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Characterization of branchial lead–calcium interaction in the freshwater rainbow trout *Oncorhynchus mykiss*

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Summary

The mechanism of branchial lead uptake and interplay with Ca^{2+} transport was investigated in the freshwater rainbow trout *Oncorhynchus mykiss*. Lead significantly reduced Ca^{2+} influx by approximately 40% and 30% after exposure to 2.3±0.1 and 1.4±0.2 μmol l^{-1} dissolved lead, respectively, for 0–48 h. Acute inhibition of Ca^{2+} influx by lead exhibited typical Michaelis–Menten kinetics with an approximate 16-fold increase in K_m, whereas J_{max} values did not significantly change, yielding an inhibitor constant ($K_{i,Pb}$) of 0.48 μmol l^{-1}. Alternative analyses suggest the possibility of a mixed competitive/non-competitive interaction at the highest lead concentration tested (4.8 μmol l^{-1}). Branchial lead accumulation was reduced with increasing waterborne Ca^{2+} concentrations, suggesting a protective effect of Ca^{2+} against lead uptake at the gill. The apical entries of Ca^{2+} and lead were both inhibited (55% and 77%, respectively) by the addition of lanthanum (1 μmol l^{-1}) to the exposure water. The use of cadmium (1 μmol l^{-1}) and zinc (100 μmol l^{-1}) as voltage-independent calcium channel competitors also reduced branchial lead uptake by approximately 56% and 47%, respectively. Nifedipine and verapamil (up to 100 μmol l^{-1}), both voltage-dependent calcium channel blockers, had no effect on gill lead accumulation. $CaCl_2$ injection reduced both Ca^{2+} and lead uptake by the gills. This suggests transport of lead through apical voltage-independent calcium channels, similar to the entry of Ca^{2+}. High-affinity Ca^{2+}-ATPase activity was not acutely affected by lead, but a significant 80% reduction in activity occurred during exposure for 96 h to 5.5±0.4 μmol l^{-1} dissolved lead, indicating a possible non-competitive component to lead-induced Ca^{2+} disruption. The effect of lead on Ca^{2+} efflux was investigated and found to be insignificant. We conclude that uptake of lead occurs, at least in part, by the same mechanism as Ca^{2+}, which results in disruption of Ca^{2+} influx and ultimately Ca^{2+} homeostasis.

Key words: waterborne lead, calcium, competition, rainbow trout, *Oncorhynchus mykiss*, branchial uptake.

Introduction

Lead is a common contaminant in the natural environment that can enter the water column through geologic weathering and volcanic action, or by various anthropogenic practices including smelting, coal burning and use in gasoline, batteries and paint (World Health Organization, 1995). Though waterborne lead concentrations do not normally exceed 0.6 μmol l^{-1} (Demayo et al., 1982), levels as high as 4.3 μmol l^{-1} have previously been reported (US Environmental Protection Agency, 1986). Contamination of water through anthropogenic practices is the primary cause of lead poisoning in fish (Sorensen, 1991).

Recent evidence has shown that the acute toxic mechanism for waterborne lead in the rainbow trout *Oncorhynchus mykiss* is ionoregulatory disruption, with observed effects on Na^+ and Cl^- balance and Ca^{2+} homeostasis (Rogers et al., 2003). These effects are manifested through an inhibition of ion influx and corresponding decreases in the plasma levels of these ions. This places lead midway between other known acute ionoregulatory toxicants like copper and silver, which affect Na^+ and Cl^- balance (Laurén and McDonald, 1985; Wood et al., 1996; Morgan et al., 1997), and zinc and cadmium, which disrupt Ca^{2+} homeostasis (Spry and Wood, 1985; Verbost et al., 1987).

Branchial uptake of Ca^{2+} is thought to be primarily by passive movement through apical voltage-insensitive channels in the ‘chloride’ cells of the fish gill (Flik et al., 1993). Once entering the chloride cell, Ca^{2+} is transported *via* Ca^{2+}-binding proteins to the basolateral membrane where it is actively extruded into the circulation by way of a high-affinity Ca^{2+}-ATPase enzyme (Flik et al., 1985; Verbost et al., 1994; Marshall 2002) and/or a Na^+/Ca^{2+} exchange mechanism (Flik et al., 1994, 1997; Verbost et al., 1997). Lead could potentially have an impact at any one of these steps of calcium entry. Recently, MacDonald et al. (2002) speculated that lead disrupts Ca^{2+}-homeostasis by competitive inhibition at apical Ca^{2+} channels in the fish gill, thus entering the fish by the same mechanism as Ca^{2+}. There is an abundance of circumstantial evidence in support of this. For example, the toxicity of

waterborne lead is greatly reduced with increasing water hardness, since the Ca^{2+} component probably exerts protective effects by inhibiting entry of lead (Sorensen, 1991). Elevated dietary Ca^{2+} levels have also been shown to reduce lead uptake in fish (Varanasi and Gmur, 1978). Once crossing the gill and entering the systemic circulation, lead accumulates in bone, suggesting similarities between the handling of lead and Ca^{2+} within the organism (Davies et al., 1976; Holcombe et al., 1976; Settle and Patterson, 1980). These indirect relationships suggest that lead may share one or multiple points of entry with calcium; however, despite such circumstantial evidence, this relationship has not been proven directly.

Using both physiological and pharmacological techniques, metals like cadmium and zinc have been shown to be Ca^{2+} antagonists. Both metals have been found to reduce rates of Ca^{2+} influx (Verbost et al., 1987; Spry and Wood, 1985) resulting in hypocalcemia. Kinetic analyses of Ca^{2+} interaction with both zinc (Spry and Wood, 1989; Hogstrand et al., 1994, 1998) and cadmium (Niyogi and Wood, in press) have demonstrated typical Michaelis–Menten relationships, i.e. increased K_m values (decreased affinity of the apical calcium channel) with little or no change in the maximal rate of uptake (J_{max}). This suggests direct competition. Additionally, apical calcium channel blockers such as lanthanum have been shown to reduce the rate of Ca^{2+}, cadmium and zinc uptake, suggesting that these metals share the same lanthanum-inhibitable route of uptake (Verbost et al., 1989; Hogstrand et al., 1995, 1996). Using the rainbow trout as a model species, the objective of the present study was to characterize the branchial interaction of lead and Ca^{2+} by incorporating kinetic analysis and the use of apical channel blockers (both voltage sensitive and voltage insensitive). The potential stimulation of Ca^{2+}-efflux, as reported in lead-exposed brown trout (Sayer et al., 1991), and possible inhibition of high-affinity Ca^{2+}-ATPase were also investigated as potential factors in lead-induced disruption of Ca^{2+}-homeostasis. Characterization of lead binding to the rainbow trout gill may aid in the development of water chemistry-based predictive models for lead, such as the Biotic Ligand Model (BLM; Paquin et al., 2002). This process requires further understanding and characterization of key binding sites involved in lead toxicity.

Materials and methods

Experimental animals

Juvenile rainbow trout *Oncorhynchus mykiss* Walbaum (3–10 g) for use in influx experiments and experiments using calcium channel blockers/competitors and $CaCl_2$ injection, larger juvenile trout (40–60 g) for use in Ca^{2+}-efflux measurements, and adult rainbow trout (200–300 g) for use in the high-affinity Ca^{2+}-ATPase assay, were obtained from Humber Springs Trout Farm in Orangeville, Ontario, Canada. Fish were held in dechlorinated City of Hamilton tapwater (from Lake Ontario) at a temperature of 7–12°C, and fed commercial trout pellets at a ration of 1% total body mass per day. Water composition (mmol l^{-1}) was Ca^{2+} 1.0, Mg^{2+} 0.2, Na^+ 0.6, Cl^- 0.8, K^+ 0.05, total lead 0.003 μmol l^{-1} (0.68 μg l^{-1}), dissolved organic carbon (DOC) 3 mg l^{-1}, hardness (as $CaCO_3$) of approximately 140 mg l^{-1}, and pH 7.9–8.0. Experiments were conducted at a temperature of 9–12°C and experimental animals were starved for 72 h prior to and throughout all experiments.

Ca^{2+} influx measurements

Ca^{2+} influx determinations were carried out using methods almost identical to those outlined by Rogers et al. (2003), differing only in lead-exposure concentrations used and duration of the pre-exposure period. In brief, Ca^{2+} influx was measured by relating the specific activity of ^{45}Ca in the exposure water to the accumulation of isotope in the fish following the flux period. In the present study, influx was measured under control conditions and at lead concentrations that were approximately 50% and 25% of the 96 h LC_{50} of 4.8 μmol l^{-1} dissolved lead determined in City of Hamilton dechlorinated tapwater by Rogers et al. (2003). The nominal lead-exposure concentrations implemented were 2.4 μmol l^{-1} and 1.2 μmol l^{-1}. Juvenile rainbow trout were subject to pre-flux exposure periods of 0 h, 12 h and 24 h before undergoing a flux period of 2 h in control water or lead-containing water.

