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Effects of acetylcholinesterase and butyrylcholinesterase on cell survival, neurite outgrowth, and voltage-dependent calcium currents of embryonic ventral mesencephalic neurons

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Abstract

The aim of this study was to investigate the effect of butyrylcholinesterase (BuChE) and acetylcholinesterase (AChE) on cell survival, neurite outgrowth and voltage-dependent calcium currents in developing rat ventral mesencephalic (VM) neurons. Both BuChE and AChE have been shown to promote neurite outgrowth in postnatnal preparations. However, the effect of these substances has never been investigated on rat embryonic VM cells, which are used in animal models of foetal transplantation as a treatment for Parkinson's disease. The effects of incubation with BuChE and tetrameric (G_4)- or monomeric (G_1)-AChE on cell survival and neurite outgrowth were characterised over a 7-day period on dopaminergic cells within embryonic VM cultures. The acute effects of these treatments on voltage-dependent calcium currents from embryonic VM cells were then investigated using whole-cell voltage-clamp recordings. The chronic effect of modulating voltage-dependent calcium channels was subsequently explored using the selective calcium channel antagonists ω-agatoxin IVA, ω-conotoxin GVIA, and nifedipine. The results presented here demonstrate firstly trophic effects of BuChE and G_4- and G_1-AChE upon dopaminergic neurite outgrowth, secondly that BuChE and G_4- and G_1-AChE have an inhibitory effect on voltage-dependent calcium currents, and finally that selective voltage-dependent calcium channel inhibitors also have trophic effects upon dopaminergic neurite outgrowth.

Keywords: AChE; BuChE; Calcium; Voltage-dependent calcium channels

Introduction

Trophic roles for both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in development are now well established (Layer, 1990; Layer et al., 1987, 1993; Layer and Sporns, 1987; Willbold and Layer, 1992). In particular AChE, independent of its hydrolytic ability, enhances neurite outgrowth of many cell types (Brimijoin and Hammond, 1996; Gupta and Bigbee, 1992; Koenigsberger et al., 1997), including postnatal dopamine cells in organotypic preparations of the substantia nigra (Holmes et al., 1997). However, the effects of AChE and BuChE have not been examined on nigral dopamine cells at earlier stages of development, i.e., from the foetus. Yet it is important to understand neurochemical dynamics at this particular time, since foetal preparations are used in transplantation as a treatment for Parkinson's disease.

Animal models of foetal transplantation have shown that grafted cells can survive and extend neurites into the host striatum (Björklund et al., 1983; Clarke et al., 1988; Nikkah et al., 1994). However, grafts in animals are not capable of restoring all behavioural deficits (Dunnett et al., 1983), and studies of postmortem animal brains that have undergone transplantation illustrate that only 5–20% of grafted dopaminergic cells (which can be referred to as TH^+ cells because they can be immunocytochemically identified using an antibody for tyrosine hydroxylase) survive (Nikkah et al., 1994; Barker et al., 1996). Clinical trials in human Parkinson's disease patients that have undergone foetal transplant

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therapy have shown that transplanted dopamine neurons can survive and are functional (Freed et al., 1992), although recovery is variable, with about 60% of patients reporting a partial recovery of motor function. The low survival rate of grafted TH^+ cells is therefore a critical drawback of the technique of foetal transplantation.

Several growth factors have been shown to improve the survival and outgrowth of TH^+ cells within both cultures and in vivo grafts, including basic fibroblast growth factor (bFGF; Mayer et al., 1993; Zawada et al., 1998).

Several studies have shown that the growth factors basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) can affect conductance through voltage-dependent calcium channels (VDCC; Rogers and Hendry, 1990; Shitaka et al., 1996; Jia et al., 1999). Developing cells are known to have a narrow range of permissive intracellular calcium concentrations, referred to as the "set point" (see Franklin and Johnson, 1992; Kater et al., 1988), which is individual to cell type. Deviation from the set point leads to changes in calcium-dependent processes, including neurite outgrowth (Franklin and Johnson, 1992; Kater et al., 1988; Rogers and Hendry 1990). Thus, alteration of intracellular calcium concentration by the actions of NGF and bFGF on VDCC is thought to be at least partly responsible for the trophic effects of these substances on cell survival and neurite outgrowth (Rogers and Hendry, 1990; Shitaka et al., 1996; Jia et al., 1999).

This study aimed firstly to characterise the actions of BuChE and tetrameric (G_4)- and monomeric (G_1)-AChE on embryonic ventral mesencephalon (VM) cells, secondly to explore any effect of these substances on cellular calcium conductance using whole-cell patch-clamp electrophysiology, and thirdly to compare any action of BuChE and G_4- and G_1-AChE with chemically disparate agents known to nonetheless modify P/Q-, N-, and L-type calcium channel conductances, respectively, ω-agatoxin IVA (Aga IVA), ω-conotoxin GVIA (GVIA), and nifedipine.

Materials and methods

Astrocyte preparation

VM cultures were grown on a layer of Type I astrocytes, as preliminary experiments demonstrated that plating of VM cells onto noncellular substrates such as laminin and polylysine resulted in very low numbers of cells adhering and growing.

Type I astrocytes were prepared. Brains were removed from decapitated 1-day-old rat pups of either sex, and cerebella removed and discarded. The remaining tissue was rolled on filter paper to remove meninges and chopped into 1-mm^3 pieces. Tissue was dissociated in 1 ml of medium consisting of DMEM (4500 mg/liter of glucose, Life Technologies Ltd., Paisley, UK) supplemented with 10% foetal calf serum (Life Technologies Ltd.) and 1% penicillin/streptomycin (Life Technologies Ltd.), and centrifuged for 6 min at 1000 rpm (167*g*). The supernatant was discarded and the pellet dissociated in 1 ml of medium which was then added to 14 ml of medium in a 75-cm^2 flask with a vented lid precoated with poly-D-lysine (Sigma-Aldrich Company Ltd., Poole, UK) and incubated at 37°C in a humidified atmosphere comprising 95% air:5% CO_2 for 7 days.

Astrocyte flasks were then passaged. Medium was removed, and the cell layer was washed with Hanks' balanced salt solution (HBSS; Life Technologies Ltd.) and digested with trypsin. The suspension was poured into 3 ml of medium and centrifuged for 6 min at 167*g*. The supernatant was discarded and the pellet dissociated in 1 ml of medium. The suspension was then placed into a fresh poly-D-lysine-coated flask containing 14 ml of medium, returned to the incubator, and allowed to reach confluency (6–8 days). Astrocyte flasks were then trypsinised as described above, plated onto poly-D-lysine-coated chamber slides for outgrowth studies or 13-mm-diameter coverslips in 30-mm-diameter petri dishes for electrophysiology experiments, and incubated for 6 days to produce a confluent extracellular matrix before addition of the dissociated VM preparation.

Cell preparation and culture

Embryonic VM cultures were prepared according to the method of Branton et al. (1998). Briefly, time-mated Wistar rats at gestational day 14 were anaesthetised (140 mg/kg of pentobarbitone; Boehringer Ingelheim Ltd., Berkshire, UK) and decapitated under anaesthesia. Uterine horns were collected in sterile HBSS. The region of the VM was removed from each embryo and stored in HBSS containing 0.05 % DNase (Sigma-Aldrich Company Ltd.) (HBSS/DNase) on ice. Pooled explants were incubated in trypsin (Sigma-Aldrich Company Ltd.) (0.1 % trypsin in HBSS/DNase) for 20 min at 37°C. The explants were then rinsed with 0.1% soyabean trypsin inhibitor (Sigma-Aldrich Company Ltd.) in HBSS/DNase prior to rinsing three times in HBSS/DNase. Explants were then triturated in a known volume of HBSS/DNase. Viability and cell density were calculated using the trypan blue exclusion method. Cells were plated at a density of 2.5×10^5 cells/cm^2 onto astrocyte-covered chamber slides or coverslips. Every 48 h, 0.75 ml of medium (of a total volume of 2 ml) was removed from each culture and replaced with 0.75 ml of fresh medium containing, where appropriate, test substances. Cultures were used to assess the effects of test substances on neurite length maintained in culture for 1 day (24 h; referred to here as "day 1 in vitro"), 3 days (72 h; referred to here as day 3 in vitro), or 7 days (168 h; referred to here as day 7 in vitro). Cultures were used for electrophysiological purposes throughout the 7-day period.

