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Different sensitivity of Ca^{2+}-ATPase and cholinesterase to pure and commercial pesticides in nervous ganglia of *Phyllocaulis soleiformis* (Mollusca)

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Abstract

We measured the effects in vitro of pure and commercial pesticides on Ca^{2+}-activated ATPase and cholinesterase (ChE) activities in the nervous system of the slug *Phyllocaulis soleiformis*. The pesticides used in this study included carbamate and organophosphates, which acts as reversible and irreversible anticholinesterases, respectively. Both enzymes were insensitive to pure carbofuran (1 mM), glyphosate (1 mM) and malathion (120 μM). However, the carbamate carbofuran, in the commercial formulation Furandan 350S, inhibited ATPase and ChE activities. The organophosphate glyphosate used in the commercial preparation of Gliz 480CS® inhibited ATPase activity and increased cholinesterase activity. These effects are likely due to the action of adjuvant substances of the chemical formulation. The commercial formulation (Malatol 500CE) did not alter enzymes activities. Our results suggest that cholinesterase present in the slug nervous tissue has a different behavior to those identified in vertebrate nervous tissue, since it was insensitive to pure compounds, known as anticholinesterases in vertebrates. Considering the insensitivity of the Ca^{2+}-activated ATPase, we suggested that the purinergic neurotransmission and other roles of ATP might not be affected by the pure pesticides tested.

Keywords: ATPase; Carbofuran; Cholinesterase; Commercial compounds; Glyphosate; Invertebrates; Malathion; Mollusks

1. Introduction

Phyllocaulis soleiformis (d'Orbigny, 1835) is a terrestrial slug belonging to the Veronicellidae, a family containing approximately 200 species. Veronicellids are of economic significance, since they represent principal agriculture and floriculture pests. Many investigators have attempted to develop efficient methods of slug control, but so far, only few advances have been made (Bennett and Andrews, 1985).

Herbicides such as organophosphate glyphosate are used in both agricultural and non-agricultural

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areas. Glyphosate is relatively effective with little to no hazard to animals (Tate et al., 1997). Glyphosate, at sublethal concentrations, affects the reproduction and development of *Pseudosuccinea columella* snails (Tate et al., 1997).

Organophosphates (OPs) and carbamate are inhibitors of cholinesterase (ChE) and, at low concentration, inhibitors of some other serum esterases, such as carboxyl or aliesterases (Ozretic and Krajnovic-Ozretic, 1992). The difference is related to the manner of inhibition, since that carbamate promotes a reversible inhibition and organophosphates act as an irreversible inhibitors of cholinesterase activity (Shore and Douben, 1994). The inhibition of cholinesterase activity has been successfully used to diagnose organophosphorous and carbamate poisoning in vertebrates. However, in invertebrates, the interaction of these compounds with cholinesterases enzymes is not homogeneous due to differences in sensitivity between different invertebrates, such as mussels and mosquitoes (Mora et al., 1999; Lee and Lees, 2001). Some authors have shown difficulty in classifying the ChE from invertebrates, since these enzymes have apparent affinity for any choline ester, suggesting the general term ChE, and not classifying them as acetylcholinesterase or butyrylcholinesterase (Bocquené et al., 1997; Mora et al., 1999).

Acetylcholine and ATP are co-released in the nervous terminals, where ATP acts as a co-transmitter or modulator of cholinergic synapses. However, it is still unclear, if the mechanism of the modulation is performed by ATP or adenosine (Cunha and Ribeiro, 2000). The life span, the duration and extent of receptor activation by acetylcholine and ATP are controlled by their hydrolysis performed by acetylcholinesterase (AChE; EC 3.1.1.7) and nucleoside triphosphate diphosphohydrolases (NTPDases), respectively, (Burnstock, 1999; Cunha and Ribeiro, 2000; Zimmermann, 2001).

Recently, we characterized an ATPase from the nervous ganglia of *P. soleiformis*, which presented a peculiar and different kinetic behavior when compared to other ATPases described in vertebrates and other invertebrates (Da Silva et al., 2002). This enzyme activity is strongly activated by $CaCl_2$ and hydrolyzes only triphosphate nucleotides, mainly ATP. It is insensitive to classical inhibitors of ATPases, such as ouabain, orthovanadate, sodium azide and *N*-ethylmaleimide.

Considering the interaction between the purinergic and cholinergic systems and the differences observed in vertebrates and invertebrates with respect to the effects of pesticides on these systems, the aim of this study was to verify the effect in vitro of three pesticides in the commercial and pure form commonly used in agriculture on the Ca^{2+}-activated ATPase and ChE activities in the nervous ganglia of a slug. We tested carbamate (carbofuran and Furadan 350S®) and OP (Malathion, Malatol 500CE®, Glyphosate and Gliz 480CS®) pesticides in order to determine the susceptibility of ATPase and ChE to these specific environmental inhibitors.

