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A NON-CHOLINERGIC, TROPHIC ACTION OF ACETYLCHOLINESTERASE ON HIPPOCAMPAL NEURONES *IN VITRO*: MOLECULAR MECHANISMS

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Abstract—In this study neurite outgrowth from cultured hippocampal neurones was increased by addition of acetylcholinesterase acting in a non-cholinergic manner. Only monomeric acetylcholinesterase, a form of acetylcholinesterase dominant in development, increased neurite outgrowth (3–10 U/ml); moreover this effect was not blocked by active site blockers (echothiophate and galanthamine) but was sensitive to the addition of peripheral site blockers (fasciculin and BW284c51). It appears therefore that acetylcholinesterase has alternative, non-cholinergic functions, one of which could be in development, via a peripheral site. The possibility of a causal relationship between neurite outgrowth and calcium influx was explored using a spectrum of acetylcholinesterase variants, inhibitors and calcium channel blockers. Acetylcholinesterase regulation of outgrowth was shown to depend on an influx of extracellular calcium specifically via the L-type voltage-gated calcium channel.

In summary, we propose that, independent of its catalytic activity, a selective form of acetylcholinesterase has a role in the development of hippocampal neurones via a selective voltage-gated calcium channel. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: neurite, outgrowth, neurotrophic, tissue culture, nimodipine.

Acetylcholinesterase (AChE, EC 3.1.1.7) is well known for its role in the hydrolysis of acetylcholine (ACh). However, it has been shown in several brain regions that there is a disparity between levels of AChE and choline acetyltransferase, a more reliable marker of cholinergic neurones (Silver, 1974). Moreover, a soluble form of AChE has been identified that is secreted, an action which is beyond the requirements of cholinergic transmission (Greenfield, 1991b). Hence AChE may have a further role distinct from its enzymatic function (Silver, 1974; Greenfield, 1984, 1991a,b, 1992; Balasubramanian and Bhanumathy, 1993; Appleyard, 1994; Darboux et al., 1996; Grifman et al., 1998; Tsigelny et al., 2000; Soreq and Seidman, 2001). More direct evidence for a non-cholinergic, developmental role for AChE has emerged from studies of cultured neurones (Gupta and Bigbee, 1992; Layer et al., 1993; Dupree and Bigbee, 1994; Jones et al., 1995; Small et al., 1995; Holmes et al., 1997). More recently, antisense technology has been used to demonstrate that AChE promotes neurite outgrowth at least in part through an adhesive function (Grifman et al., 1998; Sharma et al., 2001).

These studies have demonstrated that AChE is actively involved in neurite outgrowth since selective action of AChE inhibitors retards neuritic outgrowth and neuronal migration. Whilst a tetrameric form (G_4) is dominant within the adult CNS, lower weight forms (monomeric [G_1] and dimeric [G_2]) are most abundant in the developing CNS. Thus, in addition to the proposal that AChE has a non-cholinergic developmental role, it has also been postulated that specific developmental forms of AChE exist (Drews, 1975). In an attempt to identify selective forms of AChE responsible for these non-classical actions, this study compared for the first time the actions of monomeric and tetrameric AChE on the development of the hippocampal neurones *in vitro*.

The early survival and development of neurones is dependent on levels of calcium (Ca^{2+}) influx through voltage-gated calcium channels (VGCC)(Meir et al., 1999). Neurotransmitter-controlled Ca^{2+} influx can also direct remodelling in the mature CNS, whilst Ca^{2+} flux is a necessary step in several signal transduction pathways controlling neurite outgrowth (Mattson, 1992; Saffell et al., 1992). Based on biophysical and pharmacological data these channels can be divided into three distinct groups (Hagiwara et al., 1975; Usowicz et al., 1992; Cherksey et al., 1991): (1) low-voltage-activated channels blocked by nickel, (2) a set of moderate-conductance, high-voltage-activated channels (N, R, and P/Q) blocked by funnel web spider peptide toxin, ω-agatoxin VIA and

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BW284c51, 1:5-bis(4-allydimethylammoniumphenyl)-pentan-3-one-dibromide; DIV, days *in vitro*; Fas, fasciculin; G_1, monomeric AChE; G_2, dimeric AChE; G_4, tetrameric AChE; LDH, lactate dehydrogenase; MAP, microtubule-associated protein; NGF, nerve growth factor; P, postnatal; PAS, peripheral anionic site; rAChE, recombinant AChE; VGCC, voltage-gated calcium channel; UV, ultraviolet.

