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Leu[10] of α-conotoxin PnIB confers potency for neuronal nicotinic responses in bovine chromaffin cells

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Abstract

Two α-conotoxins PnIA and PnIB (previously reported as being ''mollusc specific'') which differ in only two amino acid residues (AN versus LS at residues 10 and 11, respectively), show markedly different inhibition of the neuronal nicotinic acetylcholine receptor response in bovine chromaffin cells, a mammalian preparation. Whereas α-conotoxin PnIB completely inhibits the nicotine-evoked catecholamine release at 10 μM, with $IC_{50} = 0.7$ μM, α-conotoxin PnIA is some 30–40 times less potent. Two peptide analogues, [A10L]PnIA and [N11S]PnIA were synthesized to investigate the extent to which each residue contributes to activity. [A10L]PnIA ($IC_{50} = 2.0$ μM) completely inhibits catecholamine release at 10 μM whereas [N11S]PnIA shows little inhibition. In contrast, none of the peptides inhibit muscle-type nicotinic responses in the rat hemi-diaphragm preparation. We conclude that the enhanced potency of α-conotoxin PnIB over α-conotoxin PnIA in the neuronal-type nicotinic response is principally determined by the larger, more hydrophobic leucine residue at position 10 in α-conotoxin PnIB.

Keywords: Conotoxin; Neuronal nicotinic receptor; Acetylcholine receptor; Chromaffin cell; Nicotinic receptor antagonist; Catecholamine release

1. Introduction

Neuronal nicotinic acetylcholine receptors have been implicated in a number of neurological disorders such as Parkinson's and Alzheimer's disease (James and Nordberg, 1995; Gotti et al., 1997; Lindstrom, 1997; Newhouse et al., 1997). Consequently, there is much interest in the functional and structural characterization of these receptors and in the development of more selective agents with therapeutic potential. The neuronal nicotinic receptors are encoded by genes specifying α subunits (α2–α9) and β subunits (β2–β4) (Galzi and Changeux, 1995). Great diversity is observed in the subunit combinations formed; functional receptors have been reported for pentameric heteroligomeric combinations of two α and three β subunits (α2–α6 and β2–β4 gene products) and homoligomers composed of five α subunits (α7–α9 gene products) (Galzi and Changeux, 1995).

A cell system that has been much favoured for studying neuronal nicotinic receptors is the bovine adrenal chromaffin cell. These cells are developmentally derived from the neural crest, and contain α3, α5, α7 and β4 subunits of the nicotinic receptor (Garcia-Guzman et al., 1995; Campos-Caro et al., 1997).

One class of molecules that is proving to be most useful for the characterization of the various nicotinic receptor subtype combinations is the α-conotoxin family (Table 1). Conotoxins are small, cysteine-rich peptides isolated from the venom of *Conus* species of predatory marine snails. α-Conotoxins are structurally related conotoxins selective for the nicotinic acetylcholine receptor. The first studied and best characterized α-conotoxins are α-conotoxin GI (Gray et al., 1981), α-conotoxin SI (Zafaralla et al., 1988) and α-conotoxin MI (McIntosh et al., 1982), all of which were isolated from piscivorous cones (*Conus geographus*, *C. striatus* and *C. magus*, respectively) and which target the *muscle*-type nicotinic receptor. By contrast, most α-conotoxins isolated from molluscivorous and vermivorous *Conus* species are selective for *neuronal*-type nicotinic receptors [e.g.. α-conotoxin ImI (McIntosh et al., 1994) and α-conotoxin EpI (Loughnan et al., 1998)].

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Table 1
Selectivity of neuronally active α-conotoxins

α-CTX	Sequence	Loop size[a]	Species	Selectivity	Reference
PnIA	G**CC**SLPP**C**AANNPDY**C**[b]	4:7	C. pennaceus	α3β2	Luo et al., 1999
PnIB	G**CC**SLPP**C**ALSNPDY**C**[b]	4:7	C. pennaceus	α7	Luo et al., 1999
				α3β4	present paper
EpI	G**CC**SDPR**C**NMNNPDY[c]**C**[b]	4:7	C. episcopatus	α3β2	Loughnan et al., 1998
				α3β4	
MII	G**CC**SNPV**C**HLEHSNL**C**[b]	4:7	C. magus	α3β2	Cartier et al., 1996
				α3β4	Broxton et al., 1997
AuIB	G**CC**SYPP**C**FATNPD-**C**[b]	4:6	C. aulicus	α3β4	Luo et al., 1998
ImI	G**CC**SDPR**C**AWR**C**[b]	4:3	C. imperialis	α7	Johnson et al., 1995
				α3β4	Broxton et al., 1999

[a]A loop size of 4:7 implies four amino acid residues between Cys^2 and Cys^3 and seven residues between Cys^3 and Cys^4.
[b]Amidated C terminus.
[c]Sulfated residue.

