl_2$ injection (11, 16, 19, 35), a response that has been demonstrated to reduce the permeability of the apical Ca^{2+} channels to Ca^{2+} and Cd (11, 28, 33, 31). Possible basolateral transfer of Zn^{2+} by the high-affinity Ca^{2+}-ATPase or the low-affinity Na^+/Ca^{2+} exchanger was tested in vitro using isolated vesicles of basolateral membranes. The same technique was used to study the possible effects of Zn^{2+} on the ATP-dependent Ca^{2+} transport across the basolateral membrane.

MATERIALS AND METHODS

Animals. Juvenile rainbow trout *Oncorhynchus mykiss* ($n = 120$, 3–10 g) for in vivo studies were purchased from Rainbow Springs Hatchery, New Dundee, Ontario. The fish were held at McMaster University, Hamilton, Ontario, for 2–5 mo before the experiments in a fiberglass tank, supplied at a rate of 1 l/min with a flow-through of dechlorinated, aerated Hamilton City tapwater [concentrations (in brackets; mM): 0.6 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 1.9 [HCO_3^-], pH 8.0; temperature = 14°C]. Rainbow trout ($n = 10$, 100–300 g) for in vitro experiments were obtained from a local hatchery and kept at the University of Nijmegen, Nijmegen, The Netherlands, for at least 2 wk before the experiments. The holding tank contained recirculated, filtered Nijmegen tap water (in mM: 0.61 [Na^+], 0.66 [Cl^-], 0.8 [Ca^{2+}], 3.15 [HCO_3^-], pH 7.5) at 10°C. Rainbow trout were fed dry pellets at a daily ration of 2% of the body weight.

Effect of La on branchial ion influxes. A total of eight polypropylene bags were filled with 3 liters each of synthetic carbonate-free water (in mM: 0.7 [NaCl], 1.9 [KCl], 1.0 [$CaNO_3$], and 1.0 µM [$ZnSO_4$], pH 6.8) and equipped with an air line. Carbonate-free water was employed because the effectiveness of La is reduced by carbonate complexation. The bags were placed in perforated polyvinylchloride supports flushed with 14°C water. Ten rainbow trout were placed in each bag, and, to one-half of the bags, 1.0 µM La was added as $LaCl_3$ from a stock solution (10 mM). Thus in total there were four pairs of bags, each consisting of one La-treated group and one control. The flux was initiated by the addition of isotope. The La-treated groups and their paired controls received (in MBq) 3.0 (81 µCi) of $^{45}Ca^{2+}$, 3.7 (100 µCi) of $^{65}Zn^{2+}$, 0.37 (10 µCi) of $^{22}Na^+$, or 0.37 (10 µCi) of $^{36}Cl^-$.

Two 5-ml water samples were withdrawn after 5 min and after 4 h, respectively, for later analysis of actual isotope activities and concentrations of total Ca^{2+}, Zn^{2+}, Na^+, and Cl^-. Measured activities and concentrations of the elements were within 10% of the nominal values.

At the end of the 4-h flux period, the fish were given an overdose of anesthetic (3-aminobenzoic acid ethyl ester, 1 g/flux bag) followed by a wash in a rinsing solution for 1 min to remove surface-bound isotope. Different rinsing solutions were used for the different groups: $^{45}Ca^{2+}$ was displaced by 10 mM $CaNO_3$, $^{65}Zn^{2+}$ by 0.1 mM of $ZnSO_4$, and $^{22}Na^+$ and $^{36}Cl^-$ by 7 mM of NaCl. The fish were then blotted dry and weighed. The fish in the Zn^{2+} flux and Na^+ flux experiments were counted for $^{65}Zn^{2+}$ and $^{22}Na^+$, respectively, in a gamma counter (MINAXI Autogamma 5000 Series, Canberra Packard). The trout in the Ca^{2+} experiment were placed in individual crucibles and ashed on a hot plate. The ash was transferred to scintillation vials, 10 ml of scintillant (ACS, Amersham) was added to each vial, and the samples were

incubated at 45°C overnight before they were counted for $^{45}Ca^{2+}$ with a scintillation counter (LKB 1217 Rackbeta, Pharmacia-LKB AB). Fish in the Cl^- flux experiment were individually frozen in liquid nitrogen then ground in a cryostatic mill (A10, IKA). The powder was weighed out in duplicate samples of 0.5 g into glass scintillation vials. Each tissue sample was digested with 2.0 ml of tissue solubilizer (Protosol, New England Nuclear) for 48 h at 45°C. The samples were neutralized with 20 µl of glacial acetic acid, diluted with 10 ml of scintillation fluor (OCS, Amersham) and counted for $^{36}Cl^-$ in the scintillation counter. In all cases, counting efficiencies were assessed by internal standardization.

