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Cholinesterase activity in the caddisfly *Sericostoma vittatum*: Biochemical enzyme characterization and *in vitro* effects of insecticides and psychiatric drugs

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ABSTRACT

Sericostoma vittatum is a caddisfly species, endemic to the Iberian Peninsula, proposed as a biomonitor species for lotic ecosystems. Since inhibition of cholinesterases' (ChE) activity has been used to evaluate the exposure of macroinvertebrates to organophosphates and carbamate pesticides, this work intended to characterize the ChE present in this species so their activity can be used as a potential biomarker of exposure. Biochemical and pharmacological properties of ChE were characterized in this caddisfly species using different substrates (acetylthiocholine iodide, propionylthiocholine iodide, and butyrylthiocholine iodide) and selective inhibitors (eserine sulfate, BW284c51, and iso-OMPA). Also, the *in vitro* effects of two insecticides (carbaryl and chlorantraniliprole) and two psychiatric drugs (fluoxetine and carbamazepine) on ChE activity were investigated. The results suggest that *S. vittatum* possess mainly AChE able to hydrolyze both substrates acetylthiocholine and propionylthiocholine since: (1) it hydrolyzes the substrate acetylthiocholine and propionylcholine at similar rates and butyrylthiocholine at a much lower rate; (2) it is highly sensitive to eserine sulfate and BW284c51, but not to iso-OMPA; and (3) its activity is inhibited by excess of substrate, a characteristic of typical AChE. *in vitro* inhibitions were observed only for carbaryl exposure while exposure to chlorantraniliprole and to relevant environmental concentrations of psychiatric drugs did not cause any significant effect on AChE activity. This study suggests that AChE activity in caddisflies can indeed be used to discriminate the effects of specific insecticides in monitoring programs. The use of non-target species such as caddisflies in ecotoxicological research in lotic ecosystems is also discussed.

1. Introduction

Acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses of both vertebrates and invertebrates. This enzyme is specifically inhibited by organophosphorus and carbamate insecticides, but can also be affected by non-specific inhibitors like some metals and detergents and even emerging pollutants such as pharmaceuticals, causing an over-accumulation of the neurotransmitter acetylcholine, and thus prolonged electrical activity at nerve endings which may ultimately lead to death (Berra et al., 2006; Damásio et al., 2011a; Domingues et al., 2010; Garcia et al., 2000; Payne et al., 1996; Pestana et al., 2009; Santos et al., 2012; Schulz and Liess, 2000; Siebel et al., 2010).

Invertebrate cholinesterase (ChE) activity has been used for decades in laboratory and in *in situ* ecotoxicity studies as biomarker of aquatic contamination (Damásio et al., 2011b; Domingues et al., 2010; Kristoff et al., 2010; Payne et al., 1996).

There are two groups of vertebrates ChE, acetylcholinesterases (AChE) and butyrylcholinestereases (BChE), depending on prefer substrate hydrolysis and sensitivity to inhibitors (Valbonesi et al., 2003). These different substrate preferences and sensitivity to specific inhibitors have been used for the biochemical and pharmacological characterization of ChE in invertebrates. This is critical since different species have several enzyme isoforms which can exhibit different sensitivities towards anticholinergic contaminants (Damásio et al., 2011a; Domingues et al., 2010; Ellman et al., 1961; Hyne and Maher, 2003; Payne et al., 1996), and thus ChE characterization should be conducted as the first step before using ChE activity as a biomarker of effect (Damásio et al., 2011b; Domingues et al., 2010; Fulton and Key, 2001; Guilhermino et al., 1996; Howcroft et al., 2011; Kristoff et al., 2010; Monteiro et al., 2005;

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Payne et al., 1996; Santos et al., 2012). Moreover, several studies performed in invertebrates have shown ChE with mixed properties and generally, invertebrates have a significance difference in acethylthiocholine and butyrilthiocholine hydrolysis. For most of the studied invertebrates acethylthiocholine was the preferred substrate (Bianco et al., 2013; Cunha et al., 2005; Forget et al., 2002; Frasco et al., 2006; García-de la Parra et al., 2006; Monserrat and Bianchini, 1998; Mora et al., 1999).

