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Characterization of Sodium, Potassium, ATPase Activity in the Gills of *Pimephales promelas* (Fathead Minnow): Influence of *In Vitro* Exposure to Lead

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The fathead minnow, *Pimephales promelas*, is a freshwater teleost useful for toxicological testing. This species is one of the test organisms employed in the assessment of acute and chronic toxicity to meet the objectives of the National Pollutant Discharge Elimination System (NPDES) permits program. While there are many practical advantages to conducting toxicity tests with the fathead minnow, relatively few studies of pollutant effects on gill ion transport have been conducted with this species. As its common name implies, the fathead minnow is a small fish; adults rarely attain a body wt greater than 5 g. Measuring low levels of transport ATPases in mg quantities of gill tissue requires a highly sensitive assay and the amount of gill tissue available can become a limitation. There is some information concerning the effects of the chlorinated hydrocarbon pesticide DDT on sodium, potassium-activated ATPase (Na^+K^+-ATPase, sodium pump) activity in fathead minnow gills. Desiah and coworkers (1975) measured the activity of gill Na^+K^+-ATPase using a 13,000 x g membrane fraction and reported an inhibitory effect of water-borne DDT after 225 days of exposure. The sensitivity of their assay method was such that it did not readily lend itself to measuring gill Na^+K^+-ATPase activity in fathead minnows younger than about 1 year of age.

The effects of water-borne lead (Pb) on gill osmoregulatory function is of particular interest considering the widespread distribution of this heavy metal in natural waters, and evidence (Preslan et al. 1993) suggesting that Pb may preferentially accumulate in gill tissue. Divalent inorganic lead (Pb^{++}), like other sulfhydryl binding agents, is known to inhibit numerous enzymes, and Pb^{++} could impair epithelial Na^+K^+-ATPase and ion transport through an interaction with a sulfhydryl close to the active site of the sodium pump. Information concerning the effect of heavy metals on Na^+K^+-ATPase activity in gills of the fathead minnow is lacking. The objectives of the present study were to develop a direct, sensitive assay for gill Na^+K^+-ATPase activity, and to characterize the activity of fathead minnows in terms of subcellular localization, pH profile and sensitivity to the reference inhibitor ouabain. In addition, we were interested in determining the effect of water-borne Pb on fathead gill Na^+K^+-ATPase using an in vitro approach that eliminated hemodynamic, endocrine and neural influences.

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MATERIALS AND METHODS

Fathead minnows were obtained from the USEPA (Region I, Lexington MA). The procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee. Adult animals (1.2 – 5.9 g) of either sex were used for all assays except for the study of pH dependence when females were used. Fish were maintained in a 125 gal aquarium (closed system) containing hard synthetic freshwater (USEPA Document 600/4-90/027F, 4th Edit. 1993), pH 7.8, with 1% Instant Ocean, under simulated natural photoperiod and constant temperature (25°C). The tank was equipped with biological filtration and NH_3 and NO_3 levels were checked periodically; water changes of 10-50% were made as needed. All animals were fed Tetramin^R flake food twice daily.

After euthanasia (decapitation), the gill arches on both sides were excised, weighed and placed in an 8 ml glass homogenizer. Tissues were homogenized in 5 volumes of ice-cold sucrose/EDTA/imidazole buffer (SEI buffer; 250 mM sucrose, 1 mM Na_2EDTA, 50 mM imidazole HCl; pH 7.4) containing 0.1% sodium deoxycholate. Homogenates were transferred into Eppendorf tubes and spun at 8,800 x g for 1 min to remove cartilagenous structures and cell debris. Supernatants were stored at –80°C until assay of Na^+K^+-ATPase activity or isolation of membrane fractions. A pool comprised of supernatants from 7 fathead minnows (2.4–5.9g, 3 males, 4 females) was subjected to differential centrifugation in order to obtain two membrane fractions. Initially, the pooled crude supernatant was centrifuged at 10,000 x g for 15 min (4°C) in a Beckman J-2M1 centrifuge. The resultant supernatant was then centrifuged at 100,000 x g for 60 min (4°C) in a Beckman ultracentifuge (Ti80 rotor). The pellets obtained at 10,000 x g and 100,000 x g ("microsomes") were suspended in 200 µl of SEI buffer and stored along with the 100,000 x g supernate (cytosol) at -80°C until assay. Freshly prepared 10,000 x g membrane fraction was analyzed by SDS-PAGE (12% acrylamide gel) and Western blot analysis using the chemiluminescence based ECL system (Amersham). Na^+K^+-ATPase (chicken kidney) α-subunit monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, made by Douglas M. Fambrough (Johns Hopkins University). Canine kidney Na^+K^+-ATPase was purchased from Sigma. Molecular weight markers (Sigma) were bovine albumin (66,000 Da), egg albumin (45,000 Da), carbonic anhydrase (29,000 Da) and trypsinogen, PMSF treated (24,000 Da).

