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In vivo effects of metals on the acetylcholinesterase activity of the *Perna perna* mussel's digestive gland

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Resumo

Efeitos *in vivo* de metais na atividade da acetilcolinesterase em glândula digestiva de mexilhões *Perna perna*. Já foi demonstrado que a enzima acetilcolinesterase (AChE) é fortemente inibida por pesticidas do tipo carbamato e organofosforado, assim como por metais. Entretanto, existem recentes indícios de que metais podem causar um aumento na atividade da AChE, em exposições agudas. Neste trabalho foi avaliada a atividade da AChE em glândulas digestivas de mexilhões *Perna perna* expostos a metais por 12, 24, 72 e 120 horas. Os mexilhões expostos a Cu e a Fe não apresentaram nenhuma diferença significativa na atividade da AChE, quando comparados aos animais controle. Mexilhões expostos a Cd por 72 horas e a Pb por 12 horas, tiveram atividade da AChE significativamente maior que os controles. Estes resultados podem indicar que em exposições agudas os metais poderiam interagir com receptores de acetilcolina afetando sua eficácia de união a estes, o que poderia causar um aumento inicial na síntese de AChE, para decompor a acetilcolina acumulada.

Unitermos: Acetilcolinesterase, mexilhões, *Perna perna*, metal, poluição

Abstract

It has been demonstrated that the enzyme acetylcholinesterase (AChE) is strongly inhibited by organophosphate and carbamate pesticides, and also by metals. However, recent reports indicate that some metals can activate AChE during acute exposure. In this work, we were interested in evaluating the effect of trace metal exposure (12, 24, 72 and 120 h) on the AChE activity of *Perna perna* mussel's digestive gland. Mussels exposed to Fe or Cu showed no changes in AChE activity during the whole period. Mussels exposed to Cd for 72 h or to Pb for 12 hours showed higher AChE activity than the control group. Based on these results, we hypothesize that under acute exposure, metals might interact with acetylcholine receptors, thereby affecting their binding efficiency and leading to a response involving an initial increase in AChE synthesis.

Key words: Acetylcholinesterase, mussel, *Perna perna*, metal, pollution

Introduction

The enzyme acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine to acetate and choline at the cholinergic synapses, terminating nerve impulse transmission. It is known that AChE is strongly inhibited by organophosphate and carbamate pesticides, and also by metals (Gill et al., 1991; Guilhermino et al., 2000; Martinez-Tabche et al., 2001). For this reason, the evaluation of AChE inhibition in marine organisms has been widely used as an indicator of marine contamination by these compounds.

Although metals have been confirmed to cause AChE inhibition, there are some studies reporting opposite effects for specific metals during acute exposure of different organisms. Gill et al. (1991) have reported increased AChE activity in skeletal muscles and brain of fish (*Barbus conchonius*) exposed to Cd for 48 hours. Zatta et al. (2002) have observed increases in AChE activity of rats treated orally with Al, and Flora and Seth (2000) and Martinez-Tabche et al. (2001) have described a stimulatory effect of Pb in rats and oligochaetes. Also, Thaker and Haritos (1989) have demonstrated that Hg (0.4 mg/L) causes a significant increase in esterase activity in shrimps (*Callianassa thyrrena*). These are substantial data that would compromise the use of AChE inhibition as an indicator of the presence of organophosphate and carbamate pesticides or metals in the environment.

Mussels represent good sentinel organisms for marine monitoring programs due to their sessile and filter-feeding habits, as well as to their inherent capability of bioaccumulating various contaminants. For this reason, much work has been carried out concerning the use of multiple biomarkers in different mussel tissues. Moreover, the kinetic and toxicological properties of AChE in bivalves have been extensively studied, allowing the optimization of the assays to measure AChE activity in different species, as well as other esterases such as butyrylcholinesterase and carboxyesterases (Basack et al., 1998; Galloway et al., 2002; Lehtonen and Leiniö, 2003; Brown et al., 2004). Regarding bivalves on the Brazilian coast, it has been demonstrated that cholinesterases from gills of *Crassostrea rhizophorae* are AChE, while extracts from gills of *Perna perna* possess both AChE and butyrylcholinesterase (BChE) activities, both presenting high sensitivity to different pesticides (Alves et al., 2002; Monserrat et al., 2002). Concerning the new discoveries of the possible effects of metals on increases in AChE activity of different organisms, we were therefore interested in assessing the effects of different metals on the cytosolic AChE activity of the *P. perna* mussel disgestive gland.