The α-conotoxins PnIA and PnIB are peptide neurotoxins from the venom of the molluscivorous marine snail *C. pennaceus*. They have previously been reported as being ''mollusc-specific'' neuronal nicotinic acetylcholine receptor antagonists because they blocked the nicotinic responses of cultured *Aplysia* neurons but did not affect neuronal-type nicotinic responses in rat brain and fish and insect bioassays (Fainzilber et al., 1994).

α-Conotoxins PnIA and PnIB have the disulfide bond framework typical of α-conotoxins. However, their sequences differ from previously described piscivorous α-conotoxins in that they possess a single negatively charged aspartate rather than a positively charged residue in the C-terminal loop. They also have additional proline residues in both loops, a property shared by α-conotoxin AuIB from *C. aulicus* (Luo et al., 1998). α-Conotoxins PnIA and PnIB have the 4:7 loop structure (that is, four amino acid residues between Cys^2 and Cys^3 and seven residues between Cys^3 and Cys^4) shared by most other known α-conotoxins from molluscivorous cones, [e.g., α-conotoxin EpI (Loughnan et al., 1998) and α-conotoxins AuIA and AuIC (Luo et al., 1998)], as compared to the 3:5 loop structures found in most α-conotoxins from piscivorous cones [e.g., α-conotoxin GI (Gray et al., 1981), α-conotoxin SI, (Zafaralla et al., 1998)].

The 1.1 Å crystal structure of synthetic α-conotoxin PnIA has been determined (Hu et al., 1996). Two disulfide bridges are in the interior of the molecule, while all side chains are oriented outwards. The compact architecture of the α-conotoxin PnIA toxin provides a rigid framework for presentation of chemical groups that are required for activity. This framework (peptide backbone) is identical to that in the structure of α-conotoxin PnIB, which presents slightly different functional groups at its surface (Hu et al., 1997).

Table 2
Sequences of α-conotoxins PnIA and PnIB and analogues

Toxin	Sequence
PnIA	GCCSLPPCA**AN**NPDYC–NH_2
[N11S]PnIA	GCCSLPPCA**AS**NPDYC–NH_2
[A10L]PnIA	GCCSLPPCA**LN**NPDYC–NH_2
PnIB	GCCSLPPCA**LS**NPDYC–NH_2

α-Conotoxins PnIA and PnIB differ in only two amino acid residues (AN versus LS at residues 10 and 11). Preliminary studies from our laboratory (Broxton et al., 1998) showed that α-conotoxin PnIB was more potent than α-conotoxin PnIA as an inhibitor of the neuronal nicotinic response of bovine chromaffin cells. Since α-conotoxins PnIA and PnIB have an identical backbone and differ only in two adjacent amino acid side chains constituting an exposed surface patch, we synthesized the structural analogues [A10L]PnIA and [N11S]PnIA to investigate the extent to which each residue contributes to changes in activity (Table 2). In this study we show that Leu 10 of α-conotoxin PnIB confers potency for neuronal nicotinic responses in bovine chromaffin cells.

2. Materials and methods

2.1. Materials

Fresh bovine adrenal glands were obtained from a local slaughterhouse. Culture media, penicillin (G, sodium, NF grade), streptomycin sulfate and fetal calf serum were obtained from Gibco BRL (NY, USA). Collagenase A was from Boehringer Mannheim (Germany) and Percoll from Pharmacia (Uppsala, Sweden). Tissue culture plates were from Sarstedt (Newton, NC, USA). Bovine serum albumin was from HÄMOSAN (Graz, Austria), perchloric acid and buffer salts were from BDH Chemicals, (Kilsyth, Australia) and nicotine was from Sigma Chemical (St Louis, USA). α-Conotoxins GI, PnIA, PnIB and the peptide analogues [A10L]PnIA and [N11S]PnIA were synthesized in the laboratory of Dr. P.F. Alewood at the Centre for Drug Design and Development, University of Queensland.