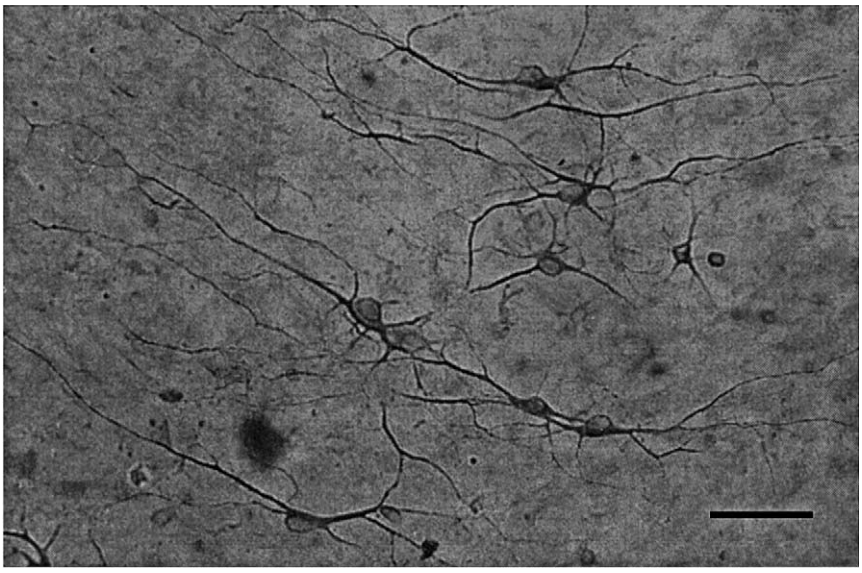

Fig. 1. Hippocampal cell culture maintained in serum-free medium 14 DIV and processed for MAP-2 immunohistochemistry. This immunochemistry formed the basis of this investigation. Scale bar = 50 μm.

(3) a large-conductance L-type channel blocked by the dihydropyridines, e.g. nimodipine.

The present study investigated the effect of AChE and VGCC blockers, nimodipine, ω-agatoxin VIA, conotoxin GVIA and soluble spider toxin (sFTX), in the development of hippocampal neurones using the following parameters: neurite outgrowth, lactate dehydrogenase (LDH) release, cell body area and primary neurite number (Holmes et al., 1995, 1997; Heng et al., 1999).

EXPERIMENTAL PROCEDURES

Purification of AChE

The purification of tetrameric AChE (G_4) was carried out by Dr Jane Loughlin (Department of Pharmacology, University of Oxford), from a commercial stock of electric eel AChE (Sigma-Aldrich, Poole, UK; type V-S, 1000 U/ml) using procainamide ECH Sepharose affinity chromatography (Novales-Li, 1994).

Mouse recombinant AChE (rAChE) was kindly provided by Professor Palmer Taylor (Department of Pharmacology, University of California, San Diego, CA, USA). The enzymes were produced from stable transfections of constructs from the mouse pcDNA-3 clone. All preparations were expressed in a HEK 293 cell line and shipped to Oxford in foetal calf serum with 20% glycerol on dry ice.

AChE purification was assessed using gel filtration with ultraviolet (UV) detection (SMART™, Fig. 1) and AChE activity for each molecular form was determined using the spectrophotometric Ellman assay (Ellman et al., 1961).

