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RESEARCH ARTICLE

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A peptide derived from acetylcholinesterase induces neuronal cell death: characterisation of possible mechanisms

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Abstract Acetylcholinesterase (AChE) exhibits functions unrelated to the catalysis of acetylcholine (ACh) in particular during development. Although the underlying mechanism(s) is presently unknown, a candidate peptide fragment (AChE-peptide) has recently been identified, and been shown to induce a continuum of apoptotic and necrotic neuronal cell death in rat hippocampal organotypic cultures. The aim of this study was to trace the cell death pathway initiated by AChE-peptide. Using specific antagonists, it was possible to track a series of cellular events following application of 1 nM AChE-peptide: NMDA receptor activation, opening of the L-type voltage gated calcium channel, activation of calcium/calmodulin kinase II, generation of reactive oxygen species and caspase activation. Pharmacological interception at any stage of this cascade blocked the effect of 1 nM AChE-peptide on neurite retraction. Lactate dehydrogenase (LDH) release, a marker for cell lysis, was unaffected by 1 nM AChE-peptide. In contrast, cell death induced by 1 mM AChE-peptide, monitored as neurite retraction and increased LDH efflux, was not offset by any drug treatment. These data suggest that nanomolar concentrations of AChE-peptide exhibit pathophysiological activity via an apoptotic pathway that could play an important role in neuronal development and neurodegeneration.

Keywords Acetylcholinesterase · Cell death · Hippocampus · AChE-peptide

Introduction

Acetylcholinesterase (EC 3.1.1.7; AChE), the familiar enzyme that hydrolyses acetylcholine (ACh), exhibits additional non-cholinergic actions related to neural development (Appleyard 1994; Greenfield 1996; Holmes et al. 1996; Day and Greenfield 2002), possibly by enhanced calcium influx (Webb et al. 1996). During development, increased calcium flux is thought to be beneficial to neuronal survival and growth; however, as a cell matures a similar benign level of calcium can prove toxic (Eimerl and Schramm 1994). It has therefore been postulated that the trophic action of AChE, under inappropriate conditions such as in a mature brain, could instead be deleterious (Greenfield and Vaux 2002).

A peptide derived from the C-terminus of AChE, AEFHRWSSYMVHWK (AChE-peptide), with structural and sequence homology to β-amyloid, has recently been described and may be responsible for some of these non-cholinergic actions (Greenfield and Vaux 2002). AChE-peptide induces both neurotrophic and neurotoxic effects when applied to organotypic hippocampal cultures, dependant on peptide concentration and the duration of treatment (Day and Greenfield 2003). A similar continuum of neurotrophic-neurotoxic actions has been reported following calcium influx mediated by *N*-methyl-D-aspartate (NMDA) receptor activation (Dickie et al. 1996), or with increasing age as calcium tolerance decreases (Eimerl and Schramm 1994).

Recent studies suggest that AChE-peptide modulates α7 nicotinic acetylcholine receptor (α7 nAChR) function (SA Greenfield, T Day, K Whyte and I Bermudez, submitted for publication), a receptor subtype selectively associated with both neuronal development (Chan and Quik 1993; Pugh and Berg 1994) and neurotoxic potential (Lukas et al. 2001). Since the α7 nAChR acts as a powerful calcium ionophore (Séguéla et al. 1993), AChE-peptide-induced cell death may be due to enhanced calcium entry.

Accordingly, the aim of this study was to elucidate, using a range of pharmacological tools, the biochemical pathway(s) that mediate the continuum of apoptotic–necrotic processes induced by AChE-peptide in rat hippocampal organotypic cultures. Since activation of the α7 nAChR by AChE-peptide provides appropriate

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depolarising voltage conditions for activation of NMDA receptor and voltage-dependant calcium channels (VDCCs), appropriate antagonists for both types of channel were tested. Regardless of the source of calcium entry, elevated intracellular calcium might be expected to induce mitochondrial dysfunction and the production of free radicals (for review, see Montal 1998); free radical scavengers were therefore tested to establish whether these processes mediate the toxic action of AChE-peptide. Since apoptosis, but not necrosis, is dependent on the activation of caspases (Harada and Sugimoto 1999), a caspase inhibitor was used to distinguish apoptotic and necrotic processes. Similarly, neuronal apoptosis is often accompanied by changes in cyclin-dependent kinase activity and cyclin expression (Park et al. 1997; Boutillier et al. 1999; Sakai et al. 1999); a cell cycle inhibitor was therefore tested to determine the role of cell-cycle dysfunction in AChE-peptide toxicity.