Material and Methods

Perna perna mussels of similar length (8-12cm) were purchased from a mussel aquaculture facility located in Florianópolis City (SC, Brazil) and divided into five groups of 20 specimens, which were placed into five tanks containing 0.5L of seawater *per* mussel. After one day of acclimatization, four groups of mussels were respectively exposed to sub-lethal doses of lead acetate (200mg/L), iron sulfate (500mg/L), cadmium acetate (200mg/L), and copper sulfate (40mg/L). These concentrations were adopted according to the LC_{50} values reported for mussels in the literature (Prakash and Rao, 1995; Viarengo et al., 1997; Viarengo et al., 1999). One group was kept under similar water conditions in the absence of metals, as the control group. The water of the control and treated groups was renewed daily. After 12, 24, 72 and 120 exposure hours, five mussels from each group were killed and their digestive glands dissected and immediately immersed in liquid nitrogen.

The tissues were weighed and homogenized with 1:5 vol of buffer (Tris-HCl 50mM, KCl 0.15M, pH 7.4), and centrifuged at 10,000 x g for 20min at 4°C. The supernatant was centrifuged at 40,000 x g for an additional 60min at 4°C, in order to obtain the cytosolic fraction used for measuring AChE activity. AChE activity was determined using Ellman's reagent DTNB (5,5'-dithio-bis(2-nitrobenzoic acid); 0.5mM) and acetylthiocholine iodide as substrate (Ellman et al., 1961), following procedures as described in previous characterizations of this enzyme in *Perna perna* (Najimi et al., 1997; Alves et al., 2002). The rate of change of absorbance at 412nm was recorded over 1.5min at 25 °C. Blank samples were taken to make sure that there was no non-specific esterase or other background activity. Protein was quantified on extracts as described by Peterson (1977), allowing the calculation of AChE as U(mmol/min)/mg protein.

Previous evidences had indicated that AChE globular forms are either readily extractable in low ionic strength buffers, or are tightly bound to the membranes, requiring detergents such as Triton X-100 for solubilization. However, there are recent works reporting an activation of inactivated AChE in samples treated with Triton X-100, as well as an elevation in the LC_{50} of different AChE inhibitors in the presence of Triton X-100 (Jaganathan and Boopathy, 1998; Marcel et al., 2000; Rosenfeld et al., 2001; White et al., 2003). For these reasons, in this work we measured AChE activities only in the citosolic fractions.

Statistical analysis was performed using Microcal Origin 6.0 software (Northampton, MA, USA). The results are presented as mean ± standard deviation. Significant differences between different groups were studied using *t*-test and one-way analysis of variance, and $p < 0.05$ was accepted as significant.

Results and Discussion

There are only few studies that have tested the *in vivo* effects of metals on the AChE activity of marine organisms. Although the literature indicates a classical inhibitory effect of certain pollutants on AChE activity, a stimulatory effect has also been reported in rats for substances such as lindane (Muñoz-Blanco et al., 1985), Al (Zatta et al., 2002), toluene, vinyl chloride and the organophosphate ethylparathion (Sanz and Repetto, 1995). According to Jackin (1974), the exposure of organisms to metals may result in stimulation, no change, or depression of the enzymes studied, depending on the duration and the metal concentration used.

As shown in figure 1, Fe and Cu exposure caused no effects on the AChE activity, under our experimental conditions. On the other hand, mussels exposed to Pb for 12 hours and to Cd for 72 hours presented a higher AChE activity (10.97 ± 2.81 and 23.90 ± 14.27U/mg protein, respectively) than the respective unexposed (control) groups (6.29 ± 1.66 and 5.30 ± 2.27U/mg protein). The results obtained from Cd are in agreement with Najimi et al. (1997), who observed higher AChE activity in *P. perna* after 3 and 7 days of Cd exposure, and after 3 and 4 days of Zn exposure. Moreover, Romani et al. (2003) observed increased AChE in the fish *Sparus auratus* exposed for 20 days to sublethal Cu concentrations, proposing that this metal could enhance the formation of the enzyme-substrate complex, increasing the activity of AChE. However, Sanz and Repetto (1995) reported that metals can affect AChE activity by decreasing its affinity for the substrate, making difficult the interpretation of these results.

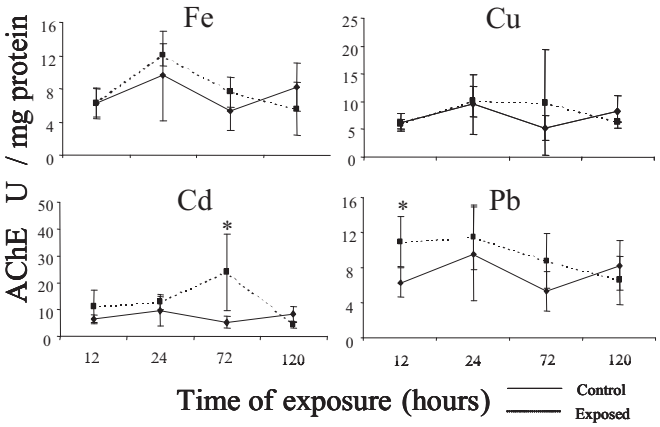

FIGURE 1: AChE activity in the digestive gland of *Perna perna* mussels exposed to Fe, Cu, Cd or Pb, compared to control groups, for different exposure periods. N=5, * indicates statistical differences ($p<0.05$).

