New Mollusc-Specific α-Conotoxins Block *Aplysia* Neuronal Acetylcholine Receptors†

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ABSTRACT: Two mollusc-specific neurotoxic peptides from the venom of the molluscivorous snail *Conus pennaceus* are described. These new toxins block acetylcholine receptors (AChR) of cultured *Aplysia* neurons. Bath application of 0.5–1 μM toxin induces 5–10-mV membrane depolarization, which recovers to the control level within 1–3 min in the presence of the toxin. This response is blocked by 1 mM hexamethonium. Concomitantly with the transient depolarization, the toxins block approximately 90% of the depolarizing responses evoked by brief iontophoretic application of acetylcholine. The pharmacology and amino acid sequences of the toxins (αPnIA, GCCSLPPCAANNPDYC-NH_2; αPnIB, GCCSLPPCALSNPDYC-NH_2) enable their classification as novel α-conotoxins. The sequences differ from those of previously described α-conotoxins in a number of features, the most striking of which is the presence of a single negatively charged residue in the C-terminal loop. This loop contains a positively charged residue in piscivorous venom α-conotoxins. In contrast to other α-conotoxins, which are selective for vertebrate skeletal muscle nicotinic ACh receptors, these *Conus pennaceus* toxins block neuronal ACh receptors in molluscs. As such they are new probes which can be used to define subtypes of ACh receptors, and they should be useful tools in the study of structure–function relationships in ACh receptors.

Conus snails are venomous marine predators which utilize a sophisticated peptidergic venom system to paralyze their prey. The vast majority of *Conus* species prey specifically upon polychaete worms; approximately 10–15% of the family envenomate specifically fish; and another 10–15%, other gastropod snails (Kohn, 1983; Kohn & Nybakken, 1975). Most previous studies on *Conus* toxins have been on the fish-eating species, in which the main paralytic components are small peptides (typically 10–30 residues) named conotoxins (Olivera et al., 1990, 1991). Conotoxins are utilized as research tools in many fields of neurobiology since they provide high-affinity antagonists for receptor and ion channel subtypes, and being small peptides, they are synthesizable and modifiable, thus increasing their availability and potential utility.

Our laboratories are interested in conotoxins as tools for the study of molluscan neuronal systems. Despite the important neurobiological research carried out on molluscan neurons, there is a lack of effective pharmacological tools for these systems. For example, the well-characterized vertebrate sodium channel blocker tetrodotoxin is less effective by 2 orders of magnitude when tested in *Aplysia* systems (Hasson et al., 1993). We have previously shown that the venoms of molluscivorous Conidae contain mollusc-specific paralytic factors (Fainzilber & Zlotkin, 1992). The main mollusc-paralyzing factors from the venom of *Conus textile neovicarius* were purified and found to be peptide conotoxins affecting sodium channels in mollusc neuronal systems (Fainzilber et al., 1991; Hasson et al., 1993). Preliminary studies on the venom of *Conus pennaceus* revealed that the venom of this molluscivorous species also contains mollusc-specific toxicity (Fainzilber & Zlotkin, 1992) and that some of the venom fractions contain acetylcholine receptor (AChR)[1] blocking activity on isolated *Aplysia* neurons (R. Oren and M. E. Spira, unpublished data). This finding was in contrast to the very weak or nonexistent effects of "classical" cholinergic blockers such as α-bungarotoxin on the responses to ACh in *Aplysia* neurons (Kehoe et al., 1976; Walker, 1986; R. Oren and M. E. Spira, unpublished results). Other studies on molluscan neurons have reported conflicting results regarding the efficacy of various cholinergic antagonists [for a review, see Walker (1986)]. A potent cholinergic blocker for molluscan neurons would provide a useful tool for neurobiologists working with these systems, and also a specific probe for AChR subtypes.

Olivera, Cruz, and colleagues have previously characterized the α-conotoxins, which are specific blockers of the nicotinic

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[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; BSA, bovine serum albumin; FMOC-Cl, 9-fluorenylmethoxycarbonyl chloride; GI, α-conotoxin-GI from *Conus geographus*; HPLC, high-pressure liquid chromatography; LSIMS, liquid matrix secondary ion mass spectrometry; PD_{50}, paralytic dose 50%; PnIA, α-conotoxin-PnIA from *Conus pennaceus*; PnIB, α-conotoxin-PnIB from *Conus pennaceus*; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; QSAR, quantitative structure–activity relationships.