All procedures 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.

Measurement of cholinesterase activity in culture medium

The method of Ellman et al. (1961) was used to determine the change in AChE and BuChE activity in samples of culture medium incubated at 37°C for 7 days, following addition of 3 U/ml of BuChE or AChE at day 0. Briefly, 25-μl aliquots of each sample were added to 175 μl of Ellman reagent (Ellman et al. 1961) in individual wells of a 96-well microtitre plate. Absorbance was read at 405 nm over a 10-min time span using a Molecular Devices plate reader (Alpha Laboratories Ltd., Hampshire, UK). For each assay, plate blanks containing HBSS were used to control for the colour of the phenol red within the culture medium. To determine whether the cholinesterase present in media samples was AChE or BuChE, the activity of the samples was measured in the presence of 100 μM tetraisopropylpyrophosphoramide (Sigma-Aldrich Company Ltd.), to inhibit BuChE, or 100 μM 1,5-bis(4-allydimethylammoniumphenyl)-pentan-3-one dibromide (Sigma-Aldrich Company Ltd.), to inhibit AChE.

Immunocytochemistry

On completion of the incubation period cultures used to assess the effects of test substances on neurite outgrowth were fixed and processed for TH immunoreactivity by the biotin–avidin peroxidase method. Cultures were rinsed with warmed phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 60 min at room temperature. Cells were then washed twice in PBS. Nonspecific binding sites were blocked by incubation with 10% goat serum (Sigma-Aldrich Company Ltd.) in PBS for 60 min at room temperature. Cultures were incubated with monoclonal anti-TH antibody raised in mouse (Chemicon International Ltd., UK) (1:1000 dilution in PBS), overnight at 4°C. Cultures were then washed for 3 $\times$ 10 min in PBS and biotinylated goat antimouse secondary antibody (Novocastra Laboratories Ltd., UK) (1:300 dilution in PBS) was applied for 2 h at room temperature. Following 3 $\times$ 10 min washes with PBS, a solution of 10% methanol and 0.3% hydrogen peroxide in PBS was used to quench any endogenous peroxidase activity (10 min at room temperature). Following further washes cultures were incubated for 60 min at room temperature with avidin–biotin complex (Novocastra Laboratories Ltd.). Cultures were then washed for 3 $\times$ 10 min with Tris-buffered saline. The complex formed between the mouse monoclonal antibody for TH, the antimouse secondary antibody, and the avidin–biotin compound was visualised using a diaminobenzidine chromagen with 0.015% hydrogen peroxide in Tris-buffered saline. Stained cultures were dehydrated by placing in successively increasing ethanol concentrations (50, 75, 90, 95, and 100%) for 2 min followed by immersion in xylene for 8 min. Cultures were then mounted in Depex (BDH/Merck Chemicals Ltd., Warwickshire, UK).

Assessment of TH^+ cell numbers and neurite length

Total cell numbers per culture well were counted for each treatment group at each time point. For all experiments, data were then normalised to the untreated control.

To assess neurite length, camera lucida drawings were made of every TH^+ cell in the chamber slide well for each treatment group at each time point. The length of each cell's longest neurite was assessed using a sports route planner (an instrument that is used by cyclists and walkers to assess the length of map routes. It has the advantage that it can accurately measure lines that twist or turn).

Experiments involving exposure of cultures to test substances were repeated a minimum of three times using foetuses from different mothers. Each treatment group contained a minimum of four chamber wells, giving a total of at least 12 cultures for each treatment group. Data were collected in a blind fashion to prevent sampling bias and values were expressed as means $\pm$ SEM. Statistical significance was assessed using analysis of variance followed by a Dunnett's multiple comparison *t* test.

Electrophysiology

Dissociated VM cultures are composed of a heterogeneous population of cells where 2–5% are TH^+ and the vast majority of the remainder stain positively for glutamic acid decarboxylase (GAD). The aim of electrophysiological experiments was to record primarily from TH^+ cells. However, as cell phenotype could not be confirmed until after the experiment was terminated, data from both TH^+ and GAD^+ cells were inevitably obtained and have been analysed.

Cultures were placed into the recording chamber and were continuously perfused with extracellular solution containing (mM) TEACl, 143; $BaCl_2$, 5; $MgCl_2$, 1; Hepes, 10; glucose, 10, and adjusted to pH 7.4 with TEAOH. The osmolarity was adjusted to 315 mOsm/kg with TEACl. Standard patch-clamp recordings (Hamill et al., 1981) (Axopatch 200A whole-cell patch-clamp amplifier, Axon Instruments; CED 1401 Cambridge Electronics; Dell XPS PC using Strathclyde Electrophysiology Whole-Cell Program Software) were made using patch-clamp pipettes fabricated from borosilicate glass, 1.0 mm o.d., 0.58 mm i.d., with an internal capillary pulled on a horizontal puller (Sutter Instruments). Pipettes were filled with (mM): CsCl, 135; $MgCl_2$, 1; Hepes, 10; triphosphocreatinine, 14; MgATP, 3.6, and 50 μl/ml of creatinine phosphokinase, adjusted to pH 7.1 with CsOH, and 290 mOsm/kg with CsCl (recipe of Gillard et al., 1997); 0.2% biocytin was then added. For experiments to control for any effect of cholinesterase being due to calcium-dependent calcium inactivation, 10 mM EGTA was incorporated into the intracellular solution. Pipettes showed resistances of 2–4 MΩ. The whole-cell patch-clamp configuration was used and cells were voltage clamped at a membrane potential of -60 mV, at which most low-voltage activated channels are inactivated.

Series resistance and cell capacitance were determined by analysing instantaneous membrane responses to 5-mV, 30-ms voltage steps. Series resistances were 4–6 MΩ and 60–85% compensation was applied. For the analysis of test substance action on voltage-activated barium currents, a standard protocol was adopted of applying 100-ms steps to a test potential of 0 mV every 30 s. Drugs were delivered to the bath in extracellular solution via a three-way tap system. Complete exchange of the bath solution occurred in approximately 1.5 min. Test substances were applied until the current had stabilised (defined by three depolarisation steps eliciting currents within 2 pA of each other). Following test substance application, the cell was perfused with extracellular solution until the current began to deteriorate. Only one test substance was applied per cell and recordings for each test substance were taken from cells at days 1 to 7 in vitro and the data were pooled. Currents were filtered by a four-pole, low-pass Bessel filter and stored on hard disk for subsequent analysis.

Postrecording cell identification

Postrecording, cultures were fixed by placing into paraformaldehyde for 60 min at room temperature. Cultures were then washed with PBS and incubated with streptavidin–Texas Red (Amersham Pharmacia Biotech. Ltd., UK; 1:100 overnight at 4°C) to label the biocytin fill. Cultures were washed for 3 × 30 min in PBS and 20% donkey serum (Jackson Immunoresearch Laboratories Inc., USA) in PBS was applied for 120 min at room temperature. Monoclonal mouse anti-TH antibody and polyclonal rabbit anti-GAD antibody (Chemicon International Ltd., UK; 1:1000 in 1% donkey serum in PBS) were then coapplied overnight at 4°C. Cultures were washed with PBS for 3 × 30 min and fluorescein-conjugated donkey antimouse and indodicarbocyanine-conjugated donkey antirabbit secondary antibodies (Jackson Immunoresearch Laboratories Inc.; 1:100 dilution in 1% donkey serum in PBS) were applied overnight at 4°C. Cultures were washed for 3 × 30 min in PBS, dipped once in distilled water, and inverted into Vectashield (Novocastra Laboratories Ltd.). After excess fluid had dried, coverslips were sealed with nail varnish and stored in the dark at 4°C until visualised.

