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

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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\(^{2+}\) 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 \(\mu\)M, 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 as possible sites of Zn\(^{2+}\) interaction with the Ca\(^{2+}\) uptake. 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\(^{2+}\) (11, 28, 33). 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 held 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\(^{2+}\)], 3.16 [HCO\(_3^{-}\)], pH 7.5) at 10°C. Rainbow trout were fed dry pellets at a daily ration of 3% of the nominal values.

**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 [Ca(NO\(_3\)]), and 1.0 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 mM 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 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), Lauren and McDonald (19), Verbost et al. (29, 33), Lauren 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-hydroxethylpiperazine-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 50 min (Beckman Ti70 rotor), and a pellet

incubated at 45°C overnight before they were counted for \(^{46}\)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 (Protocol, New England Nuclear) for 48 h at 45°C. The samples were neutralized with 20 ml of glacial acetic acid, diluted with 10 ml of scintillation fluid (OCS, Amersham) and counted for \(^{36}\)Cl\(^{-}\) in the scintillation counter. In all cases, counting efficiencies were assessed by internal standardization.

Water samples containing \(^{60}\)Zn\(^{2+}\) or \(^{22}\)Na\(^{+}\) were counted directly in the gamma counter. To the water samples containing \(^{46}\)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 pmol·kg\(^{-1}\)·h\(^{-1}\)) were calculated according to the formula

\[
J_{in} = CT/(SA \times CE \times t)
\]

where CT is the counts in tissue [counts/(min·kg)], SA is the measured mean specific activity of the water [counts/(min·mol)], 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\(_2\)O dissolved in 0.9% NaCl (wt/vol). An equal number of fish was 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.

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consisting of a firm brownish part with a lighter fluffy upper
layer was obtained. The cloudy top of the sample was resus-
pended in 30 ml of an isosmotic buffer (250 mM sucrose, 5 mM
MgCl₂, 5 mM HEPES/Tris, pH 7.4), using a Dounce homog-
enizer (100 strokes), to prepare inside-out oriented vesicles.
The resulting membrane suspension was centrifuged differen-
tially, (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⁺-Zn²⁺ exchange stud-
ies, the BLMs were resuspended in a buffer containing (in mM)
150 NaCl, 0.8 mM MgCl₂, 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 MgCl₂, 5 HEPES, 5 Tris-HCl, pH 7.4 (isosuc-
crose; for ATPase assays) or the NaCl-containing buffer (for
Na⁺/Zn²⁺-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 acetylcho-
line esterase activity (i.e., marker for inside-out vesicles)
measured in intact and permeabilized vesicles and the spec-
ific trypsin sensitivity of the cytosol-oriented part of the Na⁺-
K⁺-ATPase (i.e., marker for right-side-out vesicles). Typi-
cally, 20% of the BLMs were inside-out vesicles and 30%
right-side-out vesicles (18, 32). The remaining 50% of 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 assay 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 homog-
enate. Na⁺-K⁺-ATPase activity, defined as Na⁺- and K⁺-
dependent, ouabain-sensitive phosphorylase activity, was ana-
lyzed 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 Ca²⁺-
ATPase activity from the ER. This concentration of thapsig-
argin completely blocks ER Ca²⁺ pumps in trout gill prepara-
tions (Verbost, unpublished observations) and tilapia
(Oreochromis mossambicus) gill preparations (31).

Calcium and zinc transport assays in BLM. Ca²⁺ and Zn²⁺
transport were determined in assay media that contained
0.5 mM EGTA, 0.5 mM N-2-hydroxyethyl)-ethylendiamine-
N,N',N'-triacetatic acid (HEEDTA), and 0.5 mM nitritroacetate
acid (NTA) as a Ca²⁺ and Zn²⁺ buffering system. Free Ca²⁺,
Zn²⁺, and Mg²⁺ 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 Ca²⁺ was determined in
0.5-min incubations of membrane vesicles, which yielded
initial transport velocities. ATP-driven transport of Zn²⁺ 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 ⁴⁵Ca²⁺/stable
Ca²⁺ and/or ⁶⁵Zn²⁺/stable Zn²⁺. 150 mM KC1, 20 mM
HEPES/Tris, pH 7.4, 0.8 mM Mg²⁺ (free), and the 0.5 mM
EGTA/HEEDTA/NTA buffer system described above. This
composition of the medium is optimal for Ca²⁺ transport (18).
For studies of ATP-driven Zn²⁺ transport, two concentrations
of Zn²⁺ were used. These concentrations of free Zn²⁺, 10⁻¹⁰
and 10⁻¹² M, were chosen because the former had the highest
possible specific activity (250 mCi/mM Zn²⁺ added), and the
latter represented a concentration close to the "no ob-
served effect level" for Zn²⁺ on the Ca²⁺-ATPase (see Fig. 5).
Our results indicate that a Zn²⁺ activity as low as 50 pM may
inhibit the basolateral Ca²⁺-ATPase. It is therefore reason-
able to assume that the intracellular Zn²⁺ activity in the gill
epithelium cells never exceeds this level during physiological
conditions and that any specific Zn²⁺ transporter must oper-
ate at subpicomolar Zn²⁺ concentrations. Zinc transport
assays were run, both with no Ca²⁺ present and at the normal
cytosolic concentration of free Ca²⁺, 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 LaCl₃ (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 tempera-
ture and thereafter counted for ⁴⁵Ca²⁺ or ⁶⁵Zn²⁺ in a scintilla-
tion counter (Pharmacia-Wallac 1410). With no Zn²⁺ in the assay
medium (i.e., control conditions), the ATP-dependent Ca²⁺
transport was typically 400–400% higher than the nonspe-
cific binding.