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AChR in vertebrate muscle (Myers et al., 1991). A large homologous set of these small peptides are expected to be particularly useful for probing acetylcholine receptor structure and for identifying subtypes of acetylcholine receptors (Zafarella et al., 1988; Myers et al., 1991; Ramilo et al., 1992). In this report we describe the purification and characterization of two new mollusc-specific conotoxins from *Conus pennaceus*. These new toxins are clearly α-conotoxins, on the basis of sequence homologies and electrophysiological studies. These new α-conotoxins differ from the previously characterized members of this family in their phylogenic specificity, in unusual features of their amino acid sequences, and in their ability to block neuronal acetylcholine receptors in molluscs.

EXPERIMENTAL PROCEDURES

Toxic Substances. Conus pennaceus venom was obtained from adult specimens collected from the northern Red Sea. A total of 302 mg (dry weight) of crude venom was obtained from 44 venom ducts. α-Conotoxin-GI and α-bungarotoxin were purchased from Sigma.

Experimental Animals and Bioassays. Bioassays on limpet snails (*Patella caerulea*), fish (*Gambusia affinis*) and blowfly larvae (*Sarcophaga falculata*) were performed as previously described (Fainzilber & Zlotkin, 1992). Activity was quantified in paralytic dose units (PD_{50}), which were defined as the dose that paralyzes 50% of the test animals within the time period of the assay, and determined according to Reed and Muench (1938).

Column Chromatography. Fifty-milligram aliquots of lyophilized venom were extracted in 0.1 M ammonium acetate, pH 7.5, for 1 h in a rotatory shaker at 4 °C, followed by centrifugation at 3000*g* for 2 min. The pellet was subjected to two additional extractions under the same conditions. Supernatants from the three extracts were combined and separated on a Sephadex G-50 (Pharmacia) column as described in Figure 1A. HPLC purification of the active fractions was accomplished in two steps of reverse-phase chromatography on Vydac C18 columns as described in Figures 1B and 2.

Protein Determination. Protein content of venom fractions was determined by the procedure of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard. Purified peptides were quantified by amino acid analysis.

Amino Acid Analysis. Analysis of amino acid composition after acid hydrolysis and 9-fluorenylmethoxycabonyl chloride (FMOC-Cl) derivatization was performed on a Merck-Hitachi reverse-phase HPLC system, according to Betner and Foldi (1986). The system was calibrated prior to each analysis with FMOC-amino acid standards.

Peptide Sequencing. Purified toxins were reduced and alkylated with 4-vinylpyridine as previously described (Fainzilber et al., 1991), and the modified peptides were desalted on a Vydac C18 column in 0.1% trifluoroacetic acid with a linear gradient of 0–60% acetonitrile/2-propanol, 1:1. Desalted peptides were lyophilized, resuspended in phosphate-buffered saline, pH 7.4, and adsorbed onto poly(vinylidene difluoride) (PVDF) membranes (Speicher, 1989). Amino acid sequence analysis was performed by automated Edman degradation with an Applied Biosystems 475A gas-phase protein sequencing system at the Bletterman Laboratory for Macromolecular Research (Faculty of Medicine, Hebrew University). The chromatography system was calibrated prior to each analysis with phenylthiohydantoin (PTH) amino acid standards. Each sequence was confirmed in at least two separate determinations utilizing different batches of peptide.

Mass Spectrometry. Purified peptide samples for mass spectrometric analysis were dissolved in acetonitrile/water (1:1), and an aliquot was loaded onto the probe tip followed by addition of 1 μL of glycerol/thioglycerol containing 1% TFA. A Kratos MS-50S double-focusing mass spectrometer equipped with a high-field magnet and a cesium ion source (Aberth et al., 1982; Falick et al., 1986) was used for all liquid matrix secondary ion mass spectrometry (LSIMS) analyses.

Electrophysiology. Isolated metacerebral neurons from *Aplysia californica* or *Aplysia oculifera* (Gulf of Elat, Red Sea) were cultured as previously described (Spira et al., 1993; Benbassat & Spira, 1993; Hasson et al., 1993). The neurons were cultured at very low densities to prevent any possible synaptic interactions among them. Passive and active membrane properties of the cultured neurons were studied using conventional intracellular recording and stimulation techniques. Briefly, the cell body was impaled by a microelectrode filled with 2M KCl (5–10-MΩ resistance). This electrode was used for both current injection and voltage recording. Analysis of the resting potential, input resistance, and action potential amplitude and shape was carried out in artificial sea water (ASW) composed of 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES, at pH 7.6. Iontophoretic applications of acetylcholine were done with fire-polished microelectrodes (3–8-MΩ resistance) containing 1 M acetylcholine chloride. The duration of the iontophoretic currents was 1 ms, and the current intensity ranged between 0.1 and 0.15 μA. Venom fractions and purified toxins for electrophysiological experiments were dissolved in ASW and applied at the designated concentrations together with 1–2 mg/mL final concentration of BSA.