2.2. Synthesis of α-conotoxins GI, PnIA and PnIB and peptide analogues

The α-conotoxins GI, PnIA and PnIB and the peptide analogues were synthesized by Boc chemistry (Schnolzer et al., 1992) and purified using the protocol previously described for α-conotoxin PnIA (Hu et al., 1996). The purity of the peptides was checked by analytical reversed-phase high-pressure liquid chromatography (HPLC) and mass spectrometry.

2.3. Neuronal-type nicotinic receptor assay in bovine adrenal chromaffin cells

Chromaffin cells were isolated from bovine adrenal medullary tissue and cultured as previously described (Livett et al., 1987b). Nicotinic receptor activity was measured by stimulating the cells with nicotine (4 μM) and measuring the release of catecholamines by HPLC and electrochemical detection (Livett et al., 1987a). Experiments were conducted on single 24-well Sarstedt culture plates containing 0.4 ml medium per well (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2.5 μg/ml fluorodeoxyuridine, 2.5 μg/ml cytosine arabinoside, 2.5 μg/ml uridine, 5 μg/ml nystatin and 100 μg/ml each of penicillin and streptomycin) at a cell density of 1.25×10^6 cells/ml (5×10^5 cells/well).

Following a 3-day incubation at 37°C in an atmosphere of 5% carbon dioxide in air, the cells were allowed to equilibrate to room temperature over a period of 5 min and were then subjected to two consecutive 5-min washes with Locke's buffer of the following composition (mM): NaCl 154; KCl 2.6; K_2HPO_4 2.15; KH_2PO_4 0.85; $MgSO_4$ 1.18; $CaCl_2$ 2.2; glucose 10; 0.5% bovine serum albumin, pH 7.4. All subsequent reactions were carried out at room temperature (18–22°C). The cells were preincubated with 0.01–10 μM peptide for 5 min, and then incubated for 5 min with 4 μM nicotine in the presence of 0.01–10 μM peptide. Cells treated with buffer, 4 μM nicotine or peptide alone served as controls. To determine whether the peptides had any effect on voltage dependent catecholamine release, KCl (56 mM) was used to depolarize the cells.

The incubation medium containing the released catecholamines was acidified to 0.4 M with perchloric acid. To determine the residual catecholamine content of the cells, each well was treated for 5 min with 0.5 ml 0.01 M perchloric acid, followed by a further 0.5 ml 0.8 M perchloric acid, and centrifuged to precipitate the proteins. The supernatants were processed for catecholamines. For both the evoked release and cell content samples, the catecholamines were fractionated using a Clinical Reversed-Phase column (Bio-Rad) and mobile phase (70 mM KH_2PO_4, 0.1 mM EDTA, 0.2% sodium heptanesulfonate, 10% methanol). Quantitation was by electrochemical oxidation using a BAS LC-3A detector, at 650 mV (Livett et al., 1987a). Results were calculated as catecholamines released as a percentage of total cell content and expressed as percentage of control. Results were statistically evaluated using the two-tailed Students' *t*-test.

2.4. Rat phrenic nerve / hemi-diaphragm preparation

Male Buffalo rats (150–250 g, 3–4 months old) were killed by carbon dioxide asphyxiation, and exsanguinated. The left hemi-diaphragm and phrenic nerve were isolated as described by Bülbring (1946) and mounted under 1 g resting tension in a 25-ml organ bath, maintained at 37°C, containing a Krebs–Henseleit solution of the following composition (mM): NaCl 118; KCl 5; $CaCl_2$ 2.5; $NaHCO_3$ 25; $MgSO_4$ 1; KH_2PO_4 1.2; glucose 11, pH 7.4 aerated with Carbogen (95% O_2, 5% CO_2). The nerve was stimulated via a bipolar electrode with pulses of 0.5 ms duration and supramaximal voltage using a *Grass* S 48 stimulator with an SIU5 isolation unit. Single twitches were produced by continuous stimulation at 0.1 Hz, interspersed at 5 min intervals by train-of-four twitches (2 Hz for 1.9 s) and tetanic contractions (50 Hz for 2 s), measured alternately. Responses were detected using a *Grass* (FT 03) isometric force-displacement transducer connected to a Neotrace DC amplifier, and recorded via a MacLab/4s system using the application Chart (v3.4/s) on a Macintosh LC630 computer.

The tissue was incubated with toxin (1–10 μM) in Krebs–Henseleit solution for 60 min during which twitches, train-of-four twitches and tetani were recorded. The tissue was then washed with buffer alone, and twitches, train-of-four twitches and tetani recorded for 30 min prior to treatment with the next higher toxin concentration.

