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Cholinesterase and phenyl valerate-esterase activities sensitive to organophosphorus compounds in membranes of chicken brain

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ABSTRACT

Some effects of organophosphorus compounds (OPs) esters cannot be explained by action on currently recognized targets acetylcholinesterase or neuropathy target esterase (NTE). In previous studies, in membrane chicken brain fractions, four components (EPα, EPβ, EPγ and EPδ) of phenyl valerate esterase activity (PVase) had been kinetically discriminated combining data of several inhibitors (paraoxon, mipafox, PMSF). EPγ is belonging to NTE. The relationship of PVase components and acetylcholine-hydrolyzing activity (cholinesterase activity) is studied herein. Only EPα PVase activity showed inhibition in the presence of acetylthiocholine, similarly to a non-competitive model. EPα is highly sensitive to mipafox and paraoxon, but is resistant to PMSF, and is spontaneously reactivated when inhibited with paraoxon. In this papers we shows that cholinesterase activities showed inhibition kinetic by PV, which does not fit with a competitive inhibition model when tested for the same experimental conditions used to discriminate the PVase components. Four enzymatic components (CP1, CP2, CP3 and CP4) were discriminated in cholinesterase activity in the membrane fraction according to their sensitivity to irreversible inhibitors mipafox, paraoxon, PMSF and iso-OMPA. Components CP1 and CP2 could be related to EPα as they showed interactions between substrates and similar inhibitory kinetic properties to the tested inhibitors.

1. Introduction

Exposure to organophosphorus (OP) esters can cause several toxic effects, including acute cholinergic clinical episodes, intermediate syndrome, organophosphate-induced delayed neuropathy (OPIDN) and chronic neurological effects. The immediate effects of exposure to high levels of OPs involve inhibition of acetylcholinesterase, and they are well documented. Inhibition of acetylcholinesterase brings about changes in functions in central and peripheral nervous systems. However, the effects of long-term low doses exposure are controversial and not well known. (Sogorb and Vilanova, 2010).

Some OPs induce OPIDN after acute exposure associated with neuropathy target esterase (NTE) inhibition, followed by the so-called "aging reaction" (Williams and Johnson, 1981; Johnson, 1982). NTE is a membrane protein and chicken is the animal model, and extensive studies have been conducted into chicken brain and peripheral nerve that have used OPs compounds. NTE have been operationally measured as the PVase activity that resistant to paraoxon and sensitive to mipafox. The test involves assaying PVase activity in 2 conditions: (B) 20/30 min preincubation with 40 µM paraoxon; (C) preincubation with 40 µM paraoxon and 50 µM mipafox, being NTE activity the difference B-C.

A neurotoxic syndrome called "Intermediate syndrome" has been described after acute cholinergic crisis, which has been interpreted as the result of pre- and postsynaptic disruptions of neuromuscular transmission (Senanayake and Karalliedde, 1987). Other toxic effects with unknown molecular targets have been described but poorly defined (COT, 1999; Jamal et al., 2002).

Several enzymatic components of phenyl PVase activity have been discriminated using irreversible inhibitors: mipafox (OPIDN-inducer), paraoxon (non OPIDN-inducer) and phenylmethylsulfonyl fluoride (PMSF) (Mangas et al., 2011, 2012, 2012b, 2014) in membrane and soluble fractions. PMSF is an NTE inhibitor that protects against OPIDN development when dosed before a neuropathic dose of a neuropathic OP, but PMSF enhances neuropathy severity when dosed after a low non-neuropathic dose of a neuropathy inducer (Lotti et al., 1991; Pope and Padilla, 1990).

In the membrane fraction, four enzymatic components (EPα, EPβ, EPγ and EPδ) were discriminated using the combined information of the inhibitory kinetic properties with several irreversible inhibitors (Mangas et al., 2014). The characteristics of these fractions are

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Table 1
Phenyl valerate esterase enzymatic components in chicken brain. These are the qualitative conclusions drawn based on previously reported kinetic inhibitory properties (Mangas et al., 2014).

PVase component PVase	Paraoxon	Mipafox	PMSF
EPα (4-8%)	+ + (r)	+ +	–
EPβ (38-41%)	+	–	+ +
EPγ (39-48%) (NTE) (NTE)	–	+	+
EPδ (10 %) (IRE)	–	–	–

(+ +) the most sensitive; (+) the least sensitive; (–) resistant; (r) spontaneously reactivated after inhibition.

Table 2
Strategy to discriminate PVase components in a membrane fraction of brain chicken.

CONDITION	INHIBITOR for pre-incubation	Measured PVase component(s)
A	1000 μM PMSF	EPα + EPδ
B	50 μM mipafox	EPβ + EPδ
C	25 μM paraoxon	EPγ + EPδ
D	600 μM PMSF + 25 μM mipafox)	EPδ

summarized in Table 1. EPα is highly sensitive to mipafox and paraoxon, but is resistant to PMSF, and is spontaneously reactivated when inhibited with paraoxon. EPβ is sensitive to paraoxon and PMSF, but is resistant to mipafox. EPγ, is resistant to paraoxon, sensitive to mipafox and PMSF, and matches the operational criteria of being “NTE” (Johnson, 1982). EPδ is resistant to all the assayed inhibitors and it is related to the IRE (inhibitor resistant esterase) as described by Johnson and Richardson (1983). The strategies adopted to discriminate these components in this work are shown in Table 2.

The high sensitivity of EPα esterases to paraoxon and/or mipafox suggest that a potential role in toxicity at low dose should be considered. The spontaneous reactivation behavior after inhibition with paraoxon suggests that potential effects might be only significant for a continuous exposure. Therefore, its potential role in toxicity in the low-level long-term exposure of organophosphate compounds worth to be investigated. Alternatively, under other point of view, the binding and dephosporylation become a circle of catalytic hydrolysis and subsequent detoxication, which could have a protective role.

Moreover, the soluble fraction of chicken brain has been studied by Benabent and coworkers (2014), who showed that phenyl valerate may interact with cholinesterase activity, and several PVase components were discriminated (Eα, Eβ, Eγ). The interactions of Eα, Eβ and Eγ with PMSF (Mangas et al., 2012) suggest that they could play a role in the potentiation/promotion phenomena described by Pope and coworkers (Pope and Padilla, 1990) and by Moretto and Lotti laboratory (Lotti et al., 1991). Component Eα (PVase activity resistant to PMSF) is inhibited by acetylthiocholine, ethopropazine and iso-OMPA. This was considered to indicate that both substrates may interact on the same protein, and that all or part of Eα PVase activity might be due to butyrylcholinesterase (BuChE). However, component Eβ (resistant to mipafox) showed no interaction with acetylthiocholine. In the above work, component Eγ (resistant to paraoxon) was not studied because no cholinesterase activity was observed at the paraoxon concentration used to discriminate component Eγ. An enriched fraction with component Eα was prepared by different separation methods of the native protein (Mangas et al., 2014b), and was analyzed by LC–MS/MS and bioinformatic analyses (Mangas et al., 2017). It showed that BuChE was the only candidate responsible for virtually all Eα PVase activity. As a result of these findings, we studied the human BuChE and showed that it has PVase activity and did some characterization about their kinetic behavior (Mangas et al., 2017b). Kohli et al (2007) had observed that a biosensor containing preparation of commercial BChE and tyrosinase showed electrochemical response with phenyl valerate. This might be interpreted by a catalytic activity of BuChE with phenyl valerate. However the specific source of the enzymes in that work were commercial and no detail about it specie origin and purity were reported, and therefore, other proteins and factors may be influencing. With our current knowledge, we can interpret that those observations could be due to hydrolysis caused by BChE but other factors cannot be discarded.

