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Mechanisms behind Pb-induced disruption of Na^+ and Cl^- balance in rainbow trout (*Oncorhynchus mykiss*)

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Rogers, Joseph T., Monika Patel, Kathleen M. Gilmour, and Chris M. Wood. Mechanisms behind Pb-induced disruption of Na^+ and Cl^- balance in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 289: R463–R472, 2005; doi:10.1152/ajpregu.00362.2004.—The mechanism of Pb-induced disruption of Na^+ and Cl^- balance was investigated in the freshwater rainbow trout (*Oncorhynchus mykiss*). Na^+ and Cl^- influx rates were reduced immediately in the presence of 2.40 ± 0.24 and 1.25 ± 0.14 μM Pb, with a small increase in efflux rates occurring after 24-h exposure. Waterborne Pb caused a significant decrease in the maximal rate of Na^+ influx without a change in transporter affinity, suggesting a noncompetitive disruption of Na^+ uptake by Pb. Phenamil and bafilomycin markedly reduced Na^+ influx rate but did not affect Pb accumulation at the gill. Time-course analysis in rainbow trout exposed to 0, 0.48, 2.4, and 4.8 μM Pb revealed time- and concentration-dependent branchial Pb accumulation. Na^+-K^+-ATPase activity was significantly reduced, with 4.8 μM exposure resulting in immediate enzyme inhibition and 0.48 and 2.4 μM exposures inhibiting activity by 24 h. Reduced activity was weakly correlated with gill Pb accumulation after 3- and 8-h exposures; this relationship strengthened by 24 h. Reduced Na^+ uptake was correlated with gill Pb burden after exposures of 3, 8, and 24 h. Immediate inhibition of branchial carbonic anhydrase activity occurred after 3-h exposure to 0.82 ± 0.05 or 4.30 ± 0.05 μM Pb and continued for up to 24 h. We conclude that Pb-induced disruption of Na^+ and Cl^- homeostasis is in part a result of rapid inhibition of carbonic anhydrase activity and of binding of Pb with Na^+-K^+-ATPase, causing noncompetitive inhibition of Na^+ and Cl^- influx.

waterborne lead; Na^+-K^+-ATPase; carbonic anhydrase; ionoregulation

LEAD (Pb), a group IV element on the periodic table, is a common contaminant that enters the water column mainly through industrial practices (43). Once Pb enters the aquatic environment, Pb toxicity to freshwater fish is largely influenced by water chemistry, especially by the presence of Ca^{2+}, which is protective (6, 18, 35). Recent evidence has characterized this relationship as being predominately competitive in nature (34), whereby Pb is taken up via the same mechanism as Ca^{2+}, through voltage-independent, lanthanum-inhibitable apical Ca^{2+} channels in the gills that are susceptible to regulation by the hypocalcemic hormone stanniocalcin. This competition results in disruption of Ca^{2+} influx and homeostasis in Pb-exposed fish (33, 34), making Pb similar to other known Ca^{2+} antagonists such as Zn (16, 17, 36, 37) and Cd (38, 39). However, acute Pb toxicity involves an additional component of ionoregulatory disturbance evident in the disruption of Na^+ and Cl^- balance. This additional component is reflected in reduced rates of branchial influx, inhibition of gill Na^+-K^+-ATPase activity, and reduced plasma Na^+ and Cl^- concentrations in acutely exposed rainbow trout (33). A similar negative effect of Pb on Na^+ balance also has been reported in chronically exposed crayfish (1). These observations appear to place Pb midway between Ca^{2+} antagonists such as Zn and Cd and disruptors of Na^+ and Cl^- balance such as Ag (28, 46) and Cu (20, 32).

Metals such as Ag and Cu affect Na^+/Cl^- homeostasis by interfering with the active uptake of Na^+ from the water at apical Na^+ channels, potent inhibition of Na^+-K^+-ATPase activity at the basolateral membrane (3, 4, 28), and inhibition of carbonic anhydrase (27). Although Pb does inhibit Na^+-K^+-ATPase (33), it is unclear how this inhibition relates to Na^+ uptake, to the time course of Na^+ influx disruption, and whether this is the exclusive site of interference with Na^+ and Cl^- regulation. There may be additional binding sites for Pb that initiate the toxic effect. For example, these may include interaction at apical Na^+ channels, at the vacuolar (V-type) H^+-ATPase that energetically drives Na^+ uptake (9, 21, 22, 42), sites of Na^+/Ca^{2+} exchange at the basolateral membrane, Cl^-/HCO_3^- exchange at the apical membrane (42, 45), or intracellular carbonic anhydrase activity that provides H^+ and HCO_3^- ions for apical Na^+ and Cl^- uptake/exchange, respectively (27). Pb may also stimulate Na^+ and Cl^- efflux through disruption of branchial membrane integrity, resulting in an increase in diffusive ion losses.

It is unknown which, if any, of these mechanisms explain the effect of Pb on Na^+ and Cl^- balance in freshwater fish. Therefore, using the rainbow trout as a model species, we used both physiological and pharmacological approaches to investigate this aspect of Pb-induced ionoregulatory disruption. Characterization of the key binding sites involved in Pb toxicity is essential in developing binding models such as the biotic ligand model (8, 26, 30) that allow for the prediction of waterborne metal toxicity on a water chemistry basis. Currently, such binding models for Pb are being developed (23) but require further characterization of key binding sites contributing to Pb toxicity.

MATERIALS AND METHODS

Experimental animals. Juvenile rainbow trout (3–10 g), and in one case alevins (~100 mg), were used for Na^+ and Cl^- influx and efflux rate measurements, Na^+ competition experiments, experiments using pharmacological blockers of Na^+ transport, and assessment of Na^+-K^+-ATPase activity. Adult rainbow trout (~150 g) were used when

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assaying carbonic anhydrase. All trout were obtained from Humber Springs Trout Farm (Orangeville, ON, Canada). Fish were held in dechlorinated City of Hamilton tap water (from Lake Ontario) at a temperature of 7–12°C and fed commercial trout feed at a ration of 1% total body wt per day. Water composition was 1.0 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.6 mM Na^+, 0.8 mM Cl^-, 0.05 mM K^+, and 0.003 μM total Pb, with 3 mg/l dissolved organic carbon, ~140 mg/l hardness (as $CaCO_3$), and pH 7.9–8.0. Experiments were conducted at a temperature of 9–12°C, and experimental animals were starved 72 h before and throughout all experiments.

Measurements of Na^+ and Cl^- influx and efflux rates. Determination of Na^+ and Cl^- influx was carried out by using methods almost identical to those outlined by Rogers et al. (33), differing only in the Pb exposure concentrations used. Briefly, in the present study, measurements of influx were made by relating the specific activity of waterborne ^{22}Na or ^{36}Cl to the amount of radioisotope measured in the animal after a 2-h exposure period. Measurements of Na^+ and Cl^- net flux rates were determined using the following formula from Wood (44):

$$J_{net}^{Ion} = [([X]_i - [X]_f) \times V_{ext}]/W \times t \quad (1)$$

where $[X]_i$ and $[X]_f$ refer to initial and final concentrations of the ion in the water, V_{ext} represents the volume of the flux chamber, t is the time of the flux period, and W is the body mass of the experimental animal. Using values for influx rate obtained from the appearance of isotope in the fish and values for net flux rate obtained using the above formula, we were able to calculate efflux rates.

Unidirectional influx rate measurements were carried out over periods ranging from 0.5 to 3.0 h in the present study. To ensure that the influx rate measurements were independent of the measurement period and representative of steady-state·values, we made influx rate measurements with ^{22}Na for periods of 0.5, 1.0, 2.0, and 4.0 h under control conditions, using individual fish drawn from a common batch of trout. Mean values were 1,229 ± 126, 1,505 ± 207, 1,213 ± 205, and 1,620 + 118 $nmol \cdot g^{-1} \cdot h^{-1}$ ($n = 6$ for all groups). There were no significant differences.

