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LIZARD CHOLINESTERASES AS BIOMARKERS OF PESTICIDE EXPOSURE: ENZYMOLOGICAL CHARACTERIZATION

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Abstract—Here we report the results of a study conducted to elucidate the enzymological characteristics of lizard cholinesterases (ChEs) in order to use them as potential biomarkers for pesticide exposure. Serum and brain tissue of the lizard *Gallotia galloti* were used as ChE sources and in vitro assays were performed to identify acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities. The pH, substrate concentration, and specificity for ChE assays as well as the response of serum BChE to the reactivating agent pyridine-2-aldoxime methochloride (2-PAM) were also investigated in order to assess the possibilities of this methodology in biomonitoring programs. By the use of selective substrates and the inhibitor tetraisopropyl pyrophosphoramide, AChE and BChE activities were identified in lizard serum, while brain contained solely AChE. Likewise, butyrylthiocholine iodide was the optimum substrate for determining BChE activity and acetylthiocholine iodide for assaying both serum and brain AChE activities. The optimal ranges of pH and substrate concentrations were 7.5 to 8.0 and 5 to 10 mM, respectively. Serum was incubated with different doses of the organophosphorus (OP) compounds dichlorvos and paraoxon and subsequently incubated in the presence of two concentrations of 2-PAM (2×10^{-2} or 2×10^{-4} M). Reactivation rate of phosphorylated BChE was related to the degree of inhibition of BChE and the dose of 2-PAM. It was found that a 90-min incubation time with 2×10^{-4} M of 2-PAM satisfactorily increased the OP-inhibited BChE activity. The enzymological properties of serum BChE activity and its in vitro reactivation in the presence of 2-PAM represent the initial justification for its use in monitoring OP contamination in the field.

Keywords—Lizard Acetylcholinesterase Butyrylcholinesterase Enzymological properties Pyridine-2-aldoxime methochloride

INTRODUCTION

Cholinesterases constitute a group of hydrolases highly sensitive to toxicity by organophosphorus (OP) and carbamate pesticides [1,2]. Because of this, inhibition of cholinesterases (ChEs) is commonly used as an index of pesticide exposure in biomonitoring programs [3,4]. Acetylcholinesterase (AChE; EC 3.1.1.7) is the widely used ChE in the assessment of pesticide exposure. It participates in the regulation of cholinergic synapses of the nervous system and the neuromuscular endplate. Its inhibition by anti-ChE pesticides (OPs and carbamates) originates a wide range of clinical manifestations (e.g., convulsions, tremors, lacrimation, coma). In the last decade, blood butyrylcholinesterase (BChE; EC 3.1.1.8) emerged as a suitable biomarker for exposure to ChE-inhibiting pesticides [2,4–6] due to its high sensitivity to inhibition by this class of pesticides and its stability in various measurement techniques.

Birds, fish, and aquatic invertebrates are the organisms most often used to assess exposure to anti-ChE agrochemicals in field studies [7–14]. Tissue distribution of ChEs and the optimal conditions for their analysis are reported for some species [15–19]. Several sources of variability linked to experimental conditions, such as homogenization of tissues or factors associated with instrumentation, can affect ChE measurement [19]. In addition, the biochemical properties and tissue distribution of ChEs must be taken into account to avoid errors during processing and interpretation of data. For example, it is well known that BChE is a nonspecific ChE capable of hydrolyzing a number of cholinesters in addition to acetylcholine, such as butyryl- and propionylesters [2,5]. The use of selective substrates and inhibitors makes it possible to distinguish between AChE and BChE activities when both enzymes occur in the same target tissue.

In general, field studies of ChE activity related to pesticide-polluted environments compare the mean ChE activities among sampling areas or sampling times. Usually, a diagnostic threshold (2 SD below the mean ChE activity) is calculated in a nonexposed group of individuals [6,20]. Individuals having lower ChE activity than the threshold are considered exposed to ChE-inhibiting pesticides. It is therefore necessary to analyze a good representative control group in order to obtain an accurate diagnostic threshold. Reactivation of ChE activity using chemical agents such as pralidoxime (2-PAM) has become a complementary indicator of pesticide exposure, particularly for OPs [2]. Yet this presents a set of limitations—reactivation of OP-inhibited ChE depends mainly on OP type and time elapsed since exposure [2,5,21]. Despite these disadvantages and limited data availability, chemical reactivation of ChE activity has been recognized as a potential indicator of wildlife exposure to anti-ChE pesticides [20].

