Neuroscience Vol. 21, No. 3, pp. 991–995, 1987
Printed in Great Britain

0306-4522/87 $3.00 + 0.00
Pergamon Journals Ltd

ACETYLCHOLINESTERASE EXHIBITS TRYPSIN-LIKE AND METALLOEXOPEPTIDASE-LIKE ACTIVITY IN CLEAVING A MODEL PEPTIDE

D. H. SMALL, Z. ISMAEL and I. W. CHUBB
Department of Human Physiology, Flinders University of South Australia, Bedford Park, S.A. 5042, Australia

Abstract—Acetylcholinesterase (EC 3.1.1.7) has been shown to possess an intrinsic peptidase activity. [Chubb *et al.* (1983), *Neuroscience* **10**, 1369–1383]. To examine this activity further, the breakdown of a model hexapeptide (leu-trp-met-arg-phe-ala) LWMRFA was studied. Affinity-purified eel acetylcholinesterase rapidly cleaved the hexapeptide in a trypsin-like manner to produce two peptides (LWMR and FA). Acetylcholinesterase more slowly cleaved the *C*-terminal alanine residue from the peptide to yield LWMRF. Although the enzyme showed preference for cleaving the hexapeptide at its *C*-terminal, it was also able to cleave the *N*-terminal leucine residue from the tryptic product LWMR.

Hydrolysis of the peptide at the tryptic site (arg^4-phe^5) was strongly inhibited by the trypsin inhibitor diisopropylfluorophosphate. Cleavage of the *C*-terminal alanine was only poorly inhibited by diisopropylfluorophosphate, but more strongly inhibited by metal-ion chelating agents, and it was increased in the presence of Zn^{2+} and Co^{2+}. The pH optimum for cleavage at the tryptic site was 6, while that for the carboxypeptidase site was 8–9.

These results show that acetylcholinesterase can hydrolyse peptides like a trypsin-like endopeptidase and a Zn^{2+}- or Co^{2+}-dependent exopeptidase, and they suggest that these two peptidase activities are associated with two separate active sites on the acetylcholinesterase molecule. As both peptidase activities eluted with acetylcholinesterase from a TSK 4000SW column when it was chromatographed by high-performance liquid chromatography, it is unlikely that the presence of either peptidase activity could be attributable to a contaminant in the acetylcholinesterase preparation. We suggest that acetylcholinesterase may be involved in the breakdown of bioactive peptides or their precursors in neuroendocrine cells.

Acetylcholinesterase (AChE, EC 3.1.1.7) has recently been shown to possess peptidase activity.[3] This finding may explain why the enzyme is distributed widely in many cells and not localized solely to cholinergic neurons. AChE's ability to hydrolyze compounds like substance P and the enkephalins suggests that the enzyme may be involved in the processing of bioactive peptides.[3,7,9,12]

Although the naturally occurring peptide substrate for AChE has not been unequivocally demonstrated, we have found that AChE can rapidly degrade many of the soluble peptide constituents of bovine adrenal chromaffin granules[7] including chromogranin A, the major soluble granule protein.[12] The hydrolysis of chromogranin A by AChE results in the formation of a number of small molecular weight peptides which are similar to peptides in bovine chromaffin cells.[12] AChE can hydrolyse enkephalin precursors in retinal sections[9] and bovine chromaffin granules[7] to liberate enkephalin-like immunoreactivity. These experiments support a role for AChE in neuropeptide processing and suggest that it may act alone rather than in sequence with another peptidase when it liberates peptides from precursors.

The peptidase activity of AChE is complex, as it can act both as an exopeptidase[3] and as an endopeptidase.[7,12] Furthermore, while some peptidase inhibitors can block AChE's hydrolysis of bovine chromogranin polypeptides, they do not inhibit the generation of enkephalin immunoreactivity from enkephalin precursors in the same preparation.[7]

In this study, we have examined the ability of AChE to hydrolyse a synthetic hexapeptide (leu-trp-met-arg-phe-ala, LWMRFA). This peptide is susceptible to hydrolysis by a number of different peptidases including trypsin and amino- and carboxypeptidases.

