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A novel α-conotoxin, PeIA, cloned from *Conus pergrandis* discriminates between rat α9α10 and α7 nicotinic cholinergic receptors*

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Running title: α-CTx PeIA discriminates between α9α10 and α7 nAChRs

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The α9 and α10 nicotinic cholinergic subunits assemble to form the receptor believed to mediate synaptic transmission between efferent olivocochlear fibers and hair cells of the cochlea, one of the few examples of postsynaptic function for a non-muscle nicotinic acetylcholine receptor (nAChR). However, it has been suggested that the expression profile of α9 and α10 overlaps with that of α7 in the cochlea and in sites like dorsal root ganglion neurons, peripheral blood lymphocytes, developing thymocytes and skin. We now report the cloning, total synthesis and characterization of a novel toxin, α-conotoxin PeIA that discriminates between α9α10 and α7 nAChRs. This is the first toxin to be identified from *Conus pergrandis*, a species found in deep waters of the Western Pacific. α-Conotoxin PeIA displayed a 260-fold higher selectivity for α-bungarotoxin-sensitive α9α10 nAChRs compared to α-bungarotoxin-sensitive α7 receptors. The IC_{50} of the toxin was 6.9 ± 0.5 nM and 4.4 ± 0.5 nM, for recombinant α9α10 and wild-type hair cell nAChRs, respectively. α-Conotoxin PeIA bears high resemblance to α-conotoxins MII and GIC isolated from *Conus magus* and *Conus geographus*, respectively. However, neither α-conotoxin MII nor α-conotoxin GIC at concentrations of 10 μM, blocked acetylcholine responses elicited in *Xenopus* oocytes injected with the α9 and α10 subunits. Among neuronal non-α-bungarotoxin sensitive receptors, α-conotoxin PeIA was also active at α3β2 receptors and chimeric α6/α3β2β3 receptors. α-conotoxin PeIA represents a novel probe to differentiate responses mediated either through α9α10 or α7 nAChRs in those tissues where both receptors are expressed.

Nicotinic acetylcholine receptors (nAChRs)[1] are widely distributed in both the central and peripheral nervous system. In vertebrates, nine α subunits (α2-α10) and three β subunits (β2-β4) have been cloned. The rules of association for functional nAChRs are broadening, and now permit receptors assembled from single α subunits (α7, α8 and α9)(1-3); receptors which contain multiple α subunits both with (α2α5β2, α3α5β2, α3α5β4, α4α5β2) (4-6) and without supplemental β subunits (α7α8, α9α10) (2, 7);

receptors with single α and multiple β subunits (α3β2β4, α3β3β4) (8, 9); receptors with multiple α and β subunits (α3β2β4α5, α4α6β2β3) (10-12); as well as heteromeric nAChRs formed via pairwise combinations of α2, α3, α4 or α6 with either the β2 or β4 subunits (13-17). Thus, the number of potential molecular forms of nicotinic receptors is very large. Elucidation of the precise structure and function of various neuronal nAChRs *in vivo* is particularly challenging, in part because of the scarcity of ligands selective for specific receptor subtypes.

The venoms of predatory cone snails (*Conus*) represent a rich combinatorial-like library of evolutionarily selected, neuropharmacologically active peptides (18). There are more than 500 species of these snails. Each *Conus* venom appears to contain a unique set of 50-200 small disulfide-bonded peptides that target receptors and ion channels in a highly subtype-selective manner. For peptides where function has been determined, three classes of targets have been elucidated: voltage-gated ion channels, G-protein-coupled receptors and ligand-gated ion channels (19). Perhaps the most conserved feature of cone snail venom is the α-conotoxins; these are a series of structurally and functionally related peptides that target nAChRs. Every venom examined thus far has its own distinct complement of nicotinic receptor antagonists, suggesting that, within the genus, there are literally thousands of novel peptides that act on nAChRs. A major advance in recent years in the neuropharmacology of nAChRs has been the ability to more readily characterize particular neuronal subtypes by using specific conotoxins.

We now report the cloning of a gene encoding a novel peptide of the α-conotoxin family from *Conus pergrandis*, α-conotoxin PeIA (α-CTx PeIA). As far as we are aware, this is the first toxin to be characterized from this species found in deep waters (50-530 m) of the Western Pacific. We show that the peptide has unusual targeting specificity; it has high affinity for recombinant α9α10-containing nAChR receptors, and can readily discriminate this α-bungarotoxin-sensitive receptor from the neuronal α7 α-bungarotoxin-sensitive receptor. In addition, α-CTx PeIA blocks native cochlear hair cell nAChRs with a high potency, demonstrating an *in vivo* target for the peptide. Thus, α-CTx PeIA can be used to selectively discern between α9α10- and α7-mediated functions at those sites where both types of receptors are expressed.

EXPERIMENTAL PROCEDURES

Identification and Sequencing of Genomic Clones Encoding α-CTx PeIA-Genomic DNA was prepared from 50 mg of *Conus pergrandis* hepatopancreas using the Gentra PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN), according to the manufacturer's standard protocol. It was used as a template for polymerase chain reaction (PCR) with oligonucleotides corresponding to the conserved intron and 3′ UTR sequences of α-conotoxin prepropeptides. The resulting PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN). The eluted DNA fragments were annealed to pAMP1 vector, and the resulting products transformed into competent DH5α cells using the CloneAmp pAMP System for Rapid Cloning of Amplification Products (Life Technologies/Gibco BRL, Grand Island, NY). The nucleic acid sequences of the resulting α-conotoxin-encoding clones were determined according to the standard protocol for automated sequencing.

Chemical synthesis–α-CTx PeIA (0.45 mmol/g) was synthesized on an amide resin using Fmoc chemistry and standard side protection, except on cysteine residues. Cys residues were protected in pairs with either *S*-trityl on Cys^2 and Cys^8, or *S*-acetamidomethyl on Cys^3 and Cys^{16}. The peptide was removed from the resin and

precipitated. A two-step oxidation protocol was used to selectively fold the peptides as described previously (20). Briefly, the disulfide bridge between Cys^2 and Cys^8 was closed by dripping the peptide into an equal volume of 20 mM potassium ferricyanide, 0.1 M Tris, pH 7.5. The solution was allowed to react for 30 min, and the monocyclic peptide was purified by reverse-phase HPLC. Simultaneous removal of the *S*-acetamidomethyl groups and closure of the disulfide bridge between Cys^3 and Cys^{16} was carried out by iodine oxidation. The monocyclic peptide and HPLC eluent were dripped into an equal volume of iodine (10 mM) in H_20:trifluoroacetic acid:acetonitrile (78:2:20 by volume), and allowed to react for 10 min. The reaction was terminated by the addition of ascorbic acid, diluted 20-fold with 0.1% trifluoroacetic acid, and the bicyclic peptide was purified by HPLC on a reverse-phase C_{18} Vydac column using a linear gradient of 0.1% trifluoroacetic acid 0.092% trifluoroacetic acid, 60% acetonitrile, remainder H_2O.