RESULTS

Purification and Chemical Characterization of α-Conotoxins PnIA and PnIB. Two major peaks were obtained in the initial fractionation of *Conus pennaceus* venom on Sephadex G50 (Figure 1A). Mollusc-paralyzing activity was found in the low molecular weight peptides peak which elutes close to the total volume (V_t) of the column. This peak was subsequently refractionated by reverse-phase HPLC on a Vydac semipreparative C18 column as shown in Figure 1B. The major mollusc-paralyzing fractions are indicated by the arrows marked PnI. Additional activities were found in the peaks marked PnIII and PnVII, which will be detailed elsewhere. Amino acid composition and spectral analyses of the four PnI peaks revealed that they were of very similar composition (data not shown). In order to obtain an impression of the degree of variability in these four fractions, the earliest and latest eluting were chosen for final purification and characterization. PnIA and PnIB were purified from these two fractions in a final chromatographic step on an analytical Vydac C18 column (Figure 2). UV spectra sampled on-line with a diode array detector in the final HPLC runs revealed two very similar spectra in each peak (Figure 2, insets). Since isocratic repurifications of these peaks revealed the same spectra, their structure was examined by Edman sequencing, amino acid analyses, and mass spectrometric analyses.

The amino acid sequences of the toxins were determined by gas-phase automated Edman sequencing after reduction and pyridylethylation. Single unambiguous sequences of 16 amino acid residues were obtained for both toxins (Table 1). The sequences obtained by automated Edman degradation matched amino acid composition analyses of the two peptides (Table 2). Further confirmation of the sequence of PnIA was obtained by LSIMS. The single clean MH^+ measurement

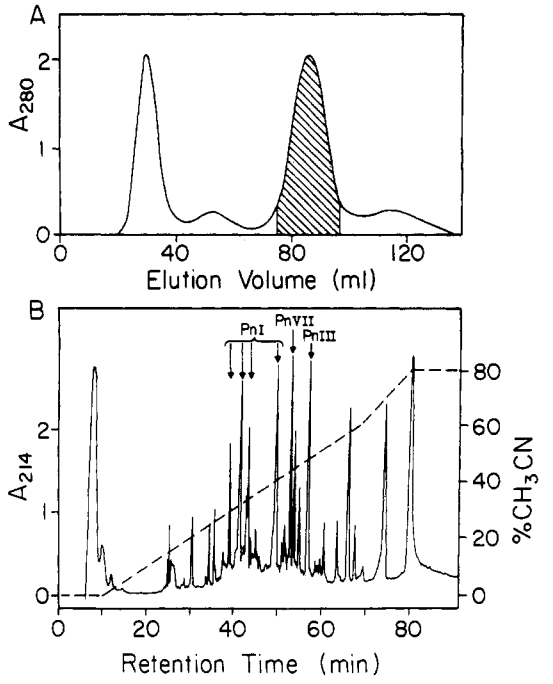

FIGURE 1: Panel A: Initial fractionation of *Conus pennaceus* venom. Fifty-milligram aliquots of lyophilized venom were extracted as described under Experimental Procedures and separated on a Sephadex G-50 column (63 × 1.42 cm), equilibrated and eluted in 0.1 M, ammonium acetate pH 7.5, at a flow rate of 5 mL/h and a temperature of 4 °C (A). The marked fraction was further fractionated by reverse-phase HPLC on a Vydac C18 semipreparative column (25 × 1 cm, 10-μm particle size) as shown in panel B. Four-milligram peptide fractions were loaded in each run and eluted at a flow rate of 2 mL/min with a gradient of acetonitrile in 0.1% TFA as shown by the dashed line.

