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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

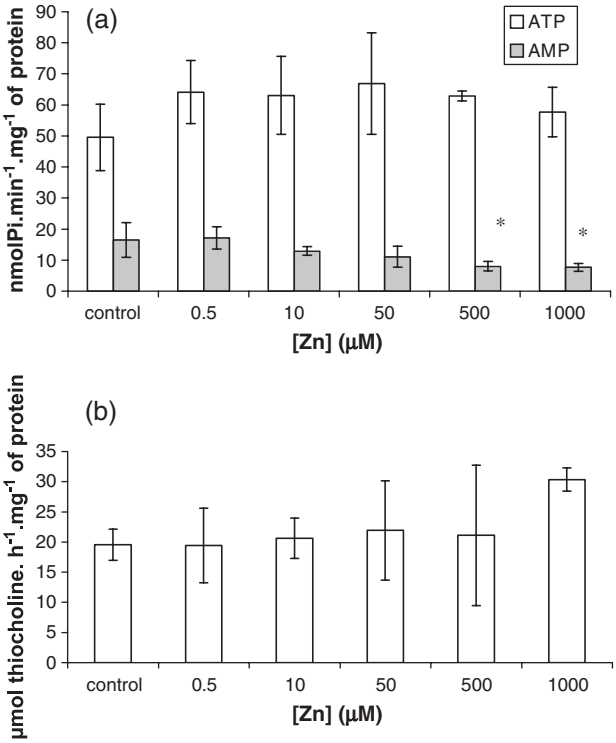

Fig. 1. In vitro effects of zinc (a) on ATP, AMP hydrolysis and (b) cholinesterase activity in the digestive gland of *H. aspersa*. Bars represent mean±S.D. of three experiments ($n=3$). *Represents statistical difference by one-way ANOVA ($P<0.05$, Duncan's test). In the control group, chemicals were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

a final volume of 200 μL. Membranes of the digestive gland of *H. aspersa* (2.5–5 μg protein) was added to reaction medium and pre-incubated with the different heavy metal concentrations for 10 min at 30 °C. The concentrations tested were: zinc (0.5, 10, 50, 500 and 1000 μM), copper (0.5, 1, 10, 25 and 50 μM) and cadmium (1, 50, 100, 500 and 1000 μM). The reaction was initiated by the addition of substrate (ATP or AMP) at final concentration of 1 mM, incubated for 20 min at 30 °C and stopped by addition of 200 μL 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min, and the inorganic phosphate (P_i) released was measured according to Chan et al. (1961). Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with TCA were used to correct for non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released min^{-1} mg^{-1} of protein. We performed three different experiments for each condition ($n=3$).

2.5. ChE assays

Digestive glands were gently homogenized in 50 vols. (w/v) of a solution of NaCl (0.65%) containing a protease inhibitor (0.1 mM PMSF). The homogenates were centrifuged for 5 min at 1000 ×*g*. The supernatant was pre-incubated for 10 min with metals in the same concentrations used for ATPase assays, in a final volume of 100 μL. ChE activity was measured using 7 mM *S*-butyrylthiocholine iodide as substrate, 50 mM phosphate buffer, pH 7.7 and 0.25 mM 5,5′-dithiobis-2-nitrobenzoic (DTNB) (Ellman et al., 1961). The reaction was initiated by the addition of aliquots with 1–3 μg of protein. Protein concentrations and incubation time were chosen to assure the linearity of the reaction. Specific activity is expressed as μmol of thiocholine released h^{-1} mg^{-1} of protein. We performed three different experiments for each condition ($n=3$).