Materials and methods

Cultures

Organotypic hippocampal cultures were prepared as detailed elsewhere (Gähwiler 1981, 1984). In brief, Wistar rats (Harlan Olac, Oxford, UK) at postnatal day 7 were killed by halothane overdose, and the brains removed. Hippocampal slices (400 µm) were prepared using a McIlwain tissue chopper and were embedded on a plasma clot. Cultures were maintained on a roller drum at 37°C. All treatments were applied in serum-free medium (Dulbecco's minimal essential media, DMEM, supplemented with B-27) containing antimitotic agents (uridine- and cytosine-β-D-furanoside, both at 1 µM; Sigma-Aldrich Company Ltd, Poole, UK) to prevent proliferation of non-neuronal cells, and Fungizone (Life Technologies Ltd, Paisley, UK) to reduce the risk of infection. AChE-peptide was purified (Mr. K. Pryor, Department of Pharmacology, University of Oxford), freeze-dried and maintained in aliquots (5 mM) at −20°C. On treatment days, fresh solutions where prepared at 1 mM in phosphate-buffered saline (PBS) and serially diluted to the required concentration in freshly filtered serum-free medium. Hippocampal cultures were treated with synthetic AChE-peptide (AEFHRWSSYMVHWK) at 1 nM and 1 mM. Cultures were fed biweekly with serum-free medium, supplemented with peptide as required. All cultures were maintained for 336 h.

Lactate dehydrogenase efflux

Membrane integrity was monitored by measuring lactate dehydrogenase (LDH) efflux according to the manufacturer's instructions (Sigma-Aldrich). Briefly, the culture medium was removed and pipetted into 96-well plates. The sample start reagent (NADH 0.194, pyruvate 16.2 mmol/l in phosphate buffer, pH 7.5) was added, and colourimetric intensity was determined at 340 nm over 3 min.

Immunohistochemistry

Microtubule associated peptide antibody (MAP2; 1:1500 dilution; Chemicon International, Harrow, UK) was used as a generic neuronal marker. AChE-peptide bioactivity was evaluated by measuring neurite outgrowth from MAP2-immunopositive neurones. Cultures were fixed with 4% paraformaldehyde for 1 h at room temperature. After washing, MAP2 antibody (100 µl) was applied to each culture for 24 h at 4°C. Following extensive washing, cultures were then exposed to the secondary antibody (1:300 dilution; goat anti-rabbit; Novocastra Labs., Newcastle-upon-Tyne, UK); immunoreactivity was detected using the ABC kit (Vector Labs., Peterborough, UK) according to the manufacturer's instructions. Camera lucida drawings were used to determine neurite outgrowth using a non-bias method involving a grid (100 μm^2) superimposed over an image of the culture. Neurite outgrowth from immunopositive neurones in every fifth square of the grid was measured using an eye piece graticule attached to a Nikon Diaphot inverted microscope.

Drugs and solutions

Test compounds were included throughout the 336 h culture period and were added in freshly filtered, pre-warmed (37°C) serum-free medium containing antimitotic agents (Sigma-Aldrich) and Fungizone (Life Technologies). Nimodipine, mimosine, Z-DEVD-fmk, sFTX-3.3, ω-agatoxin and vitamin E (CN Biosciences, Nottingham, UK), calcium/calmodulin kinase II inhibitor peptide 281–309 and melatonin (Calbiochem-Novabiochem UK Ltd, Beeston, UK) were made up to 1 mM stock solutions and stored as aliquoted fractions at −20°C. ω-Conotoxin GVIA (Tocris-Cookson Ltd, Bristol, UK) was prepared as a 1 mM stock and frozen in aliquots at −20°C. MK801, CNQX (Tocris-Cookson) and ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) were made as 1 mM stock solutions on the day of application. Compound stocks were diluted in freshly filtered serum-free medium on the treatment day and pre-warmed to 37°C prior to application.

Analysis

Each treatment group included cultures from at least three different litters and data were derived from 13–18 independent cultures. Drug treatments were generated from five independent studies, with data from each study shown in individual figures with the associated control group. Results were expressed as percentage mean ±SEM. Statistical evaluation was by analysis of variance (ANOVA) followed by Dunnett's multiple comparison *t*-tests.

Results

Effects of D-2-amino-5-phosphonovalerate and nimodipine on AChE-peptide-induced toxicity

In this experimental series, MAP2-immunopositive neurones in control cultures exhibited neurite outgrowth of 154±5.8 µm (mean ±SEM, n=134) after 14 days in vitro (DIV). LDH activity measured in supernatants from 14 DIV hippocampal cultures was 0.9±0.1 U/ml (mean ±SEM, n=134). Application of AChE-peptide for 14 DIV significantly reduced neurite outgrowth at both 1 nM and 1 mM (Fig. 1A). At 1 nM, neurones exhibited neurite outgrowth of 70.3±4.0 µm (n=131; 45% of control; P<0.01); LDH activity in culture supernatants was unaffected (Fig. 1B). Cultures treated with 1 mM AChE-peptide for 14 days exhibited neurite outgrowth of 37.6 ±3.7 µm (n=131; 24% of control; P<0.01) and increased supernatant LDH activity (to 7.2±0.1 U/ml, n=131; 800% of control; P<0.01).

