A peptide derived from the C-terminal region of acetylcholinesterase modulates extracellular concentrations of acetylcholinesterase in the rat substantia nigra

Stevan R. Emmett*, Susan A. Greenfield

University Department of Pharmacology, University of Oxford, Oxford OX1 3QY, UK

Received 3 September 2003; accepted 15 December 2003

Abstract

It is well established that acetylcholinesterase (AChE) has ‘non-classical’ functions independent of cholinergic transmission. A region of AChE distinct from the catalytic site may be responsible for these actions via a 14-residue peptide located between residues 586–599 at the C-terminus of human AChE. This AChE-peptide possesses a high amino acid sequence homology with a region of amyloid precursor protein and shares many biophysical and physiological characteristics. In this study, the effect of AChE-peptide (AEFHRWSSYMVHWK) on the extracellular levels of endogenous AChE was examined in rat substantia nigra in vitro. A chemiluminescent assay was used to continuously measure the soluble AChE concentration from tissue punches of the substantia nigra. Application of NMDA evoked an increase in extracellular AChE levels consistent with previous results obtained from in vivo models. AChE-peptide, when applied alone, had no effect on AChE release; however, when co-applied with NMDA, AChE-peptide reduced the effectiveness of NMDA to evoke release of AChE. These results indicate, in a region of the brain central to the aetiology of Parkinson’s disease, that an AChE-peptide fragment derived from AChE displays a bioactivity that could involve regulation of Ca\(^{2+}\) availability and hence the release of AChE.

q 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: Acetylcholinesterase; Substantia nigra; N-methyl-D-aspartate; In vitro release

The protein acetylcholinesterase (AChE; EC 3.1.1.7) does not only hydrolyse acetylcholine, but also has non-cholinergic functions [17]. Certain physiological and pathological correlations also exist between AChE and amyloid precursor protein (APP) [7]. Both AChE [12] and APP [16] have trophic effects on cells, whilst both AChE [20] and amyloid-Aβ-peptide (Aβ) [19] can potentiate Ca\(^{2+}\) influx into cells. Also, areas of higher AChE expression generally correlate with the brain regions that degenerate early in Alzheimer’s disease and AChE itself is associated with amyloid plaque deposits [15]. These similarities prompted the identification of a short homologous amino-acid sequence region on AChE and APP [7] located near the carboxyl terminus of AChE and the N-terminus of Aβ (Fig. 1). This peptide region is encoded within the AChE gene by exon 6, a gene region which is independent of the hydrolytic enzyme function, and instead can modify parameters such as neurite extension in glia [12]; Hence it might be that this peptide, (AEFHRWSSYMVHWK) is responsible for regulating non-cholinergic actions.

One of the non-classical, and most established, features of the now well documented phenomenon of non-cholinergic functions of AChE [17], is that it is secreted by certain non-cholinergic cell populations, such as the dopaminergic neurons of the substantia nigra (SN): once in the extracellular space, this ‘non-cholinergic’ AChE has neurotrophic effects in culture. We have therefore explored whether the AChE-peptide might influence AChE secretion and subsequent extracellular concentrations. The NMDA receptor is a potent Ca\(^{2+}\) ionophore and plays an important role in development and neuronal survival [1]. NMDA is also known to induce release of AChE within the SN [10]. Synthetically produced AChE-peptide was applied to tissue punches of the SN, in vitro, and spontaneous and/or NMDA-evoked release of AChE was continuously monitored using a chemiluminescent assay system [18] in the presence and absence of AChE-peptide.

* Corresponding author. Tel.: +44-1865-271616; fax: +44-1865-271853.
E-mail address: steve.emmett@pharm.ox.ac.uk (S.R. Emmett).
AChE peptide (AEFIHWSSYMVHVK) was synthesised at the Oxford Centre for Molecular Studies using an Applied Biosystems 430A automated peptide synthesiser, utilising the standard Fmoc methodology. The peptide was purified by reverse phase HPLC using a 90–50% water: acetonitrile gradient on a Poros R2/H column. Following purification, fractions were analysed using a Bruker Biopex 4.7T electrospray mass spectrometer or a Kratos Kompact SEQ MALDI-TOF. Peptides stocks were made to 5 mM in H₂O and stored at −20 °C. AChE-peptide, NMDA and D-AP5 (Tocris Cookson) were dissolved directly into aCSF 20–30 min before being introduced to the perfusion chamber.