From an ecotoxicological perspective, reptiles, like amphibians, have not been studied in depth [22]. Several comprehensive reviews have stressed the need for increasing the knowledge about toxic effects of environmental pollutants on herpetofauna [23–25]. Data on toxic effects of OP pesticides on the lizard *Gallotia galloti* provide evidence that this organism shows an array of desirable features that make it a suitable sentinel of pollution. Serum BChE activity of *G. galloti* is a very sensitive biomarker of pesticide exposure, with a slow recovery rate (over weeks) after acute OP exposure [26]. In addition, serum BChE activity shows a significant

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relationship with brain AChE activity after acute OP exposure, which appears to be dependent on the type of OP [27]. This may ultimately facilitate predicting inhibition of brain AChE activity in a nonlethal manner.

The aim of this study was to characterize the AChE and BChE activities in the lizard *G. galloti* in order to use them as potential biochemical biomarkers. Distribution of ChEs was determined in the target tissues, serum, and brain, commonly used for ChE activity assays, and optimal conditions for ChE measurements were established. We also investigated the response of serum ChE activity of *G. galloti* to the reactivating ability of 2-PAM as a diagnostic for OP exposure. Factors that could affect enzyme reactivation such as concentration of 2-PAM, inhibition of serum ChE, and OP type were examined.

MATERIALS AND METHODS

Reagents and tissue preparation

Acetylthiocholine iodide (AcSCh), butyrylthiocholine iodide (BuSCh), propionylthiocholine iodide (PrSCh), tetraisopropyl pyrophosphoramide (iso-OMPA), 2-PAM, and 5,5′-dithiobis-2-nitrobenzoic acid were supplied by Sugelabor (Barcelona, Spain). Organophosphate pesticides or metabolites (>98% purity) paraoxon (*O*,*O*-diethyl-*p*-nitrophenyl phosphate) and dichlorvos (2,2-dichlorovinyl dimethyl phosphate) were purchased from Chem Service (West Chester, PA, USA). Dimethyl sulfoxide and ethanol were obtained from Panreac Quimica (Barcelona, Spain). Solutions of each substrate, inhibitor, and pesticide were prepared daily as required. Pesticide solutions were initially dissolved in dimethyl sulfoxide and the final concentration of the solvent was kept below 5% in the incubation medium.

Samples of serum and brain were obtained from six adult specimens of *G. galloti* (10.0 ± 1.4 cm [mean length ± SD]) caught in the Tenerife Island (Canary Islands, Spain). Serum was separated by centrifugation at 10,000 *g* for 10 min at 4°C. Brain samples were homogenized in 0.1% Triton X-100 (Panreac Quimica) in 25 mM Tris-HCl (pH 8.0). Homogenates were then centrifuged at 5,000 *g* for 5 min at 4°C. Serum and brain homogenates were then kept at −80°C until analysis.

Cholinesterase activity assay

Cholinesterase activities were determined colorimetrically by the Ellman method [28] using a Spectronic Genesis-5 spectrophotometer (Spectronic Instruments, Rochester, NY, USA). Samples were preincubated for 2 min with 5,5′-dithiobis-2-nitrobenzoic acid (3×10^{-4} M final concentration [FC]) in 25 mM Tris-HCl, 1 mM $CaCl_2$ (pH 7.6) before the substrate was added (2×10^{-3} M FC). The variations in optical density were recorded at 410 nm for 2 min and enzyme kinetics were measured in triplicate at 25°C. Serum ChE activity was expressed as micromoles substrate hydrolyzed per minute per milliliter and brain ChE activity as micromoles per minute per milligram of total protein, using the extinction coefficient 13,600/cm/M. Protein concentrations were determined by the Bradford method [29] using bovine serum albumin as a standard.

Under these initial conditions, AChE and BChE activities were determined with the aid of selective substrates (BuSCh, AcSCh, or PrSCh) and the inhibitor iso-OMPA (believed to be selective for BChE activity). Sample aliquots were incubated with varying concentrations (from 10^{-6} to 10^{-3} M) of iso-OMPA for 30 min at 25°C and ChE activity on the different substrates was recorded.

Effect of pH and substrate concentration

Both AChE and BChE activities were assayed using a 25 mM Tris-HCl, 1 mM $CaCl_2$ buffer at pH values from 5.0 to 11.0. The AChE activity was assayed using AcSCh substrate after incubation of the samples for 30 min at 25°C in the presence of 5×10^{-4} M iso-OMPA; this concentration was found to be sufficient for full inhibition of BChE activity (see Results section).

