Cellular/Molecular

α-Conotoxin PIA Is Selective for α6 Subunit-Containing Nicotinic Acetylcholine Receptors

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Until now, there have been no antagonists to discriminate between heteromeric nicotinic acetylcholine receptors (nAChRs) containing the very closely related α6 and α3 subunits. nAChRs containing α3, α4, or α6 subunits in combination with β2, occasionally β4, and sometimes β3 or α5 subunits, are thought to play important roles in cognitive function, pain perception, and the reinforcing properties of nicotine. We cloned a novel gene from the predatory marine snail *Conus purpurascens*. The predicted peptide, α-conotoxin PIA, potently blocks the chimeric α6/α3β2β3 subunit combination as expressed in oocytes but neither the muscle nor the major neuronal nAChR α4β2. Additionally, this toxin is the first described ligand to discriminate between nAChRs containing α6 and α3 subunits. Exploiting the unusual intron conservation of conotoxin genes may represent a more general approach for defining conotoxin ligand scaffolds to discriminate among closely related receptor populations.

Key words: **nicotinic; α-conotoxin; α6; β2; β3; *Conus***

Introduction

Nicotinic acetylcholine receptors (nAChRs) in the CNS are involved in a variety of normal physiological functions, including cognition, reward, motor activity, and analgesia. They are implicated in the pathophysiology and treatment of disease states, including chronic pain syndromes, Parkinson's, and Alzheimer's. Eleven neuronal nAChR subunits have been cloned in mammals, α2–α7, α9 and α10, and β2–β4 (Lips et al., 2002). Different combinations of subunits produce distinct nAChR subtypes. Homologous subunits are organized around a central cation channel that is gated by the endogenous ligand acetylcholine and the tobacco plant toxin nicotine. β subunits and most α subunits associate to form heteropentamers, generally with a stoichiometry of two α and three β subunits. Neither α5 nor β3 subunits have been found to form nAChRs when expressed alone or in pairs with the other subunits. However, both will associate to form functional nAChRs when combined with at least one other α and one other β subunit (Ramirez-Latorre et al., 1996; Wang et al., 1996; Groot-Kormelink et al., 1998). The α5 and β3 subunits are therefore referred to as structural subunits, because they lack residues believed to be necessary to form a ligand-binding site. Instead, they are believed to assemble in a position analogous to that of the β1 subunit in muscle nAChRs.

The β2 subunit is widely expressed in the CNS and appears critical for nicotine reinforcement. β2-Containing nAChRs modulate nicotine-induced dopamine release in the striatum. This release is absent in β2 knock-out mice, which show attenuated self-administration of nicotine (Picciotto et al., 1998; Whiteaker et al., 2000). The β2 subunit can assemble with various α subunits to form distinct subtypes of nAChRs. For example, midbrain dopaminergic nuclei (substantia nigra, ventral tegmental area) are critical to reinforcement and associative motor learning (Berke and Hyman, 2000). Dopaminergic neurons within these nuclei, in addition to β2, contain α4, α6, and α3 nicotinic subunits (Klink et al., 2001). Thus, there is a need for ligands that discriminate among subpopulations of β2 subunit-containing nAChRs. α-Conotoxin MII, a peptide toxin isolated from the marine cone snail *Conus magus*, was initially thought to be selective for α3β2 nAChRs (Cartier et al., 1996). However, at the time of its characterization, no method for expressing α6-containing nAChRs was known. Subsequently, it has been shown that α-conotoxin MII also blocks α6β2* nAChRs (Kuryatov et al., 2000).

We therefore turned again to the cone snails for a more selective ligand. *Conus* is a large genus of predatory snails that feeds on fish, molluscs, and marine worms. Their venoms are complex, often containing hundreds of different peptides. Indeed, the venoms from over 500 species of snails represent a substantial combinatorial library selected over millions of years of evolution to target specific components of the nervous system. 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. Each species of cone snail examined contains at least four distinct α-conotoxins, suggesting that, within the genus, there are literally thousands of novel peptides that act on nAChRs. The diversity in these peptides is likely accounted for by the wide variety of prey types of *Conus* (five different phyla).

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Specialized genetic mechanisms appear to be used to generate peptide sequence diversity.

In this study, we describe the cloning and characterization of a novel peptide from the venom of *Conus purpurascens*, commonly known as the purple cone. The peptide has high affinity and selectivity for α6-containing nAChRs.

Materials and Methods

Identification and sequencing of genomic DNA clone-encoding α-conotoxin PIA. DNA from *C. purpurascens* hepatopancreas was isolated using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Frozen tissue was placed in 600 μl of lysis buffer, homogenized with a disposable microcentrifuge pestle, and digested overnight with 60 μg of proteinase K at 55°C. The remainder of the procedure followed the suggested protocol from the manufacturer for marine invertebrates.

The resulting genomic DNA was used as a template for PCR using oligonucleotides, which were tailed for cloning, corresponding to the 3′ end of the intron preceding the toxin region of α-conotoxin prepropeptides and the 3′ untranslated region (UTR) sequence of the α prepropeptides (Schoenfeld, 1999). The resulting PCR product was purified using the High Pure PCR Product Purification Kit (Roche Products, Indianapolis, IN) following the suggested protocol of the manufacturer. The eluted DNA fragment was annealed to plasmid (p)AMP1 vector, and the resulting product was transformed into competent DH5α cells with the CloneAmp pAMP system for rapid cloning of amplification products (Invitrogen, Grand Island, NY), following suggested protocols of the manufacturer.