AChE-peptide was investigated to establish whether it possessed its own hydrolytic capacity or was able to alter the hydrolytic kinetics of the AChE enzyme. The hydrolytic capacity of AChE-peptide and its effects on AChE were investigated using the colorimetric method of Ellman [5]. Assays were performed using a Molecular Devices UVmax plate reader.

Male Wistar rats (200–300 g) from Park Farm, Oxford, were housed in groups of 4–5, with food and water available ad libitum, on a 12 h light/dark cycle. Procedures were conducted in accordance with the Animals Scientific Procedures Act (1986), approved by Her Majesty’s Government Home Office. Rats were anaesthetised with Sagatal (6% w/v, Rhone Merieux) I.P., 3 mg kg⁻¹, until respiratory failure occurred. Rats underwent transectional perfusion using cold, gassed artificial cerebrospinal fluid (mM); NaCl, 127.5; KCl, 3; Na₂HPO₄, 0.5; NaHCO₃, 18.5; KH₂PO₄, 0.5; CaCl₂, 2.6; MgCl₂, 0.8 and d-glucose 5; aCSF. Following decapitation, the brain was removed, bathed in aCSF, and a coronal midbrain slice was isolated on a glass plate. The midbrain slice was mounted on a Vibratome (Lancer series 1000) with cyanoacrylate glue, and coronal slices of 200 μm thickness were then cut from the most posterior region of subthalmic nucleus (STN) to posterior SN. Punches of 2 mm diameter were taken from the ‘SN region’ slices using a concentric cutting device. These punches were placed onto a retaining mesh and enclosed in a perfusion chamber through which aCSF, at 20–22 °C, flowed at 20 μl min⁻¹. The nigral punches were perfused in this manner for 1–2 h, allowing a steady basal AChE release to be established before drug application. All perfusion solutions were continuously oxygenated with 95% O₂/5% CO₂.

The AChE activity in the perfusate from the tissue punches was measured ‘on-line’ in real time by means of a chemiluminescent assay [18]. A calibration curve was obtained at the start of each experiment by introducing exogenous AChE (10, 5, 2.5 and 1 mU) into the detection system.

Once a steady basal release of AChE was established, oxygenated drug media (20–22 °C) were introduced into the perfusion chamber via the aCSF inlet. Drugs were perfused for 30 min; this period allowed the chamber to fill with the drug medium, so that the concentration of drug to which the nigral punches were exposed reached a maximal level.

Four drug application protocols were followed: (i) Control experiments – following a basal release period of 1–2 h, excess potassium (10 mM) was applied for 30 min, to cause neuronal depolarisation and release of the contents of viable cells’ vesicles. (ii) NMDA (25 or 50 μM). Following a period of basal release, NMDA was applied for 30 min. A concentration of NMDA was required that was sufficiently high to evoke detectible changes in AChE levels from basal, yet avoiding NMDA-direct neurotoxicity. Twenty-five and 50 μM were chosen since these concentrations have been used in previous studies of AChE release from the substantia nigra [10]. The effects of NMDA were also examined in the presence of 50 μM D-AP5. (iii) AChE-peptide (100 μM) – as above; a period of basal release followed by a 30 min drug application. (iv) NMDA (50 μM) and AChE-peptide (100 μM) – were co-applied for 30 min following a period of basal release measurement.

Following the 30 min drug application time, the chamber was re-perfused with aCSF for 1 h to establish whether basal levels of release returned. At the end of each experiment, the perfusion chamber was disconnected from the light cell, in order to demonstrate the continuing release of AChE by the nigral punches, and hence, demonstrate punch viability.

Raw optical density data from the enzyme colorimetric assays was analysed using a paired t-test of control, versus drug-treated AChE. Significance was taken to be P = 0.05.