Reagents used

G_4-AChE, from a commercial stock of *Electrophorus electricus* AChE (Sigma-Aldrich Company Ltd.; Type V-S, 1000 U/ml), and BuChE, from a commercial equine stock (Sigma-Aldrich Company Ltd.; 500 U/ml), were purified using a procainamide (Sigma, St. Louis, MO) -ECH Sepharose 4B (Amersham Pharmacia, Uppsala, Sweden) affinity column (Ralston et al., 1985). Briefly, the washed affinity column was loaded with AChE in PBS at 12 ml per hour in a cold room and left to recycle overnight. The column was then washed again with 20 mM Na_2HPO_4 + 5 mM EDTA, pH 7.2 (solution x) and solution x + 400 mM NaCl, and AChE was eluted with 400 mM TEA in solution x and 10 mM decamethonium in solution x. The elluate was dialysed overnight at 4°C using a 10-kDa molecular weight cutoff dialysis cassette (Perbioscience, Cheshire, UK) against solution x. The dialysed AChE from affinity chromatography was applied to a column of Superdex 200 (PC 3.2/30) with a bead bed volume of 2.4 ml (Pharmacia Biotech, Uppsala, Sweden) and eluted in ascending mode, The affinity chromatography sample was monitored for protein at absorbances of 214 and 280 nm to assess the molecular species of cholinesterase present. The affinity column-eluted fractions were also assessed for protein concentration (Lowry et al., 1951) and AChE activity (Ellman et al., 1961).

G_1-AChE (truncated at amino acid 548—C-terminus) was produced from stably transfected constructs from the mouse pcDNA-3 clone expressed in a human embryonic kidney cell line kindly provided by Professor Palmer Taylor (Department of Pharmacology, University of California, San Diego, CA, USA) and purified from the medium using a *m*-trimethylaminophenylamine-coupled Sepharose affinity resin (Taylor and Jacobs, 1974). The enzyme was purified and characterised as described previously (Marchot et al., 1996).

The cholinesterase activity of each of the above purified enzymes was assessed by the method of Ellman et al. (1961), where 1 unit of activity was defined as the hydrolysis of 1 μmol of 1 mM substrate per minute at 25°C in 0.1 M sodium phosphate buffer, pH 8.0, followed by protein determination using the Lowry method (Lowry et al., 1951). Stock solutions of BuChE (450 U/ml) and G_4 (520 U/ml)- and G_1-AChE (480 U/ml) were made in 20% glycerol:80% PBS, aliquoted, and stored at −80°C. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich Company Ltd. and solutions of BSA (1 mg/ml) were made in extracellular solution immediately prior to use. Cadmium chloride and nifedipine were obtained from Sigma-Aldrich Chemical Company Ltd. Aga IVA was obtained from Calbiochem-Novabiochem Corp. (UK), and GVIA from Bachem (UK). Stock solutions of Aga IVA (100 μM), GVIA (100 μM), and cadmium chloride (100 mM) were made in distilled water. A stock solution of nifedipine (100 mM) was made in dimethyl sulfoxide. VDCC antagonist stock solutions were aliquoted and frozen at −20°C. All test substances were diluted in media or extracellular solution immediately prior to use.

Results

Changes in cholinesterase activity during incubation

A decay in cholinesterase activity was observed in samples of culture medium incubated at 37°C with either 3 U/ml of AChE or 3 U/ml of BuChE over a 7-day time period. The decay in AChE activity was 27.7% (from 2.96 to 2.14 U/ml;

$n = 6$) and the decay in BuChE activity was 31.8% (from 2.98 to 2.03 U/ml; $n = 6$).

Cell death under control (untreated) conditions

At day 1, the average number of cells per culture was 123.6 ± 12.8 (mean ± SEM, $n = 14$); at day 3 the average number of cells was 83.4 ± 7.6 ($n = 12$), which is 67.5% of the initial TH^+ cell number. At day 7, the average number of cells per culture was 69.8 ± 6.4 ($n = 15$), which is 56.5% of the initial TH^+ cell number.

Effect of test substances on cell numbers

There were no significant changes in total TH^+ cell number per culture from the untreated control with any of the treatments at any of the time points (Figs. 1A–C).

Effect of test substances on neurite outgrowth of embryonic dissociated TH^+ VM cells

As shown in Fig. 2A, at day 1 in vitro exposure of cells to 3 U/ml of BuChE (protein content, 4.98 μg/ml), 0.3 and 3 U/ml of G_1-AChE (protein content, 0.13 and 1.34 μg/ml, respectively), and 0.01 and 0.1 μg/ml of bFGF had significantly increased neurite outgrowth in comparison to the untreated control. At day 3 in vitro (Fig. 2B), exposure of cells to 3 U/ml of G_1-AChE and 0.3 and 3 U/ml of G_4-AChE (protein content, 0.52 and 5.2 μg/ml, respectively) had significantly increased neurite outgrowth, and at day 7 in vitro (Fig. 2C), 3 U/ml of G_4-AChE had significantly increased neurite outgrowth in comparison to the untreated control.

Effect of cholinesterases on voltage-dependent calcium currents elicited from embryonic dissociated VM cells

BuChE and G_4- and G_1-AChE inhibited whole-cell calcium currents elicited from TH^+ cells and GAD^+ cells (Table 1) in dissociated cultures of the VM in a concentration-dependent manner. There were no significant differences between TH^+ and GAD^+ cells in the amount of inhibition of voltage-dependent calcium currents caused by BuChE and G_4- and G_1-AChE. For both cell types perfusion of the cell with extracellular solution following application of the test substance resulted in partial recovery of the inhibited current for each of the test substances (Table 1). Representative electrophysiological traces of the inhibition of maximal peak current (I_{max}) in TH^+ cells by BuChE and G_4- and G_1-AChE are shown in Figs. 3A–C, respectively, with a representative time course for application of 3 U/ml of BuChE (Fig. 3A). Representative electrophysiological traces of the inhibition of I_{max} in GAD^+ cells by BuChE and G_4- and G_1-AChE are shown in Figs. 4A–C, respectively, with a representative time course for application of 3 U/ml of BuChE (Fig. 4A). Photographic representation of the