Identification and sequencing of a cDNA clone-encoding α-conotoxin PIA. cDNA was prepared by reverse transcription of RNA isolated from the *C. purpurascens* venom duct as described previously (Jacobsen et al., 1998). The resulting cDNA served as a template for PCR using oligonucleotides corresponding to the conserved signal sequence and the 3′ UTR sequence of α-conotoxin prepropeptides. The resulting PCR product was purified using the High Pure PCR Product Purification Kit (Roche Products) following the suggested protocol of the manufacturer. The eluted DNA fragment was ligated to *Hinc*II-linearized pTZ19U vector, and the resulting product was transformed into competent DH5α cells as described previously (Jacobsen et al., 1998). The nucleic acid sequences of the resulting clones were determined according to the standard protocol for Sequenase version 2.0 DNA sequencing kit, as described previously (Jimenez et al., 1997).

Chemical synthesis. Peptide was synthesized on an amide resin (0.45 mmol/gm; Applied Biosystems, Foster City, CA) using Fmoc (*N*-(9-fluorenyl)methoxycarboxyl) chemistry and standard side chain protection except on cysteine (Cys) 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}. Amino acid derivatives were from Advanced Chemtech (Louisville, KY). The peptides were removed from the resin and precipitated, and a two-step oxidation protocol was used to selectively fold the peptides as described previously (Walker et al., 1999). Briefly, the first disulfide bridge 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 second disulfide bridge was performed by iodine oxidation. The monocyclic peptide and HPLC eluent was dripped into an equal volume of iodine (10 mM) in H_2O:trifluoroacetic acid (TFA):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% TFA and the bicyclic product that was purified by HPLC.

Preparation of nAChR subunit cRNA. As reported previously for the human α6 subunit (Kuryatov et al., 2000), attempts to express the rat nAChR α6 subunit with β2 in *Xenopus* oocytes consistently failed; that is, no ACh-gated currents could be detected. To improve functional expression, we created a chimeric nAChR of the rat α6 and α3 subtypes. The chimeric nAChR consists of amino acids 1–237 of the rat α6 subunit protein linked to amino acids 233–499 of the rat α3 subunit protein. The chimeric junction is located at the paired arginine residues immediately preceding the M1 transmembrane segment of the α3 subunit. The resulting chimeric nAChR represents the extracellular ligand-binding domain of the α6 subunit linked to the membrane-embedded region of the α3 subunit where the membrane-spanning segments are located. The α6/α3 cDNA was constructed by introduction of *Bsp*EI sites at the chimeric junction into the α6 and α3 cDNA sequences using mutagenic primers to introduce the restriction sites through silent codon changes. The α6 and α3 segments were generated by PCR of rat brain cDNA using primers in the 5′ and 3′ untranslated regions of the corresponding cDNAs along with the internal mutagenic primers. The PCR products were digested with *Bsp*EI and ligated to generate the chimeric construct. The final chimeric construct was cloned and completely sequenced to confirm the correct cDNA sequence. To further improve expression levels, all of the 5′ and 3′ untranslated regions of the nAChR cDNA were deleted, and the chimeric construct was cloned into the *Xenopus* expression vector pT7TS, placing *Xenopus* globin 5′ and 3′ UTR regions around the nAChR cDNA. The expression construct, pT7TS/rα6α3, was transcribed with T7 RNA polymerase to generate sense-strand RNA for oocyte expression.

Electrophysiology and data analysis. Clones of nAChR subunits were used to produce cRNA for injection into *Xenopus* oocytes as described previously (McIntosh et al., 2000). A previously described chimeric α-subunit consisting of the human extracellular domain of α6 and the remaining part of the human α3 subunit was expressed with other human nAChR subunits (Kuryatov et al., 2000). The rat α6/α3 chimera (described above) was expressed with previously cloned rat subunits (Cartier et al., 1996). For most nAChR subunits, 5 ng of cRNA was injected into the oocytes. For the muscle nAChR, 0.5–2.5 ng was used. For α6/α3β2, α6/α3β4, and α6β4 nAChRs, 12.5–50 ng of cRNA was used to improve the amount of functional expression. A 30 μl cylindrical oocyte-recording chamber fabricated from Sylgard (Dow Corning, Midland, MI) was gravity-perfused with ND96A (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 μM atropine, 5 mM HEPES, pH 7.1–7.5) at a rate of ~2 per minute (Luo et al., 1998). All toxin solutions also contained 0.1 mg/ml of bovine serum albumin to reduce nonspecific adsorption of peptide. Toxin was pre-applied for 6 or 10 min (for concentrations of <10 nM). ACh-gated currents were obtained with a two-electrode voltage-clamp amplifier (OC-725B; Warner Instruments, Hamden, CT), and data were captured as described previously (Luo et al., 1998). The membrane potential of the oocytes was clamped at −70 mV. To apply a pulse of ACh to the oocytes, the perfusion fluid was switched to one containing ACh for 1 sec. This was done automatically at intervals of 1–5 min. The shortest time interval was chosen such that reproducible control responses were obtained with no observable desensitization. The concentration of ACh was 10 μM for trials with α1β1δε and 100 μM for all other nAChRs. Toxin was bath-applied for 5–10 min (10 min at concentrations <10 nM), followed by a pulse of ACh. Thereafter, toxin was washed away, and subsequent ACh pulses were given every 1 min, unless otherwise indicated. All ACh pulses contain no toxin; for it was assumed that little if any bound toxin washed away in the brief time (it takes <2 sec for the responses to peak). In our recording chamber, the bolus of ACh does not project directly at the oocyte but rather enters tangentially and then swirls and mixes with the bath solution. The volume of entering ACh is such that the toxin concentration remains at a level >50% of that originally in the bath until the ACh response has peaked (<2 sec).

The average peak amplitude of three control responses just preceding exposure to toxin was used to normalize the amplitude of each test response to obtain a "percentage response" or "percentage block." Each data point of a dose–response curve represents the average value ± SE of measurements from at least three oocytes. Dose–response curves were fit to the following equation:

$$\%\text{response} = 100/(1 + [(\text{toxin})/\text{IC}_{50}]^{n_H}),$$

where n_H is the Hill coefficient with Prism software (GraphPad Software, San Diego, CA) on an Apple Power Macintosh.

