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Identification of Acylpeptide Hydrolase as a Sensitive Site for Reaction with Organophosphorus Compounds and a Potential Target for Cognitive Enhancing Drugs

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ABSTRACT

We describe here the purification and identification of a previously unrecognized target for organophosphorus compounds. The target, acylpeptide hydrolase, was isolated as a tritiated-diisopropylfluorophosphate-reactive protein from porcine brain and purified to homogeneity using a combination of ion-exchange and gel-filtration chromatography. Biochemical characterization and internal sequence analysis confirmed identity. Acylpeptide hydrolase was found to be potently inhibited by the organophosphorus compounds chlorpyrifosmethyl oxon, dichlorvos, and diisopropylfluorophosphate (20-min IC_{50} values of 18.3 ± 2.0, 118.7 ± 9.7, and 22.5 ± 1.2 nM, respectively). The in vitro sensitivity of acylpeptide hydrolase toward these compounds is between six and ten times greater than that of acetylcholinesterase (AChE), making it a target of pharmacological and toxicological significance. We show that, in vivo, acylpeptide hydrolase is significantly more sensitive than AChE to inhibition by dichlorvos and that the inhibition is more prolonged after a single dose of inhibitor. Furthermore, using dichlorvos as a progressive inhibitor, it was possible to show that acylpeptide hydrolase is the only enzyme in the brain capable of hydrolyzing the substrate *N*-acetyl-alanyl-*p*-nitroanilide. A concentration of 154 ± 27 pmol of acylpeptide hydrolase/gram of fresh rat brain was also deduced by specific labeling with tritiated-diisopropylfluorophosphate. We also suggest that, by comparison of structure-activity relationships, acylpeptide hydrolase may be the target for the cognitive-enhancing effects of certain organophosphorus compounds. Acylpeptide hydrolase cleaves N^{α}-acylated amino acids from small peptides and may be involved in regulation of neuropeptide turnover, which provides a new and plausible mechanism for its proposed cognitive enhancement effect.

The use of acetylcholinesterase (AChE) inhibitors [organophosphorus (OP) and carbamate compounds] to treat human diseases such as myasthenia gravis and schistosomiasis has long been established (Gallo and Lawryk, 1991). A further therapeutic role for AChE inhibitors has been highlighted recently with their proposed use for the treatment of cognitive defects in Alzheimer's disease (Flicker, 1999). As well as their pharmaceutical role, organophosphorus compounds are known as one of the most widely used class of pesticides (Casida and Quistad, 1998). OP compounds exert their acute effects through progressive of AChE; leading to accumulation of the synaptic transmitter, acetylcholine. The reaction of OP compounds with their primary target, AChE, has been extensively studied by molecular modeling and detailed kinetic investigations (Ordentlich et al., 1996). The electrophilic OP initially binds to the active site of the enzyme followed by attack by the nucleophilic serine displacing the leaving group of the OP. This produces an enzyme, organophosphorylated at the active site serine (Aldridge and Reiner, 1972).

As well as providing a target for the action of pesticides, the cholinergic system plays an important role in the progression of Alzheimer's disease. There is a strong correlation between the severity of dementia and cholinergic deficits in Alzheimer's disease (Bierer et al., 1995). Such observations suggest that increasing the availability of acetylcholine might benefit Alzheimer's disease patients and logical means of achieving this is to give AChE inhibitors. At present, the anticholinergic drugs being used in the treatment of Alzheimer's disease (i.e., tacrine, E2020) rely on reversible interaction with AChE. However, the use of covalent inhibitors would produce a more sustained level of AChE inhibition. Hence, OP therapy, in particular with *O*,*O*-dimethyl-2,2,2-trichloro-1-hyroxyethyl phosphonate (metrifonate), has been advocated as an aid to improve cholinergic tone and cognitive deficits in patients with Alzheimer's disease (Knopman, 1998). However, two observations suggest that at least some of this efficacy of OP compounds may have been fortuitous. First, some good AChE inhibitors do not produce cognitive enhancement in rat models (Van der Staay et al., 1996a). Second, cognitive enhancement can be observed in rats with

ABBREVIATIONS: AChE, acetylcholinesterase; OP, organophosphorus; metrifonate, *O*,*O*-dimethyl 2,2,2-trichloro-1-hyroxyethyl phosphonate; DFP, diisopropylfluorophosphate; PAGE, polyacrylamide gel electrophoresis; AANA, *N*-acetyl-alanyl-*p*-nitroanilide; DTT, dithiothreitol; α-MSH, α-melanocyte-stimulating hormone; ACPH, acylpeptide hydrolase.

ertain OP compounds, including metrifonate, at doses lower than those required to cause significant inhibition (i.e., <20%) of brain AChE (Van der Staay et al., 1996a,b). These data strongly suggest there are additional (unintended) pharmacological targets that mediate the cognitive action of OP compounds.

