α-Conotoxin AuIB Isomers Exhibit Distinct Inhibitory Mechanisms and Differential Sensitivity to Stoichiometry of α3β4 Nicotinic Acetylcholine Receptors

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Non-native disulfide isomers of α-conotoxins are generally inactive although some unexpectedly demonstrate comparable or enhanced bioactivity. The actions of “globular” and “ribbon” isomers of α-conotoxin AuIB have been characterized on α3β4 nicotinic acetylcholine receptors (nAChRs) heterologously expressed in Xenopus oocytes. Using two-electrode voltage clamp recording, we showed that the inhibitory efficacy of the ribbon isomer of AuIB is limited to ~50%. The maximal inhibition was stoichiometry-dependent because altering α3β4 RNA injection ratios either increased AuIB(ribbon) efficacy (10αc:1β) or completely abolished blockade (1αc:10β). In contrast, inhibition by AuIB(globular) was independent of injection ratios. ACh-evoked current amplitude was largest for 1:10 injected oocytes and smallest for the 10:1 ratio. ACh concentration-response curves revealed high (HS, 1:10) and low (LS, 10:1) sensitivity α3β4 nAChRs with corresponding EC₅₀ values of 22.6 and 176.9 μM, respectively. Increasing the agonist concentration antagonized the inhibition of LS α3β4 nAChRs by AuIB(ribbon), whereas inhibition of HS and LS α3β4 nAChRs by AuIB-(globular) was unaffected. Inhibition of LS and HS α3β4 nAChRs by AuIB(globular) was insurmountable and independent of membrane potential. Molecular docking simulation suggested that AuIB(globular) is likely to bind to both α3β4 nAChR stoichiometries outside of the ACh-binding pocket, whereas AuIB(ribbon) binds to the classical agonist-binding site of the LS α3β4 nAChR only. In conclusion, the two isomers of AuIB differ in their inhibitory mechanisms such that AuIB(ribbon) inhibits only LS α3β4 nAChRs competitively, whereas AuIB-(globular) inhibits α3β4 nAChRs irrespective of receptor stoichiometry, primarily by a non-competitive mechanism.

Conotoxins are short disulfide-rich bioactive peptides that have been originally isolated from venoms of carnivorous mollusk cone snails, belonging to the genus Conus. α-Conotoxins are among the largest class of conotoxins found in the venom of most cone snail species (1). This class of conotoxins targets various subtypes of nicotinic acetylcholine receptors (nAChRs)² and is distinguished by four cysteines arranged in a CC-C-C pattern.

α-Conotoxins have attracted considerable attention as some of them, such as Vc1.1 and RgIA, have been shown to possess analgesic activity in rodent behavioral models of neuropathic pain (2, 3). Interestingly, AuIB has recently been shown to be analgesic in vivo despite the fact that it acts on the α3β4 nAChR subtype different from the α9α10 nAChR targeted by Vc1.1 and RgIA.³ Vc1.1 and RgIA have been shown to suppress N-type Ca²⁺ channel currents in dorsal root ganglion (DRG) neurons of neonatal and adult rats and wild type and α9 knock-out mice via activation of GABA₆ G protein-coupled receptors (2). Similarly, AuIB inhibits N-type Ca²⁺ channels in rat DRG neurons analogous to Vc1.1 and RgIA and its effect can be blocked with selective GABA₆ receptor antagonists.⁴ GABA₆-mediated inhibition of N-type Ca²⁺ channels is proposed as an analgesic mechanism for α-conotoxins Vc1.1, RgIA, and AuIB (2), which can reconcile obvious differences in their nAChR subtype selectivity.

α-Conotoxin AuIB has been characterized on oocyte-expressed nAChRs and shown to be selective primarily for the α3β4 nAChR subtype (3). The α3β4 nAChR subtype is a predominant subtype in autonomic ganglia, adrenal medulla, and in subpopulations of central nervous system neurons such as medial habenula and dorsal medulla (4). AuIB is a 15-residue conotoxin with an unusual 4/6 intercysteine spacing. Native AuIB peptide found in the venom, like the vast majority of other α-conotoxins, has 2–8, 3–15 (Cys₁-Cys₄, Cys₂-Cys₄) cystine globular connectivity (5). When AuIB is synthesized chemically, a disulfide bond isomer having 2–15, 3–8 (Cys₁-Cys₄, Cys₂-Cys₄) connectivity is co-produced as a by-product, which is the ribbon isomeric of AuIB (Fig. 1A) (5). Surprisingly, the ribbon isomer of AuIB has been shown previously to be ~10-fold more potent at nAChRs of rat parasympathetic ganglion neurons compared with the globular (native) peptide isoform (5). However, when AuIB(ribbon) was probed on rat nAChRs heterologously expressed in Xenopus oocytes it was reported to be less active than the globular isomer (6). The difference in

