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Effects of Elevated Calcium Concentration on Na-K-ATPase Activity of Two Euryhaline Species, *Cyprinodon variegatus* and *Mysidopsis bahia*

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The U.S. Environmental Protection Agency (EPA) and/or state regulatory agencies are requiring biomonitoring of industrial and municipal wastewater effluents as part of the National Pollutant Discharge Elimination System (NPDES) and Water Quality Based Toxics Control. Understanding the environmental tolerances of toxicity test species required for biomonitoring is important to assure that toxic substances are being acurately detected and anomalous responses are recognized. Effluent evaluations have revealed that common chemical constituents of effluents frequently considered non-toxic such as potassium, sodium and calcium may become toxic to test species at elevated concentrations.

Recently, a petroleum effluent with a salinity of 15 o/oo was tested using recommended marine species (Mysidopsis bahia [Mysid shrimp] and Cyprinodon variegatus [Sheepshead minnow]). Evaluation of the effluent with these two test species produced conflicting results (M. bahia 48-hr LC50 = 7.5% effluent dilution, C. variegatus 96-hr LC50 = 73.0% effluent dilution). Further examination of the effluent revealed a calcium concentration 12.5 times above ambient marine water concentration of 400 mg Ca/L at 32 o/oo salinity. This observation prompted our investigation on the effects of high calcium concentrations on the cellular physiology of these two test species.

A majority of the work on calcium-membrane interaction has been conducted with isolated plasma membranes. These studies have shown that calcium may (1) alter membrane surface charge; (2) lower bilayer fluidity; and (3) produce a linkage of acidic lipids to integral proteins

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(Gordon and Sauerbeber 1982). Anyone of these may alter the function of membrane bound enzymes.

Na,K-adenosine triphosphatase (Na-K-ATPase) is a transmembrane protein which has been used to study sublethal effects of toxic compounds (Shephard and Simkiss, 1978; Stagg and Shuttleworth, 1982). This protein is responsible for maintaining the Na^+ and K^+ concentrations within a cell. Maintaining the proper Na^+ and K^+ gradient is required for uptake by cells of metabolites such as glucose, amino acids, and regeneration of transmembrane potential (Haya and Waiwood, 1983). Results reported in the literature are variable regarding the effects of calcium on Na-K-ATPase activity. Benedetti and Emmelot (1968) observed a decrease in enzyme activity with all membrane fractions from rat liver (calcium 200 mg/L) while Bouting et al. (1961) observed a stimulation in enzyme activity with adult cat gray matter and human retina tissue (calcium 40 mg/L).

The objective of this study was to determine the effect of excessive calcium concentrations on the Na-K-ATPase activity of two routinely used marine test species, C. variegatus and M. bahia. The hypothesis to be tested was: There is no difference in the Na-K-ATPase activity of C. variegatus and M. bahia when exposed to elevated calcium concentrations.

MATERIALS AND METHODS

Test animals were obtained from commercial sources. All test organisms were acclimated to laboratory conditions for one week prior to conducting tests.

Two experiments were completed on each species: (1) a time-dependent study at one aqueous calcium concentration, and (2) a concentration study observing responses to various aqueous calcium concentrations for a specified time period. Calcium chloride was used as the test chemical. Calcium concentrations at the beginning of each experiment were determined using a Perkin Elmer, model 2380, Atomic Absorbtion Spectrophotometer according to Standard Methods (1985). Selection of calcium concentrations for the time-dependent studies was based on actual values observed in an industrial effluent and the resulting toxicity of the effluent to C. variegatus and M. bahia. The time-dependent study, starting with 20 o/oo saltwater, was used to determine the exposure time most affecting the Na-K-ATPase activity at one calcuim exposure concentration.

Prior to definitive testing, Na-K-ATPase activity studies were conducted to establish the quantity of tissue (C. variegatus - gill; M. bahia - entire organism) needed to produce measurable enzyme activity. Initial assays with juvenile organisms were unsatisfactory but adult organisms provided sufficient tissue to produce measureable activity. Although some differences in sensitivity may exist between juvenile and adult organisms, it was assumed that the direction of their response to increasing calcium concentration is similar.