Water samples containing $^{65}Zn^{2+}$ or $^{22}Na^+$ were counted directly in the gamma counter. To the water samples containing $^{45}Ca^{2+}$ or $^{36}Cl^-$, 10 ml of scintillation fluor (ACS, Amersham) was added.

The inward fluxes (J_{in}) for Na^+, Cl^-, Ca^{2+}, and Zn^{2+} (in µmol·kg^{-1}·h^{-1}) were calculated according to the formula

$$J_{in} = CT/(SA \times CE \times t) \qquad (1)$$

where CT is the counts in tissue [counts/(min × kg)], SA is the measured mean specific activity of the water [counts/(min × µmol)], CE is the measured relative counting efficiency of the tissue system compared with the water system, and t is the flux time (h).

Effect of $CaCl_2$ injection on influx of Ca^{2+} and Zn^{2+}. Ionic calcium was injected into juvenile rainbow trout in an attempt to reduce the apical entry of Ca^{2+} and Zn^{2+} to the gills through the stanniocalcin-controlled pathway. Previous studies have shown that an injection of 10 mg Ca^{2+}/kg body mass as $CaCl_2$ evokes stanniocalcin release from the corpuscles of Stannius followed by a fivefold increase in the plasma stanniocalcin level (16, 19, 35). Furthermore, this response persists over a 4-h period (35). Thus, in the present study, 20 fish were injected with Ca^{2+}, 10 µg/g (250 nmol/g) body wt, from an injection solution made up from 25 mM $CaCl_2$·$2H_2O$ dissolved in 0.9% NaCl (wt/vol). An equal number of fish were sham injected with vehicle only and used as controls. Ten Ca^{2+}-injected fish and ten sham-injected fish were analyzed for Ca^{2+} influx, as described in *Effect of La on branchial ion influxes.* The remaining 10 Ca^{2+}-injected fish and 10 controls were assayed for Zn^{2+} influx, also as described above.

All whole animal flux experiments followed evaluated standard procedures described by Spry and Wood (24, 25), Laurén and McDonald (13), Verbost et al. (29, 33), Perry and Flik (18), and Hogstrand et al. (8, 9). The 1-min washing step was considered effective and appropriate for removal of externally bound isotope, because it provided similar transport rates as those based on appearance of isotope in the blood plasma (8, 9, 18, 20).

Isolation of basolateral membranes from gill epithelium. The preparation of basolateral membranes (BLMs) from fish gill epithelium is a standard technique, and the fully evaluated procedure has been described previously (18, 32). In brief, adult rainbow trout (100–300 g) were killed by a blow to the head, the branchial apparatus was perfused [0.9% NaCl, 0.5 mM EDTA, 20 kU/l heparin, pH was adjusted to 7.8 with tris(hydroxymethyl)aminomethane (Tris), 4°C], and the gills were dissected out. The soft tissue of the gill arches was scraped from the cartilaginous tissue and homogenized (Polytron tissue homogenizer) in 60 ml of a hypotonic buffer [25 mM NaCl, 1 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), adjusted to pH 8.0 with Tris] to fragment the cell membranes. Cellular debris and remaining blood cells were separated from membranes by centrifugation at 550 g, 4°C, for 5 min. The supernatant was then centrifuged at 105,000 g, 4°C, for 30 min (Beckman Ti 70 rotor), and a pellet

consisting of a firm brownish part with a lighter fluffy upper layer was obtained. The fluffy top of the pellet was resuspended in 30 ml of an isosmotic buffer (250 mM sucrose, 5 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.4), using a Dounce homogenizer (100 strokes), to prepare inside-out oriented vesicles. The resulting membrane suspension was centrifuged differentially (1,000 g, 10 min and 10,000 g, 10 min, 4°C), and the resulting supernatant was centrifuged once more at 50,000 g, 4°C, for 15 min to create a final pellet of purified BLMs. In the preparation of membranes for the Na^+/Zn^{2+} exchange studies, the BLMs were resuspended in a buffer containing (in mM) 150 NaCl, 0.8 $MgCl_2$, 20 HEPES, 20 Tris·HCl, pH 7.4, to load the vesicles with Na^+, and the last centrifugation step was repeated. Finally, the pellet was resuspended in (in mM) 250 sucrose, 5 $MgCl_2$, 5 HEPES, 5 Tris·HCl, pH 7.4 (isosucrose; for ATPase assays) or the NaCl-containing buffer (for Na^+/Zn^{2+}-exchange assays). Vesicles were kept on ice and used the same day or frozen in liquid nitrogen until used.

