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COVARIATION IN REGULATION OF AFFINITY FOR BRANCHIAL ZINC AND CALCIUM UPTAKE IN FRESHWATER RAINBOW TROUT

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Summary

The possible coupling between regulation of the affinities for branchial Zn and Ca influx was investigated in juvenile rainbow trout *Oncorhynchus mykiss* acclimated to relatively hard fresh water ([Ca]=1.0 mmol l^{-1}). The K_m for branchial Ca influx was manipulated experimentally by exposing the fish to 2.3 μmol l^{-1} waterborne Zn for a total of 28 days. This procedure resulted in rapidly increased K_m values for both Ca and Zn influx, an effect that remained through the experimental period. There was a significant linear correlation (r=0.88, P<0.02) between K_m values for Ca and Zn measured at the same time points. Zn exposure caused progressively increasing maximum rate of transport, J_{max}, values for Zn relative to the control value, but there was little, if any, effect on J_{max} for Ca. These results support the idea of a shared transport site for Zn and Ca at the apical membrane of the gill epithelium and suggest that there is a certain degree of coregulation of branchial Zn and Ca uptake in rainbow trout. Removal of Ca from the water resulted in a large (six- to 24-fold) increase in affinity (decreased K_m) for Zn influx and a modest (1.1- to 1.8-fold) increase in J_{max} for Zn. Thus, Ca is a competitive inhibitor of Zn influx. In water lacking Ca, the K_m for Zn in Zn-acclimated fish was no different from that of the control fish, suggesting that the Ca^{2+}/Zn^{2+} transporter was regulated to improve Ca uptake.

Key words: fish, rainbow trout, *Oncorhynchus mykiss*, zinc, calcium, influx, uptake, acclimation, regulation, branchial, gill.

Introduction

In several recent studies, it has been suggested that Zn and Ca to some extent share a common uptake route across the gill epithelium of freshwater-acclimated rainbow trout (Spry and Wood, 1989; Hogstrand *et al.* 1994, 1995, 1996). Specifically, these studies show that Ca is a competitive inhibitor of Zn influx (Spry and Wood, 1989) and that Zn is a competitive inhibitor of Ca influx (Hogstrand *et al.* 1994, 1995). Ca^{2+} is believed to enter the gill epithelium through a voltage-insensitive 'transporter' (ion channel or facilitated diffusion), located in the apical membrane (Perry and Flik, 1988; Flik and Verbost, 1993). Experimental evidence indicates that this transporter is the site for apical Zn^{2+} transfer as well. For example, exposure to waterborne La^{3+} effectively inhibited the entry of both Zn and Ca (Hogstrand *et al.* 1996). Moreover, Zn and Ca influx were reduced when endogenous release of the calciostatic hormone stanniocalcin was stimulated by injection of Ca (Hogstrand *et al.* 1996). At least in freshwater fish, the basolateral transport of Ca^{2+} is primarily mediated by a high-affinity Ca^{2+}-ATPase (Flik and Verbost, 1993). Recent results argue against an involvement of this Ca^{2+}-ATPase in Zn transport under physiological conditions. No ATP-dependent or Na^+-gradient-driven transport of Zn could be detected in basolateral membrane vesicle preparations from rainbow trout gill epithelium (Hogstrand *et al.* 1996). Indeed picomolar concentrations of free Zn^{2+} blocked Ca^{2+}-ATPase-mediated Ca^{2+} transport in the same preparations. Thus, at this point, there is considerable pharmacological and circumstantial evidence suggesting that Zn and Ca share the same apical entry pathway, but that the basolateral transfer is carried out by separate mechanisms.

Zn is a micronutrient and, as such, its uptake and excretion are probably regulated to meet the physiological requirements on a daily and seasonal basis (for reviews, see Vallee and Falchuk, 1993; Hogstrand and Wood, 1996). For example, uptake from the water and from the food can be manipulated separately by the fish so as to achieve Zn homeostasis in the face of waterborne or dietary variations in Zn levels (Spry *et al.* 1988). Although Zn is an essential element, it is also potentially toxic to fish and, not surprisingly, the key toxic effect of waterborne Zn seems to be a disturbance of Ca metabolism (reviewed by Hogstrand and Wood, 1996). Fish chronically exposed to elevated concentrations of waterborne Zn are often able to acclimate physiologically, and this acclimation involves a progressive reduction in the branchial influx of Zn and a