Mass spectrometry-Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry was utilized.

Expression of recombinant receptors in Xenopus laevis oocytes-Capped cRNAs were *in vitro*-transcribed from linearized rat plasmid DNA templates using the mMessage mMachine Transcription Kit (Ambion Corporation, Austin, TX). The maintenance of *Xenopus laevis*, as well as the preparation and cRNA injection of stage V and VI oocytes has been described in detail elsewhere (21). Typically, oocytes were injected with 50 nl of RNase-free water containing 0.01-1.0 ng of cRNAs (at a 1:1 molar ratio when pairwise combined), and maintained in Barth's solution at 17° C.

Electrophysiological recordings were performed 2-6 days after cRNA injection under two-electrode voltage-clamp with an OC-725B oocyte clamp (Warner Instruments, Wamden, CT). Both voltage and current electrodes were filled with 3M KCl, and had resistances of ~1-2 MΩ. Data were digitized and stored on a PC computer. Data were analyzed using Clamp Fit from the pClamp 6 software (Axon Instruments Corp., Union City, CA). During electrophysiological recordings, oocytes were continuously superfused (~10 ml/min) with normal frog saline (comprised of, in mM): 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, and 10 HEPES buffer, pH 7.2, and voltage-clamped at -70 mV. In order to follow the same conditions we have previously employed to analyze the properties of α9α10 nAChRs (7), when expressing α9α10, experiments were performed in oocytes incubated with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM, 100 μM) for 3-4 hr prior to electrophysiological recordings. Drugs were applied in the perfusion solution of the oocyte chamber. All toxin solutions also contained 0.1 mg/ml bovine serum albumin to reduce nonspecific adsorption of peptide. The toxin was pre-applied for 10 min prior to the addition of the agonist. For the construction of the inhibition curves, the concentration of acetylcholine (ACh) used was near the corresponding EC_{50} for each receptor; i.e., α9α10, 10 μM; α7, 100 μM; α3β2, 10 μM; α3β4, 100 μM; α4β2, 10 μM.

Recordings from inner hair cells-Apical turns of the organ of Corti were excised from Sprague-Dawley rats at postnatal ages 8 to 10. Cochlear preparations were mounted under an Axioskope microscope (Zeiss, Oberkochem, Germany), and viewed with differential interference contrast (DIC) using a 63x water immersion objective and a camera with contrast enhancement (Hamamatsu C2400-07, Hamamatsu City, Japan). Methods to record from inner hair cells were essentially as described (22). Briefly, inner hair cells were identified visually, by the size of their capacitance (7 to 12 pF) and by their characteristic voltage-dependent Na^+ and K^+ currents, including at older ages a fast-activating

K^+-conductance (23). Some cells were removed to access inner hair cells, but mostly the pipette moved through the tissue using positive fluid flow to clear the tip. The extracellular solution was as follows (in mM): 155 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 5.6 D-glucose, and 10 Hepes buffer; pH 7.4. The pipette solution was (in mM): 150 KCl, 3.5 $MgCl_2$, 0.1 $CaCl_2$, 10 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), 5 Hepes buffer, 2.5 Na_2ATP, pH 7.2. Glass pipettes (1.2 mm i.d.) had resistances of 7-10 MΩ. Cells were held at a holding potential of -90 mV. Postsynaptic currents due to the spontaneous release of ACh from efferent synaptic terminals contacting inner hair cells are occasionally observed. Therefore, in order to study the effect of the toxin on synaptic currents in these cells, transmitter release from efferent endings was accelerated by depolarization using 25 mM external potassium saline. In this case, the pipette solution was (in mM): 150 KCl, 3.5 $MgCl_2$, 0.1 $CaCl_2$, 5 ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′- tetraacetic acid (EGTA), 5 Hepes buffer, 2.5 Na_2ATP, pH 7.2. Solutions containing 60 μM ACh (the EC_{50} in this preparation) or elevated potassium (25 mM K^+) and the toxin were applied by a gravity-fed multi-channel glass pipette (~150 μm tip diameter) positioned about 300 μm from the recorded inner hair cell. All toxin solutions also contained 0.1 mg/ml bovine serum albumin to reduce nonspecific adsorption of peptide. The extracellular solution containing the drugs was similar to that described above, except that Mg^{2+} was omitted, and the Ca^{2+} concentration was lowered to 0.5 mM to optimize the experimental conditions for measuring currents flowing through the α9α10 receptors (24, 25). To minimize the contribution of SK channel currents, 1 nM apamin, a specific SK channel blocker, was added to the external working solutions. Currents in inner hair cells were recorded in the whole-cell patch-clamp mode using (Axopatch 200B amplifier) low-pass filtered at 2-10 kHz and digitized at 5-20 kHz with a Digidata 1200 board (Axon Instruments, Union City, CA). Recordings were made at room temperature (22-25 ºC). Voltages were not corrected for the voltage drop across the uncompensated series resistance.

For constructing the inhibition curves, both in oocytes and the cochlear preparation, the average peak amplitude of three control responses just before the exposure to the toxin was used to normalize the amplitude of each test response in the presence of the toxin. Each data point of the inhibition curves represents the average value ± S.E.M of measurements from at least three experiments. Curves were fitted with the following equation: % response = 100 / (1 + $[(T)/IC_{50}]^{nH}$), where nH is the Hill coefficient, T the concentration of toxin, and IC_{50} the concentration of toxin that reduces to 50% the maximal response to ACh.

Statistical Analysis-Statistical significance was evaluated by the Student's *t* test (two-tailed, unpaired samples). A $p<0.05$ was considered significant.