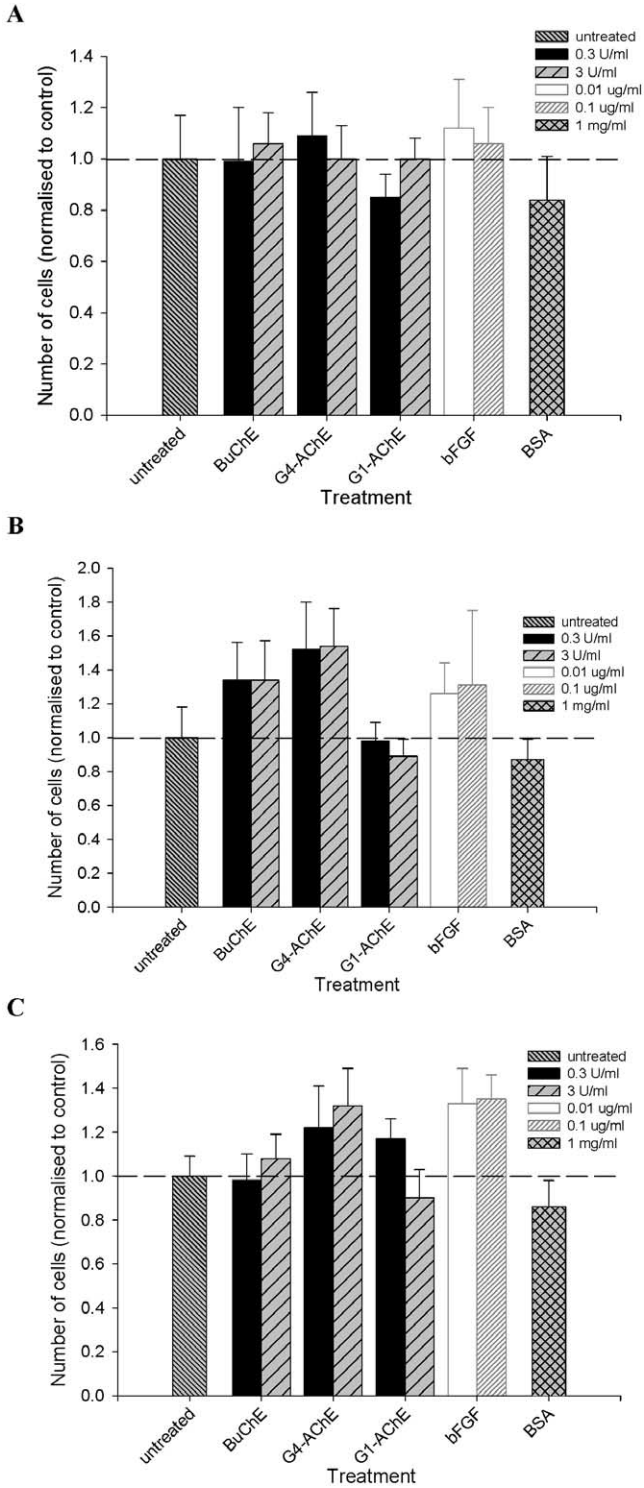

Fig. 1. The effect of BuChE, G_4- and G_1-AChE, bFGF, and BSA on TH^+ cell numbers at days 1 (A), 3 (B), and 7 (C) in vitro. Values shown are means ± sem and have been normalised to the untreated control for each time point; $n \geq 12$.

triple immunocytochemical labelling of TH^+ and GAD^+ cells carried out within these type of studies has been published previously (Whyte and Greenfield, 2002). For

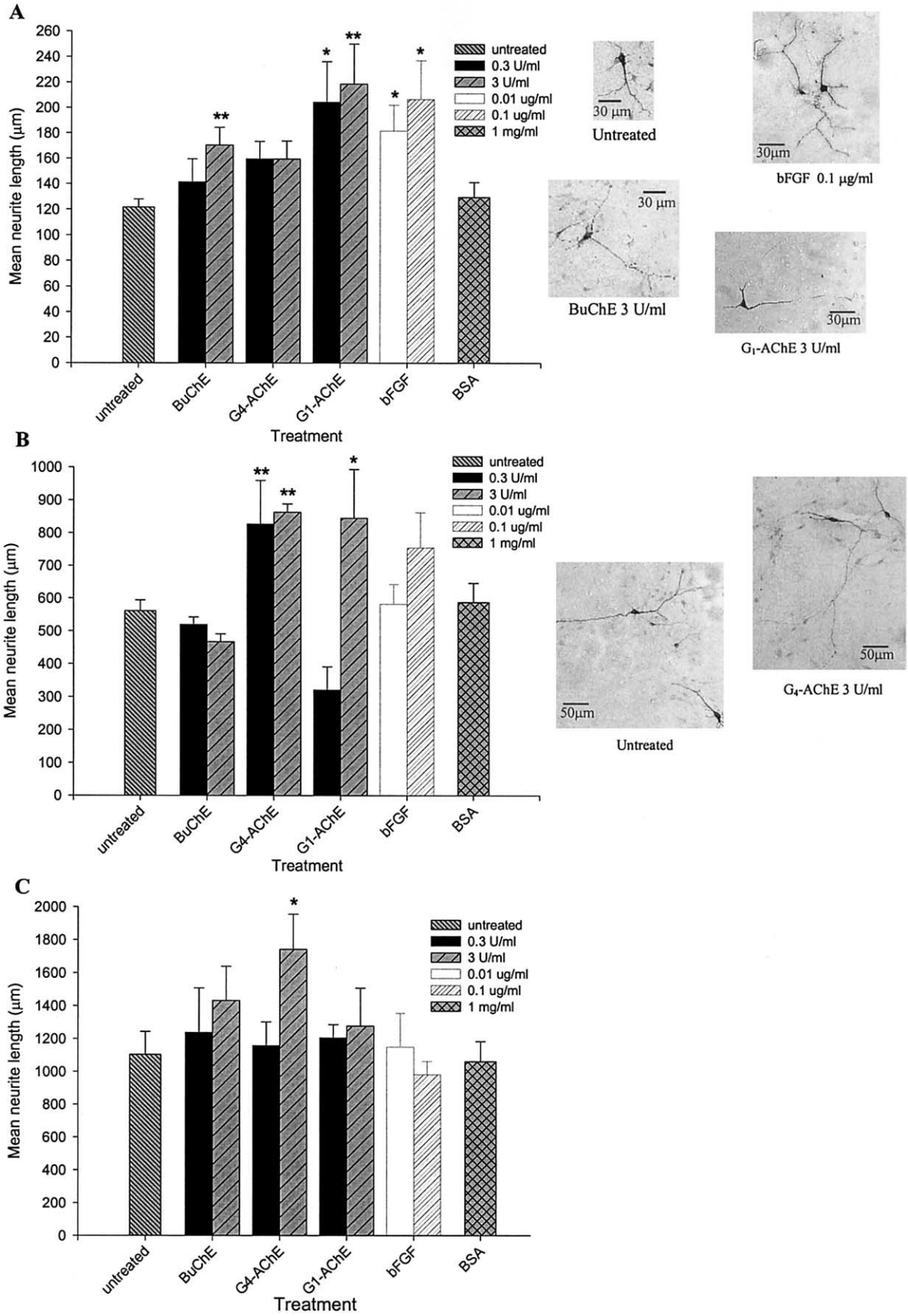

Fig. 2. The effect of BuChE, G_4- and G_1-AChE, bFGF, and BSA on neurite outgrowth of embryonic cultured dissociated TH^+ cells at days 1 (A), 3 (B), and 7 (C) in vitro. Values shown are means ± sem. $n \geq 12$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; Dunnett's multiple comparison test.

Table 1
The effect of BuChE, G_4- and G_1-AChE, and BSA on whole-cell calcium currents from TH^+ and GAD^+ cells

Treatment	TH^+ cells		GAD^+ cells	
	Inhibition of I_{max} (% ± SEM)	Recovery (% ± SEM)	Inhibition of I_{max} (% ± SEM)	Recovery (% ± SEM)
BuChE (U/ml)				
0.01	1.75 ± 0.5	94.7 ± 8.6	1.3 ± 0.3	88.3 ± 2.3
0.03	3.46 ± 0.6	86.5 ± 4.4	3.7 ± 0.6	91.1 ± 2.1
0.3	14.2 ± 1.3	64.0 ± 4.2	17.1 ± 2.0	48.1 ± 4.4
3	31.8 ± 4.8	42.6 ± 2.9	35.6 ± 2.3	26.1 ± 3.0
G_4-AChE (U/ml)				
0.01	12.8 ± 1.8	81.1 ± 3.0	13.2 ± 1.0	78.6 ± 1.6
0.03	19.4 ± 1.4	51.9 ± 3.4	21.8 ± 1.9	42.6 ± 2.2
0.3	31.4 ± 1.6	38.1 ± 2.9	36.6 ± 1.1	31.2 ± 2.2
3	43.6 ± 3.0	36.0 ± 4.3	48.5 ± 3.8	19.8 ± 5.6
G_1-AChE (U/ml)				
0.01	10.1 ± 0.9	77.4 ± 1.6	10.4 ± 0.7	72.2 ± 2.2
0.03	15.6 ± 1.0	69.5 ± 2.7	17.4 ± 1.1	58.5 ± 1.9
0.3	25.3 ± 1.0	44.1 ± 3.2	28.2 ± 0.8	30.0 ± 4.2
3	34.5 ± 1.3	33.1 ± 3.2	35.2 ± 2.6	24.3 ± 4.0
BSA (mg/ml)				
1	—	—	—	—

Values for inhibition are expressed as percentages of I_{max} ± SEM. Values for recovery are expressed as a percentage of the inhibited current. For each, $n \geq 5$.

both cell types 1 mg/ml of BSA had no effect upon whole-cell calcium currents.