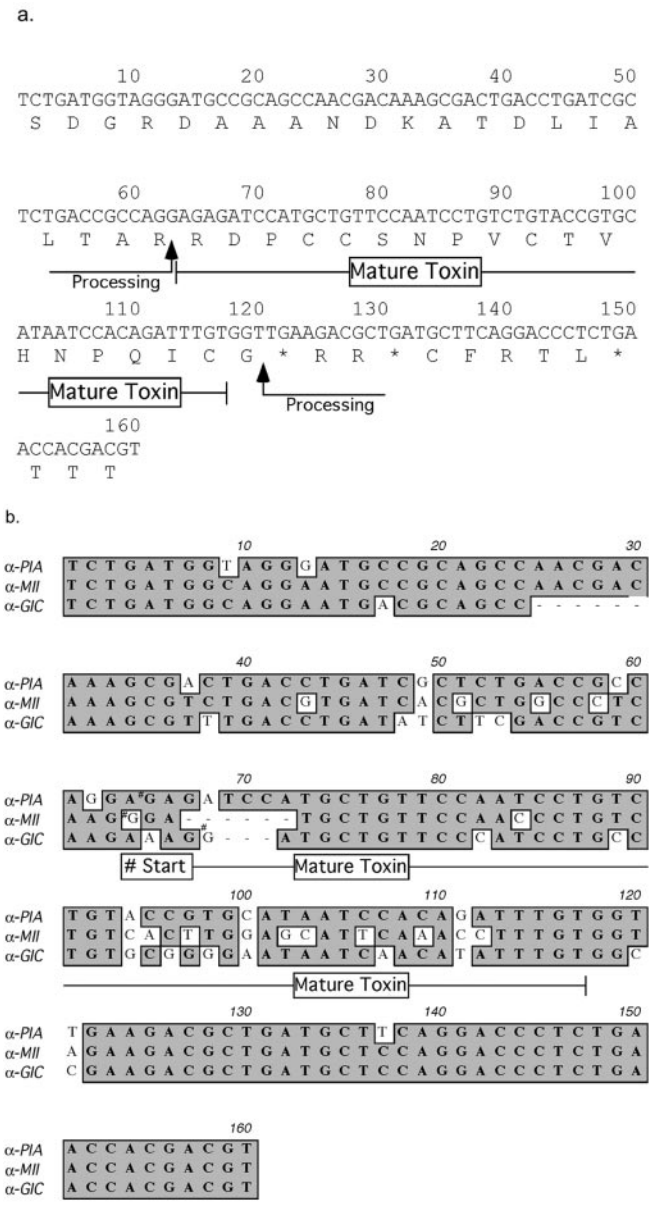


Figure 1. *a*, α-Conotoxin PIA prepropeptide and encoded toxin are shown. The putative proteolytic processing site R is indicated by the arrow. The mature toxin is demonstrated. The glycine following the C-terminal cysteine in the mature toxin is presumed to be processed to a C-terminal amide, as is the case with other known α-conotoxins. *b*, The α-conotoxin PIA prepropeptide mRNA sequence is aligned with sequences for α-conotoxin MII and α-conotoxin GIC. Note the high sequence homology in the precursor region in contrast to divergence in the mature toxin region.

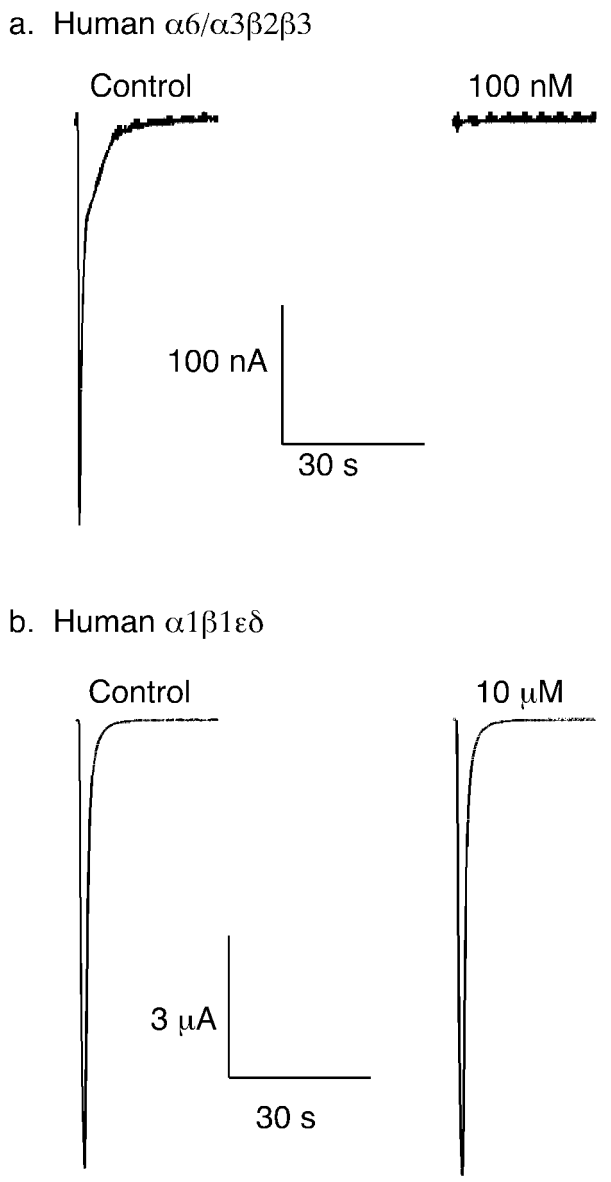
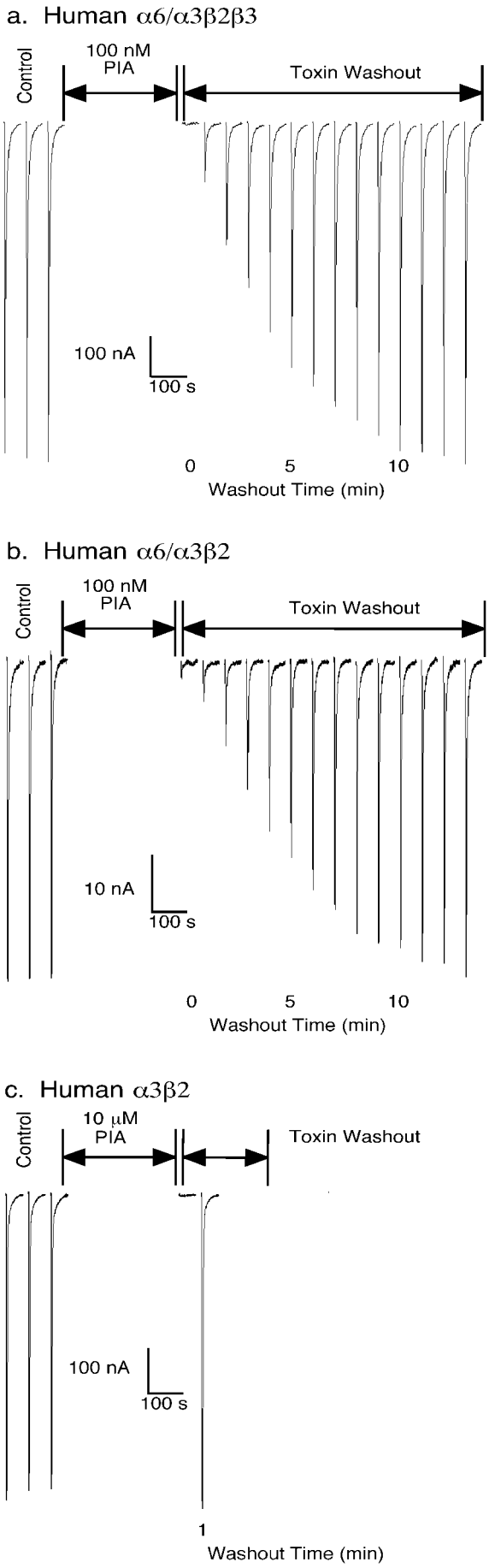