RESULTS

Cloning of α-CTx PeIA-Although the mature α-conotoxin sequences are highly variable, the organization of their encoding genes is constant across species. The α-conotoxins are proteolytically cleaved from a larger precursor protein. This prepropeptide is approximately 40 amino acids long, with the mature α-conotoxin moiety of ~13-18 amino acids located at the carboxy-terminus of the precursor. A basic amino acid, immediately preceding the mature toxin in the precursor sequence, acts as a processing site. In contrast to the highly variable sequence of the mature toxins, the precursor proteins of α-conotoxins are highly conserved. The signal sequence region is practically invariant among the different α-conotoxin precursors, even in distantly related *Conus* species. Also, sequence segments

in the 3′ untranslated region (UTR) of the α-conotoxin mRNA and in the intron immediately preceding the toxin sequence are similarly conserved (18). We utilized conserved intronic and 3′ UTR sequences of the α-conotoxin gene structure to design oligonucleotide primers for polymerase chain reaction amplification of the α-conotoxin-coding region. The resulting sequence from *Conus pergrandis* is shown in Fig. 1. The toxin was named α-conotoxin PeIA ("Pe" designating the species, *pergrandis*; Roman numeral "I" to designate the canonical α-conotoxin disulfide bond pattern; and "A" to indicate that it is the first α-conotoxin reported from this species).

Chemical synthesis of α-CTx PeIA- Solid-phase chemical synthesis of the predicted mature toxin was performed. In synthesizing the peptide, it was assumed that the disulfide bridging pattern of α-CTx PeIA was the same as all previously characterized α-conotoxins, that is, Cys^2 to Cys^8 and Cys^3 to Cys^{16} (18).The glycine at the C-terminus was assumed to be posttranslationally modified to a C-terminal amide. Cys groups were orthogonally protected in pairs to direct disulfide bond formation. Acid-labile *S*-trityl groups were removed simultaneously with peptide cleavage from the resin, and closure of the disulfide bridge between these Cys residues was accomplished with FeCN. The single-bridge peptide was purified by HPLC, and the acid-stable acetomidomethyl groups were removed; the disulfide bridges formed by iodine oxidation. The bicyclic peptide was subsequently purified by HPLC and analyzed with MALDI mass spectrometry. The mass of the synthetic peptide was consistent with the amidated sequence (monoisotopic MH^+: calculated, 1651.62; observed, 1651.6). This synthesized toxin was utilized in all subsequent experiments.

Effect of α-CTx PeIA on ACh-evoked currents through nAChRs-Conotoxins have been widely used as a pharmacological tool to characterize neuronal nAChRs (26). We therefore decided to analyze the effects of α-CTx PeIA on different nAChRs. Figure 2A shows representative responses of *Xenopus laevis* oocytes expressing either the α-bungarotoxin-sensitive α9α10 or α7 nAChRs to ACh, and blockage of responses in the presence of α-CTx PeIA. Complete block of ACh-evoked currents was obtained with 0.3 μM α-CTx PeIA in the case of α9α10, compared to 100 μM in the case of α7 nAChRs. As derived from the inhibition curves in Fig. 2B, currents elicited by ACh in α9α10 nAChRs were potently blocked by α-CTx PeIA with a mean IC_{50} and S.E.M. of 6.9 ± 0.5 nM (n = 6). In the case of α7, the IC_{50} value, 1.8 ± 0.1 μM (n = 5), was 260-fold higher than that obtained for α9α10, thus indicating a high degree of selectivity of the toxin for α9α10. The blockage produced by α-CTx PeIA on both types of receptors was reversible after washing with saline solution. Figure 3 shows the washout kinetics in the case of α9α10. The effect of α-CTx PeIA was reversed relatively rapidly: >50% recovery in 3 minutes, and total recovery after 12-15 minutes.

Cochlear outer and developing inner hair cells are the main targets of descending cholinergic olivocochlear efferent fibers (27)**.** The efferent fibers-hair cell synapse is most likely mediated by α9α10 nAChRs (7, 28, 29), providing one of the few postsynaptic functions for non-muscle nAChRs. However, α7 transcripts as well as α7 immunostaining have been reported in the mammalian organ of Corti (30, 31). Recordings from inner hair cells are an excellent tool to evaluate the effects of the toxin in native hair cell nAChRs. As shown in Fig. 4 A, α-CTx PeIA potently blocked ACh-evoked responses in inner hair cells. The IC_{50} value obtained, 4.4 ± 0.5 nM, was similar to that found in recombinant α9α10 receptors, thus confirming the α9α10 identity of this nAChR.

The physiologic conditions of neurotransmission *in vivo*, for instance a synaptic

regime, notably differ from the conditions of the oocyte recording or of a bath application of ACh to the organ of Corti. Transmitter released in a synaptic cleft, in close proximity to postsynaptic receptors reaches millimolar ranges, sufficient to activate in the millisecond range receptors with a low affinity active state and a fast desensitization rate (32). We therefore studied the effect of α-CTx PeIA on synaptic currents evoked by the release of presynaptic ACh in the presence of 25 mM KCl. As observed in Fig. 5, 30 nM α-CTx PeIA also blocked responses to synaptically released ACh, thus indicating that the toxin is a valuable tool to examine in vivo responses mediated through α9α10 receptors. Figure 5A shows K^+-evoked synaptic currents either in the absence or presence of α-CTx PeIA. The blocking effect of the toxin was rapidly reversible upon washing the preparation. The effect of α-CTx PeIA was a reduction of the amplitude (Fig. 5B) of the synaptic currents (from 57.0 ± 0.7 pA, number of events: 997, number of cells: 3, to 37.5 ± 0.7 pA, number of events: 766, number of cells: 3, $p< 0.0001$). This result confirms a postsynaptic effect of the toxin on the nAChRs present in cochlear hair cells. A reduction in the frequency of events (from 2.2 ± 0.5 Hz to 1.2 ± 0.2 Hz, data not shown) was observed. This could be due either to the fact that the blocking action of the toxin on the synaptic events of small amplitudes could not be resolved within the noise of the recordings or to an additional presynaptic effect of the toxin. The latter was not further investigated.