In principle, all serine hydrolases have the capacity to react with OP compounds, thus the characterization of members of this class of enzymes in biological systems would provide a useful resource for the identification potential OP targets. In a recent article, we have characterized some such alternative targets in rat brain homogenates (Richards et al., 1999) by reaction with the tritiated, broad-specificity OP compound, diisopropylfluorophosphate (DFP). Two major bands of [^{3}H]DFP labeling were identified, with molecular masses of 30 kDa and 85 kDa (termed OP30 and OP85, respectively). The reaction of [^{3}H]DFP with both bands could be inhibited by preincubation with particular unlabeled OP pesticides. It was found that the 30-kDa band was particularly sensitive to paraoxon and diazoxon and the 85-kDa band was found to be very sensitive toward dichlorvos. Consequently, identification of the specific proteins contained in these bands is of utmost importance to assess the physiological and toxicological effects of OP pesticides. In this article, we describe the purification and identification of the major OP-sensitive component of the 85-kDa site from porcine brain. Purification of the protein was followed by labeling with [^{3}H]DFP and its identity was established as acylpeptide hydrolase by internal sequence analysis and biochemical characterization. The reaction of this peptidase with a number of AChE inhibitors was then investigated and a possible role in cognitive function proposed.

Experimental Procedures

Materials

Scintillation fluid (Ultima Gold XR) and tissue solubilizer (Soluene-350) were obtained from Packard (Pangbourne, UK). Ultrafiltration was performed using a Viaspin concentrator of an appropriate size from Greiner (Stonehouse, UK). Trypsin was obtained from Boehringer Mannheim (Lewes, UK). Tritiated DFP was from DuPont NEN (Hounslow, UK) with a specific activity of 310 GBq/mmol. *Z*-Gly-Pro-7-amino-4-methylcoumarin was obtained from Bachem (Saffron Walden, UK). Chromatographic media were obtained from Pharmacia LKB Ltd. (St Albans, UK). OP compounds were analytical standards obtained from Chem Service Chemicals (Birkenhead, UK). Fresh porcine brains were obtained from a local abattoir and immediately placed on ice. Brains were washed in isotonic saline, meninges were removed, and stored at minus 20°C for up to 3 months. Reagents for SDS-polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad (Hemel Hempstead, UK). All other chemicals were obtained from Sigma (Poole, UK).

In Vivo Reaction of Enzymes with Dichlorvos

Male, Fischer 344 rats (180–220 g) were given i.p. doses of water or dichlorvos dissolved in water at 0.1 ml/100 g of body weight. At an appropriate time after dosing, rats were sacrificed with an overdose of anesthetic. Brains were rapidly removed and placed onto ice, and all biochemical assays were conducted within 4 h of death.

Preparation of Brain Cytosol

Samples of porcine brain (up to 75 g) were minced and homogenized in 9 volumes of ice-cold 10 mM Tris·HCl, pH 8.0, in a Waring Blender. The blender was pulsed for 5 s at 5-s intervals for a total time of 1 min. The resulting homogenate was centrifuged at 100,000*g* for 1 h at 4°C and the supernatant (brain cytosolic fraction) carefully removed. Protein was determined using the Bio-Rad DC protein assay. For rat brain, homogenization was performed on minced brain using an Ultra-Turrex T25 at 24,000 rpm for 30 s. The cytosolic fraction was prepared as for porcine brain.

Purification of OP85

OP85 was purified from porcine brain cytosol obtained from 75 g of tissue as the starting material (approximately 600 ml of cytosol). To follow the purification, a portion of the cytosol (12 ml) was removed and allowed to react with [^{3}H]DFP (final concentration, 1.2 μM) at 37°C for 30 min. The [^{3}H]DFP-labeled fraction was added back to the bulk solution and immediately precipitated by the addition of 900 ml of ice-cold, saturated ammonium sulfate and stirred at 4°C for 1 h. The precipitate was collected by centrifugation at 10,000*g* for 30 min at 4°C and the supernatant carefully removed. The protein pellet was dissolved in 50 ml of 10 mM Tris·HCl, pH 8.0, and dialyzed extensively against water and then against 10 mM Tris·HCl, pH 8.0. The above procedure was found to quantitatively recover all the ^{3}H-labeled cytosolic proteins. The resulting solution was loaded onto a Q-Sepharose column (2.5 × 15 cm) and the protein eluted with a linear gradient of 200 ml of 10 mM Tris·HCl, pH 8.0, to 200 ml of the same buffer containing 1 M NaCl. Fractions (10 ml) were collected and aliquots of the fractions assayed for radioactivity by liquid scintillation counting. Radioactive fractions were assayed for the presence of OP85 by SDS-PAGE as described below. OP85 eluted in fractions 24 to 30 ,which were pooled and exchanged into 10 mM sodium citrate, pH 6.0, by ultrafiltration. The OP85-containing sample was subsequently loaded onto a Mono-Q fast-performance liquid chromatography column (0.5 × 5 cm) equilibrated with 10 mM sodium citrate, pH 6.0. After washing the column with 10 mM sodium citrate, pH 6.0, containing 0.2 M NaCl, bound proteins were eluted with a linear gradient of 0.2 to 0.7 M NaCl in 10 mM sodium citrate over 30 min. One-milliliter fractions were collected and assayed for radioactivity by liquid scintillation counting. OP85 eluted in a single peak. Fractions containing this protein were pooled and reduced in volume using ultrafiltration. The partially purified OP85 was subjected to gel filtration chromatography on a Waters Protein Pac 300SW column (0.8 × 30 cm) and eluted with 0.1 M sodium phosphate, pH 7.0, at a flow rate of 0.25 ml/min. The relative molecular mass of OP85 was estimated to be approximately 320 kDa by gel filtration chromatography. Analysis of the OP85-containing fractions from this final purification step revealed a single protein band with a relative molecular mass of approximately 85 kDa by SDS-PAGE, indicating a homotetrameric structure. A summary of the purification is shown in Table 1.