Homogenates containing Na-K-ATPase were prepared from the gills of Cyprinodon variegatus and whole organisms for M. bahia. Each tissue sample was weighed before homogenization. Tissues were then homogenized with a Teflon-glass homogenizer using Tris-EDTA buffer pH 7.4 (sucrose 250 mM, Tris-HCl 20mM, ethylenediaminetetraacetic acid (EDTA) 2mM and 1mM dithiothreitol). Homogenates were centrifuged at 2,000 x *g* for 15 minutes to remove cellular debris. The supernate was removed and centrifuged at 10,000 x *g* for 15 minutes to obtain a fraction with high Na-K-ATPase activity. The resulting pellet was resuspended in Tris-EDTA buffer at a volume five times the initial wet weight of each sample. Protein concentrations were determined according to Bradford (1976). Na-K-ATPase activity was measured spectrophotometrically by monitoring the oxidation of NADH at 360 nm (25 C) in the coupled enzyme assay system of Swartz et al. (1969). Optimal enzyme activity was established. Each cuvette contained (in final concentration) 5mM $MgCl_2$, 100mM NaCl, 10mM KCl, 25mM Tris-Cl pH 7.5, 2.5mM Tris-ATP, 5mM NADH, 2.5 mM phosphoenolpyruvic acid (PEP) and 0.02 ml of a combined pyruvate kinase (PK, 14 units) - lactic dehydrogenase (LDH, 20 units) suspension in a final volume of 0.2ml. All additions to the cuvette were in excess except for the ATPase preparation. The assay was determined to be linear with respect to concentration and time.

Measured time intervals for the time-dependent study varied with species tested and results of previous time period studies. Tests using C. variegatus utilized three fish for each time period and calcium concentration. Due to the small size of M. bahia, each crude homogenate consisted of ten adults which were exposed in the same test chamber. There were three replicate test chambers for each time period and aqueous calcium concentration. Calcium concentrations in the time-dependent study were 5100 mg Ca/L and 990 mg Ca/L for C. variegatus and M. bahia, respectively. In the second study, calcium concentrations ranged from 352 - 5100 mg Ca/L for C. variegatus and from 317 - 1175 mg Ca/L for M. bahia.

All statistical comparisons were conducted utilizing the nonparametric Kruskal-Wallis Test.

RESULTS AND DISCUSSION

At a constant calcium exposure concentration of 5100 mg Ca/L, the teleost C. variegatus showed a gradual increase in enzyme activity (2.7-12 μmole P_i/mg protein/hr over 10 days, Fig. 1). Evaluating test calcium concentrations, there was a significant difference in enzyme activity ($p = 0.0194$) observed on day ten as compared to other test concentrations. Ten days was selected as the exposure period for enzyme activity measurements in the time-dependent study at various calcium concentrations. A statistically significant increase in enzyme activity (4.0-12.0 μmole P_i/mg protein/hr, $p = 0.0160$) was again observed with this species as exposure time was held constant and calcium concentrations increased (352 to 5100 mg Ca/L, Fig.1).

Osmotic response in the mysid, Mysid stenolepis occurs rapidly when placed in either hypo or hyperosmotic environments (Dormaar and Corey 1973). We observed a statistically significant decrease in whole-animal enzyme activity from one to six hours at a calcium concentration of 990 mg Ca/L, ($p = 0.0300$, Fig. 2), after which a stability in enzyme activity was noted for a period of 48 hours. Based on the time-dependent response, 12 hours of exposure was selected as the period of maximum activity. Increasing calcium concentrations (317-1175 mg Ca/L) for a period of 12 hours also produced a decrease in enzyme activity (4.25-1.5 μmole P_i/mg protein/hr). A statistically significant decrease in activity was observed at 940 mg and 1175 mg Ca/L ($p = 0.0200$, Fig. 2).

In these studies, gill Na-K-ATPase activity was increased in the vertebrate C. variegatus whereas whole-animal enzyme activity decreased in the invertebrate M. bahia after calcium exposure. Similar differences have been observed in vertebrate and invertebrate species when exposed to a relatively large increase in salinity (proportional increase in all ions comprising saltwater). Karnaky (1976) noted that a large increase in gill Na-K-ATPase activity accompanied adaption of C. variegatus to high salinity; enzyme activity increased 1.6 times with adaption from 50% to 100% sea water and increased 3.9 times from 100% to 200% sea water. In contrast, D'Orazio and Holliday (1985) who worked with the crustacean Uca pugilator (fiddler crab), observed a decrease in Na-K-ATPase activity in animals exposed to 150% and 200% sea water conditions. In addition, Winkler (1986), working with Na-K-ATPase in Carcinus maenas (shore crab) also reported a decrease in gill enzyme activity with increasing calcium ion concentrations.