Initial conditions for assaying ChE activities (25 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6, 25°C) were used to investigate the effect of different substrate concentrations. The BChE activity was determined using BuSCh (a selective substrate for BChE activity), and AChE activity was assayed using AcSCh after iso-OMPA incubation. Concentrations of substrates (BuSCh and AcSCh) ranged between 0.01×10^{-3} and 50×10^{-3} M FC. Butyrylthiocholine iodide was dissolved in 1:1 (v/v) dH_2O: ethanol, and the concentration of ethanol was always below 5% in the incubation medium. All determinations were made by triplicate at 25°C.

Chemical reactivation of ChEs

Chemical reactivation of serum BChE activity in the presence of 2-PAM was investigated in order to develop it as a methodology for assessing OP exposure in the lizard *G. galloti*. Time to full recovery of OP-inhibited enzyme was examined under different concentrations of 2-PAM, two types of OPs (dichlorvos and paraoxon), and different degrees of BChE inhibition. Two aliquots of serum were separately incubated with dichlorvos or paraoxon (2.2×10^{-6}, 5.4×10^{-6}, or 1.1×10^{-5} M FC) for 15 min at 25°C. A third aliquot was spiked with an equal volume of dH_2O as control. Subsequently, a solution of 2-PAM (2×10^{-2} or 2×10^{-4} M FC) was added and BChE activity was measured every 10 min over a 90-min time period.

RESULTS

Tissue distribution of ChEs

The use of selective substrates and inhibitors allowed us to distinguish between AChE and BChE activities in the analyzed tissues. The treatment of the serum samples with different concentrations of iso-OMPA demonstrated the presence of both AChE and BChE (Fig. 1A). There was an iso-OMPA-sensitive ChE fraction and an iso-OMPA-resistant fraction, the activity of which remained unalterable at iso-OMPA concentrations higher than 10^{-4} M. In addition, the iso-OMPA-sensitive fraction preferentially cleaved BuSCh, a substrate believed to be specific for BChE activity, while the iso-OMPA-resistant fraction hydrolyzed AcSCh at a higher rate than PrSCh and did not hydrolyze BuSCh. These results indicate that the iso-OMPA-resistant fraction was probably AChE activity, whereas the iso-OMPA-sensitive fraction could be BChE activity. Brain ChE activity was not affected over a wide range of iso-OMPA concentrations (10^{-3} to 10^{-6} M), its activity remaining invariable on both substrates, AcSCh and PrSCh (Fig. 1B).

In order to determine the contribution of each ChE (AChE and BChE) to total ChE activity and to ascertain which substrate is preferentially hydrolyzed by ChEs, six aliquots of serum and brain homogenates were incubated with 10^{-4} M iso-OMPA and ChE activities assayed using AcSCh, BuSCh, and PrSCh. Table 1 summarizes mean (± SD) ChE activities in both tissues. Butyrylcholinesterase represented the 83% of total serum ChE activity, while in the brain, AChE is the prin-

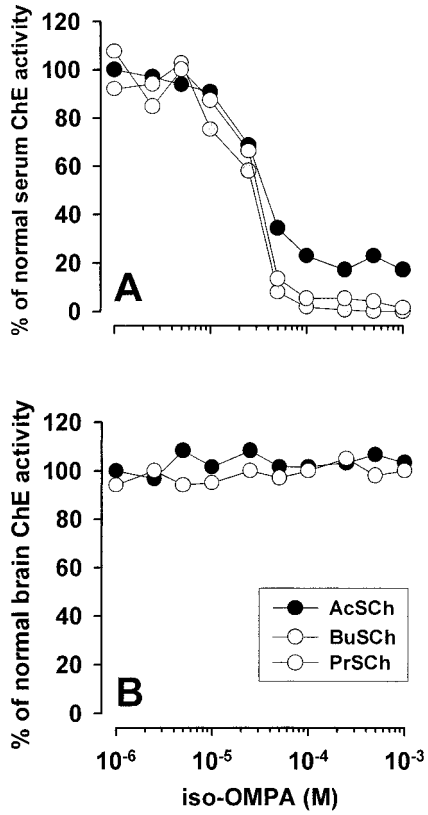

Fig. 1. Dose–response curves for the inhibitory action of tetraisopropyl pyrophosphoramide (iso-OMPA), a selective inhibitor for butyrylcholinesterase (BChE) activity, on serum (**A**) and brain (**B**) cholinesterase (ChE) activities of *Gallotia galloti*. Enzyme activity is expressed as percentage of activity using AcSCh (acetylthiocholine iodide), BuSCh (butyrylthiocholine iodide), and PrSCh (propionylthiocholine iodide) after a 30-min incubation at 30°C with iso-OMPA. Brain ChE activity using BuSCh was not detected. Each point corresponds to the average of three determinations (coefficient of variation < 4%).