Orientation of the vesicles was determined from acetylcholine esterase activity (i.e., marker for inside-out vesicles) measured in intact and permeabilized vesicles and the specific trypsin sensitivity of the cytosol-oriented part of the Na^+-K^+-ATPase (i.e., marker for right-side-out vesicles). Typically, 20% of the BLMs were inside-out vesicles and 30% right-side-out vesicles (18, 32). The remaining 50% of the the preparation consisted of membranes that were not resealed (18, 32). Protein recovery was 1.02 ± 0.19% (mean ± SE, $n =$ 8), and the protein content of BLMs used in the assays was 1.1 mg/ml. The enrichment of BLMs in the preparation was five to eight times, as determined from the Na^+-K^+-ATPase activity in the final preparation and in the crude gill homogenate. Na^+-K^+-ATPase activity, defined as Na^+- and K^+-dependent, ouabain-sensitive phosphorylase activity, was analyzed as described by Flik et al. (4). The amount of endoplasmic reticulum (ER) membranes in this type of preparation has been shown to be insignificant (18). However, as a safety measure, 1 h before use, 1 μM of thapsigargin (Sigma) was added to the membranes to inhibit any remaining Ca^{2+}-ATPase activity from the ER. This concentration of thapsigargin completely blocks ER Ca^{2+} pumps in trout gill preparations (Verbost, unpublished observations) and tilapia (*Oreochromis mossambicus*) gill preparations (31).

Calcium and zinc transport assays in BLM. Ca^{2+} and Zn^{2+} transport were determined in assay media that contained 0.5 mM EGTA, 0.5 mM N-(2-hydroxyethyl)-ethylenediamine-N,N',N'-triacetic acid (HEEDTA), and 0.5 mM nitrilotriacetic acid (NTA) as a Ca^{2+} and Zn^{2+} buffering system. Free Ca^{2+}, Zn^{2+}, and Mg^{2+} activities were calculated using a matrix computer program (22), taking into account the first and second protonations of the respective ligands (ATP, EGTA, HEEDTA, NTA). The metal-chelator stability constants were corrected for the ionic strength at the temperature of the medium. All incubations were carried out at 37°C.

The ATP-driven transport of Ca^{2+} was determined in 0.5-min incubations of membrane vesicles, which yielded initial transport velocities. ATP-driven transport of Zn^{2+} was assayed in 0.5-, 1-, and 2-min incubations. Two incubations were performed on each sample, one in the absence and one in the presence of 3 mM ATP, and the difference between these measurements was considered active transport. The assay medium contained also a varying concentration of $^{45}Ca^{2+}/$ stable Ca^{2+} and/or $^{65}Zn^{2+}/$stable Zn^{2+}, 150 mM KCl, 20 mM HEPES/Tris, pH 7.4, 0.8 mM Mg^{2+} (free), and the 0.5 mM EGTA/HEEDTA/NTA buffer system described above. This composition of the medium is optimal for Ca^{2+} transport (18). For studies of ATP-driven Zn^{2+} transport, two concentrations

of Zn^{2+} were used. These concentrations of free Zn^{2+}, 10^{-19} and 10^{-12} M, were chosen because the former had the highest possible specific activity of $^{65}Zn^{2+}$ (no cold Zn^{2+} added), and the latter represented a concentration close to the "no observed effect level" for Zn^{2+} on the Ca^{2+}-ATPase (see Fig. 5). Our results indicate that a Zn^{2+} activity as low as 50 pM may inhibit the basolateral Ca^{2+}-ATPase. It is therefore reasonable to assume that the intracellular Zn^{2+} activity in the gill epithelium cells never exceeds this level during physiological conditions and that any specific Zn^{2+} transporter must operate at subpicomolar Zn^{2+} concentrations. Zinc transport assays were run, both with no Ca^{2+} present and at the normal cytosolic concentration of free Ca^{2+}, 10^{-7} M. The transport was arrested by a 21-fold dilution of the incubate with ice-cold isotonic buffer, containing 20 mM Tris·HCl, pH 7.4, 150 mM KCl, and 1 mM $LaCl_3$ (stopping buffer). Vesicles were separated from the medium by a rapid filtration technique (27), using filters with 0.45-μm pore size (Schleicher & Schuell, ME25). After the filtration of vesicles, the filters were flushed twice with 2.0 ml of stopping buffer and transferred to scintillation vials. The filters were dissolved in 4 ml of scintillation fluor (Aqualuma) for 30 min at room temperature and then counted for $^{45}Ca^{2+}$ or $^{65}Zn^{2+}$ in a scintillation counter (Pharmacia-Wallac 1410). With no Zn^{2+} in the assay medium (i.e., control conditions), the ATP-dependent Ca^{2+} transport was typically 100–400% higher than the nonspecific binding.