To assess the effect of α-CTx PeIA on non-α-bungarotoxin-sensitive neuronal nAChRs, concentration-response analysis was conducted on rat α2β4, α2β2, α4β2, α4β4, α3β2 and α3β4 (Fig 6 and Table I). The toxin had little or no activity when bath-applied on α2β4, α2β2 and α4β4 nAChRs at concentrations as high as 10 μM. The percent response ± S.E.M. to ACh was, respectively, 98.2 ± 2.1%, 95.3 ± 1.9%, and 91.4 ± 2.3% (n = 3). Only a 39.2 ± 5.5 % (n = 4) block of α4β2 was observed in the presence of 10 μM α-CTx PeIA. On the other hand, α-CTx PeIA appeared to be effective on α3-containing receptors, displaying a higher selectivity for α3β2 (IC_{50} = 23 ± 1 nM, n = 5) than for α3β4 (IC_{50} = 0.48 ± 0.03 μM, n = 4) nAChRs. α-CTx PeIA was also tested on rat α6/α3β2β3 nAChRs, where α6/α3 is a chimeric alpha subunit containing the N-terminal 237 amino acids of α6 and the remainder of α3 (33). The toxin at 100 nM blocked 89.8 ± 1.8% of the response (n=4).

Nicotinic AChRs are members of the "Cys-loop" family of neurotransmitter-gated ion channels, which also includes $GABA_A$, $GABA_C$, glycine, $5\text{-}HT_3$ and some invertebrate anionic glutamate receptors (34). In order to assess the selectivity of α-CTx PeIA for nAChRs, we tested the effect of the toxin on some other members of the family, such as the $GABA_A$ and $5\text{-}HT_{3A}$ receptors. Concentrations of α-CTx PeIA as high as 10 μM did not modify responses to 30 μM γ-aminobutyric acid or 10 μM serotonin in oocytes injected with the respective recombinant receptors (n = 4), thus confirming the selectivity of this α-conotoxin from *Conus pergrandis* on nAChRs.

The mature toxin sequence of α-CTx PeIA bears high resemblance to α-conotoxins MII (α-CTx MII) and GIC (α-CTx GIC) isolated from *Conus magus* and *Conus geographus*, respectively (Table 2). While α-CTx MII potently targets α3- and α6-containing neuronal nAChRs, α-CTx GIC has a higher selectivity for α6-containing receptors (35). We therefore examined the effect of these toxins in α9α10-expressing oocytes. At a 10 μM concentration, neither α-CTx MII nor α-CTx GIC blocked α9α10 nAChRs (n = 3).

DISCUSSION

By utilizing conserved sequences in conotoxin genes, we have cloned a gene encoding a novel peptide of the α-conotoxin family from *Conus pergrandis*. This species was only very

rarely collected, and once regarded among the ten most valuable *Conus* species for shell collectors (36). In recent years, however, commercial collectors in the Central Philippines have collected a moderate number of specimens. α-CTx PeIA is the first toxin to be characterized form this species and adds to the arsenal of peptides active at nAChRs that have been isolated from *Conus*. Thus, *Conus pergrandis* represents a new source of useful pharmacological probes to characterize nAChRs. We note, however, that the peptide has not yet been isolated from the venom of *Conus pergrandis*, and it is possible that the native venom-derived peptide has posttranslational modifications not evident from inspection of the genetic sequence. In this report, we describe the properties of a synthetic version of the putative conotoxin. The peptide is 16 residues in length, with two disulfide bonds. α-CTx PeIA belongs to the A superfamily, as do the majority of *Conus* peptides that are known to affect the function of nAChRs (37). The spacing between Cys residues, four amino acids in the first loop and seven in the second loop, is typical of several previously isolated α-conotoxins of the α4/7 family like MII and GIC, which preferentially target non-muscle nAChRs (18, 35).

α-CTx PeIA selectively targets the α-bungarotoxin-sensitive α9α10 rather than α7 nAChRs. α9α10 nAChRs pharmacologically differ from α7 in that nicotine and ICS 205-930 block the former whereas they activate the latter (7, 38). In addition, strychnine, a well-characterized glycine antagonist, potently blocks α9α10 and α7 nAChRs, with a lower IC_{50} for α9α10 (7, 39, 40). α-CTx PeIA is the first reported conotoxin to block α9α10 nAChRs with high affinity. Similar to some previously characterized α4/7 family conotoxins, including α-CTx MII and α-CTx GIC, α-CTx PIA also potently blocks the α3β2 nAChR. In contrast, however, α-CTx MII and α-CTx GIC show no activity at α9α10 nAChRs at concentrations of up to 10 μM.

Current data supports the notion that a receptor assembled from both α9 and α10 nAChR subunits mediates synaptic transmission between efferent olivocochlear fibers and cochlear hair cells (3, 7, 28, 29, 41). However, α7 transcripts as well as α7 immunostaining have been reported in the mammalian organ of Corti (30, 31).The fact that native hair cell nAChRs were potently blocked by α-CTx PeIA, precludes the participation of α7 nAChRs in mediating ACh-evoked responses in inner hair cells. The situation for other tissues remains to be determined. Dorsal root ganglion neurons express multiple nAChR subtypes, including α7-like, α3β4-like and α4β2-like (42). They also co-express both α9 and α10 subunits (43, 44). In addition, peripheral blood lymphocytes and developing thymocytes have been shown to express cholinergic receptors, including the nAChR subunits α2–5, α7, α9, α10 and β4, that could participate at different steps in the regulation of the immune response (45-47). Finally, α7, α9 and α10 are expressed in skin keratinocytes and might be involved at different steps in the regulation of skin homeostasis (48-51). The observation that α-CTx PeIA has a 260-fold selectivity for α9α10 compared to α7, indicates that it is a useful probe to differentiate responses mediated either through α9α10 or α7 nAChRs in the above mentioned tissues where both receptors are expressed.