The effect of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents from TH^+ cells following intracellular calcium chelation

Following incorporation of 10 mM EGTA into the intracellular pipette solution to chelate intracellular calcium, values for inhibition of whole-cell calcium currents by 3 U/ml of BuChE or G_4- or G_1-AChE in TH^+ cells were 32 ± 5, 44 ± 3, and 35 ± 1 (mean I_{max} ± SEM, $n \geq 4$). These are similar to values for inhibition of TH^+ cell currents by 3 U/ml of BuChE or G_4- or G_1-AChE in experiments without intracellular EGTA (see Table 1).

Effect of VDCC antagonists on cell survival and neurite outgrowth of embryonic dissociated TH^+ VM cells

Concentrations of Aga IVA, GVIA, nifedipine, and cadmium used in incubation experiments fully inhibited the relevant VDCC component, as determined by the IC_{50} value of each inhibitor on whole-cell calcium currents from embryonic VM cells in culture (data not shown).

As illustrated by Figs. 5A–C, addition of the VDCC antagonists Aga IVA, GVIA, and nifedipine had no effect upon total TH^+ cell number per culture from the untreated control at any of the time points examined. However, addition of cadmium significantly decreased the number of TH^+ cells in comparison to the control at day 1 in vitro ($P < 0.001$). Examination of cultures incubated with cadmium at days 3 and 7 in vitro revealed a complete absence of cells at both time points.

Exposure of cells to Aga IVA, GVIA, or nifedipine for 24 h resulted in an increase in neurite outgrowth with respect to the untreated control (Fig. 6A). In contrast, exposure of cells to cadmium for 24 h significantly decreased neurite outgrowth (Fig. 6A). At day 3 in vitro, cultures incubated with Aga IVA, GVIA, or nifedipine displayed an increase in neurite outgrowth (Fig. 6B), whilst at day 7 in vitro (Fig. 6C) there were no effects on neurite outgrowth of cells incubated with Aga IVA or GVIA, but those cultures incubated with nifedipine displayed a decrease in neurite outgrowth in comparison to the untreated control. At days 3 and 7 in vitro, neurite length measurement was not possible in cultures incubated with cadmium, due to the complete absence of cells in these cultures.

Discussion

Cholinesterases within the culture system

Analysis of the decay of cholinesterase in culture medium due to incubation at 37°C revealed that although the activity of both AChE and BuChE did decay, active AChE and BuChE were still present after 7 days of incubation. Since 0.75 ml (of 2 ml) of treated culture medium was replaced every 48 h, it can be concluded that cells in cultures that were incubated with media supplemented with BuChE or AChE were constantly exposed to cholinesterase.

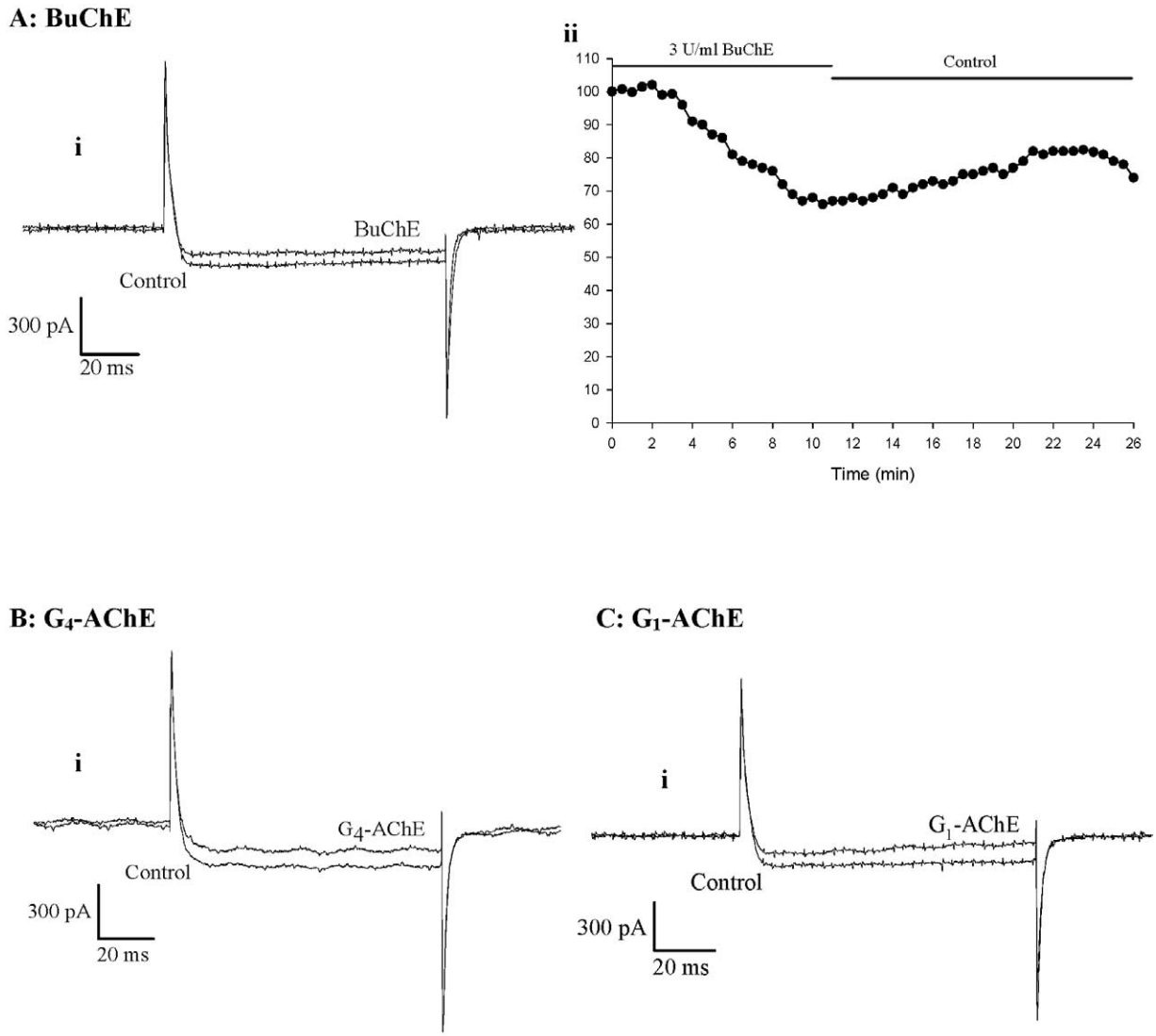

Fig. 3. Representative traces (i) and time course (ii) of the inhibitory effects of 3 U/ml of (A) BuChE, (B) G_4-AChE, and (C) G_1-AChE on whole-cell calcium currents elicited from embryonic TH^+ cells in dissociated cultures.

Survival of TH^+ cells

Cell death under control conditions

Considerable dopamine cell death occurred under control (untreated) conditions during the 7-day culture period. Similar levels of cell death over a 7-day period in dissociated VM cultures have been documented elsewhere (Fawcett et al., 1995; Branton et al., 1998). Cell death within such preparations is of particular interest as this is the method of preparing a suspension for use in foetal transplantation work, both in animal models and in humans. It is obvious that a certain amount of cell death will be attributable to traumatisation during the preparation of the suspension and by the fact that culture conditions are not precisely parallel to in vivo conditions. It is also true, however, that cells die during normal development of the nervous system. Most regions of the central nervous system, including the substantia nigra (Janec and Burke, 1993; Oo and Burke, 1997), undergo a distinct phase during which significant proportions of the constituent cells die and are rapidly removed by the surrounding glial cells (see Linden, 1994). Several reasons have been postulated for such "naturally occurring" cell death, including the possibilities that it is a mechanism for matching the size of the neuronal population to the magnitude of its target field or a procedure for eliminating erroneous projections (Cowan et al., 1984; Linden, 1994).