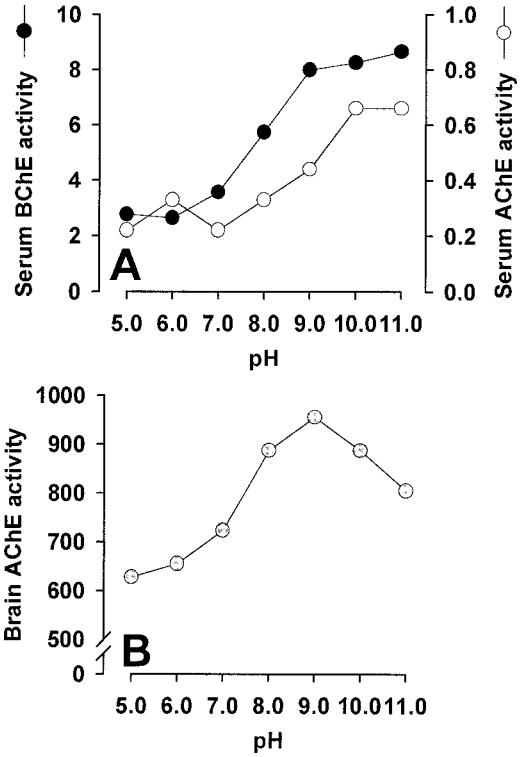

Fig. 2. Effect of pH on serum (**A**) and brain (**B**) cholinesterase activities of *Gallotia galloti*. Each point corresponds to the average of three determinations.

cipal ChE, as previously observed in the dose–response relationship experiment with iso-OMPA (Fig. 1). The AChE activity in serum (0.27 μmol/min/ml of serum, on AcSCh) was lower than BChE activity (1.33 μmol/min/ml, computed as the difference between total ChE activity on AcSCh and the remaining ChE activity after iso-OMPA treatment). Serum BChE activity using BuSCh was completely depressed after iso-OMPA treatment. This substrate was therefore sufficient to estimate BChE activity in serum samples. Brain ChE activity was considered to be exclusively AChE, with a mean (± SD) activity of 801 ± 115 μmol/min/mg of total protein. No activity using BuSCh was found in brain. The substrate AcSCh was cleaved at a higher rate than PrSCh by brain and serum AChE activities.

Effect of pH and substrate concentrations

Figure 2 shows the effect of pH on the ChE activities. Serum BChE and AChE exhibited a similar pattern of activity, with maximum activities at pH higher than 9.0. However, brain AChE activity showed a maximum activity at pH 9.0. Spontaneous nonenzymatic hydrolysis of the substrates BuSCh and AcSCh was observed at pH >8.0. Cholinesterase activity measurements were therefore corrected for spontaneous hydrolysis of the substrates.

Variations in serum AChE and BChE activities related to substrate concentrations were established using AcSCh and BuSCh, respectively, while substrate kinetics of brain AChE were investigated using AcSCh. All ChE activities showed an apparent Michaelis–Menten kinetic (Fig. 3). The values of apparent K_m and V_{max} were calculated from the Lineweaver–Burk plots. For brain AChE activity, K_m was 12.3 μM and V_{max} was 820 μmol/min/mg of total protein ($[1/V_0] = 1.22 \times 10^{-3} + 1.50 \times 10^{-5}[1/S]$, $r^2 = 0.89$). Likewise, serum ChE activities showed K_m and V_{max} values of 1.0 mM and 11.6 μmol/min/ml, respectively, for BChE ($[1/V_0]$ = 0.086 +

Table 1. Distribution and substrate specificity of cholinesterase activity[a] in brain and serum of *Gallotia galloti*

	AcSCh		BuSCh		PrSCh	
Tissue	Basal activity	10^{-4} M iso-OMPA	Basal activity	10^{-4} M iso-OMPA	Basal activity	10^{-4} M iso-OMPA
Serum	1.60 ± 0.38	0.27 ± 0.04	6.68 ± 1.02	—	5.53 ± 1.10	0.18 ± 0.03
Brain	801 ± 115	797 ± 90	—	—	596 ± 120	588 ± 86

[a] Cholinesterase (ChE) activity (mean ± standard deviation) is expressed as μmol/min/ml of serum for serum ChE or μmol/min/mg of total protein for brain ChE. AcSCh = acetylthiocholine iodide; BuSCh = butyrylthiocholine iodide; PrSCh = propionylthicholine iodide; iso-OMPA = tetraisopropyl pyrophosphoramide.