In brief, 25-μl aliquots of sample were added to 96-well microtitre plates, followed by the addition of 175 μl Ellman reagent. Absorbance was determined at 405 nm over a 10-min time span using a Molecular Devices UV plate reader (Alpha Laboratories, Hampshire, UK). All cholinesterases were expressed in U/ml.

The Ellman assay was used to determine cholinesterase levels in the culture system (culture medium and chicken plasma), to confirm the final concentration of cholinesterase in the supplemented culture medium and to confirm that the concentration of inhibitors added was sufficient to inhibit levels of cholinesterase within the culture system. The Bradford method was used to determine protein content (Dang et al., 1997).

Drugs and solutions

Cultures were maintained and all treatments were given in prewarmed, fresh filtered serum-free medium (Dulbecco's minimal essential medium plus B-12 supplement) containing antimitotic agents (Sigma-Aldrich) to prevent the over-proliferation of glia cells and Fungizone® (Life Technologies, Paisley, UK) to minimise the risk of infection. At this point it is relevant to state that B-12 is sufficient to replace serum in the medium. All inhibitors were solubilised in water at 10 mM and stored in 50-μl aliquots at −20°C. Fasciculin (Fas; Latoxan, France) was solubilised in water to give a 10 μM stock. Nimodipine, sFTX 3.3 and ω-agatoxin (CN Biosciences, Nottingham, UK) were made up as 1 M stock solutions in 0.1% dimethyl sulphoxide and stored as aliquoted fractions at −20°C. ω-Conotoxin GVIA (Tocris-Cookson, Bristol, UK) was made as 1 mM stock and maintained at −20°C.

Hippocampal slice cultures

The organotypic slice culture system was chosen to investigate the effects of cholinesterase due to the complexity of the preparation. These cultures resemble as closely as possible the physiological environment in the developing rat brain.

Hippocampal slices were prepared from postnatal day 7 (P7) Wistar rats (Harlan UK, Oxford, UK) (Gähwiler, 1981, 1984). In brief, rats were decapitated by a quick scissor cut at the level of the foramen magnum. Excess cortex and adhering meninges were removed before the hippocampi were placed ventral surface down on a McIlwain tissue chopper (The Mickle Lab., Engineering Company, Surrey, UK). Coronal sections (400 μm) were cut and attached to cleaned, sterile poly-D-lysine-coated coverslips by means of a plasma clot, formed by mixing a solution of chicken plasma with bovine thrombin.

Cultures were treated for 14 days *in vitro* (DIV) with serum-free medium supplemented with cholinesterase in the presence or absence of cholinesterase inhibitors. Ca^{2+} channel blockers were added for 24 h prior to fixation. Cultures were changed biweekly using supplemented serum-free medium, used in order to minimise the amount of cholinesterase in the system. 14 DIV was chosen as a time point prior to the cultures becoming less healthy and cell necrosis occurring.

All experiments were conducted in accordance with the Animals Scientific Procedures Act (1986), approved by Her Majesty's Government Home Office. All efforts were made to minimise the number of animals used and their suffering.

