Arch Toxicol (2017) 91:3295–3305
DOI 10.1007/s00204-017-1946-5

TOXICOKINETICS AND METABOLISM

Phenyl valerate esterase activity of human butyrylcholinesterase

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Received: 28 October 2016 / Accepted: 23 February 2017 / Published online: 15 March 2017

Abstract Phenyl valerate is used for detecting and measuring neuropathy target esterase (NTE) and has been used for discriminating esterases as potential target in hen model of organophosphorus delayed neuropathy. In previous studies we observed that phenyl valerate esterase (PVase) activity of an enzymatic fraction in chicken brain might be due to a butyrylcholinesterase protein (BuChE), and it was suggested that this enzymatic fraction could be related to the potentiation/promotion phenomenon of the organophosphate-induced delayed neuropathy (OPIDN). In this work, PVase activity of purified human butyrylcholinesterase (hBuChE) is demonstrated and confirms the novel observation that a relationship of BuChE with PVase activities is also relevant for humans, as is, therefore the potential role in toxicity for humans. The K_M and catalytic constant (k_{cat}) were estimated as 0.52/0.72 μM and 45,900/49,200 min^{-1} respectively. Furthermore, this work studies the inhibition by preincubation of PVase and cholinesterase activities of hBuChE with irreversible inhibitors (mipafox, iso-OMPA or PMSF), showing that these inhibitors interact similarly in both activities with similar second-order inhibition constants. Acethylthiocholine and phenyl valerate partly inhibit PVase and cholinesterase activities, respectively. All these observations suggest that both activities occur in the same active center. The interaction with a reversible inhibitor (ethopropazine) showed that the cholinesterase activity was more sensitive than the PVase activity, showing that the sensitivity for this reversible inhibitor is affected by the nature of the substrate. The present work definitively establishes the capacity of BuChE to hydrolyze the carboxylester phenyl valerate using a purified enzyme (hBuChE). Therefore, BuChE should be considered in the research of organophosphorus targets of toxicity related with PVase proteins.

Keywords NTE (neuropathy target esterase) · Phenyl valerate esterases; serine esterases; acetylcholinesterase · Butyrylcholinesterase · Acetylthiocholine · Organophosphorus compounds

Abbreviations

AChE	Acetylcholinesterase
AtCh	Acetylthiocholine
BuChE	Butyrylcholinesterase protein
ChE	AtCh hydrolyzing activity
DTNB	5,5′-Dithio-bis-2-nitrobenzoate
hBuChE	Human butyrylcholinesterase
k_{cat}	Catalytic constant
NTE	Neuropathy target esterase
OP	Organophosphorus
OPIDN	Organophosphate-induced delayed neuropathy
PMSF	Phenylmethylsulfonyl fluoride
PV	Phenyl valerate
PVase	Phenyl valerate esterase
SDS	Sodium dodecyl sulfate

Introduction

Exposure to organophosphorus (OP) esters can cause several toxic effects, including acute cholinergic clinical episodes, intermediate syndrome, organophosphate-induced

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delayed neuropathy (OPIDN) and chronic neurological effects. Other toxic effects with unknown molecular targets have been described (COT 1999; Jamal et al. 2002).

Several observations support the view that OPs have additional non-acetylcholinesterase (AChE) targets (Lockridge and Schopfer 2006; Terry 2012), which emerge especially given the limited correlation between certain behavioral responses and the magnitude and regional selectivity of AChE inhibition in the brain (McDaniel and Moser 2004). The wide variety of long-term neuropsychiatric symptoms that have been associated with OP exposures suggests that other non-AChE targets might be implicated (Terry 2012). Linking these sets of OP pesticide-binding proteins to their toxicological relevance is needed to understand low-dose, long-term OP neurotoxicity and OP detoxification pathways (Lockridge and Schopfer 2006; Costa 2006). However, the effects of low doses for long-term exposure remain unclear (Sogorb and Vilanova 2010).

Phenyl valerate (PV) was the substrate used in the identification and characterization of neuropathy target esterase (NTE; Johnson 1975; Chemnitius et al. 1983; Carrington and Abou-Donia 1984; Vilanova et al. 1990; Glynn et al. 1994, 1998) and other serine hydrolases of neural tissue and brain (Céspedes et al. 1997; Escudero et al. 1997; Barril et al. 1999; Estévez et al. 2004, 2010, 2011).

In soluble brain fraction of chicken, three enzymatic components with phenyl valerate esterase (PVase) activity have been discriminated using irreversible inhibitors as follows: mipafox (OPIDN-inducer), paraoxon (non OPIDN-inducer) and phenylmethylsulfonyl fluoride (PMSF) (Mangas et al. 2011, 2012): Eα, Eβ, and Eγ. PMSF is not an OP but is an irreversible NTE inhibitor that protects 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 (Pope et al. 1990; Lotti et al. 1991). Mangas and coworkers (Mangas et al. 2012) suggested that the inhibitory kinetic properties of Eα PVase activity with organophosphates are compatible with being a target of the potentiation/promotion phenomenon of PMSF on OPIDN and for understanding the effects of low-dose exposures to OPs. Benabent et al. (2014a, b) showed that PV (the substrate of PVase activity) partially inhibited cholinesterase activity in soluble brain fractions of chicken, and vice versa, acetylthiocholine (AtCh) shows some inhibition on PVase activity components. This scenario suggested that PVase components may contain enzymes that hydrolyze acetylcholine. By a proteomic analysis, we demonstrate that a fraction enriched with the Eα component of PVase activity contains butyrylcholinesterase (BuChE; Mangas et al. 2016).