restoration of normal plasma Ca^{2+} levels (Hogstrand *et al.* 1994, 1995). In two previous investigations, we found that a reduction in branchial Zn uptake occurred concomitantly with a decrease in the affinity (increased K_m) for Ca and speculated that the decreased Zn influx might be brought about by a reduction in the affinity of the shared apical Ca^{2+}/Zn^{2+} transporter (Hogstrand *et al.* 1994, 1995). These studies were carried out at an ambient water Ca concentration (1 mmol l^{-1}) yielding influx rates, J_{in}, for Ca close to the J_{max}. The Zn concentration, in contrast, was kept at a level (2.3 μmol l^{-1}) below the K_m for Zn influx (3.6 μmol l^{-1}, as determined by Spry and Wood, 1989). This means that a reduction in K_m of the Ca^{2+}/Zn^{2+} transporter should greatly decrease the influx of Zn while leaving Ca influx more or less unchanged. If the apical entry steps of Zn and Ca occur through the same transport mechanism, and if the purpose of increasing the K_m for Ca is to reduce the affinity for Zn, then, changes in K_m for Zn and Ca influx should be coordinated. Specifically, the K_m for Zn influx and the K_m for Ca influx should both increase during acclimation to waterborne Zn. Data from previous work cannot provide a direct answer to this problem because the K_m for Zn influx has not been analyzed during Zn acclimation or in parallel with Ca influx kinetics.

The present study was designed to test the hypothesis that the branchial influx of Zn can be regulated by alterations in the affinity of a mutual Ca^{2+}/Zn^{2+} transporter. The affinity for Ca was manipulated, as previously described (Hogstrand *et al.* 1994, 1995), by a long-term exposure to an elevated, but sublethal, level of waterborne Zn. To enable correlations to be made between changes in kinetic parameters for Zn and Ca influx, the kinetics of Ca influx was measured in parallel with the kinetics of Zn influx. Since Ca is a competitive inhibitor of Zn influx (Spry and Wood, 1989), analysis of Zn influx in the presence of Ca in the water produces values of the apparent K_m for Zn influx. As an additional facet of the present study, we attempted to determine the true kinetic properties of the Zn transport system by eliminating Ca from the flux medium. This analysis was carried out simultaneously with the final measurement of Zn kinetics in the presence of Ca on day 28 of the exposure.

Materials and methods

Animals

Female juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum) (1–5 g), were obtained from a local hatchery (Rainbow Springs Hatchery, New Dundee, Ontario). The fish were held in two 264 l fibreglass tanks (350 fish per tank), each supplied at a rate of 900 ml min^{-1} with a continuous flow of dechlorinated, aerated Hamilton city tapwater ($[Na^+]$=0.6 mmol l^{-1}; $[Cl^-]$=0.7 mmol l^{-1}; [Ca]=1.0 mmol l^{-1}; $[HCO_3^-]$=1.9 mmol l^{-1}; pH 7.9–8.2) at a temperature of 9–11 °C. Fish were fed dry trout pellets (Martin's Feed Mill Ltd, Ontario) at a ration of 1 % of their body mass per day.

Zinc exposure

After a 2 week period of acclimation to laboratory conditions, one of the two tanks was equipped with a dosing system which added Zn, as $ZnSO_4 \cdot 7H_2O$ (BDH Chemicals), from a stock solution (45 mg l^{-1}=690 μmol l^{-1}). The fish in the other tank served as a control. The flow rate of the added Zn stock was maintained using a pump (Masterflex), which delivered 3 ml min^{-1} to produce a final total Zn concentration in the exposure tank of 2.3 μmol l^{-1} (=150 μg l^{-1} measured concentration; 2.25±0.11 μmol l^{-1}; mean ± S.E.M., N=44). Computer modelling of the water chemistry, using the MINEQL+ program (Schecher and McAvoy, 1994) indicated that 59 % of the added Zn was present as Zn^{2+}, 25 % as $ZnCO_3$(aq), 7.5 % as $Zn(OH)_2$, 3.1 % as $ZnHCO_3^+$ and 2.0 % as $ZnOH^+$. Speciation calculations further suggested that 96 % of the Ca present was speciated as Ca^{2+} and 4 % as $CaSO_4$. The flow rates of water and Zn stock solution were checked daily and adjusted if necessary. Water samples were taken once or twice a day from the exposure tank for analysis of total Zn. The samples were acidified with HNO_3 (trace metal grade, BDH Chemicals, was used in all procedures) to a final concentration of 1 % (w/w), and Zn was measured by atomic absorption spectroscopy (AAS; Varian AA-1275) using an air/acetylene flame. The day the exposure started is referred to as day 0. The experiment continued for 28 days, during which the water temperature gradually increased from 9 to 11 °C owing to ambient conditions. Feeding was maintained as described above at 1 % of the body mass per day. No mortalities occurred during the exposure period.