Measurements were made under control conditions and at Pb concentrations ~50% and 25% of the 96-h lethal exposure dose (LC_{50}) of 4.8 μM dissolved Pb determined in City of Hamilton dechlorinated tap water by Rogers et al. (33). The nominal exposure concentrations used were 2.4 and 1.2 μM. Juvenile rainbow trout were subject to preflux exposure periods of 0, 12, 24, and 48 h before undergoing a flux measurement period of 2 h in control or Pb-exposed conditions.

Kinetic analysis of Na^+ influx rate in the presence of Pb. Experimental methods for kinetic analysis of Na^+ influx followed closely those outlined by Rogers and Wood (34) for kinetic analysis of Ca^{2+} influx. Sixteen polyethylene bags representing a control (no Pb), three Pb concentrations, and a series of four different Na^+ concentrations were filled with 3 liters each of synthetic Na^+-free water (1.0 mM $CaCO_3$, 0.2 mM $MgCO_3$, 0.36 mM $MgCl_2$, pH 8.0; Ref. 28). Each bag was fitted with an air line and placed on a water bath for temperature control. Three bags of each set were spiked with a $Pb(NO_3)_2$ stock solution (Sigma Aldrich) to obtain nominal Pb concentrations of 0.48, 2.4, and 4.8 μM Pb (control, 0 μM). Each bag was then spiked with Na_2SO_4 (Fisher Scientific, Ottawa, ON, Canada) to achieve nominal sodium concentrations of 50, 100, 600, and 1,200 μM. Finally, flux bags were injected with 10 μCi/l ^{22}Na (as NaCl; specific activity 690.19 mCi/mg; NEN Life Science Products, Boston, MA).

Juvenile rainbow trout were transferred to each of the sixteen flux bags (7 fish/bag), and an initial 15-min "settling" period was allowed for acclimation and isotopic equilibration. The exposure period was 2 h in length, with initial and final water samples (5 ml) drawn in duplicate for determination of ^{22}Na activity and total Na^+ concentration. Water samples were also drawn for determination of total Pb (unfiltered) and dissolved Pb (filtered; 0.45-μm filter; Acrodisc, Pall, MI) concentrations. These samples were acidified (1% HNO_3) and stored in plastic scintillation vials for analysis. After the 2-h flux period, fish were removed and killed with a single blow to the head. The fish were then rinsed for 1 min in deionized water (NANOpure II; Sybron/Barnstead, Boston, MA) to remove any surface-bound radioisotope. Whole bodies were then blotted dry and placed in scintillation

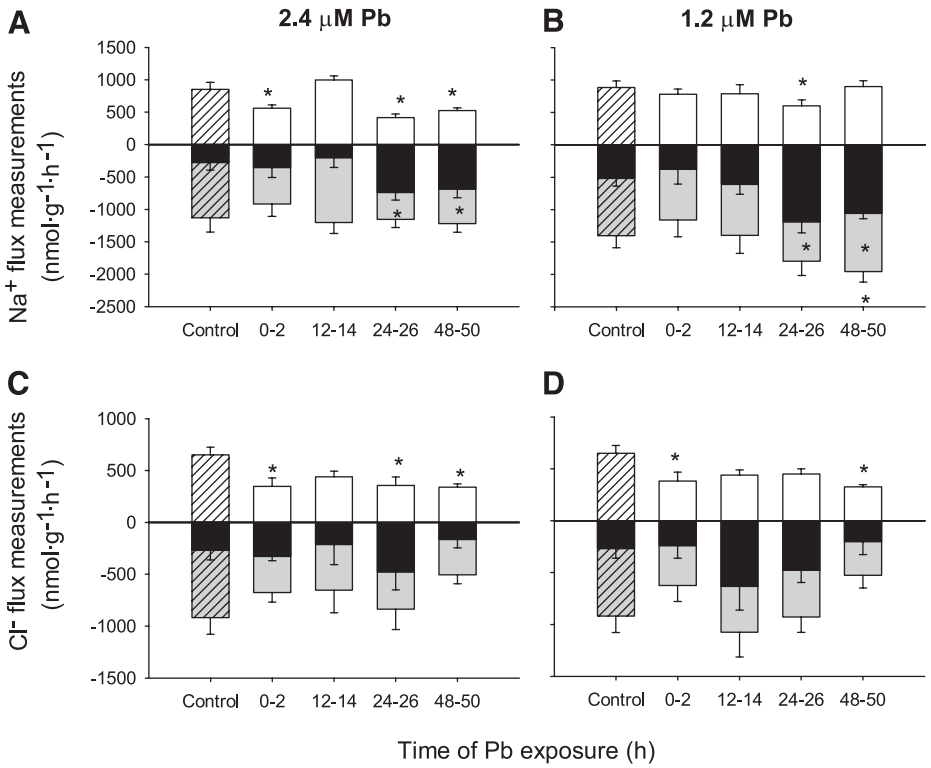

Fig. 1. Unidirectional (J_{in}, open bars; J_{out}, shaded bars; controls, hatched bars) and net flux rates (J_{net}, solid bars) of Na^+ in rainbow trout exposed to control conditions or 2.4 ± 0.2 μM dissolved Pb after exposure periods of 0, 12, 24, or 48 h (*A*), Na^+ in rainbow trout exposed to control conditions or 1.2 ± 0.1 μM dissolved Pb after exposure periods of 0, 12, 24, or 48 h (*B*), Cl^- in rainbow trout exposed to control conditions or 2.4 ± 0.2 μM dissolved Pb after exposure periods of 0, 12, 24, or 48 h (*C*), or Cl^- in rainbow trout exposed to control conditions or 1.2 ± 0.1 μM dissolved Pb after exposure periods of 0, 12, 24, or 48 h (*D*). Data are expressed as means ± SE ($n = 8$ per treatment). *$P < 0.05$, significant differences from 0 h control means.

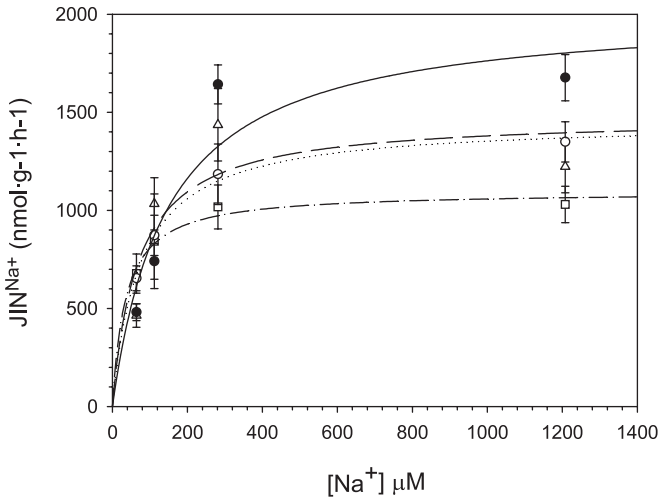

Fig. 2. Unidirectional branchial Na^+ influx rates in juvenile rainbow trout at various waterborne Na^+ concentrations in synthetic water for 4 different lead concentrations. Data for control fish are shown as filled circles (solid regression line). Fish exposed to 0.48 μM are shown as open triangles (dotted regression line), to 2.4 μM Pb as open circles (dashed regression line), and to 4.8 μM as open squares (dash-dot regression line). Data are expressed as means ± SE (n = 7 per treatment at each Na^+ concentration).

vials, and ^{22}Na radioactivity in water and animals was measured by gamma counting (Minaxi γ; Canberra-Packard, Meridian, CT).