To clarify if the relationship of BuChE with PVase activity is also relevant for humans, here we test and demonstrate that purified human butyrylcholinesterase (hBuChE) is able to hydrolyze PV. The relationship between the catalytic center of PVase activity and its AtCh hydrolyzing activity (ChE) is evaluated by assessing the interactions between substrates and inhibitors.

Materials and methods

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%) acetylthiocholine iodide (purity ≥ 98) phenylmethylsulfonyl fluoride (PMSF), paraoxon, tetraisopropylpyrophosphoramide (iso-OMPA) and 10-(2-diethylaminopropyl) phenothiazine (ethopropazine) were purchased from Sigma (Madrid, Spain). *N, N*′-di-isopropylphospho rodiamidefluoridate (mipafox, purity > 98%) and phenyl valerate were attained from Lark Enterprise (Webster, MA, USA). All the other reagents were obtained from Merck SL (Madrid, Spain) and were of analytical grade.

Solutions

"Phosphate buffer", which is mentioned throughout contained 0.1 M phosphate, pH 7.4, 1 mM EDTA. A stock solution of inhibitors: 10 mM mipafox was prepared in 10 mM Tris–citrate buffer (pH 6.0); 50 mM PMSF in dimethylsulfoxide; 10 mM paraoxon in dried acetone; 10 mM iso-OMPA in dried acetone; 10 mM ethopropazine in water. All the inhibitors were diluted to the appropriate concentration in water immediately before the kinetic assays.

A stock solution of substrate PV (168 mM) was prepared in dried *N, N*-diethylformamide, and was diluted in water at the concentrations indicated in each assay immediately before the enzymatic assays. AtCh was prepared in water before use at the concentrations indicated in each assay immediately before the enzymatic assays.

To stop the enzymatic reaction and color development, the following solutions were prepared. SDS-AAP solution (for PVase activity): a 2% SDS (sodium dodecyl sulfate) that contained 1.23 mM aminoantypirine in phosphate buffer. SDS-DTNB solution (for ChE activity): a 2% SDS solution that contained 6 mM DTNB in phosphate buffer.

Enzyme

Purified human BuChE isolated from human plasma was a gift from Dr David Lenz and Dr Douglas Cerasoli [USAMRICD (US Army Medical Research Institute of Chemical

Defense), Aberdeen Proving Ground, MD, USA]. All the enzyme concentrations refer to the concentration of catalytic sites, i.e., monomers.

Measure of esterase activities

PVase activity and ChE activity were both inhibited and measured by a similar strategy and procedure to enable the comparison of the response to inhibitors and to evaluate the interaction between the substrates under comparable conditions. Enzyme preparation was pre-incubated with the inhibitor for the desired time (needed for irreversible inhibitor), the substrate was added for a fixed time of 10 min and the reaction was stopped with a mixture that contained SDS plus the color reagent (SDS-AAP or SDS-DTNB solutions for PVase or ChE, respectively).

An automated Work Station (Beckman Biomek 2000) was employed for pipetting and incubating process with inhibitors and substrates.

PVase activity

PVase activity was measured according to Mangas et al. (2011) by following a procedure based on the colorimetric method for the NTE assay developed by Johnson (1977), and using an automated Work Station (Beckman Biomek 2000) for the full procedure as follows: samples of 200-µl volume of the enzyme preparation of 2 nM human butyrylcholinesterase (buffer in blanks for spontaneous hydrolysis) were incubated with 200 µl of PV at the concentration indicated in each experiment. The mixture was incubated for 10 min at 37 °C for the enzyme reaction with the substrate. The reaction was stopped by adding 200 µl of SDS-AAP solution and after mixing. Next 100 µl of 1.21 mM potassium ferricyanide was added and left for 5 min for color development. A 300-µl volume from each microtube was transferred to a 96-well microplate. Absorbance was read at 510 nm in a microplate reader (Beckman Coluter AD 340). Blanks for the spontaneous hydrolysis (samples without hBuChE) were included and the same procedure was applied.

ChE activity

Assays were carried out according to Benabent et al. (2014b). Samples of a 200-µl volume of the enzyme preparation of 4 nM hBuChE (buffer in the blanks) were incubated with 200 µL of AtCh in ultrapure water at the concentration indicated in each experiment for 10 min at 37 °C to measure enzymatic activity. The enzymatic reaction was stopped by adding 200 µl of SDS-DTNB solution. Then 200 µL of phosphate buffer (diluted enzyme preparation in the blanks) was added. The final assay volume was 800 µL. After mixing and waiting for at least 5 min, a 300-µL volume from each microtube was transferred to a 96-well microplate to read absorbance at 410 nm.

Interactions with inhibitors

For the irreversible inhibitors (mipafox, iso-OMPA, PMSF), the inhibitor was preincubated for 30 min with the enzyme preparation before adding the substrate to measure residual activity. With the reversible inhibitor (ethopropazine) the inhibitor and substrate were added simultaneously. For the substrates interaction, both substrates PV and AtCh were added simultaneously. Then after running the enzyme substrate reaction for 10 min, PVase or ChE activity was measured. The specific conditions are shown for each experiment in the Results or in the legends of figures.