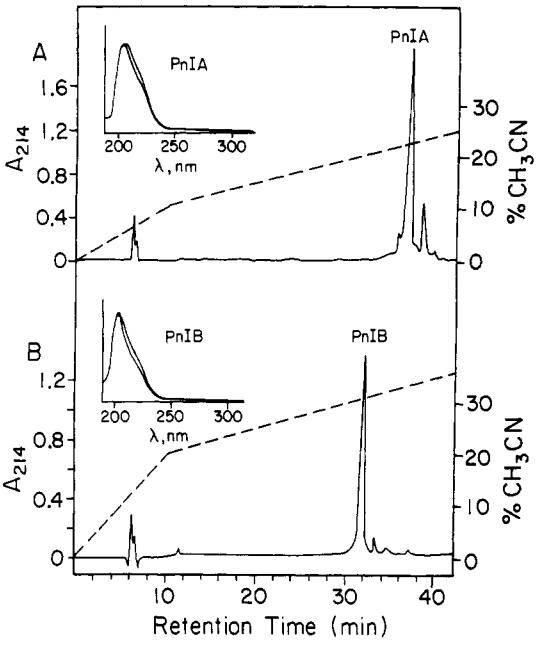

FIGURE 2: Purification of α-conotoxins PnIA and PnIB. The first PnI fraction eluting from the semipreparative C18 step (see Figure 1B) was purified on an analytical Vydac C18 column (25 × 0.46 cm, 5-μm particle size), eluted at a flow rate of 0.5 mL/min with a gradient of acetonitrile in 0.1% TFA as shown by the dashed line in panel A. The fourth PnI semipreparative C18 fraction was also purified on the analytical column, as shown in panel B. The insets in panels A and B show the UV spectra sampled by a diode array detector at different time points of the elution of the PnIA and PnIB peaks. Both toxins reveal two similar UV spectra that alternate in different regions of the peak and that may represent shifts between two conformational states.

for PnIA was 1622.8, which is consistent with a peptide of the given sequence, with all four cysteines in disulfide bridges, and an amidated C-terminus. Mass measurements of PnIB revealed the presence of a mass corresponding to the amidated sequence (MH^+ = 1637.8), as well as an 80 Da higher molecular mass signal which may correspond to a sulfated or phosphorylated derivative. This aspect is currently under further study. Although the slight variability in UV spectra in PnIB could be due to heterogeneity, the similar UV observations on the homogenous PnIA (Figure 2, insets) may suggest an oscillation of these peptides between two different conformations, as was previously shown for α-conotoxin-MI (Gray et al., 1983). As can be seen from Figure 4, the cysteine framework of PnIA and PnIB is homologous to that of the α-conotoxins, leading us to suspect that these new toxins might also belong to the same pharmacological category.

Effects of PnIA and PnIB on Isolated Cultured Aplysia Neurons. The site of action of the toxins was determined in two steps. Bath application of PnIA or -B to a final concentration of 0.5–1 μM induced a 5–10-mV membrane potential depolarization, which recovered to control levels within 1–3 min in the presence of the toxin (Figure 3A). Examination of input resistance and action potential amplitude and shape following the recovery of the toxin-induced membrane depolarization revealed that the toxins had no effects on the passive and active membrane properties of the neuron even at higher concentrations (12 μM). Bath application of the toxins (0.5–1 μM) blocked 90% of the transient depolarization induced by brief (1 ms) iontophoretic application of ACh. The blocking action was detected within a few seconds following toxin application (Figure 3A,C), throughout the recovery phase of the toxin-induced depolarization, and

Table 1: Sequence Analyses of α-Conotoxins from *C. pennaceus*

	α-conotoxin-PnIA		α-conotoxin-PnIB	
cycle	assigned residue	yield (nmol)	assigned residue	yield (nmol)
1	Gly	1.00	Gly	1.80
2	Cys[a]	0.80	Cys	1.28
3	Cys	0.78	Cys	1.33
4	Ser	0.45	Ser	1.60
5	Leu	0.63	Leu	1.20
6	Pro	0.56	Pro	1.20
7	Pro	0.50	Pro	1.40
8	Cys	0.32	Cys	1.1
9	Ala	0.63	Ala	1.15
10	Ala	0.65	Leu	0.96
11	Asn	0.46	Ser	0.97
12	Asn	0.44	Asn	0.65
13	Pro	0.30	Pro	0.58
14	Asp	0.35	Asp	0.50
15	Tyr	0.30	Tyr	0.48
16	Cys	0.16	Cys	0.24

Sequences[b]

PnIA: GCCSLPPCAANNPDYC-NH_2[c]

PnIB: GCCSLPPCALSNPDYC-NH_2

[a] Cys residues were determined in the pyridylethylated form. [b] Standard one-letter code used for summarized sequences. [c] C-terminal amidation indicated by mass-spectral analyses.