Water samples taken for the determination of total Na^+ concentration were diluted with deionized water and analyzed by flame atomic absorption spectrophotometry (FAAS; 220FS Spectra AA; Varian Australia, Victoria, Australia) . Determination of total and dissolved waterborne Pb concentrations was done using graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA; Varian Australia) against a certified multielement standard (Inorganic Ventures, Lakewood, NJ).

Effect of waterborne Na^+ on branchial Pb accumulation. The effect of waterborne Na^+ concentration on the branchial accumulation of Pb in juvenile rainbow trout was assessed by using methods similar to those employed in the kinetic analysis of Na^+ influx in the presence of Pb. However, measurements were made without the use of radioisotopes and in the presence of elevated waterborne Na^+ concentrations. These were nominally 0.5, 1, 10, and 20 mM Na^+, added from a concentrated stock solution of Na_2SO_4. After a 2-h exposure period, fish were removed from the flux bags and killed with a blow to the head, and the gills were dissected. The gills were then rinsed for 1 min in deionized water, blotted dry, weighed, and digested in a 5:1 vol/wt ratio of 1 N HNO_3 at 55°C for 48 h. The use of a 1-min rinse in deionized water was evaluated experimentally and found to yield values identical to those from a 3-min and a 5-min rinse in deionized water, as well as a 5-min rinse in 1 mM EDTA. The protocol was therefore judged to be effective in removing surface-bound Pb, thereby allowing accurate measurement of Pb transported into the gills. Samples were then homogenized by vortexing, an aliquot was removed (~1.5 ml) and centrifuged at 13,000 *g* for 10 min, and the supernatant was analyzed for total Pb concentration using GFAAS.

Experiments with pharmacological blockers of Na^+ transport. The role of apical Na^+ channels in mediating the transport of Pb across the branchial epithelium was assessed by pharmacologically manipulating these channels. Methods closely followed those outlined by Bury and Wood (4). The first approach utilized phenamil, an analog of amiloride that irreversibly blocks Na^+ influx such that its effects persist after removal of the drug from the bathing solution, allowing Pb uptake to be measured without the complicating presence of the drug (12, 19). This is important because amiloride and its analogs may bind metals, thereby reducing their bioavailability and consequent uptake by complexation, an undesired mechanism (4). In assessing the effects of phenamil on branchial Pb accumulation, a series of four containers (1,000 ml) representing a control (0 μM phenamil) and three phenamil concentrations were filled with 3 liters of synthetically modified water obtained by reverse osmosis (Na^+ and Cl^- added as NaCl = 0.7 mM), made carbonate free to reduce complexation of Pb and calcium free to reduce Pb/Ca^{2+} competition. The beakers were then spiked with phenamil from a stock solution prepared by using deionized water to achieve nominal phenamil concentrations of 1, 10, and 100 μM. Juvenile rainbow trout were then transferred into the drug-containing beakers (8 fish/beaker) for an incubation period of 2 h in the drug-containing medium, allowing time for phenamil to bind to the gill surface.

After the incubation period, animals were then transferred to each of four clean beakers containing a phenamil-free flux medium identical in water chemistry to the above treatment but containing a nominal waterborne Pb concentration of 4.8 μM [as $Pb(NO_3)_2$]. This transfer was followed by a 3-h flux period in the Pb-containing medium. After the flux period, fish were removed from the beakers, and the gills were dissected and processed using methods identical to those outlined above. By using ^{22}Na as a radiotracer, measurements of Na^+ influx rate at control and 1, 10, and 100 μM phenamil were conducted on a different set of fish using methods similar to those described above. Procedures used for determination of Na^+ influx rates were identical to those outlined above for measurements of Na^+ influx and efflux rates.

Bafilomycin A1, a blocker of the V-type H^+-ATPase that powers Na^+ uptake (4), was employed as an additional tool to assess whether the apical Na^+ channel is a route for Pb transport into the fish gill. Because of the high cost of bafilomycin, the flux volume was necessarily reduced and rainbow trout alevins (~100 mg) were used as experimental animals. In brief, four beakers, representing two control groups and two bafilomycin-exposed groups, were filled with synthetically modified water, identical in chemistry to that used in experiments with phenamil. In assessing Pb-accumulation, a control group and bafilomycin-exposed group (n = 7 per treatment) were exposed to a nominal concentration of 4.8 μM [as $Pb(NO_3)_2$] for 0.5 h. Bafilomycin was delivered using a stock solution prepared in ethanol with a final concentration in the flux medium of 0.001%. The final concentration of bafilomycin in the flux medium was 2 μM. To maintain consistency, we exposed control groups to an equal concentration of ethanol. After the flux period, whole fish were removed from the flux medium, killed with a blow to the head, rinsed in deionized water, and analyzed for Pb accumulation with the use of methods outlined above. The remaining two groups were used in measurements of Na^+ influx under control conditions and after exposure to 2 μM bafilomycin by using procedures identical to those used in experiments with phenamil, differing only in the length of the flux period (0.5 h).

Time-course analysis of gill Pb burden, Na^+ uptake, and Na^+-K^+-ATPase activity. This experiment utilized four different waterborne Pb concentrations, including one control group (0 μM). For each concentration, juvenile rainbow trout were exposed to the appropriate concentration of Pb on a flow-through basis for 3, 8, or 24 h in the

Table 1. *J_{max} and apparent K_m for unidirectional whole body Na^+ influx rates in juvenile rainbow trout at various waterborne Pb concentrations.*

Waterborne Pb, μM	J_{max}, nmol·g h^{-1}	Apparent K_m, μM
Control	2,024±383	148±85
0.48	1,476±327.7	70±58
2.4	1,454±35*	74±8
4.8	1,096±44*	36±8

Values are means ± SE (n = 7). J_{max}, peak Na^+ influx rate; K_m, Na^+ transporter affinity. *$P < 0.05$, significant difference from control mean.

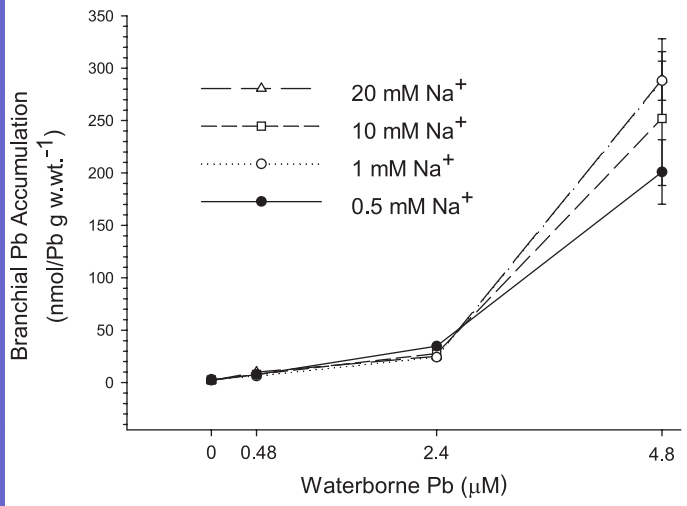

Fig. 3. Measurements of branchial Pb accumulation in juvenile rainbow trout at various waterborne Na^+ concentrations in synthetic water for 4 different lead concentrations. Data are expressed as means ± SE (n = 8 per treatment at each Pb concentration).

acclimation tap water [140 mg/l hardness (as $CaCO_3$)] by using methods identical to those employed previously (33). In brief, juvenile trout were transferred to clean tap water (flow rate per chamber = 600 ml/min) in aerated experimental chambers, which would subsequently contain each of the four nominal Pb concentrations (0, 0.48, 2.4, and 4.8 μM Pb). Animals were allowed a settling period of 24 h. At *time 0*, stock solutions prepared from $Pb(NO_3)_2$ were used to spike the exposure chambers to achieve the appropriate Pb concentrations. These stock solutions were then dripped at a rate of 1 ml/min into mixing tanks with an inflow of freshwater of ~600 ml/min. Mixing tanks then fed experimental chambers (flow rate = 600 ml/min) that were 22 liters in volume.