Tryptic Digestion of OP85

Purified OP85 was exchanged into 50 mM Tris·HCl, pH 7.5, by ultrafiltration and digested with trypsin (ratio of OP85 to trypsin, 10:1). After digestion with trypsin for 3 h at 25°C the peptides were resolved by SDS-PAGE on a 12.5% gel, transferred onto a polyvinylidene difluoride membrane and subjected to Edman sequencing (ABI automatic sequencer).

TABLE 1
Purification of OP85 from 75 g of porcine brain
Because the cytosolic fraction contains a number of DFP-labeling sites that do not represent OP85, yields were estimated after an initial fractionation on Q-Sepharose.

Step	Total ^{3}H	Total Protein	Purification Factor	Yield
	pmol	*mg*		*%*
Cytosol	29,400	2,220		
$(NH_4)_2SO_4$	28,500	1,875		
Q-Sepharose	6,300	203	31	100
Mono-Q	2,100	6.8	306	33.3
Gel filtration	740	1.4	516	11.7

Identification of OP85-Containing Samples

SDS-PAGE was conducted according to Laemmli (1970) using 10% discontinuous slab gels. Following electrophoresis, gels were stained with Coomassie Blue R-250 and lanes sliced into 5-mm sections. The sections were incubated in 0.5 ml of tissue solubilizer for 1 h at 50°C followed by the addition of 5 ml of scintillant, and the ^{3}H level was determined. OP85 was identified a major peak of DFP labeling at 85 kDa. Mass was estimated by comparison with molecular mass markers run on the same gel.

Measurement of Enzyme Activity

For all enzymes, 1 unit of enzyme activity (U) is defined as the amount of enzyme required to hydrolyze 1 μmol of substrate/min. Unless otherwise stated, enzyme activity was recorded using samples of rat brain homogenized in 9 volumes of 10 mM Tris·HCl, pH 8.0.

Acylpeptide Hydrolase. Acylpeptide hydrolase activity was measured using the chromogenic substrate *N*-acetyl-alanyl-*p*-nitroanilide (AANA; Jones and Manning, 1985). Samples containing acylpeptide hydrolase (up to 100 μl) were added to 1 ml of 0.2 M Tris·HCl, 1 mM dithiothreitol (DTT), pH 7.4, containing 4 mM AANA. The rate of hydrolysis was determined by the release of *p*-nitroaniline (ϵ_{405} = 7530 $M^{-1} \cdot cm^{-1}$) at 37°C. To monitor the elution of acylpeptide hydrolase activity from chromatography columns, assays were performed in microtiter plates. In this case, 10 μl of sample was added to 200 μl of substrate solution and the absorbance at 405 nm read after 5 min.

AChE. AChE activity was measured by the hydrolysis of *S*-acetylthiocholine iodide using the Ellman assay (Ellman et al., 1961).

Dipeptidylpeptidase IV. For dipeptidyl peptidase IV, 30 μl of rat brain homogenate was added to 1 ml of 0.5 mM Gly-Pro-*p*-nitroanilide in 50 mM Tris·HCl, pH 7.4, containing 1 mM DTT at 37°C and the release of *p*-nitroaniline was monitored by the absorbance at 405 nm.

Prolyl Oligopeptidase. Prolyl oligopeptidase enzyme activity was recorded using 30 μl of rat brain homogenate in a solution containing 0.25 mM *Z*-Gly-Pro-7-amino-4-methylcoumarin in 50 mM Tris·HCl, pH 7.4, containing 1 mM DTT and change in fluorescence recorded at an excitation wavelength of 383 nm and an emission wavelength of 455 nm at 37°C. The amount of 7-amino-4-methylcoumarin released was determined from a standard curve of the fluorophore prepared in the above buffer.

Hydrolysis of α-Melanocyte-Stimulating Hormone (α-MSH) by Acylpeptide Hydrolase. α-MSH was dissolved in 0.2 M potassium phosphate, 0.2 M NaCl, pH 7.4. Purified acylpeptide hydrolase was added and the mixture incubated at 37°C; aliquots (50 μl) were removed at various time intervals and assayed for the release of free amino groups using the fluram assay (Jones and Manning, 1985).

Inhibition of Acylpeptide Hydrolase Activity by α-MSH. α-MSH (55 μM final concentration) was added to various concentrations (0.2 to 1 mM) of AANA in 0.2 M Tris·HCl, pH 7.4. Acylpeptide hydrolase was added and hydrolysis of the substrate monitored as described above. Control reactions were also performed in the absence of α-MSH. Kinetic constants (K_m and V_{max}) for the hydrolysis of AANA by acylpeptide hydrolase were determined in the presence and absence of α-MSH.

Measurement of IC_{50} for the Inhibition of Enzyme Activity. Samples of purified acylpeptide hydrolase or brain homogenate were incubated in 50 mM Tris·HCl, pH 7.4, at 37°C with varying concentrations of OP or carbamate (eserine). The OP compounds were diluted from stock solutions in acetone just before use (final acetone concentration <1%). Residual enzyme activity was recorded after 20 min and the concentration of inhibitor producing 50% inhibition was calculated from a plot of log (% activity remaining) versus OP concentration.