lasted for as long as the toxin was present in the bathing medium. In the experiment of Figure 3A brief iontophoretic applications of ACh induced transient depolarizations (trace 1 in Figure 3A,B). Toxin application (arrow in Figure 3A) induced membrane depolarization, which gradually recovered to the control level, while concomitantly the responses to repeated iontophoretic applications of ACh were almost completely blocked (traces 2 and 3 in Figure 3A,B). Following a wash with ASW, the responses to ACh application recovered

Table 2: Amino Acid Analyses of α-Conotoxins from *C. pennaceus*

amino acid	mole ratio[a] αPnIA	αPnIB
Asp	2.9 (3)	2.1 (2)
Ser	0.9 (1)	1.7 (2)
Cys	3.7 (4)	3.6 (4)
Gly	1.1 (1)	0.9 (1)
Ala	1.8 (2)	1.1 (1)
Tyr	0.9 (1)	1.0 (1)
Pro	3.2 (3)	2.9 (3)
Leu	1.1 (1)	2.2 (2)

[a] Values in parentheses indicate the number of residues predicted from sequence analyses.

to control levels within 4–6 min (Figure 3C,D). Using identical experimental protocols, we found that the piscivorous venom conotoxin αGI (at a concentration of 2 μM) induced only 15% blockage of the response to ACh application, and the effect was rapidly washed.

Once it was clear that PnIA and -B act as AChR antagonists, we examined whether the transient depolarization observed immediately following toxin application might be due to transient activation of an AChR subtype by the toxin itself. In a series of experiments we found that preincubation of the neuron in 1 mM hexamethonium (a concentration that completely blocks the depolarization induced by iontophoretic application of ACh) also blocks the transient depolarization response induced by the toxin (data not shown). This result suggests that conotoxins PnIA and -B transiently activate a subtype of the AChR.

The acetylcholine receptor block and the sequence homologies detailed above enable us to classify PnIA and PnIB as α-conotoxins. The micromolar range potency of these toxins as blockers of neuronal AChR in *Aplysia* contrasts with the very low efficacy of classical cholinergic antagonists on these receptors. For example, d-tubocurarine blocks the response to ACh application in *Aplysia* metacerebral neurons only at 1 mM concentration, and α-bungarotoxin has no effect at concentrations of up to 10 μM (R. Oren and M. E. Spira, unpublished data).

Toxicity and Phylogenic Specificity of α-Conotoxins. The toxicity of αPnIA and αPnIB was examined in bioassays in molluscs (*Patella* snails), insects (*Sarcophaga* fly larvae), and fish (*Gambusia*) and by intracerebral injection into rats. We first quantified the paralytic activity of the toxins in molluscs by determining the PD_{50} for paralysis 5 min postinjection. We then examined the effects of doses up to 100 times higher (normalized by weight) in the other bioassay animals. In parallel we examined the effects of synthetic αGI in the same manner, after first determining its PD_{50} on fish. As shown in Table 3, both *Conus pennaceus* toxins were strictly selective for molluscs in these bioassays, in contrast to αGI, which was toxic to fish. Intracerebral injections of αPnIB in rats did not reveal any toxic or behavioral effects even at very high doses up to 40 nmol/rat. It is noteworthy that there is a good correlation between the PD_{50}'s of PnIA and -B (10–20 pmol/100 mg of snail body mass, corresponding to approximately 200–400 nM in the snail's hemolymph) and the concentrations effective in electrophysiological experiments on *Aplysia* neurons (see above).

DISCUSSION

Chemistry of α-Conotoxins. The two *Conus pennaeus* toxins described in this study are typical for the α-conotoxin family as regards their cysteine framework and overall hydrophobicity (Figure 4). They differ from all previously described α-conotoxins in (1) the lengths of their inter-cysteine loops (4- and 7-residue loops, as compared to 3 and 4 residues in other α-conotoxins; Figure 4); (2) the presence of additional proline residues in both inter-cysteine loops; and (3) their content of only one negatively charged residue, Asp-14, as compared to the positive charges in other α-conotoxins (Figure 4). The increased size of the inter-Cys loops and the presence of additional proline residues are expected to cause differences in the conformations of PnIA and PnIB versus previously described α-conotoxins. The positioning of the negative charge in the C-terminal loop of these peptides may also be of functional significance, since this loop contains a positively charged residue in all the typical piscivorous α-conotoxins. A number of studies on the conformation and structure–function characteristics of α-conotoxins have emphasized the importance of the positioning of the C-terminal loop positive charge for the toxins' activity (Pardi et al., 1989; Kobayashi et al., 1989; Zafaralla et al., 1988). In this context it is interesting to note that in αSI a relative lack of activity on mammals could be correlated with the appearance of a Pro residue in the C-terminal loop and a shift in the position of the positive charge in this loop (Zafaralla et al., 1988). Furthermore, the related toxin αSIA has a toxicity to mice which is intermediate between those of SI and the relatively potent αGI and αMI. SIA lacks the extra Pro found in SI, and its C-terminal loop positive charge is in the same position as those of GI and MI (Myers et al., 1991); however, in the same loop it contains also a negatively charged Asp residue, homologous to the mollusc-specific PnIA and PnIB (see Figure 4). It should be noted, however, that the recently described atypical three-loop α-conotoxin-SII does not contain any charged residues in its sequence, and although it is toxic to fish, it lacks paralytic activity in mice (Ramilo et al., 1992). The conformations of αSII and αPnIA and -B will most likely prove to be somewhat different from that already determined for αGI (Pardi et al., 1989; Kobayashi et al., 1989). This aspect should be considered in future analyses of structure–function relations in these peptides.