In this work, considering the hypothesis that some fractions of PVase activity might be related with protein containing cholinesterase (ChE) activity in the membrane fraction, we studied the interaction of acetylthiocholine with PVase and phenyl valerate with ChE activities. The results suggested that these substrates could interact in the same proteins. Several enzymatic components of cholinesterase activity have also been discriminated in membrane fractions according to their sensitivity to several irreversible inhibitors (mipafox, paraoxon, PMSF and iso-OMPA). Consequently, the relationship between the PVase components and the ChE activity components was established in a membrane fraction of chicken brain.

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulfate (SDS; purity 99%) was obtained from Panreac Química S.L.U. (Barcelona, Spain). Ellmant’s reagent, 5,5′-dithio-bis-2-nitrobenzoate (DTNB, purity 99%) was acquired from Sigma-Aldrich Quimica SL (Madrid, Spain). Acetylthiocholine iodide (purity ≥ 98) was ordered from Sigma-Aldridge Quimica SL (Madrid, Spain). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma (Madrid, Spain). Phenyl valerate and *N, N′*- di-isopropylphosphorodiamidefluoridate (mipafox, purity 98%) were attained from Lark Enterprise (Webster, MA, USA). 1,5-Bis (4- allydimethylammoniumphenyl) pentan- 3 – one dimobride (BW284c51), tetraisopropylpyrophosphoramide (iso-OMPA) and 10-(2-diethylaminopropyl) phenothiazine (ethopropazine) were obtained from Sigma-Aldrich Quimica SL (Madrid, Spain). All the other reagents were bought from Merck SL (Madrid, Spain) and were of analytical grade.

2.2. Solutions

The buffer for tissue homogenization was 50 mM Tris–HCl (pH 8.0), which contained 1 mM EDTA. The “phosphate buffer” mentioned throughout the paper contained 0.1 M phosphate (sodium), pH 7.4 (measured at 25 °C), 1 mM EDTA.

Note that in all cases, the expression of %, when applied to concentration, is referred to w/w

The following reagent stocks solutions were prepared:

- 10 mM mipafox was prepared in 10 mM Tris-citrate buffer (pH 6.0), and diluted to the appropriate concentration in the buffer used for the enzyme reaction immediately before the kinetic assays.
- 50 mM PMSF was prepared in DMSO (dimethylsulfoxide), and diluted before use in the appropriate buffer.
- substrate phenyl valerate (16.8 mM), in dried *N, N*-diethylformamide, was diluted at 0.56 mM in ultrapure water immediately before the enzymatic assays. The quality of the solution and the level of spontaneous hydrolysis were checked by measuring in each experiment the phenol concentration in the controls.
- 11 mM BW284C51 in ultrapure water was diluted immediately before the assays.
- 20 mM iso-OMPA in ethanol was diluted immediately before the assays.
- 11 mM ethopropazine in ultrapure water was diluted immediately before the assays.
- acetylthiocholine in water immediately before use took the concentrations indicated in each assay.

- SDS-AAP solution, containing 2% SDS (sodium dodecyl sulfate) and 1.23 mM aminoantypirine, was prepared in water and was used to stop the enzymatic reaction of PVase activity and color development.
- The SDS-DTNB solution containing 2% SDS solution and 6 mM DTNB was prepared in phosphate buffer and was used to stop enzymatic activity and color development in the ChE assays.

2.3. Tissue preparation and subcellular fractionation for preparing membrane fraction

Chicken brains were obtained from a commercial slaughtering house immediately after killing the animals and were kept in cold (0–5 °C) homogenization buffer until use. They were homogenized in a Polytron homogenizer (Kinematica GmbH, Germany) using a PTA 10S head at 70% power (3 × 30 s) at a concentration of 200 mg of fresh brain tissue/ml in the same buffer.

The homogenized tissue was centrifuged at 1000 *g* and 4 °C for 10 min to precipitate fibers and nuclei. The supernatant was centrifuged at 100,000 *g* for 60 min to obtain a precipitate (membrane fraction) consisting of mitochondrial and microsomal fractions. Membrane brain fractions were resuspended in buffer at the same volume of the original tissue homogenate and, therefore, it contains the membranes from the 200 mg fresh tissues per mL. Finally, the resuspended membrane fraction was frozen and stored in liquid nitrogen until use.

This material was considered the “membrane fraction” that originated from 200 mg of tissue per ml. To use it, it was thawed at room temperature and diluted in the corresponding buffer at the concentration needed in each experiment, as indicated in the Results. The concentration of the diluted tissue preparation was expressed by indicating the mg of fresh tissue that derived from each mL of solution (i.e: the membranes that correspond to 30 mg fresh tissue per mL).

2.4. Preincubation of samples with inhibitor solutions (PMSF, mipafox, paraoxon) for discriminating PVase components

In a 1-ml microtube, 20 μL of inhibitor solution were incubated with 200 μL of the diluted brain membrane fraction (buffer in blanks for spontaneous hydrolysis). The mixture was incubated at 37 °C for 30 min.

Table 2 shows the strategy to discriminate the PVase component in a membrane fraction of brain chicken. Four pre-incubation conditions were used (A. B, C, and D). EPδ was the activity that was resistant to all the inhibitors (observed for condition D). This strategy and the appropriate experimental conditions (inhibitor, concentration and time) were designed based on the kinetic inhibitory properties that had been previously reported (Mangas et al., 2014)

2.5. Interaction between substrates in the membrane PVase activity components

A 200-μL volume of the membrane samples was pre-incubated with 20 μL of inhibitor to achieve the condition A, B, C, or D indicated in Table 2, and was prepared as indicated in Section 2.4. After a 30-minute pre-incubation, a 200-μL volume of a mixture of phenyl valerate and acetylthiocholine was added to obtain the concentrations indicated in each experiment. The mixture was incubated for 10 min for the enzyme reaction with the substrates.