Chemiluminescent signal data was checked and subsequently replayed through a Gould 420 oscilloscope, which had built-in area under curve-measuring facilities, for analysis. A calibration curve of Area Under Curve (Vs) against absolute activity of AChE (mU) using standards was also plotted. The calibration curve was then used to calculate absolute AChE activity released from the punches. Changes in the concentration of AChE are expressed as percentage change from basal release levels. All data were normalised, and the basal release level of AChE was taken as 100%. Data was analysed using a two-way analysis of variance (ANOVA) with factors of NMDA and AChE-peptide, with a Bonferroni post-hoc test; significance was again taken as P = 0.05. All statistical analyses were carried out using Graphpad Prism software.

To ensure that the correct region of the SN had been employed in the release studies, punches were labelled fluorescently for tyrosine hydroxylase. Briefly, tissue punches were submersion fixed in 4% para-formaldehyde made in 0.05 M Tris–HCl buffer, pH 7.4, then washed and...
immersed for 20 min in 3% H_{2}O_{2} and incubated for 60 min at room temperature in 4% normal fetal calf serum (Sigma) in 0.05 M Tris–HCl, pH 7.4, containing 0.1% Triton X-100 (Sigma). Tissue was incubated overnight at 4 °C with rabbit anti-TH polyclonal primary antibody (1:1000; TE101; Eugene Tech, NJ, USA), washed with 0.05 M Tris–HCl, and then incubated for 90 min with anti-mouse FITC secondary antibody (Jackson Immunological). Punches were subsequently slide-mounted in Vectashield (Vector Laboratories) and analysed using a Leica Diaplan microscope or a Leica TCS confocal microscope (using TCS-NT software).

Low levels of impurities were present following the synthesis of peptides. These impurities were primarily formed from the synthesis method. Following purification by HPLC, the largest peak corresponded to the peptide of interest; AChE-peptide. Over an eluting gradient of 10–90% acetonitrile in water the peak of AChE-peptide eluted after 11 min (Fig. 2A(i)) at approximately 75% acetonitrile. A purity of 96–98% was obtained for AChE-peptide. Purified peaks of AChE-peptide were analysed using mass spectrometry and were found to have a molecular weight of 1864.21 (Fig. 2A(ii)). This corresponded closely to the theoretically calculated molecular weight of 1864.12.

AChE-peptide possessed no hydrolytic activity and did not significantly differ from control solutions (0.1 M K+ phosphate buffer) (P > 0.05, paired t-test, n = 6). Acetylthiocholine was not hydrolysed at any of the concentrations of AChE-peptide that were examined (10 mM, 1 mM, 0.1 mM, 10 μM, 1 μM).

One hundred mU ml⁻¹ of AChE was selected for investigating the effects of AChE-peptide on AChE activity. This concentration of AChE was then assayed over time (5 min) in the absence or presence of AChE-peptide (1 mM, 0.1 mM, 10 μM, and 1 μM). AChE-peptide at concentrations of 1 mM, 0.1 mM, 10 μM, and 1 μM had no significant effect on the residual hydrolytic activity of 100 mU ml⁻¹ AChE (P > 0.05, paired t-test, n = 5).

Prior to studies on the release of AChE in vitro, the chemiluminescent AChE detection system was calibrated. Fig. 3 shows a typical trace of the light signal resulting from the introduction of exogenous AChE into the chemiluminescent assay system. Application of AChE caused a dose-dependent increase in the light signal that was linear over the range of AChE activities tested (far left; 1–10 mU ml⁻¹). The area under the curve for each AChE concentration was then plotted against the activity of AChE applied, giving a calibration curve at the start of each experiment.

Twelve randomised punches from bilateral SNc, placed in the tissue bath, were sufficient to detect basal and evoked AChE levels. Tissue punches perfused at the flow rate of 28 μl min⁻¹ revealed activities of AChE spontaneous release ranging from 0 to 2.4 mU ml⁻¹, with a mean of 1.6 mU ml⁻¹. This activity corresponded to a mean absolute activity of 0.09 mU which was released (where 1 mU AChE will hydrolyse 1 mM ACh per min at pH 8 and 37 °C). Evidence for the enduring viability of the tissue was obtained by disconnecting the perfusion chamber from the assay system at the end of each experiment: this caused the detected chemiluminescent signal to return to the levels seen prior to connection of the chamber, i.e., to the levels attributed to the spontaneous hydrolysis of ACh within the assay system.

