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Polyamine amides are neuroprotective in cerebellar granule cell cultures challenged with excitatory amino acids

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Abstract

Primary cultures of rat cerebellar granule cells have been used to assess the potential neuroprotective effects of philanthotoxins and argiotoxin-636 (ArgTX-636). These polyamine amides are potent antagonists of ionotropic L-glutamate (L-Glu) receptors. In granule cells loaded with fluo-3, ArgTX-636 and philanthotoxin-343 (PhTX-343) antagonised increases of intracellular free calcium concentration ($[Ca^{2+}]_i$) that were stimulated by *N*-methyl-D-aspartate (NMDA). The antagonism was use-dependent. Antagonism by PhTX-343 was fully reversible, but recovery following antagonism by ArgTX-636 was slow and only partial during the time-course of an experiment. Neither compound inhibited K^+-induced increases in $[Ca^{2+}]_i$. In excitotoxicity studies with cerebellar granule cells, the release of lactate dehydrogenase (LDH) and morphological observations were used to assess cell death. A 20–30 min exposure to 500 μM NMDA, 100 μM L-Glu or 500 μM kainate was sufficient to kill $> 90\%$ of the cells after 18–20 h. When added 5 min prior to, and during agonist exposure, PhTX-343 and ArgTX-636 provided total neuroprotection. ArgTX-636 was about 20–30 fold more potent than PhTX-343 against NMDA, but was approximately equipotent with PhTX-343 against a kainate challenge. Neither of the toxins showed any inherent toxicity even at 400 μM and 100 μM respectively. Some analogues of PhTX-343 are more potent, both in terms of antagonism of NMDA-stimulated increases of $[Ca^{2+}]_i$ and neuroprotection, than PhTX-343 and ArgTX-636.

Keywords: Polyamine amide toxin; Excitotoxicity; Neuroprotection; Cerebellar granule cell; Excitatory amino acid receptor

1. Introduction

Polyamine amide toxins are low molecular weight compounds that have been isolated from the venoms of a wide variety of spiders (e.g. argiotoxin-636; ArgTX-636), and from the Egyptian digger wasp *Philanthus triangulum* (e.g. philanthotoxin-433; PhTX-433) [10,35,69,70]. They share a similar structural design, consisting of an aromatic chromophore, a polyamine backbone, one or more amino acid residues and a terminal amino or guanidino group [70] (Fig. 1). These natural products contribute to the paralysing effects of spider and wasp venoms by non-competitively antagonising ionotropic glutamate receptors of prey such as insects [20,42]. They are also potent, non-competitive antagonists of ionotropic glutamate receptors of mammalian central nervous systems (CNS), where both *N*-methyl-D-aspartate (NMDA) and non-NMDA L-glutamate (L-Glu) receptors are antagonised [4,5,13,23,27,38,39,53,62,63]. In addition to their effects on glutamate receptors, polyamine amide toxins antagonise nicotinic acetylcholine receptors [7,61] and, at higher concentrations, voltage-sensitive calcium channels (VSCCs) [40,51,64,65,67].

Sustained agonist stimulation of the ionotropic glutamate receptors of excitable cells can lead to a form degeneration of these cells termed excitotoxicity [18,48], i.e. the agonist is excitotoxic. In humans, excitotoxic cell death may contribute to the neuronal injury that follows acute CNS insults such as ischaemia, seizures and trauma and may also be involved in the development of certain chronic neurodegenerative diseases [18,48]. Data from in vitro models suggest that the entry of Ca^{2+} plays a pivotal role in the initiation of a cascade of changes that leads eventually to cell death [17,19,25,28,29,60]. Prevention of Ca^{2+} entry, particularly through channels gated by NMDA receptors [57,68] and VSCCs [6,71] may be an efficacious neuroprotective strategy, which might be achieved using either competitive or non-competitive antagonists of these signalling proteins. However, some subtypes of non-

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NMDA receptor types are also permeable to Ca^{2+} [12]. For this reason polyamine amides might be ideally suited as neuroprotectants because they antagonise both NMDA and non-NMDA classes of L-Glu receptor as well as VSCCs [40,65,67,70]. In this study, polyamine amide-mediated antagonism of NMDA receptors and VSCCs in primary cultures of rat cerebellar granule cells has been examined by monitoring changes of intracellular free Ca^{2+} ($[Ca^{2+}]_i$). In addition, these cells undergo a marked excitotoxic degeneration in response to challenge with NMDA, L-Glu or kainate [41,46,47] thus allowing polyamine amide-mediated neuroprotection to be investigated quantitatively.

2. Materials and methods

2.1. Granule cell culture

Primary cultures of cerebellar granule cells were grown by a modification of published procedures [49]. The cerebella from 7–8 day old rats (Wistar) were pooled and finely chopped. The slices were collected in a modified Kreb's medium supplemented with trypsin and DNAse (0.25% and 0.01% w/v, respectively). After a 15 min incubation at 25°C, the trypsin solution was removed and