The activity of gill Na^+K^+-ATPase was determined by measurement of the ATP hydrolytic activity in the presence of sodium and potassium using a spectrometric assay adapted from that described by Chen and coworkers (1956) as modified by Spokas and Spur (2001). The standard reaction mixture (0.25 ml) consisted of Tris-HCl buffer (50 mM final, pH 7.4), $MgCl_2$ (5 mM), KCl (20 mM), NaCl (60 mM), and Tris-ATP (4 mM). All reagents were from Sigma (SigmaUltra grade) and were prepared in 18 megaohms/cm deionized water (dH_2O). For determination of pH

optimum, the enzyme source was the pooled homogenate from 12 fathead minnows (females, 1.5 – 2.6 g). The pH was adjusted to values of 7.3, 7.4, 7.5, 7.7, 8.0, 8.3, 8.6, and 8.9 using 50 mM (final) Tris/Tris HCl buffer (Sigma, Preset crystals). Canine kidney Na^+K^+-ATPase (EC 3.6.1.3, Grade IV, Sigma, St. Louis, MO) was routinely used for quality control (QC). The QC tube contained 0.008 units (6 μg) of the lyophilized enzyme; the reaction was prepared as above except that the concentration of NaCl was 100 mM. The reaction was carried out at 37°C in a water bath shaker at slow speed. Prior to addition of Tris-ATP substrate, each assay tube was preincubated at 37°C for 30 min. In some assays, 1 mM ouabain was added in dH_2O at the start of the preincubation period.

Nanomoles of inorganic phosphate (Pi)/tube were determined by linear regression analysis. Included in each assay were substrate blanks (complete mixture except for NaCl, KCl, and enzyme) and basal activity blanks (complete reaction mixture except for NaCl and KCl). Activity stimulated by Na^+ and K^+ was calculated by subtraction of both blank and basal values. Standard curve tubes, samples, QC tubes and blanks were run in duplicate. The reaction was terminated at selected times (10, 20 or 30 min) by transferring an aliquot (200 μl) of the reaction mixture to a tube containing 1.4 ml of 10% SDS in 0.1 M sodium acetate buffer (pH 4.0). After adding a premixed solution (400 μl total) of 1% ammonium molybdate and 10% ascorbic acid to each tube, the tubes were vortexed and allowed to stand at room temperature for at least 30 min before reading in an HP 8452A diode array spectrophotometer with ChemStation software. Optical absorbance was measured at 820 nm and compared to a series of tubes containing known concentrations of Pi (10 to 400 nmoles/tube).

The following methodology was used to evaluate the effect of lead nitrate on gill sodium pump activity in vitro. Fathead minnows (1.2 – 5.8g, n=32, 23 female, 9 male) were decapitated and gill arches were rapidly removed, weighed, and transferred to plastic flasks (Nalge) containing 10 ml of modified Cortland's saline (pH 7.8 at 22°C after bubbling with 95% O_2/5% CO_2). An aliquot (100 μL) from a stock solution of lead nitrate (either 0.12 or 1.2 mM; total of 2.5 or 25 μg lead, respectively) in dH_2O was pipeted into the experimental flasks. Separate incubations were carried out for each concentration of Pb tested (final metal concentration in experimental flasks = 0.25 and 2.5 ppm, respectively). Coincubated control flasks received 100 μl of dH_2O. At each dose level, gill tissue from 5 fish were exposed to Pb, and incubated along with control gill samples (n=5 fish). Addition of lead nitrate 12 μM (final concentration) caused no detectable change in the pH of the incubation medium. Two control and experimental tissues from each incubation were not assayed for Na^+K^+-ATPase activity but were analyzed for tissue Pb. The isolated gill preparations were incubated for 60 min at 20°C under an atmosphere of 95% O_2/5% CO_2. After the incubation, the tissue was removed from the medium with small forceps, rinsed in dH_20 and frozen (-80°C) until analysis. For measurement of tissue lead, samples were digested in 3:1 nitric/perchloric acid and analyzed by flameless atomic absorption spectrophotometry using a Perkin Elmer Z5100.

