Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels. α Subunits, together with β2 and/or β4 subunits, form ligand-binding sites at α/β subunit interfaces. Predatory marine snails of the genus Conus are a rich source of nAChR-targeted peptides. Using conserved features of the α-conotoxin signal sequence and 3′-untranslated sequence region, we have cloned a novel gene from the fish-Conus bullatus region, we have cloned a novel gene from the fish-H9251 previously unreported slow off-times, compared with the corresponding sites at H9252 meric probe for distinguishing between nAChRs with a 40,000-fold lower IC50 than αβ4 nAChRs. The kinetics of toxin unblock are dependent on the β subunit. nAChRs with a β4 subunit have very slow off-times, compared with the corresponding β2 subunit-containing nAChR. In each instance, rat αβ4 may be distinguished from rat αββ2 by the large difference in time to recover from toxin block. Similar results are obtained when comparing mouse αββ2 to mouse α3β4, and human α3β2 to human α3β4, indicating that the β subunit dependence extends across species. Thus, α-conotoxin BuIA also represents a novel probe for distinguishing between β2- and β4-containing nAChRs.

Acetylcholine acts on nicotinic acetylcholine receptors (nAChRs)1 to mediate fast excitatory neurotransmission or to modulate neurotransmitter release. nAChRs appear to be involved in pain sensation, attention, memory, learning, and development (1). Medications that affect nicotinic transmission may be useful for the treatment of pain, memory disorders, Parkinson’s disease, schizophrenia, and nicotine addiction. To maximize the ratio of therapeutic benefit to side effects, medications must discriminate between a plethora of subtypes of nAChRs.

Neuronal nAChRs are ligand-gated cationic channels composed of α and, in many cases, β subunits. These pentameric proteins have at least two ligand binding sites located at the interface of two subunits. For neuronal nAChRs, the ligand binding β subunit appears to be either β2 or β4. Both of these subunits are widely expressed, often within the same areas of the nervous system (reviewed in Refs. 2 and 3). Probes to distinguish among different α and β subunit-containing nAChRs are needed to identify which subtypes underlie the particular effects of nicotine.

Predatory marine snails of the genus Conus utilize nAChr antagonists to immobilize and capture their prey. The ~500 species of cone snails prey upon a broad diversity of organisms (five different phyla). Each Conus species appears to have a unique complement of nAChr antagonists, making Conus a rich source of novel ligands, often with unique specificities. In this report, we describe the cloning of a gene that encodes a novel α-conotoxin that distinguishes among α subunit-containing nAChRs and kinetically discriminates between β2- and β4-containing nAChRs.

**EXPERIMENTAL PROCEDURES**

Identification and Sequencing of a cDNA Clone Encoding α-Conotoxin BuIA—cDNA was prepared by a reverse transcription of RNA isolated from the Conus bullatus venom duct as described previously (4). 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 Applied Science) following the suggested protocol of the manufacturer. The eluted DNA fragment was annealed to plasmid pAMP1 vector, and the resulting product was transformed into competent DH5α cells, with the clone AMP pAMP System for Rapid Cloning of Amplification Products (Invitrogen) following the suggested protocols of the manufacturer. The resulting product was transferred into competent DH5α cells as described (4). 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 (5).

Chemical Synthesis—The peptide was synthesized, 0.45 mmol/kg, on a Forte one amino resin using Fmoc chemistry and standard side protection except on cysteine residues. Cys residues were protected in pairs with either S-trityl on Cysβ and Cysβ, or S-acetamidomethyl on Cysβ and Cysβ. The peptide was removed from the resin and precipitated. A two-step oxidation protocol was used to selectively fold the peptides as described previously (6). Briefly, the disulfide bridge between Cysβ and Cysβ 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 Cysβ and Cysβ was carried out by iodine oxidation. The monocyclic peptide and HPLC eluant was measured.
dripped into an equal volume of iodine (10 ml) in H2O/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 C4 Vydac column using a linear gradient of 0.1% trifluoroacetic acid 0.092% trifluoroacetic acid, 60% acetonitrile, retailer H2O.

**Mass Spectrometry**—Measurements were performed at the Salk Institute under the direction of Jean Rivier. MALDI-TOF/MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) was utilized.