Mathematical models

Irreversible inhibitors

Exponential decay models were fitted to the fixed-time inhibition data of PVase and ACth activity of one, two sensitive enzymatic components, or more, with or without a resistant fraction, using the version 8 of Sigma Plot software. The general model equation for inhibition is as follows:

$$E = E1_0 \cdot e^{-(k1 \cdot t \cdot I)} + E2_0 \cdot e^{-(k2 \cdot t \cdot I)} + \cdots + En_0 \cdot e^{-(kn \cdot t \cdot I)} + R$$

where $k1$, $k2$, $k3$,…, kn are the second-order inhibition constants; t is the inhibition time (30 min in fixed the inhibition time experiments); $E1_0$, $E2_0$,…, En_0 and R are the proportion (amplitude) of enzymatic components $E1$, $E2$, En,… and R, respectively. For the purpose of obtaining a coherent solution in the interactive computing estimation, some restrictions were applied: (1) all the parameters (rate constants and amplitudes) should have positive values (>0); (2) the following complementary restriction was also applied: $E1_0 + E2_0 + E3_0 + \cdots + En_0 + R = 100\%$.

The best fitting model (according to the F test) is shown in the "Results" section.

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

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

where ki is the second-order rate of inhibition.

If the spontaneous hydrolysis of PMSF was considered, then the general model was as follows (Estévez et al. 2012):

$$E = E1_0 \cdot e^{\left(e^{-kh \cdot t} - 1\right) \cdot \frac{k1}{kh} \cdot I_0} + E2_0 \cdot e^{\left(e^{-kh \cdot t} - 1\right) \cdot \frac{k2}{kh} \cdot I_0} + \cdots + En_0 \cdot e^{\left(e^{-kh \cdot t} - 1\right) \cdot \frac{kn}{kh} \cdot I_0} + R$$

where *kh* is the chemical hydrolysis constant of the PMSF and t is the inhibition time (30 min in the fixed inhibition time experiments). The I_{50} (30 min) values in the PMSF fixed-time inhibition experiments were obtained by applying the following equation:

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

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

Reversible inhibitors

For the reversible inhibitors (ethopropazine), the following model equations were applied:

When considering a sensitive enzymatic component, the mathematical model was as follows (Copeland 2000, 2005)

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

where I is the inhibitor concentration and I_{50} is the concentration that inhibits 50% of total activity.

When considering a sensitive enzymatic component plus other one resistant, the mathematical models were as follows.

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

When considering two sensitive enzymatic components and another one resistant, the mathematical models were as follows:

$$\%\text{Activity} = E_1 \cdot \frac{1}{1 + \frac{I}{I1_{50}}} + E_2 \cdot \frac{1}{1 + \frac{I}{I2_{50}}} + R$$

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

Determination of variability

Each point in the graphs represents the mean of three replicates (SD < 5%). Different experiments were done on distinct days with various preparations of enzyme, substrates and inhibitors. The "Results" section shows the kinetic parameters obtained in two independent experiments, while the figures show one of these experiments.

Results

Hydrolysis of PV by h-BuChE

Incubation of PV at concentrations within the 0.001–3.000 mM range with 2 nM hBuChE (1 nM in reaction volume) yielded a behavior of a rectangular hyperbola (plot with black circles in Fig. 1) and activity up to ~40 μM min^{-1} (up to 3 mM PV). The estimated V_{max}, K_M, and catalytic constant (k_{cat}) are shown in Table 1.

Hydrolysis of AtCh by hBuChE

Incubation of AtCh at concentrations within the 0.005–1 mM range with 4 nM hBuChE (2 nM in the reaction volume) showed activity up to ~53 μM·min^{-1}. Saturation was not reached under the experimental conditions (plot with black circles in Fig. 2), so it was not possible to estimate the V_{max} and K_M.

Inhibition of the PVase activity with AtCh

PVase activity was inhibited by 0.5 and 1 mM of AtCh (inhibition by 1 mM of AtCh was 7% in the presence of 0.5 mM PV).

Figure 1 shows the 3D fit to the data (Estévez et al. 2004) with the mathematical equation deduced from the

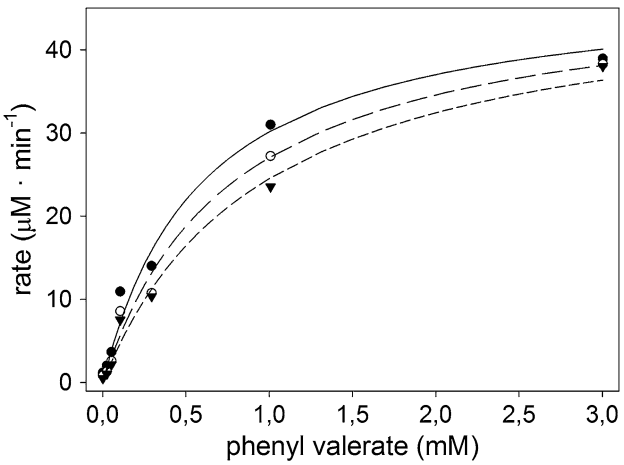

Fig. 1 Inhibition of PVase activity by AtCh. The procedure is described in "Materials and methods". PV concentrations (substrate): 0.00126, 0.0252, 0.0525, 0.105, 0.294, 1.008 and 3.003 mM in 420 μl of the enzyme–substrate reaction volume. Inhibitor concentrations (AtCh): 0 (*black circles*), 0.5 (*white circles*) and 1 mM (*black triangles*) in 420 μl of the enzyme–substrate reaction volume. The level *lines* obtained from 3D fit for 0, 0.5 and 1 mM of AtCh