Kinetic analysis of the interaction between lead and Ca^{2+}

The differential effects of lead on unidirectional calcium influx were assessed *in vivo* using methods similar to those outlined in Zohouri et al. (2001). Sixteen polyethylene bags representing a series of four different calcium concentrations in control and three lead concentrations, were filled with 3 l calcium-free synthetic water (0.7 mmol l^{-1} Na^+ and Cl^- added as NaCl, 1.9 mmol l^{-1} $KHCO_3$, pH 8.0). Each bag was fitted with an airline and placed on a water bath for temperature control (approximately 10°C). Three bags of each set were spiked with a $Pb(NO_3)_2$ stock solution (Sigma; Aldrich, Oakville, ON, Canada) to obtain nominal lead concentrations of 0.48, 2.4 and 4.8 μmol l^{-1} lead (control contained 0 μmol l^{-1} lead). Each bag was then spiked with $Ca(NO_3)_2$ (Fisher Scientific, Markham, ON, Canada) to achieve nominal calcium concentrations of 150, 300, 600 and 1200 μmol l^{-1}. Finally, flux bags were injected with 7 μCi l^{-1} ^{45}Ca (as $CaCl_2$, specific activity 0.14 μCi mol^{-1}; Perkin-Elmer, Boston, MA, USA).

Juvenile rainbow trout were transferred to each of the 16 flux bags (seven fish per bag) and an initial 15 min period was allowed for ‘settling’ and isotopic equilibration. The exposure period was 3 h; initial and final water samples (5 ml) were drawn in duplicate for determination of ^{45}Ca activity and total calcium concentration. Water samples were also drawn for determination of total lead (unfiltered) and dissolved lead (filtered; 0.45 μm filter) concentrations. These samples were stored in plastic scintillation vials in 1% HNO_3 for analysis. Following the 3 h flux period, fish were removed and killed by a single blow to the head. The fish were then rinsed for 1 min in 1 mmol l^{-1} EDTA (Sigma-Aldrich) followed by a 1 min rinse in a 5 mmol l^{-1} cold Ca^{2+} solution [$Ca(NO_3)_2$; Sigma-Aldrich] to remove all surface-bound ^{45}Ca. Whole bodies were

blotted dry, placed in scintillation vials, and digested in 1 mol l^{-1} HNO_3 (Fisher Scientific; trace metal grade) at 55°C for 48 h. Samples were then homogenized by vortexing, a sample (1.5 ml) was removed, centrifuged at 13 000 ***g*** for 10 min, and the supernatant (1 ml) added to 5 ml of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT, USA). ^{45}Ca radioactivity was measured by scintillation counting (Rackbeta 1217; LKB Wallac, Turku, Finland). Water samples taken for determination of ^{45}Ca radioactivity were added to 10 ml of aqueous counting scintillant (ACS™: Amersham, Piscataway, NJ, USA) and scintillation counted as above. ^{45}Ca radioactivity was quench-corrected to the same counting efficiency as water samples by the method of external standard ratios, using a ^{45}Ca quench curve generated from the tissue of interest in the same counting cocktail.

Water samples taken for the determination of total [Ca^{2+}] were diluted with 0.2% lanthanum and analyzed by flame atomic absorption spectrophotometry (FAAS) using the Varian 220FS Spectr AA (Varian, Mulgrave, VC, Australia). Determination of total and dissolved waterborne lead concentrations was done using graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA) against a certified multi-element standard (Inorganic Ventures, Inc., Ventura, CA, USA).

The effect of calcium on branchial lead accumulation

The effect of waterborne calcium concentration on the branchial accumulation of lead in juvenile rainbow trout was assessed using methods similar to those implemented in the kinetic analysis of lead and Ca^{2+}, though precision was lower due to the lack of a suitable lead radioisotope. Following a 3-h exposure period, experimental animals were removed from the flux bags, killed by a blow to the head, and the gills dissected. The gills were rinsed for 1 min in deionized water, blotted dry, weighed and digested in 1 mol l^{-1} HNO_3 at 55°C for 48 h. Samples were then homogenized by vortexing, a sample (approximately 1.5 ml) was removed, centrifuged at 13 000 ***g*** for 10 min, and the supernatant analyzed for total lead concentration using GFAAS.

Calcium-channel blocker experiments

The role of apical calcium channels in mediating branchial lead uptake was investigated by the use of the voltage-independent blocker, lanthanum, antagonists of voltage-independent Ca^{2+} uptake, cadmium and zinc, and the voltage-dependent channel blockers nifedipine and verapamil. In assessing the effects of lanthanum on branchial lead accumulation, a series of six polyethylene bags representing one control (0 μmol l^{-1} lanthanum) and five lanthanum concentrations were filled with 3 l of synthetically modified water obtained by reverse osmosis (0.7 mmol l^{-1} Na^+ and Cl^- added as NaCl), made carbonate-free to reduce complexation of waterborne lanthanum (Verbost et al., 1987; Hogstrand et al., 1996) and calcium-free to maximize branchial lead accumulation. Flux bags were then spiked with a $LaCl_3$ (Sigma-Aldrich) stock solution to achieve nominal waterborne lanthanum concentrations of 0.0001 to 1 μmol l^{-1}. Juvenile rainbow trout were transferred to each of the six flux bags (seven fish per bag) and allowed a 10-min settling period for equilibration with waterborne lanthanum. The bags were subsequently spiked with a $Pb(NO_3)_2$ stock solution to achieve a nominal lead concentration of 4.8 μmol l^{-1}, followed by an additional settling period to allow for lead equilibration. Water samples were taken, filtered and unfiltered, for determination of total and dissolved lead concentrations. The fish were then exposed for 3 h. Following exposure, fish were removed from flux bags and the gills were dissected. Processing of the dissected gills for total lead concentrations used procedures identical to those outlined above. Using ^{45}Ca as a radiotracer, simultaneous measurements of Ca^{2+} influx were made at control and 1 μmol l^{-1} lanthanum to assess the effect of lanthanum on Ca^{2+} uptake. Influx measurements were performed using a waterborne Ca^{2+} concentration of 600 μmol l^{-1}. Further procedures used were identical to those outlined above under 'calcium influx measurements'. The effects of cadmium and zinc on branchial lead accumulation and Ca^{2+} influx were assessed using methods almost identical to those used for lanthanum. For cadmium, flux bags were spiked with a stock solution of $Cd(NO_3)_2$ to achieve nominal concentrations of 0.0001, 0.001, 0.01, 0.1 and 1 μmol l^{-1} cadmium. For zinc, bags were spiked with $ZnSO_4$ to achieve nominal concentrations of 0.01, 0.1, 1, 10 and 100 μmol l^{-1} zinc. These experiments also included measurements of gill cadmium burden from the same gill digests analyzed for total lead, using GFAAS.

The use of nifedipine and verapamil as L-type calcium channel blockers was evaluated using methods almost identical to those used for lanthanum, cadmium and zinc with the exception of waterborne concentrations employed. For nifedipine, bags were spiked with a stock solution made using 75% ethanol (to solubilize the blocking agent; final ethanol concentration in the flux medium was 0.1%) to achieve concentrations of 0.1, 1, 10 and 100 μmol l^{-1} nifedipine. A control was run by spiking nifedipine-free water with ethanol to control for the effects of ethanol on lead accumulation and to maintain consistency between blocker experiments. For verapamil, flux bags were spiked with a stock solution made using deionized water (NANOpure II; Sybron/Barnstead, Boston, MA, USA) to achieve final concentrations of 0.1, 1, 10 and 100 μmol l^{-1} verapamil.

Effect of $CaCl_2$ injection on Ca^{2+} influx and branchial lead accumulation

Methods used to test the effects of $CaCl_2$ injection on Ca^{2+} influx and branchial lead accumulation followed closely those outlined in Hogstrand et al. (1996). Ionic calcium was injected into juvenile rainbow trout with the goal of reducing uptake of Ca^{2+} and lead through the stanniocalcin-regulated pathway. In the present study, fish were injected intraperitoneally with 0.22 μmol l^{-1} Ca^{2+} g^{-1} body mass using an injection solution made from $CaCl_2.H_2O$ dissolved in 0.9% NaCl. Control fish

were sham-injected with 0.9% NaCl (vehicle only). Approximately 30 min following injection, measurements of Ca^{2+} influx were performed on experimental and control fish (N=8 per treatment) using methods described in the previous section. Branchial lead accumulation was measured in a separate group of control and experimental fish (N=8 per treatment), again using methods outlined above.