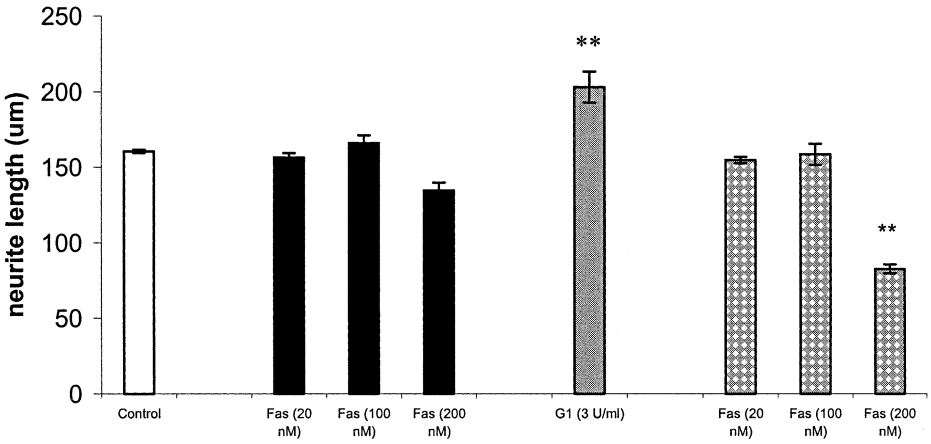

Fig. 2. Serum-free medium supplemented with 3 U/ml G_1 rAChE significantly increased neurite outgrowth of hippocampal cultures, maintained for 14 DIV when compared to a medium control group. Addition of Fas to the AChE peripheral site blocker alone had no effect on any parameter studied. Fas added with G_1 rAChE (3 U/ml) blocked the neurotrophic action. $^{**}P < 0.01$ using a Dunnett's multiple comparisons *t*-test. $n = 150$. Results are expressed as mean neurite outgrowth (μm) ± S.E.M.

Measurements and analysis

On completion of the incubation period, immunohistochemical staining for microtubule-associated protein (MAP-2) was performed using the avidin–biotin–peroxidase method (Hsu et al., 1981). MAP-2 was the antibody of choice to measure the dendritic growth of pyramidal cells in culture. We did not want to distinguish between dendrites and axons in this system; therefore only one measure of neurite growth was employed. Morphogenic analysis was by camera lucida drawing. Dendrite length was measured from cell body to dendrite tip and expressed as percentage of control cultures.

The quantitative determination of LDH kinetics was via spectrophotometry. During the reduction of pyruvate by LDH, an equimolar amount of NADH is oxidised to NAD^+. The oxidation of NADH results in a decrease in the light absorbance at 340 nm wavelength. The rate of this decrease during this reaction is directly proportional to LDH activity in the sample.

Experimental design and statistical analysis

Experiments were repeated a minimum of three times using different litters. Each treatment group contained a minimum of five cultures, with a minimum of 10 cells counted per culture ($n \geq 150$). Results were expressed as percentage mean ± S.E.M. of a medium only control group. Statistical analysis was by analysis of variance followed by Dunnett's multiple comparisons *t*-test, used to compare treatment groups with control groups.

RESULTS

The effect of cholinesterase on neurones of the hippocampus in vitro

Control cultures maintained in serum-free medium had a mean neurite length of 160.6 ± 0.1 μm (mean ± S.E.M., $n = 330$), a mean cell body area of 85.3 ± 2.4 μm^2 (mean ± S.E.M., $n = 330$) and a mean primary neurite number of 4.9 ± 0.3 (mean ± S.E.M., $n = 150$). Addition of G_1 rAChE (3 and 10 U/ml, protein content 1.34 and 4.47 μg/ml, respectively) significantly increased neurite length by 26.4 ± 2.6% (203.1 ± 10.2 μm, mean ± S.E.M., $n = 150$, $P < 0.01$, Dunnett's multiple comparisons *t*-test) and by 28.7 ± 0.4% (206.9 ± 6.6 μm, mean ± S.E.M., $n = 150$, $P < 0.01$, Dunnett's multiple comparisons *t*-test) respectively. Interestingly, G_1 rAChE had no effect on any other parameter measured including LDH release, primary neurite number and cell body area, relative to control cultures. The addition of G_4 AChE or butyrylcholinesterase (BChE) (Sigma-Aldrich) had no effect on any of the parameters measured in this culture system.

The effect of cholinesterase inhibitors on neurones of the hippocampus in vitro

The stimulatory effect observed by an increase in neurite outgrowth following the addition of G_1 rAChE-supplemented medium was blocked by the addition of AChE inhibitors that blocked the peripheral site of AChE. Fas (a potent peripheral site blocker) alone had no effect on neurite development *in vitro* (Fig. 2). However, when co-applied with G_1 rAChE, Fas blocked the trophic effect (20–100 nM). Increasing the concentration of Fas (500 nM) caused a significant decrease in neurite outgrowth (82.7 ± 2.9 μm, mean ± S.E.M., $P < 0.01$, Dunnett's multiple comparisons *t*-test, $n = 150$), when compared to control cultures. Although at this concentration there was a tendency for Fas (500 nM) to increase LDH release into the medium, this was not significant and no higher concentrations were tested.