Zinc influx kinetics

Michaelis–Menten kinetics of unidirectional Zn influx were analyzed on days 1, 4, 8, 16 and 28 of the experiment according to the procedure described by Hogstrand *et al.* (1994). This method, which is based on the appearace of ^{65}Zn in the body of the fish over a 24 h period, was found to provide the same results as that developed by Spry and Wood (1989), which is based on the appearance of ^{65}Zn in the blood plasma (data not shown). The flux chambers were black polypropylene bags filled with 15 l of dechlorinated Hamilton tapwater. Each of these flux bags was equipped with an airline and placed in a plastic basket for support. The flux bags were placed in a waterbath which was adjusted so that the water inside the bags was at the same temperature (9–11 °C) as that in the fish tanks. Unidirectional influx of Zn was analyzed at total nominal water Zn concentrations of 50, 100, 250, 600 and 1500 μg l^{-1} (0.76, 1.5, 3.8, 9.2 and 23 μmol l^{-1}); relative speciation was identical to that tabulated above (MINEQL+; Schecher and McAvoy, 1994) at all Zn concentrations. 5 min before the fish were placed in the flux bags, Zn was added to the water from a $^{65}Zn/ZnSO_4$ stock solution (Zn=22.9 mmol l^{-1}, 1.66 kBq μmol^{-1}). Eight fish were used for each concentration of Zn. At the end of the flux period, the fish were lightly anaesthetized with tricaine methanesulphonate (MS 222, 0.1 g l^{-1}) and transferred to a beaker of dechlorinated tapwater containing an overdose of MS 222 (1.0 g l^{-1}) and a high concentration of 'cold' Zn (150 mg l^{-1}=2.3 mg l^{-1}) to displace surface-bound ^{65}Zn. After a 1 min rinse in this solution, the fish were blotted dry, placed in individual scintillation vials, and

assayed for ^{65}Zn activity in a γ-counter (MINAXI Auto-Gamma 5000 Series, Canberra-Packard). Water samples were similarly counted for ^{65}Zn activity, and total [Zn] in the water was measured by atomic absorption spectroscopy (AAS) (Varian 1275). The influx of Zn was calculated from the counts in the whole body divided by the measured specific activity of ^{65}Zn in the water, the duration of the flux period and the mass of the fish.

On day 28 in both control and Zn-exposed groups, Michaelis–Menten analysis of Zn influx was carried out without Ca present in the water, in addition to the analysis (with Ca) described above. The objective was to obtain values of J_{max} and K_m for Zn influx in the absence of the competitive inhibitor, Ca. The Ca-free water used for these flux analyses consisted of 0.7 mmol l^{-1} NaCl and 1.9 mmol l^{-1} $KHCO_3$, pH 8.1. The nominal Zn concentrations used in the assay were 6.25, 12.5, 25, 50 and 100 μg l^{-1} (0.096, 0.19, 0.38, 0.76 and 1.5 μmol l^{-1}). In all other aspects, the flux analysis without Ca present was carried out in the same way as that described above for Zn influx in the presence of 1 mmol l^{-1} Ca.

Calcium influx kinetics

The kinetics of unidirectional Ca influx were determined on days 1, 7, 16 and 28 of the experiment. Kinetic analysis was performed as described by Hogstrand *et al.* (1994). For the control group, eight fish were put into each of six polypropylene flux bags containing 3 l of synthetic water ([NaCl]=0.7 mmol l^{-1}; [$KHCO_3$]=1.9 mmol l^{-1}; pH 8.0) with a designated concentration of Ca achieved by adding an appropriate volume of a $^{45}Ca/Ca(NO_3)_2$ stock solution (150 mmol l^{-1}; specific activity 148 kBq mmol^{-1}). The six flux bags represented a geometric series of increasing [Ca], with approximate values of 25, 50, 150, 350, 750 and 1500 μmol l^{-1}. The activity of Ca^{2+} in the medium was calculated to be 99.9 % of the total Ca concentration (MINEQL+, Schecher and McAvoy, 1994). For the experimental group, unidirectional influx of Ca^{2+} was measured in the presence of 150 μg Zn l^{-1} (2.3 μmol l^{-1}), yielding values of apparent K_m and J_{max} (i.e. determined in the presence of the competitor). The unidirectional Ca influx was calculated from the appearance of ^{45}Ca radioactivity in the whole body over a 4 h period, as described by Hogstrand *et al.* (1994). The fish were killed, rinsed for 1 min in 10 mmol l^{-1} $Ca(NO_3)_2$ to displace surface-bound ^{45}Ca, and processed for scintillation counting as described by Hogstrand *et al.* (1994).

All whole-animal flux experiments followed evaluated standard procedures described by Perry and Wood (1985), Lauren and McDonald (1986), Spry and Wood (1989) and Hogstrand *et al.* (1994). The 1 min washing step was considered effective and appropriate for the removal of externally bound radioactive isotope, because it was found to provide similar transport rates (data not shown) to those based on the appearance of isotope in the blood plasma (Perry and Wood, 1985; Hogstrand *et al.* 1994, 1995; Perry and Flik, 1988). The data presented on Ca and Zn influx are likely to represent the branchial pathway, because oesophageal ligation experiments have eliminated the intestine as a significant route of Ca and Zn uptake during conditions identical to the flux experiments performed in the present study (Perry and Wood, 1985; Spry, 1987).