² The abbreviations used are: nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine-binding protein; HF, hydrogen fluoride; HS, high sensitivity α3β4 nAChR; LS, low sensitivity α3β4 nAChR; RP-HPLC, reversed phase-high performance liquid chromatography; PDB, Protein data bank.
³ M. J. Christie, personal communication.
⁴ B. Callaghan and D. J. Adams, unpublished observations.
**Stoichiometry-dependent Block of α3β4 nAChRs by AuIB Isomers**

FIGURE 1. A, diagram showing the disulfide bond connectivity of the globular and ribbon isomers of α-conotoxin AuIB. B, analytical RP-HPLC trace of the crude oxidation profile of AuIB in 0.1 M NH₄HCO₃ buffer at pH 8.2 showing that ribbon and globular isomer are formed in a similar ratio. Inset, mass spectrometry confirms the folding of AuIB. C, circular dichroism spectra of the two AuIB isomers and their respective three-dimensional structures (inset) (PDB 1MXN and 1MXP), showing the loss of the secondary structure (α-helix) in the ribbon isomer.

activity of the two AuIB isomers on native versus recombinant nAChRs remains to be elucidated. Taken together, there is an incomplete understanding of diversity of mechanisms of action of α-conotoxin AuIB and its isoforms. Here, we further explore the inhibitory mechanisms of globular and ribbon isomers of AuIB on rat α3β4 nAChRs expressed in *Xenopus* oocytes.

Manipulations of the α3β4 subunit ratios show that α3β4 nAChRs expressed in oocytes are present in different subunit stoichiometries and inhibition by AuIB(ribbon), but not AuIB(globular), is limited to one of the receptor stoichiometries. Inhibition by AuIB(ribbon) is consistent with a competitive antagonism, whereas AuIB(globular) inhibits α3β4 nAChRs via a non-competitive mechanism.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—The peptide was assembled on a 4-methylbenzhydrylamine resin by manual tert-butoxycarbonyl solid-phase peptide synthesis using 2-(1H-benzoazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-mediated *in situ* neutralization protocol with *N*,*N*-dimethylformamide as solvent (7). HF deprotection and cleavage was performed by treatment of the dried peptide resin (300 mg) with 10 ml of HF/p-cresol/p-thio-cresol (18:1:1, v/v/v) for 2 h at 0 °C. Following evaporation of the HF, the peptide was precipitated and washed with cold ether, filtered, and re-dissolved in 30 ml of 50% acetonitrile, 1% trifluoroacetic acid, and lyophilized. Oxidative folding was carried out in 0.1 M NH₄HCO₃ (pH 8.2, concentration = 0.1 μM) resulting in formation of the globular and ribbon isomers of AuIB (Fig. 1B). Oxidation was monitored by RP-HPLC, liquid chromatography mass spectrometry, and mass spectrometry, and the peptide isomers were isolated using preparative C₁₈ RP-HPLC.

**Circular Dichroism (CD) Spectroscopy**—CD spectroscopy was performed on a Jasco J-810 spectropolarimeter. Spectra were recorded at room temperature under nitrogen atmosphere. Peptides were dissolved in 20 mM phosphate buffer, containing 30% trifluoroethanol at pH 7. The peptide concentration was determined by quantitative RP-HPLC. The peptides were transferred into a 0.01-cm path length demountable cell and data were recorded over 5 scans, from 260 to 185 nm at 10 nm/min, with a resolution of 1 nm and a response time of 0.25 s. CD data in ellipticity was converted to mean residue ellipticity ([θ]R) using the equation: [θ]R = θ/(10 × C × Np × l), where θ is the ellipticity in millidegrees, C is the peptide molar concentration (M), l is the cell path length (cm), and Np is the number of peptide residues. CD was used to confirm the globular and ribbon AuIB structure. AuIB(globular) showed CD spectra with α-helical content as opposed to that of AuIB(ribbon), which has less secondary structure (Fig. 1C).