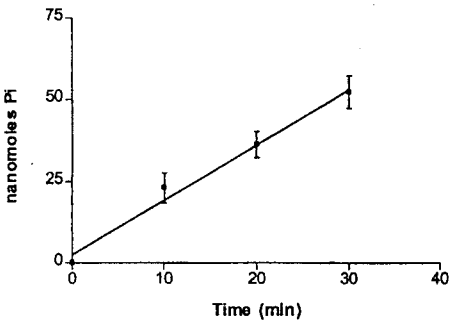

Figure 1. Kinetic assay of Na^+K^+-ATPase activity in fathead minnow gills. Means $\pm$ standard errors are given for assays using homogenates from 3 fish.

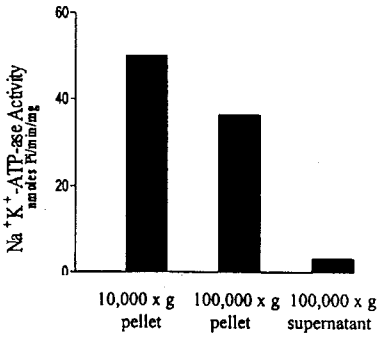

Figure 2. Na+K+-ATPase activity in subcellular fractions isolated from gill homogenates pooled from 7 fathead minnows.

RESULTS AND DISCUSSION

The conditions for measurement of Na^+K^+-ATPase activity in gill homogenates were optimized in several initial experiments, using various cation concentrations (triplicate assays of 4 different combinations). Greatest activity was found using 60 mM NaCl and 20 mM KCl (mean activities for combinations of 75 mM NaCl, 15 mM KCl; 100 mM NaCl, 20 mM KCl; 50 mM NaCl, 10 mM KCl; and 60 mM NaCl, 20 mM KCl were 0.02, 0.033, 0.036, and 0.042 nmol Pi/min/mg wet wt, respectively). In all subsequent assays, stimulated activity was measured using 60 mM NaCl and 20 mM KCl.

The mean rate of Na^+- and K^+- stimulated ATP hydrolysis, as determined in three kinetic assays each conducted using gill homogenate from a single fish, is shown in **Figure 1**. Linear reaction kinetics were observed under the standard conditions (60 mM NaCl, 20 mM KCl, 4 mM ATP) for at least 30 min. At 30 min, Na^+ and K^+-dependent Pi generation by the fathead gill homogenates averaged 52 $\pm$ 5 nmol. Thirty min was routinely used as the incubation time for the remainder of the study. Inhibition of the crude gill Na^+K^+-ATPase by ouabain, 1 mM, was incomplete, averaging 67 $\pm$5% (n=3). By contrast, the catalytic rate with the dog kidney Na^+K^+-ATPase was about four fold higher (221 mean, 123 - 327 nmoles at 30 min, n=13) and was inhibited 93 $\pm$ 2% (n=3) by ouabain, 30 μM.

When the gill homogenate was subjected to differential centrifugation, both membrane fractions contained substantial Na^+K^+-ATPase activity **(Fig. 2)**. However, the specific activity of the 10,000 x g pellet was almost 40% greater than the microsomal fraction. Less than 5% of the activity was found in the cytosol.

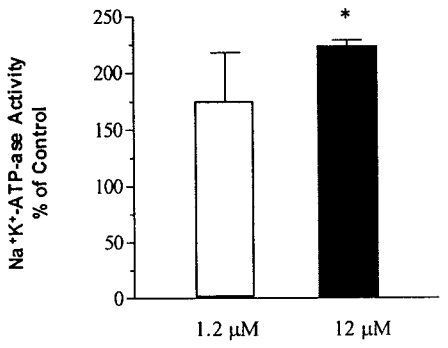

Figure 3. Effect of in vitro exposure to lead nitrate on gill Na^+K^+-ATPase activity of fathead minnow (n=3 fish per dose). *p.<.05, relative to co-incubated controls.

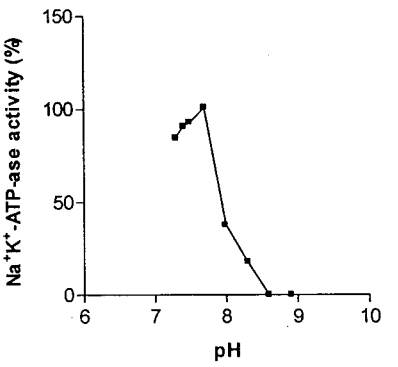

Figure 4. Activity of Na^+K^+-ATPase in fathead minnow gills as a function of pH. Results are expressed as % of maximum.