Calculations and statistical methods

Non-linear regression was used to obtain values of J_{max} and K_m for unidirectional influx of Zn and Ca. The Levenberg–Marquardt algorithm was used to fit J_{in} at different substrate (Zn or Ca) concentrations to the Michaelis–Menten equation (Leatherbarrow, 1987). Calculations of J_{in} were based on measured concentrations of total Zn or Ca (i.e. not free Zn^{2+} or Ca^{2+}). Significant differences between control and experimental groups at each flux measurement were evaluated by Student's *t*-test (two-tailed, unpaired). The number of observations for flux kinetics was considered to be the same as the number of concentrations in each kinetic series (i.e. N=5 for Zn and N=6 for Ca), rather than the total number of fish in each series (i.e. 40 for Zn and 48 for Ca). This conservative strategy was chosen because the fish in the same flux chamber were exposed to the same water and could not be regarded as totally independent samples. Groups were considered significantly different at $P<0.05$.

Results

Unidirectional influx of Zn and Ca both followed saturation kinetics and were well described by the Michaelis–Menten equation. Fig. 1 shows the Michaelis–Menten kinetic curves for influx of Zn (Fig. 1A) and Ca (Fig. 1B) on day 28 of the experiment. In control fish, J_{max} was approximately 200 times higher for Ca than for Zn, but the K_m for Zn influx was 80 times lower than that for Ca influx. Thus, the uptake system has a higher capacity for Ca transport than that for Zn, but the affinity is greater for Zn than for Ca. Exposure to 2.3 μmol l^{-1} (150 μg l^{-1}) waterborne Zn increased the K_m for influx of both Zn and Ca (i.e. decreased affinity). Zn exposure resulted in a substantial increase in the J_{max} for Zn ($P<0.001$), while the J_{max} for Ca showed a slight depression (on day 28 only, $P<0.05$; Fig. 1). The kinetic variables J_{max} and K_m for both Zn and Ca influx in control fish varied considerably over the experimental period (Figs 2, 3). However, simultaneous flux measurements of both controls and Zn-exposed fish allowed for comparison between the groups at each time point. The maximum transport rate, J_{max}, for Zn in control fish ranged from 0.24 to 0.41 μmol kg^{-1} h^{-1} (Fig. 2A). Control J_{max} values for Ca were between 12.5 and 43.5 μmol kg^{-1} h^{-1} (Fig. 3A). The K_m for Zn in controls varied between 3.6 and 7.8 μmol l^{-1} (Fig. 2B), and the control K_m for Ca ranged from 11 to 40 μmol l^{-1} (Fig. 3B). Thus, J_{max} and K_m were always much higher for Ca than for Zn.

The Zn-induced increase in K_m for Zn and Ca appeared to be present throughout the entire experimental period (Figs 2B, 3B), with the exception of day 1, where the elevation in K_m for Ca influx in Zn-exposed fish was not statistically significant. The J_{max} for Ca remained relatively unaffected by the treatment with the exception of a slight but significant inhibition on day 28 (Fig. 3A). Generally, J_{max} for Ca of Zn-

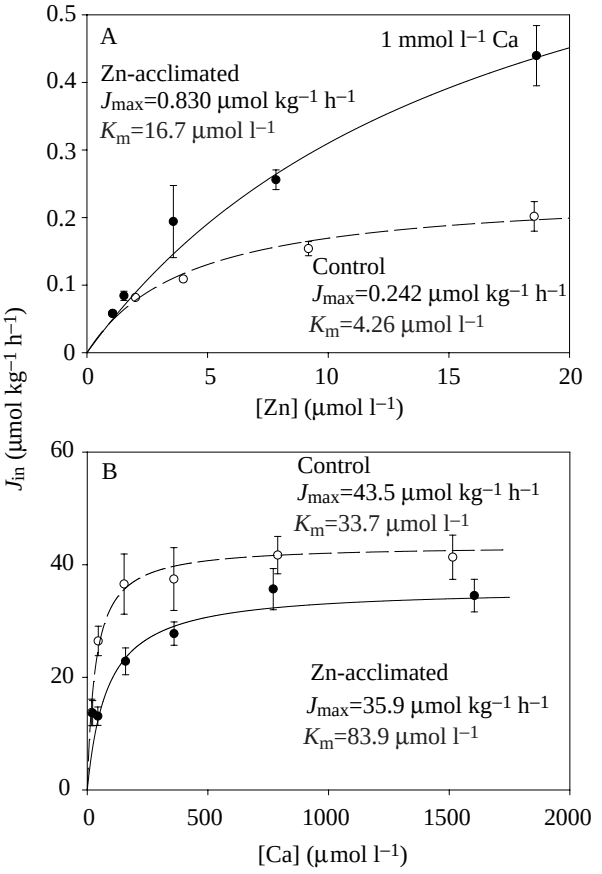

Fig. 1. Michaelis–Menten kinetics of unidirectional Zn and Ca influx across the branchial epithelium of rainbow trout after acclimation to 2.3 μmol l^{-1} waterborne Zn for 28 days and in control fish, tested simultaneously. The graphs show the substrate-dependency of (A) Zn influx in the presence of 1 mmol l^{-1} Ca and (B) Ca influx in the presence (Zn-acclimated fish) and absence (control fish) of waterborne Zn. Solid lines and filled circles show data from Zn-acclimated fish; dashed lines and open circles represent control fish. Each point is the mean value of eight fish. Vertical bars denote 1 S.E.M. J_{in}, rate of influx; J_{max}, maximal rate of influx; K_m, affinity constant.