Address for correspondence: Dr D. H. Small, School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; BW, 1,5-bis-(4-allylmethylammoniumphenyl)-pentane-3-one; *p*-CMS, *p*-chloromercuriphenylsulphonic acid DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetra-acetic acid; HPLC, high-performance liquid chromatography; PITC, phenylisothiocyanate; TFA, trifluoroacetic acid; SBTI, soya bean trypsin inhibitor.

EXPERIMENTAL PROCEDURES

Materials

The synthetic hexapeptide (LWMRFA) and smaller fragments of the same sequence were purchased from Serva Feinbiochemica (Heidelberg, F.R.G.) and further purified by reverse-phase high-performance liquid chromatography (HPLC) using methods described below. *o*-Phenanthroline was from Merck (Darmstadt, F.R.G.), disodium ethylenediaminetetra-acetic acid (EDTA) from Ajax

Chemicals (Sydney, Australia), diisopropylfluorophosphate (DFP), 1,5-bis-(4-allyldimethylammoniumphenyl)pentane-3-one (BW), soya bean trypsin inhibitor (SBTI), pepstatin A, *p*-chloromercuriphenylsulphonic acid (*p*-CMS), acetylcholine iodide (ACh) and choline chloride were all from Sigma (St. Louis, Missouri). Phenylisothiocyanate (PITC) was from Pierce (Rockford, Illinois).

Purification of acetylcholinesterase

Purified eel AChE was obtained from Sigma (Type V-S) and further purified by chromatography on columns of arginine–Sepharose (Pharmacia, Uppsala, Sweden), edrophonium–Sepharose and SBTI–Sepharose (Sigma) to eliminate any traces of contaminating proteases or other proteins.[7] It has been argued elsewhere[3] that the AChE used in these studies is not contaminated by other peptidases.

Incubations

Unless otherwise specified, all incubations used a 50 mM sodium phosphate buffer (pH 7.4) and contained 30 μg hexapeptide and 5 μg AChE or 0.05 μg trypsin in a total volume of 0.1 ml. Incubations were at 37°C for 4 h, after which they were stopped by adding 5 μl glacial acetic acid.

High-performance liquid chromatography of peptides and acetylcholinesterase

The products of peptide hydrolysis were analysed by reverse-phase chromatography using a linear gradient of acetonitrile [0–60% (v/v) acetonitrile over 60 min] as outlined previously.[12] The column effluent was monitored for absorbance at 215 nm. Peptides were identified by chromatography of authentic standards as well as by amino acid analysis. Peak fractions were dried *in vacuo* and hydrolysed in 6 N HCl for 18 h. Acid hydrolysates were analysed by derivatizing the amino acids with PITC and separating the resulting PTC amino acids by reverse-phase HPLC.[1]

For gel-exclusion chromatography of AChE by HPLC, the enzyme (16 U or 8 μg in 50 μl) was applied to a TSK 4000 SW column (0.75 × 30 cm) at a flow rate of 1 ml/min. The column was eluted with 100 mM sodium phosphate buffer (pH 7.0). Fractions (0.5 ml) were collected and assayed for AChE or sampled (0.05 ml) for incubation with the synthetic peptide. The recovery of AChE activity from the column was about 90%.