The reaction was stopped by adding 200 μL of 2% SDS/1.23 mM AAP solution and 1.21 mM potassium ferricyanide, following the description indicated in Section 2.5 for measuring PVase activity based on the released phenol.

2.6. Interaction between substrates of the membrane ChE activities

A 200-μL volume of membrane preparation (or buffer in blanks) was pre-incubated with 20 μL of inhibitors to achieve the condition A, B, C or D, as described in Table 2. Then a 200-μL volume of a mixture of acetylthiocholine and phenyl valerate was added and incubated for 10 min for the enzyme reaction. ChE activity was measured according to Benabent et al. (2014b). For this purpose, the enzymatic reaction was stopped by adding 200 μL of 2% SDS/6 mM DTNB solution. Then 200 μL of phosphate buffer (or the diluted enzyme preparation in the blanks) were added. The final assay volume was 820 μL. After mixing and waiting for at least 5 min, a 300-μL volume from each microtube was transferred to a 96-well microplate. An automated Work Station (Beckman Biomek 2000) was employed. Absorbance was read at 410 nm in a microplate reader (Beckman Coulter AD 340).

2.7. Fixed-time inhibition curves of ChE activity with mipafox, paraoxon, PMSF, iso-OMPA, BW284c51 or ethopropazine in brain membrane fractions

A 200-μL volume of membrane fraction (buffer in blanks) was incubated with 20 μL of the inhibitor at 37 °C for a 30-min inhibition time. Then 200 μL of 2.1 mM acetylthiocholine in ultrapure water were added and incubated for 10 min at 37 °C to measure residual enzymatic activity following the procedure described by Benabent et al. (2014b). The reaction was stopped by adding 200 μL of 2% SDS/6 mM DTNB solution following the description indicated in Section 2.9. The results were expressed as a percentage of activity over the control without an inhibitor and were plotted versus concentration.

2.8. Kinetic data analysis

We used nonlinear regression with the Enzyme Kinetics Module to obtain the Michaelis-Menten graphic, and the lineal double reciprocal Lineawever-Bürk plot to determine the best fitting kinetic inhibition model and to calculate kinetic parameters Vmax, Km (Sigma Plot software 12.5, Systat Software Inc, Chicago, USA, for Windows).

For the irreversible inhibition with paraoxon, mipafox and iso-OMPA, model equations were fitted to the experimental fixed-time inhibition data by a non linear computerized method based on the least squares principle using version 12.5 of the Sigma Plot software. The model equations for a system with several enzymatic components, with or without a resistant fraction, as described by Estévez and Vilanova (2009), were as follows:

$$E = E1_0 \cdot e^{-k1 \cdot I \cdot 30} + E2_0 \cdot e^{-k2 \cdot I \cdot 30} + E3_0 \cdot e^{-k3 \cdot I \cdot 30} + \ldots + E_R$$

or included the chemical hydrolysis constant (kh) in PMSF inhibition, according to Estévez et al. (2012):

$$E = E1_0 \cdot e^{(e^{-kh \cdot 30}-1) \cdot \left(\frac{k1}{kh}\right) \cdot I} + E2_0 \cdot e^{(e^{-kh \cdot 30}-1) \cdot \left(\frac{k2}{kh}\right) \cdot I} + E3_0 \cdot e^{(e^{-kh \cdot 30}-1) \cdot \left(\frac{k3}{kh}\right) \cdot I} + \ldots + E_R$$

where 30 is the fixed inhibition time, k1, k2, k3,…, are the second-order rate constants; $E1_0$, $E2_0$,…, are the amplitude or proportion of the enzymatic sensitive components at the initial time and E_R is the resistant component.

The I50 (30 min) values in the mipafox, paraoxon and iso-OMPA fixed-time inhibition experiments were obtained by applying the following equation:

$$I_{50}^{30} = Ln\,2/(ki \cdot 30)$$

where ki is the second-order rate constant of inhibition.

The I50 (30 min) values in the PMSF fixed-time inhibition experiments were obtained by applying the following equation:

$$I_{50}^{30} = Ln\,2/\,\{(e^{(-kh \cdot 30)} - 1) \cdot \frac{ki}{kh}\}$$

where ki is the second-order rate of inhibition and kh is the rate constant of chemical hydrolysis.

For the reversible inhibitors, the following model equations were applied:

- For a system with an enzymatic component (Copeland, 2000, 2005), the mathematical model was as follows

$$\%Activity = 100\cdot\frac{1}{1+\frac{I}{I_{50}}}$$

where I is the inhibitor concentration and I_{50} is the concentration that inhibits 50% of total activity.
- For a system with several enzymatic components, the mathematical model was as follows

$$\%Activity = E_1\cdot\frac{1}{1+\frac{I}{I1_{50}}} + E_2\cdot\frac{1}{1+\frac{I}{I2_{50}}} + \ldots + E_n\cdot\frac{1}{1+\frac{I}{In_{50}}} + E_R$$

where E_1, E_2, and E_n are the proportions of activity of the sensitive enzymatic components, E_R is the resistant component, I is the inhibitor concentration, and $I1_{50}$, $I2_{50}$ and In_{50} are the concentrations that inhibit 50% of the activity of each sensitive component.

3. Results

3.1. Inhibition of PVase activities of the membrane in the presence of acetylthiocholine

Fig. 1 shows the Michaelis-Menten graphics of the data obtained in each experiment. Only for component EPδ the kinetic parameter was estimated, as in condition D only a single component was measured, giving a Km 0.8 mM.

The PVase activity measured under conditions B, C or D were not altered with acetylthiocholine (Fig. 1B–D). It was concluded that PVase components EPβ, EPγ and EPδ did not interact with acethylthiolocine,

However, the PVase activity measured under condition A (resistant to 1000 µM PMSF) lowered when the acetylthiocholine concentration increased, similarly to the non competitive inhibition model (Fig. 3A). Under this condition, activity was due to Epα + EPδ. However, EPδ were not affected by acetylthiocholine. Therefore, we concluded that the observed effect of acethylthiocholine should be by the interaction with component EPα.