Figure 2. α-Conotoxin PIA blocks human α6/α3β2β3 but not human muscle nAChRs. α-Conotoxin PIA was applied to oocytes heterologously expressing the indicated nAChRs as described in Materials and Methods. Traces from individual oocytes are shown. Note that 100 nM PIA abolishes the ACh response at the human α6/α3β2β3 subunit combination, whereas 10 μM α-conotoxin PIA had no effect on the adult form of the human muscle nAChR.

Results

Cloning of α-conotoxin PIA

Conus venoms are extremely complex, with an estimated 200 peptides in the venom of a single *Conus* species. There are ~500 species of cone snails; in each species, there appears to be on average one-half dozen α-conotoxins targeted to nAChRs. Although the mature α-conotoxin sequences diverge considerably, the organization of α-conotoxin genes reveals considerable conservation across species. The intron immediately preceding the toxin sequence, the signal sequence within the exon, and the 3′ untranslated region show a high degree of conservation. Proteolytic processing of the larger precursor protein leads to the formation of the mature α-conotoxin. Conserved features of the α-conotoxin gene structure were exploited to isolate a novel α-conotoxin gene from *Conus purpurascens*. Two complementary approaches were used. In the first, direct amplification of the α-conotoxin gene sequence from genomic DNA was performed. The coding region of the signal sequence and most of the propeptide region was separated from the toxin-coding region and 3′ untranslated region by a single intron. Primers were designed to this conserved intron sequence and used in conjunction with primers to the conserved 3′ untranslated region to PCR-amplify the toxin-coding portion of the α-conotoxin gene. In the second approach, reverse-transcription PCR of venom duct RNA was performed. This was accomplished by designing oligonucleotide primers on the basis of conserved regions in the signal sequence and the 3′ untranslated region. Both methods yielded clones encoding α-conotoxin PIA. Figure 1*A* shows the results for amplification of genomic DNA. Clones of α-conotoxin MII from *Co-*



nus magus and α-conotoxin GIC are shown for comparison in Figure 1*B*; as can be appreciated, the prepropeptide regions of the three peptides are highly conserved. This is in contrast to the mature toxin regions that are much more divergent, particularly in the C-terminal portions of the peptides.

Chemical synthesis of α-conotoxin PIA

The α-conotoxin gene sequences were conceptually translated to identify the conotoxin peptide. Because of the highly conserved nature of the cysteine residues in α-conotoxins and conserved proteolytic processing sites, the mature toxin sequences can be reliably identified from the gene sequence. Complete chemical synthesis of the predicted mature toxin was undertaken. It is important to note that α-conotoxin PIA has not yet been isolated from *Conus purpurascens* venom. Therefore, it is possible that there are post-translational modifications in the native peptide. Such modifications could influence the properties of the native peptide compared with the synthetic peptide described in this study.

To synthesize the peptide, two assumptions were made. First, it was assumed that the disulfide-bonding pattern of α-conotoxin PIA was the same as that of all previously characterized α-conotoxins, that is, Cys^1 to Cys^3 and Cys^2 to Cys^4. Cys residues were orthogonally protected to direct the formation of disulfide bonds in this configuration. The first and third cysteine residues were protected with acid-labile groups, which were removed first after a cleavage from the resin; ferricyanide was used to close the first disulfide bridge. Reverse-phase HPLC was used to purify the monocyclic peptide; subsequently, the acid-stable acetometomethyl groups were removed from the second and fourth cysteines by iodine oxidation, which also closed the second disulfide bridge. The fully folded peptide was purified again by HPLC. Laser desorption mass spectrometry (reflectron) of synthetic α-conotoxin PIA was consistent with the amidated sequence (monoisotopic MH^+: calculated, 1980.83; observed, 1980.71). Synthetic peptide was used in all subsequently described studies.