Assays for Na⁺/Zn²⁺-exchange activity (i.e., uptake of Zn²⁺
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⁺/Ca²⁺ exchange. Five microliters of vesicles, equili-
ibrated with 150 mM NaCl, was added to 120 μl of medium
containing either 150 mM NaCl (blank) or 150 mM KCl and a
varying concentration of ⁶⁵Zn²⁺/stable Zn²⁺ and Ca²⁺. The
Zn²⁺ concentrations tested were 10⁻¹⁰, 10⁻¹², and 10⁻¹⁰ M.
Each of these Zn²⁺ concentrations was run with either 5 pM
Ca²⁺ or no Ca²⁺ 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 mem-
brane. In addition to the 150 mM NaCl or KCl, the assay
medium contained 20 mM HEPES/Tris, pH 7.4, 0.8 mM Mg²⁺
(free), and 0.5 mM EGTA/HEEDTA/NTA. After 1-min incuba-
tion, the reaction was arrested by addition of stopping buffer,
as described above. Vesicles were collected by rapid filtration,
rinsed, and counted for ⁶⁵Zn²⁺. The difference in ⁶⁵Zn²⁺
accumulation in K⁺- and Na⁺-containing media was taken as
a measure of Na⁺ gradient-driven Zn²⁺ transport.

The Kₘ and maximal velocity (Vₘₚ) 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 pro-
gram 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
Zn²⁺ on Ca²⁺ 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 kg\(^{-1}\) h\(^{-1}\), respectively (means ± 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 \(\mu\)M 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 kg\(^{-1}\) h\(^{-1}\) (means ± 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 mg\(^{-1}\) min\(^{-1}\) for ATP-driven Zn\(^{2+}\) transport and 10\(^{-23}\) mol mg\(^{-1}\) 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 \(V_{\text{max}}\) 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 \(V_{\text{max}}\) 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 \(V_{\text{max}}\) became significantly reduced only at the highest tested concentration of free Zn\(^{2+}\), 500 pM (Table 1). At the same Zn\(^{2+}\) activity, the increase in \(K_{M}\) was eightfold, and the \(V_{\text{max}}\) 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.
MECHANISM OF ZINC UPTAKE IN RAINBOW TROUT

Fig. 2. Effect of Ca2+ injection on the influx of Ca2+ and Zn2+ in juvenile rainbow trout. Bars show mean influx of Ca2+ and Zn2+ of 10 fish, expressed as percentage of the control mean. Error bars denote SE. Absolute control means of Ca2+ and Zn2+ influx were 44 ± 4.9 and 0.0927 ± 0.00493 (means ± SE) nmol kg⁻¹ h⁻¹, respectively. *Significant difference compared with the control value (P < 0.05).

Table 1. Effect of free Zn²⁺ on V_max and K_M for the transport kinetics of ATP-driven Ca²⁺ transport in isolated basolateral membrane vesicles of adult rainbow trout

<table>
<thead>
<tr>
<th>Zn²⁺, pM</th>
<th>V_max, nmol mg⁻¹ min⁻¹</th>
<th>K_M, pM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.05 ± 0.51</td>
<td>0.13 ± 0.03</td>
<td>7</td>
</tr>
<tr>
<td>60</td>
<td>4.36 ± 1.16</td>
<td>0.57 ± 0.34</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>3.70 ± 0.91</td>
<td>0.43 ± 0.08*</td>
<td>6</td>
</tr>
<tr>
<td>500</td>
<td>2.13 ± 0.49*</td>
<td>1.03 ± 0.19*</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 4–7 individuals). Values are derived from nonlinear regression curves of individual samples, describing the Michaelis-Menten equation. The Zn²⁺ concentration is the free Zn²⁺ 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).

Two independent approaches were used to investigate whether Zn²⁺ and Ca²⁺ cross the apical membrane of the gill epithelium by the same mechanism. The first approach was to block the Ca²⁺ uptake sites with La, a classic Ca²⁺-channel blocker (36). La treatment was an effective inhibitor of both Ca²⁺ and Zn²⁺ influxes, suggesting common uptake sites for Ca²⁺ and Zn²⁺ 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 Ca²⁺/Zn²⁺ 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 Zn²⁺ most likely enters the fish through the Ca²⁺-transporting system in the apical membrane of the chloride cells.

The second approach used to demonstrate the intimate relationship between the uptake mechanisms for Ca²⁺ and Zn²⁺ was to give the fish a signal to reduce Ca²⁺ influx and then observe the effect on branchial Zn²⁺ influx. Such a signal was given by injecting ionic Ca²⁺. From previous research it is firmly established that a consequence of Ca²⁺ 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 Ca²⁺ (11, 28, 33, 34). This treatment had the predicted inhibitory effect on the branchial Zn²⁺ influx, and it also significantly reduced the influx of Zn²⁺. The results provide an independent line of evidence that the apical passage for Zn²⁺ takes place through the Ca²⁺-transporting system in the apical membrane of the chloride cells.

After both La treatment and Ca²⁺ injection the inhibition of Ca²⁺ influx was more pronounced than that of Zn²⁺. This difference could be accounted for if the affinity of the uptake sites were higher for Zn²⁺ than for Ca²⁺. Previous investigations do, in fact, show that the K_M for Zn²⁺ 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 \(J_{\text{max}}\) 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 \(Zn^{2+}\) 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 LC50 for \(Cd\) and \(Zn\) to rainbow trout has been determined to be 0.3 and 14 \(\mu M\), 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 blockade (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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