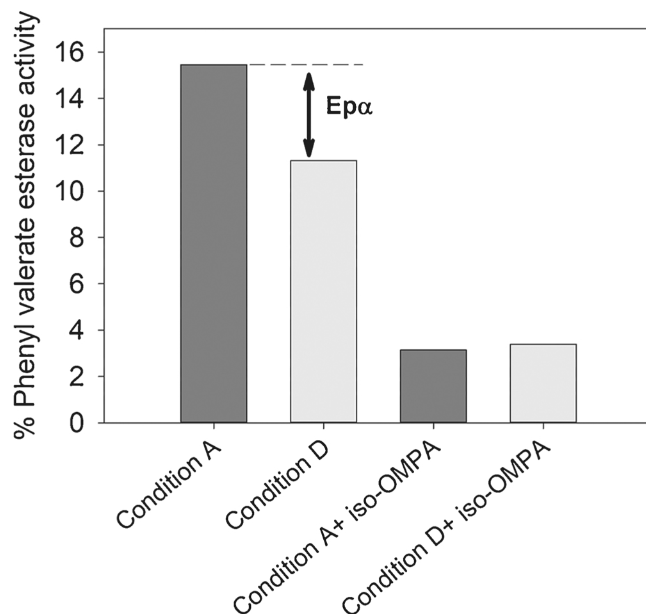

Fig. 2. Inhibition by iso-OMPA of membrane PVase activity EPα and EPδ. The preparation which contained the membrane fraction of 63 mg fresh tissue/ml in 200 µl was pre-incubated as indicated under condition A (dark gray) and D (light gray) in Table 2. Enzymatic activity was assayed by incubating the substrate phenyl valerate and iso-OMPA (900 µM in a 420 µl reaction volume) for 10 min. Percentages of activity refer to the control activity without inhibitors. Each bar represents the mean of three replicates (SD $< 5\%$).

3.2. Interactions of BW284C51, ethopropazine or iso-OMPA with membrane PVase components EPα and EPδ

As EPα seemed to be the only PVase component inhibited by acetylthiocholine, the effect of typical cholinesterase inhibitors on it was

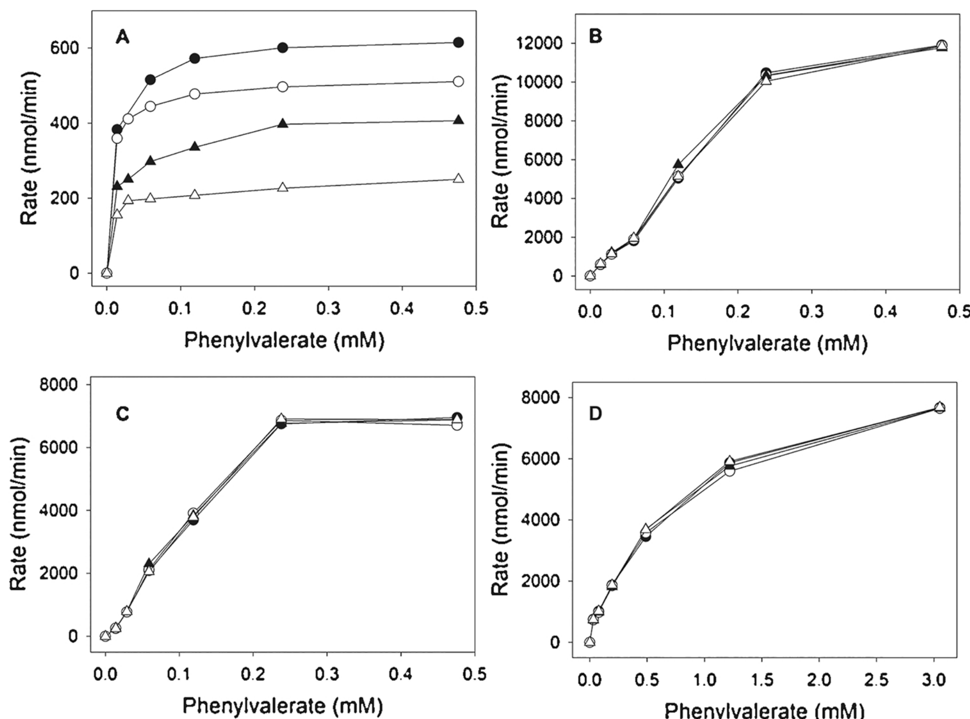

Fig. 1. Inhibition by acetylthiocholine of the PVase activity of the membranes pre-treated under conditions A, B, C and D described in Table 2. The procedure was followed with a 200 µl diluted tissue preparation containing chicken brain membrane. Panel A, 30 mg of fresh tissue per ml. Panels B, C, and D, 15 mg of fresh tissue per ml. Substrate concentrations (phenyl valerate): Panel A, 0.03, 0.08, 0.2, 0.3, 1.2 and 3 mM in 420 µl of the enzyme-substrate reaction volume; Panels B, C, and D 0.01, 0.03, 0.06, 0.12, 0.24 and 0.48 mM. The inhibitor concentrations (acetylthiocholine) were: Panel A, B and C, 0, 1.42, 3.33 and 7.14 mM; Panel D, 0, 0.7, 1.42, 2.14 mM; indicated by symbols △, ▲, ○ and ●, respectively.