2. Material and methods

2.1. Phyllocaulis soleiformis

P. soleiformis (Mollusca, Gastropoda, Veronicellidae) were collected all year long from gardens (pesticide free) of metropolitan region of Porto Alegre, RS, Brazil. Animals were maintained in plastic boxes at 25 ± 5 °C with control of humidity ($\pm 70\%$). The specimens collected weighed to approximately 2.8 ± 1 g. They were fed on a mixture of vegetables (pesticide free) and maintained in a room for at least seven days before the experiments.

2.2. Chemicals

ATP and Trizma base were purchased from Sigma (St. Louis, MO, USA). The kit for ChE activity was obtained from Wiener Lab. The pure compounds were kindly donated by FMC Química do Brasil LTDA (96%, carbofuran; 2,3-Dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate), Monsanto do Brasil LTDA (glyphosate; *N*-(Phosphonomethyl)glycine) and Indol do Brasil (malathion; [(Dimethoxyphosphinothioyl)thio] butanedioic acid diethyl ester). Malatol 500CE® (95%, BioCarb-Industria Química LTDA), Gliz 480CS® (99%, Sanachem Brasil-Comércio LTDA) and Furadan 350S® (FMC do Brasil Industria e Comércio S.A) were purchased from commercial suppliers. All others reagents were of the highest purity available. The concentration used for commercial compounds is close to the concentrations recommended for the dilution of technical products, when used in the agricultural area.

2.3. Isolation and homogenization of the nervous ganglia

The nervous system of *P. soleiformis* is composed of ganglia and their connectives fused into a circumoesophageal ring (South, 1992). The ring was isolated under the stereomicroscope with a single razor cut in the anterior region of the animal. Ganglia were then weighed and gently homogenized in 100 (ATPase assays) or 25 (ChE assays) volumes of 0.65% NaCl with a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged for 3 min at $4000 \times g$ and the supernatant was used in enzyme assays. All preparations were done at 4 °C.

2.4. ATPase assays

Ca^{2+}-activated ATPase was assayed as described previously (Da Silva et al., 2002). The standard reaction medium contained 50 mM TRIS–HCl, pH 7.2, 5 mM $CaCl_2$ in a final volume of 200 μl. Fractions of nervous ganglia of *P. soleiformis* (10–20 μg protein) were added to the reaction medium and pre-incubated with the different agricultural chemical concentrations for 10 min at 30 °C. The pure compounds used were the OP glyphosate (99%) and the carbamate carbofuran (96%) at the concentrations 10, 100, 500 and 1000 μM and the OP malathion (95%) at the concentrations 7.5, 15, 30, 60 and 120 μM. The commercial compounds used were Malatol 500CE® (malathion) at 0.003, 0.0075, 0.015, 0.03 and 0.12 nM, Gliz 480CS® (glyphosate) at 1.4, 14, 56 and 112 nM and Furadan 350S® (carbofuran) at 0.8, 1.6, 8, 16, 32 and 64 nM. The reaction was initiated by the addition of substrate (ATP) to a final concentration of 1 mM and stopped after 10 min by adding 200 μl 10% trichloroacetic acid. Incubation time and protein concentrations were chosen in order to assure the linearity of the reaction. The samples were chilled on ice for 10 min before inorganic phosphate (Pi) released was measured (Chan et al., 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released min^{-1} mg^{-1} of protein. We performed at least four different experiments, each in triplicate.

2.5. ChE assays

Fractions of the nervous ganglia of *P. soleiformis* were pre-incubated for 10 min with pesticides 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 at least four different experiments, each in triplicate.

2.6. Protein determination

Protein was determined by a 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 the 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

In this study, we demonstrate the effect in vitro of commercial and pure pesticides on the ChE and ATPase activities of the fractions from the nervous ganglia of *P. soleiformis*. The effect of the three pure compounds on the ChE and ATPase activities of the nervous ganglia of *P. soleiformis* is demonstrated in the Figs. 1 and 2, respectively. There were no significant changes in ChE and ATPase activities in any concentration tested of glyphosate (10–1000 μM), carbofuran (10–1000 μM) and malathion (7.5–120 μM). It was found a different sensitivity to commercial compounds between the ATPase and ChE since that the ATPase was inhibited by low concentration of Furadan 350S® and the ChE was inhibited only at the highest concentration tested (Figs. 3 and 4). Another interesting difference between these enzymes in relation to the commercial compounds can be