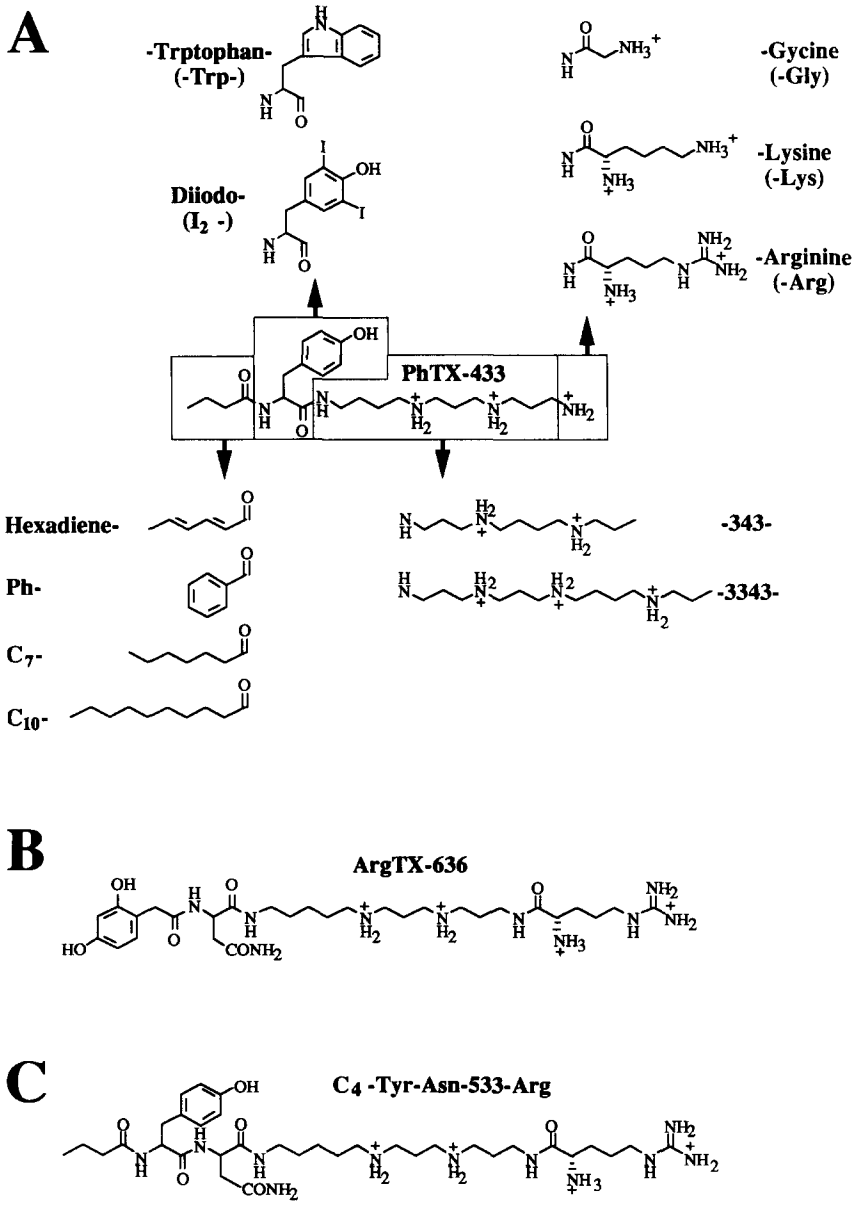

Fig. 1. Structures of the polyamine amides used in these studies. A: structure of the wasp toxin PhTX-433, and substitutions. B: structure of the spider toxin ArgTX-636. C: structure of a hybrid philanthotoxin-argiotoxin molecule C_4-Tyr-Asn-533-Arg. Note that the numbers in PhTX-433 and analogues and in the hybrid molecule refer to the number of methylenes between the amino groups. In ArgTX-636 they represent the molecular weight of the toxin.

replaced with culture medium (Minimum Essential Medium (MEM) with Earle's salts, supplemented with 10% foetal calf serum, 2 mM glutamine, 5 μg/ml insulin, 3.5 g/l D-glucose, KCl to a final concentration of 25 mM, penicillin 10 IU/ml and streptomycin 10 μg/ml) supplemented with 0.01% DNAse. The tissue was then dissociated by gentle trituration through a graded series of fire-polished Pasteur pipettes. The dissociated cells were collected by a brief centrifugation (1000 × *g* max 5 min) and resuspended in fresh culture medium. The cell suspension was passed through a 77 μm steel mesh to remove undissociated tissue and cell clumps and diluted to ~ 1.5×10^6 cells/ml. Cells were plated in 24-well plates (0.5 ml/well) coated with poly-D-lysine for toxicity studies or on to 22 mm square coverslips (No. 1) coated with poly-D-lysine and placed in 35 mm Petri dishes for Ca^{2+} studies. The cultures were incubated at 37°C in humidified 95% air/5% CO_2. After 18–24 h, the culture medium was replaced with fresh medium containing 10 μM cytosine-β-D-arabinofuranoside to prevent the replication of non-neuronal cells. The medium was not changed again during the culture period. Cells were used after 9–14 days in vitro for Ca^{2+} studies and at 12–14 days for toxicity studies.

2.2. Calcium studies

Measurements of changes of $[Ca^{2+}]_i$ were made using the Ca^{2+}-sensitive fluorescent dye fluo-3 essentially as described by Richardson et al. [59]. The dye was introduced into the granule cells by a 30–40 min incubation with 2.5 μM fluo-3 AM in serum-free culture medium supplemented with 0.1% BSA and 25 mM KCl at 37°C. After loading, the cells were clamped in a perfusion chamber and perfused (2 ml/min) for ~ 10 min at 22–25°C with a HEPES-buffered Locke's solution (154 mM NaCl, 5.6 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM HEPES, pH 7.4) prior to measurements being taken. Fluorescence measurements were made using a modified Leitz 'Diavert' microscope with epifluorescence optics. Recordings were made from small groups of granule cells (5–10 cell bodies) which were identified with a 100 ×, 1.32 NA objective and the field diaphragm closed so that only these cells were exposed to the excitation light (380–490 nm, 100 W tungsten halogen bulb). Only a single group of cells on each coverslip was examined. A shutter was installed in the excitation light path to prevent photodamage and bleaching of the dye. Cells were illuminated for 40 ms at a frequency of approximately 0.7 Hz. Fluorescence intensity (> 515 nm) was monitored using a photomultiplyer tube (PMT) attached to the camera port. The output from the PMT was fed to a sample/hold amplifier synchronised to the open shutter and the amplified signal was displayed and recorded on a PC via a Thurlby digital storage adaptor. Drugs were applied in the perfusion saline with an exchange time of ~ 7 s. The stimulation conditions used were such that reproducible responses could be routinely recorded from successive stimulations of the same group of cells (see Results). Calibration of these data using ionomycin and Mn^{2+} quenching proved unreliable, with fluorescence increases in the presence of ionomycin/Ca^{2+} being unstable and not well-maintained. Consequently, these results have been expressed as a change in the fluorescence intensity relative to baseline (i.e. $\Delta F/F$ where ΔF is the change in fluo-3 fluorescence intensity and F is the baseline fluorescence measured after subtraction of the background signal recorded following lysis of cells by Triton-X100). Fluo-3 was used in these studies because of constraints imposed by the available single wavelength microfluorimetry equipment. Subsequently however we have obtained preliminary data from granule cells loaded with the ratiometric Ca^{2+} sensitive dye fura-2. Measurements of $[Ca^{2+}]_i$ obtained with a duel wavelength fluorescence imaging system and IonVision software (Image processing and Vision Company Ltd.) have been fully supportive of the data presented in this report and will be the subject of future publications.