Assays for Na^+/Zn^{2+}-exchange activity (i.e., uptake of Zn^{2+} by BLM vesicles driven by a transmembrane Na^+ gradient) were performed similarly to the method described by Flik et al. (4) and Verbost et al. (32), using conditions shown optimal for Na^+/Ca^{2+} exchange. Five microliters of vesicles, equilibrated with 150 mM of NaCl, was added to 120 μl of medium containing either 150 mM NaCl (blank) or 150 mM KCl and a varying concentration of $^{65}Zn^{2+}/$stable Zn^{2+} and Ca^{2+}. The Zn^{2+} concentrations tested were 10^{-19}, 10^{-12}, and 10^{-10} M. Each of these Zn^{2+} concentrations was run with either 5 μM Ca^{2+} or no Ca^{2+} present. Thus, in the 150 mM KCl medium, a NaCl gradient across the vesicles was created. The inside contained 150 mM NaCl, and the outside contained 6.2 mM NaCl. In the blank (used to assess nonspecific binding), the NaCl concentration was 150 mM on both sides of the membrane. In addition to the 150 mM NaCl or KCl, the assay medium contained 20 mM HEPES/Tris, pH 7.4, 0.8 mM Mg^{2+} (free), and 0.5 mM EGTA/HEEDTA/NTA. After 1-min incubation, the reaction was arrested by addition of stopping buffer, as described above. Vesicles were collected by rapid filtration, rinsed, and counted for $^{65}Zn^{2+}$. The difference in $^{65}Zn^{2+}$ accumulation in K^+- and Na^+-containing media was taken as a measure of Na^+ gradient-driven Zn^{2+} transport.

The K_M and maximal velocity (V_{max}) values of the transport systems were derived from nonlinear regression analyses of the measured velocities of individual preparations as a function of substrate concentration, using a computer program with the Levenberg-Marquardt algorithm (14). Values for duplicate samples of each membrane preparation were averaged.

Statistical methods. Statistical differences between paired groups were tested by the Mann-Whitney U test before transformation of data to percentage figures. Statistical significance was accepted at the level $P < 0.05$. The effect of Zn^{2+} on Ca^{2+} transport in BLM was statistically assessed by testing equality against ordered alternatives (15). Each treated group was then tested in paired fashion against the control, using the Mann-Whitney U test and a significance level of $P < 0.01$.

RESULTS

Passage of Zn^{2+} across the apical membrane. The influxes of Na^+, Cl^-, Ca^{2+}, and Zn^{2+} in untreated rainbow trout were 251 ± 22.0, 185 ± 7.9, 40.0 ± 4.5, and 0.0949 ± 0.0128 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively (means $\pm$ SE, $n = 10$). The hypothesis that Zn^{2+} enters the chloride cells of the gill epithelium through the same ion channels as Ca^{2+} was tested by measuring the influx of Ca^{2+} and Zn^{2+} in the presence of a Ca^{2+}-channel blocker, La, in the water. The specificity of the blockade was investigated by analysis of the effects of La on the influx of Na^+ and Cl^-. Addition of 1 μM La to the experimental water significantly decreased the influx of Ca^{2+} (to 22% of control mean; Fig. 1A) and Zn^{2+} (to 53% of control mean; Fig. 1A) but had no effect on Na^+ and Cl^- influx (Fig. 1B).