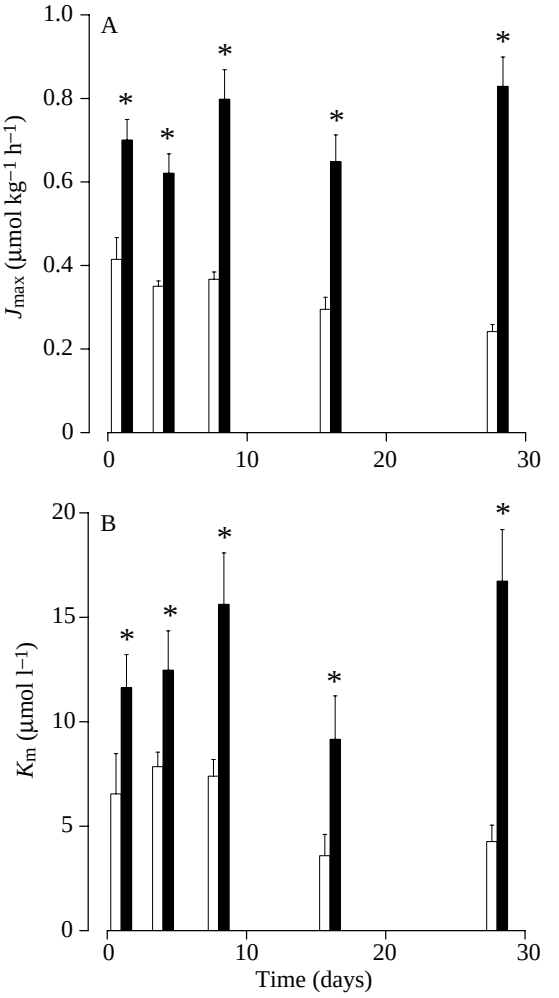

Fig. 2. Changes in (A) J_{max} and (B) K_m for unidirectional influx of Zn (in the presence of Ca) during acclimation to 2.3 μmol l^{-1} waterborne Zn. Filled columns show values for Zn-exposed fish and open columns represent controls. Values are shown as means +1 S.E.M. Means were calculated from five groups of eight fish each (N=5). Asterisks indicate statistically significant differences from the control value at the same time point. J_{max}, maximal rate of influx; K_m, affinity constant.

exposed fish followed the same pattern of changes as that of the control group (Fig. 3A). In contrast, the J_{max} for Zn in the experimental group increased progressively over time, relative to that of the control group (Fig. 2A). This trend was more evident when the J_{max} for Zn in Zn-exposed fish was expressed as a percentage of the simultaneous control value (Table 1).

Possible covariance between the affinities for Zn and Ca transport was evaluated by plotting the K_m for Zn influx against that for Ca (Fig. 4). Because of the natural temporal variations in K_m for both elements, only data from the same days were used for this analysis. Regression analysis suggested a close (r=0.88) and statistically significant (P<0.020) linear relationship between K_m for Zn and Ca influx. If K_m values for Ca influx on day 7 and for Zn influx on day 8 were introduced as a data pair, the correlation coefficient was slightly decreased (r=0.793), whereas the level of significance was further increased (P<0.0188; data not shown).

In order to determine the true, rather than the apparent, values of K_m and J_{max} on day 28, additional kinetics series for Zn influx were determined in the absence of the competitive inhibitor Ca. Acute removal of Ca from the water had several notable effects. In contrast to the flux measurements performed with Ca present in the water, there were mortalities during the 24 h in water without Ca; survival varied from 75 to 100 % (0–2 dead fish per flux bag) in a fashion that was not obviously related to the presence of Zn. Kinetic curves for Zn influx were very different in the absence of Ca (compare Fig. 5 with Fig. 1A and note the difference in scales of the axes). Removal of Ca caused the K_m for Zn influx in control fish to decrease from 4.3±0.8 μmol l^{-1} (mean ± S.E.M., N=5) to 0.68±0.10 μmol l^{-1} (mean ± S.E.M, N=5), indicating a large (sixfold) increase in affinity for Zn (Figs 1A, 5). The K_m for Zn in fish exposed to Zn for 28 days was 16.7±2.5 μmol l^{-1}