2.3. Toxicity studies

Acute exposures to excitotoxic agents (500 μM NMDA, 100 μM L-Glu and 500 μM kainate) were conducted at 22–25°C in HEPES-buffered Locke's solution. For exposures to NMDA and L-Glu, Mg^{2+} was omitted. The cultures were washed twice in this solution before the test agents were added. Antagonists, when present, were added 5 min prior to an agonist and were present throughout the period of exposure to agonist. All compounds were added from 10-fold, concentrated stock solutions. After incubation, a sample of the exposure medium was reserved; that remaining was replaced by a low-serum, maintenance medium (MEM supplemented with 10% granule cell culture-conditioned medium from 9–14 DIV cultures, 0.1% bovine serum albumin, 4.5 g/l glucose and KCl to a final concentration of 25 mM) before the cultures were returned to the incubator. Cell death was monitored 18–24 h after the initiation of the excitotoxic insult. The cultures were routinely examined by phase contrast microscopy and by fluorescence microscopy after vital staining with fluorescine diacetate (FDA) and propidium iodide (PI) (5 μg/ml and 10 μM respectively in maintenance medium). Following exposure to excitotoxins cell death was quantified by measuring the release of lactic dehydrogenase (LDH) into the medium [43].

LDH activity in the medium was measured essentially as described by Johnson [37]. Aliquots of the incubation media, generally 0.2 ml, were added to 0.8 ml of a saline solution (140 mM NaCl, 1 mM $MgCl_2$, 25 mM Tris[hydroxymethyl]aminomethane, pH adjusted to 7.4 with HCl) supplemented with nicotinamide dinucleotide (reduced form) and sodium pyruvate to give final concentrations of 100 μM and 900 μM respectively. After a 1 min equilibration period the reaction was followed spectrophotomet-

rically at 340 nm for 3 min using an LKB Ultrospec II controlled by the LKB 'multiple reaction rate measurement' programme. The difference between the rate of change of OD in control and experimental samples was used directly as a measure of cell death.

2.4. Materials

Polyamine amides were synthesised according to methods already described [30,36]. The following compounds were used: Tyrosyl-butanoyl-spermine (PhTX-343), tyrosyl-butanoyl-spermine-glycine (PhTX-343-Gly), tyrosyl-butanoyl-spermine-lysine (PhTX-343-Lys), tyrosyl-butanoyl-spermine-arginine (PhTX-343-Arg), 2,4-diiodo-tyrosyl-butanoyl-spermine-arginine (I_2-PhTX-343-Arg), tryptophan -butanoyl-spermine-aginine (C_4-Trp-343-Arg), tyrosyl-decanoyl-spermine (C_{10}-PhTX-343), tyrosyl-decanoyl-spermine-arginine (C_{10}-PhTX-343-Arg), tryptophan-decanoyl-spermine-arginine (C_{10}-Trp-343-Arg), tyrosyl-heptanoyl-3343 (C_7-PhTX-3343), tyrosyl-heptanoyl-spermine-lysine (C_7-PhTX-343-Lys), tyrosyl-heptanoyl-spermine arginine (C_7-PhTX-343-Arg), tyrosyl-benzoyl-spermine (Ph-PhTX-343), tyrosyl-hexadienol-spermine (Hexadiene-PhTX-343), tyrosyl-butanoyl-asparaginyl-polyamine (533)-arginine (C_4-Tyr-Asn-533-Arg) (the digits refer to the number of methylene groups separating the amino moieties of the polyamine), 2,4-dihydroxyphenyl-acetyl-asparaginyl-polyamine (533)-arginine (ArgTX-636). For long-term storage, compounds were dissolved in sterile H_2O, divided into aliquots, freeze-dried and stored desiccated at −20°C. Aliquots were dissolved as needed in appropriate solutions and subsequently stored frozen at −20°C between experiments. Fluo-3 AM was obtained from Molecular Probes Inc. NMDA, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and D,L-2-amino-5-phosphonopentanoic acid (D,L-AP5) were obtained from Tocris-Cookson, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) was from Research Biochemicals International. All other chemicals were from Sigma.

3. Results

3.1. Polyamine amide antagonism of NMDA-stimulated increases of $[Ca^{2+}]_i$

In the absence of Mg^{2+}, stimulation of cultured cerebellar granule cells with 50 μM NMDA caused only a small increase of $[Ca^{2+}]_i$, as indicated by an increase of the fluo-3 fluorescence intensity. This effect was greatly potentiated by μM concentrations of glycine. Co-application of 50 μM NMDA and 50 μM glycine in the absence of Mg^{2+} caused a rapid increase of $[Ca^{2+}]_i$ which was maintained during the period of exposure to agonist, but which fell rapidly to baseline values upon washing with