Twitches and tetanic amplitude were expressed as a percentage of their respective control values. Train-of-four twitches were expressed as the T4/T1 ratio. Tetanic fade was expressed as a percentage of the maximum tetanic force. In control preparations neither tetanic nor train-of-four twitch fade were measurable (Blount et al., 1992).

The protocol complies with the NH and MRC guidelines for the use of animals and animal tissues, and approval for these experiments was obtained from the Animal Experimentation Ethics Committee of the University of Melbourne.

3. Results

3.1. Neuronal-type nicotinic acetylcholine receptor responses

3.1.1. α-Conotoxin PnIA

Synthetic α-conotoxin PnIA produced a relatively weak concentration-dependent inhibition of nicotine-evoked catecholamine release from chromaffin cells (Fig. 1). At the

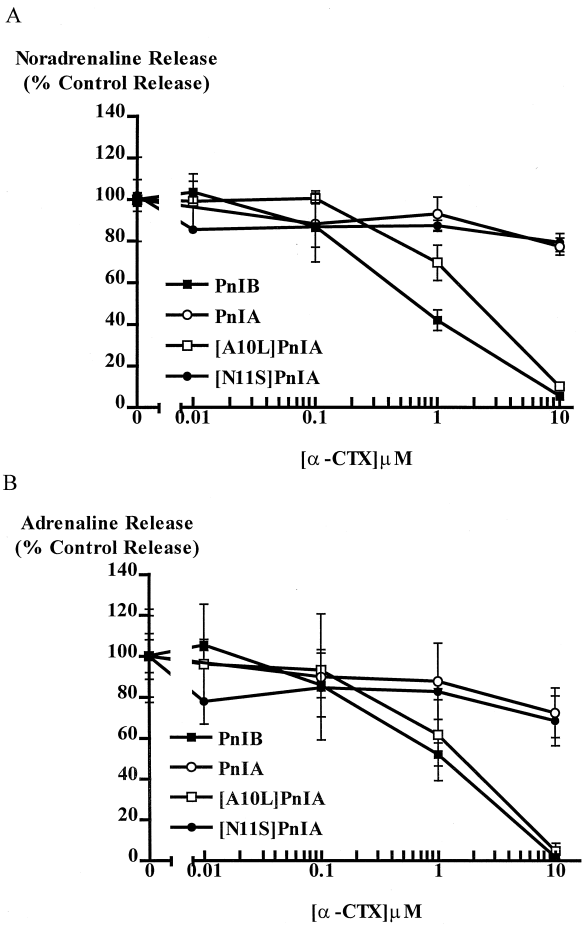

Fig. 1. Effect of α-conotoxin PnIA, PnIB, [A10L]PnIA and [N11S]PnIA on nicotine evoked catecholamine release from bovine adrenal chromaffin cells. Inhibition of nicotine (4 μM) evoked release of noradrenaline (A) and adrenaline (B) from bovine chromaffin cells by α-conotoxin PnIA (○), PnIB (■), [A10L]PnIA (□) and [N11S]PnIA (●). Cells were preincubated with α-conotoxin (0.01–10 μM) for 5 min and then incubated with nicotine (4 μM) together with α-conotoxin (0.01–10 μM) for 5 min. Results are expressed as percent of control (maximum release, nicotine alone). Data represent mean ± S.D., ($n = 3$–4). Figure is based on data from one representative experiment. Three independent experiments produced qualitatively similar results. Statistical probability was calculated using Students' t–test.

highest concentration tested (10 μM), PnIA inhibited the noradrenaline release by 20% and adrenaline release by 30%. In contrast, when KCl (56 mM) was used to depolarize the cells, α-conotoxin PnIA at concentrations up to 10 μM did not inhibit the K^+-evoked catecholamine release (Fig. 2).

3.1.2. α-Conotoxin PnIB

Synthetic α-conotoxin PnIB almost completely inhibited nicotine-evoked catecholamine release from the chromaffin cells with an IC_{50} value of 0.7 μM for noradrenaline release (Fig. 1A) and 1 μM for adrenaline release (Fig. 1B). Inhibition of adrenaline release was complete (and of noradrenaline almost complete) with 10 μM α-conotoxin PnIB (Fig. 1). In contrast, K^+-evoked release of catecholamines was not inhibited by α-conotoxin PnIB at concentrations up to 10 μM (Fig. 2).