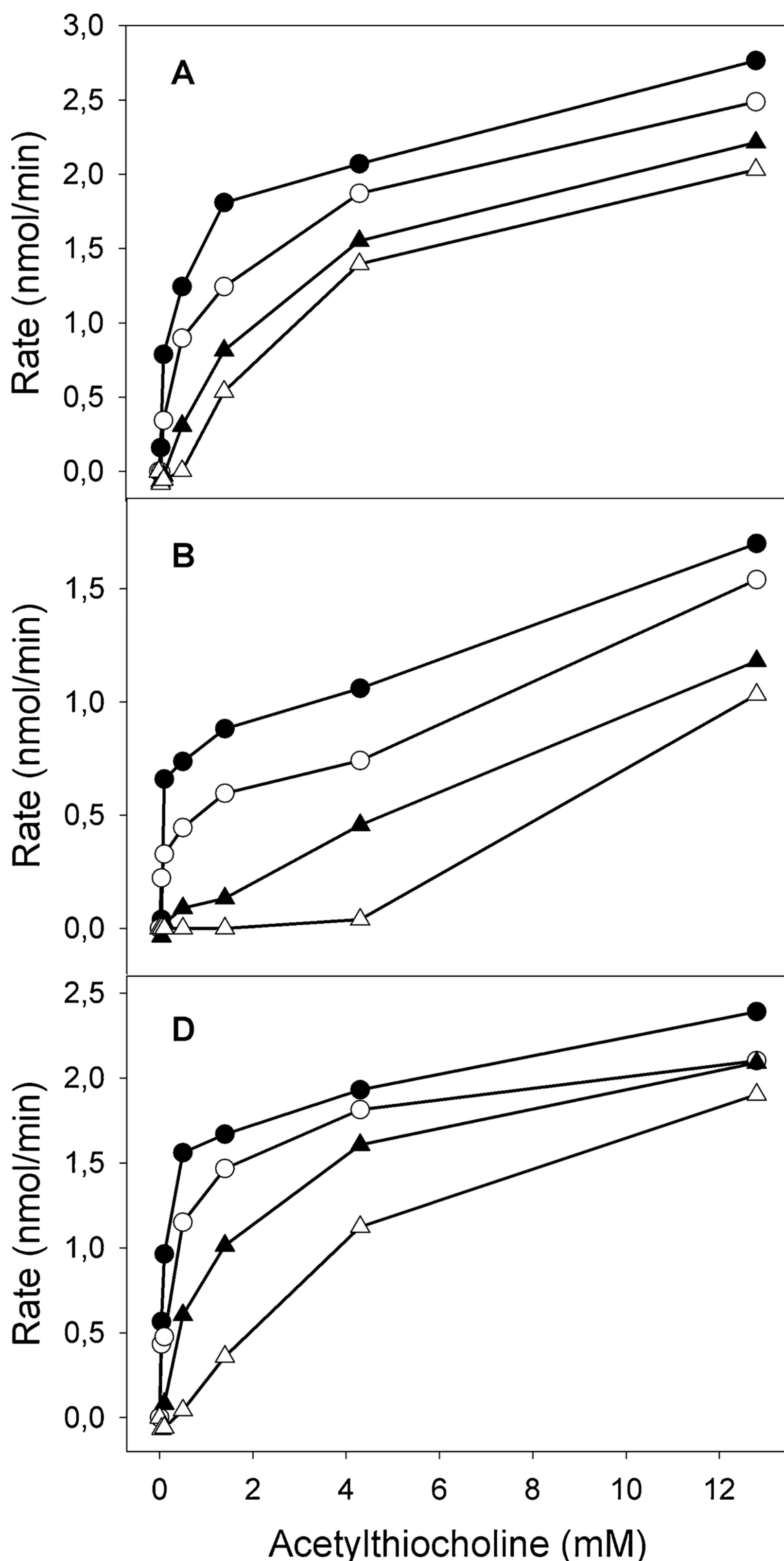

Fig. 3. Inhibition by phenyl valerate of the cholinesterase activity of the membranes pre-treated under conditions A, B and D described in Table 1. Condition C inhibited all ChE activity. The procedure was followed with a 200-μl diluted tissue preparation, which contained the chicken brain membrane fraction that corresponded to: Panel A, 20 mg, 25 mg and 15 mg of fresh tissue per ml for B and D, respectively. Substrate concentrations (acetylthiocholine): 0.04, 0.1, 0.5, 1.4, 4.3 and 12.8 mM in 420 μl of the enzyme-substrate reaction volume. The inhibitor concentrations (phenyl valerate) were 0, 1.4, 4.3 and 8.6 mM. Each point represents the mean of three replicates (SD < 5%).

studied.

The PVase activity measured under condition A in the membrane fraction showed no inhibition with 50 μM BW284C51. Therefore, no further studies were done with this inhibitor.

Fig. 2 shows the results of the interaction of iso-OMPA during the substrate reaction in the sample pretreated under conditions A and D to discriminate EPα and EPδ.

The preparation that contained the membrane fraction of 63 mg fresh tissue/ml in 200 μL was preincubated with 20 μL of inhibitor for conditions A and D, as indicated in Table 2. Afterward, 20 μL of μM iso-OMPA (final concentration of 900 μM) were added and incubated for 10 min. Then enzymatic activity was assayed by adding 200 μL of 0.56 mM phenyl valerate in ultrapure water to be incubated for 10 min. The reaction was stopped by adding 200 μL of 2% SDS/1.23 mM AAP

solution and 1.21 mM potassium ferricyanide, following the description indicated in Section 2.5. The percentage of activity referred to activity without iso-OMPA.

Note that EPδ was measured under condition D, while the mixture of EPα + EPδ was observed under condition A. Therefore, EPδ was estimated by the A–D difference, which was zero when pretreated with iso-OMPA. It was concluded that EPα was almost completely inhibited by 900 μM iso-OMPA, while EPδ was not completely inhibited and was, thus, less sensitive than EPα.

3.3. Behavior of the cholinesterase activities of the membrane in the presence of phenyl valerate

Cholinesterase activity was measured under the same A, B, C and D conditions used to discriminate the PVase components, and the effects of phenyl valerate on the observed cholinesterase were tested.

Fig. 3 shows the Michaelis-Menten graphics of the data obtained in each experiment.

No activity was found under condition C (25 μM paraoxon pre-incubation). The cholinesterase activities observed under conditions A (resistant to 1000 μM PMSF), B (50 μM mipafox) or C (600 μM PMSF + 25 μM mipafox) showed how the rate lowered when the phenyl valerate concentration increased. It was not possible to reach Vmax at the highest tested acetylthiocholine concentration.

3.4. Fixed-time inhibition curves of ChE activity with irreversible inhibitors in a brain membrane fraction

Chicken brain membrane fractions were incubated by mipafox, paraoxon, PMSF or iso-OMPA for 30 min at 37 °C. The fixed time inhibition curves of cholinesterase activity were fitted with exponential decay models for inhibition with no spontaneous reactivation of one, two, three, four or five sensitive enzymatic components, either with or without a resistant fraction, as indicated in the Materials and Methods. The results are shown in Fig. 4 and the proportions of the components, their kinetic parameters and the I50 values are found in Table 3.

The best-fitting model (according to the F-test) consisted of two sensitive enzymatic components when iso-OMPA was used as the inhibitor.

When mipafox or paraoxon were assayed, the best-fitting model consisted of one sensitive enzymatic component and another resistant one, which can be considered negligible because total activity was around 2.7–3.8%.

When PMSF was used as the inhibitor, the best-fitting model (according to the F-test) consisted of one sensitive enzymatic component.

3.5. Inhibition curves of cholinesterase activity with reversible inhibitors in a brain membrane fraction

Chicken brain membrane fractions were incubated by ethopropazine and BW284c51 for 30 min at 37 °C. The inhibition curves of cholinesterase activity were fitted with models for reversible inhibition (Copeland, 2000, 2005) of one, two, three, four or five sensitive enzymatic components, either or without a resistant fraction (see the Materials and Methods)

The results are shown in Fig. 4 and the proportions of the different components and kinetic parameters obtained for the best fitting model according to the F-test are provided in Table 3.

Two enzymatic components were discriminated with ethopropazine: a sensitive one and a resistant one. Three enzymatic components were estimated in the inhibition with BW284c51: two sensitive ones and a resistant one.

4. Discussion

This work studied the interaction of acetylthiocholine with PVase activity and the interaction of phenyl valerate with ChE activity of the chicken brain membrane fraction to establish if a relationship exists between both enzymatic activities.

The membrane PVase activity components were discriminated in this work by pre-incubating with PMSF, mipafox, paraoxon or PMSF + mipafox to irreversibly inhibitPVase activity to discriminate the enzymatic components, (EPα, EPβ, EPγ and EPδ). The concentrations employed to discriminate the membrane enzymatic components of PVase activity were based on the kinetic properties reported by Mangas et al. (2012b; 2014). In those works, EPα was the activity resistant to PMSF, but was sensitive to mipafox or paraoxon. EPβ was resistant to mipafox, but sensitive to paraoxon or PMSF related to the known NTE. EPγ was resistant to paraoxon, but sensitive to mipafox or PMSF. EPδ was the resistant activity to all the inhibitors related to IRE.