To our knowledge, and despite some research on ChE activity in caddisflies (Damásio et al., 2011b), ChE characterization has not been performed in this insect order.

The caddisfly *Sericostoma vittatum* Rambur (Trichoptera: Sericostomatidae) is an endemic species of the Iberian Peninsula. The aquatic larvae of *S. vittatum* occur all year round, and are among the most abundant and conspicuous detritivores in many streams of this region (Damásio et al., 2011a; Feio and Graça, 2000; Tessier et al., 2000). For this reason, they play a key role in the fragmentation of allochthonous organic matter in streams (Diamantino et al., 2003; Gonzalez and Graça, 2003) and, as other caddisflies, are considered good model species to be used in ecotoxicological studies for the assessment of pesticide and metal pollution in lotic ecosystems (Berra et al., 2006; Damásio et al., 2011a; Payne et al., 1996; Pestana et al., 2009; Schulz and Liess, 2000; Xuereb et al., 2007).

The aim of this study is to characterize the ChE present in *S. vittatum* by determining substrate preferences and selective inhibitor effects. Cholinesterase activity was firstly measured *in vitro* by using different substrates (acetylthiocholine (ATCh), propionylthiocholine (PTCh) and butyrylthiocholine (BTCh)) and specific inhibitors (eserine for total ChE, BW284c51 for AChE; iso-OMPA for BChE). Secondly, *in vitro* effects on ChE activity were studied for carbaryl, a model insecticide which specifically inhibits the enzyme acetylcholinesterase (Gunasekara et al., 2008) and chlorantraniliprole, a recently developed anthranilic diamide insecticide acting as an activator of insect ryanodine receptors causing uncontrolled release of calcium from the intracellular stores (Lahm et al., 2009). Two psychiatric drugs, fluoxetine and carbamazepine which are commonly prescribed worldwide and found in many surface waters in concentration up to 44 and 1160 μg/L respectively in rivers receiving effluents from sewage treatment plants (Gonzalez Alonso et al., 2010), were also studied in order to assess their potential *in vitro* inhibitory effects in this insect species. Fluoxetine is an antidepressant drug acting by inhibiting the re-uptake of serotonin (Lamichhane et al., 2013) while carbamazepine is a commonly prescribed antiepileptic and anticonvulsant drug which act by inhibiting sodium channel activity in mammals (Ambrósio et al., 2002). Although not fully understood, it is conceivable that the main mode of action of these drugs and its metabolites is the same in invertebrates (Brooks et al., 2003; Lamichhane et al., 2013).

2. Material and methods

2.1. Test organisms

S. vittatum Rambur (1842) larvae were collected from Ribeira S. João, Serra da Lousã, Central Portugal (40°06′N, 8°14′W), using a hand net. Organisms were acclimated in plastic containers with 1–2 cm river sand and 4–5 cm of water column for two weeks to laboratory conditions: 20° C, light–dark cycle of 14:10 h, in aerated American Society for Testing Materials (ASTM) hard water and were fed *ad libitum* with unconditioned alder (*Alnus glutinosa*) leaves.

2.2. Cholinesterase characterization

Three replicates, each containing five *S. vittatum* larvae of similar size, selected directly from the rearing tanks, were frozen in liquid nitrogen and then ultra-sound homogenized in potassium-phosphate buffer (0.1 M, pH 7.2). The tissue homogenate of each sample was centrifuged for 20 min at 10,000*g* (4 °C) to isolate the post-mitochondrial supernatant (PMS).

Protein concentration was determined according to the Bradford method (Bradford, 1976; Gagnaire et al., 2008), adapted from BioRad's Bradford microassay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard.

The ChE activity was determined in quadruplicates in the PMS for each sample previously diluted (final protein concentration of 0.8 mg/mL), by the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996).

In these assays 250 μl of the reaction solution [30 ml potassium-phosphate buffer (0.1 M pH=7.2), 1 ml of reagent 5,5′-dithiobis-(2-nitrobenzoic acid) 10 mM (DTNB) and 200 μl of substrate] were added to 50 μl of the diluted PMS. The absorbance was measured at 412 nm during 5 min (25 °C). All spectrofotometric measurements were performed in the microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA).

