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In vitro exposure of heavy metals on nucleotidase and cholinesterase activities from the digestive gland of *Helix aspersa*

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Abstract

Zinc, copper and cadmium are important environmental contaminants and differences in purinergic and cholinergic systems of invertebrates have been described when compared to characteristics of these signaling systems in vertebrates. Here we evaluate the effect in vitro of these metals on the ATPase, 5′-nucleotidase and cholinesterase (ChE) activities in the digestive gland of *Helix aspersa*. Zinc (500 and 1000 μM) promoted a significant decrease in 5′-nucleotidase activity. However, it did not induce changes in ATP hydrolysis. Copper (25 and 50 μM), inhibited significantly ATPase activity, but did not alter 5′-nucleotidase when compared to control (no metal added). In relation to effects of cadmium, an inhibitory effect on ATP hydrolysis has been observed at concentrations of 100, 500 and 1000 μM and a similar decrease of AMP hydrolysis was observed at 500 and 1000 μM. However, there were no significant changes in ChE activity from homogenates of the digestive gland of *H. aspersa* for all metals tested. This study demonstrated that zinc, cadmium and copper affect ATPase and 5′-nucleotidase in digestive gland, but not ChE, suggesting that the purinergic system may be a target related to toxicity induced by these metals and a possible indicator of biological impact of exposure to these contaminants.

Keywords: Mollusks; *Helix aspersa*; Nucleotidases; Digestive gland; Heavy metals; Cholinesterase

1. Introduction

Metals occur naturally in the environment, but since the industrial revolution the distribution and availability of metals to biological systems have increased significantly (Hopkin, 1989). There are many anthropogenic sources of metal pollution, such as mining activities, traffic, smelting, combustion of fossil fuels, and certain agricultural activities (Soon, 1981; Hopkin, 1989; Gummow et al., 1991).

Due to their high potential for accumulation of pollutants (Coughtrey et al., 1979; Hopkin, 1989; Jones, 1991), snails and slugs may provide important links in transfer of chemicals from vegetation or plant litter to carnivores. Such transfer along food chains is an important aspect of ecotoxicology. They are able to accumulate bioavailable metals in their organs and they present an important organotropism for the digestive gland and the kidney (Berger and Dallinger, 1993; Pihan, 2001). It is well known that heavy metals are accumulated to very high concentrations in, especially, the digestive gland of mollusks, but the concentration of copper in foot and digestive gland are similar (Hamza-Chaffai et al., 1998; Marigomez et al., 1998; Blasco and Puppo, 1999). The effects of such accumulated heavy metals and other pollutants on the molluscan digestive gland cell structure and the possible use of such cellular changes as biomarkers of exposure to xenobiotics have been investigated (Marigomez et al., 1998; Etxeberria et al., 1994).

Van Straalen et al. (1987) suggested that the main differences in the ecophysiology of metals are due to their essentiality versus non-essentiality to organisms. Nutritional metals, such as zinc and copper, are regulated and xenobiotics, as cadmium, are accumulated. However, zinc, copper and cadmium can be potentially toxic to organisms if they occur at high concentrations (Harris, 1991; Beyer and Storm, 1995).

Extracellular ATP has been established as a signaling molecule, which mediates its actions through two subclasses of P2-purinoceptors: metabotropic P2Y receptors and ionotropic P2X

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receptors (Bodin and Burnstock, 2001; Khakh et al., 2001). Acetylcholine and ATP are co-released, where ATP acts as a co-transmitter or modulator of cholinergic transmission. The administration of AMP, ADP and ATP and acetylcholine in the snail *Helix aspersa* produced concentration-dependent contractions in the rectum and esophagus, suggesting that purinoceptors and cholinergic receptors are important for these responses in mollusks (Knight et al., 1992). Hoyle and Greenberg (1988) analyzed species belonging to several different invertebrate phyla and observed that the effects of the agonists to purinoceptors were extraordinarily varied, when compared to effects observed in vertebrates.

Signaling actions of nucleotides and acetylcholine require effective mechanisms for inactivation (Zimmermann, 1996, 2001). The inactivation of extracellular ATP to AMP is mediated mainly by a family of ectonucleotidases named NTPDases (nucleoside triphosphate diphosphohydrolase), that are ubiquitous enzymes with a broad phylogenetic distribution (Zimmermann, 1996). This family of enzymes consists of eight members that includes NTPDase 1 (ATP diphosphohydrolase, EC 3.6.1.5) and NTPDase 2 (ecto-ATPase, EC 3.6.1.3). The nucleotide AMP is hydrolyzed to adenosine by the action of a 5′-nucleotidase (CD73, EC 3.6.1.5). Recently, Borges et al. (2004) have demonstrated an ATPase activity in nervous ganglia and digestive gland of *H. aspersa*. Despite some differences related to pH optima, substrate specificity and K_M and V_{max} values, the presence of this nucleotide-metabolizing enzyme in mollusk tissues may play a similar role when compared to vertebrate NTPDases, contributing to the modulation of nucleotide and nucleoside levels and controlling their actions on specific purinoceptors in these species. Acetylcholine effects can be inactivated by the action of an acetylcholinesterase activity (Patocka et al., 2004; Aldunate et al., 2004). However, some authors have shown difficulty in classifying cholinesterase from invertebrates, since these enzymes have apparent affinity for any choline ester, suggesting that they should be classified generally as cholinesterases (ChE) (Bocquené et al., 1997; Mora et al., 1999).