**Voltage Clamp Recording**—Oocytes were harvested and injected with cRNA encoding nAChR subunits as described previously (7). The oocyte recording chamber was fabricated from Sylgard and was 300 μm in volume. Oocytes were gravity-perfused with ND96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 5 mM HEPES, pH 7.1–7.5)

![FIG. 1](image)

**FIG. 1.** Prepropeptide and encoded toxin of α-BuIA. Post-translational processing sites are indicated by the arrows, and the mature toxin is indicated. The glycine following the C-terminal cysteine in the arrow is indicated. The arrows translational processing sites are indicated by the arrows.

**FIG. 2.** Concentration response analysis of α-BuIA. A, α-BuIA was tested on β2-containing heteromeric nAChRs and homomeric α7 nAChRs heterologously expressed in Xenopus oocytes as described under “Experimental Procedures.” B, α-BuIA effect on β4-containing nAChRs. r, rat. Error bars are the S.E. Data are from 3–12 oocytes. IC50 and Hill slope values are shown in Table I.

**Table I**

| nAChR | IC50 (nM) | CI | Hill slope | CI |
|-------|-----------|----|------------|----|
| aβ2j2 | 800       | 567–1130 | 0.850     | 0.591–1.11 |
| aβ3j2 | 5.72      | 4.57–7.16 | 1.48      | 1.04–1.92 |
| aβ4j2 | 10,400    | 4,930–21,900 | 0.525 | 0.171–0.879 |
| aβ6α3j2 | 0.258 | 0.207–0.320 | 0.963 | 0.815–1.11 |
| α7    | 272       | 243–304  | 1.21      | 1.10–1.32 |
| aβ2j4 | 121       | 92.2–160 | 1.31      | 0.615–2.01 |
| aβ3j4 | 27.7      | 22.3–34.5 | 1.52      | 1.01–2.04 |
| aβ4j4 | 69.9      | 47.9–102 | 1.15      | 0.738–1.57 |
| aβ6α3j4 | 1.54   | 1.32–1.78 | 1.40      | 1.12–1.68 |

* 95% confidence interval.
average value ± S.E. of measurements from at least three oocytes. Data fits were performed with Prism software (GraphPad Software, San Diego, CA).

Cloning of Mouse α3, β2, and β4 cDNAs—The mouse β2 cDNA was cloned as described previously (9). For the cloning of the α3 and β4 cDNAs, first strand cDNAs were generated from 2 μg each, DBA/2Jbg adrenal gland total RNA and P19 teratocarcinoma cell total RNA using AMV reverse transcriptase (Promega, Madison, WI), 1× reverse transcriptase buffer (Promega) 200 μM each, dATP, dCTP, dGTP, and dTTP, 5 mM MgCl₂, and 2.5 μM random hexamers (Promega). cDNA synthesis was performed for 1 h at 42 °C. Following synthesis, the first strand cDNAs were purified using the Qiagen (Valencia, CA) PCR purification protocol according to the manufacturer's instructions. The α3 (1612 bp) and β4 (1578 bp) cDNAs subsequently were amplified from the DBA/2Jbg adrenal gland first strand cDNA and P19 teratocarcinoma cell first strand cDNA, respectively, using Pfu Turbo Polymerase (Stratagene, La Jolla, CA), 1× Pfu buffer, 200 μM each, dATP, dCTP, dGTP, dTTP, and 0.4 μM each, α3- and β4-specific forward and reverse primers.
Primers used for cDNA amplification were as follows: α3 (Forward: 5'-GCTTAGGTGCTTCGTTGTTG-3', Reverse: 5'-CTTTCTAGCCACAGGTAGAGC-3') and β4 (Forward: 5'-CTATTGGGTTAGGGGACCCGTCAGCC-3', Reverse: 5'-GTGGGTTATGATGACGACGTCAG-3'). Following amplification, cDNAs were inserted into the vector pCRBluntII-TOPO (Invitrogen) and sequenced. A minimum of 6 cDNAs per subunit were sequenced in order to determine the consensus sequence of each subunit cDNA. cDNA clones containing the consensus sequence subsequently were subcloned into either pcDNA3.1zeo (Invitrogen) or pcDNA3.1hygro (Invitrogen) (β4). Transcription with T7 RNA polymerase yielded sense-strand cRNA for injection into oocytes.