D-2-Amino-5-phosphonovalerate (D-APV, 1 and 10 µM), a specific NMDA receptor antagonist (Davies

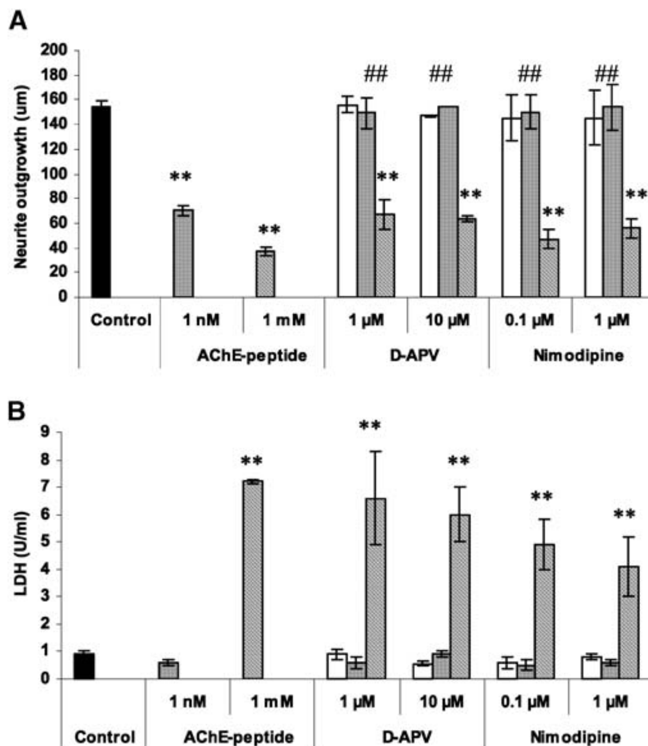

Fig. 1A,B Effects of specific NMDA receptor antagonist D-APV (1 and 10 μM) and L-type voltage-dependant calcium channel blocker nimodipine (0.1 and1 μM) on AChE-peptide-induced alterations in **A** MAP2-positive neurite outgrowth, and **B** lactate dehydrogenase (LDH) efflux, from rat hippocampal organotypic cultures following 14 days in vitro. Values are means ±SEM (n=131–134); *vertical-hatched bars* 1 nM AChE-peptide, *diagonal-hatched bars* 1 mM AChE-peptide, *open bars* D-APV or nimodipine treatment alone, *solid bars* control. **Significant difference from control (P<0.01), ##significant difference from 1 nM AChE-peptide (P<0.01); ANOVA followed post hoc Dunnett's multiple comparisons t-test

and Watkins 1982), did not affect neurite outgrowth or LDH release when applied alone for 14 DIV (Fig. 1A,B). While 1 and 10 μM D-APV reversed the effect of 1 nM AChE-peptide on neurite outgrowth (to 97% and 100% of control, respectively, n=134; Fig. 1A), the alterations in neurite outgrowth (43% and 41% of control, respectively, n=134; Fig. 1A) and LDH efflux (733% and 667% of control, respectively, n=134; Fig. 1B) induced by 1 mM AChE-peptide were unaffected. Co-application of a non-NMDA receptor antagonist (CNQX, 1 and 5 μM; Heng et al. 1999) failed to inhibit the affects of either concentration of AChE-peptide (data not shown).

Nimodipine (0.1 and 1 μM), an L-type voltage-dependant calcium channel (L-VDCC) blocker (Catterall 2000; Mironov and Richter 2000) did not elicit neurite outgrowth or LDH efflux when applied alone for 14 DIV (Fig. 1A,B). The reduction in neurite outgrowth observed following application of 1 nM AChE-peptide was blocked by co-application of 0.1 or 1 μM nimodipine (97 and 100% of control, respectively; Fig. 1A). In contrast, nimodipine did not influence the effects of 1 mM AChE-peptide on neurite outgrowth (30% and 36% of control, respectively, n=131; Fig. 1A) or LDH efflux (544% and 465% of control, respectively, n=131; Fig. 1B). Blockers of other calcium channel subtypes, such as ω-conotoxin GVIA (N-type VDCCs, at 1 and 10 μM; Spedding and Paoletti 1992; Heng et al. 1999), ω-agatoxin IVA (P-type VDCCs, at 100 and 300 nM; Spedding and Paoletti 1992; Heng et al. 1999) and sFTX-3.3 (N-, P- and T-type VDCCs, at 1 and 10 μM; Moya and Blagbrough 1996) were ineffective on the response to either concentration of AChE-peptide (data not shown).

Effects of EDTA and calcium/calmodulin kinase II inhibitor peptide 281–309 on AChE-peptide-induced toxicity

In this series, MAP2-immunopositive neurones in 14 DIV control cultures exhibited neurite outgrowth of 151.4 ±5.8 μm (mean ±SEM, n=131); supernatant LDH activity was 0.9±0.1 U/ml (mean ±SEM, n=134). Application of 1 nM AChE-peptide for 14 DIV reduced neurite outgrowth (73.3±6.2 μm, n=131; 48% of control, P<0.01), with no effect on supernatant LDH activity (Fig. 2A,B). Using 1 mM AChE-peptide, neurite outgrowth was reduced to 31.6±3.9 μm (n=131; 21% of control, P<0.01; Fig. 2A) and supernatant LDH activity was 6.1±0.1 U/ml (n=131; 667% of control, P<0.01; Fig. 2B).