Electrophysiology of Neuronal AChR in Molluscs. PnIA and PnIB clearly act as efficient blockers of AChR in cultured *Aplysia* neurons. This was demonstrated by their ability to block 90% of the acetylcholine-induced depolarization at concentrations of 0.5–1 μM (Figure 3), 3 orders of magnitude lower than the concentrations required for vertebrate AChR antagonists in this system. Bath application of PnIA and -B to cultured metacerebral neurons initially induced membrane depolarization of 5–10 mV, which recovered to control level within several minutes (Figure 3A); concomitantly, the responses to iontophoretic application of ACh were blocked. Both actions are observed at PnIA and -B concentrations of 0.5–1 μM. As the depolarizing effect of PnIA and -B is blocked by hexamethonium, it is reasonable to assume that the toxins act as blockers of one AChR subtype and activate another receptor subtype which rapidly inactivates in the presence of the toxin. Because of the limited amounts of the toxins, we have not yet analyzed the precise mechanisms underlying the nature of the transient toxin-induced depolarization. Comparative experiments with 2 μM αGI revealed only a slight (15%) and rapidly washed block, although this concentration is 20-fold higher than the effective concentration required for blockade of pure muscle subtype nicotinic AChR expressed in *Xenopus* oocytes (Luetje et al., 1990).

It is of interest to compare these data with previous studies on the pharmacology of molluscan AChR. Studies to