Before investigating the effects of AChE-peptide on AChE release, the peptide was directly introduced into the assay system, in order to ascertain whether AChE-peptide per se interfered with any of the reactants which were part of the chemiluminescent assay. In a similar manner to results obtained by colorimetric methods [5], introduction of 250 or 100 μM AChE-peptide into the chemiluminescent assay system had no effect on the chemiluminescent reaction.

NMDA (25 and 50 μM) caused a dose-dependent increase in release of 17.71 ± 3.2% (t = 5.34, df 26, P < 0.01) and 39.4 ± 4.2% (t = 9.36, df 26, P < 0.01) above basal levels respectively. After NMDA application, when the punches were re-perfused with aCSF and the

![Fig. 2. Reverse phase HPLC and mass spectrometry of AChE peptides. Following solid-phase organic synthesis peptides were purified using reverse phase HPLC with a Poros R2/H column and then assessed for purity on an analytical Poros R2/H column. AChE-peptide (A(i)) purity was assessed by UV absorbitivity at 214 and 280 nm. To further characterise the purified peptides, AChE-peptide (A(ii)) was examined by MALDI-TOF mass spectrometry.](image)

![Fig. 3. The light signal from one representative experiment. The numbers 1–4 correspond to the introduction of exogenous AChE (10, 5, 2.5 and 1 mM ml⁻¹, respectively) into the chemiluminescent system, for calibration. The ‘c’ corresponds to the connection of the perfusion chamber (containing the tissue punches) to the system; the asterisk shows the point at which, in this experiment, AChE-peptide was applied.](image)
The perfusion chamber had filled with aCSF, AChE activity levels returned to baseline. Perfusion of 50 μM NMDA in the presence of 50 μM D-AP5 significantly antagonised the agonist effect by 29.1 ± 8.6% (t = 3.39, df 6, P < 0.01). Application of AChE-peptide (100 μM) alone to nigral tissue, did not alter AChE release from basal levels (Bonferroni t = 1.054, df 32, P > 0.05); release changed neither during exposure of the punches to the peptide, nor after the peptide had been removed and the punches were re-perfused with aCSF. However, the application of AChE-peptide together with 50 μM NMDA caused an increase in release of 14.6 ± 3.6% above basal levels, some 24.8% less than that from NMDA applied alone. This co-application indicated a significant reduction in 50 μM NMDA effectiveness (Bonferroni t = 4.54, df 32, P < 0.001). On re-perfusion with aCSF, release of AChE returned to basal levels; this occurred as soon as the perfusion chamber had re-filled with aCSF.

No drug applications were carried out after a 2 h period. After 2 h all tissue was tested for responsiveness to a 10 mM KCl application; if this stimulus was unable to evoke AChE release, data collected for that experiment was excluded. Following a sustained perfusion period, application of 10 mM KCl caused a 45.5 ± 5.3% increase in the extracellular concentration of AChE above basal levels (t = 8.81, df 26, P < 0.001; Fig. 4). Moreover, in all experimental groups, following applications of peptide and/or NMDA, exposure to 10 mM KCl after a wash period still caused an increase in extracellular concentrations of AChE.

Using immunohistochemistry to identify TH immunoreactive (TH-ir) cells, it was verified that the tissue punches contained dopaminergic neurons (Fig. 5). The TH-ir neurons were normally located within a sub-region of the punch with an average of 193 ± 42 cells per punch (n = 12). The TH-ir neurons were characterised by their relatively large cell body diameter of 20.1 ± 2.8 μm (n = 61) and three to five primary dendrites that extended for several hundred micrometers through the punch.