In the present work, the membrane PVase components were measured as the residual activity under conditions A, B, C and D shown in Table 2. Under condition D, only EPδ was measured. However, it was not possible to study isolated EPα, EPβ and EPγ because EPδ was resistant to all the used inhibitors. Nevertheless, they could be evaluated by studying the activity under condition A, B or C by taking into account that EPδ was included.

The fixed-time inhibition experiments with irreversible inhibitors were performed in membrane acetylthiocholine-hydrolyzing activities to discriminate the enzymatic components and to study if a relationship between PVase activity and ChE activity existed.

4.1. A membrane PVase activity component (EPα) shows inhibition with acetylthiocholine

From the observations made in this work, it was deduced that membrane PVase component EPα was inhibited by acetylthiocholine (Fig. 1), while the other PVase components (EPβ, EPγ and EPδ,) did not interact with acetylthiocholine.

EPδ PVase activity (measured under condition D) did not interact with acetylthiocholine. This behavior was also found in the PVase activities under condition B or C, representing EPβ + EPδ or EPγ + EPδ. According to these results, it was deduced that PVase components EPβ, EPγ and EPδ do not interact with acetylthiocholine (Fig. 1).

The PVase activity of the membrane measured under condition A, representing components EPα + EPδ, was inhibited by the presence of acetylthiocholine in the medium, similarly to the non-competitive inhibition model, where Vmax lowered without Km being altered. According to the results obtained for component EPδ (no interaction with acetylthiocholine), it was deduced that PVase component EPα interacted with acetylthiocholine in a more complex interaction than a competitive inhibition (see Fig. 1A). It was not possible to estimate the Km and Ki of component EPα because the obtained kinetic data were the sum of the activities of both PVase components Epα + EPδ. Furthermore, both components were resistant to BW284c51 and more resistant to ethopropazine than human butyrilcholinesterase in cortex and plasma (I50 of 0.21 and 0.3 μM, respectively; Atack et al., 1989).

EPγ is paraoxon-resistant, sensitive to micromolar concentrations of mipafox and meets the operational criteria of being "NTE" (Mangas et al., 2012b). EPγ did not interact with acetylthiocholine. Escudero and coworkers (Escudero et al., 1997) have proposed that SNTE-1 (soluble component Eγ) to be a membrane-bound NTE form, solubilized at pH 8.0, but not at pH 6.8, following the homogenization procedure. However, this soluble component Eγ interacted with acetylthiocholine. Therefore, such behavior revealed that membrane component EPγ (NTE) and soluble component Eγ (S-NTE1) could be different enzymatic entities.

4.2. Is there a relationship between PVases activities and cholinesterases?

Conversely, phenyl valerate inhibited the resistant cholinesterase activity measured under conditions B, C and D, which suggests that

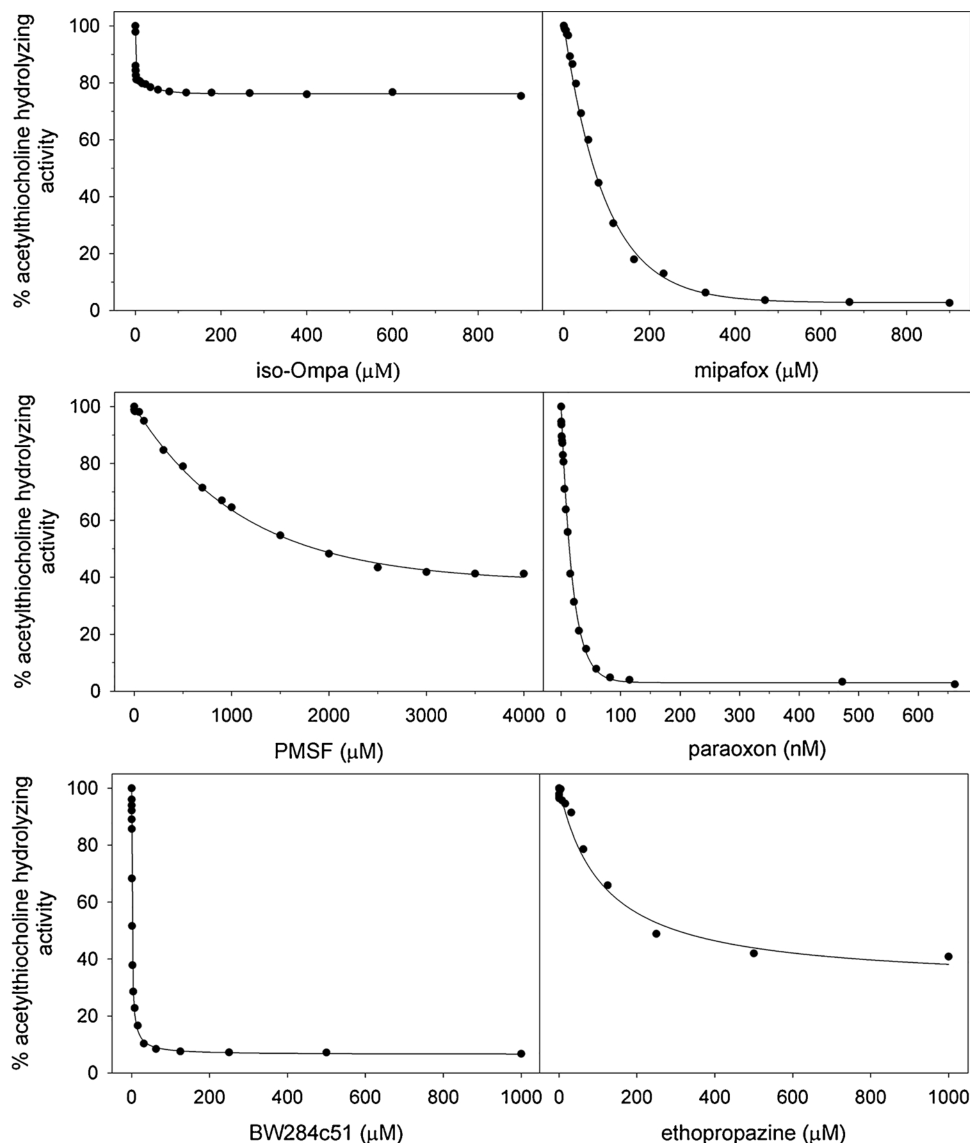

Fig. 4. The 30-min fixed-time inhibition curve of cholinesterase activity in brain membranes. A 200-μl volume of brain membrane fraction from 21 mg tissue/ml was incubated with iso-OMPA, mipafox, PMSF, paraoxon, BW284c51 or ethopropazine for 30 min at 37 °C. Residual cholinesterase activity was measured. Curves were fitted to a model with the sensitive and resistant components shown in Table 1. Each point represents the mean of three replicates (SD $<$ 5%).