Sampling procedures were similar to those used by Morgan et al. (27). Briefly, at 3, 8, and 24 h, eight fish from each Pb concentration were sampled for branchial Pb accumulation and whole body Na^+ uptake rate by using ^{22}Na as a radiotracer. An additional eight fish were killed at each time point for determination of branchial Na^+-K^+-ATPase activity.

Methods used for Na^+ influx rate determination were similar to those outlined above. For each Pb concentration, eight fish were transferred to a 1,000-ml Pyrex glass beaker with 600 ml of Pb-containing water. Water samples for the determination of ^{22}Na radioactivity and waterborne Na^+ concentration (5 ml) were drawn at the beginning and end of a 1-h flux period. After the flux period, fish were removed from the flux medium, killed with a single blow to the head, and rinsed in deionized water to remove surface-bound Pb and ^{22}Na radioactivity. Gills and whole bodies were then counted separately, and the activity was summed for determination of whole body Na^+ influx rate. Gills used for Na^+ influx measurements were then digested in 1 N HNO_3 at 55°C for 48 h and centrifuged at 13 000 *g* for 10 min, and the supernatant was drawn off (~1 ml) for determination of total Pb concentration by GFAAS.

Gills used for measurements of Na^+-K^+-ATPase activity were dissected from fish on ice, placed in 2-ml plastic centrifuge tubes, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. The protocol used for the determination of Na^+-K^+-ATPase activity followed that outlined by McCormick (25).

Effect of waterborne Pb on carbonic anhydrase activity. In assessing the effects of Pb on the activity of branchial carbonic anhydrase, the gills of adult rainbow trout were perfused to eliminate enzyme activity contained in red blood cells. Briefly, trout were exposed to control conditions or to nominal waterborne Pb concentrations of 1.2 and 4.8 μM for 3, 8, or 24 h using methods identical to those outlined above. The gill perfusion technique used was identical to that outlined by Rogers and Wood (34) whereby the perfusate (cold saline containing 20 U/ml sodium heparin) was delivered via the bulbus arteriosus to the gill tissue by using a pressure of 60 cmH_2O applied through a catheter (PE 60 tubing). After perfusion, gill baskets were excised and filaments dissected from the gill arches on ice. Only fully perfused gill tissue was collected for assaying carbonic anhydrase. Gill filaments were then homogenized in 1 ml of buffer containing 10 mM Tris base, 225 mM mannitol, and 75 mM sucrose and brought to a pH of 7.4 with 20% orthophosphoric acid. After homogenization, samples were centrifuged at 13,000 *g* for 5 min, and the supernatant was frozen at −80°C until analysis. Carbonic anhydrase activity was assayed using methods outlined by Gilmour et al. (11). Protein was measured using the Bradford protein assay with bovine serum albumin protein standards (Sigma).

Statistical analysis. In kinetic experiments, Michaelis-Menten analysis of the relationship between rates of Na^+ influx ($J_{in}^{Na^+}$) and waterborne Na^+ (substrate) was performed using nonlinear regression of the kinetic plots (SigmaPlot 2000). Data are expressed as means ± SE of n values. Experimental means were compared with corresponding control mean values at the same time point by using an unpaired, two-tailed Student's t-test. Time-dependent and dose-dependent responses in both control and experimental groups were tested against initial 0-h or control measurements by using a one-way ANOVA with a two-sided Dunnett's post hoc comparison. All statistical significance was calculated at $P < 0.05$.

RESULTS

Effect of Pb on Na^+ and Cl^- flux. Juvenile rainbow trout exposed to 2.4 μM Pb (measured dissolved = 2.4 ± 0.2 μM Pb) showed significant inhibition of Na^+ influx (Fig. 1*A*). Effects were immediate, with an approximate 34% inhibition

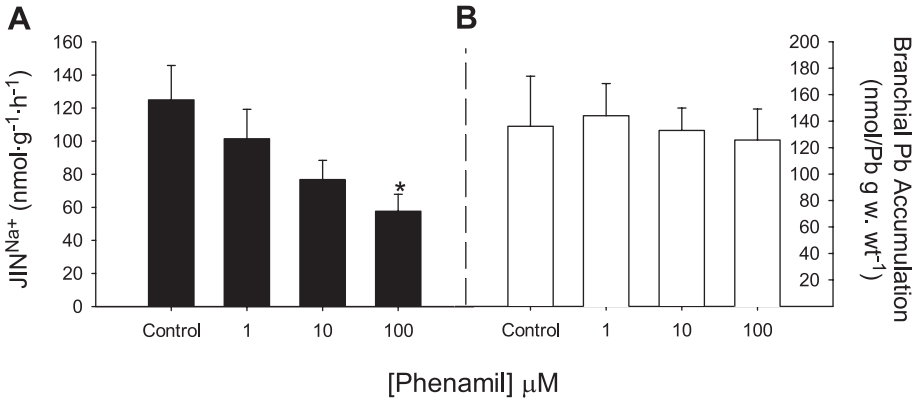

Fig. 4. Effect of phenamil pretreatment on unidirectional Na^+ influx (*A*) and branchial Pb accumulation (*B*). Data are expressed as means ± SE (n = 7). *$P < 0.05$, significant difference (1-way ANOVA with a 2-sided Dunnett's post hoc comparison) from corresponding control mean.

occurring at 0–2 h of Pb exposure compared with control values. Although this effect was not evident at 12–14 h, disruption of uptake was again significant at 24–26 h and 48–50 h. Elevated levels of Na^+ efflux were not observed; however, net loss of Na^+ by Pb-exposed fish increased by ~60% after 24–26 h and 48–50 h of exposure (Fig. 1*A*). Na^+ balance in rainbow trout exposed to 1.2 μM Pb (dissolved = 1.2 ± 0.1 μM Pb) was also disrupted with a significant 31% reduction in influx rate after 24–26 h of Pb exposure (Fig. 1*B*) but not at the other times. Efflux rates remained conserved after 2 and 14 h of exposure but showed a slight, nonsignificant increase after 26 h and a significant increase at 48–50 h. This contributed to an approximate 50% increase in net Na^+ loss after these prolonged exposure periods (Fig. 1*B*).

Exposure to 2.4 ± 0.2 μM Pb resulted in significant reductions in Cl^- influx rates after 0–2, 24–26, and 48–50 h compared with control values (Fig. 1*C*). An immediate influx inhibition of ~47% occurred after 2 h of Pb exposure. Although influx rates were disrupted, efflux measurements in both control and experimental groups were undisturbed, contributing to relatively stable net flux rates in Pb-exposed fish compared with controls. Exposure to 1.2 ± 0.1 μM dissolved Pb resulted in reductions in Cl^- influx similar in magnitude to those observed at twice the Pb concentration (Fig. 1*D*). Significant impacts were observed after 0–2 and 48–50 h of exposure. Again, efflux rates in both control and experimental fish remained constant, contributing to a conserved rate of net flux in experimental fish that did not differ significantly from controls despite slight, nonsignificant increases in net Cl^- loss after 12–14 and 24–26 h of Pb exposure (Fig. 1*D*).