Effect of test substances on cell numbers of dissociated embryonic TH^+ VM cells

The number of embryonic dopamine cells per culture was not affected by the addition of BuChE, G_1- or G_4-AChE, bFGF, or BSA. It is interesting that none of the factors decreased cell death over the observed time period, and yet AChE, BuChE, and bFGF increased neurite out-

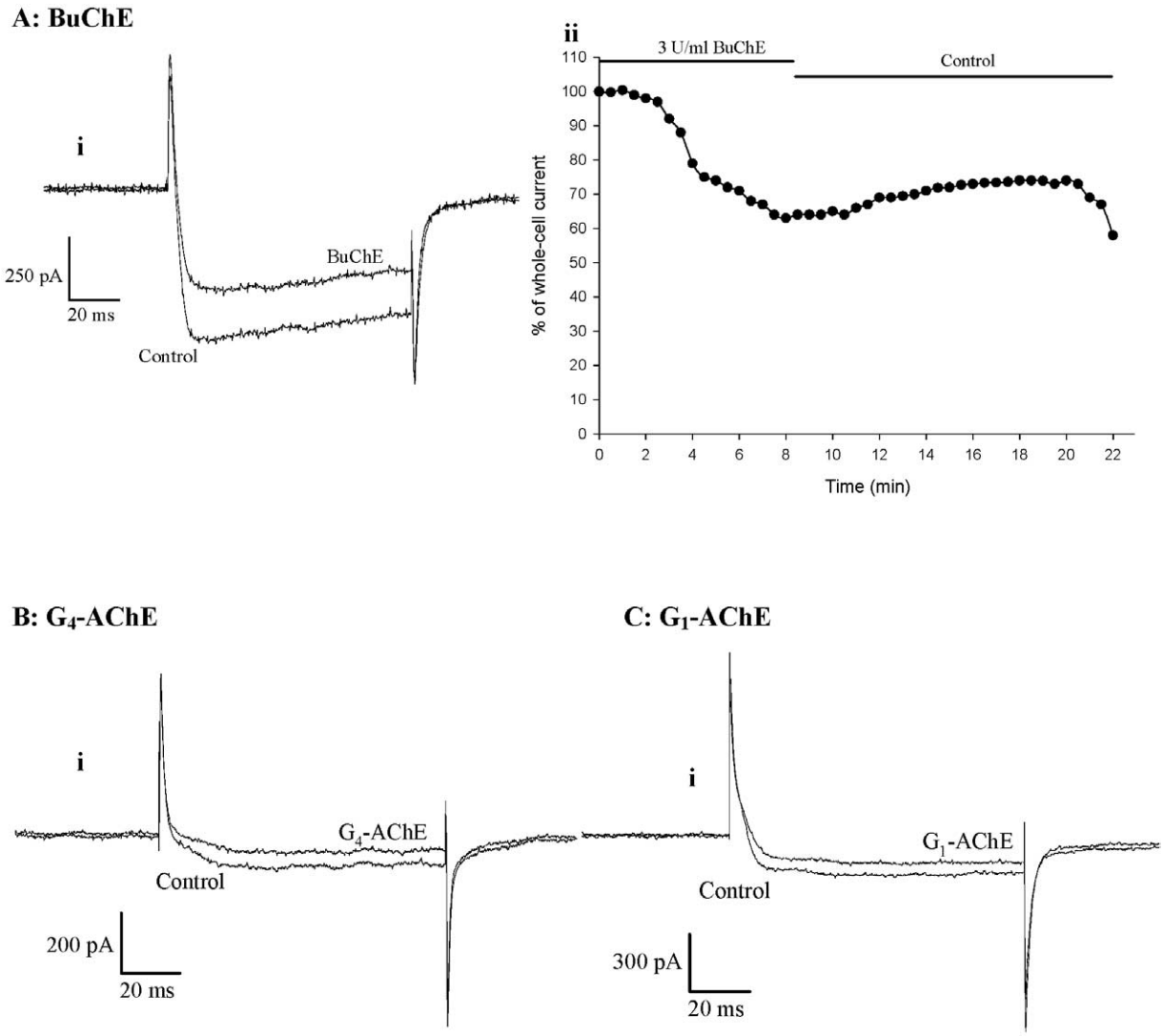

Fig. 4. Representative traces (i) and time course (ii) of the inhibitory effects of 3 U/ml of (A) BuChE, (B) G_4-AChE, and (C) G_1-AChE on whole-cell calcium currents elicited from embryonic GAD^+ cells in dissociated cultures.

growth and therefore may be considered to have a trophic role in this system. It could be postulated that upon application of the trophic factor, 2 h after plating, the mechanisms causing cell death, whether a result of natural occurrence or due to traumatisation, are sufficiently advanced to prevent reversal by the addition of a trophic factor.

Effect of cholinesterases on neurite outgrowth of dissociated embryonic TH^+ VM cells

Neurite outgrowth in cell culture is a widely used parameter for assessing the effect of a substance upon the development of cells in culture (Holmes et al., 1997; Ronn et al., 2000). Addition of BuChE, G_4- and G_1-AChE, and bFGF significantly enhanced neurite outgrowth of embryonic dopamine cells in comparison to the untreated control. It is unlikely that these increases were due to a nonspecific protein effect, as no effect upon outgrowth was seen with BSA. The trophic actions of bFGF are well documented (Morrison et al., 1988; Silani et al., 1994) and validate the culture system as used here as viable for investigating potentially trophic test substances.

The stimulatory effects on neurite outgrowth of BuChE and G_4- and G_1-AChE presented here are in agreement with those of another group that has used embryonic tissue (Layer, 1990; Layer et al., 1987, 1993; Willbold and Layer, 1992). In the fetal VM studied here, the trophic effects of BuChE and G_1-AChE precede a trophic response to G_4-AChE. It is possible that the difference in the time course of effects of BuChE, G_1-AChE, and G_4-AChE are due to the difference in species origin (BuChE from horse, G_1-AChE from mouse, and G_4-AChE from *Electrophorus*). However, it is also possible that this sequence of events corresponds to the in vivo situation in the developing embryo, i.e., the temporal response to factors in vitro may reflect the sequen-