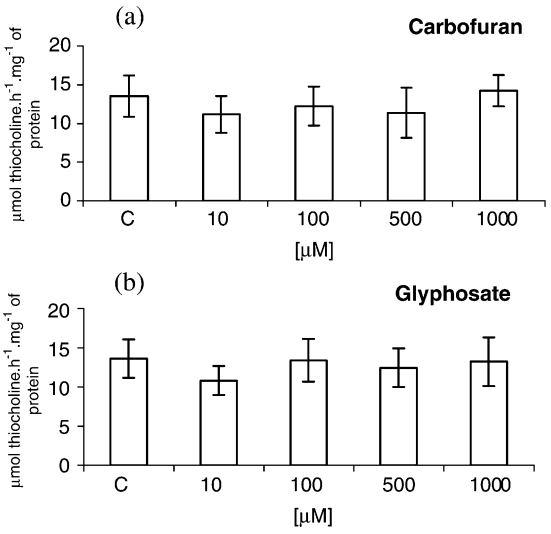

Fig. 1. In vitro effects of pesticides on the cholinesterase activity in the nervous ganglia of *P. soleiformis*. (a) carbofuran (96%) and (b) glyphosate (99%). Bars represent mean ± S.D. of at least four experiments. (c) Signifies controls where chemicals were not added.

verified when Gliz 480CS was tested since that on the ATPase activity there was an inhibition and on the ChE activity was observed an activation when the Gliz 480CS was tested at 14 nM (Fig. 3).

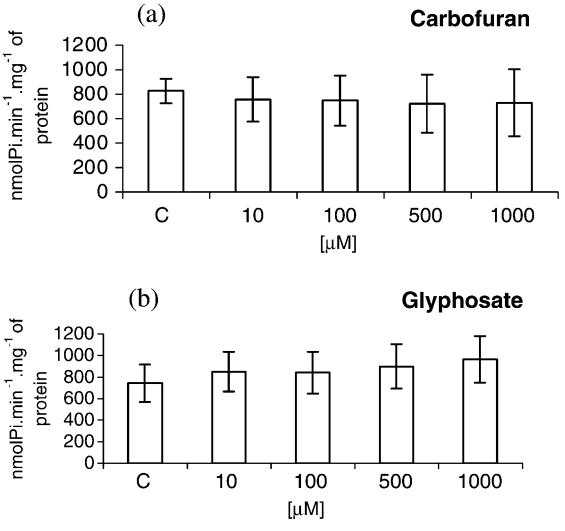

Fig. 2. In vitro effects of pesticides on the Ca^{2+}-activated ATPase in the nervous ganglia of *P. soleiformis*. (a) carbofuran (96%) and (b) glyphosate (99%). Bars represent mean ± S.D. of at least four experiments. (c) Signifies controls where chemicals were not added.

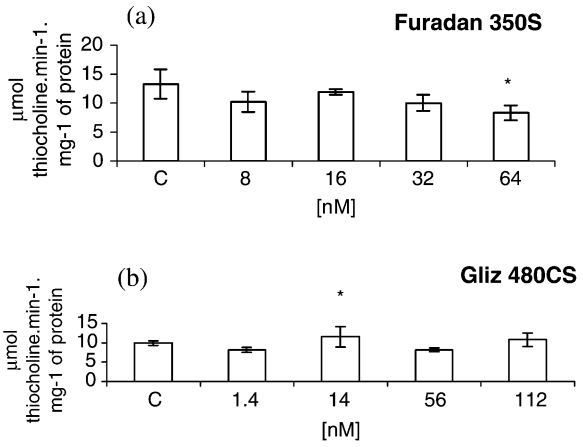

Fig. 3. In vitro effects of pesticides on the cholinesterase in the nervous ganglia of *P. soleiformis*. (a) Furadan 350S® and (b) Gliz 480CS®. Bars represent mean ± S.D. of at least four experiments. (c) signifies controls where chemicals were not added. $*P < 0.05$.

Malatol 500CE® was also unable to inhibit these enzyme activities (data not shown).

4. Discussion

The inhibition of ChE activity is the first toxic action of carbamates and OPs, and this effect is one of the most important biomarkers (Shore and Douben, 1994). Land invertebrates, including gas-

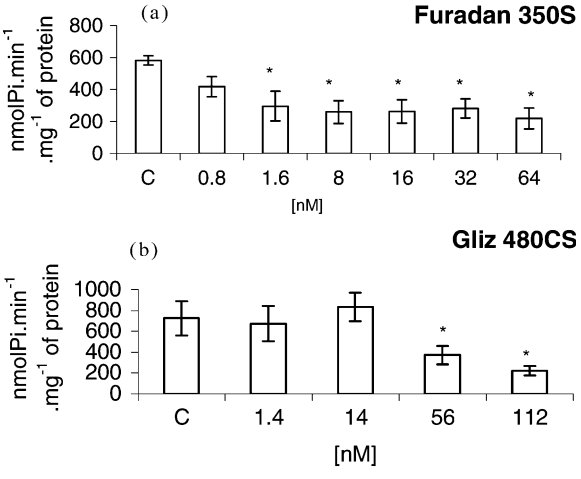

Fig. 4. In vitro effects of pesticides on the Ca^{2+}-activated ATPase in the nervous ganglia of *P. soleiformis*. (a) Furadan 350S® and (b) Gliz 480CS®. Bars represent mean ± S.D. of at least four experiments. (c) Signifies controls where chemicals were not added. $*P < 0.0001$.