BW284c51 (Sigma-Aldrich), a potent AChE inhibitor that binds to both the active site and the peripheral site of AChE, alone caused toxic effects on hippocampal cell *in vitro*. At 100 μM, BW284c51 caused a significant decrease in outgrowth (83.7 ± 2.7 μm, mean ± S.E.M., $P < 0.01$, Dunnett's multiple comparisons *t*-test, $n = 160$) (Fig. 3) and a significant decrease in cell body area (56.5 ± 7.5 μm^2, mean ± S.E.M., $P < 0.05$, Dunnett's multiple comparisons *t*-test, $n = 160$). A significant increase in LDH release was seen at 10–100 μM BW284c51 (6.1 ± 2.9 U/ml, $P < 0.01$, and 5.2 ± 1.7 U/ml, $P < 0.05$ respectively, mean ± S.E.M., Dunnett's

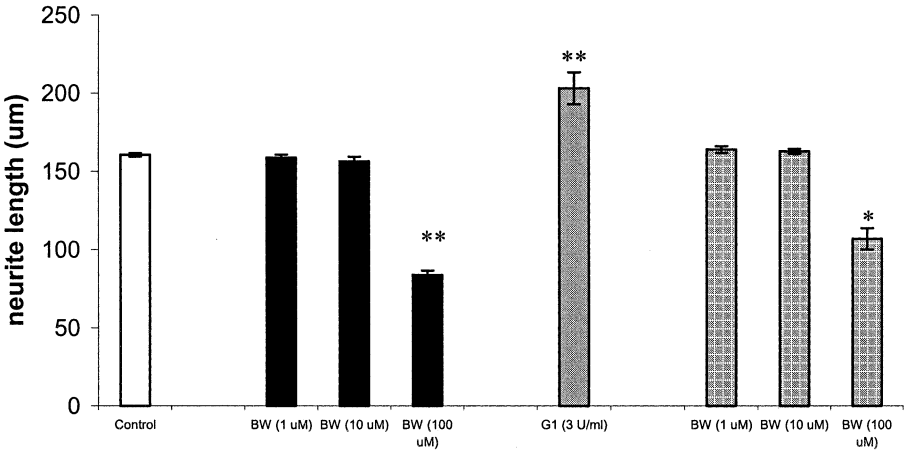

Fig. 3. Serum-free medium supplemented with 3–10 U/ml G_1 rAChE significantly increased neurite outgrowth of hippocampal cultures, maintained for 14 DIV when compared to a medium control group. Addition of BW284c51 (BW), which blocks both the AChE active site and peripheral anionic site, caused a significant decrease in neurite outgrowth at the maximum concentration tested (100 μM). BW284c51 applied with G_1 rAChE (3 U/ml) blocked the neurotrophic action. $^{**}P < 0.01$, $^{*}P < 0.05$ using a Dunnett's multiple comparisons *t*-test. $n = 160$. Results are expressed as mean neurite outgrowth (μm) ± S.E.M.

multiple comparisons *t*-test, $n = 150$). This result was not disregarded due to reports of a similar nature showing a toxic action of high BW284c51 concentrations (Holmes et al., 1997). At all concentrations, BW284c51 blocked the trophic effect of G_1 rAChE (Fig. 3). Addition of the active site inhibitors galanthamine (Sigma-Aldrich) and echothiophate and the BChE inhibitor tetraisopropyl pyrophosphoramide (Sigma-Aldrich) had no effect when applied alone or when co-applied with G_1 rAChE on this slice culture system.

There is a possibility that due to the purification procedure used, procainamide may bind to the anionic site, even in the case of the ionic site inhibitors, thereby inducing a block attributable to the peripheral anionic site (PAS), rather than to the actual anionic site. However, given that these inhibitors did not have an effect and, indeed, were different from those peripheral site blockers, such a possibility seems unlikely.