Analysis of branchial Pb and Na^+ interaction. Consistent with the immediate inhibition of Na^+ influx outlined above, kinetic analysis at a series of waterborne Na^+ concentrations and at four different Pb concentrations revealed an immediate inhibition of Na^+ uptake over a 2-h exposure period, with the inhibition increasing with waterborne Pb concentration (Fig. 2). The saturable nature of Na^+ influx with increasing substrate concentration allowed for typical Michaelis-Menten analysis by nonlinear regression. Significant reductions in J_{max} occurred with increasing waterborne Pb concentration (Table 1). Although J_{max} was reduced, K_m values, which were quite variable, especially in the control treatment, were not significantly altered in Pb-exposed fish.

The effect of elevated waterborne Na^+ concentrations on branchial Pb accumulation is shown in Fig. 3. Consistent with the relative stability of Na^+ transporter affinity (K_m) in the presence of elevated waterborne Pb concentrations, increasing Na^+ concentrations of 0, 0.5, 1, 10, and 20 mM did not significantly impact gill Pb burden. Although significant accumulation of Pb occurred with increasing waterborne Pb concentration, there were no significant differences among Na^+ treatments. A marked change in the slope of the gill Pb accumulation vs. exposure Pb concentration occurred at each waterborne Na^+ concentration upon exposure to the highest concentration, 4.8 μM Pb.

Experiments with pharmacological blockers of Na^+ transport. Preexposure to phenamil, a blocker of Na^+ channels, caused a dose-dependent decrease in Na^+ influx rate over the range of 1, 10, and 100 μM (Fig. 4*A*) that was significant at the highest phenamil concentration (~54% inhibition). In contrast, increasing phenamil concentrations did not significantly affect gill Pb burden compared with controls (Fig. 4*B*).

Bafilomycin A1 (2 μM), a pharmacological blocker of the vacuolar-type ATPase that creates a favorable electrochemical gradient for Na^+ influx, caused a significant 37% reduction in Na^+ influx rate in trout alevins (Fig. 5*A*). Despite an impact on Na^+ homeostasis, bafilomycin treatment did not reduce whole body Pb accumulation (Fig. 5*B*).

Time-course analysis of gill Pb burden, Na^+ uptake, and Na^+-K^+-ATPase activity. Juvenile rainbow trout were subjected to control conditions or to a series of waterborne Pb concentrations for various times up to 24 h. Exposure to dissolved Pb concentrations of 0.48 ± 0.1, 2.9 ± 0.03, and 5.3 ± 0.9 μM resulted in significant increases in branchial Pb burden compared with controls after 3 h (Fig. 6*A*). Accumulation continued in a time-dependent fashion, with gill burden greatly increasing (~25- to 30-fold) after 8 and 24 h. Significant differences in accumulation also occurred between Pb treatments, with fish exposed to 5.3 ± 0.9 μM showing significantly higher accumulation than those at 0.48 ± 0.1 μM at 3, 8, and 24 h. Gill Pb burden measured in fish exposed for 8 h to 2.9 ± 0.03 μM was significantly lower than in those exposed to the highest Pb concentration for 8 h.

Although Pb accumulation at the gill increased significantly after only 3 h, branchial Na^+-K^+-ATPase activity at the same time point was only reduced by the highest waterborne Pb concentration, with an observed inhibition of ~50% (Fig. 6*B*). Figure 6*B* displays the time course of changes in Na^+-K^+-ATPase activity in trout exposed to 0.48 ± 0.1 and 2.9 ± 0.03 μM dissolved Pb, respectively. Activity remained stable in both treatments for up to 8 h, after which significant inhibition of enzyme activity occurred. In addition to inhibition after 3 h,

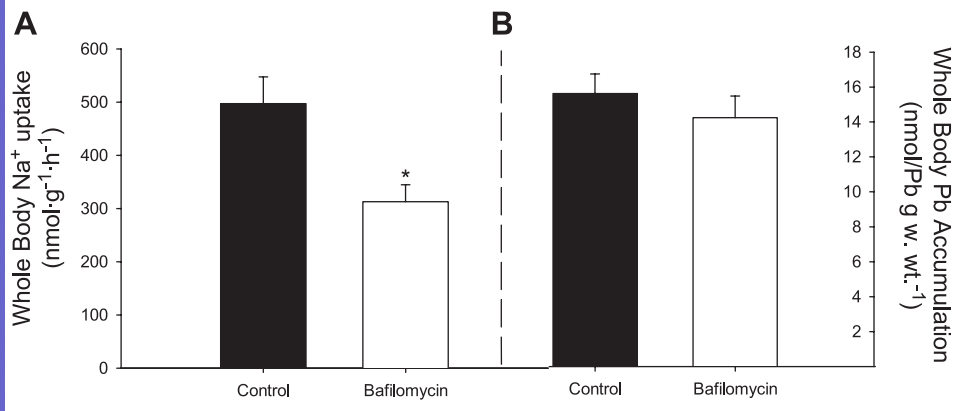

Fig. 5. Effect of bafilomycin A1 on unidirectional Na^+ influx rates in rainbow trout alevins exposed to control conditions or 2 μM bafilomycin (*A*) and branchial Pb accumulation in rainbow trout alevins exposed to 4.8 μM Pb under control conditions or 2 μM bafilomycin (*B*). Data are expressed as means ± SE (n = 7). *$P < 0.05$, significant difference (2-tailed Student's t-test) from corresponding control mean.