tropod mollusks, are considered as potentially useful indicators of environmental contamination by organic and metallic compounds (Coeurdassier et al., 2001). In agreement with our results, there are some reports questioning the utilization of this parameter for invertebrates, mainly for two reasons. The first reason is that ChE should not be used as a specific biomarker for OPs and carbamates intoxication, since there are many chemicals that inhibit this enzyme, such as detergents and metallic compounds. Guilhermino et al. (1998) showed that ChE from the hemolymph of the mussel, *Mytilus galloprovincialis*, is inhibited by the detergents dodecyl benzyl sulfonate (DBS at concentrations >12.5 mg/l) and sodium dodecyl sulfate (at concentration >50 mg/l) and by metals such as chromium (at concentration >25 mg/l). Secondly, a large number of animals demonstrate ChE insensitivity to carbamates and OPs and the existence of many isoenzymes with different levels of inhibition by these compounds. Bocquené et al. (1997) demonstrated the presence of two isoenzymes in the gills of the common oyster *Crassostrea gigas*, one sensitive and the other resistant to carbamates and OPs. Mora et al. (1999) described a ChE that is poorly inhibited by OPs in marine mussels (*M. galloprovincialis* and *M. edulis*) and in a freshwater bivalve (*Corbicula fluminea*), suggesting the use of the ChE activity of this bivalve as a biomarker for acute rather than chronic contamination by these compounds.

The majority of the investigations about pesticides effects are focused to in vivo experiments. However some investigations with biological preparations from vertebrates and invertebrates have been conducted in vitro. Experimentation in vitro in carp brain with chlorfenvinphos, chlorpyrifos diazinon and carbofuran indicated that several concentrations inhibited 50% of the AChE activity, ranging from 4.1×10^{-7} to 8.12×10^{-4} M in both single inhibitory action and joint inhibitory effect (Dembele et al., 2000). Barata et al. (2001) observed that the inhibition in vitro by ethyl parathion was more marked than in vivo, indicating that not all the pesticides undergo metabolism or only a portion of the pesticide in the active form is in direct contact with the AChE. However, Barata et al. (2001) concluded that clonal differences in pesticide metabolism are the best explanation for the differences observed. In the present work, the differences between commercial and pure pesticides open a new question about the contribution of the excipient from the commercial formulation for the toxicity of pesticides.

In regard to the effect of pesticides upon on ATP hydrolysis, it is known that the ATPase activity can be taken as an important index of cellular activity and toxicological tool (Rahman et al., 2000). Moreover, ATPases are target enzymes for organochlorine chemicals that affect conduction of nerve impulses (Jinna et al., 1989). However, there are few investigations regarding the effect of OPs and carbamates on the ATPase activity. Rahman et al. (2000) demonstrated an inhibition of AChE, Na^+, K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase from rat brain in a dose- and time-dependent manner in the presence of a phosphorothionate. In vitro studies performed in synaptosomes from hen brain showed different effects of some organophosphates on the Ca^+-stimulated ATPase (Barber et al., 2001). Barber et al. (2001) demonstrated that following in vitro exposure to 10^{-3}–10^{-5} M OP compounds, Ca^{2+}-stimulated ATPase activity was inhibited by chlorpyrifos, chlorpyrifos-oxon, phenyl saligenin phosphate (PSP) and tri-*o*-tolyl phosphate (TOTP), but not by parathion, paraoxon or diisopropyl fluorophosphate. In our study, the Ca^{2+}-activated ATPase of the nervous ganglia of the *P. soleiformis* was insensitive to the three pure pesticides, which leads us to propose that the ATP hydrolysis may be resistant to this chemical aggression. The interesting inhibition caused by Gliz 480CS® and Furadan 350S® when compared to equivalent pure compounds suggests a possible action of the excipient substances present in the commercial formulation on the enzyme responsible for the ATP hydrolysis.

Our investigation evaluated the relationship between pesticides, recognized or not as anticholinesterasic agents and the enzymes responsible for the hydrolysis of the neurotransmitters ATP and acetylcholine. In addition, we compared pure and commercial compounds and observed that there is a different sensitivity of ATPase and ChE activities to these compounds. This investigation aimed to shed light upon the biochemical and toxicological differences between invertebrates and vertebrates, and the requirement of further investigations to study the purinergic and cholinergic systems in mollusks and other invertebrates.

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