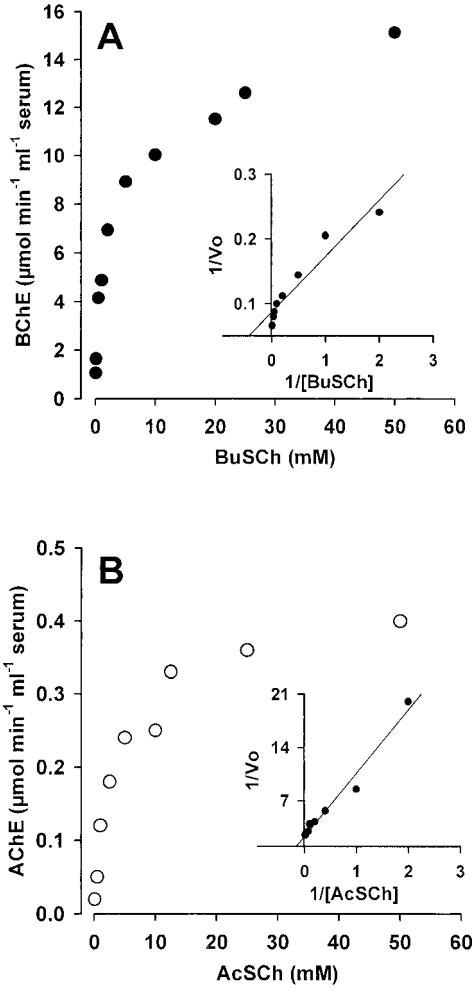

Fig. 3. Serum butyrylcholinesterase (BChE, (**A**)) and acetylcholinesterase (AChE, (**B**)) activities of *Galliotia galloti* as a function of substrate concentration. Each point corresponds to the average of three determinations. Insets are Lineweaver–Burk plots of both ChE activities for butyrylthiocholine iodide (BuSCh) and acetylthiocholine iodide (AcSCh) concentrations.

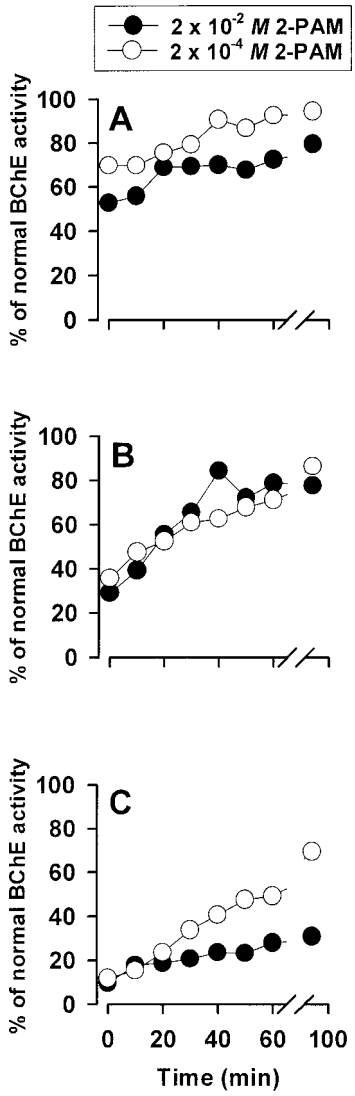

Fig. 4. Reactivation of paraoxon-inhibited serum butyrylcholinesterase (BChE) activity in the presence of pralidoxime (2-PAM). Aliquots of serum were previously incubated with 2.2×10^{-6} (**A**), 5.4×10^{-6} (**B**), and 1.1×10^{-5} M (**C**) of paraoxon for 15 min at 25°C and subsequently treated with two concentrations of 2-PAM (2×10^{-2} and 2×10^{-4} M, final concentration). Enzyme activity was determined serially for varying time periods after addition of 2-PAM and is expressed as percentage of remaining activity. Each point corresponds to the average of three determinations.