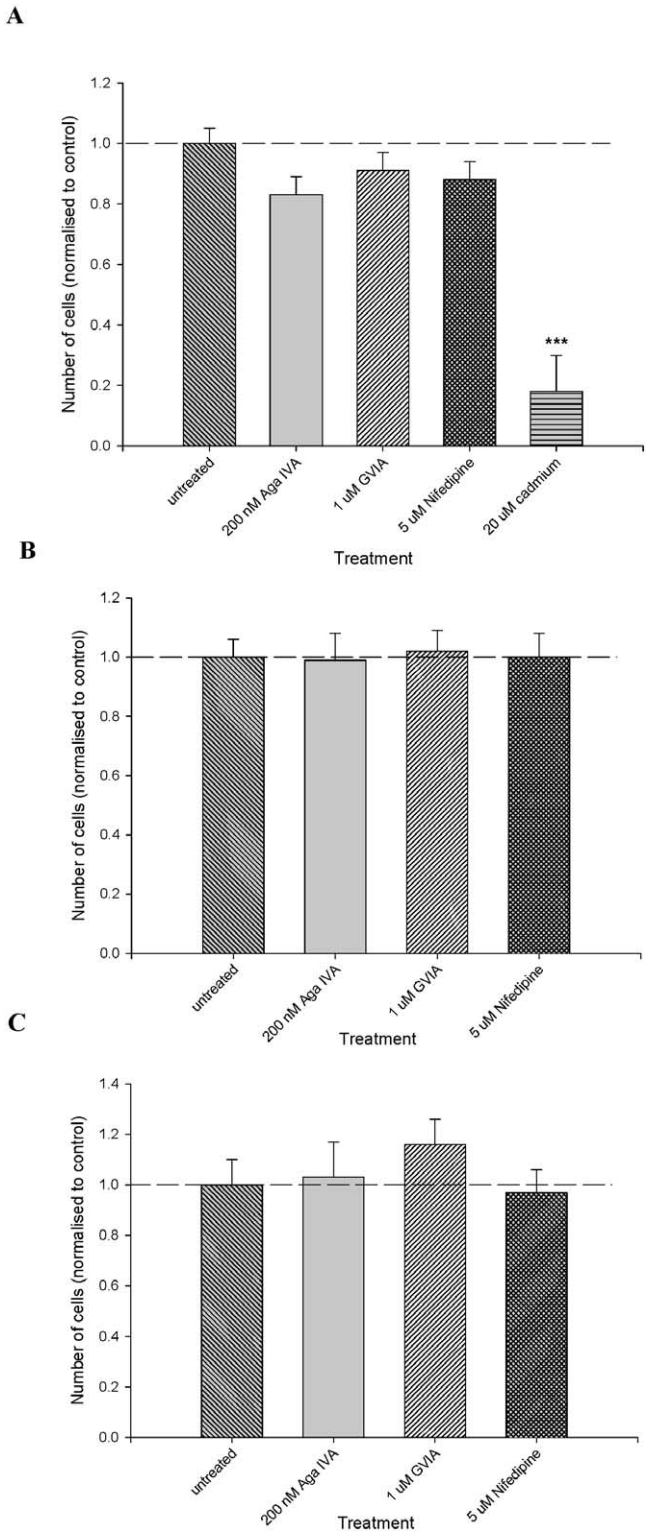

Fig. 5. The effect of VDCC antagonists Aga IVA, GVIA, nifedipine, and cadmium on TH^+ cell numbers at days 1 (A), 3 (B), and 7 (C) in vitro. Examination of cultures incubated with cadmium at days 3 and 7 revealed a complete absence of cells. Values shown are means ± sem and have been normalised to the untreated control for each time point; $n \geq 12$. ***$P < 0.001$; Dunnett's multiple comparison test.

tial expression of the same factors in vivo. For example, in the developing avian nervous system, BuChE expression foreshadows AChE expression (Layer et al., 1987, 1988; Willbold and Layer, 1992). A temporal shift also occurs in the expression of soluble globular AChE from G_1 and G_2 forms to the G_4 form (Arendt et al., 1992; Inestrosa et al., 1994; Layer et al., 1987).

The results presented here also show that test substances which increased neurite outgrowth at days 1 or 3 in vitro, with the exception of 3 U/ml of G_4-AChE, lost effect over the remainder of the 7-day incubation period. This may again parallel the in vivo situation, whereby cells may respond to the transient expression of stimulatory factors. Prolonged exposure in vitro may be of little consequence if the cells have such a "window" of response to particular factors in vivo.

A difficulty of the cultures used in these experiments, however, is that they comprised a heterogeneous population of cells. In order to optimise growth of VM cells, an astrocyte matrix was used, and due to the cultures being composed of the VM (rather than the sn alone), other cells types were also present—primarily GAD^+ cells. The possibility that the effects of trophic factors on TH^+ cells were mediated through other cell types present in the cultures cannot be discounted.

Effect of cholinesterases on VDCC of dissociated embryonic VM cells

BuChE and G_4- and G_1-AChE all inhibited whole-cell calcium currents elicited from both TH^+ and GAD^+ embryonic VM cells in a dose-dependent manner. It is unlikely that this inhibition resulted from a nonspecific protein effect, as BSA had no effect upon whole-cell calcium currents elicited from either cell type.

It is unlikely that the effects of BuChE and G_4- and G_1-AChE on whole-cell calcium currents are a result of cholinergic activity. The cultures were continuously perfused during recording, and as they comprised a single layer of cells, acetylcholine from normal inputs such as the pedunculopontine nucleus would be unlikely to remain in the extracellular space for such a protracted period. Furthermore, acetylcholine inhibits voltage-dependent calcium currents (Higashida et al., 1990); therefore, it is unlikely that AChE, in working as an enzyme, would have the same effect as its substrate, acetylcholine.

The possibility that BuChE and G_4- and G_1-AChE caused an increase in intracellular calcium concentration which resulted in inhibition of the voltage-dependent calcium current (the well-recognised phenomenon of calcium-dependent calcium inactivation; see Hering et al., 2000) can be excluded by the demonstration here that inhibitory effects of cholinesterase persisted following intracellular calcium chelation by incorporation of EGTA into the intracellular pipette solution.

The inhibitory effects of BuChE and G_4- and G_1-AChE on calcium currents from GAD^+ cells as well as TH^+ cells