Determination of Ca^{2+}-efflux

The possibility of lead-induced stimulation of Ca^{2+}-efflux was investigated by following closely the methods of Perry and Flik (1988). Fish were subject to either no pre-flux exposure (control), or a pre-exposure period of 24 or 48 h to nominal waterborne lead concentrations close to the 96 h LC_{50} of 4.8 μmol l^{-1}. Lead exposure was carried out by dripping a stock solution of $Pb(NO_3)_2$ (Sigma-Aldrich) dissolved in deionized water (NANOpure II; Sybron/Barnstead, Boston, MA, USA) at a rate of 1 ml min^{-1} into a mixing tank fed with 500 ml min^{-1} of City of Hamilton dechlorinated tapwater. The mixing tank then fed an exposure tank (~200 l) holding 20 rainbow trout. At t=0 h, the exposure tank was spiked with the lead stock solution to achieve the appropriate lead concentration.

After the appropriate pre-exposure period, rainbow trout were anaesthetized using MS-222 and given an intra-peritoneal injection of a ^{45}Ca solution (30 μCi ^{45}Ca in 1 ml saline) in preparation for efflux measurements. A period of 8–12 h was allowed for recovery of the fish, and for isotopic equilibration. Fish were then transferred to darkened, Plexiglass boxes (volume 450 ml), each fitted with an air supply. The boxes were filled with either lead-free water or water spiked with $Pb(NO_3)_2$ to achieve a nominal lead concentration of 4.8 μmol l^{-1}, and placed in a water bath for temperature control. Following a 0.5 h settling period, a 3 h Ca^{2+} flux measurement was started. Initial water samples were taken in duplicate for ^{45}Ca activity and total calcium concentration (5 ml), and for determination of dissolved lead concentrations (10 ml). At the end of the flux period, comparable final water samples were drawn, fish were removed from their respective exposure boxes and anaesthetized with MS-222. A terminal blood sample was taken from each fish by caudal puncture, centrifuged at 13 000 ***g***, and the plasma frozen for measurement of ^{45}Ca activity and total calcium concentration. Ca^{2+}-efflux J was calculated using the following formula:

$$J_{out}^{Ca^{2+}} = \frac{(R_i - R_f)V}{SAMt}, \quad (1)$$

where R_i and R_f are initial and final water radioactivities in c.p.m. ml^{-1}, V is the volume of the external flux medium in ml, SA is the t=3 h specific activity of the plasma in c.p.m. μmole, t is the duration of the flux period in h, and M the fish mass in kg. Water samples were analysed using methods identical to those outlined in the lead/Ca^{2+} kinetic experiments.

The effect of lead on high-affinity Ca^{2+}-ATPase activity

High-affinity Ca^{2+}-ATPase activity was assayed in adult rainbow trout exposed to control conditions or to a nominal lead concentration of 4.8 μmol l^{-1} for 3 h, 24 h or 96 h in City of Hamilton dechlorinated tapwater. Lead exposure was carried out using methods identical to those used in measurements of Ca^{2+}-efflux. Isolation of the basolateral membrane from gill epithelium was carried out to assay enzyme activity. Procedures followed closely those outlined in Perry and Flik (1988). Trout were anaesthetized with MS-222 and injected intravenously with 2500 U of sodium heparin dissolved in 1 ml of saline. After 20 min, the fish were decapitated just posterior to the pectoral fins. The head was placed onto an operating table where the gills, irrigated with freshwater, were perfused with ice-cold isotonic saline (0.6% NaCl) containing 20 U ml^{-1} of sodium heparin. Perfusion was carried out at a pressure of 60 cmH_2O *via* a catheter (PE 60 tubing) inserted into the bulbus arteriosus until the gills appeared to be free of trapped red blood cells. Following perfusion, the gill basket was quickly excised. Further preparations were carried out on ice (0–4°C) from this point.

The gill epithelium was scraped from the gill arches onto a glass plate using a glass microscope slide. The scrapings were then placed into a douncer with a loose pestle and homogenized in 15 ml of a hypotonic buffer consisting of 25 mmol l^{-1} NaCl and 20 mmol l^{-1} Hepes-Tris (pH 7.4). The volume was brought to 50 ml and the homogenate centrifuged for 15 min at 550 ***g*** (Beckman Ti 70 Rotor, Palo Alto, USA) to remove nuclei and cellular debris. Membranes were then collected by ultracentrifugation of the remaining supernatant at 30 000 ***g*** for 30 min. The resulting pellet, fixed to the centrifuge tube, was resuspended with 100 strokes of a douncer in 15 ml of an isotonic buffer containing 250 mmol l^{-1} sucrose, 12.5 mmol l^{-1} NaCl, 0.5 mmol l^{-1} H_2EDTA, and 5 mmol l^{-1} Hepes-Tris (pH 7.5). The volume of the suspension was then brought to 30 ml with the same buffer. The resulting suspension was then centrifuged differentially in the following manner: 1000 ***g*** for 10 min, 10 000 ***g*** for 10 min, 30 000 ***g*** for 30 min. The resulting pellet was obtaining by adding 300 μl of a buffer containing 20 mmol l^{-1} Hepes-Tris (pH 7.4) and 200 mmol l^{-1} sucrose, and resuspended by passing the mixture through a 23-gauge syringe needle. The membrane preparations contained approximately 3 mg ml^{-1} of bovine serum albumin protein equivalents and were used on the same day of isolation. High-affinity Ca^{2+}-ATPase activity was assayed in the presence of oligomycin B (5 μg ml^{-1}) and 5 mmol l^{-1} sodium azide, at 1.0 μmol l^{-1} and 0 μmol l^{-1} free Ca^{2+} in an assay medium containing 100 mmol l^{-1} NaCl, 0.1 mmol l^{-1} ouabain, 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} *N*-hydroxyEDTA, 5 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Na_2ATP and 20 mmol l^{-1} Hepes-Tris (pH 7.4). Membrane vesicles were permeabilized with 20 μg saponin mg^{-1} membrane protein. High-affinity Ca^{2+}-ATPase activity was measured as the difference in inorganic phosphate release (P_i) in the presence and absence of Ca^{2+} in the assay medium.

The *in vitro* effect of lead on Ca^{2+}-ATPase activity was tested by adding lead [from a $Pb(NO_3)_2$ stock solution] to the final membrane suspension, just prior to addition to the assay

reaction medium, at concentrations similar to those measured in the final membrane suspension from the *in vivo* exposure. Finally, branchial lead accumulation was measured in a separate group of fish, similar in size and exposed to control conditions or to 4.8 μmol l^{-1} lead for 3 h, 24 h or 96 h. Procedures used for measurement of gill lead burden were identical to those outlined in the section 'Calcium-channel blocker experiments' above.

Statistical analysis

In kinetic experiments, Michaelis–Menten analysis of the relationship between the rate of Ca^{2+} influx ($J_{in}^{Ca^{2+}}$) and waterborne calcium (substrate) were performed using Lineweaver–Burk linear regression (double reciprocal plots), where J_{max} (maximum rate of influx) is the inverse of the *y*-intercept of the regression line, and $-K_m$ (Michaelis constant) is the inverse of the *x*-intercept of the regression line. For comparison, data were also analysed by Eadie–Hofstee plots and by non-linear regression (Sigmaplot 2000).

Calculated data are expressed as means ± 1 S.E.M. (*N*). Experimental means were compared to corresponding control mean values by an unpaired two-tailed Student's *t*-test. Both time-dependent and dose-dependent responses in both control and experimental groups were tested against initial 0 h or control measurements using a one-way analysis of variance (ANOVA) with a two-sided Dunnett's *post hoc* multiple comparison. All statistical significance was calculated at $P<0.05$.

Results

Water chemistry

As an integral component to the characterization of lead/Ca^{2+} interaction in the rainbow trout, speciation analysis was conducted for these waterborne ions using MINEQL+, a computer-based geochemical modeling program (Schecher and McAvoy, 1994). For each experiment, simulations were run taking into account all chemical species present in the medium. These analyses indicated that 98% of the calcium in exposure water was present in the free ionic form, Ca^{2+}. In determining the speciation of lead in the exposure water, it was found that >95% of waterborne lead was present in the bioavailable forms Pb^{2+} (~50%), $Pb(OH)^{+}$ (~30%) and $Pb(OH)_2$ (~15%), with a small component being bound to carbonate ($PbCO_3$; 3–5%). These data are consistent with dissolved lead concentrations measured using filtration with a 0.45 μmol l^{-1} filter.