The effect of calcium channel blockers on neurones of the hippocampus in vitro

The stimulatory effect observed by an increase in neurite outgrowth following the addition of G_1 rAChE-supplemented medium was blocked by the addition of nimodipine (1–100 nM) (Fig. 4), the L-type VGCC blocker. This effect was not observed with the addition of agatoxin VIA (N-type), GVIA (P-type) or sFTX 3.3 (N-, P-, T-, R-type). The addition of these agents, which selectively block Ca^{2+} influx through voltage-dependent membrane channels, had no effect alone on hippocampal cells in culture.

DISCUSSION

Cholinesterases within the organotypic culture system

The literature surrounding a 'non-classical role' for AChE is vast and many good reviews exist (Greenfield, 1991a,b; Soreq and Seidman, 2001). For these studies endogenous cholinesterases in the preparation might have been accountable for the effects observed. In an attempt to minimise cholinesterase levels in the culture system, tissue was maintained in a serum-free medium. However, despite this strategy, cholinesterase would still be present in the chicken plasma used to secure the tissue section. However, this background cholinesterase was predominantly BChE which was shown to be ineffective at increasing neurite length in this system. None the less, inclusion of a group with no additional exogenous cholinesterase adequately controlled for the presence of endogenous cholinesterase in the culture system.

A neurotrophic role for monomeric AChE

The addition of G_1 rAChE (3 U/ml) (protein content, 1.34 μg/ml) to developing and regenerating neurones of the hippocampus within the slice culture system significantly increased the length of MAP-2 positive neurones. The concentrations of G_1 rAChE, which elicited a stimulatory response (3–10 U/ml, protein content of 1.34–4.47 μg/ml), were relatively high when compared to doses at which other neurotrophic factors are active. The survival promoting effect of nerve growth factor (NGF) on GABAergic cells of the hippocampus can be seen at 100 ng/ml (Arimatsu and Miyamoto, 1991). The more modest potency of G_1 rAChE could be due to the fact that the hippocampal slice culture was embedded in a plasma clot. Diffusion experiments have showed that AChE was capable of permeating the plasma clot. However, it is likely that the concentration reaching the neurones in the cultured hippocampus would be greatly reduced.

In contrast tetrameric AChE (G_4) (3–10 U/ml) had no significant effect on the growth of hippocampal neurites *in vitro*. One possible explanation for this would be the species difference. The monomeric AChE used was