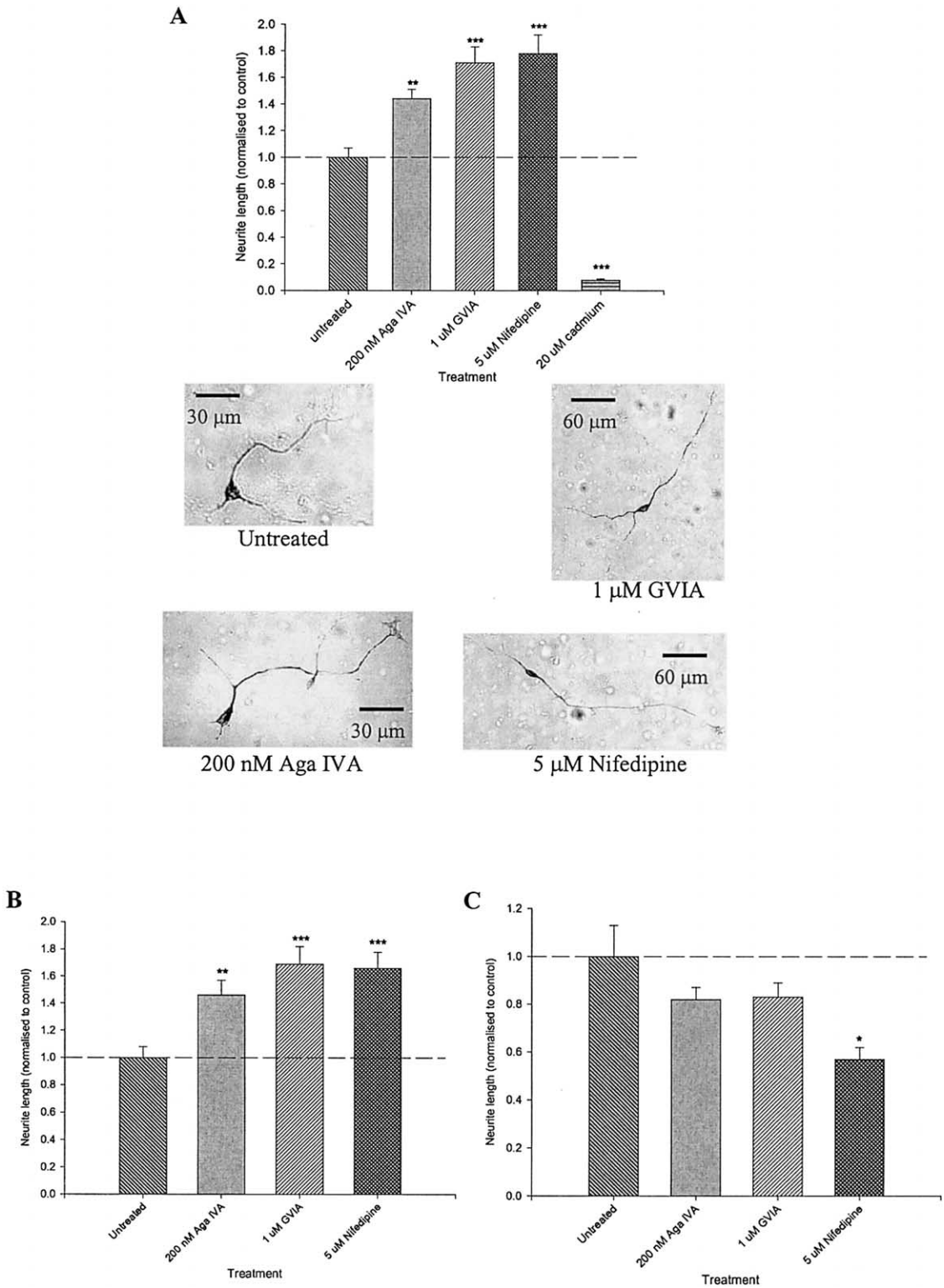

Fig. 6. The effect of VDCC antagonists Aga IVA, GVIA, nifedipine, and cadmium on neurite outgrowth of embryonic cultured dissociated TH^+ cells at days 1 (A), 3 (B), and 7 (C) in vitro. Neurite length measurement at days 3 and 7 was not possible in cultures incubated with cadmium, due to the complete absence of cells. Values shown are means ± sem and have been normalised to the untreated control for each time point; $n \geq 12$. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; Dunnett's multiple comparison test.

demonstrate that the effects of these substances on voltage-dependent calcium currents are not cell specific. As the aim of this project was to provide further information about the potential of cholinesterases to act as trophic factors for embryonic TH^+ cells, neurite outgrowth experiments did not examine the effects of test substances upon the cultured

GAD^+ cell population. However, it has recently been shown in postnatal hippocampal organotypic cultures that are known to have a large population of GAD^+ cells (Streit et al., 1989) that enhanced neurite outgrowth by G_1-AChE could be inhibited by L-type VDCC antagonists (Day and Greenfield, 2002), suggestive of a role for VDCC in AChE-mediated outgrowth in these cells.

A weakness of the data presented here is that values for the effect of each test substance were obtained and pooled from cells recorded on different days in culture. The pooling of data from several days may have repercussions for interpretation of the results obtained. We have recently published data to illustrate that the density of certain VDCC subtypes in embryonic VM cells changes over the 7-day culture period (Whyte and Greenfield, 2002). If the inhibitory effect of BuChE and G_4- and G_1-AChE on calcium currents are a result of an interaction of each with a VDCC subtype or subtypes, then application of the same concentration of these substances at different days could produce disparate results due to changes in the density or conductance of the relevant VDCC subtype(s). Pooling of data from different time points would mask such discrepancies, and this may explain why different concentrations and times of exposure of BuChE, G_4-AChE, and G_1-AChE have different effects on the neurite outgrowth of TH^+ cells within cultures and yet inhibit calcium currents to a similar extent.

It is not possible to speculate on the mechanism of action for cholinesterases on VDCC. It could be that BuChE and G_4- and G_1-AChE modulate the activity of calcium channels directly, by interacting with the α_1- pore-forming subunit, or generate some other signal that in turn decreases voltage-dependent calcium conductance. Nevertheless, given that is has been shown that growth factors NGF and bFGF can affect conductance through voltage-dependent calcium channels (Rogers and Hendry, 1990; Shitaka et al., 1996; Jia et al., 1999), the results presented here are interesting. However, demonstration by electrophysiology of an acute, partially reversible action of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents is not evidence that chronic incubation with these substances causes changes in intracellular calcium concentration, which in turn increase neurite outgrowth. Therefore, it was important to establish whether chronic incubation of embryonic VM cultures with VDCC antagonists affects the neurite outgrowth of TH^+ cells.

Effects of VDCC antagonists on dissociated embryonic TH^+ VM cells

Fig. 5 demonstrates that incubation of cultures with 20 μM cadmium for 24 h results in a significant decrease in TH^+ cells in comparison to the untreated control. By 72 h of exposure to cadmium, cultures were devoid of TH^+ cells and it was also apparent that there was a complete absence of all cells. These results illustrate the importance of calcium to the developing cell. Entry of calcium into the developing cell activates a number of secondary messenger enzymes that influence many fundamental cellular processes. VDCC constitute the primary route of entry of calcium into the cell in response to depolarisation, and complete inhibition of VDCC by cadmium will therefore severely deprive the developing cell of calcium. The consequence of calcium deprivation will be a loss of calcium-dependent developmental processes, including those fundamental to cell–substrate attachment and cell survival.

Addition of the selective VDCC antagonists Aga IVA, GVIA, and nifedipine to culture medium had no effect upon TH^+ cell numbers at any of the time points examined. It could be hypothesised that inhibition of individual VDCC subtypes did not reduce calcium levels sufficiently to detrimentally affect cell survival.

Given the stimulatory effect of Aga IVA, GVIA, and nifedipine on TH^+ cell neurite outgrowth, it is interesting that these substances had no stimulatory effect upon survival of TH^+ cells at any time point examined. It was also the case that addition of BuChE and G_4- and G_1-AChE to culture medium of embryonic VM cells increased TH^+ neurite outgrowth, but had no effect upon cell numbers. It is possible, as discussed above, that the death of TH^+ cells in culture is a result of both naturally occurring cell death and death due to traumatisation during the culture procedure. It was hypothesised that the lack of effect of BuChE and G_4- and G_1-AChE upon TH^+ cell numbers may be due to the delay of 2 h in the addition of these factors to cultures. This may also be the case with the addition of VDCC antagonists, as the experimental procedure was the same as for the BuChE and G_4- and G_1-AChE experiments.

Incubation of embryonic VM cultures with cadmium for 24 h resulted in a decrease in neurite outgrowth of TH^+ cells, and by 72 h, cultures were devoid of all cells, suggesting that cadmium may be toxic to the cells or that calcium is of critical importance for cell survival. Addition of the selective P/Q-, N-, and L-type VDCC antagonists Aga IVA, GVIA, and nifedipine, respectively, to culture medium for 1–3 days had a stimulatory effect on TH^+ neurite outgrowth, suggesting that the intracellular calcium concentration required for optimal neurite outgrowth (the set point) of TH^+ cells at days 1 and 3 in vitro in this culture system is *lower* than the normal physiological intracellular calcium concentration in these cells.

After incubation with Aga IVA and GVIA for 7 days, however, no further increases in neurite length were detected in comparison to the untreated control, but incubation with 5 μM nifedipine had decreased neurite outgrowth. In cultured embryonic TH^+ cells at day 7 in vitro, the contribution to the whole-cell current made by the L-type channel, which is antagonised by nifedipine, is greater than the contribution from the P/Q- and N-type channels (Whyte and Greenfield, 2002). It is possible that by day 7 in vitro the intracellular calcium concentration of TH^+ cells is similar to the set point. Reduction of the whole-cell calcium current by addition of Aga IVA or GVIA may not, at this particular time point, dissociate intracellular calcium concentration

from the range required for optimal neurite outgrowth. However, inhibition of the L-type VDCC by nifedipine in TH^+ cells by day 7 in vitro may sufficiently deprive cells of calcium to result in disruption of the processes required for neurite outgrowth.

In conclusion, the findings reported here add further support to a developmental action of cholinesterases, possibly with staged roles for BuChE and G_4- and G_1-AChE during development. An acute inhibitory action of BuChE and G_4- and G_1-AChE on voltage-dependent calcium currents is then reported. It is possible that the actions of BuChE and G_4- and G_1-AChE on calcium conductance may contribute to the trophic effect of these substances on embryonic TH^+ cell outgrowth, a suggestion which is not disproved by the final set of data presented, that incubation of embryonic cultures with selective VDCC antagonists also increases TH^+ neurite outgrowth. These findings are intriguing, and work will now focus on identifying whether there is truly a connection between the inhibition of calcium currents by BuChE and G_4- and G_1-AChE and the increase in neurite outgrowth seen upon incubation of cultures with these substances.

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