The carnivorous marine snails of the genus *Conus* are a rich source of peptides targeted to nAChRs. A major component of the complex venomous arsenal that the fish-eating *Conus* employ, are toxins that act at the muscle nicotinic receptor type (18). Why might these snails have evolved a toxin with high affinity for α9α10 receptors? *Conus pergrandis* is found in deep waters (50-530 m) of the Western Pacific. The feeding habits of *Conus pergrandis* are unknown, but *Conus* species in general prey upon fish,

mollusks and/or worms. Little is known about the nAChR subtypes found in these prey. However, the *Fugu rubripes* (pufferfish) genome contains three candidate α7, two α8 and four α9 subunit genes. Moreover, it has been described that this fish genome contains the largest family of vertebrate nAChR subunits reported to date (52). In addition three α9 subunit genes have been described in the rainbow trout (*Oncorhynchus mykiss*) (53). Although, none of the *Fugu* nAChR sequences show close identity to the mammalian or avian α10 subunits, three of the α9-like subunits possess glycosylation sites also found in the higher vertebrate α10 subunit (52), suggesting that these α9-like subunits might co-assemble to form a functional nAChR, much like the higher vertebrate homomeric α9 and/or heteromeric α9α10 nAChRs. Higher vertebrate α9 and α9α10 nAChRs mediate efferent cholinergic inhibition at cochlear and vestibular hair cells (3, 7, 28, 29, 41). The cholinergic pharmacology of efferent block in the fish lateral line organ is similar to that in hair cells of the cochlea, indicating that the same nicotinic cholinergic receptor is likely involved (54) (55). The proper orientation of mechanosensory hair cells along the lateral-line organ of a fish is essential for the animal's ability to sense directional water movements. This sensory system appears to be important in many behavioral tasks such as prey capture, orientation with respect to external environmental cues, navigation in low-light conditions, and mediation of interactions with nearby animals (56-59) Thus, block of efferent modulation of the lateral line activity by *Conus* toxins could facilitate prey capture by these predatory snails.

α-CTx PeIA shows considerable sequence similarity to the α4/7 α-conotoxins MII and GIC (26). The comparison of the structure of α-CTx PeIA, MII and GIC, indicates that the four Cys residues are identically placed having the same disulfide connectivity. Moreover, a conserved proline and the identical placement of an histidine and an asparagine that are known to either initiate or immediately precede an α-helix in GIC and MII, indicate that the peptide backbone topology of α-CTx PeIA is likely similar to that of the other two α-conotoxins. Indeed, when compared as a group, there are only four non-conservative amino acid substitutions among the three peptides (see bold residues in Table 2). The α4/7 toxins have a common structural scaffold. Their polypeptide backbones appear to be virtually identical: the canonical α helical structure and two β turns are prominent structural features of the family. Given the near identity of the peptide backbones the ability of the different α4/7 *Conus* peptides to discriminate between different neuronal nAChR subtypes must clearly be mediated through their divergent side-chain groups (18). Although all three toxins, MII, GIC and PeIA, have high affinity for the α3β2 nAChR (35, 60), only α-CTx PeIA has high affinity for α9α10. Thus, the amino acids shown in bold in Table 2 are likely structural determinants of the high selectivity of α-CTx PeIA for α9α10 nAChRs.

Conclusion-A remarkable accomplishment of the *Conus* genus has been the evolution of nAChR antagonist of diverse subtype specificities. The diversity of their venom products is likely a consequence of the complex marine environment in which these slow-moving and otherwise unarmed predators must compete. *Conus* hunt a broad range of organisms (five different phyla) and also must defend themselves against crustaceans and other predators. We have now identified a novel peptide, α-CTx PeIA, that selectively targets the most recently identified nAChR subunits, α9 and α10. Thus, isolation of peptides from *Conus* venoms continues to prove useful to identify novel pharmacological tools to characterize the nAChR family.

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FOOTNOTES

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[1]The abbreviations used are: nAChRs, nicotinic acetylcholine receptors; α-CTx PeIA, α-conotoxin PeIA; α-CTx MII, α-conotoxin MII; α-CTx GIC, α-conotoxin GIC; PCR, polymerase chain reaction; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; acetylcholine, ACh; MALDI, Matrix-assisted laser desorption ionization.

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Fig. 1. Prepropeptide and encoded toxin of α-Ctx PeIA. A putative proteolityc processing site following the basic residue R is denoted. The mature toxin is indicated. The glycine following the C-terminal cysteine in the mature toxin is presumed to be processed to a C-terminal amide.

Fig. 2. Effect of α-CTx PeIA on α9α10 and α7 nAChRs. ***A***, representative traces of the response to ACh either alone or in the presence of α-CTx PeIA. ***B*****,** inhibition curves performed by the co-application of 10 μM (α9α10) or 100 μM ACh (α7) and increasing concentrations of α-CTx PeIA. Oocytes were incubated with each concentration of the toxin for 10 min prior to the addition of ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and S.E.M. of five to six experiments per group are shown.

Fig. 3. Wash-out kinetics of α-CTx PeIA from α9α10 receptors. After a control response to 10 μM ACh was obtained, 1 μM α-CTx PeIA was applied to α9α10 expressing oocytes for 10 minutes. The oocyte was then continuously perfused with saline solution without toxin while responses to ACh were recorded. Similar results were obtained in three other experiments.

Fig. 4. Effect of α-CTx PeIA on ACh-evoked currents of inner hair cells. ***A***, representative traces to 60 μM ACh either alone or in the presence of α-CTx PeIA. ***B*****,** inhibition curves performed by the co-application of 60 μM ACh and increasing concentrations of α-CTx PeIA. Cells were incubated with each concentration of the toxin for 10 min prior to the addition of ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and S.E.M. of four to six cells per point are shown.

Fig. 5. Effect of α-CTx PeIA on inner hair cell synaptic currents. ***A***, representative traces of the effect of 30 nM **α-**CTx PeIA on synaptic currents evoked by 25 mM KCl. The insets show synaptic currents on an expanded time scale. *B,* bar diagram showing the effect of 30 nM **α-**CTx PeIA on the amplitude of synaptic currents. The recordings are from three independent inner hair cells and the number of analyzed events were 997 and 766, either in the absence or the presence of **α-**CTx PeIA, respectively. The asterisk denotes a significant difference, $p < 0.0001$.

Fig. 6. Effect of α-CTx PeIA on α3β2, α3β4 and α4β2 nAChRs. Inhibition curves obtained by the co-application of 10 μM (α3β2), 100 μM (α3β4) or 10 μM ACh (α4β2) and increasing concentrations of α-CTx PeIA. Oocytes were incubated with each concentration of the toxin for 10 min prior to the addition of ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean and S.E.M. of four to five experiments per group are shown.