0.086[1/*S*], $r^2 = 0.92$), and 3.50 mM and 0.42 µmol/min/ml for AChE ($[1/V_0] = 2.34 + 8.32[1/S]$, $r^2 = 0.97$).

The BuSCh substrate suggests the optimal concentration for determining serum BChE activity is 10 mM while the AcSCh optimal concentration for assaying serum and brain AChE activity is 5 mM. Nonenzymatic hydrolysis was observed at high concentrations of substrate (>25 mM BuSCh or >8 mM AcSCh). Furthermore, no inhibition of BChE and AChE activities was detected at high substrate concentrations (up to 50 mM and 17 mM for BuSCh and AcSCh, respectively).

Chemical reactivation of BChE with 2-PAM

The possibility of serum BChE reactivation in the presence of a reactivating agent such as 2-PAM was investigated. In a first experiment, aliquots of serum were incubated with three concentrations of paraoxon (2.2×10^{-6}, 5.4×10^{-6}, or 1.1×10^{-5} M FC) and the reactivation rate was examined using two concentrations of 2-PAM (2×10^{-2} or 2×10^{-4} M FC). Figure 4 shows that BChE activity increased progressively in the presence of 2-PAM over 90 min of incubation, irrespective of the degree of BChE inhibition. However, the concentration of 2-PAM had a pronounced effect on the recovery rate of BChE. This enzyme activity was almost completely recovered with the lowest dose of 2-PAM for the three OP doses tested, while it remained below 80% of normal activity when 2×10^{-2} M of 2-PAM was used or even <40% in the samples previously treated with the highest dose of paraoxon (Fig. 4C).

In a second experiment, serum was incubated with 2.2×10^{-6}, 5.4×10^{-6}, or 1.1×10^{-5} M FC of dichlorvos (Fig. 5). As for paraoxon-phosphorylated BChE, positive reactivation was observed in the samples previously incubated with dichlorvos at the three different concentrations, and the lowest dose of 2-PAM appeared to be more effective than 2×10^{-2} M. A delay in the reactivation of 2-PAM was found when the highest dose (1.1×10^{-5} M FC) of dichlorvos was used (Fig. 5C). An increase of BChE activity was not observed after 20 and 40 min of incubation with 2×10^{-4} and 2×10^{-2} M of 2-PAM, respectively.

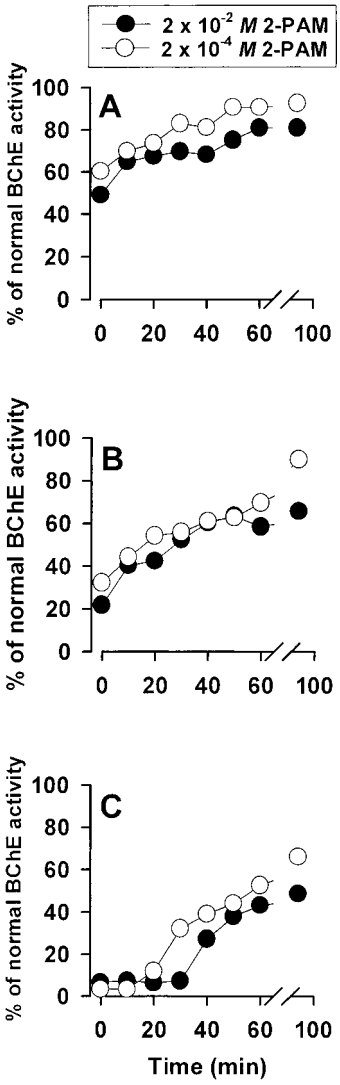

Fig. 5. Reactivation of dichlorvos-inhibited serum butyrylcholinesterase (BChE) activity in the presence of pralidoxime (2-PAM). Aliquots of serum were previously incubated with 2.2×10^{-6} (**A**), 5.4×10^{-6} (**B**), and 1.1×10^{-5} M (**C**) of dichlorvos for 15 min at 25°C and subsequently treated with two concentrations of 2-PAM (2×10^{-2} and 2×10^{-4} M, final concentration). Enzyme activity was serially determined for varying time periods after addition of 2-PAM and expressed as percentage of remaining activity. Each point corresponds to the average of three determinations.