Construction of Rat α6/α3 Chimeras—cDNA clones encoding rat nAChR subunits were provided by S. Heinemann (Salk Institute, San Diego, CA) with plasmid constructs as described (10). The rat α6 subunit does not express with the rat β2 subunit (11). We therefore used a chimera that contains the N-terminal extracellular α6 subunit sequence linked to the remaining portion of the α3 subunit protein as a model of activity at α6 subunit-containing nAChRs. The α6/α3 chimera was provided by James Garrett (Cognetix Inc., Salt Lake City, UT). 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. The α6/α3 cDNA was constructed by introduction of BstEII sites at the chimeric junction into the α6 and α3 cDNA sequences using mutagenic primers to introduce restriction sites through silent codon changes. The α6 and α3 segments were generated by PCR of rat brain cDNA clones 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 BstEII and ligated to generate the chimeric construct. The final chimeric construct was cloned and completely sequenced to confirm the correct cDNA sequence. The protein sequence was an exact match to the rat α6 and α3 sequences in GenBank™, except for a valine to alanine change at amino acid 278 in the chimeric construct. To further improve expression levels, most of the 5'- and 3'-untranslated regions of the nAChR cDNA were deleted, leaving only 12 bp of 5'-UTR and 34 bp of 3'-UTR sequence. 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/α6α3, was transcribed with T7 RNA polymerase to generate sense-strand RNA for oocyte expression. To improve expression levels of the α6/α3 chimera, it was co-injected with β2 and β4 subunits (provided by Charles Luetje) that were engineered into the pGEMHE high expression vector as described (12).

**RESULTS**

Cloning of α-Conotoxin BuIA—In common with other known families of conotoxins, the α-conotoxins are proteolytically processed from a larger precursor protein. In the case of the α-conotoxins, this prepropeptide is ∼40 amino acids long, with the mature α-conotoxin moiety of 13–18 amino acids located at the C terminus of the precursor. A processing site, usually consisting of a basic amino acid, immediately precedes the mature toxin in the precursor sequence. An unusual feature of the conotoxins is that while the mature toxin peptides are highly variable in sequence, the precursor proteins are highly conserved. The signal sequence region is practically invariant among the different α-conotoxin precursors, and this remains true even for phylogenetically distant Conus species (13, 14). Also, the 3'-untranslated region of the α-conotoxin mRNA is highly conserved. We utilized the conserved features of the α-conotoxin gene structure to design oligonucleotide primers for polymerase chain reaction amplification of the α-conotoxin-coding region. The resulting cDNA clone from *C. bellus* is shown in Fig. 1.

Chemical Synthesis of α-BuIA—Solid phase chemical synthesis of the predicted mature toxin was undertaken. The glycine at the C terminus was assumed to be post-translationally modified to a C-terminal amide. It was also assumed that the disulfide bridging of α-conotoxin BuIA was analogous to all previously characterized α-conotoxins, that is, Cys² to Cys⁸ and

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**FIG. 4. Recovery from α-BuIA block in mouse and human nAChRs.** α-BuIA was perfusion-applied to oocytes expressing the indicated mouse and human nAChRs. Recovery is prolonged for the β4-containing nAChRs. *m*, mouse; *h*, human. Error bars are the S.E.
Cys$^3$ to Cys$^{13}$. Cys groups were orthogonally protected in pairs to direct disulfide bond formation in this configuration. Acid-labile S-trityl was removed simultaneously with peptide cleavage from the resin and closure of the disulfide bridge between these Cys residues was accomplished with FeCN. The monocyclic peptide was purified by HPLC, and the acid-stable acetomidomethyl groups were removed, and the disulfide bridges formed by iodine oxidation. The folded peptide was subsequently analyzed with matrix-assisted laser desorption mass spectrometry. The mass of the synthetic peptide was consistent with the amidated sequence (monoisotopic MH$^+$ calculated, 1311.5; observed, 1311.4).