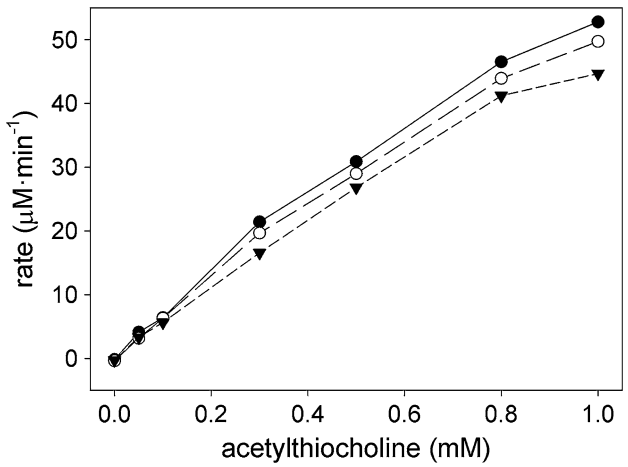

Fig. 2 Inhibition of ChE activity by PV. The procedure is described in the Materials and Methods. AtCh concentrations (substrate): 0, 0.05, 0.1, 0.3, 0.5, 0.8, and 1 mM in 420 µl of the enzyme–substrate reaction volume. PV concentrations: 0 (*black circles*), 0.5 (*white circles*) and 1 mM (*black triangles*) in 420 µl of the enzyme–substrate reaction volume

model of two substrates that compete for a same active site according to the Michaelis–Menten kinetic reaction (Cornish-Bowden 2004). This mathematical equation was as follows:

$$V = \frac{V_{\max} \cdot [S1]}{K_M1 \cdot \left(1 + \frac{[S2]}{K_M2}\right) + [S1]} \quad (1)$$

where $V_{\max}$ is the maximun rate, $S1$ is PV concentration, $S2$ the AtCh concentration, K_M1 is the Michaelis–Menten constant for PVase activity and K_M2 for ChE activity. The $V_{\max}$ estimated in two independent experiments was 48.0/53.8 µM min^{-1}. K_M1 was 0.59/0.88 mM and K_M2 was 1.62/1.95 mM.

Inhibition of ChE activity with PV

ChE activity was inhibited by 0.5 and 1 mM of PV (~19% inhibition for 0.05 mM AtCh and in the presence of 1 mM PV, and ~15% for 1 mM AtCh in the presence of 1 mM PV). As Fig. 2 illustrates, inhibition of ChE activity took place at all PV concentrations. The rate values did not reach a plateau, so it was not possible to estimate the $V_{\max}$ and Km for the AtCh and PV hydrolisis reactions.

PVase and ChE activities inhibition by iso-OMPA, mipafox and PMSF

hBuChE was pre-incubated at different concentrations with iso-OMPA (up to 1818 µM), mipafox (up to 50 µM) or PMSF (up to 50 µM) for 30 min at 37 °C. Then substrate (2.7 mM PV or 1 mM AtCh in the reaction volume) was added to measure residual PVase or ChE activity (Fig. 3 for PVase activity and Fig. 4 for ChE activity). Exponential decay models were fitted to the fixed time inhibition data of PVase or ChE activity.

The best fitting model (according to the '*F*' test) for iso-OMPA was $E = E_0 \cdot e^{-k \cdot 30 \cdot I}$ for both activities, which consisted of a sensitive component; for mipafox it was $E = E1_0 \cdot e^{-k1 \cdot 30 \cdot I} + E2_0 \cdot e^{-k2 \cdot 30 \cdot I}$ for both activities, which consisted of two sensitive components; for PMSF it was $E = E_0 \cdot e^{\left(\frac{ki}{kh}\right) \cdot I \cdot e^{-kh \cdot 30}}$ (Estévez et al. 2012) for both activities, which consisted of a sensitive component.

The kinetic parameters are shown in Table 2.

Inhibition by ethopropazine of ChE and PVase activities

hBuChE was incubated with ethopropazine and 1 mM AtCh (Fig. 5a) or 2.7 mM PV (Fig. 5b). The mathematical models used to fit the inhibition data were those shown in the "Materials and methods" section. The best fitting model (according to the '*F*' test) for ChE activity was as follows:

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

which consisted of a sensitive enzymatic component.

The best fitting model (according to the '*F*' test) for PVase activity was as follows:

$$\%\text{Activity} = E_1 \cdot \frac{1}{1 + \frac{I}{I_{50}}} + E_R$$

which consisted of a sensitive component plus a resistant one of around 3.5% of total activity.

The estimated *I*50s are shown in Table 2.

Discussion

The novelty of this work is that it has proven that human BuChE hydrolyzes PV. This work confirms the relevance to humans of previous published works done in a chicken brain fraction of Eα PVase activity (Benabent et al. 2014a, b; Mangas et al. 2016). PV is a substrate which has been used to detect and describe proteins considered potential targets of OP toxicity. It was also observed that AtCh inhibited PVase activity and PV inhibited ChE activity. Different irreversible inhibitors were used. The results of the inhibition experiments showed that these inhibitors interact similarly with both activities, which suggests that both are related with the same active catalytic center. However, ChE activity is more sensitive than PVase activity to reversible inhibitor ethopropazine.