Table 3
Kinetic constants (k1 and k2) and the proportions of components of cholinesterase activity, obtained from the different inhibition experiments run with PMSF, iso-OMPA, mipafox, ethopropazine, BW284c51 or paraoxon on membrane brain fractions. I50 is deduced from the rate constant (k1 or k2) for 30 min inhibition time, except for ethopropazine and BW284C51 for which the inhibition is not time dependent. The chemical hydrolysis constant for PMSF is shown as kh. Two values from two independent experiments are given.

	Component 1 (most sensitive)			Component 2 (less sensitive)			Resistant Component	Degradation compound
	E1 (%)	k1 (M^{-1} min^{-1})	I_{50} (μM)	E2 (%)	k2 (M^{-1} min^{-1})	I_{50} (μM)	E3 (%)	kh (min^{-1})
PMSF	61.7/ 61.6	$1.0·10^2$/ $9.6·10^1$	847/ 839	–	–	–	38.3/ 38.4	0.108/ 0.100
				–	–	–		
iso-OMPA	18.3	$1.12·10^5$	0.21	5.5	$8.0·10^2$	28.8	76.2	–
mipafox	96.8/ 96.3	$3.0·10^2$/ $2.5·10^2$	67.3/ 90.1	–	–	–	2.7/ 3.2	–
				–	–	–		–
paraoxon	97.0/ 96.2	$1.75·10^6$/ $1.96·10^6$	0.013/ 0.012	–	–	–	3.0/ 3.8	–
				–	–	–		–
ethopropazine	69.0/	–	115.0/	–	–	–	31.0/	–
	83.1	–	171.8	–	–	–	16.9	–
BW284C51	17.9/	–	7.7/	75.1/	–	0.8/	7.0/	–
	22.2	–	5.4	71.3	–	0.6	6.5	–

some chicken brain cholinesterases might possess PVase activity (Fig. 3). No cholinesterase activity was observed for condition A. AChE was reported to be inhibited by acetylthiocholine in excess (Szegletes et al., 1999). However, under our experimental conditions, cholinesterase activities were not inhibited at the highest acetylthiocholine concentration (Fig. 3) and Vmax was not reached in all the experiments. Thus, our interpretation is that these cholinesterase activities are due to similar enzymes to BuChE, or to similar enzymes, because BuChE often shows substrate activation at high substrate concentrations (Tormos et al., 2005).

PVase component EPα is more sensitive to iso-OMPA than EPδ (Fig. 2) and resistant to BW284c51. This finding indicates that it might be, or may contain, BuChE. However, these conclusions have to be carefully considered because this component showed low sensitivity to ethopropazine, and iso-OMPA can inhibit other activities apart from BuChE activity. Furthermore, different sensitivity to reversible inhibitors can be expected because reversible inhibition depends on the nature of the substrate, and on the interaction between the substrate and inhibitor.

4.3. Cholinesterase activity components in membrane fractions of chicken brain discriminated with irreversible inhibitors

It is possible to discriminate several enzymatic components by taking into account the proportions (percentages) obtained in the inhibition experiments as a whole (Estévez et al., 2010, 2011, 2012; Mangas et al., 2011, 2012, 2012b, 2014).

Fig. 5 is showing a schematic summary with our interpretation of the detected components of cholinesterase activity as deduced from the results of experiments with the different inhibitors showed in Table 3. Globally considered, 4 cholinesterase components were deduce named as CP1, CP2, CP3 and CP4.

Two sensitive ChE enzymatic components were detected in the inhibition experiment with iso-OMPA: CP1 (18.3%) very sensitive and other CP2 (5.5%) less sensitive. The remaining activity (76.2%) was resistant, like the usually known acetylcholinesterases in mammals (Table 3, Fig. 5).

With BW284c51, about 71–75% was highly sensitive, which matched the resistant proportion to iso-OMPA, while about 18–22% was less sensitive and others resistant (7%), which matched the same proportion of components CP1 and CP2 discriminated with iso-OMPA.

About 62% of ChE activity was sensitive to PMSF and the remaining percentage was resistant. This suggests that the 72% resistant to iso-OMPA (and highly sensitive to BW284c51) seems to contain two enzymatic components: CP3 and CP4 (see Fig. 5).

Considering the properties of component EPα PVase, which is inhibited by acethylthiocholine, is resistant to PMSF and BW284c51, and is sensitive to iso-OMPA, we hypothesize that components CP1 and CP2 ChE form part of PMSF-resistant cholinesterase activity. Therefore, the remaining 15% could form part of the iso-OMPA resistant component, which we call CP3. Thus CP4 would be the 62% activity that is sensitive to PMSF and resistant to iso-OMPA (see Fig. 5).

Therefore, if we take all these results into account, we hypothesize that at least four components can be considered in the membrane cholinesterase activity of the brain (Fig. 5):

- CP1 (18%) is highly sensitive to iso-OMPA, sensitive to mipafox, BW284c51 and paraoxon, but resistant to PMSF;
- CP2 (5.5%) is sensitive to iso-OMPA, to mipafox and paraoxon, but is resistant to PMSF and BW284c51;
- CP3 (15%) is resistant to iso-OMPA, BW284c51 and PMSF, but is sensitive to mipafox and paraoxon;
- CP4 (62%) is resistant to iso-OMPA, highly sensitive to BW284c51 and sensitive to mipafox, paraoxon and PMSF.

The I50 of the inhibition by mipafox or paraoxon on total membrane cholinesterase activity was comparable to the I50 reported by Lotti and Johnson (1978). However, it was not possible to discriminate different enzymatic components with these inhibitors.

According to sensitivity to iso-OMPA, it was possible to establish a relationship among CP1, CP2 and BuChE, while CP3 and CP4 could be considered different isoforms of AChE or other enzymes that hydrolyze acetylcholine. The I50 obtained for the inhibition of components CP1 and CP2 with iso-OMPA was comparable to BuChE in human cortex and

<table>
<tr><th>COM-PONENT</th><th>iso-OMPA (I50 μM) (30 min)</th><th>BW284c51 (I50 μM)</th><th>PMSF (I50 μM) (30 min)</th><th>paraoxon (I50 μM) (30 min)</th><th>mipafox (I50 μM) (30 min)</th></tr>
<tr><td>CP1 18-22 %</td><td>E1 18% (0.21 μM)</td><td>E1 18/22% (7.7/5.4 μM)</td><td rowspan="3">E3 38/38% (Resistant)</td><td rowspan="4">E1 96/97% (0.012/0.013 μM)</td><td rowspan="4">E1 96/98%) (67/90 μM)</td></tr>
<tr><td>CP2 5.5-7.0 %</td><td>E2 5.5% (28.8 μM)</td><td>E3 6/7% (Resistant)</td></tr>
<tr><td>CP3 10-15 %</td><td rowspan="2">E3 76% (Resistant)</td><td rowspan="2">E2 71/75% (0.8/0.6 μM)</td></tr>
<tr><td>CP4 61-62 %</td><td>E1 61/62% (847/839 μM)</td></tr>
</table>

Fig. 5. Discrimination of four enzymatic components in the cholinesterase activity of membrane brain fraction by considering globally all the inhibition parameters with iso-OMPA, BW284c51, PMSF, mipafox and paraoxon showed in Table 3. Left column are showing the deduced components with the range of estimated proportion of the total activity. The discriminated component in each experiment with individual inhibitors are showed ordered for showing in parallel the corresponding components observed with the other inhibitor. Inside each box, the corresponding related component with the related values in Table 3 are indicated.