Other procedures

Protein was assayed by the method of Bradford.[2] AChE was assayed by the method of Ellman *et al.*[5]

RESULTS

When the hexapeptide LWMRFA was incubated with AChE or trypsin, the peptide was cleaved to yield a number of small peptides. These peptides were separated and identified by HPLC (Fig. 1). After 1 h of incubation, AChE had cleaved the hexapeptide internally at the arg[4]–phe[5] bond (tryptic site), yielding two peptides, LWMR and FA, which eluted on HPLC at 25 and 12 min, respectively (Fig. 1A). Trypsin cleaved the hexapeptide similarly to produce the same two products (Fig. 1B). However, unlike trypsin, more prolonged incubation with AChE (up to 24 h) resulted in the further breakdown of the hexapeptide and its two digestion products. AChE cleaved the *C*-terminal alanine residue from the hexapeptide to produce LWMRF, which eluted on HPLC at 30 min. Although AChE preferred to cleave the *C*-terminal residue from the hexapeptide, it also cleaved the smaller peptide LWMR at the *N*-terminal

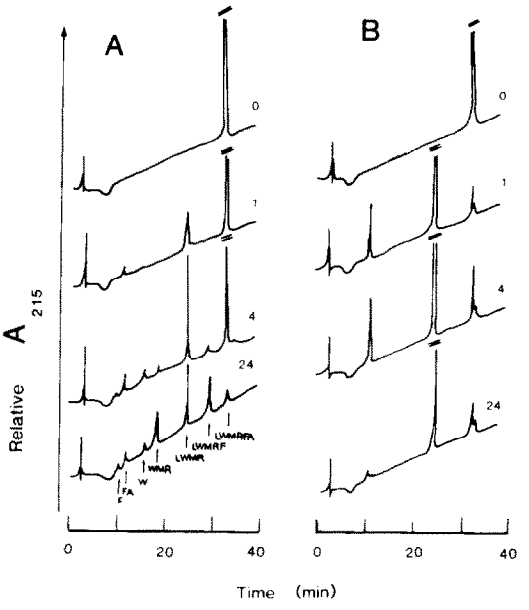

Fig. 1. Elution profile of peptides produced by the digestion of a model peptide (LWMRFA) by AChE (A) and trypsin (B). Model peptide (30 μg) was incubated with either AChE (5 μg) or trypsin (0.05 μg) for 0, 1, 4 and 24 h and the resulting peptides were separated by reverse-phase HPLC.

residue to produce WMR. The appearance of phenylalanine and tryptophan in the incubations after 24 h presumably resulted from the further digestion of the peptides FA and WMR.

Effects of peptidase inhibitors

A number of peptidase inhibitors were examined for their effects on AChE's digestion of the hexapeptide (Table 1). The amount of inhibition by each inhibitor was related to the type of bond hydrolysed. The trypsin inhibitor DFP strongly inhibited (60%) cleavage at the tryptic site (arg[4]–phe[5]), while SBTI was less potent (about 20% inhibition). In contrast, cleavage of the hexapeptide at the *C*-terminal (phe[5]–ala[6]) was inhibited most strongly by metal-ion chelating agents (EDTA and *o*-phenanthroline, 70 and 60% inhibition, respectively) and by the acid protease inhibitor pepstatin A (70%). Compounds which have been shown to interact with AChE or inhibit its activity (ACh, choline, BW) had no effect on the rate of cleavage at either site.

Effect of divalent metal ions

Because metal-ion chelating agents inhibited AChE's ability to cleave the hexapeptide on the *C*-terminal, a number of metal ions were tested for their effects on AChE's hydrolysis of the hexapeptide (Table 1). The effect of each metal ion was related to the type of bond cleaved. Hydrolysis at the tryptic site was partially inhibited by Fe^{2+}, Co^{2+} and Cu^{2+}, while cleavage of the *C*-terminal alanine was partially inhibited by Ca^{2+}, Fe^{2+}, and completely inhibited by Cu^{2+}. The rate of cleavage of alanine was increased

Table 1. Influence of various compounds and metal ions on acetylcholinesterase's digestion of a model peptide (LWMRFA)