Table 1: Effects of α-Ctx PeIA on nAChRs

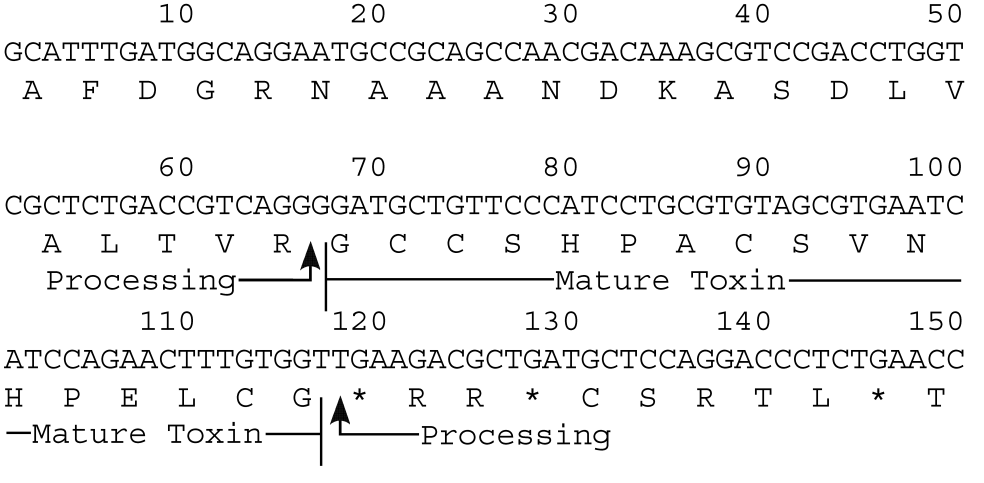
	IC_{50} (nM)	CI	Hill slope	CI	n
α9 α10	6.9	5.2 - 9.1	0.88	0.74 - 1.02	6
α7	1,800	1,396 – 2,206	1.10	0.86 - 1.33	5
α3β2	23	19.2 - 27.5	1.25	0.97 - 1.50	5
α3β4	480	372 - 640	1.23	0.89 - 1.58	4
α4β2	11,600	8,811 – 15,170	3.02	1.76 - 7.81	3

IC_{50}: concentration of toxin that produces 50% of block; CI: 95% confidence interval; n: number of experiments. In the case of α4β2, the IC_{50} was derived from the regression.

Table 2. Sequence comparison of α-conotoxins

α-Conotoxin	Sequence	IC_{50} α9α10 (nM)
PeIA	GCCSHPAC**SV**NH**PE**LC	6.7
MII	GCCSNPVCHLEHSNLC	>10,000
GIC	GCCSHPACAGNNQHIC	>10,000

Figure 1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A F D G R N A A A N D K A S D L V
60 70 80 90 100
CGCTCTGACCGTCAGGGGATGCTGTTCCCATCCTGCGTGTAGCGTGAATC
A L T V R G C C S H P A C S V N
Processing
Mature Toxin
110 120 130 140 150
ATCCAGAACTTTGTGGTTGAAGACGCTGATGCTCCAGGACCCTCTGAACC
H P E L C G * R R * C S R T L * T
Mature Toxin
Processing
ACGACGT
T T