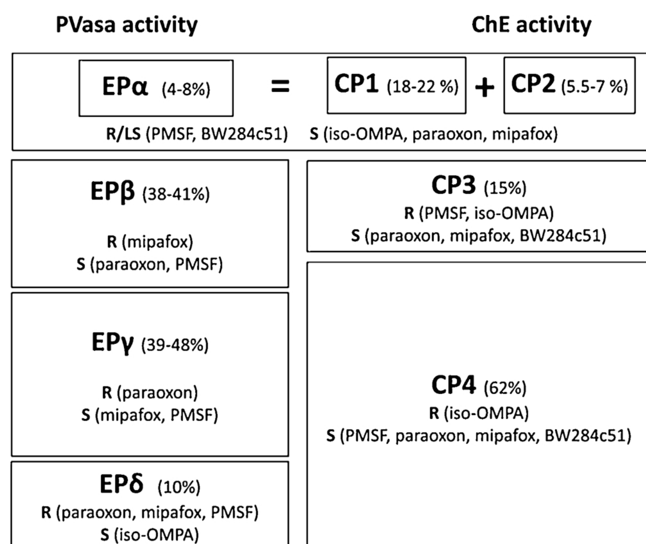

Fig. 6. Components of PVase and ChE activities. Each box represents a PVase or cholinesterase enzymatic component with the characteristic sensitivity to inhibitors indicated as follows: R = resistant; S = sensitive; LS = low sensitive. The properties indicated for the PVase components are based on the previously published data referred in Table 1. The indicated properties for the corresponding cholinesterase components are based on the data showed in Table 3 and summarized in Fig. 5, and the observed substrate interactions described in Figs. 2 and 3 are taken into consideration. Note that % values are over their respective activity and therefore, the values for PVase cannot be correlated with those for AChE end vice versa.

plasma (6.7 and 1 mM, respectively; Atack et al., 1989). The I50 obtained for CP3 and CP4 in the inhibition with this inhibitor was comparable to the I50 obtained for AChE in human erythrocytes and cortex, and electric eel (340, 180 and > 1000 mM, respectively; Atack et al., 1989).

However, CP1 and CP2 were resistant to inhibition with PMSF, and CP3 could be considered resistant to PMSF inhibition if its PMSF I50 is compared to the I50 deduced for mouse AChE (I50 28.1 μM; Kraut et al., 2000). Mouse BuChE and AChE were inactivated by PMSF, but Torpedo californica AChE was resistant to PMSF inhibition (Kraut et al., 2000). Chicken BuChE was able to interact with PMSF in a different manner to mouse BuChE, which occurs with AChE of different species.

Two components were discriminated with ethopropazine in cholinesterase activity: one is by sensitive around 69–73 % and other is resistant by around 17–34 %. The sensitive component showed a higher I50 of ethopropazine than I50 for BuChE in human cortex and plasma (0.21 and 0.3 μM, respectively), and showed a similar I50 to AChE in human erythrocytes and cortex, and electric eel (260, 210 and 120 μM, respectively) as reported by Atack et al. (1989) for 30 min preincubation (temperature not reported). However, the I50 estimated in this work were comparable to inhibitory profile reported for BuChE in chicken brain (Treskatis et al., 1992), which was estimated to lie between 10 and 100 μM, and to take a higher value if compared to human BuChE. Note that comparison of I50 values could be done only approximately as in some articles; temperature of time preincubation is not reported.

We suggest that the discrimination of the main cholinesterase activity components in a membrane brain fraction can be performed by simple assay testing inhibition for 30 min under the following conditions: (A) no inhibitors: (B) 1.5 μM iso-OMPA; (C) 191 μM iso-OMPA; (D) 5.6 mM PMSF; (E) 5.6 mM PMSF + 191 μM iso-OMPA. Components may be estimated according to: CP1 = A–B; CP2 = B–C; CP3 = A–D; and CP4 = A–E.

4.4. Relationship between PVase and cholinesterase enzymatic components

EPα is the PVase enzymatic component in a chicken brain membrane fraction that is sensitive to mipafox and paraoxon, but is resistant to PMSF. The present work also shows that EPα PVase activity interacts with acetylthiocholine, is sensitive to iso-OMPA, shows low sensitivity to ethopropazine and is resistant to BW284c51.

The general inhibitory profile PVase and cholinesterase activity components are showed in a simplified summary in Fig. 6, indicating to which compound, the component is resistant (R) or sensitive (S). For that, we compared the inhibitory properties of the cholinesterase activity showed in this paper (Table 2, Figs. 3 and 4, and summarized in Fig. 5), versus the inhibitory properties of the PVase components described by Mangas et al (2014), summarized in Table 1 for paraoxon, mipafox and PMSF and in this paper for iso-OMPA in Fig. 2.

The profile of EPα correlates well with the profile of membrane ChE activity components CP1 and CP2, which were discriminated with iso-OMPA and had similar I50 to BuChE in human cortex and plasma, and a lower I50 than the obtained for AChE in human erythrocytes and cortex, and electric eel (Atack et al., 1989).

4.5. Conclusions

According to the approach of this work, four enzymatic components (CP1, CP2, CP3 and CP4) were discriminated in the ChE activity of a brain membrane fraction. Our approach establishes a relationship between cholinesterase activity components CP1 and CP2, and PVase activity component EPα, but they could be different isoforms of BuChE, while CP3 and CP4 could be molecular structures of AChE, and no relationships with PVase activity have demonstrated. With the approach of this work, although we cannot exclude them to have some PVase activity, bur the different inhibitory properties allow to conclude that CP3 and CP4 are different entities that the reported PVase components.

The results of this paper have clarified the profile of the PVase and ChE activity components in the brain membrane fraction of chicken, the animal model for testing the OP delayed neurotoxicity; This will allow further research for the molecular identification of proteins interacting with OP and understanding their neurotoxicity under a multi-target approach of their adverse output pathways.

Further studies are needed to molecularly identifying all the discriminated cholinesterase activity components in soluble and membrane fractions of chicken brain.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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