EDTA (1 and 10 μM), a calcium chelator, and peptide 281–309 (10 and 100 nM), a calcium/calmodulin kinase II inhibitor (Soderling et al. 1990), did not affect neurite outgrowth or LDH efflux when applied alone for 14 DIV (Fig. 2A,B). Co-application of EDTA (1 and 10 μM) blocked the effect of 1 nM AChE-peptide on neurite outgrowth (101% and 102% of control, respectively, n=134; Fig. 2A), but again failed to prevent the reduction in neurite outgrowth (42% and 51% of control, respectively, n=134; Fig. 2A) or the increase in LDH efflux (744% and 689% of control, respectively, n=134; Fig. 2B) induced by 1 mM AChE-peptide. Similarly, peptide 281–309 (10–100 nM), attenuated the affect of 1 nM AChE-peptide on neurite outgrowth (101% and 101% of control, respectively, n=134; Fig. 2A), but failed to reverse the effects of 1 mM AChE-peptide on neurite outgrowth (51% and 32% of control, respectively, n=134; Fig. 2A) or LDH efflux (744% and 844% of control, respectively, n=134; Fig. 2B).

Effects of vitamin E and melatonin on AChE-peptide mediated toxicity

In this series of studies, MAP2-immunopositive neurones in 14 DIV control cultures exhibited neurite outgrowth of 158.4±5.8 μm (mean ±SEM, n=131), and supernatant LDH activity of 0.7±0.1 U/ml (mean ±SEM, n=134). Application of 1 nM AChE-peptide for 14 DIV reduced neurite outgrowth (79.3±4.1 μm, n=131; 50% of control, P<0.01; Fig. 3A), without affecting supernatant LDH activity (Fig. 3B). Cultures treated with 1 mM AChE-peptide for 14 DIV exhibited reduced neurite outgrowth

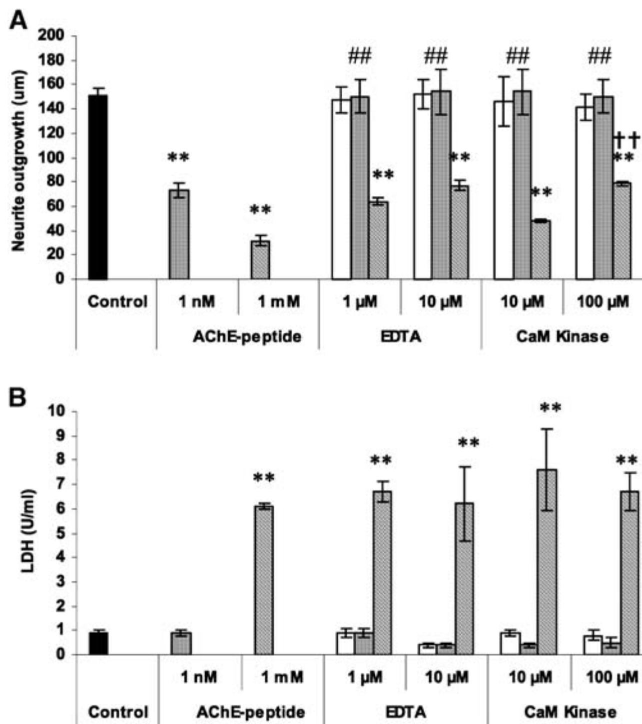

Fig. 2A,B Effects of calcium chelator EDTA (1 and 10 µM) and calcium/calmodulin kinase II inhibitor peptide 281–309 (*CaM Kinase*; 10 and 100 µM) on AChE-peptide-induced alterations in **A** MAP2-positive neurite outgrowth and **B** LDH efflux, from rat hippocampal organotypic cultures following 14 days in vitro. Values are means ±SEM (n=131–134); *vertical-hatched bars* 1 nM AChE-peptide, *diagonal-hatched bars* 1 mM AChE-peptide, *open bars* EDTA or peptide 281–309 treatment alone, *solid bars* control. **Significant difference from control levels (P<0.01), $^{\#\#}$significant difference from 1 nM AChE-peptide (P<0.01), and ††significant difference from 1 mM AChE-peptide (P<0.01); ANOVA followed by post hoc Dunnett's multiple comparisons t-test