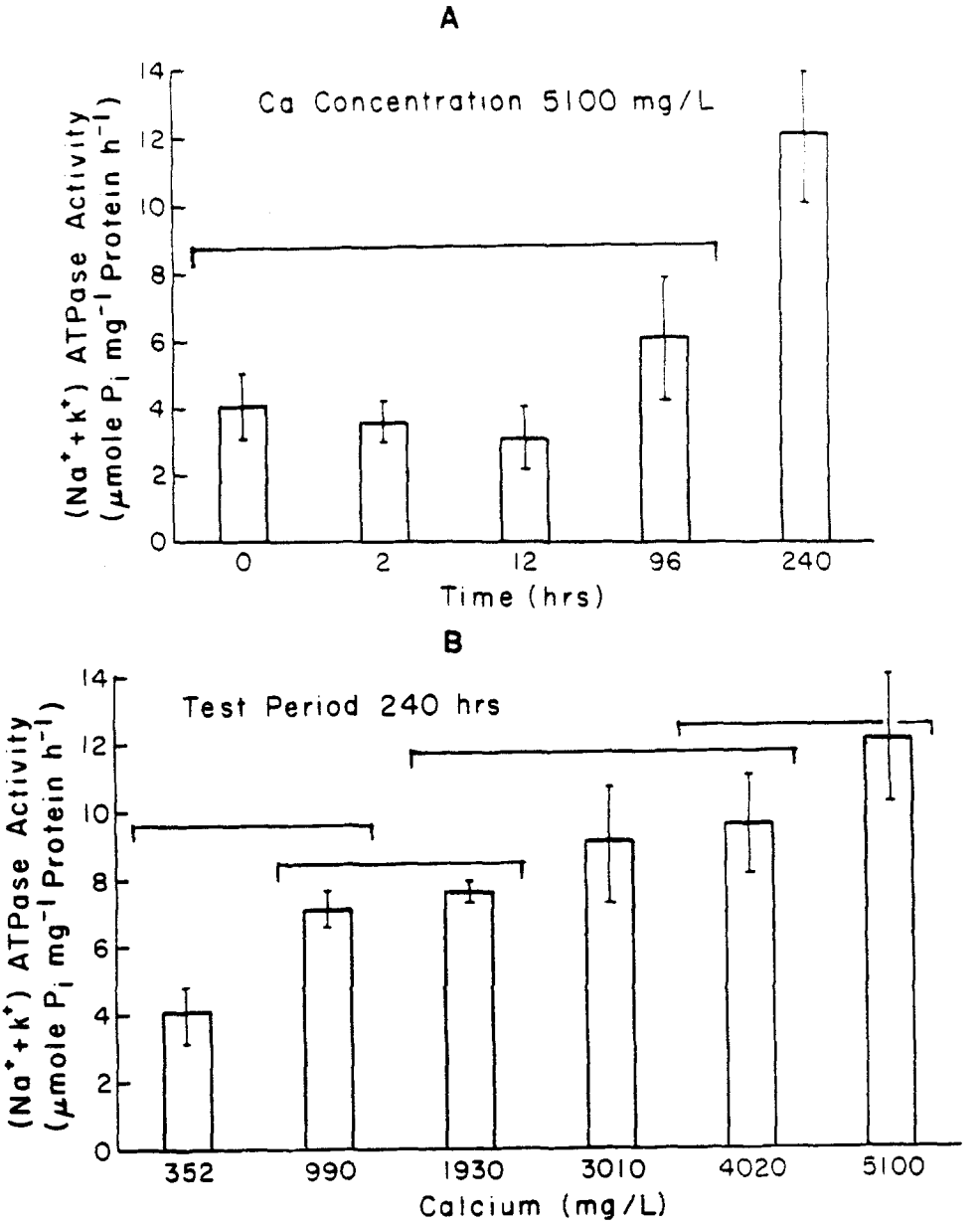

Figure 1. Na-K-ATPase activity in homogenates of C. Variegatus gills. (A) Activity at a constant calcium concentration (5100 mg Ca/L) for various time intervals. (B) Activity for various calcium concentrations after 240 hr exposure. All concentrations $\bar{x} \pm$ SD. Activity measurements not statistically different ($p \leq 0.05$) are enclosed by brackets.