A concentration-dependent increase of gill Na^+K^+-ATPase activity was observed **(Fig. 3)** after in vitro exposure of whole gill preparations to concentrations of lead nitrate of 1.2 and 12 μM. At 1.2 μM, the activity of the Na^+K^+-ATPase tended to increase (average change from control = +75%) although this change was not statistically significant. The mean specific activity of gills exposed to the higher dose was significantly elevated ($p<0.05$) relative to coincubated controls. In order to control for the possible influence of the nitrate ion, the effect of sodium nitrate was evaluated in experiments identical to those described. Sodium nitrate at a final concentration of 40 μM did not significantly affect gill Na^+K^+-ATPase activity (mean specific activity after 60 min of incubation with sodium nitrate = 46.8 ± 9.4 nmoles Pi/min/mg protein, n=4; mean specific activity of coincubated dH_20 controls = 30.3 ± 10.7 nmoles Pi/min/mg protein, n=4; average change = +54%, $p>.05$). Tissue lead concentrations of gills exposed to 1.2 μM and 12 μM lead nitrate (2 fish per dose level), averaged 2.7 and 13.6 μg/g wet weight, respectively. The mean lead concentration of coincubated control gills (n=4) was 0.7 ± 0.4 μg/g wet weight.

Figure 4 illustrates the influence of pH on the Na^+K^+-ATPase activity of pooled gill homogenates. At pH 8.6 and above, no detectable Na^+K^+-ATPase activity was observed. The catalytic activity was maximal at a pH of 7.7, and fell only slightly (≤15%) from this peak over the range of 7.5 to 7.3. These results indicate that the pH dependence of the fathead minnow enzyme is similar to the mammalian renal Na^+K^+-ATPase (Jorgensen 1986; Park 1983) and to coho salmon gill Na^+K^+-ATPase as determined using homogenates (McCormick and Bern 1989).

Western blot analysis was performed on freshly isolated 10,000 x g membrane

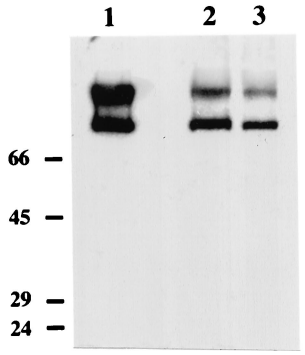

Figure 5. Western analysis of Na+K+-ATPase. Total protein in a membrane preparation (10,000 x g fraction) from fathead minnow gill and purified canine kidney Na^+K^+-ATPase were separated on a 12% SDS-polyacrylamide gel and analyzed by Western blotting using monoclonal antisera specific for the α-subunit. *Lane 1*, canine kidney Na^+K^+-ATPase (20 μg); *lane 2* (100 μg) and *lane 3* (50 μg) minnow membrane fraction. *Left margin*, molecular weight markers.

fraction pooled from five fathead minnow gills to determine if the Na^+K^+-ATPase α-subunit could be identified using antisera specific for chicken kidney α-subunit **(Fig. 5)**. The results show the monoclonal antibody detected a protein band of approximately 100 kDa in both minnow (lanes 2 and 3) and control samples (lane 1, Na^+K^+-ATPase purified from canine kidney). The mammalian kidney α-subunit has been previously estimated at 100 kDa (Jorgensen 1986). The lower band present in all samples (approximately 80 kDa) is most likely a degradation product. Identification of the α-subunit in fathead minnow gills using antisera directed against the relatively distant avian α-subunit suggests the enzyme is highly conserved.

The present report shows that the ascorbic acid method (Chen et al. 1956, Spokas and Spur 2001) for micro-determination of Pi is a sensitive technique for measuring Na^+K^+-ATPase activity in the gills of small teleost fish. Measurements can be made with short incubation times using 50 mg wet wt of gill tissue (filaments and arches). In our study population, this corresponds to a body wt of about 1.5 – 2.5 g. For many studies (e.g., Desaiah et al. 1975; McCormick 1993; Marshall et al. 1999) of teleost gill Na^+K^+-ATPase activity, investigators have used a kinetic assay in which the rate of NADH disappearance is monitored using a spectrophotometer or microplate reader. Compared to techniques that monitor NADH oxidation, the ascorbic acid method for assay of liberated Pi involves relatively few pipetting steps, does not require addition of multiple substrates and enzymes, and can be performed using a simple spectrometer without kinetics software.

The NADH oxidation assay technique was employed by Desaiah et al. (1975) who measured gill Na^+K^+-ATPase activity using a 13,000 x g membrane fraction isolated from relatively large (approx. 1 year of age) fathead minnows. A microplate version of the assay was used by Marshall and coworkers (1999) to assess changes in gill Na^+K^+-ATPase activity during salinity adaptation studies with *Fundulus heteroclitus*. In the latter study, unfractionated gill homogenate was also used as the enzyme source. Some progress was made in the present study towards defining the membrane fraction which was enriched in Na^+K^+-ATPase activity; i.e., the 10,000 x g pellet was relatively enriched in the catalytic activity.