Effect of lead on Ca^{2+} influx

Juvenile rainbow trout exposed to 2.3±0.1 μmol l^{-1} dissolved lead (measured) showed significant reductions in Ca^{2+}-influx rate compared to control fish (Fig. 1A). Effects on influx were immediate, with an approximate 40% inhibition occurring at 0–2 h of lead exposure, this effect carrying through to 24–26 h, though non-significant at 12–14 h. Exposure to 1.4±0.2 μmol l^{-1} dissolved lead resulted in similar inhibition of Ca^{2+} influx (Fig. 1B), though significant only at 12–14 h where the reduction was 32%. The concentration of total lead in control water was 0.002±0.0001 μmol l^{-1}.

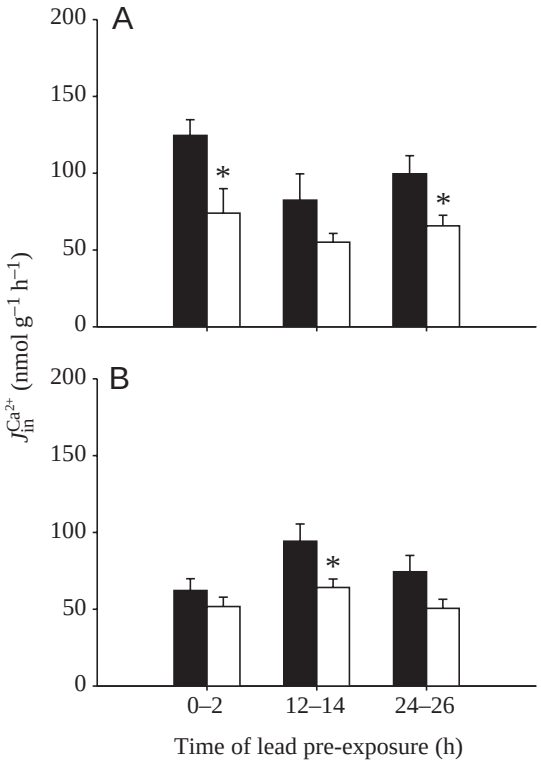

Fig. 1. (A) Calcium influx rates $J_{in}^{Ca^{2+}}$ in juvenile rainbow trout in City of Hamilton dechlorinated tapwater in control conditions (black bars) or water containing 2.3±0.1 μmol l^{-1} dissolved lead (white bars) after exposure for 0, 12 or 24 h. (B) Calcium influx rates in control water (black bars) or water containing 1.4±0.2 μmol l^{-1} dissolved lead (white bars) after exposure for 0, 12 or 24 h. Values are means ± 1 S.E.M. (N=8 throughout). *Significant difference ($P<0.05$; two-tailed Student's *t*-test) from corresponding control means.

Kinetic analysis of the interaction between lead and Ca^{2+}

The saturable nature of calcium influx with increasing waterborne calcium concentrations obeyed typical Michaelis–Menten kinetic analysis, in the presence and absence of lead (Fig. 2). Measured waterborne lead concentrations were 0.46±0.03 μmol l^{-1}, 2.5±0.2 μmol l^{-1} and 4.9±0.3 μmol l^{-1}. Consistent with the immediate inhibition of Ca^{2+}-influx documented in the previous experiment, waterborne lead exposure resulted in inhibition of calcium influx in juvenile rainbow trout over a 3 h exposure period, the inhibition increasing with increased lead concentrations. From analysis of a double reciprocal Lineweaver–Burk plot (Fig. 3A), it is apparent that K_m values increased significantly with increasing waterborne lead concentration (Table 1), for example, 4.8 μmol l^{-1} lead exposure resulted in an approximate 16-fold increase in K_m value from control levels. By contrast, J_{max} was not significantly altered as a function of waterborne lead concentration (Table 1). Kinetic analysis

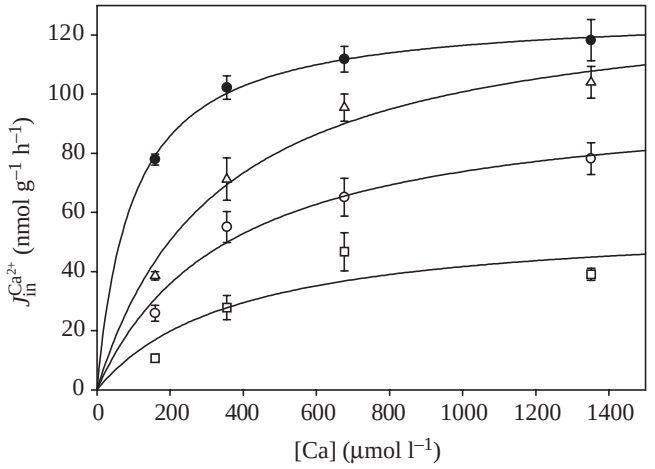

Fig. 2. Unidirectional branchial calcium influx rates $J_{in}^{Ca^{2+}}$ in juvenile rainbow trout at various waterborne calcium concentrations in synthetic water for four different lead concentrations. Control fish, black circles; fish exposed to 0.46±0.03 μmol l^{-1} dissolved lead, white triangles; 2.5±0.2 μmol l^{-1} lead, white circles; 4.9±0.3 μmol l^{-1} lead, white squares. Values are means ± 1 S.E.M. (N=7 per treatment).

using Eadie–Hofstee and non-linear regression also indicated significant increases in K_m; however, unlike the results obtained by Lineweaver–Burk regression, calculations revealed a significant decrease (approximately 57%) in J_{max}, but only at the highest concentration, 4.8 μmol l^{-1} lead (Table 1).

Because of the competitive nature of Ca^{2+}-influx inhibition by lead, an inhibitor constant was determined from a regression plot of apparent K_m (measured K_m values from Table 1)/J_{max} *vs*. waterborne lead concentration (Fig. 3B; Segel, 1976). The $K_{i,Pb}$ (–K_i, x-intercept of the regression line) was calculated to be 0.48 μmol l^{-1} lead.

The effect of calcium on branchial lead accumulation

The effect of waterborne calcium on branchial lead uptake is shown in Fig. 4. Overall a protective effect of calcium was observed, with the amount of branchial lead accumulation decreasing with increasing waterborne Ca^{2+} concentration. At a nominal waterborne lead concentration of 0.48 μmol l^{-1}, accumulation was consistent over the range of Ca^{2+} concentrations (150–1300 μmol l^{-1}). With increases in waterborne lead concentrations to 2.4 (dissolved 2.5±0.2 μmol l^{-1}) and 4.8 μmol l^{-1} (dissolved 4.9±0.3 μmol l^{-1}), the concentration of waterborne Ca^{2+} required to yield the same protective effects increased. For example, upon exposure to 4.8 μmol l^{-1} lead (dissolved 4.9±0.3 μmol l^{-1}), lead accumulation at 150 μmol l^{-1} Ca^{2+} was