DISCUSSION

Characterization and tissue distribution of ChEs

Both AChE and BChE are commonly used as biomarkers for pesticide exposure. They can be distinguished easily by the use of specific substrates and inhibitors [2,30,31]. In order to validate these enzymes as reliable biomarkers, it is necessary to know their tissue distribution, their enzymological behavior, optimal conditions for their activity, and even other features (e.g., relationship between field OP exposure and inhibition of ChEs, in vivo sensitivity of ChEs to OPs, stable inhibition response of ChEs, etc). For example, muscle tissue in several species of teleost fish have both AChE and BChE activities, while other species have AChE solely [30]. Both types of ChEs are present in the serum of many vertebrate species, with different sensitivity to the inhibitory action of OPs [4]. In order to use the most suitable enzyme as a biomarker, it is therefore necessary to characterize both ChEs. To our knowledge, this is the first report on enzymological characterization of lizard ChEs. Current results point out the existence of AChE and BChE activities in the analyzed tissues of *G. galloti*. Both AChE and BChE activities were found in serum, the latter being the primary ChE activity. Brain contained exclusively AChE activity. Regardless of the tissue, both ChE activities showed features typical of mammalian AChE and BChE, i.e., AChE hydrolyzed AcSCh but not BuSCh (a selective substrate for BChE activity) and was resistant to the inhibitory action of iso-OMPA (a specific inhibitor for BChE activity) and BChE activity hydrolyzed BuSCh at a higher rate than AcSCh or PrSCh and furthermore was drastically inhibited by iso-OMPA.

Measurement of serum BChE activity did not require the use of iso-OMPA pretreatment. The use of the substrate BuSCh was sufficient to estimate this enzyme activity because AChE activity was not able to hydrolyze it. Serum BChE activity is suggested to be a better biomarker than serum AChE activity for assessing exposure to anti-ChE pesticides. The high activity (on BuSCh) of serum BChE with respect to AChE, together with the previously reported [26] response to the inhibitory effect from OPs (high sensitivity and slow recovery rate after acute OP exposure), justify the use of serum BChE activity as an acceptable biomarker of pesticide exposure in *G. galloti*.

Effects of pH and substrate concentration

Nonenzymatic hydrolysis of BuSCh and AcSCh takes place at pH above 8.0 as well as at relatively high substrate concentrations. Lizard ChEs, however, showed maximum activities at pH higher than this value. Despite this, it is preferable to use pH values between 7.5 and 8.0 for the ChE determinations to avoid undesirable interferences by nonenzymatic hydrolysis of substrates. The reason for a different pH-dependent pattern of response of brain and serum AChE compared with mammals is not clear.

Like ChEs in other organisms, lizard BChE and AChE activities showed a Michaelis–Menten behavior with pronounced differences in K_m and V_{max} between them. Although comparison with data reported in the literature is difficult because of differences in the analytical procedures, brain AChE activity showed a relatively high affinity by AcSCh (K_m = 12.3 μM) in comparison with K_m values (160–650 μM) of brain AChE activity of teleost fish, for instance [30]. However, it was in the same order of magnitude of the ChE K_m for mollusks (30 μM, [16]).

The AChE activity, but not BChE activity, is generally inhibited by high substrate concentrations [2,17,30]. This property can help distinguish AChE from BChE in addition to the use of selective substrates and inhibitors. However, other authors found that ChE activity of marine mussels is not inhibited by high substrate concentration (up to 20 mM AcSCh). Brain and serum AChE activities of *G. galloti* did not show inhibition by high concentrations of AcSCh (up to 50 mM; Fig. 3). Although initial experimental conditions for lizard ChE determinations were carried out using a substrate concentration of 2 mM, current results support that the optimal substrate concentration is 10 mM for determining serum BChE activity and 5 mM for serum and brain AChE activities. Substrate concentrations higher than these values could lead to nonenzymatic hydrolysis of substrates.

Table 2. Optimal conditions for cholinesterase measurements in *Gallotia galloti*; data of some species are also reported for comparisons