The role of the apical Ca^{2+} transporter in Zn^{2+} uptake was further studied by measuring the influx of Ca^{2+} and Zn^{2+} in Ca^{2+}-injected rainbow trout. Ca^{2+} injection evokes an increased stanniocalcin production, which decreases the permeability of the apical membranes for Ca^{2+} (11, 33). The branchial influx of Ca^{2+} and Zn^{2+} in sham-injected (NaCl) fish was 44.0 ± 4.9 and 0.0927 ± 0.00493 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (means $\pm$ SE), respectively. These values were similar to those measured in untreated fish (see above). Injection of Ca^{2+} significantly reduced both Ca^{2+} and Zn^{2+} influx (Fig. 2). The influx of Ca^{2+} was decreased to 45% of the mean control value and the influx of Zn^{2+} to 68%.

Passage of Zn^{2+} across the BLM. The presence of basolateral ATP or Na^+ gradient-driven transport systems for Zn^{2+} was tested in vitro in isolated BLM vesicles. None of the various assay conditions tested resulted in any detectable active transport of Zn^{2+} across the vesicles. In the system used, the detection limit of the rate of active transport, based on the specific activity of $^{65}Zn^{2+}$ in the incubation medium, was 10^{-20} $mol \cdot mg^{-1} \cdot min^{-1}$ for ATP-driven Zn^{2+} transport and 10^{-23} $mol \cdot mg^{-1} \cdot min^{-1}$ for Zn^{2+} transport driven by a Na^+ gradient.

Effect of Zn^{2+} on the basolateral Ca^{2+} transport. The basolateral high-affinity Ca^{2+}-ATPase was found to be sensitive to Zn^{2+}. The Zn^{2+} inhibition was mixed, competitive and noncompetitive, in its nature, although the competitive component prevailed (Fig. 3 and Table 1). Quantitatively, our results vary slightly depending on the methods used to calculate V_{max} and K_M. Figure 3 is a traditional Eadie-Hofstee plot where the unweighted regression lines are fitted to the averages of measurements from four to seven individuals, whereas the V_{max} and K_M values presented in Table 1 were obtained from nonlinear regressions of data from individual samples. Although Fig. 3 allows a graphic perception of the Zn^{2+} inhibition, the constants shown in Table 1 are more accurate mathematically. The effect on K_M was significant ($P < 0.01$) at a Zn^{2+} activity of 100 pM (Table 1). The V_{max} became significantly reduced only at the highest tested concentration of free Zn^{2+}, 500 pM (Table 1). At the same Zn^{2+} activity, the
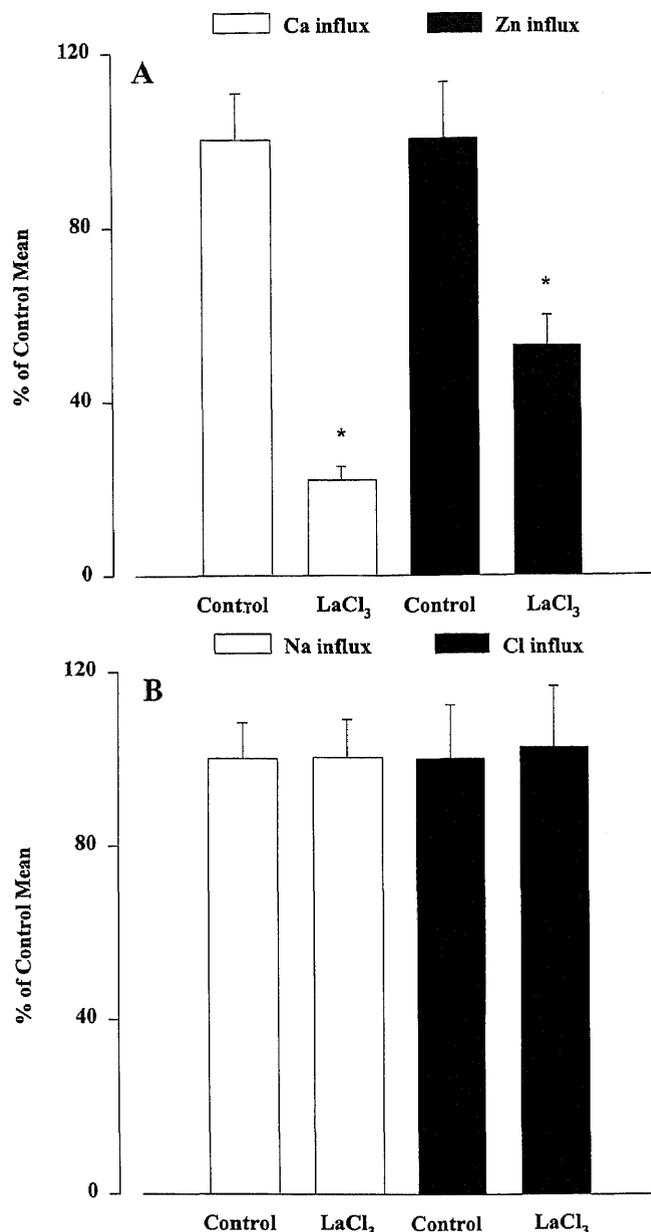