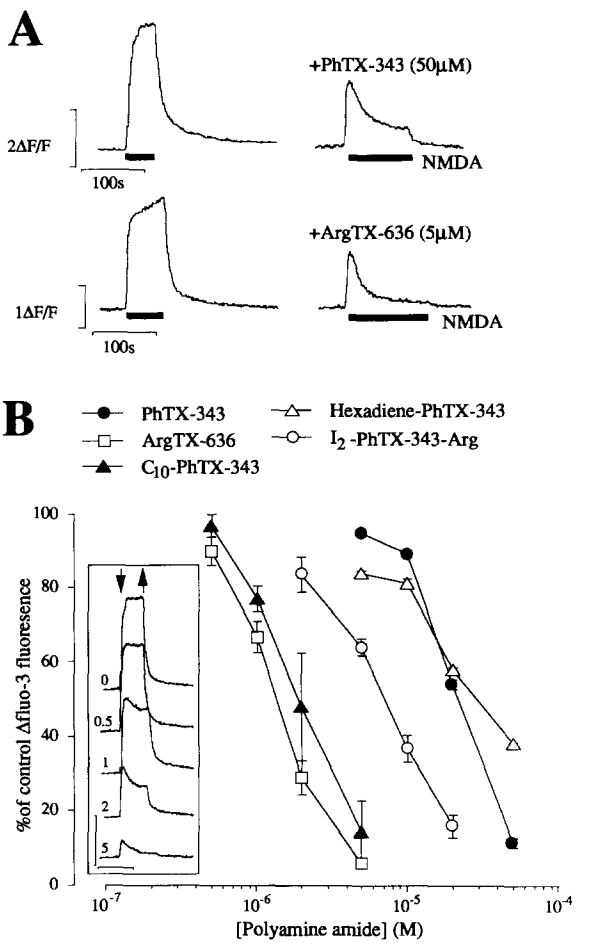

Fig. 2. Antagonism by polyamine amides of NMDA-stimulated increases of $[Ca^{2+}]_i$ in cultured cerebellar granule cells. A: antagonism of NMDA-stimulated increases of $[Ca^{2+}]_i$ by PhTX-343 (top) and ArgTX-636 (bottom). Fluo-3 fluorescence was monitored as described in Materials and methods. Control NMDA (50 μM) stimulations (left) were carried out in the presence of a saturating concentration of glycine (50 μM) in Mg^{2+}-free saline. After a 5 min wash, the agonist was re-applied simultaneously with the polyamine amide. B: dose-inhibition curves for polyamine amide antagonism of NMDA-stimulated increases of $[Ca^{2+}]_i$. The data are expressed as a percentage of the control responses to 50 μM NMDA plus 50 μM glycine and are calculated from the plateau phase of the response. The data are means ± S.D. for at least 3 determinations at each concentration point. The inset shows the recordings for a typical dose-inhibition experiment for ArgTX-636. Sequential NMDA responses were recorded in the presence of the indicated concentrations (μM) of ArgTX-636 (scale bars represent 2 ΔF/F units and 100 s). Note that the peak response to the agonist in the presence of 5 μM ArgTX-636 is smaller than that in (A) (bottom, left). This is because, in the former case, the toxin was applied more than once and recovery from ArgTX-636 block is slow and only partial (see Fig. 3)

Mg^{2+}-containing NMDA-free medium (Fig. 2). The increases of $[Ca^{2+}]_i$ were strongly inhibited by 1 mM Mg^{2+}, 100 μM D,L-AP5 or 0.5 μM MK-801 (data not shown). The polyamine amides antagonised the NMDA-stimulated increases of $[Ca^{2+}]_i$ (Fig. 2). Reproducible responses could be routinely obtained from a single group of cells by using a protocol where 60–100 s applications of 50 μM NMDA

plus 50 μM glycine were preceded by a 60 s perfusion with Mg^{2+}-free saline and were separated by 5-min wash periods. Dose-response data for a series of polyamine amides were obtained in this way (Fig. 2A,B). None of the polyamine amides significantly affected the resting $[Ca^{2+}]_i$ when applied alone at the concentrations referred to in Fig. 2B (for an example see Fig. 3C). However, all of the compounds antagonised the NMDA-stimulated increase of $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 2B). Although accurate estimations of inhibition constants cannot be made from these data because they have not been calibrated to $[Ca^{2+}]_i$ values (see section 2.2), the order of potency ArgTX-636 > C_{10}-PhTX-343 > > I_2-PhTX-343-Arg > > PhTX-343 is clear (Fig. 2B).

Simultaneous application of the NMDA stimulus and a polyamine amide resulted in a characteristic biphasic response; i.e. $[Ca^{2+}]_i$ increased rapidly to a peak value then slowly decayed to a plateau (Fig. 2A). The plateau was maintained until washout of the NMDA returned $[Ca^{2+}]_i$ to its baseline value. A similar response was obtained when antagonist was applied prior to, and during, application of NMDA (not shown). Recovery from antagonism by PhTX-343 and by analogues of this toxin reached 90–100% of control values during a subsequent control stimulation (Fig. 3A). In contrast, recovery from antagonism by ArgTX-636 was slow and always incomplete even after prolonged (20 min) washing in toxin-free, NMDA-free saline (Fig. 3B). Recovery was improved with repeated NMDA-stimulations but still did not reach control values (Fig. 3D). When ArgTX-636 applied in the absence of NMDA, for periods of 2–3 min, subsequent NMDA-stimulated responses elicited after washout of the ArgTX-636 were not inhibited (Fig. 3C).

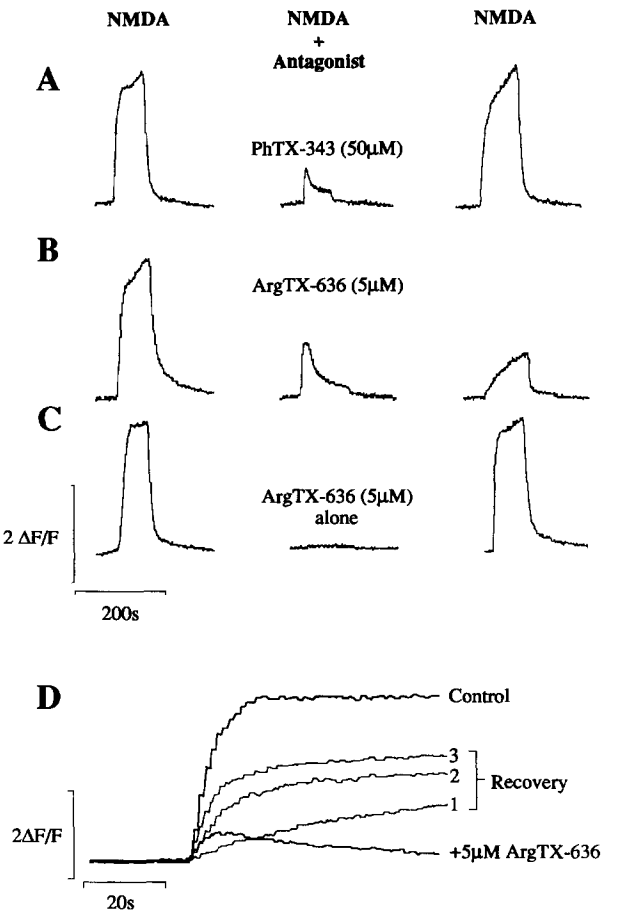

Fig. 3. Recovery of NMDA-stimulated increases of $[Ca^{2+}]_i$ from polyamine amide-mediated antagonism. Control, antagonised and recovery responses to NMDA (50 μM) plus glycine (50 μM) are shown for PhTX-343 (A) and ArgTX-636 (B). Note the slow recovery following ArgTX-636 antagonism. In (C), ArgTX-636 was applied in the absence of agonist, but this did not affect subsequent responses to NMDA. In (D) control and antagonised (5 μM ArgTX-636) NMDA responses are shown as are three subsequent control responses each separated by a 5-min wash in NMDA and toxin-free saline. Each stimulation lasted for ~ 100 s. The data are shown on an increased time base. The data are from four different groups of granule cells from different coverslips and are representative of at least two other similar experiments in each case.