Analysis of the Turnover Number of Purified Porcine Brain Acylpeptide Hydrolase. Known activities (measured by the hydrolysis of AANA) of purified acylpeptide hydrolase were allowed to react with an excess of [^{3}H]DFP (5 μM final concentration) at 37°C in 50 mM Tris·HCl, pH 7.5, containing 0.75 mg/ml bovine serum albumin as a carrier protein. After incubation for 30 min, protein was precipitated by the addition of 5 volumes of ice-cold acetone and the protein pellet was washed repeatedly in acetone until no more unbound [^{3}H]DFP was released, as determined by liquid scintillation counting of fractions of the supernatant. After washing, the protein pellet was dissolved in 1% SDS and the amount of tritium quantified by liquid scintillation counting.

Results

Identification of Porcine Brain OP85 as Acylpeptide Hydrolase by Internal Sequencing. OP85, originally identified as a major OP-sensitive band of DFP reactivity on SDS-PAGE gels of rat brain proteins (Richards et al., 1999), was purified to homogeneity as a [^{3}H]DFP labeled polypeptide as described under *Experimental Procedures*.

Two peptides were produced from the tryptic digest of OP85, with relative masses of 57 and 26 kDa. The smaller had a blocked N terminus and the larger revealed the sequence TXXPDQAIKGDQFLY. A BLAST search (Altschul et al., 1997) revealed that this sequence corresponded to residues 194 to 209 of porcine liver acylpeptide hydrolase (EC 3.4.19.1) with a single amino acid difference at residue 208 (a Tyr instead of a Phe). By comparison with porcine liver acylpeptide hydrolase, it can be inferred that trypsin cleaves after Arg-193. Acylpeptide hydrolase is a 732-residue serine hydrolase with a calculated molecular mass of 81.2 kDa. It is known to be a homotetramer with an apparent mass of 340 kDa by gel filtration chromatography (Mitta et al., 1997), which is consistent with our results for the brain form of the enzyme (see *Experimental Procedures*).

Analysis of Acylpeptide Hydrolase Activity in Rat and Porcine Brain. To confirm that acylpeptide hydrolase is found in porcine brain, acylpeptide hydrolase activity and OP85 radioactive content were monitored during the fractionation of porcine brain cytosol. It was found that acylpeptide hydrolase activity and OP85 coeluted both during ion-exchange (Fig. 1a) and gel-filtration (Fig. 1b) fractionation of cytosol. In a separate experiment, acylpeptide hydrolase was purified from porcine brain using a published procedure (Jones and Manning, 1985). Pure acylpeptide hydrolase was analyzed by SDS-PAGE where it was found to comigrate with OP85 (results not shown). From these data, it is evident that OP85 and acylpeptide hydrolase are highly similar proteins.

Reaction of OP and Carbamate Compounds with Porcine Brain Acylpeptide Hydrolase. OP85, now demonstrated to be acylpeptide hydrolase, was first identified by its reaction with OP compounds, in particular dichlorvos (Richards et al., 1999). The reaction of a number of OP pesticides with purified porcine-acylpeptide hydrolase was therefore determined (Table 2). It can be seen that there is a large range of nearly 5 orders of magnitude in the sensitivity of acylpeptide hydrolase to a number of OP compounds. With the exception of mipafox, all these compounds are good inhibitors of mammalian AChE. Thus, the reaction of acylpeptide hydrolase with this class of pesticide seems to have its own very distinctive structure-activity relationship. As well as DFP, the widely used pesticide chlorpyrifos-methyl oxon (the active metabolite of chlorpyrifos-methyl) and dichlorvos are strong inhibitors of acylpeptide hydrolase activity. At the

other end of the acylpeptide hydrolase reactivity order, malaoxon [the active metabolite of diethyl (dimethoxythiophosphorylthio)succinate (Malathion; Chem Service Chemicals, Birkenhead, UK)] shows nearly a 5000-fold selectivity ratio for AChE over acylpeptide hydrolase. The carbamate eserine showed no detectable inhibition of acylpeptide hydrolase activity. The IC_{50} value (20-min incubation) for the inhibition of acylpeptide hydrolase activity by dichlorvos was found to be 118.6 ± 9.7 and 110 ± 8.0 nM for porcine and rat brain acylpeptide hydrolase, respectively. These values are in close agreement with the calculated value for the inhibition of rat brain OP85 by the same compound (118 nM; Richards et al., 1999). This again supports the identity of OP85 as acylpeptide hydrolase. Because dichlorvos is a potent inhibitor of acylpeptide hydrolase activity, it can be used to titrate enzyme activity in rat brain. Inhibition of AANA-splitting activity in brain homogenates by dichlorvos displayed pseudo–first-order kinetics to 97% inhibition, showing it to be the only dichlorvos-sensitive, AANA-hydrolyzing enzyme present in rat brain (Fig. 2).