Peptide Effect on nAChRs—$\alpha$-Conotoxin BuIA was tested on various subunit combinations of neuronal nAChRs heterologously expressed in Xenopus oocytes. Concentration response analysis indicated that, unlike some $\alpha$-conotoxins, $\alpha$-BuIA was active against a broad spectrum of nAChR subtypes. The $\alpha$ nAChR subunit had a profound influence on the effect of BuIA. The peptide was most potent on nAChRs containing the extracellular portion of the $\alpha 6$ subunit and next most potent on the closely related $\alpha 3$ subunit-containing nAChRs. The ligand had little effect on $\alpha 4\beta 2$ nAChRs (IC$_{50} > 10 \mu M$). Results are shown in Fig. 2 and Table I.

Effects of BuIA on $\beta 2$- versus $\beta 4$-containing nAChRs—The rate of unblock by toxin was monitored subsequent to washout of ligand. The results are shown in Fig. 3. Recovery from toxin block was markedly slower for $\beta 4$ versus $\beta 2$ subunit-containing nAChRs. This effect is particularly noticeable when nAChRs containing the same $\alpha$ subunit are compared. We further investigated this effect on mouse $\alpha 3\beta 2$ versus mouse $\alpha 3\beta 4$ nAChRs, and human $\alpha 3\beta 2$ and human $\alpha 3\beta 4$ nAChRs. As was the case for rat nAChRs, the rate of recovery from toxin block was significantly slower for $\beta 4$- versus $\beta 2$-containing nAChRs (Fig. 4).

Although the off-rate for $\beta 4$-containing nAChRs was much slower, surprisingly in several instances the corresponding IC$_{50}$ values of $\alpha$-BuIA for $\beta 2$-containing nAChRs was lower than that of the $\beta 4$-containing receptors. Since affinity is a ratio of off-rate to on-rate, this implies that the on-rate for $\beta 2$-containing nAChRs is faster than that of the corresponding $\beta 4$-containing nAChRs. Time to steady state for toxin block of rat $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs was examined by perfusing the toxin over the oocyte and then assessing a response to ACh (Fig. 5). The results are consistent with a markedly faster on-rate for the $\beta 2$-containing receptor. For $\alpha x\beta 2$ nAChRs the recovery $t_{1/2}$ was longest for $\alpha 6/\alpha 3\beta 2$ nAChRs. The $K_b$ of functional block of $\alpha 6/\alpha 3\beta 2$ nAChRs, calculated from $k_{off}/k_{on}$ (Fig. 6A) is consistent with the IC$_{50}$ determined from concentration response analysis (0.69 nM versus 0.26 nM respectively). Kinetics of block and unblock were difficult to accurately quantitate for $\alpha x\beta 4$ receptors due to the very long on- and off-times combined with limitations of oocyte life span and a tendency for the ACh response to drift over extended periods of time. However, kinetic constants for the $\alpha 2\beta 4$ nAChR were determined (Fig. 6B) and the calculated $K_b$ of functional block by BuIA (62.9 nM) is consistent with the IC$_{50}$ value (121 nM) determined by concentration response experiments. We further assessed kinetic constants for all $\alpha x\beta 4$ nAChRs by determining $k_{on}$ of block by three different toxin concentrations (Fig. 6C). The $k_{on}$ and $k_{off}$ for $\alpha 2\beta 4$ were $1.99 \times 10^5$ min$^{-1}$ M$^{-1}$ and 0.0125 min$^{-1}$, respectively, and this compares well to the value determined from the toxin wash in and washout experiments ($1.84 \times 10^5$ min$^{-1}$ M$^{-1}$ and 0.0106 min$^{-1}$, Fig. 6B). The $K_b$ values determined by this method were also consistent with IC$_{50}$ values determined by concentration response analysis (Fig. 6C and Table II). However, whereas the on-rates determined by this method had reasonable 95% confidence intervals, the off-rates did not. The off-rate is determined by the y-intercept and for these toxins the y-intercept is near the origin (Fig. 6C). When the confidence interval includes the origin, the off-rate range becomes infinite. Therefore, as an additional check, we estimated off-rates by using the $k_{on}$ value determined by linear regression analysis of $k_{obs}$ values (Fig. 6C) and by assuming that the $K_b$ is approximately equal to the IC$_{50}$. $k_{off}$ values determined by this method were consistent with those obtained by the analysis of $k_{obs}$ values (Table II). For $\alpha 2\beta 2$, $\alpha 3\beta 2$, and $\alpha 4\beta 2$ nAChRs the toxin off-rate was rapid ($t_{1/2} < 1$ min, see Fig. 2); we therefore were unable to further quantitate $k_{off}$ for these receptor subtypes due to receptor desensitization that occurs from ACh exposure more frequent than once per minute.