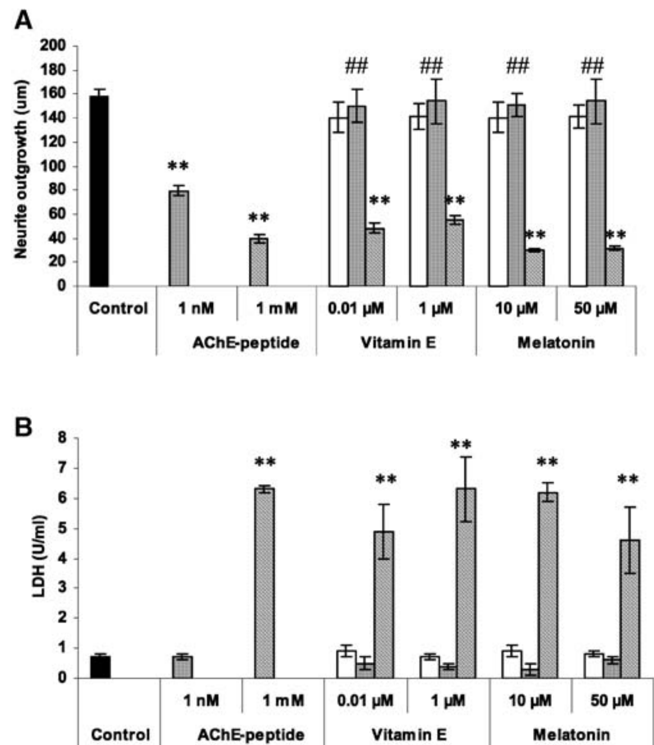

Fig. 3A,B Effects of free radical scavenger vitamin E (0.01 and 1 µM) and antioxidant melatonin (10 and 50 µM) on AChE-peptide induced alterations in **A** MAP2-positive neurite outgrowth and **B** LDH efflux, from rat hippocampal organotypic cultures following 14 days in vitro. Values are means ±SEM (n=131–134); *vertical-hatched bars* 1 nM AChE-peptide, *diagonal-hatched bars* 1 mM AChE-peptide, *open bars* vitamin E or melatonin treatment alone, *solid bars* control. **Significant difference from control levels (P<0.01), $^{\#\#}$significant difference from 1 nM AChE-peptide (P<0.01); ANOVA followed by post hoc Dunnett's multiple comparisons t-test

(39.6±3.2 µm, n=131; 25% of control, P<0.01) and increased supernatant LDH activity (6.3±0.1 U/ml, n=131; 900% of control, P<0.01) (Fig. 3A,B).

Vitamin E (0.01 and 1 µM), a free radical scavenger (Grundman 2000), and melatonin (10 and 50 µM), an antioxidant (Marshall et al. 1996; Reiter et al. 1997, 1999), did not affect neurite outgrowth or LDH release when applied alone for 14 DIV (Fig. 3A,B). Vitamin E (0.01 and 1 µM) attenuated the reduction in neurite outgrowth induced by 1 nM AChE-peptide (19% and 103% of control, respectively, n=134; Fig. 3A), but did not influence the effect of 1 mM AChE-peptide on either neurite outgrowth (Fig. 3A; 31% and 35% of control, respectively, n=134) or LDH efflux (700% and 900% of control, respectively, n=134; Fig. 3B). Melatonin (10 and 50 µM) also blocked the reduction in neurite outgrowth induced by 1 nM AChE-peptide (95% and 102% of control, respectively, n=134; Fig. 3A). The reduction in neurite outgrowth induced by 1 mM AChE-peptide was unaffected by 10 and 50 µM melatonin (19 and 20% of control, respectively, n=134; Fig. 3A), as was the associated increase in supernatant LDH activity (886% and 657% of control, respectively, n=134; Fig. 3B).

Effects of Z-DEVD-fmk on AChE-peptide mediated toxicity

In this series (Fig. 4A,B), MAP2-immunopositive neurones in 14 DIV control cultures exhibited neurite outgrowth of 156.4±5.6 µm (mean ±SEM, n=131), and supernatant LDH activity of 0.7±0.1 U/ml (mean ±SEM, n=134). Application of 1 nM AChE-peptide for 14 DIV (Fig. 4A) reduced neurite outgrowth to 75.8±5.9 µm (n=131; 49% of control, P<0.01), without affecting supernatant LDH activity (0.7±0.1 U/ml, n=131; Fig. 4B). In cultures treated with 1 mM AChE-peptide for 14 DIV, neurite outgrowth was reduced to 38.0±11.6 µm (n=131; 24% of control, P<0.01) with LDH release of 6.3±0.1 U/ml (n=131; 900% of control, P<0.01) (Fig. 4A,B).

While exposure to a caspase inhibitor (Z-DEVD-fmk, 1–10 µM) for 14 DIV did not affect neurite outgrowth or LDH efflux in control cultures (Fig. 4A,B), co-application with 1 nM AChE reversed the reduction in neurite outgrowth induced by exposure to peptide alone (80% and 93% of control, respectively, n=134; Fig. 4A). The effects of 1 mM AChE-peptide on neurite outgrowth was unaffected by 1 and 10 µM Z-DEVD-fmk (32% and 27%