3.2. Polyamine amide effects on K^+-stimulated increases of $[Ca^{2+}]_i$

Exposure of cerebellar granule cells to saline containing 40 mM KCl (high-K^+ saline) resulted in a rapid increase of $[Ca^{2+}]_i$ that peaked and then decayed to a plateau which was maintained until the high-K^+ saline was removed. $[Ca^{2+}]_i$ fell rapidly to basal levels upon removal of the high-K^+ saline (Fig. 4). When either PhTX-343 (20 μM) (Fig. 4A) or ArgTX-636 (10 μM) was co-applied with high-K^+ saline, the increase of $[Ca^{2+}]_i$ was not inhibited. In contrast, nifedipine (1 μM), an L-type VSCC antagonist, was an effective inhibitor of the plateau phase of this response (Fig. 4B).

3.3. Polyamine amide antagonism of excitotoxicity in granule cell culture

Brief exposures (25–30 min) of cerebellar granule cells to 100 μM L-Glu, 500 μM NMDA or 500 μM kainate were sufficient to kill > 90% of the cells over the following 18–24 h period. This cell death was easily recognised using phase contrast microscopy (Fig. 5). It was accompanied by a 3- to 4-fold increase in the LDH activity of the cell culture medium compared to control cultures. In contrast to L-Glu and NMDA, kainate elicited a significant release of LDH during the period of exposure to this agonist, a result observed previously [41]. Therefore, for kainate, granule cell death was determined from the total release of LDH activity; i.e that released during the excitotoxic challenge combined with the LDH activity released during the 18–24 h post-exposure incubation [41]. Excitotoxicity induced by 500 μM NMDA or 500 μM kainate was completely prevented by D,L-AP5 (100 μM) and DNQX (50 μM) respectively (data not shown).

There was no cell death when granule cell cultures were exposed to either PhTX-343 (400 μM) or ArgTX-636 (50

μM) for 30 min or 24 h in maintenance medium. Even 24 h after the continuous exposure to these compounds, the granule cells appeared morphologically normal, they excluded PI and the medium LDH activity was no different from controls. However, the two polyamine amides proved to be effective antagonists of the toxicity induced by NMDA L-Glu and kainate. When applied 5 min prior to and during application of one of these amino acids, the polyamine amides prevented both the initial cell swelling and the subsequent cell degeneration which characterise excitotoxicity (Fig. 5). Quantitative analysis of cell death using the release of LDH as a marker, showed that neuroprotection by the polyamine amides is dependent on the dose of the antagonist (Fig. 6) and that complete protection was obtained with the higher concentrations of these compounds (Fig. 6). ArgTX-636 was more potent (~ 30 fold) than PhTX-343 in providing protection against NMDA and L-Glu insults (Fig. 6; Table 1). However, whereas PhTX-343 antagonised both NMDA- and kainate-induced cell death with similar potency, ArgTX-636 showed a marked selectivity for antagonism of NMDA- and L-Glu-mediated toxicity (Table 1).

Recently, there has been much interest in defining the type of neuronal death, i.e. necrotic versus apoptotic, that occurs following an excitotoxic challenge. In granule cells, both forms might be expected to contribute according to the severity and time course of the excitotoxic exposure [3,72]. Thus prolonged exposures to glutamate appear to involve a necrotic cell death whereas brief exposures involve an early necrotic followed by a delayed apoptotic cell death [3,72]. Since our exposure paradigm, a brief 20–30 min application of agonist, was very similar to that of Ankarcrona et al. [3], we could assume that both

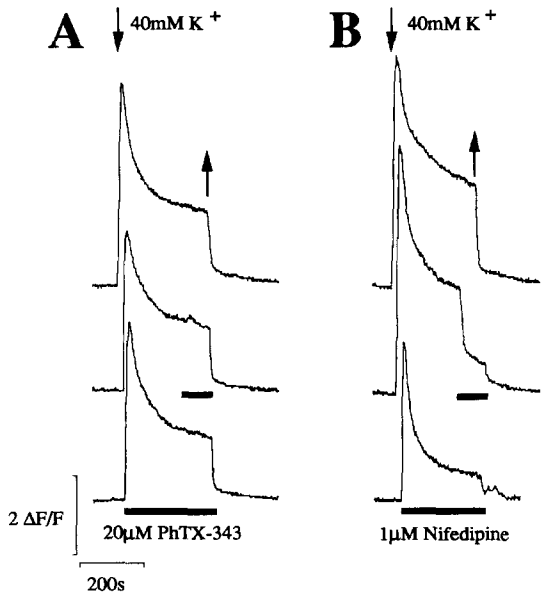

Fig. 4. Effect of PhTX-343 and nifedipine on K^+-stimulated increases of $[Ca^{2+}]_i$. A: 40 mM K^+ was added for 300 s (between arrows); PhTX-343 was added at the times indicated by the horizontal bars. B: as in (A) except that nifedipine was substituted for the polyamine amide. The responses in each panel were recorded sequentially from a single group of granule cells in each case, and are representative of at least 2 other similar experiments in each case.