**DISCUSSION**

*C. bullatus* is found from Mozambique and Zanzibar to Marquesas and Hawaii. It lives in muddy sand, coral rubble and gravel, often beneath dead coral rocks outside and inside the reef. It is known to feed on fish and molluscs, and is preyed upon by skates and stingrays as well as mollusc-eating cone snails (15). In this report, we describe the first
toxin to be isolated from this species. The toxin-encoding gene is homologous to peptides that are members of the A-superfamily of *Conus* toxins. This superfamily consists of peptides that act on nAChRs, potassium channels and sodium channels (14, 16, 17).

α-Conotoxin BuIA is a 13-amino acid peptide with homology to α-conotoxins isolated from other cone species. It is unusual however in the spacing between Cys residues. Previously isolated α-conotoxins fall into three broad categories. There are those that are referred to in the literature as having a 3/5 spacing, indicating that there are three and five amino acids, respectively, between Cys residues in the two loops of the toxin; these are paralytic toxins isolated from Indo-Pacific cone snails that hunt fish, and potently inhibit the muscle nicotinic receptor. A second group is made up of those toxins having a 4/7 spacing; these conotoxins predominately target neuronal subtypes of nicotinic receptors. The third group are the 4/3 peptides, isolated thus far only from *Conus imperialis* (18, 19) (Table III). α-Conotoxin BuIA has unusually broad specificity for different subtypes of neuronal nAChRs compared with previously characterized α-conotoxins. It is unknown whether the unique 4/4 spacing influences specificity.

Total chemical synthesis of the new peptide was carried out assuming the disulfide bond configuration of previously characterized α-conotoxins. We note that native α-conotoxin BuIA has never been isolated from venom. Therefore, it is possible that there are post-translational modifications present in the native peptide that could influence the properties reported for the synthetic peptide described in this report.

α-Conotoxin BuIA distinguishes among αβ2 nAChRs with a rank order potency of α6>α3>α2>α4 and there is a greater than 40,000-fold difference in IC_{50} between α6/α3β2 and α4β2 nAChRs. The α6 subunit appears to be important in both normal and pathophysiological conditions. α6 is expressed in catecholaminergic neurons and in retina (20, 21). α6β2* nAChRs appear to be involved in the modulation of dopamine release (22) and are decreased in both primate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine models as well as human Parkinson’s disease (23, 24).

Another striking feature of α-conotoxin BuIA is its ability to discriminate, on the basis of off-rate kinetics, between β2- and β4-containing nAChRs. Off-rates are very slow for β4-containing nAChRs, in contrast to the relatively rapid off-rates for β2-containing receptors. The β2 and β4 subunits are found within both the central and peripheral nervous systems. Within the central nervous system, the transcripts for the β2 subunit have widespread expression, whereas the β4 subunit expression is more restricted. The areas within the brain where these two subunits are co-localized include the habenula-interpeduncular pathway, the locus ceruleus, and a few structures within the sensory and motor areas of the brainstem (25, 26). Within the peripheral nervous system, both β2 and β4 subunits are found in the autonomic ganglia (27, 28) and contribute to autonomic ganglionic neurotransmission (29–31). Knockout studies in mice suggest a more prominent role for the β4 subunit in regulation of certain visceral functions, such as cardiac and intestinal autonomic regulation and bladder contractility (29, 31, 32). However, mice that lack either the β4 or the β2 subunit grow to adulthood with no visible phenotypical abnormalities, thus indicating a degree of redundancy between