Quantification of Rat Brain Acylpeptide Hydrolase. Because there is a 1:1 reaction stoichiometry between DFP and acylpeptide hydrolase, the turn-over number for acylpeptide hydrolase can be deduced by labeling purified acylpeptide hydrolase activity with [^{3}H]DFP. Thus, 1 mol of acylpeptide hydrolase was found to hydrolyze 19,305 ± 3,540 mol of AANA/min at pH 7.5, 37°C. With Fisher 344 rats, an acylpeptide hydrolase activity of 2.97± 0.16 μmol/min/g of brain was determined, which corresponds to a value of 154 ± 27 pmol of acylpeptide hydrolase/g of fresh rat brain (assuming similar turnover numbers for rat and porcine acylpeptide hydrolase).

Reaction of Prolyl Oligopeptidases with OP and Carbamate Compounds. Acylpeptide hydrolase is a member of a small family of related peptidases (the prolyl oligopeptidase family; Rawlings et al., 1991). To assess the possibility that the other members of this family (dipeptidyl peptidase IV

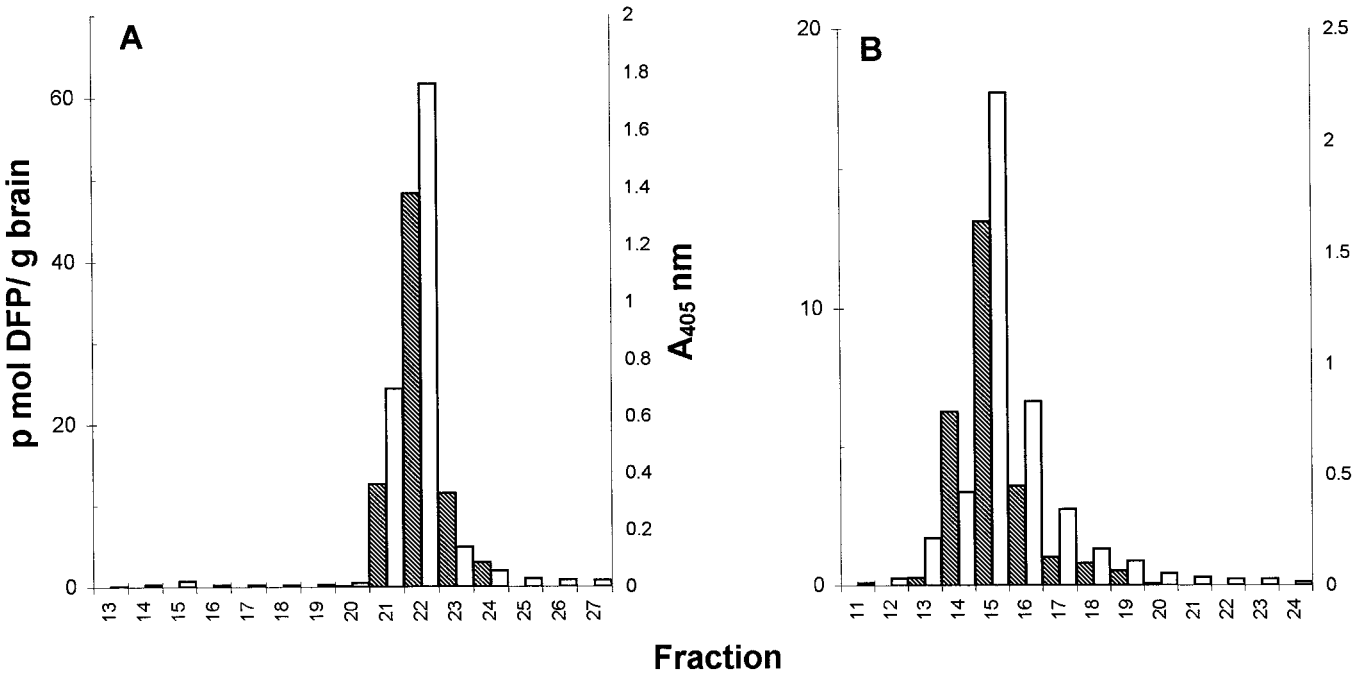

Fig. 1. Coelution of OP85 and acylpeptide hydrolase. Analysis of acylpeptide hydrolase and OP85 was performed either by Mono-Q FPLC (A) or gel filtration chromatography (B). Fractions were monitored for acylpeptide hydrolase activity (shaded bars) and OP85 (open bars) as described under *Experimental Procedures*.

TABLE 2
The in vitro reaction of AChE inhibitors with acylpeptide hydrolase, prolyl oligopeptidase, and dipeptidyl peptidase IV (mean ± S.D.) AChE

Compound	IC_{50} (20 min, 37°C, pH 7.4)			
	Acylpeptide hydrolase	AChE	Prolyl oligopeptidase	Dipeptidyl peptidase IV
		nM		
Chlorpyrifos-methyl oxon[a]	18.3 ± 2.1	108.3 ± 1.7	—[b]	—
Dichlorvos	118.6 ± 9.7	787.6 ± 25.1	19,254 ± 4,278	>300,000
DFP	22.5 ± 1.2	239 ± 66	770.1 ± 102.6	14,440 ± 4211
Mipafox	3,013 ± 20	66,777 ± 8,234	—	—
Diazoxon[a]	1,386 ± 133	268.6 ± 39.6	—	—
Paraoxon[a]	3,805 ± 316	41.8 ± 3.5	>70,000	>70,000
Eserine	>100,000	270.7 ± 103.6	>100,000	>100,000
Chlorfenvinphos	1,386 ± 200	66.6 ± 10.2	—	—
Malaoxon[a]	$1.4 \times 10^6 \pm 4 \times 10^5$	296.2 ± 93.6	—	—

[a] These compounds are the active oxon metabolite (P = O bond) of the thion (P = S bond) form of the pesticide.
[b] Not determined.

and prolyl oligopeptidase) were targets for AChE inhibitors in the brain, we studied inhibition of catalytic activity by a number of such compounds. In vitro screening of rat brain homogenates for enzyme inhibition by DFP, dichlorvos, paraoxon, and eserine revealed very low rates of reaction, relative to AChE, for dipeptidyl peptidase IV and prolyl oligopeptidase with all four compounds (Table 2). These results contrast with that of acylpeptide hydrolase, which is strongly inhibited both by dichlorvos and DFP.