Considering the interaction between the purinergic and cholinergic systems in invertebrate tissues and that the molluscan digestive gland accumulates metals and performs multiple functions in the physiology of the animal, here we evaluate the effect in vitro of zinc chloride, copper sulfate and cadmium acetate on the ATPase, 5′-nucleotidase and ChE activities in the digestive gland of *H. aspersa*.

2. Material and methods

2.1. Experimental model

Adult *H. aspersa* snails were collected all year long from gardens of metropolitan region of Porto Alegre, RS, Brazil. All snails used in the experiments were adults and weighed approximately 6±1.5 g. Animals were maintained in plastic boxes (68×60×22 cm) at 25±5 °C, in a photoperiod of 12 h light/12 h dark for at least 7 days. Snails were fed ad libitum with lettuce (*Latuca sativa*).

2.2. Chemicals

ATP and Trizma base were purchased from Sigma-Aldrich (St. Louis, MO, USA). The kit for ChE activity was obtained from Wiener Lab. The salts metal cadmium acetate [$Cd(CH_3COO)_2$; CAS number 5743-04-4], zinc chloride ($ZnCl_2$, CAS number 7646-85-7) and copper sulfate ($CuSO_4$, CAS number 7758-98-7) were purchased from Merck. All the other reagents were of the highest purity available.

2.3. Membrane preparation of the digestive gland

The animals were cryoanesthetized, the shells were removed and the digestive glands were isolated. The membrane preparations were made according to Barnes et al. (1993). Briefly, the digestive gland was homogenized in 5 volumes (w/v) in a solution of NaCl (0.65%) containing a protease inhibitor (0.1 mM PMSF). The homogenate was centrifuged at 1000 ×*g* for 10 min, the pellet discarded, and the supernatant centrifuged for 20 min at 40,000 ×*g*. The pellet was frozen in liquid nitrogen for 10 s, thawed, resuspended twice and centrifuged for 20 min at 40,000 ×*g*. The membrane was prepared fresh daily and maintained at 4 °C throughout the preparation and experiment.

2.4. Enzyme assays

Enzyme activity was assayed in standard reaction medium containing 50 mM Tris–HCl, pH 7.2, 5 mM $CaCl_2$ or $MgCl_2$ in

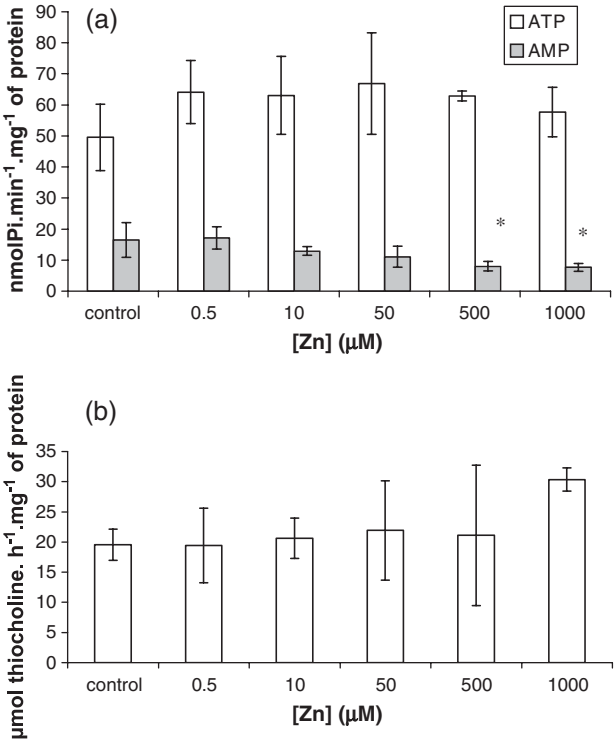

Fig. 1. In vitro effects of zinc (a) on ATP, AMP hydrolysis and (b) cholinesterase activity in the digestive gland of *H. aspersa*. Bars represent mean±S.D. of three experiments ($n=3$). *Represents statistical difference by one-way ANOVA ($P<0.05$, Duncan's test). In the control group, chemicals were not added.