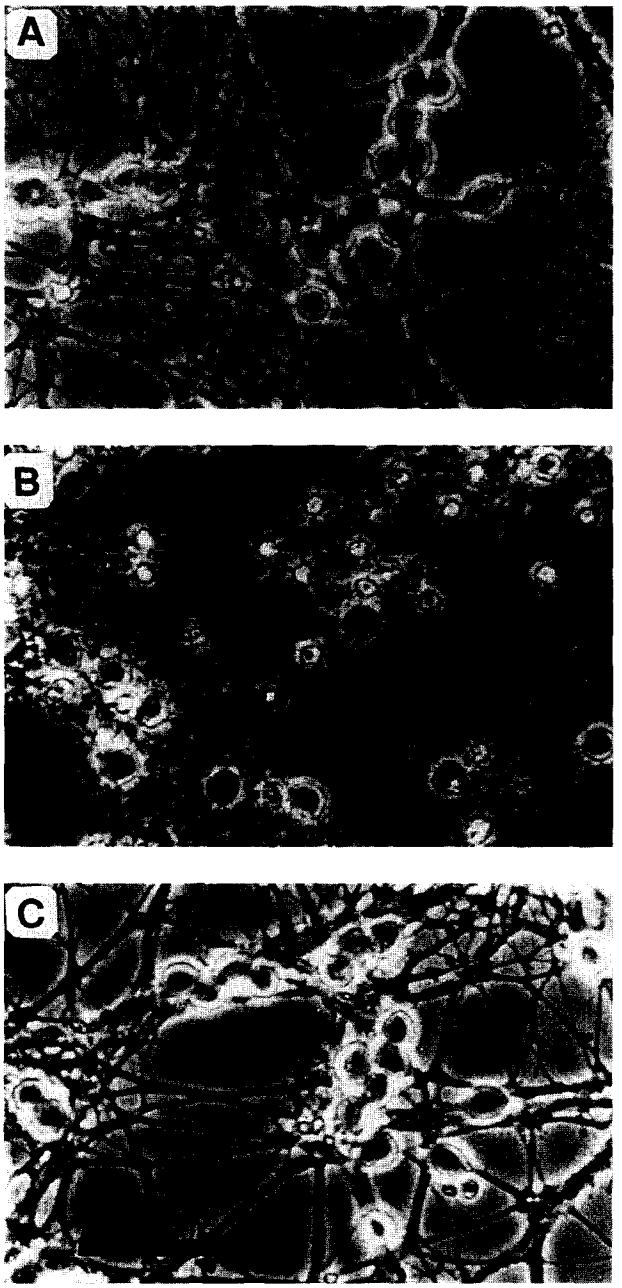

Fig. 5. PhTX-343 antagonises NMDA-mediated excitotoxicity in cultured cerebellar granule cells. Exposure to NMDA and PhTX-343 was as described in Materials and methods. A: control cultures 24 h after a 30-min incubation in Mg^{2+} free Locke's solution. Note the rounded cell bodies and the well-defined neurite network. B: 24 h after exposure to NMDA (500 μM plus 50 μM glycine for 25 min) in Mg^{2+}-free Locke's solution, the neurite network and most of the cell bodies have degenerated. C: 24 h after PhTX-343 (50 μM) was applied 5 min prior to, and during, a 25 min exposure to NMDA (500 μM plus 50 μM glycine). Note that the cell bodies and neurite network remain intact. The scale bar in (C) represents 50 μm.

Table 1
Neuroprotective potencies of PhTX-343 and ArgTX-636

Polyamine amide	Neuroprotective potency IC_{50} values (μM)		
	L-Glutamate	NMDA	Kainate
PhTX-343	40 ± 8.9	30 ± 13	47 ± 12
ArgTX-636	0.95 ± 0.18	1.4 ± 1.3	34 ± 2 *
C_4-Tyr-Asn-533-Arg		11 ± 0.8 *	

Dose inhibition curves were constructed using LDH release as a quantitative measure of cell death. The neuroprotective potency of the polyamine amides was calculated from the curves and is given as an IC_{50} value; i.e. the concentration of the polyamine amide required to inhibit 50% of the agonist induced release of LDH. Exposure conditions and concentrations of agonist are detailed in Materials and methods. The data are presented either as means ± S.D. (n = 3–7) or as means ± ranges * (n = 2).

necrosis and apoptosis are responsible for the cell death seen after 24 h (Fig. 5). However, because we did not examine our cultures for the characteristic markers of apoptotic cell death we have not tested the possibility that delayed cell death seen in L-Glu challenged granule cell cultures is apoptotic.

3.4. Neuroprotection by PhTX-343 analogues

The neuroprotective properties of some analogues of PhTX-343 (Fig. 1) were also investigated. Elongation of the polyamine chain of PhTX-343 and the addition of an extra charge, as in the lysine and arginine analogues, produced a modest 2–3 fold increase in potency. When changes were made additionally in the aromatic region (i.e. I_2-PhTX-343-Arg and C_4-Trp-343-Arg), potency was not increased (Table 2). Marked increases in potency were obtained when substitutions were made in the butyryl region of the molecule. Elongation of this region, as in the C_7- and C_{10}-analogues, increased potency beyond that of their C_4-counterparts (Table 3). The hexadiene substitution resulted in a fall in potency in both the Ca^{2+} and toxicity experiments (Table 3; Fig. 6); this suggests that flexibility in the butyryl side-chain is important. Compounds incorporating both the C_{10} and arginine substitutions were the most potent analogues of PhTX-343 (Table 3; Fig. 6).

3.5. Toxicity of PhTX-343 analogues

Although the C_{10}-substitution produced some of the most potent polyamine amide antagonists tested in these studies, this substitution also introduced some toxicity. Concentrations of 30 μM of all of the C_{10}-analogues tested (Table 3) were sufficient to cause a marked degeneration of granule cell cultures, killing between 20 and 80% of the cells as judged by the release of LDH activity and FDA/PI staining (data not shown). Higher concentrations resulted in complete degeneration of the cultures, with both the granule cells and the small population of non-neuronal cells being affected. The C_6-hexadiene analogue showed occasional toxicity, but only at the highest concen-

Table 2
Neuroprotective potencies of philanthotoxin analogues characterised by modifications at the terminal amino group of PhTX-343

Polyamine amide	Neuroprotective potency IC_{50} values (μM) and potency relative to PhTX-343					
	L-Glutamate		NMDA		Kainate	
PhTX-343-Gly	87 ± 2 *	0.46	71	0.42		
PhTX-343Lys	16	2.5	13 ± 2 *	2.3		
PhTX-343-Arg	12 ± 1.9	3.3	16 ± 3*	1.9	21	2.2
C_4-Trp-343-Arg	16 ± 3.4	2.5	10 ± 1*	3	14 ± 6 *	3.3
I_2-PhTX-343-Arg	12 ± 3.2	3.3	9.2 ± 2.3	3.3	19 ± 8.6	2.5

The data are shown as IC_{50} values and as potencies relative to that of PhTX-343 (see legend to Table 1 for details), and are presented as means ± S.D. (n = 3) or means ± ranges * (n = 2).

Table 3
Neuroprotective potencies of philanthotoxin analogues characterised by modifications at the butyryl group of PhTX-343

Polyamine amide	Neuroprotective potency IC_{50} values (μM) and potency relative to PhTX-343					
	L-Glutamate		NMDA		Kainate	
Hexadien-PhTX-343	64.6 ± 11.5	0.62	61.6	0.49		
Ph-PhTX-343			75	0.4		
C_7-PhTX-3343			0.76 ± 0.4 *	42		
C_7-PhTX-Lys			6.9 ± 0.6 *	4.8		
C_7-PhTX-Arg			1.8 ± 0.25 *	15		
C_{10}-PhTX-343	3 ± 1.1	13	1.9	16		
C_{10}-PhTX-343-Arg	1.0 ± 0.43	40				
C_{10}-Trp-343-Arg	1.1 ± 0.40	36	0.63 ± 0.1	47	1.3 ± 0.8	36

The data are shown as IC_{50} values and as potencies relative to that of PhTX-343 (see legend to Table 1 for details). The data are presented either as means ± S.D. (n = 3) or as means ± ranges * (n = 2).