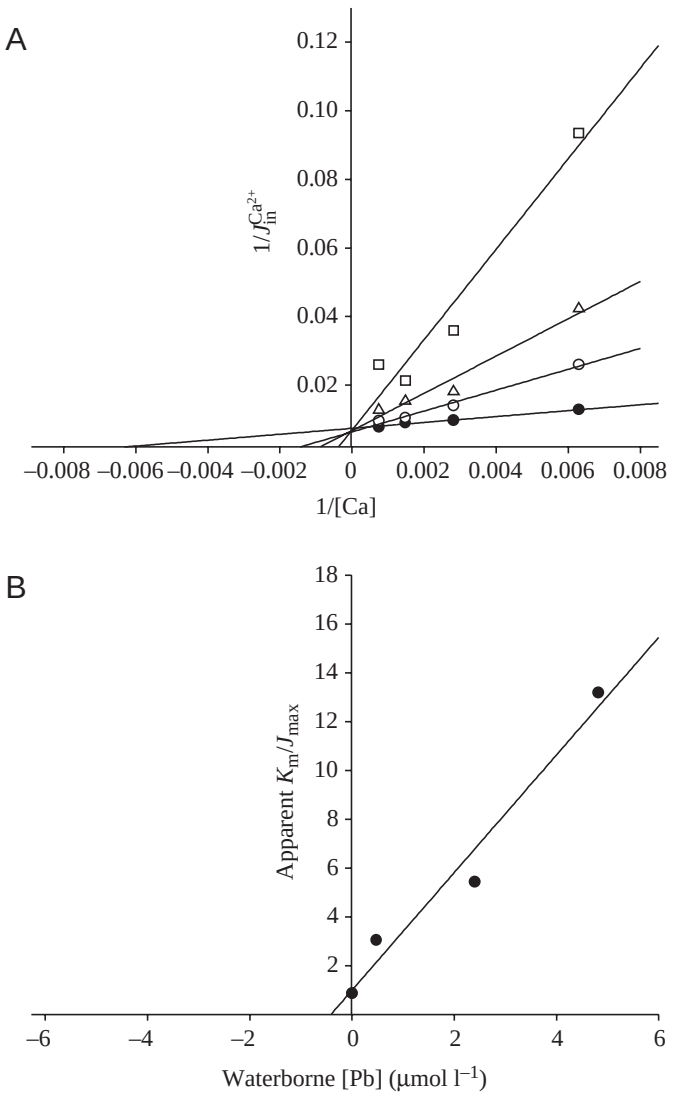

Fig. 3. Analytical plots for data presented in Fig. 2 using (A) Michaelis–Menten analyses by Lineweaver–Burk regression (double reciprocal plot) illustrating the competitive relationship between lead and calcium uptake in the rainbow trout, and (B) regression analyses of apparent K_m/J_{max} *vs.* waterborne lead concentration (using apparent K_m and J_{max} values presented in Table 1. The regression equation was y=2.38x+1.12 (r^2=0.95). The inhibitor constant (–$K_{i,Pb}$, the x-intercept of the regression line) was 0.48 μmol l^{-1} lead.

Table 1. J_{max} *and apparent* K_m *for unidirectional branchial calcium influx rates in juvenile rainbow trout at various waterborne lead concentrations*

Waterborne	J_{max} (nmol g^{-1} h^{-1})			Apparent K_m (μmol l^{-1})		
[Pb] (μmol l^{-1})	LB	EH	NLR	LB	EH	NLR
Control	135.1	128.5	127.9	166.2	100.5	98.0
0.48	156.3	141.0	134.3	475.6*	385.4*	332.6*
2.4	147.1	102.2	100.4	798.7*	391.4*	360.2*
4.8	144.9	54.4*	56.5*	1911.7*	490.0*	395.0*

Values are means.

Kinetics were characterized by Lineweaver–Burk regression (LB), Eadie–Hofstee regression (EH) and non-linear regression (NLR).

*Significant difference (P<0.05) from control (N=7).

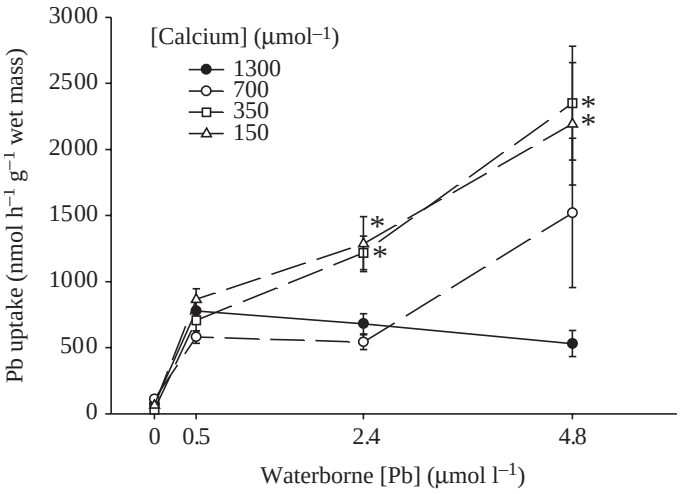

Fig. 4. Measurements of branchial lead accumulation in juvenile rainbow trout at various waterborne calcium concentrations in synthetic water for four different lead concentrations. Values are means ± 1 S.E.M. (N=7 per treatment). *Significant difference (P<0.05; two-tailed Student's t-test) from corresponding lead uptake in the presence of 1300 μmol l^{-1} calcium.

approximately 35 times higher than control (0 μmol l^{-1} lead) accumulation, while at 1300 μmol l^{-1} Ca^{2+}, accumulation was only 6 times greater than control gill lead accumulation. At a waterborne Ca^{2+} concentration of approximately 1300 μmol l^{-1}, branchial lead accumulation measured after exposure to 0.48, 2.4 and 4.8 μmol l^{-1} lead did not differ significantly.

The effect of Ca^{2+} channel blockers on branchial lead accumulation

The effect of lanthanum, a voltage-independent Ca^{2+}-channel inhibitor, on Ca^{2+} uptake and branchial lead accumulation is shown in Fig. 5A,B. From Ca^{2+} influx measurements determined for control conditions at 600 μmol l^{-1} Ca^{2+} and 1 μmol l^{-1} lanthanum (added as $LaCl_3$), an approximate 55% reduction in Ca^{2+} uptake occurred in the presence of 1 μmol l^{-1} lanthanum. Upon exposure to 2.3±0.2 μmol l^{-1} dissolved lead, waterborne lanthanum significantly reduced gill lead accumulation in a dose-dependent fashion. In the presence of 0.0001 μmol l^{-1} lanthanum, an approximate 39% reduction in lead accumulation occurred compared to control fish exposed to the same lead concentration in the absence of lanthanum. This

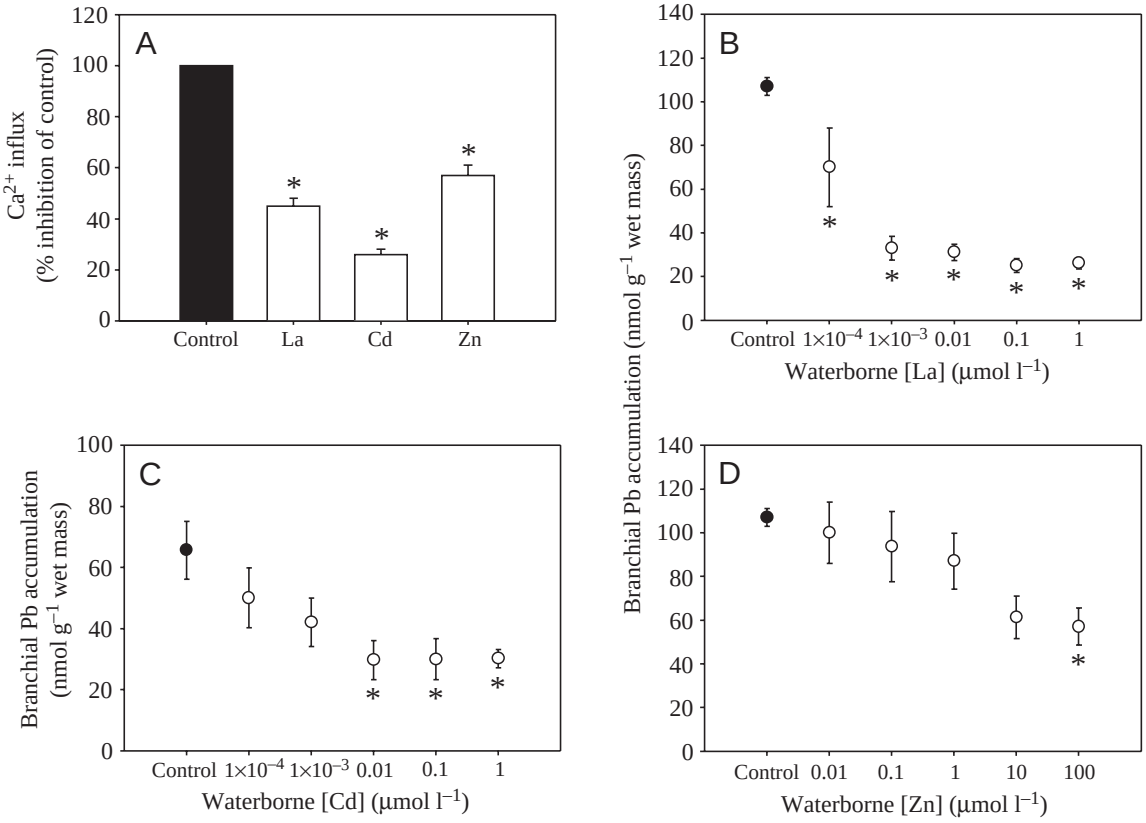

Fig. 5. (A) Percentage inhibition of calcium uptake in juvenile rainbow trout exposed to control conditions (black bar) or 1 μmol l^{-1} lanthanum, 1 μmol l^{-1} cadmium or 100 μmol l^{-1} zinc (white bars) in synthetically modified water (600 μmol l^{-1} Ca^{2+}). (B–D) Branchial lead accumulation in juvenile rainbow trout exposed to control conditions (black circles) or (B) a series of waterborne lanthanum concentrations, (C) a series of waterborne cadmium concentrations, and (D) a series of waterborne zinc concentrations. Values are means ± 1 S.E.M. (N=7 per treatment). *Significant difference (P<0.05; two-tailed Student's t-test) from corresponding control mean.