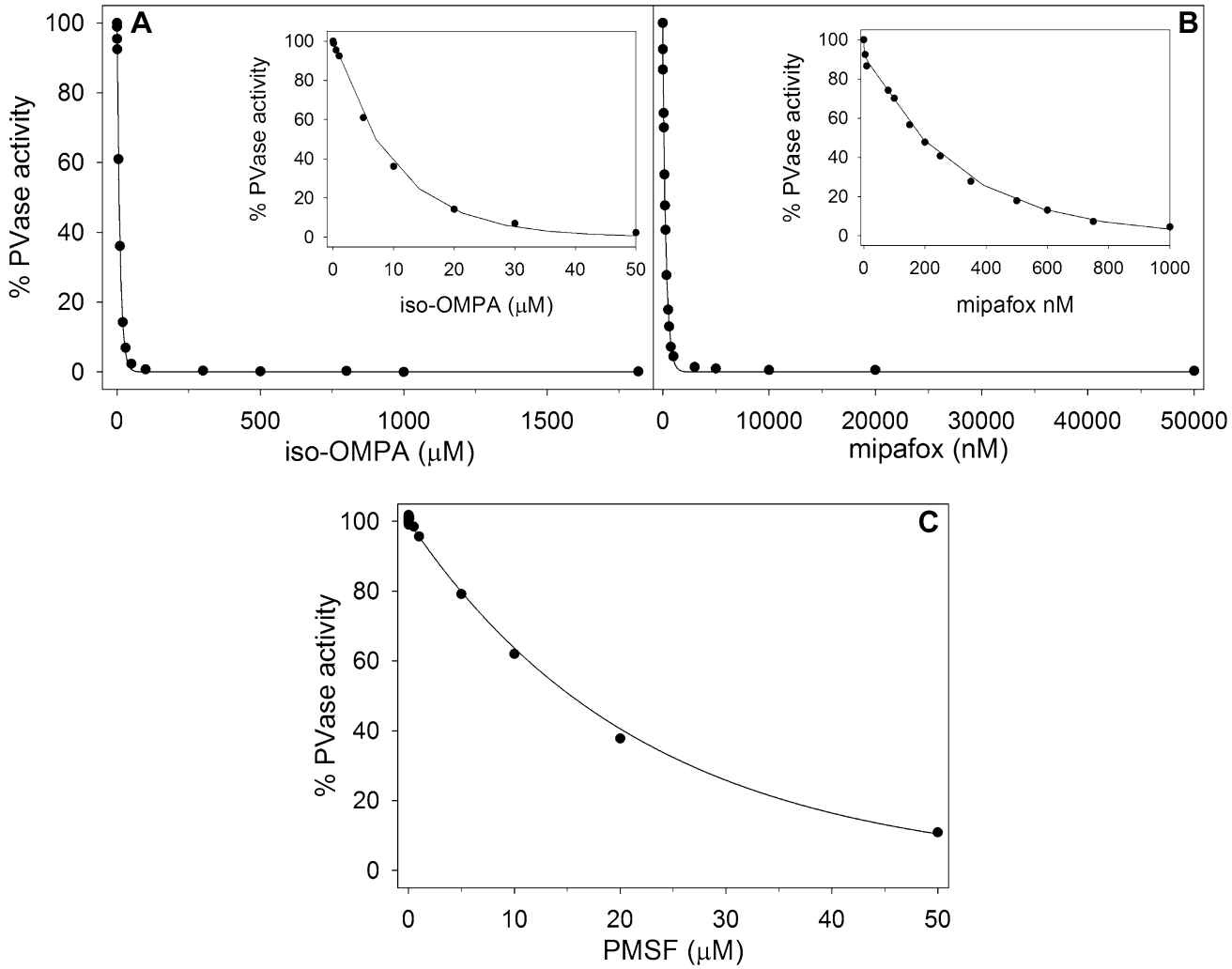

Fig. 3 Fixed time inhibition curve of PVase activity with irreversible inhibitors. A 100-µl volume of 0.2 nM hBuChE was incubated with a 10-µl volume of **a** iso-OMPA, **b** mipafox or **c** PMSF for 30 min at 37 °C, then the mixture was incubated with 100-µl volume of 5.4 mM PV for 10 min at 37 °C and residual PVase activity was measured. The *curves* show the model that best fitted the data provided in the "Results" section. *Each point* represents the mean of three replicates (SD < 5%). The *insets* in *panels* **a** and **b** show the inhibition within a low concentration range

Hydrolysis of PV by hBuChE

Mangas and coworkers (Mangas et al. 2011, 2012) kinetically distinguished an enzymatic component of PVase activity in chicken brain soluble fraction called Eα. This component interacted with AtCh (Benabent et al. 2014a, b) and was sensitive to mipafox (I50 for 30 min, 0.004 µM) and to paraoxon (I50 for 30 min, 0.009–0.011 µM), iso-OMPA and ethopropazine, but was resistant to PMSF and BW284C51. A subsequent molecular identification study showed that BuChE was the only candidate responsible for PVase activity in component Eα (Mangas et al. 2014, 2016).

The results of this work confirm that the human enzyme (hBuChE) also displays PVase activity. No activation or inhibition by the substrate was observed under the assay conditions.

Comparison with other reported substrates of hBuChE

Other substrates have been reported to be hydrolyzed by BuChE. The K_M estimated for PV (0.52/0.72 mM) was similar to the K_M obtained for the reaction with other substrates like o-nitrophenylacetate, α-naphthylacetate, propionylthiocholine, o-nitrophenylbutyrate, acetylcholine, AtCh and butyrylthiocholine at a high substrate concentration, but higher than the K_M of butyrylthiocholine and AtCh at low substrate concentration (Table 1). The estimated k_{cat} was similar to the k_{cat} estimated in the literature for the reaction with butyrylthiocholine, with AtCh, phenylacetate,