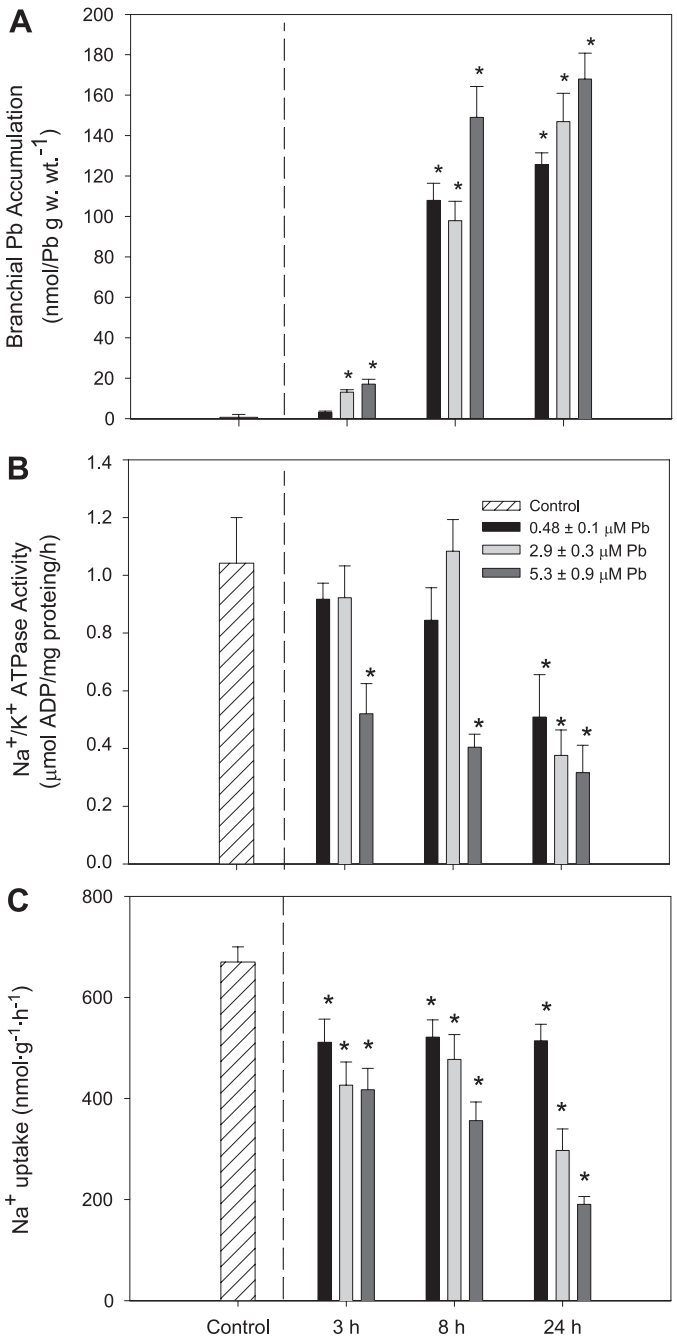

Fig. 6. *A*: time course of branchial Pb accumulation in rainbow trout exposed to control conditions (t = 0 h) or to a series of dissolved Pb concentrations for 3, 8, and 24 h. Data are expressed as means ± SE (n = 8). Exposure to dissolved Pb resulted in significant increases in branchial Pb accumulation compared with controls after 3 h. There were also differences in burden between Pb treatments, with fish exposed to 5.3 ± 0.9 μM accumulating more Pb than those at 0.48 ± 0.1 μM at 3, 8, and 24 h. Gill Pb burden measured in fish exposed for 8 h to 2.9 ± 0.03 μM was markedly lower than in fish exposed to the highest Pb concentration for 8 h. Branchial Na^+-K^+-ATPase activity (*B*) and rates of Na^+ influx are shown for rainbow trout exposed to control conditions (t = 0 h) or to dissolved Pb concentrations of 0.48 ± 0.1, 2.9 ± 0.03, and 5.3 ± 0.9 μM for 3, 8, and 24 h. Data are expressed as means ± SE (n = 8). *$P < 0.05$, significant difference (1-way ANOVA with a 2-sided Dunnett's post hoc comparison) from corresponding control mean.

the highest Pb treatment (5.3 ± 0.9 μM dissolved Pb) also reduced activity at 8 and 24 h of exposure.

Measured rates of Na^+ influx were also reduced in trout exposed to 0.48 ± 0.1, 2.9 ± 0.03, and 5.3 ± 0.9 μM dissolved Pb (Fig. 6*C*). Compared with controls, inhibition of Na^+ uptake was significant at all time points and for all exposure concentrations used. The highest waterborne Pb concentration caused a time-dependent reduction in Na^+ influx rates that was significant at 3, 8, and 24 h.

Inhibition of Na^+-K^+-ATPase activity did not strongly correlate with branchial Pb accumulation at 3 h (r^2 = 0.65, $P > 0.05$; Fig. 7*A*) and 8 h (r^2 = 0.61, $P > 0.05$; Fig. 7*C*) of waterborne Pb exposure. Despite a weak negative relationship between gill Pb burden and enzyme activity, reductions in Na^+ influx were significantly correlated with gill Pb accumulation at 3 h (r^2 = 0.78, $P < 0.05$; Fig. 7*B*) and 8 h (r^2 = 0.83, $P < 0.05$; Fig. 7*D*). After 24 h of Pb exposure, there was a strong negative correlation between Na^+-K^+-ATPase activity and branchial Pb levels (r^2 = 0.99, $P < 0.05$; Fig. 7*E*), and the relationship between Pb concentration on the gill and reduced Na^+ uptake was again significant (r^2 = 0.80, $P < 0.05$; Fig. 7*F*).

Effect of waterborne Pb on carbonic anhydrase activity. Exposure to waterborne Pb significantly reduced carbonic anhydrase activity compared with controls. A dissolved Pb concentration of 0.82 ± 0.05 μM caused an immediate 25% reduction in activity at 3 h, after which inhibition of 71 and 60% occurred at 8 and 24 h, respectively (Fig. 8). Exposure to 4.3 ± 0.05 μM dissolved Pb had similar effects, with carbonic anhydrase activity reduced by 41% after 3 h (Fig. 8). Further inhibition of ~60% was observed at 8 and 24 h of Pb exposure.

DISCUSSION

Mechanisms behind Pb-induced disruption of Na^+ and Cl^- balance. Similar to metals like Zn (16, 17, 36, 37) and Cd (38, 39), Pb has been characterized as an analog of Ca^{2+} in freshwater teleost fish. This is supported by an abundance of circumstantial evidence suggesting similarities in Pb and Ca^{2+} uptake and handling (6, 15, 18, 23, 33) and, more recently, by direct evidence showing a competitive Pb/Ca^{2+} interaction at apical voltage-independent Ca^{2+} channels in the gills and an inhibition by Pb of high-affinity Ca^{2+}-ATPase in the basolateral membrane. The latter is the active Ca^{2+} extrusion mechanism from ionocyte to blood (10, 40). Acute Pb toxicity, however, does not appear exclusive to disruption of Ca^{2+} homeostasis but includes disturbances in Na^+ and Cl^- balance evident in reductions in ion influx rates and decreases in plasma Na^+ and Cl^- concentrations (33) upon exposure to Pb concentrations approaching 4.8 μM. This property of Pb toxicity places it midway between metals that disrupt Ca^{2+} homeostasis (Cd and Zn) and metals such as Ag (28, 46) and Cu (20, 32) that are potent inhibitors of Na^+ and Cl^- influx.

The characterization of the toxic mechanism of Pb as being "midway" between other metals refers to its ability to influence both Na^+/Cl^- and Ca^{2+} homeostasis This is likely based on chemical properties of Pb that allow for intermediary interaction with multiple enzymes involved in ionoregulation. The softness index is a chemical property often related to toxicity (24). Lead is considered "borderline" in this respect, which enables binding to both sulfur groups and oxygen-containing