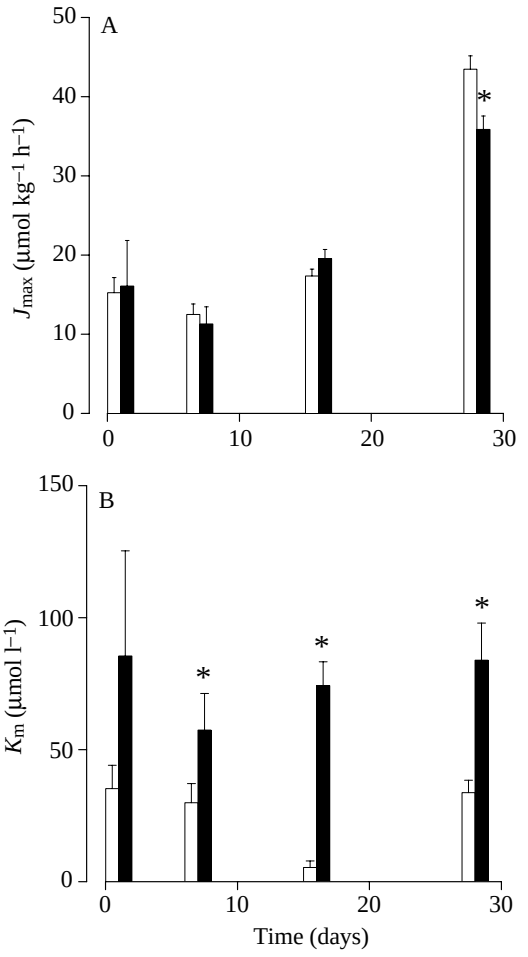

Fig. 3. Changes in (A) J_{max} and (B) K_m for unidirectional influx of Ca during acclimation to 2.3 μmol l^{-1} waterborne Zn. For the experimental group, the influx of Ca was analyzed in the presence of 2.3 μmol l^{-1} Zn, whereas no Zn was present during the analysis of control Ca influx. Filled columns show values for Zn-exposed fish and open columns represent controls. Values are shown as means +1 S.E.M. Means were calculated from six groups of eight fish each (N=6). Asterisks indicate statistically significant differences from the control value at the same time point. J_{max}, maximal rate of influx; K_m, affinity constant.

(mean ± S.E.M, N=5) in the presence of 1 mmol l^{-1} of Ca compared with 0.71±0.28 μmol l^{-1} (mean ± S.E.M, N=5) when Ca was removed. Thus, the difference in K_m between Zn-exposed fish and control fish disappeared when Ca was withdrawn from the water. In control fish, the removal of Ca caused a twofold increase in J_{max} for Zn from 0.24±0.02 to 0.43±0.05 μmol kg^{-1} h^{-1} (compare Fig. 5 with Fig. 1A). There were no changes in J_{max} for Zn in Zn-acclimated fish following withdrawal of Ca (0.83±0.07 μmol kg^{-1} h^{-1} with Ca and 0.93±0.22 μmol kg^{-1} h^{-1} without Ca).

Discussion

The present study is the first to explore the Michaelis–Menten kinetics of Zn influx during exposure of fish

Table 1. *Maximum transport rate, J_{max}, for Zn influx in rainbow trout exposed to 2.3 μmol l^{-1} waterborne Zn*

	Day 1	Day 4	Day 8	Day 16	Day 28
J_{max} for Zn (% of control)	169±12	177±13	217±19	220±22	343±29

The values are expressed as a percentage of the simultaneous control mean ± 1 S.E.M. (N=8).

All values were significantly different from those of the control at P<0.001.

to an elevated level of waterborne Zn. It has been shown previously that waterborne Zn induces a reduction in the affinity of the Ca transport system and that physiological acclimation to waterborne Zn is associated with a decreased Zn influx (Hogstrand *et al.* 1994, 1995). Furthermore, there is direct experimental evidence suggesting that apical entries of Zn and Ca into the gills occur by a shared transport mechanism, which may be regulated by the calciostatic hormone stanniocalcin (Hogstrand *et al.* 1996). The present study ties the information from these previous investigations together by showing an intimate connection between the regulation of Zn influx and Ca influx. The strong positive correlation between the K_m values for Zn and Ca strongly supports the hypothesis that Zn and Ca share a common apical transporter and suggests that there is a certain degree of coregulation in Zn and Ca influx. The documented downregulation of Zn influx during acclimation to waterborne Zn in hard water (Hogstrand *et al.* 1994, 1995) is probably caused by a decreased affinity of this branchial Ca^{2+}/Zn^{2+} transporter for both elements.

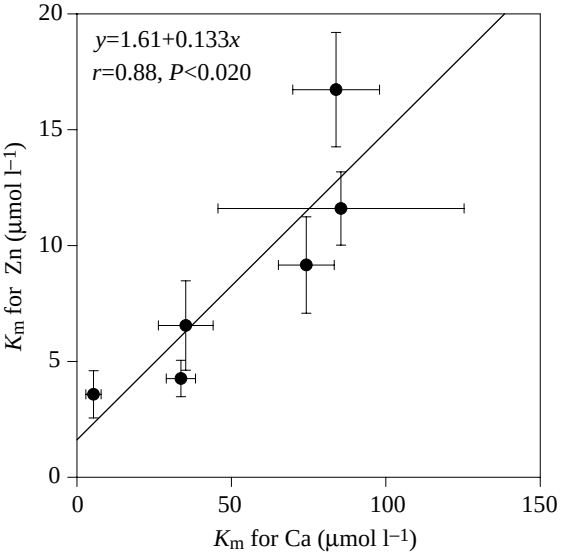

Fig. 4. Correlation between the K_m (inverse of affinity) for Zn and Ca influx in control fish and in fish (Zn-exposed) with experimentally increased K_m values for both elements. Because of cyclic changes in rates of Zn and Ca transport (see text), only data pairs generated on the same days (days 1, 16 and 28) were used. Values are shown as means ± 1 S.E.M.