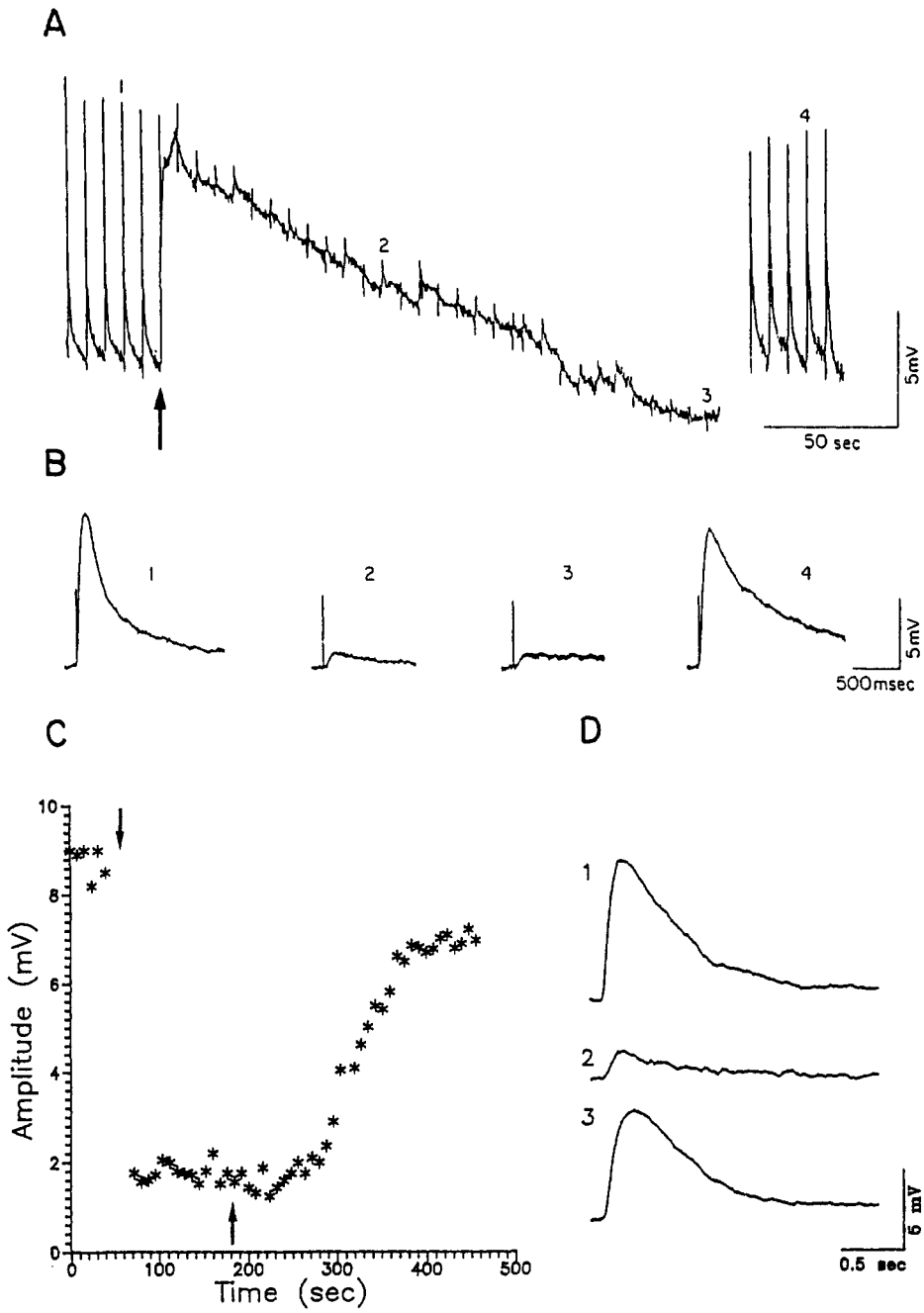

FIGURE 3: PnIA induces transient membrane depolarization and concomitantly blocks AChR. Repeated ACh application by brief (1 ms) iontophoretic current pulses induces transient depolarizations (A1 and the corresponding record B1). Bath application of PnIA (arrow in A) induces membrane depolarization which recovers to the control level in the presence of the toxin (A). Concomitantly the amplitudes of the depolarizations induced by ACh iontophoresis are markedly reduced (A2 and A3 and the corresponding records B2 and B3). Following a wash with ASW, the responses to ACh applications recover (A4 and the corresponding record B4). (C) Kinetics of the antagonistic action of PnIA to ACh applications (downward arrow, bath application of the toxin; upward arrow, ASW wash). These data are from an experiment different from that shown in panels A and B. (D) Sample recordings from the experiment shown in panel C: D1, before toxin application; D2, after bath application of PnIA; D3, recovery upon wash with ASW.

Table 3: Phylogenic Specificity in α-Conotoxins

	PD_{50} (pmol/100 mg of body mass)		
toxin	mollusc (*Patella*)	fish (*Gambusia*)	insect (*Sarcophaga*)
PnIA	14.6	>1540[a]	>1600
PnIB	18.1	>1985	>2000
GI	>1460	29.2	>2000

[a] > designates no toxic effects at indicated dose.

characterize the pharmacological nature of *Aplysia* AChR indicated that potent vertebrate AChR antagonists are less efficient in the *Aplysia* nervous system (Kehoe, 1972; Kehoe et al., 1976). The effects of different snake venoms on excitatory and inhibitory responses to acetylcholine in *Aplysia* neurons were studied by Parmentier and Carpenter (1976). Most of the venoms tested blocked some of the responses to acetylcholine, and the venom of *Bungarus multicinctus* seemed to be the most potent. However, even the effects of *B. multicinctus* venom were readily reversible upon washing (Parmentier & Carpenter, 1976). Although an earlier report had suggested that α-bungarotoxin blocked all three types of ACh responses in *Aplysia* (Shain et al., 1974), Kehoe et al. (1976) observed a reversible block only of the Cl^--mediated response. In our hands pure α-bungarotoxin at concentrations up to 10^{-5} M had no effect on the acetylcholine-induced depolarization in cultured *Aplysia* metacerebral neurons. It

Toxin	Sequence	Charge	π
PnIA	G C C S L P P C A *A* *N* N P D Y C -NH_2	0	3.43
PnIB	G C C S L P P C A *L* *S* N P D Y C -NH_2	0	5.38
GI	E C C N • P A C G • R H Y • S C -NH_2	+1.5	-0.17
MI	G R C C H • P A C G • K N Y • S C -NH_2	+3.5	-0.52
SI	I C C H • P A C G P K Y S • • C -NH_2	+2	3.61
SIA	Y C C H • P A C G • K N F D • C -NH_2	+1.5	1.55