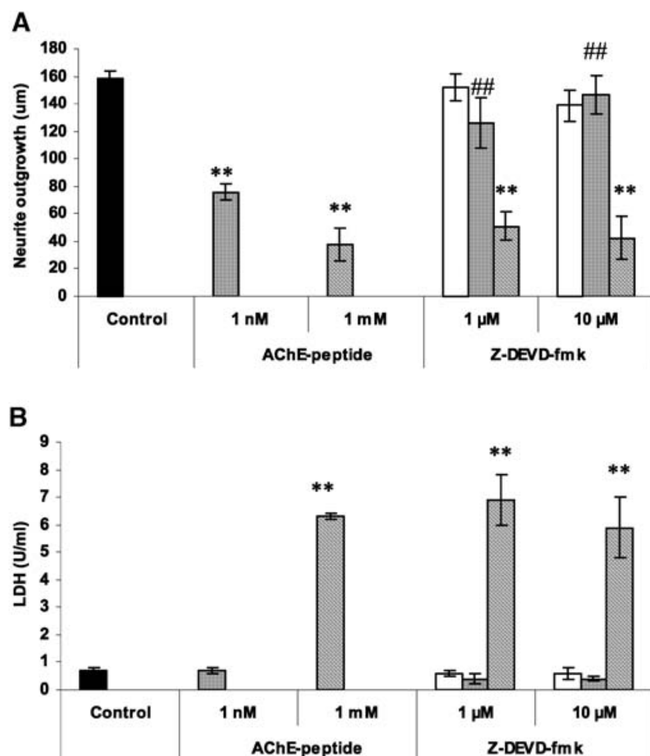

Fig. 4A,B Effects of caspase inhibitor Z-DEVD-fmk (1 and 10 µM) on AChE-peptide induced alterations in **A** MAP2-positive neurite outgrowth and **B** LDH efflux, from rat hippocampal organotypic cultures following 14 days in vitro. Values are means ±SEM (n=131–134); *vertical-hatched bars* 1 nM AChE-peptide, *diagonal-hatched bars* 1 mM AChE-peptide, *open bars* Z-DEVD-fmk treatment alone, *solid bars* control. **Significant difference from control levels (P<0.01), ##significant difference from 1 nM AChE-peptide (P<0.01); ANOVA followed by post hoc Dunnett's multiple comparisons t-test

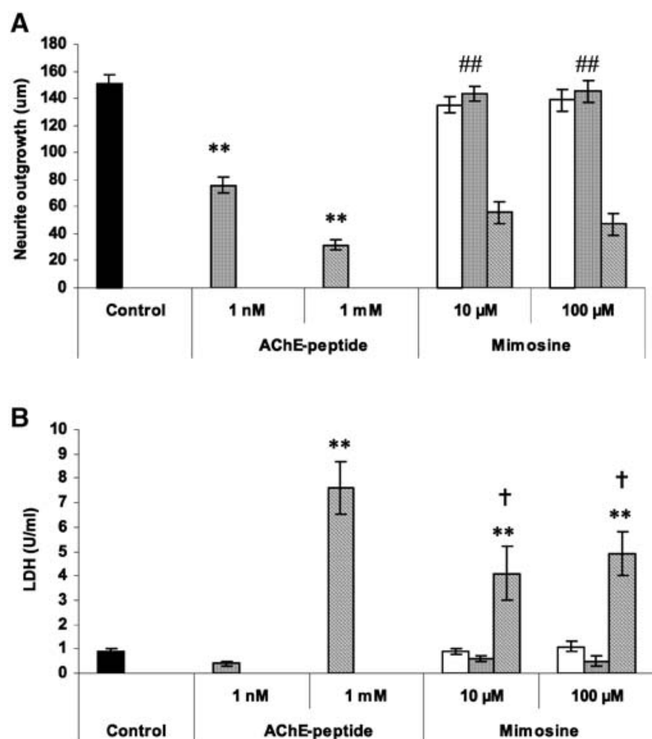

Fig. 5A,B Effects of cell-cycle inhibitor mimosine (10 and 100 µM) on AChE-peptide induced alterations in **A** MAP2-positive neurite outgrowth and **B** LDH efflux, from rat hippocampal organotypic cultures following 14 days in vitro. Values are means ±SEM (n=131–134); *vertical-hatched bars* 1 nM AChE-peptide, *diagonal-hatched bars* 1 mM AChE-peptide, *open bars* mimosine treatment alone, *solid bars* control. **Significant difference from control levels (P<0.01), ##significant difference from 1 nM AChE-peptide (P<0.01), and †significant difference from 1 mM AChE-peptide (P<0.05); ANOVA followed by post hoc Dunnett's multiple comparisons t-test

of control, respectively, n=134; Fig. 4A) and LDH efflux was similarly unaffected (985% and 843% of control, respectively, n=134; Fig. 4B).

AChE-peptide induced toxicity is mediated via cell-cycle re-entry

In this study, MAP2-immunoreactive neurones in 14 DIV control cultures exhibited neurite outgrowth of 151.4 ±4.2 µm (mean ±SEM, n=131), and supernatant LDH activity of 0.9±0.1 U/ml (mean ±SEM, n=134). Application of 1 nM AChE-peptide for 14 DIV reduced neurite outgrowth to 75.3±6.2 µm (n=131; 50% of control, P<0.01; Fig. 5A) without affecting supernatant LDH activity (Fig. 5B). Using 1 mM AChE-peptide, neurite outgrowth was reduced to 31.6±3.9 µm (n=131; 21% of control, P<0.01; Fig. 5A), with supernatant LDH activity of 7.6±1.1 U/ml (n=131; 844% of control, P<0.01; Fig. 5B).

Mimosine (10 and 100 µM), which blocks the G1/S stage of the cell cycle (Park et al. 1997), did not affect neurite outgrowth or supernatant LDH activity when applied alone for 14 DIV. However, 10 and 100 µM mimosine reversed the effect of 1 nM AChE-peptide on neurite outgrowth (96% and 94% of control, respectively, n=134; Fig. 5A). In contrast, mimosine failed to block the reduction in neurite outgrowth (37% and 31% of control, respectively, n=134; Fig. 5A) or increase in LDH efflux (456% and 544% of control, respectively, n=134; Fig. 5B) induced by 1 mM AChE-peptide.