Compound	Percentage inhibition arg⁴–phe⁵	phe⁵–ala⁶
Peptidase inhibitors		
DFP (1 mM)	58	25
SBTI (100 μg/ml)	18	20
Pepstatin A (100 μM)	−1	70
p-CMS (1 mM)	7	32
o-Phenanthroline (1 mM)	−3	60
EDTA (1 mM)	4	70
Metal ions (1 mM)		
Mg^{2+}	−4	7
Ca^{2+}	−1	52
Fe^{2+}	35	67
Zn^{2+}	1 (±7)	−33 (±11)
Co^{2+}	16 (±28)	−183 (±67)
Mn^{2+}	−4	−2
Cu^{2+}	65	100
Cholinergic agents		
BW (1.5 μM)	0	−2
ACh (1 mM)	−1	7
Choline (1 mM)	0	7

Model peptide (30 μg) was incubated with AChE (5 μg) and the rates of cleavage at the arg⁴–phe⁵ and phe⁵–ala⁶ bonds were measured by the respective amounts of LWMR and LWMRF produced in a 4 h incubation. Values are the means of two determinations, with the exception of the values for Co^{2+} and Zn^{2+}, where $n = 3$ (±SE).

in the presence of Co^{2+} and Zn^{2+} (183 and 33%, respectively), although these two ions did not enhance the rate of hydrolysis at the tryptic site.

pH dependence

The pH dependence of AChE's peptidase activity was also related to the site of cleavage (Fig. 2). The optimum pH for cleavage at the tryptic site was 6, while for the *C*-terminal cleavage it was 8–9. AChE activity was also highest at alkaline pH, with maximum activity between pH 8 and 9. Similar results to those shown in Fig. 2 have been obtained in two separate experiments.

High-performance liquid chromatography of acetylcholinesterase

It has been argued elsewhere[3] that the AChE used in this study is pure and that the peptidase activity is intrinsic to the AChE molecule. The peptidase activity could not be separated from AChE by gel-exclusion chromatography (Fig. 3). Peptidase and AChE activities co-eluted from a TSK 4000 SW column with an apparent molecular weight of about 280,000 daltons. A much smaller second peak of peptidase activity eluted at 12 ml. This peak probably resulted from the breakdown of AChE by autolysis, as storage of the enzyme at 4°C in aqueous solution for several weeks resulted in a large increase in the size of this lower molecular weight peak. We have previously shown that AChE has the capacity to degrade itself with loss of protein and AChE activity.[7]

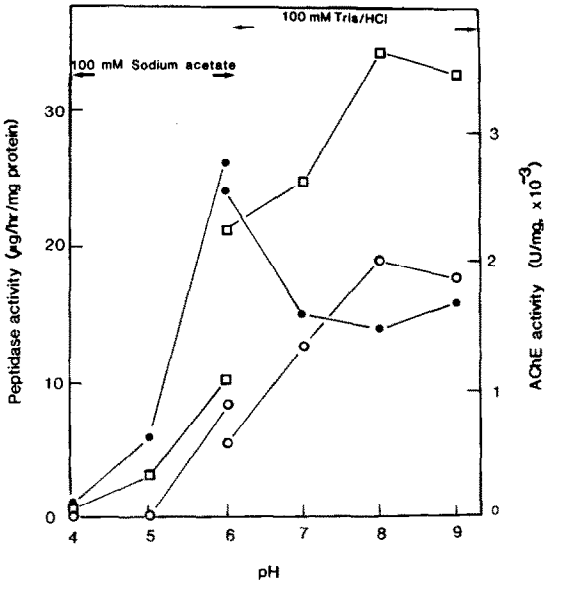

Fig. 2. pH dependence of AChE activity (□) and of the rate of cleavage of LWMRFA at the tryptic site (arg⁴–phe⁵) (●) and at the *C*-terminal end (phe⁵–ala⁶) (○) by AChE. Units of peptidase activity are μg model peptide hydrolysed per h. For the sake of comparison, the carboxypeptidase activity is shown as 10 × calculated values.