It is known that the pro-drug for dichlorvos, metrifonate, is unreactive toward AChE and that only when it has undergone a spontaneous dehydrochlorination to form dichlorvos does it have any capacity to inhibit this enzyme (Reiner et al., 1975). Reaction of acylpeptide hydrolase with metrifonate at pH 6.0 and pH 7.4 revealed inhibition only at the higher pH (Fig. 3), which is known to favor the conversion to dichlorvos (Reiner et al., 1975). Conversion of metrifonate to dichlorvos is also required for the inhibitory potential of metrifonate toward acylpeptide hydrolase. Comparison of the rates of reaction of dichlorvos with acylpeptide hydrolase at pH 6.0 and 7.4 revealed little difference in the rates of phosphorylation (results not shown) demonstrating that conversion of metrifonate to dichlorvos is the rate-limiting step.

In Vivo Reactivity of Prolyl Oligopeptidases with Dichlorvos. Considering the relatively large differences in IC_{50} values between acylpeptide hydrolase and AChE, the former would be expected to be a potential target in vivo. To investigate this, rats were dosed with dichlorvos at 4 mg/kg, i.p. and sacrificed after 1 h (within the time range of optimum AChE inhibition). Rats showed no obvious cholinergic signs at this dose level. The rats showed an average of 47% inhibition of AChE activity and 93% inhibition of acylpeptide hydrolase, whereas prolyl oligopeptidase and dipeptidyl peptidase IV were not significantly different from control activities (Table 3). The dose-response of acylpeptide hydrolase toward dichlorvos showed that, at a range of doses of dichlorvos producing moderate levels of AChE inhibition, a marked inhibition of acylpeptide hydrolase was seen (Fig. 4a). The in vivo experiments confirmed the in vitro results and, as expected, the sensitivity of acylpeptide hydrolase was greater than that of AChE toward dichlorvos.

Dichlorvos also produced a longer-lasting inhibition of acylpeptide hydrolase. Thus, a single dose of dichlorvos (4 mg/kg, i.p.) in rats produced a marked inhibition of both AChE and acylpeptide hydrolase activities. However, AChE activity had recovered by 24 h postdose, but acylpeptide hydrolase activity returned relatively slowly, with a half-life of about 5 days (Fig. 4b).

Does Acylpeptide Hydrolase Hydrolyze α-MSH? Suggestions have been made that acylpeptide hydrolase plays a specific role in the degradation of *N*-acetylated neuropeptides such as α-MSH (Jones et al., 1986). However, we found that incubation of α-MSH (0.155 mM) with purified acylpeptide hydrolase gave only 23% hydrolysis after 20 h incubation at 37°C. Inhibition studies with α-MSH revealed no significant changes in the K_m or V_{max} values of acylpeptide-catalyzed hydrolysis of AANA in the presence of 55 mM α-MSH, showing little competition for the active site with a synthetic substrate. We conclude that full-length α-MSH is a poor substrate for acylpeptide hydrolase.

Discussion

We have shown here that acylpeptide hydrolase is potently inhibited by certain OP compounds, representing a novel target for these widely used chemicals. OP compounds have traditionally been associated with the inhibition of esterase activity (Mounter et al., 1963), so the finding that a peptidase is also a very sensitive target is somewhat surprising.

The results with different peptidases demonstrate that within the same family of serine hydrolases, showing sequence similarity in their C-terminal catalytic domain, large variations in the rate of reaction occur with a particular

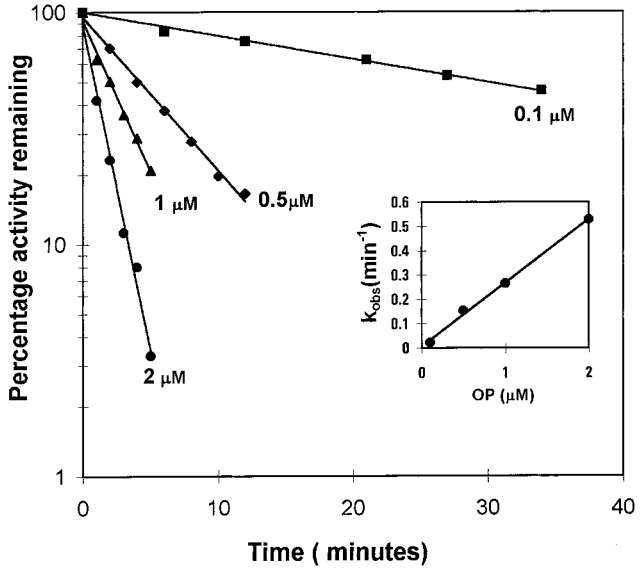

Fig. 2. Kinetics of ACPH inhibition by dichlorvos. Rat brain homogenate was allowed to react with various concentrations of dichlorvos as described under *Experimental Procedures*. Residual ACPH activity is plotted on a semilog scale, and pseudo-first-order rates (k_{obs}) of reaction (slope of line) are shown as a function of OP concentrations (inset). Reaction of highly purified porcine brain ACPH with dichlorvos gave essentially the same results as rat brain homogenate.