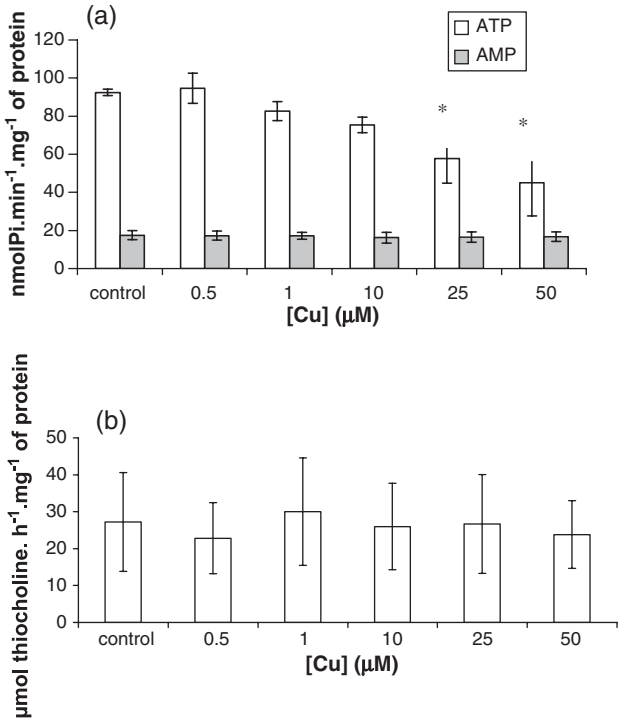

Fig. 2. In vitro effects of copper (a) on ATP, AMP hydrolysis and (b) cholinesterase activity in the digestive gland of *H. aspersa.* Bars represent mean±S.D. of three experiments ($n=3$). *Represents statistical difference by one-way ANOVA ($P<0.05$, Duncan's test). In the control group, chemicals were not added.

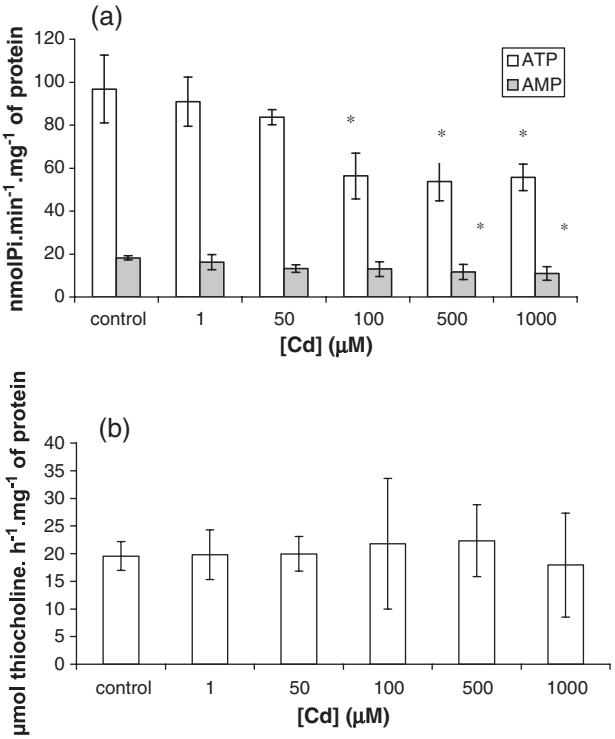

Fig. 3. In vitro effects of cadmium (a) on ATP, AMP hydrolysis and (b) the cholinesterase activity in the digestive gland of *H. aspersa.* Bars represent mean± S.D. of three experiments ($n=3$). *Represents statistical difference by one-way ANOVA ($P<0.05$, Duncan's test). In the control group, chemicals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

2.6. Protein determination

Protein was determined by Coomassie Blue method using bovine serum albumin as a standard (Bradford, 1976).

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan test, considering a level of significance of 5%. All analyses were performed using the Statistical Package for Social Science (SPSS) software program.

3. Results

The in vitro effects of zinc chloride, copper sulfate and cadmium acetate on ATPase, 5′-nucleotidase and ChE activities from the digestive gland of *H. aspersa* were evaluated. Zinc chloride promoted a significant decrease of 51% and 53% on 5′-nucleotidase activity at 500 and 1000 μM, respectively (Fig. 1a).

However, zinc chloride did not induce changes in both ATPase and ChE activities at any concentrations tested (Fig. 1a and b).

Copper sulfate, at 25 and 50 μM, significantly inhibited the ATPase activity (37% and 52%, respectively) of membrane preparations from the digestive gland of *H. aspersa* (Fig. 2a). In contrast, this metal did not alter 5′-nucleotidase and ChE activities at any concentration tested, when compared to control (no metal added) (Fig. 2a and b).

In relation to in vitro effects of cadmium, it was possible to observe an inhibitory effect on ATP hydrolysis at concentrations of 100, 500 and 1000 μM cadmium acetate (41%, 44% and 42%, respectively) (Fig. 3a). A similar decrease of AMP hydrolysis was observed at 500 and 1000 μM cadmium acetate (36% and 40%, respectively) (Fig. 3a). However, there were no significant changes in ChE activity from homogenate of the digestive gland of *H. aspersa* at any concentrations tested (Fig. 3b).