Toxin effects on heterologously expressed nAChRs

Injection of human α6 subunits into oocytes either alone or in combination with β2 or β3 subunits does not reliably produce functional nAChRs (Kuryatov et al., 2000; our observations). In these studies, we therefore used a previously reported chimera, wherein the large extracellular domain of α6 is joined to the transmembrane and intracellular portion of the closely related human α3 subunit (Kuryatov et al., 2000). Similarly in these studies, we failed to achieve expression of rat α6 when its cRNA was injected into oocytes either alone or in combination with β2 or β3. We therefore created and used a chimera of the rat α6 and α3 subunits analogous to the human chimera described above to improve functional expression.

The mature toxin sequence of α-conotoxin PIA bears some resemblance to α-conotoxins MII and GIC isolated from *Conus magus* and *Conus geographus*, respectively. The latter two peptides potently target neuronal subtypes in preference to the muscle subtype of nAChR (Table 1). We therefore examined the effect

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Figure 3. Washout kinetics of α-conotoxin PIA. α-Conotoxin PIA was bath-applied to oocytes expressing the indicated human nAChR subtypes heterologously expressed in oocytes. After the 5 min application, toxin was washed out, and responses to a 1 sec pulse of ACh were measured each minute. *a*, A 100 nM concentration of toxin on α6/α3β2β3. *b*, A 100 nM concentration of toxin on α6/α3β2. *c*, A 10 μM concentration toxin on α3β2. Note that recovery from toxin block is rapid in the case of the human α3β2 nAChR in contrast to the human α6/α3β2β3 and α6/α3β2 nAChR.

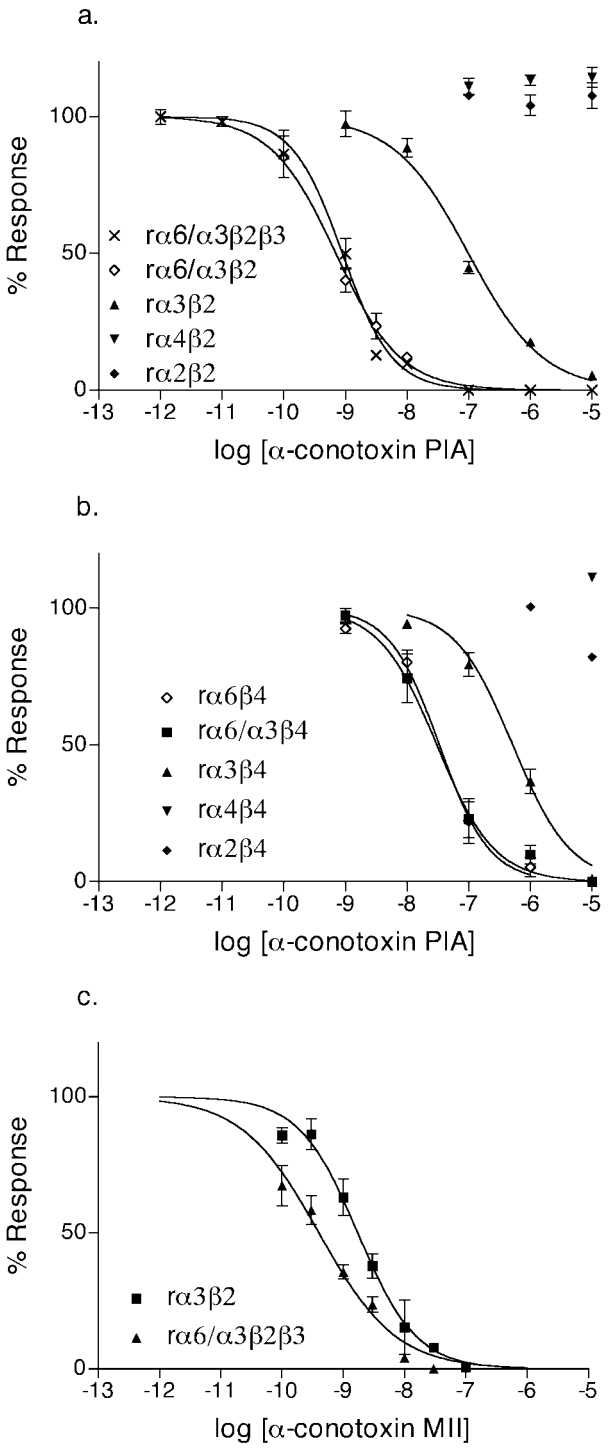

Figure 4. α-Conotoxins were bath-applied to oocytes expressing the indicated subtype for 5 min, and then the response to a 1 sec application of ACh was measured. The results are summarized in Table 2. *a*, Block of β2-containing rat nAChRs by α-conotoxin PIA. α-Conotoxin PIA blocked both α6/α3- and α3-containing nAChRs but had no measurable effect on α4- or α2-containing nAChRs. *b*, Block of β4-containing nAChRs by α-conotoxin PIA. Toxin potently blocked rat α6β4 and α6/α3β4 nAChRs and had less or no effect on other rat β4-containing nAChRs. *c*, Block of β2-containing nAChRs by α-conotoxin MII. Each data point represents the average of 3–5 oocytes. Error bars indicate SEM.

of α-conotoxin PIA on heterologously expressed neuronal and muscle nAChRs in *Xenopus* oocytes. As shown in Figure 2, 100 nM α-conotoxin PIA abolishes the ACh-induced response on human α6/α3β2β3 nAChRs. In contrast, 10 μM α-conotoxin PIA has no effect on the adult form of the human muscle nAChR, indicating a high degree of preference for the neuronal nAChR. The effect of α-conotoxin PIA was reversed only after several minutes of toxin washout in the case of human α6/α3β2β3 and human α6/α3/β2 nAChRs. In contrast, although high concentrations of α-conotoxin PIA also blocked the human α3β2 nAChR, this effect was rapidly reversed after toxin washout (Fig. 3).