Fig. 1. Effect of apical Ca^{2+}-channel blockade by waterborne La on the influx of Ca^{2+} and Zn^{2+} (A) and Na^+ and Cl^- (B) in juvenile rainbow trout. Bars show mean influx of Ca^{2+}, Zn^{2+}, Na^+, or Cl^- of 10 fish, expressed as percentage of the control means. Error bars denote SE. Absolute control means of Ca^{2+}, Zn^{2+}, Na^+, and Cl^- influx were 40.0 ± 4.5, 0.0949 ± 0.00128, 251 ± 22.0, and 185 ± 7.9 (means $\pm$ SE) $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively. *Significant difference compared with the control value ($P < 0.05$).

increase in K_M was eightfold, and the V_{max} was decreased by a factor of two.

DISCUSSION

Previous research has given strong indications that Ca^{2+} and Zn^{2+} compete for the same uptake sites on the gills of freshwater-adapted rainbow trout (8, 9, 25). In the present study, we provide direct evidence that Zn^{2+} may interfere with the branchial Ca^{2+} uptake at several stages and that the two elements very probably share the same apical entry mechanism.
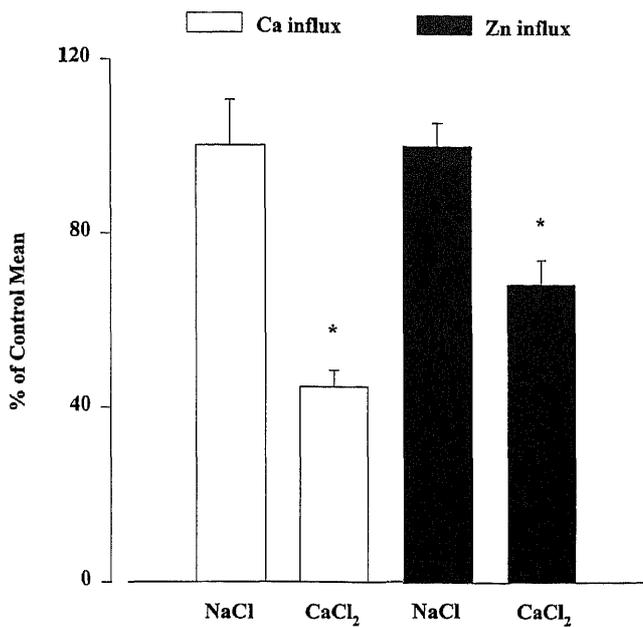
Fig. 2. Effect of Ca^{2+} injection on the influx of Ca^{2+} and Zn^{2+} in juvenile rainbow trout. Bars show mean influx of Ca^{2+} and Zn^{2+} of 10 fish, expressed as percentage of the control mean. Error bars denote SE. Absolute control means of Ca^{2+} and Zn^{2+} influx were 44 ± 4.9 and 0.0927 ± 0.00493 (means $\pm$ SE) $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively. *Significant difference compared with the control value ($P < 0.05$).

Two independent approaches were used to investigate whether Zn^{2+} and Ca^{2+} cross the apical membrane of the gill epithelium by the same mechanism. The first approach was to block the Ca^{2+} uptake sites with La, a
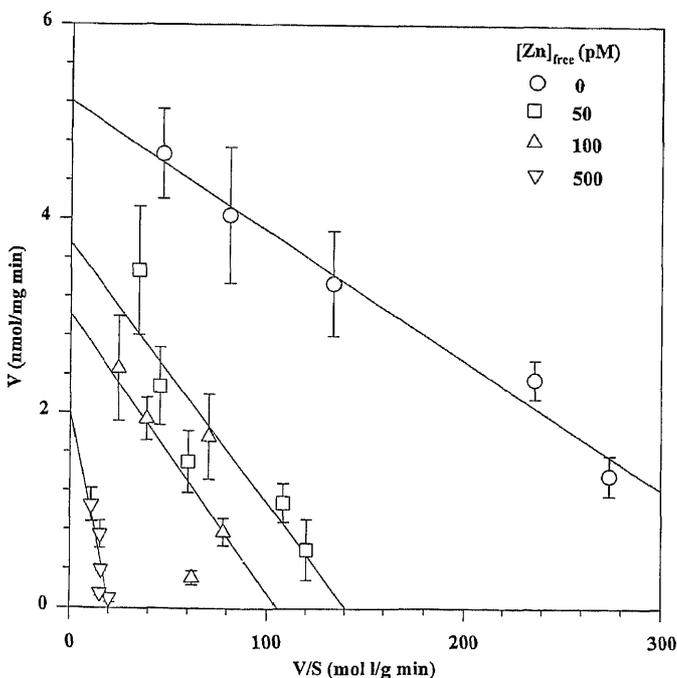