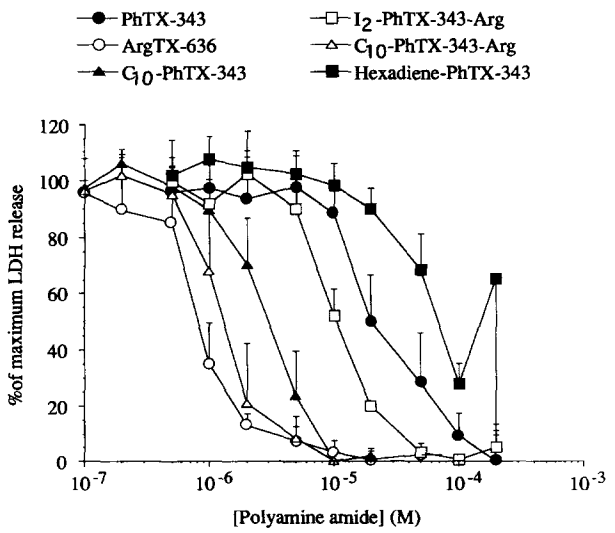

Fig. 6. Dose-inhibition curves for polyamine amide-mediated antagonism of granule cell death induced by L-Glu (100 μM plus 50 μM glycine). Experiments were conducted as described in Materials and methods, and cell death was quantified by measurement of released LDH activity. The results are expressed as a percentage of the LDH activity released by L-Glu alone, and are shown as means ± S.D. from 3–7 independent experiments. High concentrations (200 μM) of hexadiene-PhTX-343 resulted in occasional toxicity hence the large error bar associated with this point.

tration tested (200 μM) (see Fig. 6). However, there was no cell death following exposure of cerebellar granule cells to PhTX-343 and analogues containing either a C_7 or C_4 alkyl moiety at concentrations up to 200 μM (C_7-PhTX-3343) or 400 μM (PhTX-343).

4. Discussion

Polyamine amides antagonise NMDA-stimulated and L-Glu- induced increases of granule cell $[Ca^{2+}]_i$ and thus are effective neuroprotectants against excitotoxic insults by these compounds. L-Glu toxicity in cerebellar granule cells cultured in media containing 25 mM KCl (the conditions used here) is mediated predominantly by NMDA receptor activation [22,45,50]. Presumably, non-NMDA receptors (AMPA/kainate receptors) are not involved because they are rapidly desensitised by L-Glu [16]. It has been shown convincingly using an invertebrate model that desensitization of L-Glu receptors is a powerful protectant against L-Glu-induced toxicity [25]. In contrast to the action of L-Glu, kainate excites but does not desensitize mammalian AMPA receptors [16], so it seems reasonable to assume that the excitotoxicity obtained with this compound may be due to Ca^{2+} entry through channels gated by AMPA receptors [12,15,44]. It was of interest, therefore, to test the ability of polyamine amides to antagonise the toxicity caused by kainate. Polyamine amides were found to be effective antagonists of kainate-induced toxicity, thus demonstrating that they may be potentially useful neuroprotectants in excitotoxic paradigms involving both NMDA and non-NMDA ionotropic L-Glu receptors. That L-Glu toxicity is mediated by NMDA receptors in cerebellar granule cell cultures is further supported by the results obtained here for ArgTX-636 which showed similar potency for protection against NMDA and L-Glu challenges but a considerably lower potency for protection against a kainate challenge. PhTX-343 and its analogues, and to a lesser extent ArgTX-636, also protected cultured cerebellar granule cells against kainate-mediated toxicity, thus demonstrating their efficacy at both NMDA and non-NMDA receptors in these cells. However, unlike ArgTX-636, the philanthotoxins did not discriminate between NMDA- or kainate-mediated toxicity. The presence of agonist was clearly important for at least a major part of the antagonism of granule cell NMDA receptors. Antagonism by ArgTX-636 was only slowly reversible over the time course of the experiments conducted here, and recovery, like antagonism appeared to have a use-dependent component with repeated application of NMDA facilitating recovery. These results are consistent with previous suggestions that there is a use-dependent component to polyamine amide antagonism of mammalian L-Glu receptors [13,53].

Although polyamine amide toxins have been shown to be effective antagonists at vertebrate ionotropic L-Glu receptors, a consistent view on their L-Glu receptor subtype selectivity has failed to emerge [70]. PhTX-433 specifically antagonises responses to non-NMDA agonists in rat spinal and brainstem neurones in vivo [38,39] and selectively antagonises these responses in rat brain RNA injected oocytes [13], yet its selectivity appears to be for NMDA receptors in CA1 pyramidal neurones [27] and in oocytes injected with adult rat brain RNA [56]. Likewise, ArgTX-636 selectively antagonises NMDA-induced currents in cultured rat cortical neurones [53] and oocytes injected with rat brain RNA [23,24], yet it exhibits similar activity at both NMDA and non-NMDA receptors on rat spinal neurones in vivo [39] and oocytes injected with RNA from 14-day-old rat brain [13]. Additionally, in hippocampal slices, ArgTX-636 blocks excitatory neurotransmission in the CA1 region in response to stimulation of the Schaffer collaterals [5], responses presumably mediated by non-NMDA receptors. Advances in the molecular biological characterisation of ionotropic L-Glu receptors have recently revealed a possible explanation for these divergent selectivity data. Expression of L-Glu receptor subunits in homomeric and heteromeric combinations using the *Xenopus* oocyte system has revealed a marked subunit selectivity to polyamine amide toxin antagonism of both non-NMDA and NMDA receptors. Thus homomeric receptors composed of GluR1, GluR3 or GluR4 subunits are sensitive to polyamine amide antagonism [11,13,31], whereas heteromeric receptors containing GluR2 are strongly resistant to antagonism [11,13,31]. The well-characterised Q/R site in these subunits is a major determinant

of Ca^{2+} permeability and also an important determinant of polyamine amide antagonism [11,31]. Homomeric NR1 [13] and heteromeric NR1/NR2A and NR1/NR2B receptors [55] are sensitive to ArgTX-636 antagonism, whereas heteromeric receptors containing the NR2C subunit are much less sensitive [55]. Although the Q/R site of non-NMDA subunits can affect polyamine amide potency [11,31], this site is occupied by asparagine in both NR1 and NR2A-C. This suggests that other factors contribute to the polyamine amide subunit selectivity seen with NMDA receptors [55].