To assess the effect of the toxin on rodent nAChRs, concentration–response analysis was conducted on rat α6/α3β2 (with and without β3), α3β2, α4β2, and α2β2 subunit combinations heterologously expressed in *Xenopus* oocytes. The results are shown in Figure 4 and Table 2. In contrast to previously isolated α-conotoxins and other known nAChR antagonists, α-conotoxin PIA shows selectivity for heteromeric nAChRs containing the extracellular α6 subunit sequence in comparison with α3, α4, and α2 subunits. We note that the β3 subunit, when coinjected with chimeric α6/α3 and β2, substantially increased expression in oocytes. However, the β3 subunit did not significantly affect the IC_{50} of the α-conotoxin PIA (Fig. 4, Table 2). β3 is also believed to be present in native α6-containing nAChRs, where it presumably acts as a structural subunit (Lena et al., 1999). Concentration–response analysis for α-conotoxin PIA was also performed on human chimeric α6/α3β2β3 nAChRs and gave similar results (IC_{50}, 1.7 nM; 95% confidence interval; 1.1–2.8 nM) to rat chimeric α6/α3β2β3 (IC_{50}, 0.95 nM; 95% confidence interval; 0.72–1.3 nM)

α-Conotoxin PIA was also tested on β4-containing nAChRs. Potency was comparatively low on α2β4, α3β4, and α4β4 nAChRs. Although the α6β4 subunit combination is present in the chick (Vailati et al., 1999), it has not been identified yet in mammalian systems. Nevertheless, we assessed the toxin affinity for this subtype. No currents were seen using rat α6/α3β4 subunits when 5 ng of cRNA per subunit was injected; therefore, cRNA amounts were increased to 10–25 ng per subunit. α-Conotoxin PIA potently blocked this combination for both rat and human subunits (Table 2). Larger amounts of cRNA (40 ng) were also needed to see expression of rα6β4. Expression was small, usually leading to currents <5 nA, and occurred only in a minority of oocytes at 7 d after injection. α-Conotoxin PIA blocked rat α6β4 and chimeric α6/α3β4 with similar potencies (Fig. 4, Table 2). Thus, PIA selectively blocks α6/α3β2 versus α3β2 nAChRs, and selectively blocks α6/α3β4 versus α3β4 nAChRs, indicating the α6 versus α3 preference of this peptide. In contrast, α-conotoxin MII does not distinguish well between α6/α3β2β3 and α3β2 nAChRs. The IC_{50} was 0.40 nM (95% confidence interval; 0.29–0.54 nM) for α6/α3β2β3, and the IC_{50} for α3β2 was 1.7 nM (95% confidence interval; 1.3–2.4 nM).

Toxin off-rates were examined for rat α6/α3β2β3, α6/α3β2, and α3β2 nAChRs. As in the case of the human nAChRs, the effect of α-conotoxin PIA is much more slowly reversed during washout from α6/α3β2 (with or without β3) versus α3β2 nAChRs (Fig. 5*a*). Thus, α-conotoxin PIA distinguishes between these two nAChRs, both in potency and kinetics of unblock. In addition, the toxin also dissociates slowly from rat β4-containing subunit combinations (which under the described conditions may lead to a small underestimation of its potency at these subtypes) (Fig. 5*b*). However, for both rα3β4 and rα6/α3β4, preincubation with α-conotoxin PIA for 20 min did not significantly change the IC_{50} compared with that obtained with 10 min preincubation (data not shown).

Discussion

Toxins represent powerful tools for distinguishing among various subtypes of ligand- and ion-gated channels. In particular,

Table 1. Sequence comparison of α-conotoxins

α-Conotoxin	Sequence	AChR preference	Reference
PIA	RDPCCSNPVCTVHNPQIC*	$\alpha6\beta2\beta3 > \alpha3\beta2$	This study
MII	GCCSNPVCHLEHSNLC*	$\alpha6\beta2\beta3 \approx \alpha3\beta2$	This study
GIC	GCCSHPACAGNNQHIC*	$\alpha6\beta2\beta3 \approx \alpha3\beta2$	McIntosh et al., 2002; our unpublished observations
GID	IRDγCCSNPACRVNNOHVC	$\alpha7 \approx \alpha3\beta2 > \alpha4\beta2$	Nicke et al., 2003
EpI	GCCSDPRCNMNNPCY†C*	Intracardiac ganglia, adrenal chromaffin cells	Loughnan et al., 1998
AuIB	GCCSYPPCFATNPDC*	$\alpha3\beta4$	Luo et al., 1998
ImI	GCCSDPRCAWRC*	$\alpha7 > \alpha9$	Johnson et al., 1995
MI	GRCCHPACGKNYSC*	$\alpha1\beta1\delta\gamma$	Johnson et al., 1995

* Amidated C terminus.

Y † Sulfotyrosine; γ-gammacarboxy glutamate.

Table 2. Effect of α-conotoxin PIA on nAChR subtypes

nAChR	IC_{50} (in nM)	95% CI	n^H	95% CI
rα6/α3β2β3	0.95	0.71–1.3	1.1	0.70–1.6
hα6/α3β2β3	1.72	1.1–2.8	0.98	0.56–1.4
rα6/α3β2	0.69	0.48–0.99	0.81	0.58–1.0
rα3β2	74.2	49–110	0.8	0.55–1.1
rα2β2	>10,000		ND	
rα4β2	>10,000		ND	
hα1β1ϵδ	>10,000		ND	
hα6/α3β4	12.6	8.4–19	1.2	0.64–1.7
rα6/α3β4	30.5	20–47	0.93	0.62–1.2
rα6β4	33.5	23–49	1.1	0.73–1.4
rα3β4	518	374–718	0.93	0.63–1.2
rα2β4	>10,000		ND	
rα4β4	>10,000		ND	

h, Human; r, rat; ND, not determined; CI, confidence interval; n^H, hill slope.