DISCUSSION

This study shows that a highly purified preparation of AChE hydrolyses a model hexapeptide by cleaving at several sites in the molecule. AChE behaved like a trypsin-like endopeptidase in hydrolysing the peptide at its arg⁴–phe⁵ bond. This cleavage was strongly

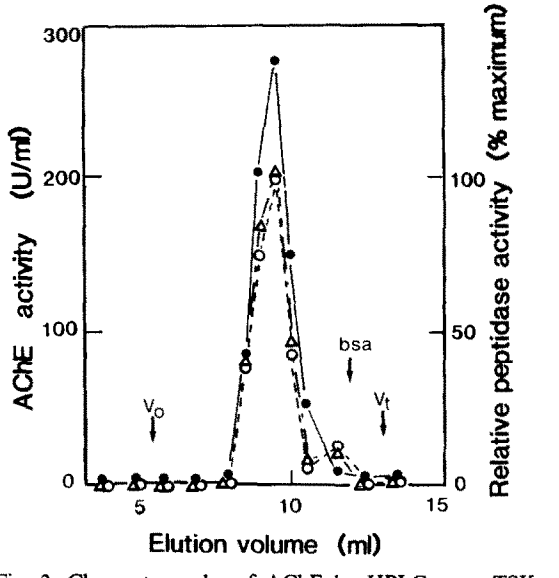

Fig. 3. Chromatography of AChE by HPLC on a TSK 4000 SW column (0.75 × 30 cm). Fractions (0.5 ml) were collected and assayed for AChE activity (●) and peptidase activity, which was determined from the rate of cleavage of LWMRFA at the tryptic site (○) and at the *C*-terminal end (△). The figure shows the elution volume of bovine serum albumin (bsa, molecular weight = 70,000).

inhibited by the trypsin inhibitor DFP. The trypsin-like action of AChE probably explains our finding[12] that in degrading the chromaffin granule protein chromogranin A, at least two of the products formed by AChE possessed similar molecular weights to those formed by trypsin.

It is unlikely that the esteratic site on AChE is involved in the peptidase activity since the peptidase activity was not inhibited by cholinergic agents such as BW or ACh. Furthermore, the esteratic site is rapidly blocked by low (micromolar) concentrations of DFP,[11] while much higher concentrations were required to inhibit the trypsin-like activity.

AChE also cleaved the hexapeptide like an exopeptidase. The exopeptidase activity was distinct from the trypsin-like activity as it was inhibited by metal-ion chelating agents, stimulated by Zn^{2+} or Co^{2+}, and the optimum pH for its activity was 8–9, compared with a pH of 6 for the trypsin-like activity. Despite the presence of two distinct peptidase activities, both peptidases co-eluted along with AChE when chromatographed by gel-exclusion HPLC.

AChE has also been shown to cleave the *N*- and *C*-terminal amino acids from other peptides, including the enkephalins.[3] EDTA also inhibited (80–90%) the exopeptidase action of AChE on the enkephalins.[3] The results from the present study suggest that for AChE to cleave peptides like an exopeptidase, it requires a metal ion, either Zn^{2+} or Co^{2+}. A number of exopeptidases require either Zn^{2+} or Co^{2+} for their activity.[10] For example, bovine pancreatic carboxypeptidase A (EC 3.4.17.1), which releases hydrophobic residues from the *C*-terminal ends of peptides, has Zn coordinately bonded to it, which participates in the catalysis.[8]

The identification of two distinct peptidase activities associated with AChE explains our previous observations[3,7,12] that the ability of peptidase inhibitors to inhibit AChE's peptidase activity is related to the nature of the substrate. For example, we have found that the breakdown of bovine chromogranins is inhibited by serine peptidase inhibitors such as DFP.[7] However, DFP does not inhibit the generation of enkephalins from their precursor molecules in the same incubations. The breakdown of chromogranins is probably attributable to the trypsin-like activity of AChE. However, the generation of enkephalin-like immunoreactivity could have been due, at least in part, to AChE's exopeptidase-like action on some of the enkephalin precursor fragments.