Discussion

The aim of this study was to investigate the pathway of AChE-peptide-induced cell death in rat hippocampal organotypic cultures using inhibitors of various cellular processes. MAP2 immunochemistry was used as a general marker of neuronal viability, allowing morphological analysis of neurite outgrowth, and LDH efflux into culture supernatants was monitored to determine membrane integrity. The study was conducted in five independent experimental series; comparison of control data from each series revealed a reassuring internal consistency, suggesting that antagonist effects were robust.

Recent data suggest that nanomolar, pathophysiological concentrations of AChE-peptide induce neuronal damage

via a predominantly apoptotic mechanism involving α7 nAChR activation (Day and Greenfield 2003; SA Greenfield, T Day, K Whyte and I Bermudez, submitted for publication). In contrast, an α7 nAChR-independent process is involved in the primarily necrotic cell-death process noted using higher AChE-peptide concentrations (Day and Greenfield 2003; SA Greenfield, T Day, K Whyte and I Bermudez, submitted for publication). The present study aimed to further characterise and define the two cell-death processes using selective biochemical tools.

Calcium entry mediates the neuronal effects of nanomolar AChE-peptide

While previous studies have demonstrated that α7 nAChR activation mediates the bioactivity of nanomolar AChE-peptide (SA Greenfield, T Day, K Whyte and I Bermudez, submitted for publication), calcium entry via the α7 nAChR receptor will result in membrane depolarisation, which will in turn activate other voltage-sensitive membrane ionophores. The present data lend support to this suggestion since a specific NMDA receptor antagonist, but not non-NMDA glutamate receptor antagonists, reversed the effect of 1 nM AChE-peptide; this effect is not surprising because membrane depolarisation will remove the voltage-dependant blockade of the NMDA ionophore by magnesium (Sombati et al. 1991). Data generated using VDCC blockers suggest additional routes of calcium entry, which may be activated by membrane depolarisation resulting from α7 nAChR and/or NMDA receptor activation. Nimodipine, a specific inhibitor of L-type VDCCs, reversed the effects of nanomolar AChE-peptide, suggesting that calcium entry via this channel contributes to AChE-peptide neurotoxicity (Day and Greenfield 2002). Interestingly, the effect was channel specific since inhibitors of other VDCCs were ineffective. The effect of nimodipine might be explained by the selective involvement of L-type VDCCs in AChE-peptide-induced neurite retraction. Alternatively, L-type VDCCs may be an indirect consequence of free radical formation (see below), rather than the result of membrane depolarisation. These data, together with the findings that the calcium chelator EDTA and an inhibitor of calcium/calmodulin kinase II reverse the effect of 1 nM AChE-peptide, strongly suggest that neurite retraction is calcium-mediated (Pugh and Berg 1994).

Intracellular cascades involved in nanomolar AChE-peptide bioactivity

Oxidative stress and excitotoxic stimuli, such as excess activation of NMDA receptors, may represent a final common pathway for cell vulnerability in the brain (Lei et al. 1992; Coyle and Puttfarcken 1993; Dykens 1994; Le et al. 1995; Perez-Velazquez et al. 1997; Anegawa et al. 2000). The present data demonstrate that the effects of 1 nM AChE-peptide are attenuated by co-application of the free radical scavengers vitamin E and melatonin, suggesting that the AChE-peptide-induced cell death pathway involves the production of free radicals and that oxidative stress free radical production may also be linked to L-type VDCC activation since membrane lipid peroxidation increases calcium channel permeability and passive calcium leak (Okabe et al. 1988; Clague and Langer 1994), an effect inhibited by both L-VDCC blockers and free radical scavengers. Moreover, activation of VDCCs by β-amyloid is reported to involve free radical production (Ueda et al. 1997), raising the possibility that L-VDCC activation is an indirect consequence of free radical production, rather than an effect of membrane depolarisation (Ohkuma et al. 2001).

Previous observations suggest that nanomolar AChE-peptide induces neuronal apoptosis (Day and Greenfield 2003). A potential step in the process that commits a cell to apoptosis is an attempt to re-enter the cell cycle, perhaps as an adherent developmental response to cell stress. This process has been noted in the brains of patients suffering from chronic neurodegenerative diseases such as Alzheimer's dementia (Nagy et al. 1998; Giovanni et al. 1999; Raina et al. 1999, 2000; Jellinger and Stadelmann 2001). Since mimosine, a cell-cycle inhibitor, reversed the effect of nanomolar AChE-peptide, it would appear that attempted cell-cycle re-entry may play a role in the apoptotic response to AChE-peptide. A recent study has also demonstrated that G1/S blockers prevent cell death induced by nerve growth factor (NGF) withdrawal in PC12 cells, an effect attenuated by caspase inhibitors (Park et al. 1997). While these data appear complementary, data interpretation is conflated by the antioxidant properties of mimosine.