The chemiluminescent detection system used in the current in vitro experiments for the assessment of AChE possessed equal sensitivity and similar temporal resolution to previous reports when used in conjunction for in vivo analysis of released AChE concentrations [18]. The current results revealed that the tissue was viable for a sustained period of time and was responsive to drug applications over a period of 2 h. The chemiluminescent cascade reaction for the detection of AChE has a number of stages where compounds introduced into system may react with reagents [8]. Neither NMDA, D-AP5 or AChE-peptide interfered with the detection of AChE. The basal release of AChE from 12 punches per experiment remained constant (1.6 mU ml⁻¹) throughout each experiment, and was reduced when punches were disconnected from the perfusion system, indicating that the tissue remained viable throughout the course of the experiment. Within the SN, the majority of soluble AChE is released from these DA neurons [6]. TH immunolabelling revealed that the tissue punches obtained from sections of the SNc contained the AChE releasing DA neurons. Hence, it is likely the AChE released from the tissue punches in this study is indeed from dopaminergic cells.

The dopaminergic neurons of the SN themselves express NMDA receptors [13]. There are also glutamatergic afferent projections from the STN and pedunculopontine tegmental nucleus; the former of these nuclei modulate the extracellular concentrations of AChE in vivo, within the SN [10]. In the current study, for the first time in vitro using on-line detection of AChE, NMDA has been shown to increase extracellular concentrations of AChE, presumably by acting either directly on the dopaminergic neurons themselves, or indirectly via the glutamatergic afferent projections which may be operational even in a tissue punch. Such a concentration-dependent effect of NMDA was physiologically as concentrations were well outside the range of neurotoxicity previously reported by Dickie et al. [4] for in vitro studies. Interestingly APP and Aβ, like AChE in the current study, can modulate NMDA excitatory synaptic transmission [11] and potentially AChE release.

The AChE-derived peptide did not modify release of AChE alone, so any non-specific membrane effects of bulk peptide can be excluded, as well as any neurotoxic effects observed with Aβ1–40 studies [9]. Within the SN,
full length AChE has been shown to hyperpolarise neurones via the NMDA receptor [20]; an effect which would lead to reduced extracellular concentrations of AChE. The work of Webb was carried out in brain slices where there is likely to be tonic innervation of the AChE releasing neurones; in the punch experiments currently employed such innervation is less likely. Given AChE-peptide did not modify basal AChE concentrations it is probable there is no tonically active neurones; only in the presence of an NMDA stimulus do AChE-peptide effects become unmasked.

On endogenous transmitter systems, in the presence of NMDA, AChE-peptide modulated the extracellular concentration of AChE compared to the control NMDA treatment; since the release of AChE is a Ca\(^{2+}\) dependent process [14] AChE-peptide is presumably modifying AChE release by affecting Ca\(^{2+}\) signalling. AChE itself causes activation of Ca\(^{2+}\) channels [3], whilst AChE-peptide enhances Ca\(^{2+}\) potentials when NMDA receptors are antagonised [2]. However, AChE-peptide decreases the amplitude of NMDA-induced depolarisation of Ca\(^{2+}\)-spikes in hippocampal neurons [2]. In both this study of hippocampus and the current preparation of nigral tissue, the most parsimonious explanation is that additional Ca\(^{2+}\) influx, triggered by both NMDA plus AChE-peptide, leads to Ca\(^{2+}\) channel inactivation.

AChE-peptide is shown to be bioactive within the SN, where it may play an important role as a modulator of Ca\(^{2+}\) entry and regulate extracellular concentrations of AChE. Hence, there is a potential feedback mechanism for autocontrol of soluble AChE levels which in turn can modify the activity of nigral neurones. Since the dopaminergic cells of the SN degenerate in Parkinson’s disease this homeostatic system may be of fundamental relevance to our understanding of mechanisms of cell signalling underlying cell death.

Acknowledgements

SRE and SAG wish to thank Synaptica Ltd, UK for the funding of this work carried out within the University of Oxford, UK. We would also like to acknowledge the contributions of Maureen Pitkeithley at OCMS, Carolyn Carr at The Dyson Perrins laboratory, Oxford and Kevin Prior at the Department of Pharmacology, Oxford.

References