Fig. 3. Eadie-Hofstee plot of the effect of Zn^{2+} on the ATP-driven Ca^{2+} transport in rainbow trout gill basolateral membrane. Regression lines are unweighted fits to the average velocities of preparations from 4 to 7 individuals. Vertical error bars denote SE. Zn^{2+} concentrations ($[Zn^{2+}]$) given are the activities of free Zn^{2+} in the assay medium. V, velocity of Ca^{2+} transport; S, concentration of substrate ($[Ca^{2+}]_{free}$) in the assay medium.

Table 1. *Effect of free Zn^{2+} on V_{max} and K_M for the transport kinetics of ATP-driven Ca^{2+} transport in isolated basolateral membrane vesicles of adult rainbow trout*

Zn^{2+}, pM	V_{max}, $nmol \cdot mg^{-1} \cdot min^{-1}$	K_M, μM	n
0	5.05 ± 0.51	0.13 ± 0.03	7
50	4.36 ± 1.16	0.57 ± 0.34	6
100	3.70 ± 0.91	$0.43 \pm 0.08^*$	6
500	$2.13 \pm 0.49^*$	$1.03 \pm 0.19^*$	4

Values are means $\pm$ SE ($n = 4-7$ individuals). Values are derived from nonlinear regression curves of individual samples, describing the Michaelis-Menten equation. The Zn^{2+} concentration is the free Zn^{2+} activity in the assay medium. The decrease in maximal velocity (V_{max}) and the increase in the Michaelis-Menten affinity constant (K_M) were found significant by testing equality against ordered alternatives (14). *Significances of individual groups compared to control (Mann-Whitney U test, $P < 0.01$).

classic Ca^{2+}-channel blocker (36). La treatment was an effective inhibitor of both Ca^{2+} and Zn^{2+} influxes, suggesting common uptake sites for Ca^{2+} and Zn^{2+} at the apical surface. The specificity of the blockade was demonstrated by the unaffected influxes of Na^+ and Cl^- in La-exposed fish. If La had acted in a nonspecific manner to block all negatively charged sites of the gill surface, then it is probable that also the Na^+ influx would have been reduced. During these relatively short exposure times and at the concentration used (1 μM), La does not enter the gill epithelium but accumulates on the surface of the chloride cells (18). Thus it could be argued that the effect of La is specific to fluxes across chloride cells in general and not to the Ca^{2+}/Zn^{2+} uptake sites. However, the unaltered Cl^- influx is a control showing that the La blockade was not merely a physical cover of the chloride cell surface. These control experiments substantiate our finding that Zn^{2+} most likely enters the fish through the Ca^{2+}-transporting system in the apical membrane of the chloride cells.

The second approach used to demonstrate the intimate relationship between the uptake mechanisms for Ca^{2+} and Zn^{2+} was to give the fish a signal to reduce Ca^{2+} influx and then observe the effect on branchial Zn^{2+} influx. Such a signal was given by injecting ionic Ca^{2+}. From previous research it is firmly established that a consequence of Ca^{2+} injection in fish is an increased release of the calciostatic hormone stanniocalcin (11, 16, 19, 35), which, in turn, leads to a decreased permeability of the apical chloride cell membrane to Ca^{2+} (11, 28, 33, 34). This treatment had the predicted inhibitory effect on the branchial Ca^{2+} influx, and it also significantly reduced the influx of Zn^{2+}. The results provide an independent line of evidence that the apical passage for Zn^{2+} takes place through the Ca^{2+} uptake sites and also suggest that Zn^{2+} influx may be regulated by stanniocalcin in vivo.