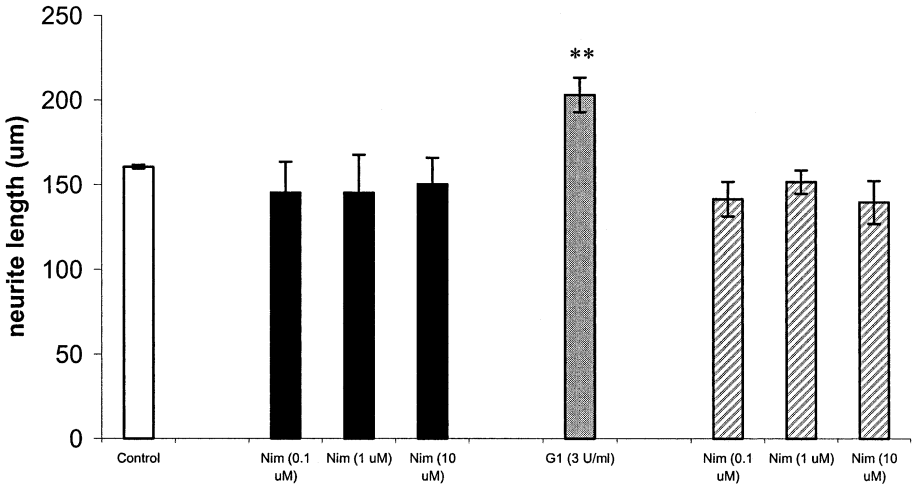

Fig. 4. Serum-free medium supplemented with 3–10 U/ml G_1 rAChE significantly increased neurite outgrowth of hippocampal cultures maintained for 14 DIV, when compared to a medium control group. Nimodipine (Nim; L-VGCC blocker) was added to the culture system for 24 h prior to tissue fixation. Alone nimodipine had no significant effect on neurite outgrowth. However, when applied with G_1 rAChE (3 U/ml) nimodipine blocked the neurotrophic action. $^{**}P < 0.01$ using a Dunnett's multiple comparisons *t*-test. $n = 150$. Results are expressed as mean neurite outgrowth (μm) ± S.E.M.

recombinant mouse whereas the tetrameric form was eel. There is 91% identity between rat and mouse AChEs, whereas the identity between eel and mouse is only 59%. However, Holmes et al. (1995) used organotypic cultures of the substantia nigra to show that there is an effect of eel G_4 on rat neurones in culture. Clearly, therefore, eel AChE in a similar preparation can exert a significant bioactive effect. Hence the discrepancy seen here, where no effect was observed, is more likely to be attributable to a difference in the brain region (hippocampus rather than substantia nigra), than in the species. It has been demonstrated that AChE is released from the dendrites of neurones in the substantia nigra, independently of cholinergic transmission, almost exclusively from the dopaminergic containing cells of the pars compacta (Greenfield, 1991a,b). The dopaminergic cells of the substantia nigra may therefore be more sensitive to the effects of 'non-cholinergic' AChE. The efficacy of PAS inhibitors, and hence the involvement of the PAS in this non-cholinergic action of AChE, might also explain the enhanced stimulatory effect of G_1 over G_4. Bourne et al. (1999) have shown that there is a partial occlusion of the peripheral site in the tetramer that presumably would not apply in the monomeric form.

In comparison addition of BChE (3–10 U/ml) had no significant effect on the survival of neurones in this slice culture system. This finding provides further proof that this action of AChE is not via the hydrolysis of ACh. None the less, BChE has been postulated to have a role in development with an involvement in cell proliferation (Layer, 1991; Willbold and Layer, 1994; Mack and Robitzki, 2000). AChE and BChE are expressed at different stages of development and their expression is mutually exclusive: therefore the lack of an effect of BChE on this slice culture system does not preclude a developmental role for BChE elsewhere or at other times in development (Drews, 1975; Layer, 1991; Mack and Robitzki, 2000).

Implications for a neurotrophic role of monomeric AChE

Under the current culture conditions it would appear that the stimulatory effect of G_1 rAChE was mediated via the peripheral site as Fas and BW284c51, both of which block the peripheral site, attenuated this stimulatory effect. This effect was not replicated by the addition of the active site blockers echothiophate or galanthamine, further confirming a non-cholinergic action of AChE. In the case of BW284c51, at the highest concentration (100 μM), there was a non-specific toxic action as reflected by an increase in LDH release and a decrease in neurite outgrowth. However, in the remaining range of BW284c51 used, since it had no effect on its own, its blockage of the AChE should be interpreted as a specific blocking action. Analysis of the molecular composition of embryonic AChE reveals that an increase in the ratio of G_4:G_1/G_2 occurs during development (Inestrosa et al., 1994).

A cell adhesion function of AChE has also been correlated with the peripheral site (Jones et al., 1995; De-Farrari et al., 1998; Sharma and Bigbee, 1998; Johnson and Moore, 1999). Experiments on rat spinal cord cultures, PC12 cell lines and dopaminergic pedal ganglion cells also suggest that the polypeptide domains, which form the peripheral site microdomain, mediate the trophic effects of AChE (Srivatsan and Peretz, 1997; Johnson and Moore, 1999; Muñoz et al., 1999).