Figure 2

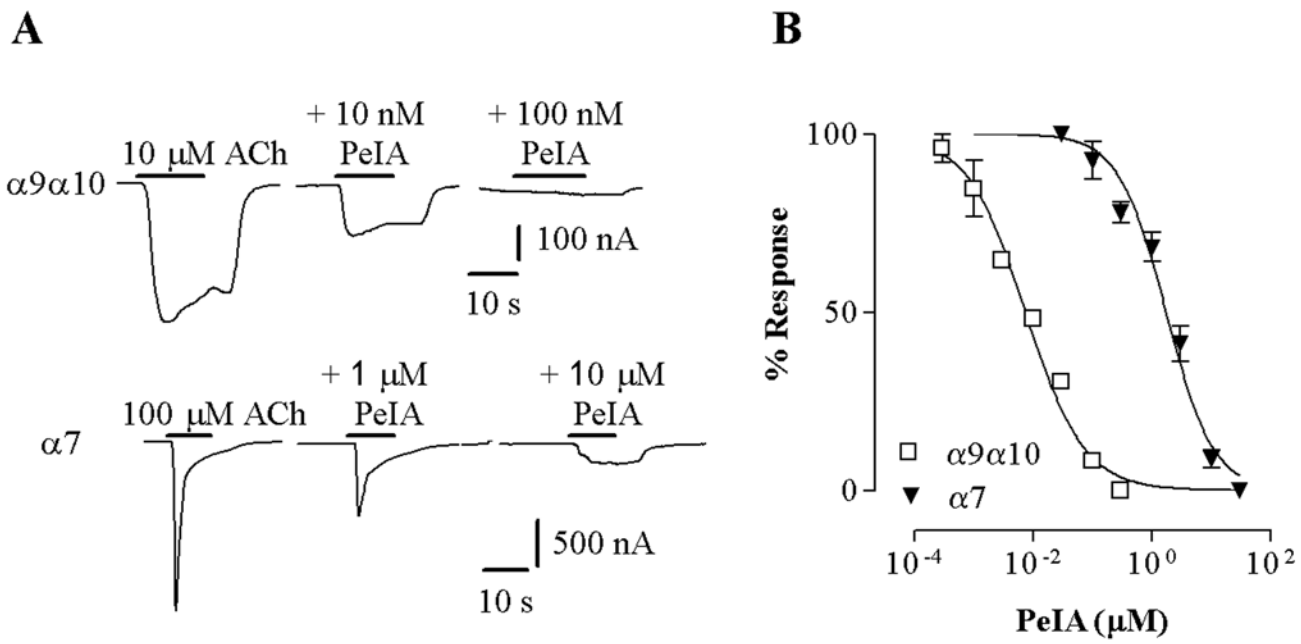


Figure 3

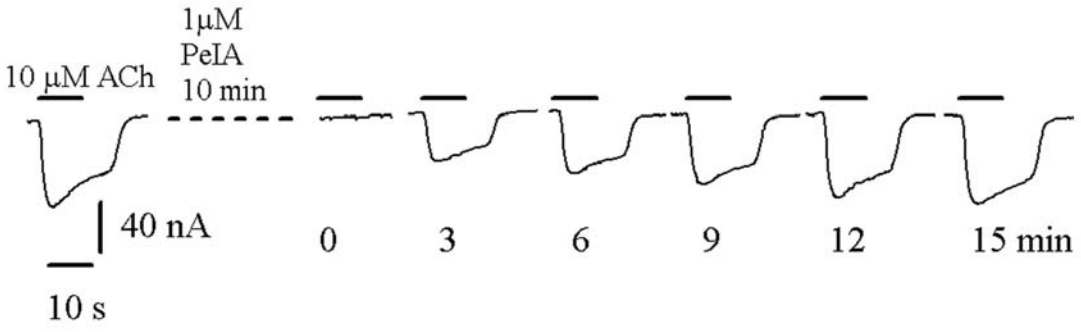


Figure 4

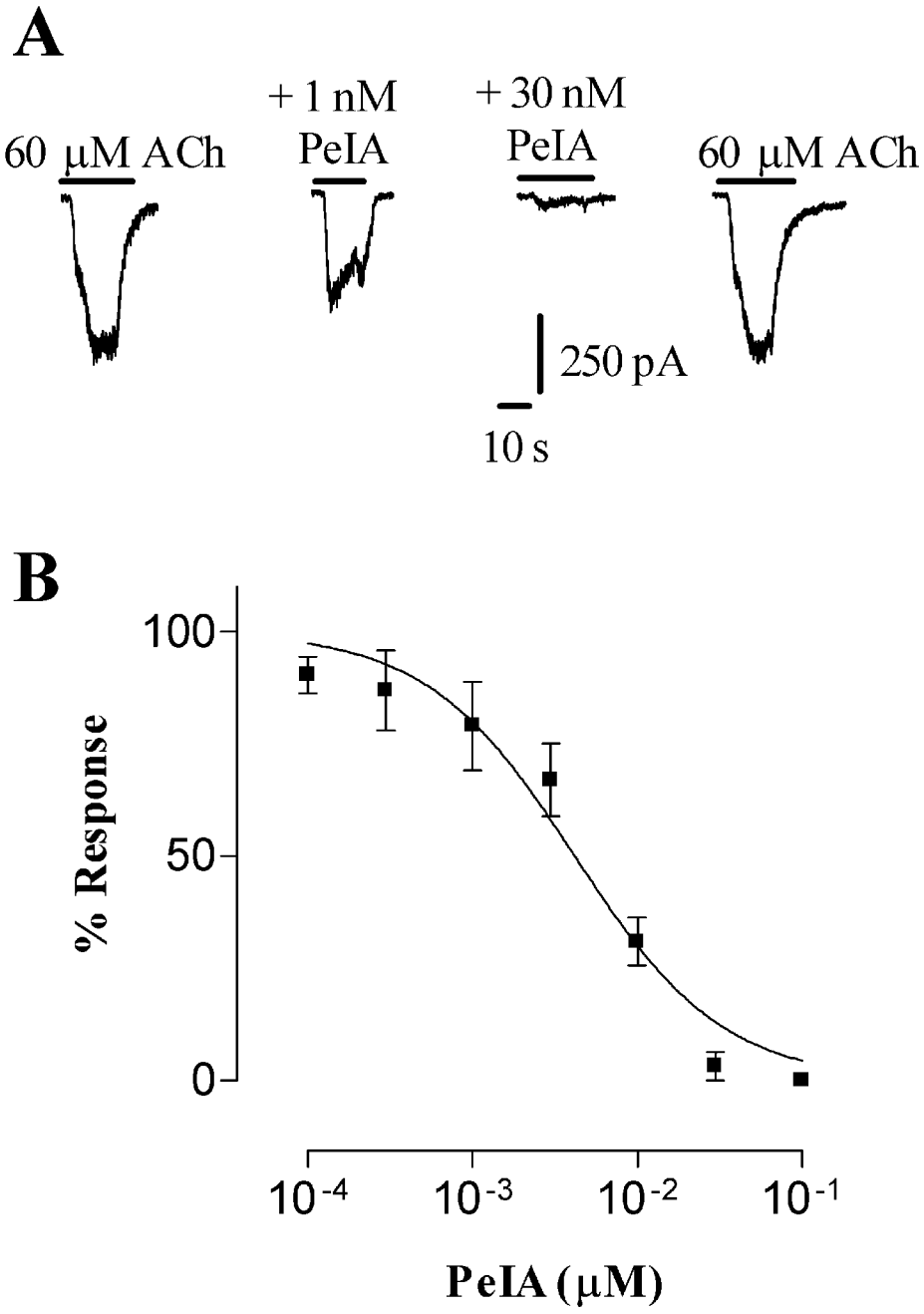


Figure 5

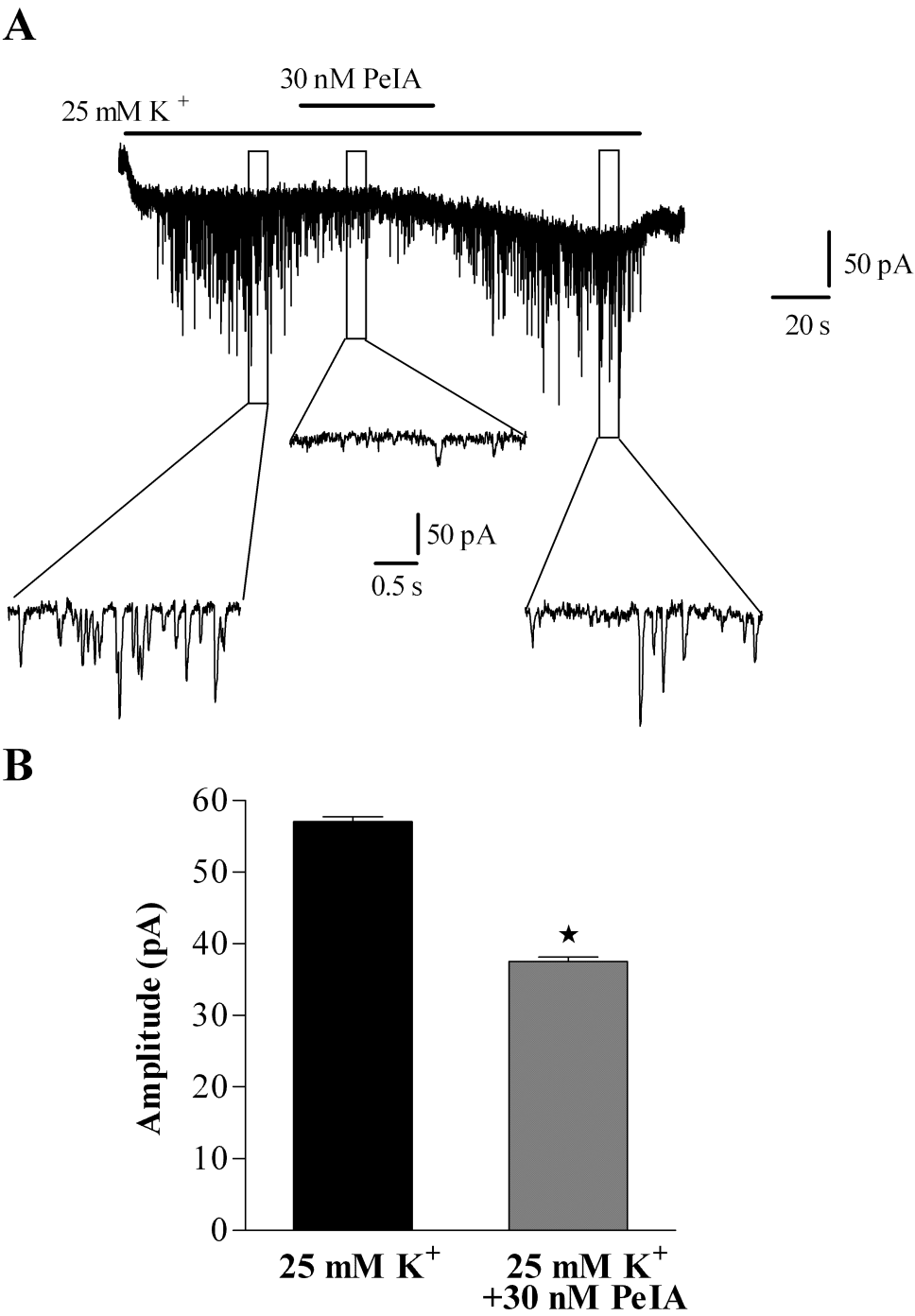
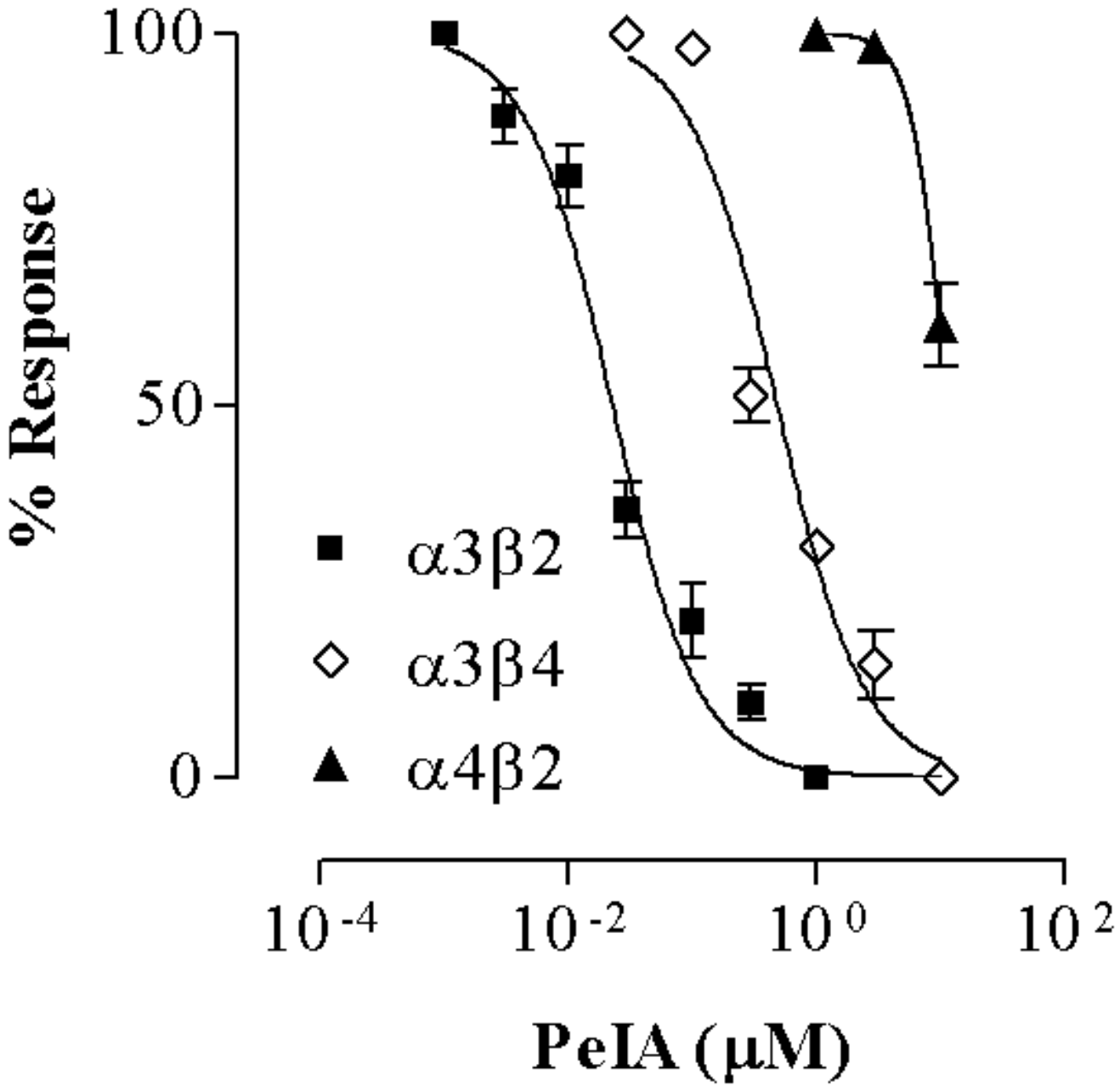

A
30 nM PeIA
25 mM K +
50 pA
20 s
50 pA
0.5 s
B
Amplitude (pA)
60
50
40
30
20
10
0
25 mM K+
25 mM K+
+30 nM PeIA

Figure 6



A novel α-conotoxin, PeIA, cloned from Conus pergrandis discriminates between rat α9α10 and α7 nicotinic cholinergic receptors

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