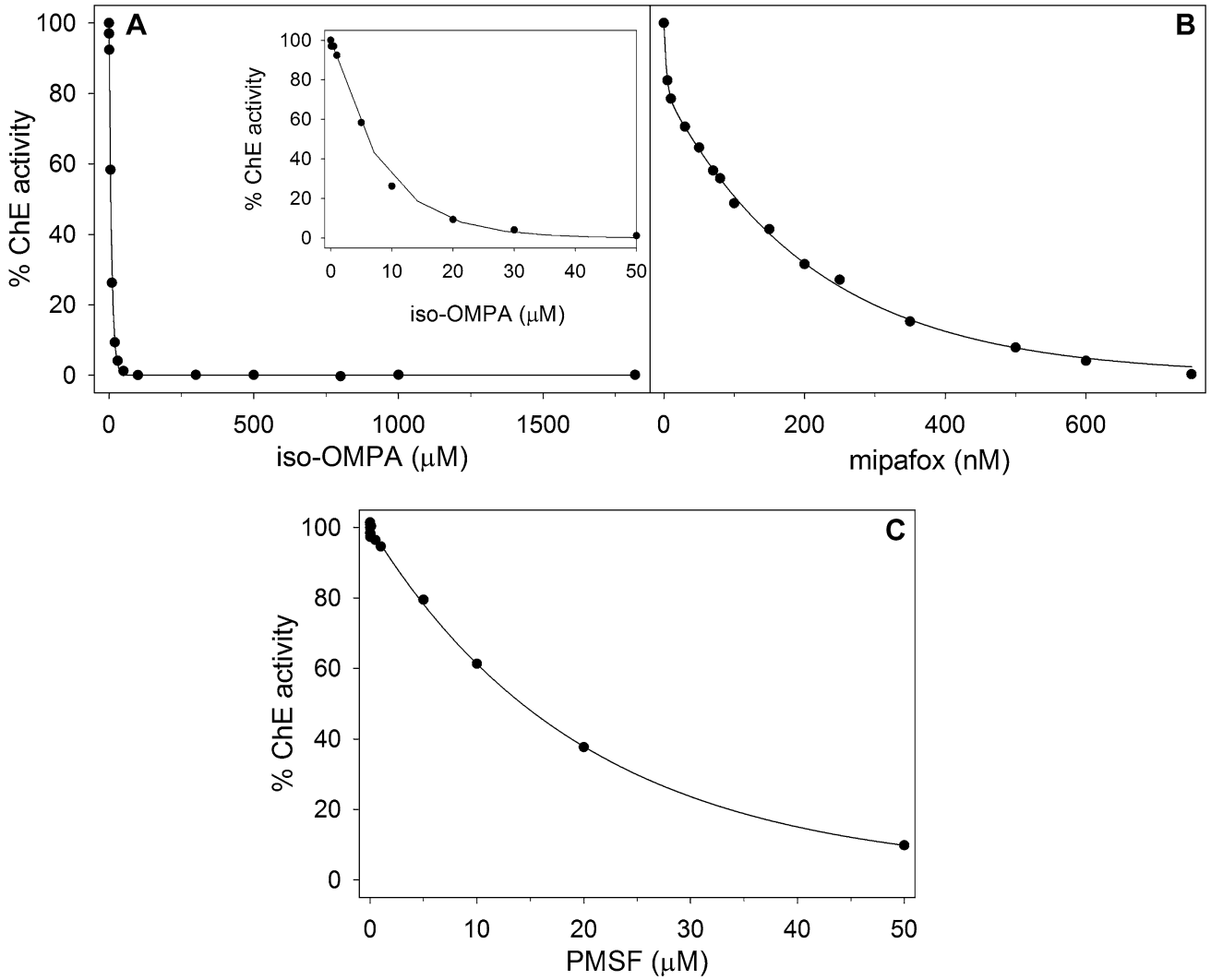

Fig. 4 Fixed time inhibition curve of ChE activity with irreversible inhibitors. A 100-µl volume of 0.4 nM hBuChE was incubated with a 10-µl volume of **a** iso-OMPA, **b** mipafox or **c** PMSF for 30 min at 37 °C, then the mixture was incubated with 100-µl volume of 2 mM AtCh for 10 min at 37 °C, and residual ChE activity was measured. The *curves* show the model that best fitted to the data provided in the "Results" section. *Each point* represents the mean of three replicates (SD < 5%). The *inset* in *panel* **a** shows the inhibition within the 0–50 nM range

o-nitrophenylbutyrate and the reaction with acetylcholine (Table 1).

Inhibition of PVase and ChE activities with AtCh and PV

PVase activity was inhibited by the presence of AtCh in the medium and ChE activity was inhibited with PV, which suggests that both substrates interact at the same active site. According to the Michaelis–Menten kinetic reaction (Cornish-Bowden 2004), the K_M estimated for AtCh in the PVase reaction was higher than the highest AtCh concentration used in the ChE assay (Fig. 2). However both reactions, or only one of them, could result in non-Michaelis–Menten kinetic reaction because it was not possible to estimate K_M in the ChE assay since the rate values for ChE assay did not reach a plateau (Fig. 2). Therefore, kinetic behavior could be more complex than the model applied to fit the data in the PVase assay. It is known that hBuChE shows substrate activation at high AtCh concentrations (Masson et al. 1993), but no activation was found under the experimental conditions in the PVase assay.

Inhibition of PVase and ChE activities by irreversible inhibitors (iso-OMPA, mipafox and PMSF)

An enzymatic component was estimated in the inhibition with iso-OMPA and PMSF in the assays of PVase