After both La treatment and Ca^{2+} injection the inhibition of Ca^{2+} influx was more pronounced than that of Zn^{2+}. This difference could be accounted for if the affinity of the uptake sites were higher for Zn^{2+} than for Ca^{2+}. Previous investigations do, in fact, show that the K_M for Zn^{2+} influx is at least a factor of 10

below the K_M for Ca^{2+} influx (10). There is, however, a second possible explanation, which is that the apical Ca^{2+}-uptake sites might not be the sole branchial pathway for Zn^{2+} to enter the fish.

Even though the apical entry of Ca^{2+} and Zn^{2+} in the gills seem to occur via the same pathway, we were unable to show any active transport of Zn^{2+} driven by ATP or the transmembrane Na^+ gradient. Because the basolateral transfer of Ca^{2+} occurs via a Ca^{2+}-ATPase (2, 3, 6, 18) and, at least in tilapia gills, to some extent via a Na^+/Ca^{2+} exchanger (32), we conclude that Zn^{2+} is not transported across the BLM via the Ca^{2+} transporters during physiological conditions. The mechanism for basolateral Zn^{2+} transport is yet to be elucidated.

Our results suggest that the basolateral Ca^{2+}-ATPase of the fish gill epithelium is not involved in Zn^{2+} transport, but we did find evidence that Zn^{2+} binds to this Ca^{2+} pump. Indeed, the present data suggest that Zn^{2+} can interfere very powerfully with the ATP-dependent basolateral transport of Ca^{2+}. As little as 100 pM of free Zn^{2+} was able to inhibit the high-affinity Ca^{2+}-ATPase in isolated vesicles from the basolateral membrane. This concentration is 10^7 times lower than the normal total Zn^{2+} concentration of the gill in rainbow trout (9). Thus it is evident that the intracellular regulation of Zn^{2+} activity must be rigorously controlled to ensure that there is virtually no free Zn^{2+} present in the cytoplasm.

Although the inhibition of the Ca^{2+}-ATPase by Zn^{2+} was mixed, the competitive component of the inhibition dominated markedly. It was interesting to note that the nature of the inhibition of the Ca^{2+}-ATPase in vitro resembled the effect of elevated waterborne Zn^{2+} on the in vivo influx of Ca^{2+}, where, again, there is a large competitive and a small noncompetitive inhibition (8, 9). We speculate that the inhibitory effect of Zn^{2+} on J_{max} for branchial Ca^{2+} transport in the in vivo situation is due to an inhibition of the Ca^{2+}-ATPase. The increased K_M in vivo for Ca^{2+} transport caused by the presence of Zn^{2+}, on the other hand, could represent a competition for binding sites both at the apical Ca^{2+} uptake sites and the basolateral Ca^{2+}-ATPase. The inhibitory effect of Ca^{2+} on the branchial influx of Zn^{2+} does not have any noncompetitive component (25) and is likely to represent a simple competition for binding sites at the level of the apical membrane.

Cadmium is one of the most toxic metals to fish, substantially more toxic than Zn. In the same water quality as that used in the present study, the 96-h LC_{50} for Cd and Zn to rainbow trout has been determined to 0.3 and 14 µM, respectively (17). Thus Cd is ~50 times more toxic than Zn despite the fact that the two ions have equal inhibitory effects on the basolateral Ca^{2+}-ATPase and at least partially share the same transcellular transport pathway. The pronounced difference in in vivo toxicity between the two metals must, therefore, lay either in the accumulation rate in the gill tissue or on the intracellular handling of the metals. Because Zn^{2+} is an essential micronutrient that is normally taken up through the gill, whereas Cd^{2+} is not, the system appears to be much better designed to handle Zn^{2+}.

Perspectives

Ca^{2+} is believed to enter the chloride cells through a voltage-independent channel in the apical membrane driven by the electrochemical gradient (5). Although this "Ca^{2+} channel" has never been isolated or patch clamped, there is a wealth of indirect evidence supporting its existence (5). Pharmacological blockage (i.e., La treatment) and endocrine inactivation (i.e., stanniocalcin) of this putative channel reduced the branchial influx of both Ca^{2+} and Zn^{2+}. Previous work provides evidence that Cd^{2+} also passes the apical membrane through the same channel (33). Thus the apical Ca^{2+} channel of the gill epithelium may be a relatively nonspecific divalent cation channel. Electrophysiological and/or molecular studies are required to verify this hypothesis.

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