AChE fulfills many of the requirements for an enzyme involved in neuropeptide processing.[4] Both trypsin-like endopeptidases and amino- and carboxypeptidases have been implicated in neuropeptide synthesis[4] and degradation.[14] In the adrenal medulla, the 30,000 mol. wt enkephalin precursor proenkephalin is cleaved at dibasic amino acid residues by a trypsin-like enzyme and then further trimmed by a carboxypeptidase B-like enzyme to yield the final product, enkephalin.[6] Thus, the presence of trypsin-like and carboxypeptidase-like activities associated with AChE may explain how the enzyme can generate enkephalin-like immunoreactivity in a preparation enriched in enkephalin precursors.[7]

In the adrenal gland and in other neurosecretory tissues, AChE could act as a prohormone processing enzyme. A function for the enzyme other than in cholinergic neurotransmission would explain why the enzyme is not localised solely in cholinergic tissues, but is also found in noncholinergic cells.[11] The intracellular location of AChE in many cells (endoplasmic reticulum, Golgi apparatus; see Refs 11 and 13) is also consistent with such a role, as prohormones and many of the chromaffin granule constituents may be processed by proteolysis and post-translationally modified in these subcellular structures.[4,13]

Acknowledgements—The authors gratefully acknowledge the assistance of D. Bridges This work was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

1. Bidlingmeyer B. A., Cohen S. A. and Tarvin T. L. (1984) Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **336,** 93–104.
2. Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248–254.
3. Chubb I. W., Ranieri E., White G. H. and Hodgson A. J. (1983) The enkephalins are amongst the peptides hydrolysed by purified acetylcholinesterase. *Neuroscience* **10,** 1369–1377.
4. Docherty K. and Steiner D. F. (1982) Post-translational proteolysis in polypeptide hormone biosynthesis. *Ann. Rev. Physiol.* **44,** 625–638.
5. Ellman G. L., Courtney K. D., Andres V. and Featherstone R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmac.* **7,** 88–95.
6. Fleminger G., Ezra E., Kilpatrick D. L. and Udenfriend S. (1983) Processing of enkephalin-containing peptides in isolated bovine adrenal chromaffin granules. *Proc. natn. Acad. Sci. U.S.A.* **80,** 6418–6421.
7. Ismael Z., Millar T. J., Small D. H. and Chubb I. W. (1986) Acetylcholinesterase generates enkephalin-like immunoreactivity when it degrades the soluble proteins (chromogranins) from adrenal chromaffin granules. *Brain Res.* **376,** 230–238.
8. Lipscomb W. N. (1983) Structure and catalysis of enzymes. *Ann. Rev. Biochem.* **52,** 17–34.
9. Millar T. J. and Chubb I. W. (1984) Treatment of sections of chick retina with acetylcholinesterase increases the enkephalin and substance P immunoreactivity. *Neuroscience* **12,** 441–451.
10. Neurath H. (1984) Evolution of proteolytic enzymes. *Science* **224,** 350–357.

11. Silver A. (1974) *The Biology of Cholinesterases*. North-Holland, Amsterdam.
12. Small D. H., Ismael Z. and Chubb I. W. (1987) Acetylcholinesterase hydrolyses chromogranin A to yield low molecular weight peptides. *Neuroscience* **19**, 289–295.
13. Somogyi P., Hodgson A. J., Depotter R. W., Fischer-Colbrie R., Schober M., Winkler H. and Chubb I. W. (1984) Chromogranin immunoreactivity in the central nervous system. Immunochemical characterization, distribution and relationship to catecholamine and enkephalin pathways. *Brain Res. Rev.* **8**, 193–230.
14. Turner A. J. (1984) Neuropeptide processing enzymes. *Trends Neurosci.*, July 258–260.

(*Accepted* 8 *October* 1986)