Srivatsan and Peretz (1997) demonstrated a neurotrophic action for AChE in *Aplysia* hemolymph. Using cultured dopaminergic neurones from the *Aplysia*, exogenously applied hemolymph caused an increase in neurite outgrowth which was subsequently blocked using AChE inhibitors of the catalytic and peripheral sites but not by a catalytic site blocker alone. Grifman et al. (1998) used antisense suppression of AChE on PC12 cells to demonstrate redundant neurite outgrowth-promoting activities for AChE and implicate

AChE-like proteins as potential mediators of cytoarchitectural changes supporting neuritogenesis. These papers support the current data presented in this study and we further demonstrate a possible mechanism for the action of AChE in neurite outgrowth.

The role of the calcium channel and neurite outgrowth

Critical developmental processes such as appearance of neurotransmitters and ion channels, neurite outgrowth, synaptogenesis and intrinsic firing patterns all depend in part on developmental changes in voltage-dependent Ca^{2+} conductances (Lipton and Kater, 1989; Heng et al., 1999). Regulation of neuronal Ca^{2+} channels is important because entry of Ca^{2+} through VGCC is a major mechanism by which changes in membrane potential can influence cellular processes (Shitaka et al., 1996). Ca^{2+} affects the assembly states of both microtubules and microfilaments and is a prime candidate as an intracellular regulator of neurite elongation and motile growth cone structures (filopodia and lamellipodia).

Many neurotrophins such as NGF, brain-derived neurotrophic factor and basic fibroblast growth factor have been shown to exert their trophic action by their ability to regulate intracellular Ca^{2+} via the VGCC (Black, 1999; Defazio et al., 2000; Gysbers et al., 2000; Kang and Schuman, 2000). AChE has been implicated in differentiation and survival of neuronal cells of various brain regions. Ca^{2+} is a critical signal in neuronal development and electrophysiological studies suggest that AChE may cause Ca^{2+} influx (Webb et al., 1996), which could in turn result in altered growth cone activity during development (Kater and Mills, 1991). In our hands the trophic action of AChE was attenuated by co-application of AChE with nimodipine, the L-type VGCC blocker. Application of AChE with other respective blockers of other VGCC failed to block this non-classical effect. Hence it appears that AChE exerts a non-cholinergic trophic action, selectively via L-type VGCC. This finding is in agreement with that of Luo et al. (1994).

Early studies on avian pectoral muscle cultures have shown that the dihydropyridine Ca^{2+} channel blocker nifedipine caused a reduction in AChE secretion rate (Decker and Berman, 1990). Results indicated a reduction in AChE biosynthesis and a link in skeletal muscle between transcription and post-transcriptional processing of mRNA and ligand occupation of the dihydropyridine receptor. Later studies attempted to ascertain the importance of the signalling mechanism during muscle development (Luo et al., 1994, 1996). Such studies indicate that L-VGCC play an important role in the regulation of AChE expression during skeletal muscle development *in vivo*.

Since AChE enhances calcium entry (Webb et al., 1996) and since calcium can subsequently promote AChE mRNA expression (Luo et al., 1994, 1996; Decker and Berman, 1990), it is possible that there could be a feed-forward mechanism involving neurite outgrowth, calcium and AChE.

CONCLUSION

The transient expression of AChE during the development of many brain regions has been correlated with stages of neurite outgrowth. (Dupree and Bigbee, 1994; Karpel et al., 1996; Holmes et al., 1997; Bataille et al., 1998; Sternfeld et al., 1998). AChE has also been postulated in a role of axonal pathfinding and synaptogenesis (Anderson and Key, 1999), while high expression of AChE in migrating germ cells is lost after their target is reached (Drews, 1975).

In conclusion, the current findings provide further evidence for a non-classical trophic role for AChE and suggests a role for the exposed peripheral site on the G_1 form of AChE. Moreover, for the first time this study demonstrates that only the L-type VGCC channel has a role in this action, adding powerful support to the idea that the trophic action of AChE is via Ca^{2+} influx. We still have much to understand concerning the non-cholinergic action of AChE, yet identification of the mechanism as provided here might lead to more selective manipulation of the phenomenon, and hence insights into its physiological and pathological implications.

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