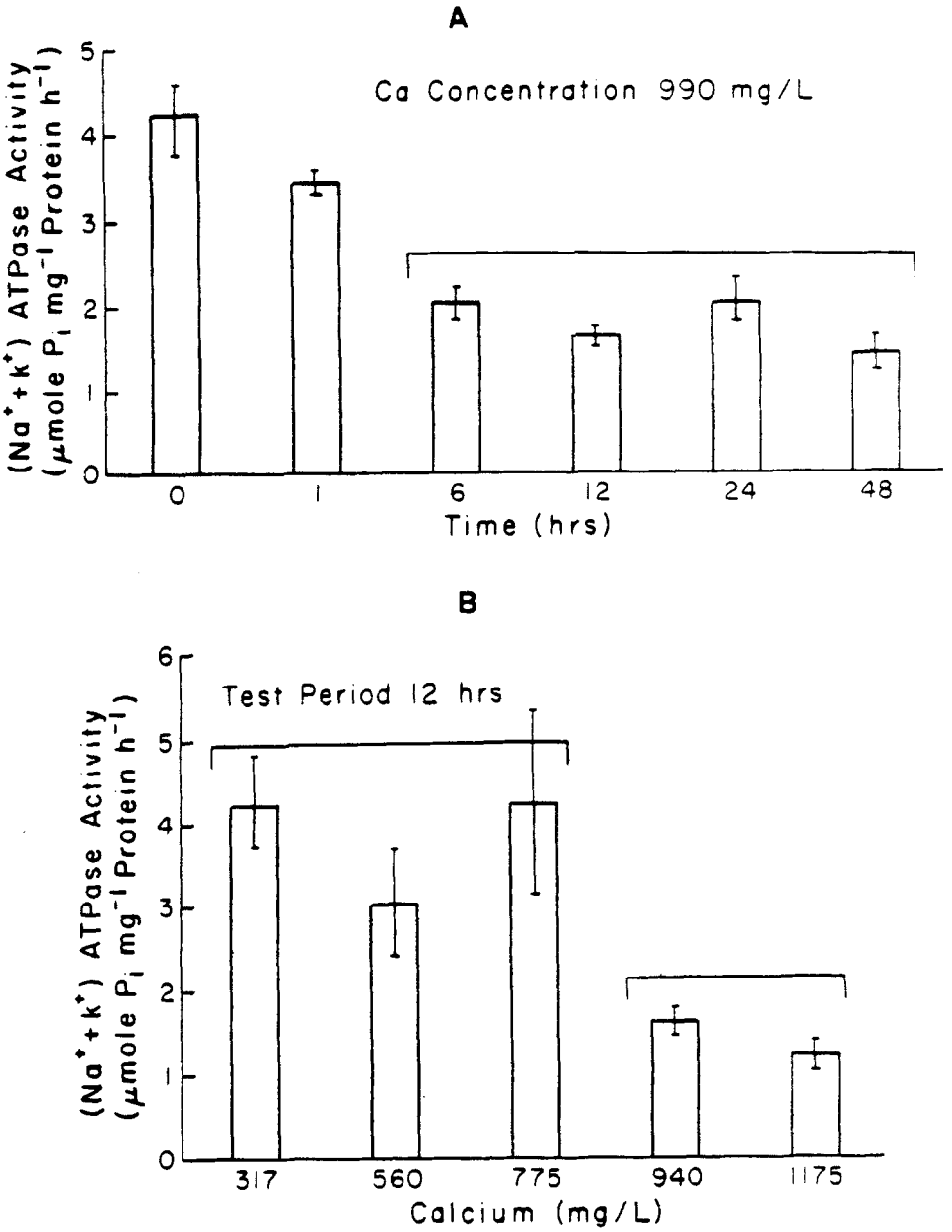

Figure 2. Na-K-ATPase activity in homogenates of M. bahia. (A) Activity at a constant calcium concentration (990 mg Ca/L) for various time intervals. (B) Activity for various calcium concentrations after 12 hr exposure. All concentrations $\bar{x} \pm$ SD. Activity measurements not statistically different ($p \leq 0.05$) are enclosed by brackets.

Calcium is a component of saltwater and therefore contributes to the measured salinity. The interaction of salinity and calcium ion concentration on an organism's response to a changing environment is not clearly defined. These studies have shown that C. variegatus and M. bahia had opposing responses to increases in calcium, an increase and decrease being observed in Na-K-ATPase enzyme activity, respectively. Acute calcium toxicity tests, however, have demonstrated that calcium concentration plays a significant role in an organisms ability to survive test conditions. Further studies are needed to illuminate the interaction of calcium concentration, salinity, and different responses of different phyla to this changing environment, as might occur in dilutions under toxicity testing conditions. Salinity is a multi-component parameter and may not be sufficiently specific to use as the sole parameter in selection of appropriate test species for toxicity testing.

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