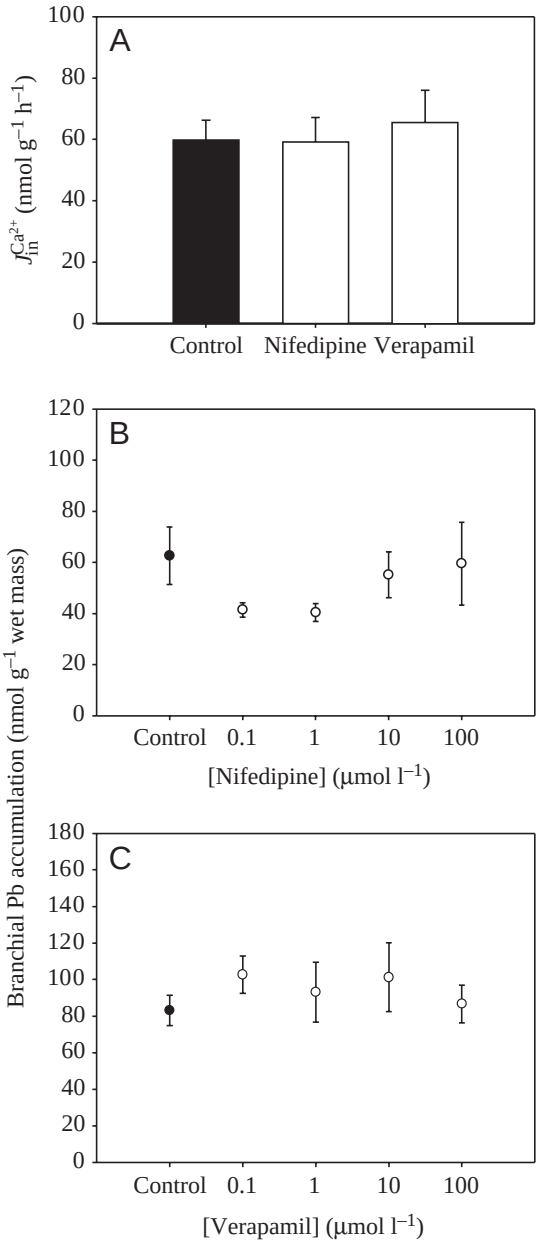

Fig. 6. (A) The effects of the voltage-dependent Ca^{2+} channel blockers nifedipine and verapamil on calcium uptake $J_{in}^{Ca^{2+}}$ in juvenile rainbow trout exposed to control conditions (black bars) or 100 μmol l^{-1} nifedipine and 100 μmol l^{-1} verapamil (white bars) in synthetically modified water (600 μmol l^{-1} Ca^{2+}). (B,C) The effect of nifedipine on branchial lead accumulation in juvenile trout exposed to control conditions (black circle) or (B) to 0.1, 1, 10 and 100 μmol l^{-1} nifedipine (white circles), (C) to 0.1, 1, 10 and 100 μmol l^{-1} verapamil (white circles). Values are means ± 1 S.E.M. (*N*=7 per treatment).

inhibition of lead uptake was dramatic from 0.001 to 1 μmol l^{-1} lanthanum, with a reduction of approximately 77% (compared to control) in lead accumulation in the presence of these waterborne lanthanum concentrations.

Waterborne cadmium had similar effects on Ca^{2+} uptake and branchial lead accumulation. A waterborne concentration of 1 μmol l^{-1} cadmium reduced the rate of Ca^{2+} influx by approximately 74% compared to controls (Fig. 5A). Branchial cadmium accumulation occurred in a dose-dependent fashion when waterborne cadmium concentration was increased from control levels to 1 μmol l^{-1}, with the largest increase occurring at the highest concentration (data not shown). This dose-dependent uptake corresponded with reduced levels of branchial lead accumulation (Fig. 5C). While gill cadmium accumulation increased by approximately 20-fold, lead uptake was reduced by 56% upon exposure to 2.3±0.2 μmol l^{-1} dissolved lead. Results using zinc as a calcium-channel blocker were similar. Ca^{2+} uptake was reduced by approximately 43% in the presence of 100 μmol l^{-1} zinc (Fig. 5A). This inhibition corresponded with a reduction of approximately 47% in branchial lead accumulation in fish exposed to 2.3±0.2 μmol l^{-1} dissolved lead in the presence of zinc (Fig. 5D). Similar to cadmium, zinc also accumulated dose-dependently at the gill, with the highest accumulation occurring at 100 μmol l^{-1} zinc (data not shown).

Voltage-dependent, L-type calcium-channel blockers did not appear to affect Ca^{2+} influx or branchial lead accumulation. Exposure to 100 μmol l^{-1} nifedipine or 100 μmol l^{-1} verapamil did not significantly reduce the rate of Ca^{2+} influx at 600 μmol l^{-1} Ca^{2+} (Fig. 6A). The effects of nifedipine on lead accumulation were found to be not significant compared to controls (Fig. 6B). Exposure to verapamil yielded similar results when comparing lead accumulation in the presence and absence of the blocking agent (Fig. 6C).

Effect of $CaCl_2$ injection on Ca^{2+} influx and branchial lead accumulation

Injection of $CaCl_2$ significantly reduced Ca^{2+} influx by approximately 52% compared to fish sham-injected with vehicle only (Fig. 7A). Inhibition of Ca^{2+} influx corresponded with a significant decrease in branchial lead accumulation in $CaCl_2$ injected fish (Fig. 7B). This reduction was approximately 37% compared to controls (NaCl injected).

The role of efflux in lead-induced hypocalcemia

The role of efflux in lead-induced disruption of Ca^{2+}-homeostasis was assessed during exposure to control conditions or 6.3±0.1 μmol l^{-1} dissolved lead. The results are summarized in Table 2. Rainbow trout exposed to control conditions (0 h) had an average Ca^{2+}-efflux ($J_{out}^{Ca^{2+}}$) of approximately 32.8±4.6 nmol g^{-1} h^{-1}. Lead-exposed fish did not show significant deviations from this value after exposures of 3, 27 and 51 h to the experimental conditions.

High-affinity Ca^{2+}-ATPase activity

Adult rainbow trout were exposed to a dissolved lead concentration of 5.5±0.4 μmol l^{-1}. When compared to values for unexposed fish at *t*=0 h (control sampling), activity after 3 h of lead exposure was not reduced. However, prolonged exposure resulted in a reduction in activity after 24 h, and a significant 80% inhibition by 96 h of lead exposure (Fig. 8A).

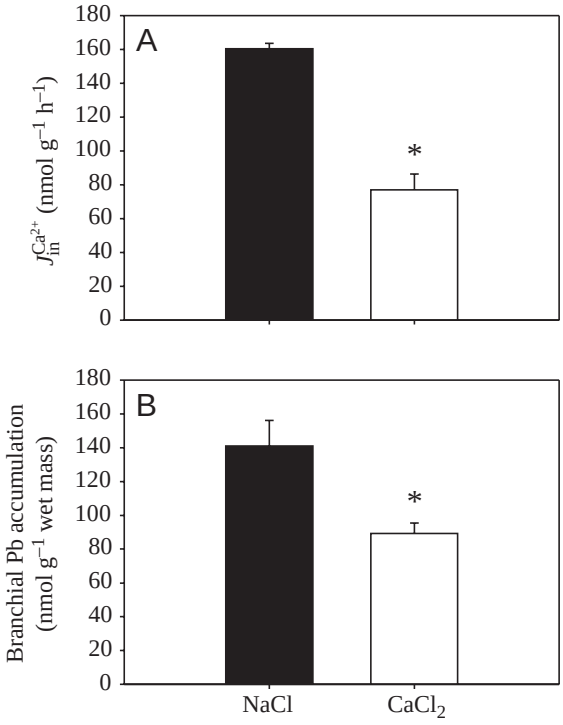

Fig. 7. The effect of $CaCl_2$ injection on (A) Ca^{2+} influx $J_{in}^{Ca^{2+}}$ in juvenile rainbow trout in synthetically modified water (600 μmol l^{-1} Ca^{2+}), and (B) branchial lead accumulation in juvenile rainbow trout. Values are means ± 1 S.E.M. (N=7 per treatment). *Significant difference (P<0.05; two-tailed Student's t-test) from corresponding control mean.