Another possibility for the AChE activation by metals would be related to a *de novo* synthesis of the enzyme as a response to an initial inhibition. Despite some reports of an *in vitro* inhibition of AChE by metals, in *in vivo* experiments, other factors might contribute to changes in synthesis and/or degradation rates of the enzyme due to the metal accumulation in the tissues (Najimi et al., 1997). In fact, a differential activation of multiple molecular forms of the esterase system has been reported previously for the shrimp *C. thyrrena* after Hg exposure (Thaker and Haritos, 1989).

Based on these considerations, we can suggest that metals, under our exposure conditions, could interact with the acetylcholine receptor and thereby affect its binding efficiency, leading to an increase in AChE synthesis, to decompose the higher levels of neurotransmitter, as an acute response. The higher AChE activity might also be related to an up-regulation of AChE gene, due to an initial inhibitory effect of metals. Nevertheless, the mechanisms involved in this study remain to be clarified. Also, the lack of differences in subsequent periods of exposure would be due to an inefficiency of this response after

chronic exposures, leading to a decrease in AChE synthesis with concomitant inactivation of the active forms of the enzyme.

Gallegos et al. (2001) observed an increase on the AChE activity in brain of rats exposed to 10 mg/kg of Pb, after 30 min, but a strong decrease in this enzyme after 24 and 72 exposure hours. Thus, increase in AChE activity in response to metals seems to be a response to acute exposure, while decreases in AChE activity would be expected after more extended exposure. In agreement with this, we observed punctual increases in AChE after Cd and Pb exposure, which did not remain elevated in the subsequent periods of exposure. Perhaps after chronic exposure, metals would be able to inhibit AChE, as already proposed. Thus, experiments concerning the exposure of mussels to metals for more than 5 days need to be carried out in order elucidate the response of AChE in mussels after chronic exposure. Studies relating the exposure of mussels to higher concentrations of Fe and Cu than those used in this work, and to other metals, should also be conducted to verify their possible effects on the AChE activity of *Perna perna* mussels.

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References

Alves, S. R. C.; Severino, P. C.; Ibbotson, D. P.; Silva, A. Z.; Lopes, F. R. A. S.; Saénz, L. A.; Bainy, A. C. D. 2002. Effects of furadan in the brown mussel *Perna perna* and in the mangrove oyster *Crassostrea rhizophorae*. **Marine Environmental Research, 54**: 241-245.

Basack, S. B.; Oneto, M. L.; Fuchs, J. S.; Wood, E. J.; Kestenm E. M. 1998. Esterases of *Corbicula fluminea* as biomarkers of exposure to organophosphorous pesticides. **Bulletin of Environmental Contamination and Toxicology, 61**: 569-576.

Brown, M.; Davies, I. M.; Moffat, C. F.; Redshaw, J.; Craft, J. A. 2004. Characterization of choline esterases and their tissue and subcellular distribution in mussel (*Mytilus edulis*). **Marine Environmental Research, 57**: 155-169.

Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. **Biochemical Phrmacology, 7**: 88-95.

Flora, G. J.; Seth P. K. 2000. Alterations in some membrane properties in rat brain followed by exposure to lead. **Cytobios, 103**: 103-109.

Gallegos, M. E. H.; Zannatha, M. M. I.; Osornio, E. G.; Sanches, A. S., Rio F. A. P. 2001. Immediate and delayed effects of lead on AChE, GSH-T and thiols in the substantia nigra, neostriatum and cortex of the rat brain. **Journal of Applied Toxicology, 21**: 397-401.

Galloway, T. S.; Millward, N.; Browne, M. A.; Depledge, M. H. 2002. Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. **Aquatic Toxicology, 61**: 169-180.

Gill, T. S.; Teware, H.; Pande, J. 1991. *In vivo*, and *in vitro* effects of cadmium on selected enzymes in different organs of the fish *Barbus conchonius* Ham. (rosy barb). **Comparative Biochemistry and Physiology Part C**, **100**: 501-505.

Guilhermino, L.; Lacerda, M. N.; Nogueira, A. J. A.; Soares, A. M. V. M. 2000. *In vitro* and *in vivo* inhibition of *Daphnia magna* acetylcholinesterase by surfactant agents: possible implications for contamination biomonitoring. **The Science of the Total Environment, 247**: 137-141.