The selective interactions of polyamine amides with L-Glu receptor subunits has important implications for the neuroprotective properties of these compounds. For example, by antagonising the Ca^{2+} permeable AMPA/kainate receptors, i.e. those lacking GluR2 subunits, they could reduce the rise of $[Ca^{2+}]_i$ that would accompany a challenge with L-Glu [12,15,44], but only if those receptors were not rapidly desensitised by agonist. Receptors containing the GluR2 subunit would not be expected to contribute to the primary signal associated with a Ca^{2+}-mediated excitotoxicity. Also, NMDA receptors containing the NR2C subunit, i.e. those with low affinities for polyamine amides, contribute little to the development of excitoxicity in transfected HEK 293 cells [1]. Significantly, in vivo, the granule cell layer of the cerebellum, an area which predominantly expresses the NR2C subunit, is relatively resistant to ischaemic insults [54]. It follows from the above comments that polyamine amide could be used to target those receptors most directly involved in the generation of L-Glu-mediated neuronal injury.

Cerebellar granule cells grown in depolarising media (containing 25 mM KCl) show an upregulation of NR2A mRNA [9,58] and protein [58] levels. Additionally, the time course of expression of NR2C mRNA is attenuated by this treatment, as compared to cells grown in lower (10 mM) KCl media [58]. GluR1 mRNA and protein levels are also considerably increased in granule cells grown in depolarising media, whilst GluR2 mRNA and GluR2/3 protein levels are lower than in cells grown in low KCl media [21]. These results correlate well with the ability of AMPA to produce a larger increase Ca^{2+} influx in granule cells grown in depolarising media, implying a greater contribution of the Ca^{2+} permeable GluR1 subunit to receptors in these cells [21]. In this study, NMDA- and kainate-mediated toxicities are both sensitive to polyamine amide inhibition suggesting a major involvement of NR1/NR2A/NR2B and GluR1/3/4 subunits. However, the polyamine amide affinities were generally lower than those obtained elsewhere from electrophysiological experiments possibly indicating some interactions with the less sensitive configurations of L-Glu receptors. Additionally, the differences in affinities could be partially explained by the known voltage-dependence of polyamine amide mediated antagonism of these receptors [13,26,53], with any agonist induced depolarisation of the granule cells tending to relieve polyamine amide block of L-Glu receptors, hence reducing their neuroprotective potency.

In granule cell cultures, polyamine amide neuroprotection appears to be mediated solely by antagonism of L-Glu receptors. However, antagonism of VSCCs could contribute to the prevention of $[Ca^{2+}]_i$ elevation and the initiation of toxicity in other models involving excitotoxic neuronal death [6,71]. PhTX-433 and its dideaza- analogue have been found to antagonise Ca^{2+} currents in hippocampal CA1 neurones with 10 μM concentrations reducing the peak current by 28 and 60% respectively. Additionally, ArgTX-636 antagonises Ca^{2+} currents in dorsal root ganglion (DRG) cells by about 35% at 10μM, the maximally effective concentration in these cells [67]. In cultures of cerebellar granule cells, sFTX-3.3, a synthetic polyamine-arginine compound, inhibited by $\sim$25% the high-voltage activated currents [51], and in DRG cells produced a selective block of the low voltage activated Ca^{2+} currents [65]. Pocock et al. [52] however, monitoring changes of $[Ca^{2+}]_i$ in cultured cerebellar granule cells in response to K^+ depolarisation, found no antagonism with either sFTX-3.3 (10 μM) or spermine (10 μM). The results presented here, showing that ArgTX-636 and PhTX-343 lack activity at granule cell VSCCs, extend these findings and suggest that in cultured cerebellar granule cells the VSCCs contributing to K^+-stimulated increases of $[Ca^{2+}]_i$ are resistant to polyamine amide containing antagonists. Jackson and Parks [34] however, citing unpublished observations, report that at high concentrations polyamine amide toxins may inhibit L-type VSCCs. Since there is a major contribution from L-type VSCCs to the K^+-stimulated increases of $[Ca^{2+}]_i$ in cultured granule cells it would appear that the concentrations of polyamine amide toxins used here may have been insufficient for the observation of any antagonism of these channels.

Many structural analogues of PhTX have been produced and their activities have been determined in both biochemical (inhibition of MK-801 binding) [2] and physiological [8,14] assays of L-Glu receptor function. A number of regions of the PhTX-343 molecule contribute to potency. Elongation of the polyamine chain or substitution of the terminal polyamine amino group, with the addition of an extra charge, gave compounds with a modest increase in potency. The position of the charges along the polyamine chain and the ability of the delocalisation of the guanidino group +ve charge in these analogues has been suggested to enhance the interactions with anionic centres within the ion channel pore thus giving an increase in potency [14,70]. The greatest increase of potency was achieved by substitutions of the butyryl region of the molecule, with flexible hydrophobic substitutions being the most effective. However, when these involved a C_{10}-chain the analogues were cytotoxic.Their amphiphilic structure may have introduced detergent-like properties leading to their disruption of cell membranes. In contrast, the C_7-analogues, whilst having relatively high affinity at NMDA receptors, were not toxic.

In this report we have shown that the polyamine amide containing toxins and a number of their analogues can act as neuroprotective agents in an in vitro model of excitotoxicity. Preliminary data have also shown these compounds to have efficacy in in vivo models of L-Glu receptor function. Thus, intracerebroventricular injections of a polyamine amide found in Joro spider venom prevents L-quisqualate-induced but not kainate-induced or NMDA-induced seizures [32,33]. Systemic administration of a fraction from *Agelonopsis aperta* venom containing the polyamine amide α-agatoxin or of ArgTX-636 antagonises, respectively, kainate-induced seizures in rats [34] and audiogenic- and NMDA-mediated seizures in mice [66]. These data imply that polyamine amides may cross the blood brain barrier. These results and the findings of the present study show that the polyamine amides are potentially useful neuroprotective agents worthy of further study.

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