Table 2. *Unidirectional calcium efflux rates in juvenile rainbow trout in City of Hamilton dechlorinated tapwater in control water or after exposure to 6.3±0.14 μmol l^{-1} dissolved lead for 0, 3, 27 or 51 h*

Time of Pb exposure (h)	$J_{out}^{Ca^{2+}}$ (nmol g^{-1} h^{-1})
0 (Control)	32.8±4.6
0–3	39.6±7.2
24–27	37.1±4.0
48–51	48.6±8.8

Values are means ± S.E.M. (N=7). There were no significant differences.

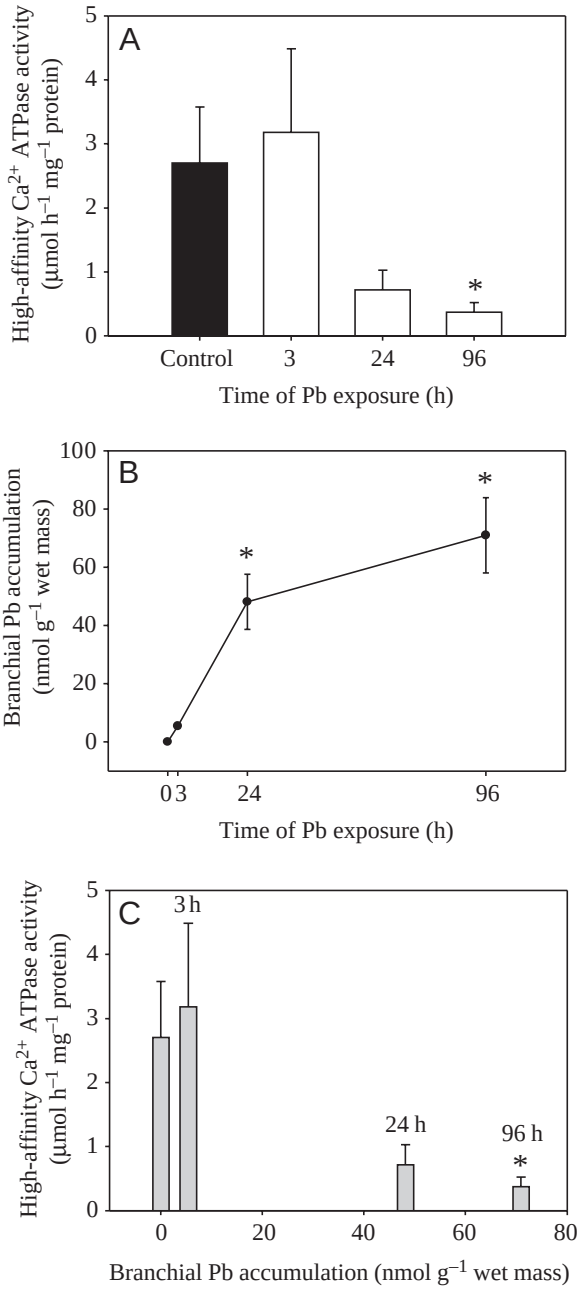

Fig. 8. Time course analysis of (A) branchial high affinity Ca^{2+}-ATPase activity in adult rainbow trout, and (B) branchial lead accumulation, and (C) plot of high-affinity Ca^{2+}-ATPase activity *versus* branchial lead accumulation in rainbow trout exposed to control conditions and to 5.5±0.4 μmol l^{-1} dissolved lead for 3 h, 24 h and 96 h. Values are means ± 1 S.E.M. (N=6). *Significant difference (P<0.05; one-way ANOVA with Dunnett's *post-hoc* multiple comparison) from control sampling mean.

Branchial lead accumulation increased significantly after 24 h and 96 h in fish exposed to an identical waterborne lead concentration (Fig. 8B). This accumulation appeared to correlate well with the inhibition of high-affinity Ca^{2+}-ATPase activity (Fig. 8C). The *in vitro* effects of directly added $Pb(NO_3)_2$ on the Ca^{2+}-ATPase assay were insignificant.

Discussion

Previous research has presented circumstantial evidence that lead may compete with Ca^{2+} for the same binding sites on the gills of freshwater fish (Davies et al., 1976; Varanasi and Gmur, 1978; Sorensen, 1991; MacDonald et al., 2002; Rogers et al., 2003). In the present study we provide direct evidence suggesting that lead does in fact compete with calcium for uptake and that interference with branchial Ca^{2+} transport

includes sharing of at least two phases of this transport: an immediate interaction with apical entry and a more slowly developing interaction with high-affinity Ca^{2+}-ATPase.

The observed rapid inhibition of calcium influx (Fig. 1) after exposure to 1.4 and 2.3 μmol l^{-1} lead is consistent with previous studies demonstrating reduced Ca^{2+} influx after exposure to lead concentrations close to 4.8 μmol l^{-1} (Rogers et al., 2003). This is contrary to findings presented by Sayer et al. (1989, 1991) that lead exposure does not result in disruption of Ca^{2+} influx. From the present data (Figs 1 and 2), it is apparent that at exposure concentrations (0.48 μmol l^{-1} upwards) that approach environmentally relevant or normal levels (0.003–0.58 μmol l^{-1} lead; Demayo et al., 1982), the hypocalcemic effects of lead are still prominent. Additionally, influx inhibition occurs immediately, suggesting that the interaction may be competitive in nature, similar to metals like cadmium (Verbost et al., 1987, 1989) and zinc (Spry and Wood, 1985; Hogstrand et al., 1994, 1996). Therefore, measurements of Ca^{2+} influx were useful for validating a kinetic approach to the analysis of the relationship between lead and Ca^{2+}.

The construction of uptake curves at a number of waterborne substrate (Ca^{2+}) concentrations using various waterborne lead levels allowed for characterization of the effect of lead on branchial Ca^{2+} influx kinetics. From these curves, K_m and J_{max} values were calculated for each lead concentration used (Table 1). These data indicated a typical Michaelis–Menten competitive relationship with a significant increase in K_m value with increasing waterborne lead concentrations. Increases in K_m suggest that as waterborne lead concentrations are elevated, the affinity of the calcium-binding site for the calcium ion decreases, due to the presence of a competitor. Consequently, the amount of Ca^{2+} required to achieve half maximum transport is elevated. The stability of J_{max} values obtained from this kinetic study confirmed the competitive nature of the lead/Ca^{2+} relationship, as the maximum rate of Ca^{2+} transport, or the integrity of Ca^{2+} binding sites, remained unchanged. These findings are consistent with those made for zinc (Hogstrand et al., 1994) and for cadmium (Niyogi and Wood, in press), metals that are known calcium antagonists.

It is also important to note, however, that exposure to the highest lead concentration of 4.8 μmol l^{-1} may have caused a reduction in the maximum rate of Ca^{2+} influx. This was not detected by Lineweaver–Burk analysis, but was indicated by significant decreases in J_{max} value when using Eadie–Hofstee and non-linear regression to interpret kinetic data. This would suggest that the branchial interaction between lead and Ca^{2+} might be described as a mixed competitive and non-competitive interaction upon exposure to elevated waterborne lead concentrations. This seems to be consistent with data presented in Fig. 2, that suggest the possibility of a J_{max} decrease, Fig. 4, which suggests that exposure to 4.8 μmol l^{-1} waterborne lead results in a linear or diffusive component to lead influx when the waterborne concentration of Ca^{2+} is reduced (350 and 150 μmol l^{-1}), and Fig. 8, which indicates that a high concentration of accumulated lead in the gill may inhibit baso-lateral high-affinity Ca^{2+}-ATPase activity, albeit over a longer exposure period.

Kinetic analysis of lead and Ca^{2+} interaction allowed calculation of an inhibitor constant ($K_{i,Pb}$) for calcium influx of 0.48 μmol l^{-1}. Based on this value, it is evident that the affinity of lead for Ca^{2+} binding sites on the fish gill is less than that for cadmium, but greater than that for zinc. Niyogi and Wood (in press) reported an inhibitor constant of 0.12 μmol l^{-1} for cadmium in rainbow trout, a value 4 times lower than that reported in the present study for lead. From the data of Hogstrand et al. (1994, 1998), the inhibition constant of zinc appears to be above 2 μmol l^{-1}. This variation in affinity for Ca^{2+} binding sites could explain the difference in potency between lead, cadmium and zinc in terms of acute toxicity; cadmium is the most toxic with a 96 h LC_{50} of 0.2 μmol l^{-1} (Hollis et al., 1999) compared to an LC_{50} of 4.8 μmol l^{-1} for lead (Rogers et al., 2003) and 13.3 μmol l^{-1} for zinc (Alsop et al., 1999). All values were determined in the same water quality.