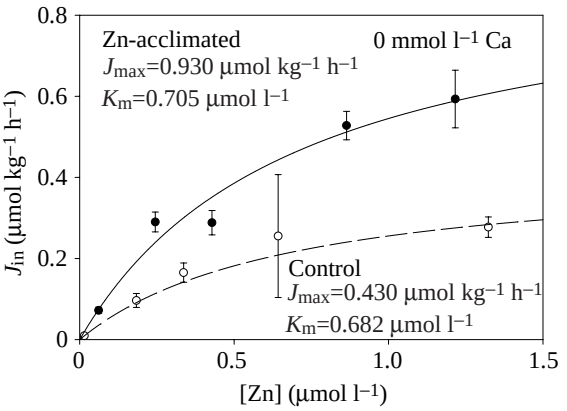

Fig. 5. Michaelis–Menten kinetics of unidirectional Zn influx across the branchial epithelium of rainbow trout after acclimation to 2.3 μmol l^{-1} waterborne Zn for 28 days and in control fish, tested simultaneously. In contrast to the experiment shown in Fig. 1A, Ca was omitted from the water. Note the different axes scales compared with Fig. 1A. Each point represents the mean value for 6–8 fish; error bars show ±1 S.E.M. Other details are as in the legend to Fig. 1. J_{in}, rate of influx; J_{max}, maximal rate of influx; K_m, affinity constant.

The effect of waterborne Zn on J_{max} for Ca seems to be modest at the Zn concentration used in the present study. At the last flux measurement only (i.e. after 28 days of exposure), there was a small depression of the J_{max} for Ca. Similar results have been obtained in previous studies (Hogstrand *et al.* 1994, 1995), indicating that there is no major disruption in the total number of functional Ca^{2+} transport sites at this level of Zn exposure. In contrast to the affinity constant, K_m, there was no apparent correlation between the maximum transport rate, J_{max}, for Zn and Ca. This finding is in accordance with previous findings that changes in J_{max} for Ca have no direct influence on Zn influx (Hogstrand *et al.* 1994, 1995). The partial coupling between Zn and Ca influx (dependent K_m but independent J_{max}) may form the basis for discriminating the regulation of levels of these two elements. In hard water, the Ca concentration (1 mmol l^{-1}) greatly exceeds the K_m for Ca influx in Zn-exposed fish (50–250 μmol l^{-1}; Hogstrand *et al.* 1994, 1995; present study) and the actual rate of influx, J_{in}, is modified mainly by changes in J_{max}. In contrast, influx of Zn is very dependent upon the K_m because the Zn concentration of natural waters (Eisler, 1993; Hogstrand and Wood, 1996) rarely exceeds the K_m for Zn influx (>3 μmol l^{-1}; Spry and Wood, 1989; present study). In soft water, in which the active J_{in} for Ca would be more dependent upon the K_m, the fish may have greater difficulty in separating Zn and Ca influx. Further research with soft-water-acclimated fish would be required to test this hypothesis.

A number of aspects of Zn *versus* Ca transport interactions remain unclear at present. For example, Spry and Wood (1989) reported that exposure to high waterborne [Ca] increased J_{max} for Zn influx. Similarly, a novel and curious finding of the present study is that J_{max} for Zn influx was greatly increased by the presence of 2.3 μmol l^{-1} Zn in the water. This response was evident at the first flux measurement on day 1 (note that Zn influx analysis started on day 1 and ended 24 h later) and became gradually stronger during the course of the experiment (Table 1). Neither the Ca-induced nor the Zn-induced increases in J_{max} for Zn influx are easily explained with current knowledge. The two phenomena may or may not be related. However, we speculate that, in the latter case, exposure to an elevated [Zn] may have disabled Ca^{2+}-specific transport sites despite the unaffected J_{max} for Ca. New apical Ca^{2+}/Zn^{2+} transporters may have been activated, exposed or introduced to compensate for a loss of Ca uptake. In such a scenario, the number of Zn transport sites (J_{max} for Zn) would increase while the net number of *functional* Ca^{2+} transporters (J_{max} for Ca) would remain unchanged. Waterborne Zn is thought to interfere with branchial Ca transport on both sides of the epithelium. At the apical membrane, Zn and Ca seem to compete for the same uptake site, but at the basolateral membrane Zn effectively blocks the high-affinity Ca^{2+}-ATPase by mixed inhibition (reduced J_{max} and increased K_m) without being transported itself by this enzyme (Hogstrand *et al.* 1996). It is possible that a blockage of basolateral Ca^{2+}-ATPases leads to a compensatory increase in the number of apical Ca^{2+}/Zn^{2+} transporters and, thus, an increased J_{max} for Zn. More research is needed to clarify the nature and significance of this increase in J_{max} for Zn during exposure to elevated concentrations of Zn in the water.