**RNA Preparation**—Plasmid DNAs encoding rat α3 and β4 nAChR subunits were obtained from J. Patrick (Baylor College of Medicine, Houston, TX). After multiplication plasmid DNA was linearized with appropriate restriction enzymes and cRNA was synthesized *in vitro* using a SP6 *in vitro* transcription kit (mMessage mMACHINE; Ambion, Foster City, CA). RNA for α3 and β4 subunits was synthesized in parallel on the same day using identical procedures to maximize consistency between subunits in concentration and purity. RNA concentration was controlled spectrophotometrically for each new aliquot of RNA and before injections. Total amount of RNA injected per oocyte was ~5 ng in ~50 nl/volume. Stage V to VI oocytes obtained from *Xenopus laevis* were subsequently incubated for 2–8 days at 18 °C before electrophysiological recordings as described previously (6).

**Electrophysiological Recordings and Data Analysis**—Oocytes were transferred to the recording chamber (~50 μl volume) and perfused at 3–5 ml/min with ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5) by a gravity fed perfusion system. nAChR-mediated currents were evoked by pipetting 100 μl of ACh-containing solutions into the bath with the perfusion stopped. Oocytes were preincubated with the peptide for ~5 min prior to ACh application and subsequently ACh was co-applied together with the peptide. Two-electrode voltage clamp recordings from *Xenopus* oocytes were made at room temperature (21–23 °C) using a
Stoichiometry-dependent Block of α3β4 nAChRs by AuIB Isomers

GeneClamp 500B amplifier (Molecular Devices Corp., Sunnyvale, CA) at a holding potential of −80 mV. ACh-induced currents were recorded and their peak amplitudes were measured with pClamp 9 software (Molecular Devices Corp.). ACh-evoked currents recorded following exposure to peptides were normalized to an average of 2–6 control ACh-induced currents. Data from several experiments were pooled and each data point represents the average of 3–8 cells ± S.E. Estimates of toxins potencies (IC50, n50) were obtained by fitting data points to the equation: % response = 100/[1 + ([toxin]/IC50)n50] with SigmaPlot8 (Systat Software Inc., San Jose, CA).

Molecular Modeling and Docking—The molecular model of rat α3 subunit was generated using α1 nAChR subunit (Protein Data Bank (PDB) code 2QCI (8)). The rat β4 subunit homology model was generated using the β chain of Torpedo acetylcholine receptor (PDB ID 2BG9 (9)), as several attempts by using high resolution AChBP crystal structures were not satisfactory. The open and closed C-loop conformations of α3 subunit were resembled using co-crystal structures of AChBP in complex with different molecular sizes of ligands (Ac-AChBP- Tx1A(A10L) (PDB code 2UZ6 (10)), Ac-AChBP-methyllycaconitine (PDB code 2BYR (11)), Ac-AChBP-nicotine (PDB code 1UW6 (12)), and Ls-AChBP-HEPES (PDB code 1I9B (13)). Templates and interfaces were assembled using Aplysia californica acetylcholine-binding protein (PDB code 2BR8 (14)) as the template, with programs Modeler 9 version 2 (15) and Sculptor, and/or by docking simulations using the program HEX 5.1 (16). All sequence alignments were generated using ClustalW (17) and were further manually adjusted based on secondary structure alignment. The subunit α3 and β4 and the pentamers (α3)β4 and (α3)3β4 were validated individually using online server Verify3D (18) and Ramachandran plot available from ProFunc data base (19). The solvent accessible area of each interface was calculated using online server CASTp (20). In addition, we further validated our homology models by performing a global docking simulation with ACh. The outcome showed that ACh only docked to the classical agonist binding site in the α(+)/β(−) interface and was unaffected by subunit stoichiometry (supplemental Fig. S1).

Each of the top 20 lowest energy NMR structures of globular (PDB code 1MXN (5)) and ribbon (PDB code 1MXP (5)) AuIB were docked to all (α3)β4 and (α3)3β4 pentamer models (assembled by both approaches with different C-loop conformations) individually using the program HEX 5.1, followed by energy minimization. The solutions that disagreed with the known constraints, such as binding at the transmembrane region or inside the ion permeation pathway were excluded. All docking simulation with (α3)β4 and (α3)3β4 pentamer models were successful, except for docking of ribbon AuIB to (α3)3β4. Global docking (without energy minimization) of ribbon AuIB to (α3)3β4 pentamer showed that ribbon AuIB docked in the C-loop binding site. However, no solution could be obtained after energy minimization. Therefore, we tried the α(+)/β(−) dimer extracted directly from the (α3)β4 pentamer models, an approach used previously for docking simulations of AChBP-derived models of nAChRs. The docking solutions after energy minimization showed that ribbon AuIB docked at the same location as the global docking in the (α3)β4 pentamer. For consistency, dimer extracted from (α3)β4 and (α3)3β4 pentamer models were also used for docking simulation with globular and ribbon AuIB. Outcomes of docking simulations of globular and ribbon AuIB to dimer (α3)β4 and (α3)3β4 models are consistent with results obtained using pentameric models. The top 20 energetically favorable docking solutions of each combination were selected and presented in Fig. 8.