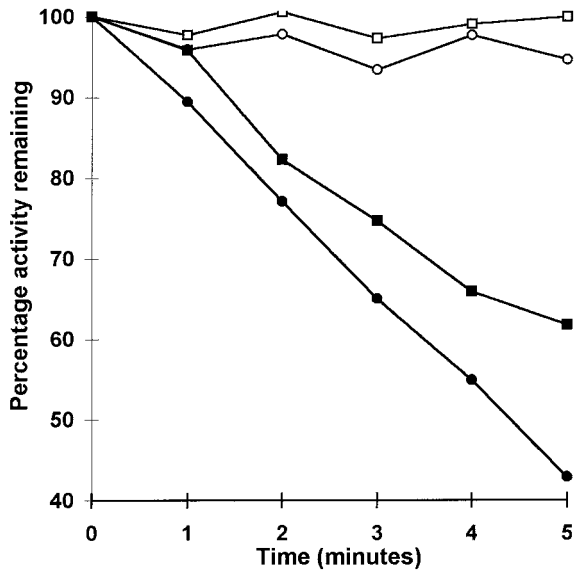

Fig. 3. Kinetics of ACPH inhibition by metrifonate. Rat brain homogenate was allowed to react with either 48 μM (●, ○) or 91 μM (■, □) metrifonate at either pH 7.4 (●, ■) or 6.0 (○, □). Residual ACPH activity was measured as described under *Experimental Procedures*.

inhibitor. Of potential pharmacological significance is the higher rate of reaction of dichlorvos with acylpeptide hydrolase. This compound is the active component of the proposed Alzheimer therapeutic, metrifonate. The result is unexpected as dichlorvos was selected as a specific inhibitor of cholinesterases and this is the basis for its parent compound, metrifonate, being proposed as a therapeutic in the treatment of Alzheimer's disease (Knopman, 1998).

Despite being described nearly 30 years ago (Witheiler and Wilson, 1972), the exact biological function of acylpeptide hydrolase remains unknown. Acylpeptide hydrolase catalyzes the hydrolysis of N^{α}-acylated amino acids from short peptides to form an acylamino acid and a peptide with a free NH_2-terminus. Preference for *N*-acetyl-methionyl-, alanyl-, glycyl-, and seryl-containing peptides (common N-terminal residues for cytosolic proteins) has led to the suggestion that the enzyme is important in protein catabolism or removal of *N*-acylated amino acids from nascent polypeptide chains emerging from the ribosome (Raphel et al., 1999). N-terminal acetylation is a common post-translational modification in proteins, with up to 80% of cytosolic proteins displaying this modification (Tsunasawa and Sakiyama, 1984). A gene homologous to mammalian acylpeptide hydrolase has been found in the thermophilic archaeon *Pyrococcus horikoshii* (Ishikawa et al., 1998). The gene product from *P. horikoshii* has a high hydrolytic activity for acylpeptides, suggesting conservation of mechanisms within protein synthesis or degradation from archaea to mammals. Specific inhibitors of acylpeptide hydrolases would greatly facilitate in the study of this family of enzymes.

We now have a number of highly active inhibitors of this enzyme that can be used to chemically "knock out" acylpeptide hydrolase activity and have used such compounds to titrate and quantify acylpeptide hydrolase activity in rat brain. The pesticides chlorpyrifos methyl oxon and dichlorvos both have IC_{50} values in the nanomolar range, making them orders of magnitude more potent than compounds specifically designed as good acylpeptide hydrolase inhibitors, such as acetyl leucine chloromethyl ketone (Scaloni et al., 1992) and *p*-nitrophenyl-*N*-propyl carbamate (Scaloni et al., 1994). Future work will concentrate on the elucidation of the endogenous substrates for acylpeptide hydrolase and the effects of their accumulation after inhibition of acylpeptide hydrolase activity.

The most interesting finding of this work is the high sensitivity of acylpeptide hydrolase toward dichlorvos and DFP and its correlation with an unknown target for a novel cognitive effect of these OP compounds (Van der Staay et al., 1996a,b). We have shown that in the rat, acylpeptide hydrolase is a good candidate for this effect, because it is substantially more sensitive than AChE to OP compounds that elicit noncholinergic cognitive enhancement and is poorly inhibited by those compounds that do not (UK patent application 9807931.2). We have shown that at the level of cholinesterase inhibition proposed for Alzheimer's therapy (50 to 80% inhibition of red-cell cholinesterase; Cummings et al., 1998), acylpeptide hydrolase activity would be depressed in a sustained manner to <10% of normal activities. The OP compounds dichlorvos and DFP, which elicit cognitive enhancement at doses too low to inhibit AChE, reacted with acylpeptide hydrolase 6.6 and 10.6 times faster, respectively, than with AChE (Table 2). Conversely, eserine and paraoxon,

TABLE 3
In vivo inhibition of peptidases
Rats were dosed at 4 mg/kg with dichlorvos i.p. and sacrificed after 1 h. Enzyme activity was recorded in brain homogenates.