3.1.3. [A10L]PnIA

The peptide analogue [A10L]PnIA almost completely inhibited the nicotine-evoked release of catecholamines from chromaffin cells, with an IC_{50} value of 2 μM for noradrenaline release (Fig. 1A) and approximately 1.5 μM for adrenaline release (Fig. 1B). Inhibition of the catecholamines was nearly complete at 10 μM [A10L]PnIA. In contrast, release of catecholamines evoked by 56 mM K^+ was not inhibited by [A10L]PnIA at concentrations up to 10 μM (Fig. 2).

3.1.4. [N11S]PnIA

The peptide analogue [N11S]PnIA, like α-conotoxin PnIA, was essentially inactive. At a peptide concentration

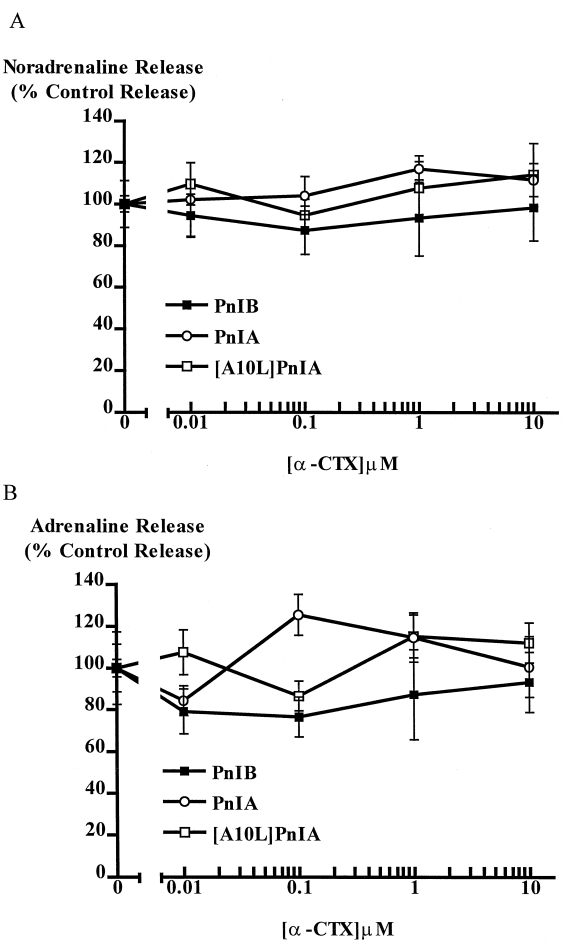

Fig. 2. Effect of α-conotoxin PnIA, PnIB and [A10L]PnIA on K^+-evoked catecholamine release from bovine adrenal chromaffin cells. Inhibition of K^+ (56 mM) evoked release of noradrenaline (A) and adrenaline (B) from bovine chromaffin cells by α-conotoxin PnIA (○), PnIB (■) and [A10L]PnIA (□). Cells were incubated with α-conotoxin (0.01–10 μM) together with 56 mM KCl for 5 min. Results are expressed as percent of control (maximum release, K^+ alone). Data represent mean ± S.D., ($n = 3$–4). Figure is based on data from one representative experiment. Three independent experiments produced qualitatively similar results. Statistical probability was calculated using Students' t–test.

of 10 μM it caused 20% and 30% inhibition of nicotine-evoked noradrenaline and adrenaline release, respectively (Fig. 1). Due to its lack of activity against nicotine-evoked release, [N11S]PnIA was not tested on K^+-evoked catecholamine release.

3.2. Muscle-type nicotinic acetylcholine receptor responses

The conotoxins and analogues were tested on the rat phrenic nerve/hemi-diaphragm preparation. After incubation for 30 min over the concentration range 1–10 μM, α-conotoxins PnIA, PnIB and analogues did not inhibit single twitches, train-of-four twitches or tetanic contractions. Under the same conditions α-conotoxin GI (5 μM) completely blocked these tension parameters in the rat neuromuscular junction assay (Fig. 3).

4. Discussion

The discovery that some α-conotoxins are able to discriminate between neuronal- and muscle-type nicotinic receptors and between various neuronal nicotinic receptor subtypes has prompted studies on the structural basis of these selectivities. α-Conotoxins PnIA and PnIB have been reported to be selective for the neuronal-type nicotinic receptor and ''mollusc specific'' (Fainzilber et al., 1994).