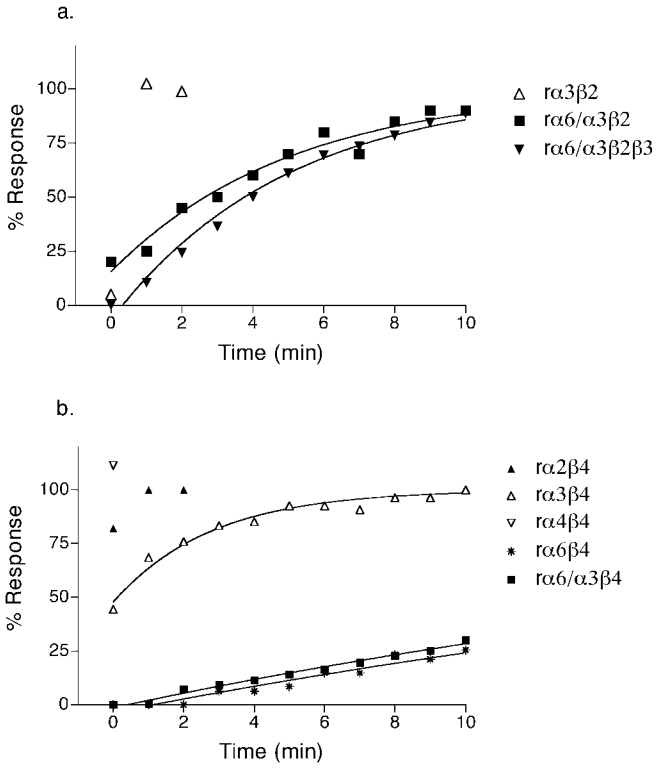

Figure 5. Off-rate kinetics of α-conotoxin PIA. *a*, α-Conotoxin PIA was bath-applied for 5 min to oocytes heterologously expressing the indicated rat β2-containing nAChRs. The concentration of toxin was 10 nM for α6/α3β2, 100 nM for α6/α3β2β3, and 10 μM for α3β2. k_{off} for α6/α3β2β3 = 0.17 min^{-1}. k_{off} for α6/α3β2 nAChRss was similar (0.19 min^{-1}). In contrast, the block of α3β2 nAChRs by α-conotoxin PIA was fully reversed within 1 min of toxin washout. *b*, α-Conotoxin PIA was applied as described above to rat β4-containing nAChRs. The toxin concentration was 1 μM for α6β4, α6/α3β4, and α3β4, and 10 μM for rα2β4 and α4β4. k_{off} for α3β4 was 0.44 min^{-1}. Recovery from block for α6β4 and α6/α3β4 was comparatively slow, with only 25 and 30% recovery, respectively, after 10 min.

polypeptide toxins from land and sea snakes have proven invaluable for characterizing nAChRs. Certain alkaloids such as methyllycaconitine, isolated from the seeds of the larkspur plant, are also quite useful. More recently, small peptides from the venomous gastropod *Conus* have been shown to discriminate between various forms of nAChRs. Previous studies of α-conotoxin MII indicated its ability to discriminate among α3β2 versus other nAChR subtypes. However, at the time of initial characterization of α-conotoxin MII, the pharmacology of α6-containing nAChRs was unknown. The α6 subunit is structurally closely related to α3. This study indicates that α-conotoxin MII discriminates poorly between α3β2 nAChRs and α6β2* nAChRs. α6β2* nAChRs appear central to important physiological and possibly pathological conditions. α6β2 nAChRs appear to be involved in the modulation of dopamine release (Champtiaux et al., 2002) and are significantly reduced or eliminated in primate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine models of Parkinson's disease (Quik et al., 2001). We therefore wished to investigate whether other species of *Conus* snails produce toxins that have the ability to distinguish α6β2* from other β2-containing nAChRs. We used conserved features of the *Conus* genome to probe for novel peptide ligands.

Conus purpurascens, also known as the purple cone, is moderately common in shallow water from the Gulf of California to Peru. It is often found in tide pools and on rocky ledges where it hunts fish (Keen, 1972; Walls, 1979). We used both genomic cloning and cDNA libraries in *C. purpurascens* to isolate a gene encoding a novel α-conotoxin. On the basis of the clone, we synthesized the predicted mature peptide toxin. It should be noted that the native toxin has not been isolated yet from the venom of *Conus purpurascens*. Post-translational modifications of the native peptide, if present, may confer different properties than those indicated for the synthetic peptide described in this study. The predicted structure is a 16 amino acid peptide with two disulfide bonds. The disulfide bonds were folded in the arrangement of Cys^1–Cys^3, Cys^2–Cys^4, because this conformation is present in all previously characterized venom-isolated α-conotoxins.

Because the α6 subunit did not express with β2 or β2 plus β3, we used a chimeric α6/α3 subunit to allow expression. The putative structural subunit, β3, did not substantially affect either toxin potency or off-rate kinetics. That is, the block by α-conotoxin PIA of α6/α3β2β3 nAChRs was not significantly different from that of α6/α3β2 nAChRs. Both rat α6 with β4 and the rat chimeric α6/α3 with β4 gave similar IC_{50}s with

α-conotoxin PIA. This indicates that, as expected, the toxin binds to determinants on the extracellular portion of the nAChR and that the remaining α3 portion of the chimeric α6/α3 subunit does not substantially affect peptide binding. Also note that the IC_{50} for α-conotoxin MII for chimeric rat α6/α3β2β3 determined in this study (0.4 nM) is in reasonable agreement with the affinity of α-conotoxin MII (1.3 nM) for rat α6β2* nAChRs immunoprecipitated from the striatum (Zoli et al., 2002). This again suggests that the α6/α3 chimera as expressed in *Xenopus* oocytes provides a good approximation of the native α6 extracellular binding site. In null mutant mice, [^{125}I]α-conotoxin MII binding is abolished in α6−/− mice, yet primarily retained in α3−/− mice (Champtiaux et al., 2002; Whiteaker et al., 2002). Given the similar affinity of α-conotoxin MII for α6/α3β2 and α3β2 nAChRs found in this study, we interpret the results with radiolabeled MII to indicate that the predominant CNS binding site of MII is an α6β2* nAChR.