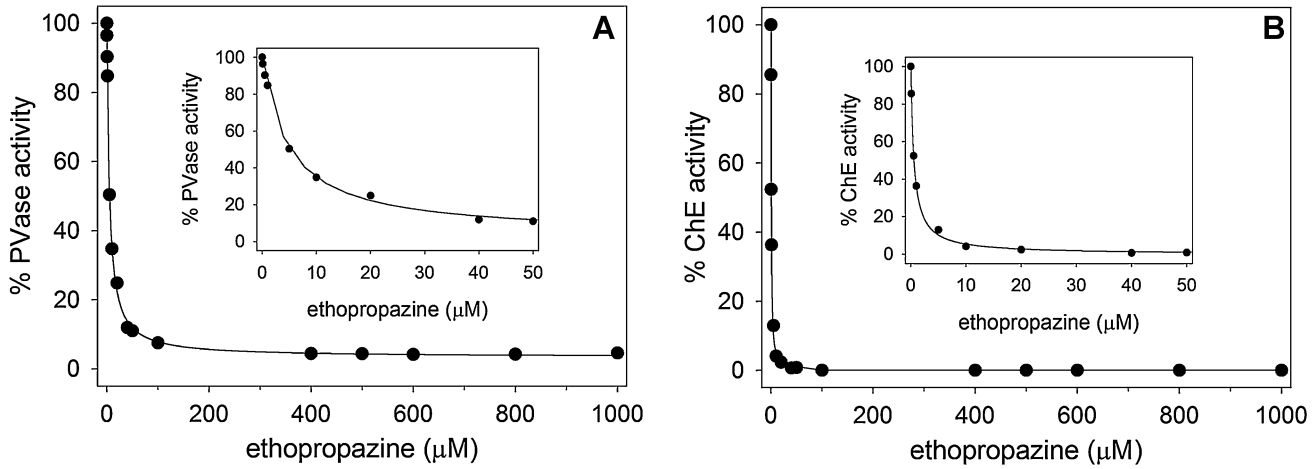

Fig. 5 Inhibition of PVase and ChE activities with ethopropazine. A 100-µl volume of 0.4 nM hBuChE was incubated with ethopropazine and 100-µl volume of **a** 5.4 mM PV or **b** 1 mM AtCh for 10 min at 37 °C, and residual activities were measured. The *curves* show the model that best fitted the data provided in the "Results" section. *Each point* represents the mean of three replicates (SD < 5%).The *insets* in *panels* **a** and **b** show the inhibition within the low concentration range

Table 1 Kinetic parameters for substrates of hBuChE

Substrate	V_{max} (µM/min)	K_M (mM)	k_{cat} (min^{-1})	References
Phenyl valerate	45.9/49.2	0.52/0.72	45,900/49,200	In this paper
o-Nitrophenylacetate		0.48		Masson et al. (1993)
α-Naphthylacetate		0.34		Masson et al. (1993)
Propionylthiocholine		0.76		Reiner et al. (1995)
o-Nitrophenylbutyrate		0.140	48,000	Lockridge and La Du (1977)
		0.125		Masson et al. (1993)
Phenyl acetate			32,000	Masson and Lockridge (2010)
Acetylcholine		0.148	61,200	Hou et al. (2013)
			13,000	Masson and Lockridge (2010)
Acetylthiocholine		0.49[a]		Masson et al. (1993)
		0.049[b]		Masson et al. (1993)
		0.04	50,000	Kaplan et al. (2001)
		0.033	20,200	Hou et al. (2013)
Butyrylthiocholine		0.26[a]		Masson et al. (1993)
			24,000[b]	Masson and Lockridge (2010)
			76,800[a]	Masson and Lockridge (2010)
		0.050	110,000	Kaplan et al. (2001)
		0.021	27,000	Weingand-Ziade et al. (2001)
		0.023[b]		Masson et al. (1993)
			33,900	Sun et al. (2001)
		0.017	29,500	Hou et al. (2013)

For the phenyl valerate tested in this study, two values from two independent experiments are shown

[a]At high substrate concentrations

[b]At low substrate concentrations

and ChE activities. However, two sensitive components were estimated in the inhibition assays with mipafox in both activities. The second-order rate constants and I50 estimated for each irreversible inhibitor were similar in both activities, which indicates that these irreversible inhibitors could alter PVase and ChE activities similarly and their interaction with hBuChE is independent of the nature of the substrate.

For iso-OMPA, both activities showed similar I50 (30 min) to the human cortex and plasma BuChE with

Table 2 Kinetic parameters and the best fitting kinetic model deduced from the experiments described in Figs. 3–5

Irreversible inhibitor	Substrate	*E*1 (%)	*k*1 (μM^{-1} min^{-1})	I_{50} (μM)	*kh* (min^{-1})	*E*2 (%)	*k*2 (μM^{-1} min^{-1})	I_{50} (μM)
Iso-OMPA	Phenyl valerate	100/100	$3.3 \times 10^{-3}/3.2 \times 10^{-3}$	7.1/7.2	–	–	–	–
	Acetylthiocholine	100/100	$3.9 \times 10^{-3}/3.8 \times 10^{-3}$	5.9/6.2	–	–	–	–
Mipafox	Phenyl valerate	6.9/6.9	9.6/ 18.3	$2.4 \times 10^{-3}/1.3 \times 10^{-3}$	–	93/93	$81 \times 10^{-3}/110 \times 10^{-3}$	0.286/0.211
	Acetylthiocholine	12/19	5.0/9.5	$4.6 \times 10^{-3}/2.4 \times 10^{-3}$	–	88/81	$125 \times 10^{-3}/156 \times 10^{-3}$	0.185/0.148
PMSF	Phenyl valerate	100/100	$5.5 \times 10^{-3}/6.4 \times 10^{-3}$	15.5/15.4	0.12/0.14	–	–	–
	Acetylthiocholine	100/100	$4.9 \times 10^{-3}/ 5.5 \times 10^{-3}$	15.1/14.8	0.10/0.11	–	–	–
Reversible inhibitor								
Ethopropazine	Phenyl valerate (2.7 mM)	96.7/96.4		4.9/4.8		3.3/3.6		
	Acetylthiocholine (1 mM)	100/100		0.5/0.4				

Two values from two independent experiments are shown. *E*1 and *E*2 indicates the proportion of activity, *k*1 and *k*2 are the second-order rate of inhibition and *kh* is the chemical hydrolysis constant for PMSF

butyrylthiocholine as a substrate (6.7 and 1 μM, respectively; Atack et al. 1989).