Species	Suitable tissue or organ	Incubation temperature (°C)	Incubation pH range	Substrate[a]	Substrate concn. (mM)	Reference
Reptiles						
Gallotia galloti	Brain	25	7.5–8.0	AcSCh	5	This study
	Serum	25	7.5–8.0	BuSCh	10	This study
	Serum	25	7.5–8.0	AcSCh	5	This study
Fish						
Cyprinus carpio	Brain	25	7.5–8.5	AcSCh	2	[30]
Rutilus rutilus	Brain	37	7.1–8.5	AcSCh	1.8	[30]
Cyprinodon variegatus	Brain	22	7.0–7.5	AcSCh	10	[30]
Perca fluviatilis	Brain	37	7.1–8.5	AcSCh	1.8	[30]
Cymatogaster aggregata	Brain	37	7.0–7.5	AcSCh	1.8	[30]
Acerina cernua	Brain	37	7.1–8.5	AcSCh	2	[30]
Invertebrates						
Mytilus edulis	Gills	20–34	6.5–8.5	AcSCh	—	[33]
Palaemon serratus	Muscle	20–34	6.5–8.5	AcSCh	—	[33]
Crangon crangon	Cephalothorax	20–34	6.5–8.5	AcSCh	—	[33]
Nereis sp.	Whole animal	20–34	6.5–8.5	AcSCh	—	[33]
Mytilus galloprovincialis	Digestive gland, gills	37–42	6.0–8.5	AcSCh	—	[15]
Perna perna	Digestive gland, muscle	28–40	6.0–8.5	AcSCh	—	[15]
Mytilus galloprovincialis	Gills	25	7.2–9.2	AcSCh	2	[16]
Corbicula fluminea	Whole animal	25	8.0–9.2	PrSCh	5	[16]
Chironomus riparius	Whole animal	30	8.0–8.5	AcSCh	4	[18]

[a] AcSCh = acetylthiocholine iodide; BuSCh = butyrylthiocholine iodide; PrSCh = propionylthicholine iodide; — = data not reported.

2-PAM reactivation

Oximes, in particular 2-PAM, are known reactivating agents of phosphorylated ChEs. They are clinically used, in addition to atropine, as enzyme reactivators for OP intoxications. This property has been tentatively used for diagnosing field exposure to OP pesticides [14,20]. However, this methodology presents a set of limitations. The ability of 2-PAM to reactivate phosphorylated ChE is dependent on the time elapsed since OP exposure. The progressive aging of the phosphorylated enzyme, leading to the loss of one OP alkyl group, interferes with the ability of 2-PAM to reactivate the enzyme. The reactivation of OP-inhibited ChEs is, at least in birds, dependent on the OP type [21]. However, our results with two OPs did not support this assumption because no substantial differences were observed among OPs in reactivation.

The concentration of 2-PAM was a determinant in achieving full recovery of enzyme activity. A concentration 2×10^{-4} M 2-PAM in the incubation medium was considered as optimum. In fact, large doses of oximes can cause inhibition of AChE activity [32]. Inhibition of serum BChE was observed by Thompson et al. [21] when samples of starlings exposed to demeton-*S*-methyl were incubated with 12.5 mM of 2-PAM. These investigators concluded that this enzyme inhibition in the presence of 2-PAM might be due to a combination of two factors, i.e., aging of enzyme and/or high residual levels of demeton-*S*-methyl or its metabolites. No decrease in phosphorylated BChE activity of *G. galloti* was recorded after treatment with 2-PAM. However, the ability of 2-PAM to reactivate BChE was delayed when the enzyme was inhibited with dichlorvos (see Fig. 5C). Moreover, the delay was dependent on the 2-PAM dose. Positive reactivation was found after 20 min of incubation with 2×10^{-4} M, whereas an increase in BChE activity was observed after 40 min of incubation with the highest dose of 2-PAM. The reason for this might be the inhibitory effect that high concentrations of 2-PAM produce, as dichlorvos concentration was the same throughout the experiment (1.1×10^{-5} M).

In summary, in vitro experiments have demonstrated that chemical reactivation of phosphorylated BChE in the presence of 2-PAM is a good index of OP exposure. A 90-min incubation time was sufficient to detect a significant increase of BChE activity after OP exposure, and 10^{-4} M 2-PAM concentration was optimal for satisfactory enzyme reactivation. Furthermore, optimal conditions for ChE measurements in *G. galloti* are in the pH range of 7.5 to 8.0 and the substrate concentrations of 5 mM (serum and brain AChE) or 10 mM (serum BChE) (Table 2). Although not examined in this study, an incubation temperature of 25°C was considered as optimal for ChE measurements, as previously reported for this lizard species [26]. Likewise, data on the most favorable conditions for determining ChE activity in some species of aquatic invertebrates and fish are also reported in Table 2 for comparison. We have chosen assay conditions for the lizard that yield satisfactory ChE activities and minimize nonenzymatic hydrolysis of substrates. These optimal conditions appear to be in line with those reported for other organisms (Table 2).

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