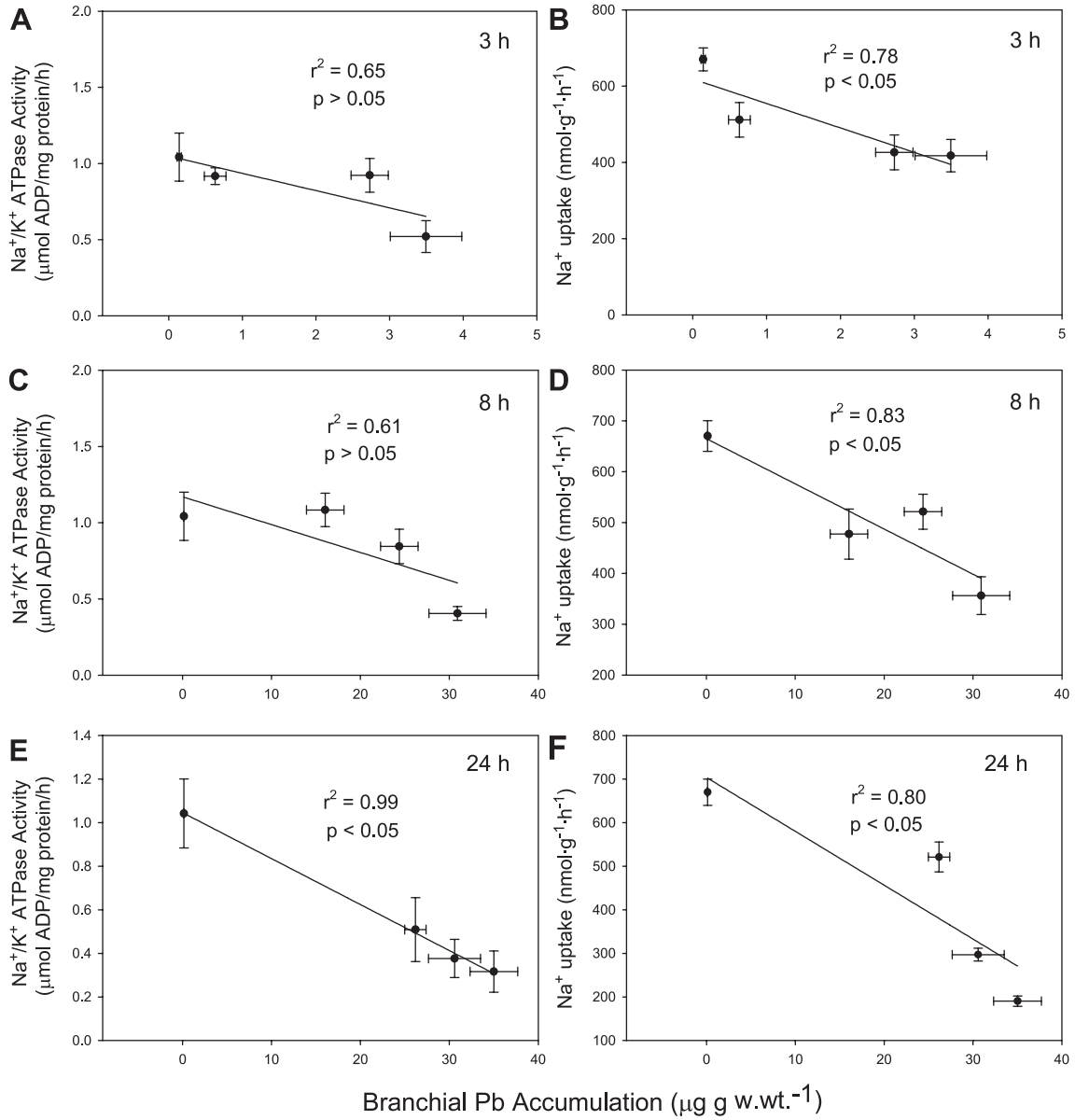

Fig. 7. Relationships between inhibition of Na^+-K^+-ATPase activity and branchial Pb accumulation (*A*, *C*, and *E*) and whole body Na^+ influx and branchial Pb accumulation (*B*, *D*, and *F*) in fish exposed to $Pb(NO_3)_2$ for 3 (*A* and *B*), 8 (*C* and *D*), and 24 h (*E* and *F*). Lines indicate linear regression analyses.

groups with intermediary strength. Consequently, interaction at multiple enzymes involved in ion transport, such as Na^+-K^+-ATPase, Ca^{2+}-ATPase, and carbonic anhydrase, may dictate Pb-induced ionoregulatory toxicity.

The approach taken in the present study was to mechanistically characterize the effect of Pb on Na^+ and Cl^- balance, focusing predominately on possible interactions with Na^+ and Cl^- uptake pathways (see Introduction). Present measurements of influx (Figs. 1 and 2) show that at Pb concentrations that approach environmentally realistic levels (up to 0.58 μM; Ref. 7), inhibition of Na^+ and Cl^- influx is still prominent. Pb-exposed fish showed an immediate inhibition of Na^+ and Cl^- uptake at a concentration of 2.4 ± 0.2 μM dissolved Pb; however, only Cl^- uptake showed an immediate reduction in influx upon exposure to 1.2 ± 0.1 μM dissolved Pb. Interestingly, the disruption of Cl^- influx by Pb exposure was quantitatively larger and more rapidly developing than that of Na^+. This finding suggests that Pb-induced disruption of Na^+ and Cl^- could be initiated, at least in part, by interference with Cl^- uptake mechanisms.

Although uptake was affected, changes in Na^+ and Cl^- efflux rates appeared to be minimal initially. However, prolonged exposure (24–48 h) did result in an increase in Na^+ efflux that contributed to a significant net Na^+ loss compared with controls. This could reflect an increase in gill membrane permeability via disruption of tight junctions and/or displacement of membrane-bound Ca^{2+} by Pb. Increased efflux could also reflect diffusional Na^+ loss due to a gradual increase in "inside" transepithelial potential resulting from Pb inhibition of Na^+-K^+-ATPase activity at the basolateral membrane (1, 2, 33) (Figs. 6*B* and 7). Reduced rates of Cl^- uptake may also be a contributing factor in observed Na^+ loss. This rapid disrup-

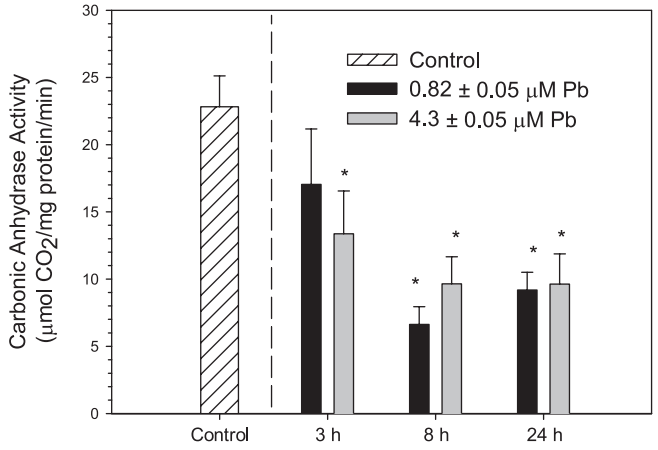

Fig. 8. Effect of waterborne Pb on carbonic anhydrase activity in adult rainbow trout exposed to control conditions or to dissolved Pb concentrations of 0.82 ± 0.05 and 4.3 ± 0.05 μM for 3, 8, or 24 h. Data are expressed as means ± SE (n = 5–6). *$P < 0.05$, significant difference (1-way ANOVA with a 2-sided Dunnett's post hoc comparison) from control mean.

tion in influx and resulting net ion efflux is similar to the action of Ag^+ in disrupting Na^+ balance in freshwater rainbow trout (28). Future experiments investigating Pb-stimulated increases in Na^+ efflux should include the impact of Pb on the mechanism of Na^+/Ca^{2+} exchange. Should Pb act as a strong substitute for Ca^{2+}, the action of this exchanger may be increased, resulting in losses of Na^+. Again, further studies in this area are needed.

Michaelis-Menten analysis of Na^+ uptake kinetics demonstrated that increasing the waterborne Pb exposure did not significantly reduce the affinity of Na^+ binding sites for the substrate (i.e., K_m values did not increase) but, rather, reduced J_{max}, indicating a noncompetitive rather than a competitive inhibition of Na^+ influx. From data presented in Table 1, it could be interpreted that K_m values show a decreasing trend with increasing Pb levels; however, this is likely an artifact caused by the high variability in control K_m measurements. A reduction in J_{max} is indicative of a Pb-induced loss of Na^+ "transport sites" that could include the apical Na^+ channels or the basolateral Na^+-K^+-ATPase. In addition, interaction at the apical H^+-ATPase, an enzyme that creates a favorable electrochemical gradient for Na^+ uptake (9, 31, 42), or at carbonic anhydrase, which plays a role in providing protons for apical Na^+ uptake, could contribute to a reduction in maximal Na^+ uptake. Exposure of rainbow trout to Ag results in a similar reduction in J_{max}, predominately due to a potent inhibition of Na^+-K^+-ATPase activity (27, 28). The results of the present analyses of Pb/Na^+ interaction are in strong contrast to the competitive relationship between Pb and Ca^{2+}, which is manifested as a decrease in affinity of Ca^{2+} binding sites (increase in K_m) with little or no change in J_{max} (34).

Our conclusion that the interaction between Pb and Na^+ is mostly noncompetitive is supported by the data presented in Fig. 3. Despite elevated levels of Na^+, ~35-fold higher than those in the acclimation tap water, branchial Pb accumulation was completely unaffected by the presence of this potentially competing cation. This suggests that the initial entries of Na^+ and Pb across the apical gill-cell surface are independent of each other.