4. Discussion

This study has shown that in vitro exposure to metals, such as zinc, cooper and cadmium promoted a significant inhibition in nucleotidase activities in the digestive gland of *H. aspersa*, but did not alter ChE activity. The in vitro studies do not reproduce all the complexity of what happens in nature, but they do give short-term indications at the impact of the contaminants on key species, which have an important function in the ecosystem due to their biology and distribution, and this does not follow that they can be bred for experiments (De Vaufleury and Pihan, 2000).

Terrestrial mollusks have been widely used as indicators of environmental pollution (Pihan and de Vaufleury, 2000; Snyman et al., 2005). Dallinger (1993) considered snails as macro-concentrators of zinc and cadmium. However, Laskowski and Hopkin (1996) have shown that zinc was not accumulated in snail tissues to concentrations exceeding its levels in food, but copper and cadmium were clearly concentrated in soft tissues in comparison to their concentrations in food. Studies have shown that cadmium is a non-essential metal and is ten times more toxic than zinc, an essential metal involved in important metabolic process (Walker et al., 1996; Jackson, 1989; Depledge et al., 1994). In our experiments, cadmium, zinc and copper promoted differential effects on ATPase and/or 5′-nucleotidase activities in the digestive gland of *H. aspersa.* ATPase activity was significantly inhibited by copper and cadmium and 5′-nucleotidase activity was inhibited by zinc and cadmium. Considering the essentiality and non-essentiality of these metals and the diversity of morphological and metabolic changes induced by these compounds, it is possible to suggest that nucleotidase pathway could be a bioindicator to detect these pollutants. However, further studies in vivo would be required in order to evaluate the sensitivity of these enzyme activities to these metals. Our findings have shown that ChE activity is not altered by any metal at any concentrations tested. Studies have shown that ChE is widely sensitive to many chemicals that inhibit this enzyme, such as detergents, metallic compounds carbamates and organophosphorous (Mineau et al., 1990; Guilhermino et al., 1998; Perez et al., 2004). In agreement with our results, there are some reports questioning the utilization of this parameter for invertebrates, since a large number of animals demonstrate a ChE less sensitivity to agricultural chemicals (Bocquené et al., 1997; Couerdassier et al., 2002). The existence of many isoenzymes with different levels of inhibition by these compounds can impair the utilization of this enzyme as a biomarker in invertebrates (Bocquené et al., 1997).

Extracellular nucleotides are important messengers both in physiological as well in pathological conditions. After its release, ATP can be degraded to ADP, AMP and adenosine. Studies have demonstrated that purines can induce cytotoxic effect (Chow et al., 1997; Inoue, 2002). However, the effects of agonists to purinoceptors in invertebrates were varied when compared to the effects promoted by these agonists in vertebrates, presenting only some similar effects (Hoyle and Greenberg, 1988). It is possible to hypothesize that changes in the nucleotidase activities induced by the exposure to heavy metals can promote alterations in the extracellular nucleotide concentrations. Previous studies have shown that heavy metals (Hg^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}) at micromolar concentrations strongly inhibit the Ca^{2+}-ATPase activity present in the plasma-membrane obtained from the gill cells of *Mytilus galloprovincialis* Lam (Viarengo et al., 1993). The authors suggest that the copper strongly stimulates the lipid peroxidation damage of the gill plasma-membranes, a result that may explain the high copper cytotoxicity (Viarengo et al., 1993). Furthermore, our findings have shown that cholinesterase activity is insensitive to the metals tested in digestive gland. Previous studies have shown that ChE activity is inhibited by pesticide, and to a less extent, by metal exposure in the gills of the clam *Ruditapes decussatus* under various environmental conditions (Bebianno et al., 2004). Therefore, the impairment in the control of nucleotide levels may evoke an imbalance of purinergic neurotransmission, affecting nucleotide-mediated signal transduction, which could contribute to toxic effects promoted by these contaminants. Furthermore, the measurement of biochemical parameters, such ATPase and 5′-nucleotidase activities in the digestive gland of *H. aspersa* could be used as biomarkers of exposure to these metals.

Based on the data presented herein, this study demonstrated that zinc, cadmium and copper affect ATPase and cadmium affects 5′-nucleotidase in digestive gland, but not ChE, suggesting that purinergic system can be a target related to toxicity induced by these metals and a possible indicator of biological impact of exposure to heavy metal contaminants.

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