We show here that α-conotoxin PnIB is a potent inhibitor of the neuronal nicotinic response of bovine adrenal chromaffin cells, a mammalian preparation, causing almost complete inhibition of catecholamine release at 10 μM. In contrast, α-conotoxin PnIA was at least an order of magnitude less potent producing only 20–30% inhibition at 10 μM.

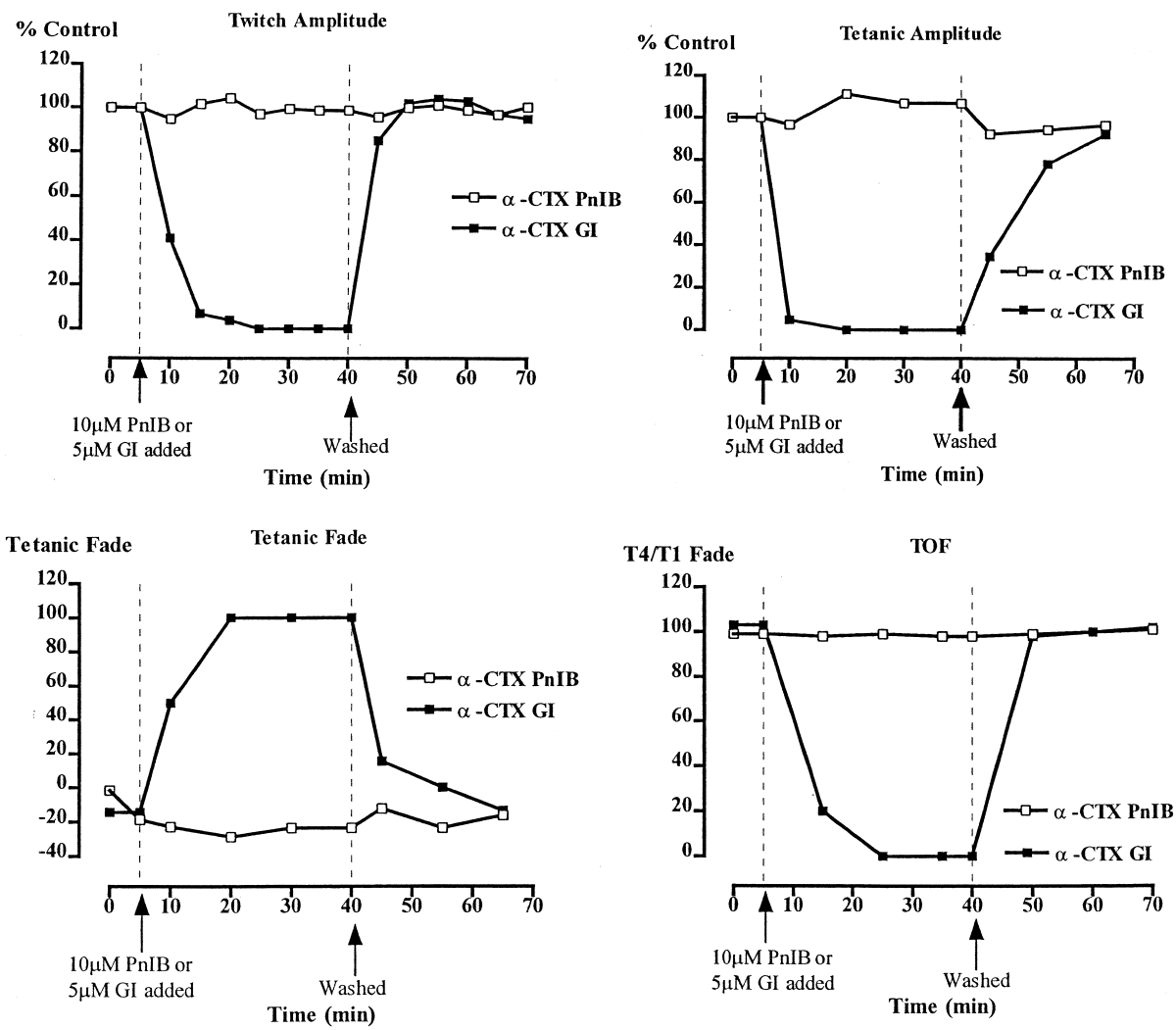

Fig. 3. Effect of α-conotoxin PnIB and α-conotoxin GI on the tension parameters of the rat phrenic nerve/hemi-diaphragm preparation. Effect of α-conotoxin PnIB (10 μM) (□) and α-conotoxin GI (5 μM) (■) on single twitch amplitude, tetanic amplitude, tetanic fade and T4/T1 ratio. Single twitch and tetanic amplitude are expressed as percent of the control values (before addition of α-conotoxin). Tetanic fade is expressed as the percentage by which the amplitude decreases during the tetanus and T4/T1 ratio is expressed as the percentage diminution of the fourth contraction relative to the first in a train of four contractions.