PMSF seemed to respond differently with BuChE of distinct species. Kraut and coworkers (2000) reported that the second-order inhibition constant of PMSF in mouse BuChE was 10×10^{-3} μM^{-1} min^{-1} at 25 °C, without considering the hydrolysis constant of PMSF. This second-order inhibition constant is comparable to the constants deduced at 37 °C in this work. Mangas and coworkers (2016) identified BuChE in the enriched fraction of PVase activity Eα in chicken brain, which was resistant to PMSF. However, hBuChE is sensitive to PMSF. Mouse BuChE and AChE were inactivated by PMSF, but Torpedo californica AChE was found to be resistant to PMSF inhibition (Kraut et al. 2000). Human BuChE could interact with PMSF differently from chicken brain BuChE, which occurs with AChE of different species. Nevertheless, the published results of inhibition with PMSF should be considered cautiously because PMSF is quickly hydrolyzed and the reported results could be differently affected by this possible artifact according to data published by other authors who did not take this effect into account.

With mipafox, according to *F* test the best mathematical model to fit the inhibition data showed two enzymatic entities with similar I50 in both activities. This could be interpreted as a more complex molecular mechanism than the molecular mechanism proposed in the irreversible inhibition reaction. The most sensitive component observed in both activities is compatible with a reaction in which mipafox is binding to hBuChE to slow down the activity, but to not totally inactivate it. The least sensitive component is the proportion of the activity that could be irreversibly inactivated by mipafox. The second-order inhibition rate constant of the most sensitive component is comparable to the constant estimated by Kropp and Richardson (2007), with butyrylthiocholine used as substrate in horse serum (1.28 μM^{-1} min^{-1}). Chemnitius and coworkers estimated inhibition second-order rate constants with the same substrate in porcine left ventricular heart muscle (8.5×10^{-3} μM^{-1} min^{-1}; Chemnitius et al. 1997) and human left ventricular heart muscle (5.3×10^{-3} μM^{-1} min^{-1}; Chemnitius et al. 1999). These constants are comparable to the constants for the least sensitive component estimated herein (Table 2). It is also comparable to the *I*50 estimated by Petroianu and coworkers in

human blood plasma with AtCh (0.824 µM) and butyrylthiocholine (0.35 µM) as substrates (Petroianu et al. 2004) for 10 min at 25 °C.

Inhibition by ethopropazine (reversible inhibitor) of the ChE and PVase activities

An enzymatic component was estimated in the inhibition with ethopropazine of ChE activity. However, two enzymatic components were estimated in PVase activity, one sensitive and the other resistant, which only represented 3% of total activity. Different sensitivity can be expected because reversible inhibition depends on the nature of the substrate and the interaction between the substrate and inhibitor.

Relationship with previous observations of the PVase activity of BuChE

In the soluble fraction of chicken brain, three main enzymatic components of PVase activity (Eα, Eβ, and Eγ) were discriminated by inhibitory kinetic approaches using an inducer (mipafox), a noninducer (paraoxon) and an enhancer (PMSF) of neuropathy. PVase component Eα was inhibited with AtCh, iso-OMPA and ethopropazine but not with BW284C51 (Benabent et al. 2014a, b). A detailed proteomic analysis of a fraction enriched in Eα activity demonstrated that the only protein responsible for all PVase activity in component Eα was probably BuChE (Mangas et al. 2016). Eα is spontaneously reactivated after inhibition with paraoxon. Due to the high sensitivity of Eα esterases with paraoxon and/or mipafox, it has been suggested that it might either play a role in toxicity in the low-level long-term exposure of organophosphate compounds or have a protective effect in relation to the spontaneous reactivation of some OPs, such as paraoxon, and other di-ethyl/dimethyl phosphates, which may be considered a biodegradation reaction (Mangas et al. 2014). PMSF can interact with Eα at a concentration that does not inhibit, but which strongly modifies its sensitivity to other esterase inhibitors. These results were interpreted as the covalent irreversible interaction of PMSF at sites other than the substrate catalytic center because PMSF was quickly removed through chemical hydrolysis. It was suggested that this kind of interaction should be considered to interpret the OPIDN potentiation/promotion phenomenon of PMSF (Mangas et al. 2012). These esterases have been suggested to be possible secondary targets of OPs, whose inhibition may be related to some neurotoxicological effects of OPs with unidentified targets (Mangas et al. 2011). Therefore, BuChE should be considered among the candidates of possible targets of OP related to the mechanism of the potentiation/promotion phenomenon of OPIDN, and also in the understanding of the toxic neurological effect of low-dose chronic exposure to OPs.

Final remarks

The results of this work show that human BuChE possesses PVase activity, which supports the findings obtained in soluble chicken brain. Relevance for humans is confirmed and, therefore, the potential role in toxicity hypothesized for PVase proteins may be extended to human cholinesterase. The capacity of BuChE to hydrolyze the carboxylester PV has been definitively established and confirmed using a purified enzyme (hBuChE). Therefore, BuChE should be considered in research into OP targets of toxicity related with PVase proteins, which would be especially interesting for understanding the mechanism of the potentiation/promotion phenomenon of OPIDN, as well as the toxic neurological effect of low-dose long-term exposure to OPs.

Acknowledgements We are most grateful to Dr David Lenz and Dr Douglas Cerasoli [USAMRICD (US Army Medical Research Institute of Chemical Defense), Aberdeen Proving Ground, MD, USA] for the supply of hBuChE.

Compliance with ethical standards

Conflict of interest None declared.

Funding sources statement Institutional funds.

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