RESULTS

Concentration-Response Relationships Obtained for Inhibition of ACh-evoked Currents by Globular and Ribbon Isomers of AuIB—Concentration-response curves were obtained for the inhibition of rat α3β4 nAChRs expressed in Xenopus oocytes by globular and ribbon isomers of AuIB α-conotoxin AuIB. Note that maximal block (efficacy) of the ribbon isomer is only −50%.

FIGURE 2. A, representative superimposed ACh-evoked currents obtained in the absence (Control) and presence of AuIB(globular) and AuIB(ribbon). The time course of ACh application is indicated by the bars above the current traces. B, concentration-response curves for inhibition of rat α3β4 nAChRs expressed in Xenopus oocytes by globular and ribbon isomers of α-conotoxin AuIB. Note that maximal block (efficacy) of the ribbon isomer is only −50%.

(α3)β4 pentamer. For consistency, dimer extracted from (α3)β4 and (α3)3β4 pentamer models were also used for docking simulation with globular and ribbon AuIB. Outcomes of docking simulations of globular and ribbon AuIB to dimer (α3)β4 and (α3)3β4 models are consistent with results obtained using pentameric models. The top 20 energetically favorable docking solutions of each combination were selected and are presented in Fig. 8.
2.48 and 0.77 μM, respectively (Table 1). We hypothesized that the difference in inhibitory action of the two AuIB isomers may be due to specific action of the AuIB(ribbon) on one of the two likely ((α3)2(β4)3 versus (α3)2(β4)2) α3β4 nACHR stoichiometries. The concentration–response curves shown in Fig. 2 were obtained on oocytes injected with equal amounts of α3 and β4 RNA (1:1 injection ratio). Theoretically, a 1:1 injection ratio may yield equal occurrence of the (α3)2(β4)3 and (α3)2(β4)2 stoichiometries, which would result in maximal inhibition of 50% of the whole cell ACh-evoked current amplitude if blockade of α3β4 nACHRs by AuIB(ribbon) is stoichiometry dependent.

Different α3:β4 Ratios Reveal Stoichiometry-dependent Inhibition of α3β4 nACHRs by AuIB(ribbon) but Not AuIB-(globular) Isomer—To probe the stoichiometry sensitivity of α3β4 nACHR inhibition by AuIB isomers, experiments were carried out on oocytes injected with different amounts of RNA encoding α3/β4 subunits. Concentration-response curves for inhibition of ACh-evoked currents by AuIB isomers in oocytes injected at 10:1 versus 1:10 α3:β4 ratios are shown on Fig. 3A. The efficacy of inhibition (maximal block) by AuIB(ribbon) at α3-dominant (10α:1β) nACHRs increased to 73.2 ± 3.0% (n = 8, p < 0.001) at 10 μM (Fig. 2A) but the IC50 of 0.86 μM remained approximately the same as for 1:1 injected oocytes (IC50 = 0.77 μM, Table 1). In contrast, in β4-dominant (1α:10β) nACHRs, the ribbon isoform of AuIB exhibited a dramatic decrease in inhibitory effect averaging only 14.2 ± 1.1% maximal block at 30 μM AuIB(ribbon) (Fig. 3A, panel ii). In comparison, maximal block by AuIB(globular) was unaffected by varying the α3:β4 ratios at both α3-dominant (10:1 ratio) nACHRs and β4-dominant (1:10 ratio) nACHRs (Fig. 3A, panels i and ii). We note that nACHR inhibition by AuIB(ribbon) in oocytes injected with the 1:10 ratio of mRNAs was incomplete (Fig. 3B, panel ii). This incomplete block likely reflects expression of a population of (α3)2(β4)3 receptors insensitive to the ribbon isomer (Fig. 3A, panel ii).