The structural basis for this difference in potency at the neuronal nicotinic receptor of bovine chromaffin cells was investigated by constructing two analogue peptides — [A10L]PnIA and [N11S]PnIA — to observe the extent to which each of the residues 10 and 11 contribute to activity in these otherwise identical conotoxins. We found that [A10L]PnIA was similar in activity to α-conotoxin PnIB at inhibiting neuronal-type nicotinic receptor responses in bovine chromaffin cells, whereas [N11S]PnIA was relatively inactive in this preparation. Following completion of this investigation, similar observations were reported for neuronal nicotinic receptors in two experimental preparations, rat sympathetic neurons (Hogg et al. 1999) and rat neuronal nicotinic receptors expressed in frog oocytes (Luo et al. 1999).

A main structural feature of α-conotoxin PnIB is an alpha-helical region (two turns of helix) formed by residues 5–12 (Hu et al., 1997). This is the region occupied by the two variant amino acids at positions 10 and 11. Hydrogen bonds are formed intramolecularly between the Ser 11 side chain and Pro 7 and Cys 8 main chains. When this Ser is replaced by Asn as in [N11S]PnIA, hydrogen bonding would be affected and could contribute to the decreased potency of [N11S]PnIA. Comparison of the three-dimensional structure of α-conotoxin PnIB with other conotoxins specific for the neuronal nicotinic receptors revealed similarities in backbone conformations and solvent-accessible surface areas. Whereas the rigid hydrophobic core scaffold was conserved, the residues in solvent-exposed positions varied greatly (Rogers et al., 1999).

Our results with the peptide analogues demonstrate that the residue essential for the greater activity of PnIB over that of PnIA is the Leu at position 10. When Ala^{10} in α-conotoxin PnIA was replaced by Leu the resulting peptide [A10L]PnIA exhibited activity similar to that of α-conotoxin PnIB, causing almost complete inhibition of catecholamine release. In contrast replacement of Asn^{11} of α-conotoxin PnIA by Ser resulted in a peptide with PnIA-like activity.

Neither conotoxin PnIA nor PnIB (or their analogues [N11S]PnIA and [A10L]PnIA) have any net charge and each shares the same backbone structural fold at positions 10 and 11 (Hu et al., 1996; 1997). The difference in potency could be due to the sidechain substituents at residues 10 and 11. For all four compounds in this study, the hydrophobicities (π, Fauchère et al., 1988) of residues 10 and 11 were summed.

Higher values of π_{10+11} were associated with lower IC_{50} values, a trend which persisted (save for α-conotoxin EpI) for other 4:6 and 4:7 loop structure α-conotoxins investigated in the same assay under identical conditions (Table 3). α-Conotoxin EpI alone of these compounds has loop 1 homology (S–D–P–R) with α-conotoxin ImI, in which the triad D–P–R has been shown to be essential for potency at α7/5-HT-3 chimeric neuronal-type nicotinic receptors (Quiram and Sine, 1998). It is probable that α-conotoxin EpI owes its enhanced activity in our assay to a greater involvement of the N-terminal loop at the receptor than is the case with the other six peptides, in which the critical loop 1 triad D–P–R is absent.

These results suggest that the enhanced potency of α-conotoxin PnIB over α-conotoxin PnIA for inhibition of the neuronal nicotinic response of bovine chromaffin cells is related to the increase in hydrophobicity at residues 10 and 11, the major part of which is due to the very hydrophobic leucine residue at position 10 in PnIB.

Our finding that α-conotoxin PnIB and (to a lesser extent) α-conotoxin PnIA inhibit the nicotinic response in bovine adrenal chromaffin cells challenges their previously proposed ''mollusc specific'' status (Fainzilber et al., 1994) and indicates that their neuronal activity extends beyond molluscs. In contrast to α-conotoxin GI from the piscivorous cone snail *C. geographus*, the α-conotoxins PnIA and PnIB from the molluscivorous *C. pennaceus* were inactive at the rat neuromuscular junction, consistent with the earlier reports of their selectivity for neuronal- over muscle-type nicotinic receptors. Fainzilber et al. (1994) reported that α-conotoxins PnIA and PnIB blocked 90% of the acetylcholine-induced depolarization in cultured *Aplysia* neurons but had no effect when injected intracerebrally into the rat brain, and were inactive in insect and fish bioassays.