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F = k_{on} \times C + k_{off}
\]

where \(C\) is the free toxin concentration, the graph of \(F\) versus \(C\) has a slope of \(k_{on}\) and a y-intercept of \(k_{off}\).
the β2 and β4 subunits (28, 29, 31, 32). Only the deletion of both the β2 and β4 subunits is lethal (29), and results in death soon after birth, therefore suggesting important contributions of both subunits to autonomic function. However, since few ligands can distinguish between the two subunits, it has been difficult to determine the exact contribution of each subunit to the ligand binding interface. The binding of other ligands is difficult to determine the exact contribution of each subunit to subunit-dependent ligand selectivity (34). The agonist cytisine selectively activates rat α4β2 versus α3β2 nAChRs expressed in Xenopus oocytes (35). k-Bungarotoxin blocks rat α3β2 nAChRs with slow off-rate kinetics compared with α3β4 nAChRs. Subunit residue number 59 (threonine in β2 and lysine in β4) appears to be the major determinant of k-bungarotoxin binding differences between these subunits (36). Cocaine, in addition to blocking the dopamine transporter, preferentially blocks β4-containing versus β2-containing nAChRs (37). Likewise, substance P, a small peptide that acts at neurokinin type 1 receptors, also noncompetitively blocks nAChRs with a 20- to 30-fold higher affinity for β4- versus β2-containing rat nAChRs (38). Species differences in nAChR subunits may have substantial effects on toxin binding. For example, dihydro-β-erythroidine blocks rat α3β2, α4β2, and α4β4 nAChRs expressed in Xenopus oocytes with nearly equal IC₅₀ values (in μM, 0.41, 0.37, and 0.19, respectively) (39). In contrast, dihydro-β-erythroidine blocks human α3β2, α4β2, and α4β4 nAChRs expressed in Xenopus oocytes with ~10–100-fold differences in IC₅₀ values (in μM, 1.62, 0.11, and 0.01, respectively) (40). For this reason, we examined the off-rate kinetics of block by α-conotoxin BuIA in three species. We demonstrate in this report that the ability of α-conotoxin BuIA to kinetically discriminate between α3β4 and α3β2 nAChRs is true for rat, mouse, and human subtypes as expressed in Xenopus oocytes. For each species, the t½ is less than 1 or 2 min for the α3β2 subtype, and greater than 30 min for the α3β4 subtype. Previously analyzed α-conotoxins have been shown to bind to determinants on both the α and β subunits of the nAChR. Receptor mutation analysis indicates that α-conotoxin MII interacts with Lys¹⁸⁵ and His¹⁸⁸ of the α3 subunit, and Thr³⁹ of the β2 subunit (41). Analysis of α-conotoxin PnIA indicates that it interacts with overlapping but distinct residues on the α3 subunit, including Pro¹⁸², Ile¹⁸⁸, and Gly¹⁹⁸ (42). It should be possible using a similar approach to identify residues on the β4 subunit that confer slow off-rate kinetics for α-conotoxin BuIA.

| α-Conotoxin | Sequence | nAChR preference | Ref. |
|-------------|----------|------------------|------|
| α4/4        | BuIA     | GCSTPPPCAVLYC⁵   | See Table I | This work |
| α3/5        | MI       | GRCHPPACGKNSC⁶   | mααβ, tααγ | (43, 44) |
|             | GI       | ECCNAPACGRHYSC⁷  | mαβ, tαγ  | (45, 46) |
| α4/7        | MII      | GCCSNPVCHLEHSNLC⁹ | roα3β2, roβ6x | (7, 11) |
|             | PsIA     | GGCSSPLCPAAANPPYC⁹ | roβ6 > roα1 | (48) |
|             | PIA      | RDPCCSNPVCTHNPYC⁹ | roβ6 > α3β2 | (11) |
|             | GIC      | GGCSPAPGNNQHC⁹   | raβ3β2 > raβ6 | (49) |
|             | Epl      | GCCSDRCCMNNPIDY⁵⁵ | intracardiac ganglia | (50) |
|             | GID      | IRDYCCSNPACVNNOHVC | α7 > α3β2 > α4β2 | (51) |
|             | EI       | RDCCYHTCCMNPSNPC⁹ | mαβ > maαγ, taαβ | (44) |
| α4/3        | ImI      | GCSDSPCRACWRC⁶   | ra7 > ra9 | (10)  |
|             | ImII     | ACCDRRRCRWC⁶     | ra7⁷ | (19)  |

⁵ Amidated C terminus.
⁶ m, mouse.
⁷ t, Torpedo.
¹⁵ r, rat.
¹⁶ Y, sulfotyrosine.
¹⁷ Y, γ-carboxyglutamate.