Comparison of the concentration-response relationships obtained from oocytes with different α3β4 nACHR subunit ratios indicates distinct modes of action of AuIB(ribbon) versus AuIB(globular) (Fig. 3). Shifting α3:β4 ratios did not change the maximal inhibition as well as the other parameters of the AuIB-(globular) concentration–response curve (Fig. 3B, panel i). Half-maximal inhibitory concentrations (IC50) and Hill slopes (nH) obtained for AuIB(globular) inhibition of ACh-evoked currents were 1.1 (1.38), 2.48 (1.37), and 3.0 μM (1.0) for 10:1, 1:1, and 1:10 ratios, respectively (Table 1). In contrast, when the subunit ratio changed from 10:1 to 1:10, the concentration-response curve of AuIB(ribbon) flattened out to exhibit no significant inhibition at β-dominant oocytes (Fig. 3B, panel ii). These results suggest an intrinsic difference in the mechanisms of action of the two isomers of α-conotoxin AuIB.

α3β4 Subunit Ratio Affects ACh-evoked Current Amplitudes and ACh Concentration-Response Curves—Varying the α3:β4 subunit ratio in oocytes in either direction had a marked effect on the ACh-induced current amplitude. Application of 50 μM ACh elicited the largest currents in oocytes injected at a 1:10 ratio, and the smallest current amplitudes in oocytes injected with a 10:1 α3:β4 ratio. Injecting all three different ratios of RNA in oocytes resulted in a significant difference between the average ACh-evoked current amplitudes in the three

### TABLE 1

Comparison of concentration-response curves parameters of AuIB analogues obtained in oocytes injected with different α3 and β4 nACHR subunit ratios

| α3:β4 ratio | AuIB(globular) | AuIB(ribbon) |
|-------------|----------------|--------------|
|             | IC50 (μM) | nH | IC50 (μM) | nH |
| 10:1        | 1.1        | 1.38 | 0.86  | 0.94 |
| 1:1         | 2.48       | 1.37 | 0.77   | 0.97 |
| 1:10        | 3.0        | 1.0  | NA     | NA   |

a Maximal efficacy ~50%. Data were acquired with 50 μM ACh used as agonist.
b NA, not active.

**FIGURE 3.** Different α3:β4 RNA injection ratios reveal dependence of AuIB(ribbon) inhibition on α3β4 nACHR stoichiometry and indicate the difference in the inhibitory mechanisms of the two AuIB isoforms. A, concentration-response curves obtained for AuIB(globular) and AuIB(ribbon) inhibition of α3- (A, panel i) and β-dominated oocytes (A, panel ii) (10:1 versus 1:10 α3:β4 ratio, respectively). B, concentration-response curves obtained for AuIB(globular) (B, panel i) and AuIB(ribbon) (B, panel ii) inhibition in oocytes injected with different α3:β4 ratios. Note the efficacy change for the ribbon isomer at different α3:β4 injection ratios and absence of this effect in AuIB(globular) concentration-response curves.
Stoichiometry-dependent Block of $\alpha3\beta4$ nAChRs by AuIB Isomers

![Graph A](image)

![Graph B](image)

FIGURE 4. A, perturbing 1:1 $\alpha3\beta4$ ratio alters amplitudes of ACh-induced currents. A, bar graph of the averaged currents induced by 50 $\mu$M ACh in all three groups of oocytes. B, ACh concentration-response curves for all three $\alpha3\beta4$ injection ratios. Note that 50 $\mu$M ACh used in previous experiments is below $EC_{50}$ in 1:1 and above $EC_{50}$ in 1:10 injected oocytes.

The average amplitude of ACh-evoked currents in the 10:1 ratio injected oocytes was 1.66 ± 0.14 $\mu$A and 13.74 ± 2.26 $\mu$A in 1:1, and 23.33 ± 3.48 $\mu$A in 1:10 ($n = 17–21$). The current amplitudes in the 10:1 and 1:10 ratio injected oocytes were significantly different from the current amplitudes obtained for the 1:1 ratio injected oocytes ($p < 0.001, 10:1; p < 0.05 1:10$, unpaired t test) (Fig. 4A). In a series of experiments on 10:1 ratio injected oocytes, 2.5 mM ACh elicited current amplitudes similar to those observed for 1:10 injected oocytes (data not shown) indicating that the smaller current observed in response to 50 $\mu$M ACh in 1:10 oocytes was not due to reduced nAChR expression.