There is a high degree of identity between the extracellular portion of human α6 and α3, and between rat α6 and α3 nAChR subunits. Despite these similarities, α-conotoxin PIA has an ~75-fold lower IC_{50} for α6/α3β2β3 nAChRs compared with α3β2 nAChRs. In the extracellular regions, there is also considerable homology between rat α6, α4, and α2 subunits. Nevertheless, α-conotoxin PIA has an IC_{50} >10 μM for the rat α4β2 and α2β2 subunit combinations heterologously expressed in oocytes. α-Conotoxin PIA also potently blocks α6/α3β4 versus α2β4, α3β4, and α4β4 nAChRs, also demonstrating that toxin selectivity determinants are located on the extracellular portion of the α6 subunit. α6β2* nAChRs are located in the striatum, where they are believed to modulate dopamine release (Zoli et al., 2002). We note that α-conotoxin PIA potently blocks nicotine-stimulated dopamine release in rat striatal synaptosomes with low nanomolar potency (Azam et al., 2002a).

Structure–function studies of the α3 nAChR subunit and α-conotoxin MII indicate that lysine 185 and isoleucine 188 of α3 are critical amino acids for toxin binding (Harvey et al., 1997). These are conserved between α3 and α6 subunits. Because α-conotoxin PIA, unlike α-conotoxin MII, is able to distinguish α6 from α3, it appears that at least some of the nAChR-binding determinants for the two toxins are distinct. Table 3 shows residues that are present in α6 but not identical or homologous to residues in aligned positions of α2, α3, and α4 subunits. These residues account for ~10% of the total amino acids in the extracellular region. Only one of the identified residues differs between rat and human α6. This conserved nature of the α3 and α6 subunits suggests that a nAChR mutagenesis approach would be useful to establish the residues in α6 responsible for high-affinity α-conotoxin PIA binding.

α-Conotoxins PIA and MII have identical spacing of Cys residues, disulfide connectivity, and the SNPV (serine, asparagine, proline, valine) sequence in the first peptide loop. Therefore, it follows that differences in either the N terminal or loop 2 sequences account for the differences in selectivity between α6 and α3 subunits. In addition, whereas MII has some affinity for muscle and α4β2 subtypes (IC_{50}, 430 nM and 3.7 μM, respectively) (Cartier, 2002), PIA has no measurable effects on these subtypes at 10 μM (Figs. 2, 4).

Differences between α-conotoxin MII and α-conotoxin PIA also result in differences in off-time kinetics between the two toxins. Both toxins show a relatively long off-time for α6/α3β2β3 nAChRs (this study; our unpublished observations). In contrast, whereas α-conotoxin MII shows a relatively long off-time for α3β2 nAChRs (Cartier et al., 1996), block by α-conotoxin PIA reverses quickly (Fig. 5). Thus, block by α-conotoxin PIA of α6/α3β2β3 versus α3β2 nAChRs may be distinguished in two ways. The first is the 75-fold lower IC_{50} for α6/α3β2β3 nAChRs; the second is the prolonged k_{off} relative to α3β2. Off-rate kinetics for α-conotoxin PIA was dependent on the β subunit. The k_{off} for α-PIA was substantially longer for α6β4 than α6/α3β2. Likewise, the k_{off} for α3β4 was longer than that of α3β2 (Fig. 5). This suggests that α-conotoxin PIA has binding determinants on the β subunit, as demonstrated previously for α-conotoxin MII (Harvey et al., 1997). In addition, it indicates that the β subunit influences both the toxin on-rate and off-rate. The range of kinetic values for α-conotoxin PIA is consistent with that of α-conotoxins reported previously (Cartier et al., 1996; McIntosh et al., 2002). The IC_{50} of α-conotoxin PIA was also related to the β subunit. For both α3 and α6 subunits, the presence of a β2 versus β4 subunit led to a lower IC_{50} (Table 2).

To our knowledge, this is the first demonstration that α6 versus non-α6-containing nAChRs may be pharmacologically distinguished by an antagonist. This has implications for several pathophysiological states. The α6 subunit is highly expressed in CNS regions including the nigrostriatal pathway, which is pathologically affected in Parkinson's disease, vision-related nuclei, the locus ceruleus implicated in attention, and ventral tegimental area that modulates reward and addictive behavior (Le Novère et al., 1996, 1999; Vailati et al., 1999; Klink et al., 2001; Azam et al., 2002b; Champtiaux et al., 2002).

Table 3. nAChR α-subunit comparison

α6 Residue number	rα2	rα3	rα4	rα6	hα6
6	K	Q	K	H	H
11	G	D	G	H	H
20	P	A	A	E	E
27	I	I	L	T	T
33	S	S	S	A	A
39	D	K	D	N	N
55	Q	Q	Q	H	H
101	H	D	H	G	G
112	T	E	R	V	M
126	S	K	S	P	P
127	I	I	I	M	M
136	Q	Y	Q	H	H
141	M	M	M	L	L
152	K	K	K	E	E
164	L	L	Q	M	M
165	K	K	L	N	N
170	S	S	S	N	N
171	G	G	G	S	S
174	A	A	V	E	E
200	Y	—	A	S	S
202	V	—	I	Y	Y

r, Rat; h, human.

Indicated residues are those present in the α6 subunit but are neither identical nor homologous to the corresponding residue in the α2, α3, or α4 subunits.

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