Of possible importance to future studies regarding the uptake of Pb at the gill and interaction with competing cations such as Na^+ or Ca^{2+} is the change in the slope of Pb uptake curves generated at a series of waterborne Na^+ concentrations (Fig. 3). At dissolved Pb concentrations close to 4.8 μM, we observed a major increase in gill Pb burden, which appears to be independent of waterborne Na^+ concentration. This may indicate the presence of two populations of receptors: high-affinity sites that are bound at lower waterborne Pb levels (≤2.4 μM) and low-affinity sites bound when waterborne Pb concentrations increase above this range.

The use of pharmacological agents in the characterization of metal-ion interactions has contributed to mechanistic studies investigating metal binding and toxicity (4, 5, 16, 34, 38, 39, 41). Phenamil, an analog of amiloride that specifically and irreversibly blocks apical Na^+ channels at the gill (12, 19), had no effect on branchial Pb accumulation despite causing a dose-dependent inhibition of Na^+ influx in synthetic water (Fig. 4). These observations suggest that unlike Ag (4) and Cu (13), whose entry into the gill is blocked in the presence of phenamil, Pb is not likely transported through apical Na^+ channels. This remains consistent with the above outlined results that suggest a noncompetitive disruption of Na^+ influx by Pb and, furthermore, is supported by the lack of effect of bafilomycin on gill Pb accumulation. Inhibition of the apical vacuolar-type ATPase associated with Na^+ transport reduced Na^+ influx but had no impact on Pb accumulation (Fig. 5), in agreement with data from the previous experiments. This finding again contrasts with results reported by Bury and Wood (4) and Grosell and Wood (13), who observed inhibition of Ag and Cu influx, respectively, in the presence of bafilomycin.

Time-course analysis of branchial Pb accumulation showed gill Pb burden to be both time dependent and concentration dependent with Pb binding increasing from background levels at 3, 8, and 24 h of exposure (Fig. 6*A*). Na^+-K^+-ATPase activity was impacted by all Pb exposure concentrations, differing only in the time to inhibition. This likely reflects the time required to accumulate a sufficient Pb burden to interfere with enzyme function. The highest exposure concentration (4.8 μM) was the first to reduce Na^+-K^+-ATPase activity (Fig. 6*B*). Inhibition of Na^+-K^+-ATPase activity was largely dependent on the time of Pb exposure. At 3 h, enzyme inhibition was weakly correlated with branchial Pb concentrations (Fig. 7*A*). The same held true after 8 h (Fig. 7*C*). This relationship strengthened, however, as prolonged Pb exposure (24 h) resulted in increased inhibition of enzyme activity that was highly correlated with branchial Pb accumulation (Fig. 7*E*). These observations could reflect the time needed for Pb entering the gill ionocytes to travel to the basolateral membrane, where it would interact with Na^+ transport enzymes. A similar relationship has been reported in Ag-exposed juvenile rainbow trout (3, 27).

Interestingly, enzyme inhibition occurred more rapidly than previously shown by Rogers et al. (33), who showed a reduction in activity by 24 h; however, measurements were not taken at 3 and 8 h. In addition, the present observed inhibition occurs at a faster rate than inhibition of high-affinity Ca^{2+}-ATPase reported by Rogers and Wood (34) for Pb and by Verbost et al. (39) for Cd. Previous work on the effect of Pb on red blood cell Na^+-K^+-ATPase suggests a strong binding of enzyme carboxyl groups by Pb (29). It is

possible that interaction at high-affinity Ca^{2+}-ATPase is fundamentally different.

The inhibition of Na^+ uptake during the time-course experiment agrees with previous data reported by Rogers et al. (33) showing that disruption in Na^+ influx rates occurred almost immediately (i.e., after only 2 h of exposure). Although the relationship between gill Pb accumulation and Na^+-K^+-ATPase activity was developed slowly, reduced Na^+ uptake at all time points (3, 8, and 24 h) was correlated with branchial Pb concentrations (Fig. 7, *B*, *D*, and *F*). Similarly to metals such as Ag and Cu, inhibition of Na^+-K^+-ATPase activity is likely the major contributing factor to the observed disruption in plasma Na^+ levels in Pb-exposed rainbow trout (33). However, given the slow developing nature of Na^+-K^+-ATPase inhibition, it is possible that there is another, more immediate, component to reduced Na^+ influx.

On the basis of data presented in Fig. 8, this more rapid mechanism is likely inhibition of branchial carbonic anhydrase activity by Pb. The action of this enzyme was impacted after only 3 h of exposure to two different lead concentrations (0.82 and 4.3 μM), with significant inhibition occurring through to 24 h of exposure. The result would be a decrease in the hydration and catalysis of intracellular CO_2, resulting in a shortage of H^+ and HCO_3^- required for active Na^+ uptake and Cl^-/HCO_3^- exchange (14). This would point to Cl^- disruption as the early determining factor in Pb-induced ionoregulatory disruption. Data presented in Fig. 1 would agree with this hypothesis, because Cl^- uptake appeared to be more severely impacted than did Na^+ influx in the presence of identical waterborne Pb concentrations. Similarly to the action of Pb, immediate inhibition of Na^+ uptake in Ag-exposed fish has been linked to early inhibition of cytosolic carbonic anhydrase by newly accumulated Ag (27). Interestingly, despite an approximate fivefold difference in Pb exposure concentration used, the degree of carbonic anhydrase inhibition was similar between the two Pb treatments, suggesting a low threshold of carbonic anhydrase sensitivity to Pb. Further research pertaining to Cl^- uptake kinetics, exchange with HCO_3^-, and the interaction of Pb with carbonic anhydrase is warranted.

Perspectives

Comparison of the data presented in this study with previous findings by Rogers et al. (33) and Rogers and Wood (34) shows that the threshold Pb concentrations eliciting effects on Ca^{2+} influx are lower than those that affect Na^+ and Cl^- uptake. This is especially evident when comparing ion fluxes and plasma concentrations of Ca^{2+}, Na^+, and Cl^- upon exposure to equal Pb concentrations, as in Rogers et al. (33). Ca^{2+} influx was inhibited by 65% upon exposure to 4.8 μM, whereas Na^+ and Cl^- fluxes were reduced by ~50 and 40%, respectively. Consequently, this was reflected in plasma ion concentrations in that plasma Ca^{2+} was reduced by 30% in fish exposed to 4.8 μM. This hypocalcemic effect of Pb was likely the major cause of mortality. Plasma Na^+ and Cl^-, however, were reduced by 18 and 15%, respectively, which is considered below the lethal threshold (28). Therefore, we conclude that the acute toxicity of Pb can be attributed primarily to effects on Ca^{2+} homeostasis and secondarily to impacts on Na^+ and Cl^- balance. Regarding Na^+ and Cl^- disruption, it is believed that contributing mechanisms likely occur exclusively at the gill. Recent evidence has shown that Na^+ and Cl^- regulation at the kidney is not impacted during prolonged exposure to waterborne Pb (Rogers JT, Patel M, and Wood CM, unpublished data). From measurement of ion excretions rates and plasma clearance rates in adult catheterized rainbow trout, it is apparent that the kidney retains function in compensating for the Pb-induced loss of Na^+ and Cl^- in the plasma. In contrast, the mechanisms of Ca^{2+} homeostasis at the kidney level are specifically impacted, supporting current evidence that suggests Pb-induced disruptions of Ca^{2+} and Na^+/Cl^- homeostasis occur by fundamentally different mechanisms. These findings are important in predictive modeling of waterborne Pb toxicity in fish (23).

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