2.3. Substrate preferences

S. vittatum ChE substrate preference was investigated by determining the enzyme activity of three replicates, in quadruplicate, at 12 increasing concentrations, from 0.012 to 20.48 mM, of the substrates acetylthiocholine iodide (ATCh), s-butyrylthiocholine iodide (BTCh) and propionylthiocholine iodide (PTCh). Cholinestrase activity in the presence of these substrates was determined as described in the previous sub-section, with 200 μl of each substrate being dissolved in the reaction buffer (Howcroft et al., 2011). Blank reactions were specifically made for each substrate concentration using the same volume of potassium-phosphate homogenization buffer (0.1 M, pH 7.2) instead of sample.

2.4. Cholinesterase inhibitors

Eserine sulfate, iso-OMPA and BW284C51 were used as selective inhibitors of ChE, BChE and AChE, respectively. Cholinesterase activities of the three samples were measured as described above, in quadriplicate, using 200 μl of ATCh 0.075 M solution, at six increasing concentrations from 0.781 to 800 μM for eserine sulfate and BW284C51, and from 0.016 to 16 mM for iso-OMPA, which were dissolved in the reaction buffer. Blank reactions were specifically made for each inhibitor concentration using the same volume of homogenization buffer potassium-phosphate buffer (0.1 M, pH 7.2) instead of sample. Controls (maximum ChE activity in the three samples, with each substrate) were made in the absence of inhibitors.

2.5. *in vitro* effects of selected toxicants on cholinesterase activity

The remaining PMS after the ChE characterization was used to determine the *in vitro* effects of carbaryl, chlorantraniliprole, fluoxetine and carbamazepine on ChE activity. Cholinesterase activity was measured as described in Section 2.4, using the test compounds instead of the specific inhibitors, at 25 °C during 5 min, and based on the substrate preferences assessed before – using acetylthiocholine 0.075 M as a substrate.

Fluoxetine hydrochloride (Cas no. 56296-78-7), carbamazepine (Cas no. 298-46-4), carbaryl (Pestanal® analytical standard 99% pure, Cas no. 63-25-2) and chlorantraniliprole (Pestanal® analytical standard 98% pure, Cas no. 500008-45-7) were acquired from Sigma-Aldrich. Stock solutions of the different compounds were prepared in dimethyl sulfoxide (DMSO) [500 mg/L, 1 mg/L, 500 mg/L and 23 g/L], for carbaryl, chlorantraniliprole, fluoxetine and carbamazepine, respectively and then were diluted in the reaction buffer in order to obtain the final concentrations, which ranged from 0.244 μg/L to 2.5 mg/L in carbaryl, from 24.4 ng/L to 100 μg/L in chlorantraniliprole, from 2.43 μg/L to 40 mg/L in fluoxetine and from 12.2 μg/L to 25 mg/L in carbamazepine. The stock solutions of the different compounds were prepared 1–2 hbefore use and kept in the dark at 4 °C. Because the aim of this work was not an ecotoxicological assessment and given the range of concentrations used and the fact that *in vitro* effects were assessed over short exposures (5 min), only nominal concentrations of the compounds are presented.

Blank reactions were made for each contaminant concentration using the same volume of potassium-phosphate homogenization buffer (0.1 M, pH 7.2) instead of sample. Water controls and solvent controls (same solvent concentrations as the maximum tested pollutant concentration), without test contaminants in the reaction buffer, were also made.

2.6. Statistical analysis

Data for substrate affinity were analyzed by fitting experimental curves (monotonic increase part of the curve) using the Michaelis–Menten equation, in order to determine the ChE kinetic parameters: maximal velocity (V_{max}), Michaelis–Menten constant (K_m) and their ratio (V_{max}/K_m), indicating the catalytic efficiency of the enzyme.

Data from *in vitro* exposures to specific inhibitors and to pollutants were analyzed with GraphPad Prism statistical software (version 6.0) using ANOVA's followed by Dunnett's multicomparison test to discriminate significant differences between tested concentrations and the control/solvent control at a significance level of 0.05. Whenever statistical effects were identified in the post-hoc tests, *in vitro* inhibition concentration (IC50) was calculated using a nonlinear four parameter logistic curve.