Enzyme	Control Activity	4 mg/kg i.p. Dichlorvos	Mean % Inhibition
	U/g tissue		
AChE	10.60 ± 0.24 (n = 9)	5.68 ± 0.49 (n = 5)*	47
Acylpeptide hydrolase	2.97 ± 0.16 (n = 9)	0.21 ± 0.08 (n = 5)*	93
Dipeptidyl peptidase IV	0.078 ± 0.03 (n = 6)	0.098 ± 0.05 (n = 5)	0
Prolyl oligopeptidase	0.31 ± 0.02 (n = 5)	0.34 ± 0.027 (n = 5)	0

* $P < .001$ versus controls: one-tailed Student's t test.

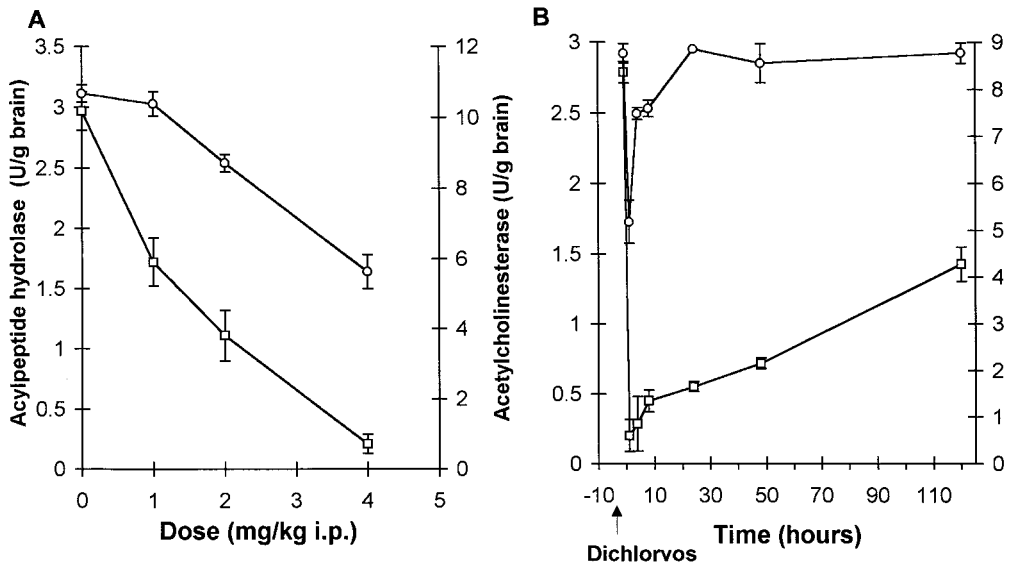

Fig. 4. A, in vivo effect of dichlorvos on AChE (○) and acylpeptide hydrolase (■) activity (activity was measured 4 h after dosing). B, in vivo time course for acylpeptide hydrolase and AChE inhibition by dichlorvos. Rats were dosed with dichlorvos (4 mg/kg, i.p.) on day 1. Acylpeptide hydrolase and AChE activity was followed over a 5-day period. Data are expressed as mean ± S.D. (n = 3 to 9 individuals).

which are poor inhibitors of acylpeptide hydrolase, do not promote noncholinergic cognitive enhancement despite being potent inhibitors of the apparent target, AChE (Van der Staay et al., 1996a) (Table 2). Thus, although cognitive enhancement with dichlorvos can be produced at levels of AChE inhibition that do not seem be to be pharmacologically significant (i.e., <20%), these are associated with a marked inhibition of acylpeptide hydrolase (Fig. 4a).

The mechanism by which acylpeptide hydrolase could be involved in cognitive function is unknown. Other members of the prolyl oligopeptidase family have a role in pro-hormone processing (Fuller et al., 1988), so it is not unreasonable to infer that acylpeptide hydrolase may perform a similar function in the brain. It is also known that a related enzyme in *Trypanosoma cruzi* (oligopeptidase B) is responsible for the generation of an active Ca^{2+} agonist in the cytosol, which is subsequently taken up into secretary vesicles and released during host-cell invasion (Caler et al., 1998). Such a mechanism has obvious parallels with neurotransmitter release.

In conclusion, we have found an unexpected target, acylpeptide hydrolase, to be particularly sensitive toward a number of OP compounds. Selective reaction of OPs with acylpeptide hydrolase may better explain their pharmacological properties as cognitive enhancers than an action on AChE. The reaction of OPs with acylpeptide hydrolase and other as-yet-uncharacterized target proteins may contribute both to such effects as cognitive enhancement and also to the putative neuropsychological effects of long-term exposure to OP pesticides (Stephens and Spurgeon, 1995). Acylpeptide hydrolase is also markedly more sensitive toward DFP than is AChE. Several authors have suggested that the neurotoxicity of DFP cannot fully be explained by its reaction with AChE (Kohen et al., 1980; Kant et al., 1983; Berndt et al., 1984). Therefore, the discovery of acylpeptide hydrolase as a new target for this compound offers an alternative site of action. Seeking new targets for the action of OP compounds should therefore aid our understanding of the beneficial and adverse effects of these chemicals.

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