Under our reaction conditions, Na^+K^+-ATPase activity, taken as the difference

between total and basal Mg^{++}-ATPase activities, was incompletely inhibited (67%) by 1 mM ouabain. The reason for the inability of ouabain to completely inhibit ATP-hydrolysis stimulated by Na^+ and K^+ is uncertain. Under very similar reaction conditions, canine kidney Na^+K^+-ATPase was inhibited 93 $\pm$ 2 % by 30 μM ouabain (IC_{50} = 1.7 μM, n=3 assays). A ouabain-insensitive sodium-stimulated Mg^{++}-ATPase with an acidic pH optimum has been described (Ventrella et al. 1990) in the gills of the gilthead bream *(Sparus auratus)*. Evidence has also been obtained for the existence of a putative ouabain-insensitive Na^+ or H^+-ATPase in the medial renal tubules of the freshwater shrimp, *Macrobrachium olfersii* (McNamara and Torres 1999). A modest contribution of such "monovalent cation ATPases" to our measured catalytic activity cannot be excluded.

Sodium pump activity more than doubled when isolated fathead gills were incubated with 12 μM lead nitrate (2.5 ppm Pb). The observed stimulatory effect of Pb on gill Na^+K^+-ATPase activity was unexpected and seems to contrast with the results of other studies. Significant reductions of gill Na^+K^+-ATPase were observed (Ay et al. 1999) when *Tilapia zillii* were exposed for 14 days to lead nitrate in the aquarium water at Pb concentrations of 1 to 4 ppm. Sola and coworkers (1994) examined the effect of Pb on gill Na^+K^+-ATPase activity in rainbow trout and reported that a concentration of 1 ppm caused 50% mortality after 11 days without appreciable effects on the activity of gill Na^+K^+-ATPase.

Extrapolation of our in vitro findings with Pb to the intact animal is difficult for various reasons such as lack of information on the distribution of Pb within the gill and the cell types affected. In the in vitro model, Pb may penetrate the epithelium and accumulate in a time-dependent fashion. The importance of exposure duration in this context is evident from an in vivo study by Thaker et al. (1996). These investigators evaluated the effect of chromium (VI) on gill Na^+K^+-ATPase activity of *Periophthalmus dipes*, and concluded that exposure duration was even more important than dose as regards inhibition of the enzyme. We incubated the isolated gills with Pb for a short period (60 min), relative to the duration of studies where intact fish have been exposed to Pb. Gill uptake of Pb in vivo from aquarium water, by movement across gill epithelium as opposed to delivery via blood channels, probably depends on the condition of the mucus layer and intercellular junctions, and the continuity of the thin epithelial covering of the secondary lamellae. In the isolated gill preparations, where some loss of epithelial barrier function would be expected, Pb might penetrate to cellular sites from which it is normally excluded.

Studies of the mammalian enzyme indicate that the direct action of Pb is to inhibit membrane Na^+K^+-ATPase activity. In a study of industrially exposed Pb workers (Raghaven et al. 1981), a significant inverse correlation was found between human erythrocyte membrane Na^+K^+-ATPase activity and membrane Pb content. Subsequently, Kramer et al (1986) reported that lead chloride inhibited Na^+K^+-ATPase activity in homogenates of rat renal cortex, with an IC_{50} of 70 μM. Under

our reaction conditions, incubation of purified canine renal Na^+K^+-ATPase with 12 μM lead nitrate resulted in essentially complete inhibition of Na^+K^+-stimulated activity (n=2 assays, 96% and 100% inhibition, respectively). We speculate that the stimulation seen in our experiments with isolated gills may possibly reflect generation of lipid mediators or changes in Ca^{++} homeostasis. Certain eicosanoids as well as long chain fatty acyl CoA derivatives can stimulate the purified enzyme (Ewart and Klip 1995). Alternatively, the stimulation could have resulted from protein kinase C (PKC) activation. The latter possibility is suggested by the ability of picomolar concentrations of Pb to activate PKC (Markovac and Goldstein 1988) and evidence that PKC can enhance sodium pump activity in some tissues (Ewart and Klip 1995). Further studies are needed to determine which, if any, of these mechanisms accounts for the in vitro effect of Pb on gill Na^+K^+-ATPase activity. Definitive studies of the subcellular localization of Pb in the gill, and the role of rapidly-acting local factors and kinases in the regulation of the sodium pump, are likely to clarify the mechanism.

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