FIGURE 4: Sequences of α-conotoxins PnIA and PnIB compared to those of other α-conotoxins with the same cysteine framework. Sequences are shown with spacers (•) inserted for maximum homology. The piscivorous *Conus* α-conotoxin sequences are taken from Ramilo et al. (1992). Boxed residues show homology; residues in *italics* are those which differ between PnIA and PnIB. π is the net hydrophobicity, calculated according to Fauchere et al. (1988).

is possible that the effects reported by Shain et al. (1974) resulted from a contamination of their α-bungarotoxin by other *Bungarus* toxins. In this context it is noteworthy that Chiappinelli, Grant, and colleagues have recently characterized the κ-neurotoxins, a new family of snake neurotoxins that specifically block neuronal AChR subtypes (Grant et al., 1988; Chiappinelli et al., 1990). The venom of *B. multicinctus* contains a potent representative of this group, κ-bungarotoxin. The effects of κ-bungarotoxin on cholinergic responses in *Aplysia* neurons should be examined in the future. Indeed, since both κ-neurotoxins and PnIA and -B block neuronal AChR, albeit in different phyla, it will be most interesting to find out whether there is any overlap in the subtypes or phylogenic range of AChR blocked by these different toxins.

Our data suggest that PnIA and -B are the most effective AChR antagonists described so far for *Aplysia* neurons. Nevertheless, it should be emphasized that diffusion of AChR antagonists into target areas in *in vivo* preparations is impeded by the compact organization of the ganglion. Therefore direct comparisons between our *in vitro* data on PnIA and -B action and that of other inhibitors tested *in vivo* cannot be done fully.

Subtypes of AChR—Pharmacology and Phylogenic Specificity of AChR-Blocking Toxins. Vertebrate AChRs have been categorized in the past into α-bungarotoxin-sensitive receptors found predominantly in muscle and κ-bungarotoxin-sensitive receptors found mainly in neurons (Luetje et al., 1990; Chiappinelli, 1993). Various AChR subtypes do not fit these categories, such as most insect neuronal AChRs, which are blocked by α-bungarotoxin (Sattelle, 1985), and at least one avian brain nicotinic receptor that is insensitive to both α- and κ-bungarotoxins (Sorenson & Chiappinelli, 1990). The variability of AChR subtypes is increased by the complex subunit compositions of neuronal AChRs, thus increasing the need for more selective AChR antagonists as probes for the different subtypes (Luetje et al., 1990; Chiappinelli, 1993). Furthermore, the degree of conservation or divergence of AChR subtypes over broad phylogenies is not clear at the present time. All of the α-conotoxins characterized up to now block specifically the α-bungarotoxin-sensitive receptors on vertebrate muscle and compete with α-bungarotoxin on binding (Myers et al., 1991; Ramilo et al., 1992). PnIA and PnIB are therefore unique among the α-conotoxins in their targeting of a neuronal receptor that is insensitive to α-bungarotoxin; i.e., PnIA and PnIB might be defined pharmacologically as κ-neurotoxins. However, the complete lack of toxicity of these *Conus pennaceus* toxins in rat brain and fish bioassays (Table 3; see Results) seems to rule out the possibility that they block the same AChR subtype as the κ-neurotoxins. PnIA and -B may define a new pharmacological subtype of AChR or pinpoint functionally important phylogenic variabilities within one pharmacological subtype. Phylogenic specificity in venom neurotoxins has been described for a number of toxin categories, most notably the insect-versus vertebrate-specific scorpion toxins affecting sodium channels (Zlotkin, 1987). The molecular basis for this selectivity is thought to be the specific recognition of subtle differences in target receptors between different phyla. Phylogenic specificity in α-conotoxins may enable high-resolution analyses of AChR structure, due to the small size of these peptide toxins (Myers et al., 1991), and the subtle structural differences discriminated by phylogenically specific toxins. The main significance of the new toxins described in the present work should therefore be expressed in their use as probes for new subtypes of AChR and to extend the possibilities for analysis of AChR structure. Furthermore, the sequence homologies and differences between these *C. pennaceus* toxins and other α-conotoxins may enable the study of the molecular basis for phylogenic selectivity in these small peptides by modeling and QSAR studies. This may lead to the future design of synthetic α-conotoxins with novel AChR subtype specificity.

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