Jackin, E. 1974. Enzyme responses to metals in fish. *In*: Vemberg, E. G. & Vemberg, W. B. (eds.) **Pollution and Physiology of Marine Organisms**. Academic Press, New York, USA, p. 59-65.

Jaganathan, L.; Boopathy, R. 1998. Interaction of triton X-100 with acyl pocket of butyrylcholinesterase: effect on esterase activity and inhibitor sensitivity of the enzyme. **Indian Journal of Biochemistry and Biophysics, 35**: 142-147.

Lehtonen, K. K.; Leiniö, S. 2003. Effects of exposure to copper and malathion on metallothionein levels and acetylcholinesterase activity of the mussel *Mytilus edulis* and the clam *Macoma baltica* from the Northern Baltic Sea. **Bulletin of Environmental Contamination and Toxicology, 71**: 489-496.

Marcel, V. M.; Estrada-Mondaca, S.; Magné, F.; Stojan, J.; Klaébé, A.; Fournier, D. 2000. Exploration of the *Drosophila* acetylcholinesterase substrate activation site using reversible inhibitor (Triton X-100) and mutated enzymes. **Journal of Biological Chemistry, 275**: 11603-11609.

Martinez-Tabche, L.; Ortega, M. L. A. G.; Mora, B. R.; Faz, C. G.; Lopez, E. L.; Martinez, M. G. 2001. Hemoglobin concentration and acetylcholinesterase activity of oligochaetes in relation to lead concentration in spiked sediments from Ignacio Ramirez Reservoir. **Ecotoxicology and Environmental Safety, 49**: 76-83.

Monserrat, J. M.; Bianchini, A.; Bainy, A. C. D. 2002. Kinetic and toxicological characteristics of acetylcholinesterase from the gills of oysters (*Crassostrea rhizophorae*) and other aquatic species. **Marine Environmental Research, 54**: 781-785.

Muñoz-Blanco, J.; Yusta, B.; Martinez-Luque, M.; Gonzalez, J. M. 1985. Acetylcholinesterase activity in blood plasma and various regions of the central nervous system of rats poisoned with lindane.

Effect of sodium pentobarbital. **Revista Española de Fisiología, 41**: 89-93.

Najimi, S.; Bouhaimi, A.; Daubèze, M.; Zekhnini, A.; Pellerin, J.; Narbone, J. F.; Moukrim, A. 1997. Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a biomarker of pollution in Agadir Marine Bay (South of Morocco). **Bulletin of Environmental Contamination and Toxicology, 58**: 901-908.

Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al, which is more generally applicable. **Analytical Biochemistry, 83**: 346-356.

Prakash, N. T.; Rao, K. S. J. 1995. Modulations in antioxidant enzymes in different tissues of marine bivalves *Perna viridis* during heavy metal exposure. **Molecular and Cellular Biochemistry, 146**: 107-113.

Romani, R.; Antognelli, C.; Baldracchini, F.; De Santis, A.; Isani, G.; Giovannini, E.; Rosi, G. 2003. Increased acetylcholinesterase activities in specimens of *Sparus auratus* exposed to sublethal copper concentrations. **Chemico-Biological Interactions, 145**: 321-329.

Rosenfeld, C.; Kousba, A.; Sulfatos, L. G. 2001. Interactions of rat brain acetylcholinesterase with the detergent Triton X-100 and the organophosphate paraoxon. **Toxicological Sciences, 63**: 208-213.

Sanz, P.; Repetto, M. 1995. Implicaciones toxicológicas de las enzimas colinesterasas. *In*: Repetto, M. (ed.) **Toxicología Avanzada**. Días de Santos, Madrid, Espanha, p. 117-145.

Thaker, A. A.; Haritos, A. A. 1989. Mercury bioaccumulation and effects on soluble peptides, proteins and enzymes in the hepatopancreas of the shrimp *Callianassa thyrrhena*. **Comparative Biochemistry and Physiology Part C, 94**: 199-205.

Viarengo, A.; Burlando, B.; Cavalleto, M.; Ponzano, E.; Blasco, J. 1999. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. **Journal of the American Physiology Society, 277**: 1612-1619.

Viarengo, A.; Ponzano, E.; Dondero, F.; Fabbri, R. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to mediterranean and antartic molluscs. **Marine Environmental Research, 44**: 69-84.

White, B. J.; Legako, J. A.; Harmon, H. J. 2003. Extended lifetime of reagentless detector for multiple inhibitors of acetylcholinesterase. **Biosensors & Bioeletronics, 18**: 729-734.

Zatta, P.; Ibn-Lkhayat-Idrissi, M.; Zambenedetti, P.; Kilyen, M.; Kiss, T. 2002. *In vivo* and *in vitro* effects of aluminum on the activity of mouse brain acetylcholinesterase. **Brain Research Bulletin, 59**: 41-45.