The K_m values for Zn in control rainbow trout from the present study (3.58–7.85 μmol l^{-1}) encompassed that reported for much larger (300 g) rainbow trout in the same water quality (K_m=3.6 μmol l^{-1}; Spry and Wood, 1989). The J_{max} values for Zn, in contrast, were 1.5–3 times greater than that reported by Spry and Wood (1989). This discrepancy may well have been caused by the 100-fold size difference between the fish in the two studies. A similar allometric relationship seems to exist for Ca influx in fish (compare Perry and Wood, 1985; Hogstrand *et al.* 1994, 1995; present study).

It is now well established that branchial Ca influx in small rainbow trout varies over time in a cyclic fashion and that this cycle is synchronous among individuals kept in the same general environment (Wagner *et al.* 1985, 1993; Hogstrand *et al.* 1994, 1995). The Ca influx cycle appears to be caused by changes in plasma levels of bioactive stanniocalcin, but its physiological significance is still a matter of speculation (Wagner *et al.* 1993). Periodic changes in Ca influx also occurred in rainbow trout from the present study and, in addition, we found that Zn influx varied over time. While there was little correspondence in the periodic variations of J_{max} for Zn and Ca, the K_m for Zn, even in control fish, seemed to follow the pattern of changes in K_m for Ca influx over time. Again, this correlation is suggestive of a coupling between the apical entries of Zn and Ca at the gills and suggests that stanniocalcin might be involved in the regulation of Zn influx.

It should be noted that the kinetic variables for Zn influx measured over the course of the experiment are apparent values because Ca, which competitively inhibits Zn influx, was present in the water. True J_{max} and K_m for Ca have been measured previously under conditions identical to those in the present study (Hogstrand *et al.* 1995). In Zn-acclimated fish, the true and

apparent J_{max} values for Ca influx were essentially the same, whereas the true K_m for Ca influx was lower than the apparent K_m (in the presence of 2.3 μmol l^{-1} Zn) but still higher than that of control fish (not Zn-acclimated; Hogstrand *et al.* 1995). These results were taken as evidence that there is an intrinsic change in the Ca^{2+}/Zn^{2+} transporter of Zn-acclimated fish towards lower affinity. In the present study, we show that this reduced affinity for Ca is, indeed, paralleled by a reduction in affinity for Zn. Measurement of true J_{max} and K_m for Zn influx (without Ca to inhibit transport) proved difficult. First, the removal of Ca from the water for 24 h resulted in mortalities from both groups (two out of 40 Zn-acclimated fish and five out of 40 controls died), which means that there was a selection bias in the experimental data and that individuals with a deteriorating physiology were probably studied. Second, the kinetic properties of Zn transport may have been altered in an attempt to maintain Ca influx. However, we feel that these data still provide some useful information that is in accordance with earlier observations. In at least three previous investigations, there have been indications of the enormous ability of rainbow trout to alter the affinity for branchial Ca transport over a very short period (Perry and Wood, 1985; Hogstrand *et al.* 1994, 1995). Perry and Wood (1985) found that a 24 h exposure of rainbow trout acclimated to hard water ([Ca]=0.9 mmol l^{-1}) to a much lower Ca concentration (0.025 mmol l^{-1}) resulted in a 50 % reduction in the K_m and a 500 % increase in the J_{max} for Ca. The likely functional explanation is that the fish tried to maintain Ca influx in the Ca-poor environment. Similarly, when Ca influx was acutely inhibited by the addition of Zn to the water, the true K_m for Ca (measured in the absence of Zn) exhibited a decrease of up to 70-fold (Hogstrand *et al.* 1994, 1995). The Ca affinity remained strongly elevated for approximately 3 days when the water Zn concentration was kept high (Hogstrand *et al.* 1995). Again, it is reasonable to assume that Ca^{2+} transporters are regulated to optimize Ca influx in such a situation. In the present study, we measured Zn influx in 'Ca-free' water (chelators were not added to remove trace levels of Ca) and found markedly decreased K_m values and increases in J_{max} compared with those measured in the presence of Ca. A decrease in K_m is to be expected, because Ca is a competitive inhibitor of Zn influx (Spry and Wood, 1989), but the really interesting finding was that the K_m for Zn in Zn-acclimated fish was no different from that of the control fish after 24 h in 'Ca-free' water. Thus, the increase in K_m, which signified previous exposure to Zn, was completely eliminated following removal of Ca from the water. Furthermore, when Ca was withdrawn, the J_{max} for Zn increased in both controls and Zn-acclimated fish. These results are completely in line with the idea of a shared and rapidly regulated Ca^{2+}/Zn^{2+} transporter at the apical membrane of the gill epithelium. Thus, in the 'Ca-free' environment, the requirement for a low affinity of the Ca^{2+}/Zn^{2+} transporter in water with a high Zn concentration was overridden by the requirement to take up Ca.

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