The observation that nicotinic receptor activity by α-conotoxins PnIA and PnIB from *C. pennaceus* is not restricted to molluscs but extends to mammalian neuronal nicotinic receptors is supported by studies in frog oocytes

Table 3
Potency as antagonists of the nAChR in bovine chromaffin cells of 4:6 and 4:7 loop structure α-conotoxins and analogues, compared with their local hydrophobicity at residues 10 and 11, $\pi_{(10+11)}$. All IC_{50} values are for inhibition of noradrenaline release under identical conditions, with nicotine (4 μM) as agonist

α-Conotoxin or analogue peptide	$\pi_{(10+11)}$ (Fauchère et al., 1988)	IC_{50}	Reference
α-Conotoxin PnIA	−0.29	> 20	present study
[N11S]PnIA	0.27	> 20	present study
α-Conotoxin AuIB	0.57	20	unpublished data
α-Conotoxin EpI	0.63	0.2	Loughnan et al., 1998
α-Conotoxin MII	1.06	3	Broxton et al., 1997
[A10]PnIA	1.10	2	present study
α-Conotoxin PnIB	1.66	0.7	present study

expressing rat neuronal nicotinic receptors (Luo et al., 1999) and in endogenous nicotinic receptors in rat parasympathetic neurons (Hogg et al., 1999) where the peptides produced inhibition of whole-cell acetylcholine-evoked currents. The *C. pennaceus* α-conotoxins thus appear to show specificity for ''ganglionic type'' receptors present in both rat parasympathetic neurons and bovine chromaffin cells. The lack of an observable effect following injection into the rat brain, as compared to the potent inhibition of the neuronal type nicotinic response in chromaffin cells could be due to any of a number of possibilities. The subtype of nicotinic receptor being inhibited in chromaffin cells (probably $\alpha 3\beta 4$) may be a subtype that is not, or is only weakly represented in the rat CNS, or that may have only a limited functional role in the rat brain. Alternatively, the contrasting activities may be due to species differences between the rat and bovine β4 neuronal nicotinic receptor subunits (Campos-Caro et al., 1997).

Campos-Caro et al. (1997) reported that the predominant subunit composition in bovine chromaffin cells was $\alpha 3\beta 4$, hence the observed activity of α-conotoxins [A10L]PnIA and PnIB might be expected to involve $\alpha 3\beta 4$ receptors in these cells. Through Northern blot and PCR analysis it has been showed that α7 nicotinic receptor subunits are also present in bovine adrenal chromaffin cells (Garcia-Guzman et al., 1995). Lopez et al. (1998) reported that α-bungarotoxin blocked whole cell native currents in bovine adrenal chromaffin cells generated in response to brief (1 s) pulses of acetylcholine (100 μM). These whole cell currents are thought to result from the simultaneous activation of a mixed population of α7 and $\alpha 3\beta 4$ receptors. However, with the lower concentrations of agonist and longer periods of stimulation such as those used here (4 μM nicotine, 5 min incubation) the functional response is not blocked by α-bungarotoxin (Broxton et al., 1999) and under these conditions the α7 nicotinic receptors are not thought to be involved in the secretory process.

Transcripts corresponding to the α7 subunit of the nicotinic receptor are localized exclusively to adrenaline-containing (but not to noradrenaline-containing) chromaffin cells (Criado et al., 1997), therefore [A10L]PnIA and α-conotoxin PnIB are unlikely to be acting at α7 receptors since they inhibit both noradrenaline release (Fig. 1A) and adrenaline release (Fig. 1B). We suggest that the principal site of action of [A10L]PnIA and α-conotoxin PnIB under the conditions used here is the $\alpha 3\beta 4$ nicotinic receptor.

We conclude that the α-conotoxins from *C. pennaceus* are not ''mollusc specific'' but also inhibit the mammalian neuronal nicotinic response in bovine adrenal chromaffin cells. The enhanced potency of α-conotoxin PnIB over α-conotoxin PnIA for inhibition of the neuronal nicotinic receptor response in bovine chromaffin cells is principally determined by the larger, more hydrophobic leucine residue at position 10 in α-conotoxin PnIB.

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