Activation of the caspase family is perhaps the key step in the apoptotic execution process, as confirmed by the ability of caspase inhibitors to block apoptotic cell death (Ferri and Kroemer 2001). Caspase inhibition attenuated the decrease in neurite outgrowth caused by nanomolar AChE-peptide, confirming that apoptosis plays a key role in AChE-peptide bioactivity. Similar conclusions have been drawn for β-amyloid toxicity (Jacobson 1996; Joseph et al. 1997; Guo et al. 1999; Ramassamy et al. 2000), suggesting that induction of apoptotic cell death may be a common feature shared by various neurotoxic β-sheet peptides (Monji et al. 2001; Bucclantini et al. 2002). Caspase activation could either be a direct consequence of calcium overload involving mitochondrial release of cytochrome *c* (Zhang et al. 2002) via activation of calcium-dependant proteases such as calpains (DeGarcia et al. 2002; Han et al. 2002; Moore et al. 2002), or as a consequence of free radical production (Amoroso et al. 2002; Kim et al. 2002). Regardless of the mechanism, caspase activation by 1 nM AChE-peptide presumably represents a late step that commits the neurone to apoptotic cell death.

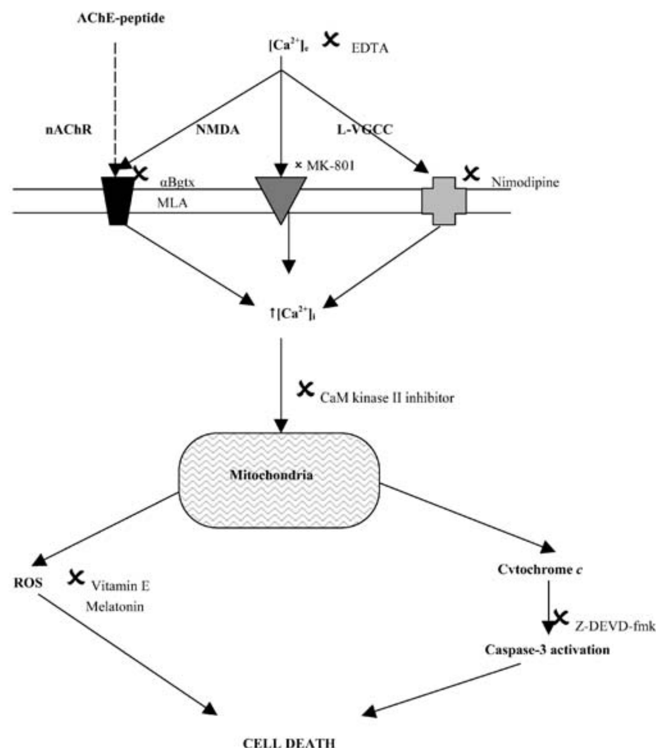

Fig. 6 Schematic representation of a potential biochemical pathway for cell death induced by AChE-peptide. Activation of α7 nAChR (*nAChR*) induces membrane depolarisation leading to the activation of the NMDA receptor. Excessive activation of NMDA receptor (*NMDA*) induces massive calcium influx and abnormal elevations in intracellular calcium ($[Ca^{2+}]_i$). This in turn causes a decrease in ATP synthesis and the opening of the mitochondrial permeation pore. Mitochondrial dysfunction elicits a further reduction in intracellular ATP pools, an increase in free radical generation, cytochrome *c* release followed by caspase-3 activation, and ultimately cell death via apoptosis. *X* Inhibition, *L-VGCC* L-type voltage-gated calcium channel, $[Ca^{2+}]_e$ extracellular calcium, *MK-801* dizocilpine, *αBgtx* α-bungarotoxin, *MLA* methyllycaconitine, *CaM* calmodulin, *ROS* reactive oxygen species

Distinct neurotoxic mechanisms at different AChE-peptide concentrations

The profile described above allows construction of a cellular hypothesis to explain the bioactivity of nanomolar AChE-peptide in hippocampal neurones (Fig. 6). At nanomolar concentrations of AChE-peptide, calcium entry via the α7 nAChR initiates a cellular process that culminates in predominantly apoptotic cell death. Calcium entry induced by AChE-peptide activation of the α7 nAChR may be further enhanced via depolarisation-induced activation of voltage-dependant NMDA receptor channels, and potentially by L-VDCCs (Huang et al. 2000). Calcium overload activates intracellular enzymes such as calcium/calmodulin kinase II, promoting mitochondrial dysfunction and free radical production, followed by abortive cell-cycle re-entry and caspase activation. In many ways these events mirror the classic description of NMDA receptor-mediated excitotoxicity involving calcium entry with consequent activation of multiple secondary cellular mediators (reviewed by Montal 1998). While at this stage the precise sequence of the intracellular cascade is difficult to predict, it is more important to distinguish this process from the cell death mechanism induced by higher (micromollar and millimolar) concentrations of AChE-peptide. The latter process is insensitive to any of the biochemical tools tested, and on the basis of morphological features has been characterised as a necrotic form of cell death (Day and Greenfield 2003). The present data regarding LDH and the lack of sensitivity to calcium chelators support this hypothesis. A picture is therefore emerging of apoptotic neuronal death induced by low nanomolar concentrations of AChE-peptide involving calcium overload and a well-defined, yet selective, intracellular cascade. Since AChE-peptide is an endogenous amino acid sequence resident in AChE, this process may be relevant to neuronal development and/or neurodegenerative disease.

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