Branchial lead accumulation was largely dependent upon waterborne Ca^{2+} concentrations, as shown in Fig. 4. At the lowest waterborne Ca^{2+} concentration of 150 μmol l^{-1}, lead accumulation significantly increased as waterborne lead concentrations increased from control to 4.8 μmol l^{-1}. This relationship changed as Ca^{2+} concentration increased. Accumulation at 0.48, 2.4 and 4.8 μmol l^{-1} lead was not significantly different at higher levels of waterborne Ca^{2+}, demonstrating strong protective effects of waterborne Ca^{2+} against branchial lead uptake. This confirms other existing evidence highlighting the protective effects of water hardness in lead toxicity (Sorensen, 1991) and suggests that it is the Ca^{2+} component of hard water that dictates the toxicity of dissolved lead to fish. Richards and Playle (1999) reported similar protective effects of Ca^{2+} against cadmium accumulation on the gills in synthetically modified soft water. Alsop and Wood (1999) demonstrated reduced zinc uptake and toxicity with increasing waterborne Ca^{2+} concentration. Considering the differential branchial lead accumulation and kinetic lead/Ca^{2+} analysis from the present study, it is evident that the gill is probably the major site of a predominately competitive interaction between lead and Ca^{2+} that contributes to lead toxicity in the rainbow trout.

Further evidence supporting the existence of a competitive relationship between lead and Ca^{2+} was acquired through the use of apical calcium-channel blockers. While inhibiting Ca^{2+} uptake (Fig. 5A), waterborne lanthanum, a classic Ca^{2+} channel blocker (Weiss, 1974), significantly reduced the amount of lead accumulation on the gill (Fig. 5B). This suggests that apical entry of lead into the chloride cells probably occurs through a lanthanum-sensitive, voltage-independent apical calcium channel. These observations are similar to those reported for zinc by Hogstrand et al. (1996), with an approximate 53% decrease in zinc uptake occurring in the presence of 1 μmol l^{-1} lanthanum compared to controls. Comhaire et al. (1998) reported a 48% inhibition of cobalt uptake at low-level lanthanum; however, 1 μmol l^{-1}

waterborne lanthanum exposure resulted in a stimulation of cobalt influx. A similar effect was observed in the present study at waterborne lanthanum concentrations exceeding 1 μmol l^{-1} (data not shown). Stimulated lead accumulation may have been due to disruption of apical membrane integrity by high lanthanum concentrations, resulting in an increase in the diffusive component of lead uptake. Finally, the results of the present study are consistent with those of Verbost et al. (1987), demonstrating an inhibition of cadmium influx by lanthanum, and leading to the conclusion that cadmium and Ca^{2+} share the same apical entry sites. This has led to the use of cadmium for the purpose of calcium-channel antagonist experiments (Comhaire et al., 1998), as in the present study.

The effects of cadmium on Ca^{2+} influx were significant (Fig. 5A). As gill cadmium accumulation increased with increased waterborne cadmium concentrations, gill lead accumulation was reduced in a dose-dependent fashion compared to uptake observed in the absence of the blocker (Fig. 5C). This is consistent with the results of Comhaire et al. (1998), who reported significant inhibition of cobalt uptake upon exposure to similar waterborne cadmium concentrations. The inhibition of lead accumulation by cadmium, a metal that traverses the chloride cell apical membrane through voltage-independent, lanthanum-inhibitable channels (Verbost et al., 1989), is further evidence supporting a similar entry route for lead. In further support of this theory is the effect of zinc on lead accumulation (Fig. 5D). The ability of zinc, a metal believed to enter fish *via* Ca^{2+} uptake pathways (Spry and Wood 1985; Hogstrand et al., 1994, 1995, 1996, 1998), to reduce branchial lead accumulation suggests a similar route of entry for these metals.

The possibility of lead and Ca^{2+} transport through voltage-dependent, L-type calcium channels at the apical surface of the chloride cell was discounted by the lack of effect of the voltage-dependent Ca^{2+} channel blockers verapamil and nifedipine (Perry and Flik, 1988) (Fig. 6A–C). Perry and Flik (1988) reported similar results when assessing the effect of nifedipine on Ca^{2+} uptake, leading to the suggestion that Ca^{2+} flux across the apical membrane occurs through voltage-independent Ca^{2+} channels.

Injection of ionic Ca^{2+} was also employed to manipulate apical Ca^{2+} channels so as to inhibit Ca^{2+} influx *via* the stanniocalcin-controlled pathway. Previous studies have shown that activation of this pathway results in a decreased permeability of chloride cells to Ca^{2+} entry, thereby reducing influx (Perry et al., 1989; Verbost et al., 1993). In the present study, the hypocalcemic effects of stanniocalcin induction reduced both Ca^{2+} influx and lead accumulation (Fig. 7A,B). This provides further evidence that lead uptake is by the same mechanism as Ca^{2+} uptake. These results are similar to those reported by Hogstrand et al. (1996), where both Ca^{2+} and zinc influx rates were reduced by injection of Ca^{2+}.

The other significant process in the transepithelial uptake of Ca^{2+} is the movement across the baso-lateral membrane against an electrochemical gradient *via* a high affinity Ca^{2+}-ATPase mechanism (Perry and Flik, 1988). The importance of this enzyme to calcium transport makes it a vulnerable target for Ca^{2+} antagonists such as cadmium, which, following sufficient accumulation within the chloride cell, inhibits Ca^{2+}-ATPase activity contributing to disturbances in Ca^{2+} homeostasis (Verbost et al., 1989). The present study revealed that prolonged lead exposure results in significant inhibition of high affinity Ca^{2+}-ATPase activity (Fig. 8A). This reduction in activity occurred after 24–96 h of lead exposure, suggesting that similar to cadmium, lead must accumulate in sufficient amounts at the gill to have a negative effect on Ca^{2+}-ATPase activity (Fig. 8C). Accumulation at the gill was significantly elevated after 24 h and 96 h of lead exposure (Fig. 8B), the same time points where substantial inhibition of enzyme activity was observed. This mechanism of Ca^{2+} homeostasis disruption is also similar to that proposed for zinc (Hogstrand et al., 1996). With increased amounts of branchial lead accumulation following prolonged exposure, a slower, non-competitive component to disruption of Ca^{2+} homeostasis would occur. This would ultimately have an effect on J_{max}, decreasing the overall efficiency of the Ca^{2+} transport system.

Ca^{2+} efflux was much less sensitive to lead than Ca^{2+} influx (Table 2). This is evident by the absence of efflux stimulation at a waterborne lead concentration (6.3 μmol l^{-1}) that significantly reduced Ca^{2+} influx (Rogers et al., 2003) and is at least 4 times higher than the minimum concentration required to elicit Ca^{2+} influx inhibition. These findings are consistent with those for cadmium (Verbost et al., 1987; Reid and McDonald, 1988). Verbost et al. (1987) found the effect of cadmium on Ca^{2+} efflux was insignificant, except for measurements made at the highest cadmium concentration. Reid and McDonald (1988) found no effect of cadmium on branchial Ca^{2+} efflux at circumneutral pH and at moderately acidic pH (4.8). While significant stimulation of efflux by lead was not observed, a slight, non-significant increase in efflux rate did occur after 48 h of lead exposure. This could reflect a secondary effect of lead on the integrity of paracellular routes, through which Ca^{2+} efflux is thought to occur (Perry and Flik, 1988).

Conclusions

To our knowledge, this study is the first to present direct evidence that the uptake of waterborne lead by freshwater adapted rainbow trout is by the same mechanism as Ca^{2+}. The uptake involves competitive inhibition of apical entry at lanthanum-sensitive Ca^{2+} channels and interference with the function of the ATP-driven baso-lateral Ca^{2+} pump. Similarities are evident between lead and other known calcium antagonists such as cadmium, zinc and cobalt, suggesting avenues for further research that include characterization of the interaction between lead and Ca^{2+}-ATPase as well as the intracellular handling of lead by Ca^{2+} transport proteins such as calmodulin.

This study could have potential implications for a predictive modelling approach such as the Biotic Ligand Model (BLM) (Paquin et al., 2002), which is based on a toxic metal binding to the gills in competition with protective and

nutrient ions in the water column (Playle et al., 1993; Playle, 1998; Paquin et al., 2002). Currently, binding models for lead are being developed (MacDonald et al., 2002), and based on their predictions and the physiological evidence presented in this study, it appears that lead is highly capable of out-competing Ca^{2+} for specific transport sites at the freshwater fish gill.

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