We hypothesized that the sensitivity to ACh ($EC_{50}$) may account for different current amplitudes observed for the three different subunit ratios. To test this possibility, ACh sensitivity of oocytes injected with different $\alpha3\beta4$ subunit ratios was examined. The ACh concentration-response curves obtained for 10:1 and 1:10 ratio groups were strikingly different with an $EC_{50}$ of 176.9 ± 23.9 $\mu$M ($n = 4–7$) for 10:1 ratio injected oocytes and 22.56 ± 14.1 $\mu$M ($n = 3–8$) for the 1:10 oocyte group (Fig. 4B). The Hill slope was also different between the two ratio groups (2.1 ± 0.5 for 10:1 and 1.00 ± 0.1 for 1:10) suggesting that the 10:1 $\alpha3\beta4$ nAChR may bind 2 ACh molecules, whereas the 1:10 $\alpha3\beta4$ nAChR binds a single agonist molecule (Fig. 4B). The $\beta$-dominated nAChRs (1:10) are high sensitivity receptors and $\alpha$-dominated nAChRs (10:1) are low sensitivity $\alpha3\beta4$ nicotinic receptors. The ACh concentration-response curve for the 1:1 injected oocytes was fit with an $EC_{50}$ of 92.8 ± 6.6 $\mu$M and $n_H = 1.3 ± 0.1$ ($n = 3–8$), which was between the values obtained for 1:10 and 1:10 ratio groups (Fig. 4B).

The parameters obtained from the ACh concentration-response curves for the three subunit ratios are summarized in Table 2. These findings indicate that the different stoichiometries of $\alpha3\beta4$ nAChRs expressed in oocytes underlie the differences in $EC_{50}$, Hill slope, and ACh-evoked current amplitudes observed for the three groups.

### Table 2

Agonist sensitivity of different $\alpha3\beta4$ nAChR stoichiometries

| $\alpha3\beta4$ ratio | $EC_{50}$ | $n_H$ |
|-----------------------|----------|-------|
| 10:1                  | 176.9 ± 23.87 | 2.14 ± 0.58 |
| 1:1                   | 92.8 ± 6.61 | 1.32 ± 0.13 |
| 1:10                  | 22.56 ± 1.41 | 1.00 ± 0.06 |

Inhibition of Low Sensitivity $\alpha3\beta4$ nAChRs (10:1) by AuIB(Ribbon), but Not AuIB(Globular), Is Dependent on ACh Concentration—The ACh concentration-response curves determined for the three different $\alpha3\beta4$ ratios indicate that 50 $\mu$M ACh used in our initial experiments is below the $EC_{50}$ for the 10:1 ratio and close to saturation for the 1:10 ratio injected oocytes (Fig. 5). The difference in the $EC_{50}$ may account for the differential inhibitory effect of AuIB(ribbon) tested on all three injection groups. The ACh dependence of the inhibition of 10:1 ratio $\alpha3\beta4$ nAChRs by both AuIB isomers was investigated for three different ACh concentrations: 50, 175 ($\sim EC_{50}$), and 500 $\mu$M. Concentration-response curves for AuIB isomers obtained at different ACh concentrations are shown in Fig. 5. The inhibition of $\alpha3\beta4$ nAChRs by AuIB(globular) was not strongly dependent on ACh concentration: $IC_{50}$ values of 1.1, 0.9, and 1.3 $\mu$M were obtained for 50, 175, and 500 $\mu$M ACh, respectively (Table 3). Overall, increasing the agonist concentration produced a small rightward shift of the concentration-response curve only at saturating ACh concentration (500 $\mu$M) without affecting maximal inhibition achieved. In contrast, the efficacy of $\alpha3\beta4$ nAChRs inhibition by AuIB(ribbon) decreased with increasing ACh concentration (Fig. 5B). 10 $\mu$M AuIB(ribbon) inhibited $\alpha3\beta4$ nAChRs by 73.2 ± 3.0% using 50 $\mu$M ACh, 34.2 ± 2.1% at 175 $\mu$M, and 22.2 ± 3.1% at 500 $\mu$M ACh (Fig. 5B).

Inhibition of High Sensitivity $\alpha3\beta4$ nAChRs by AuIB Isomers at Different ACh Concentrations—The effect of ACh concentration on the inhibition of $\beta4$-dominant oocytes (1:10 ratio) by AuIB isomers was examined. AuIB(globular) inhibition of $\beta4$-dominant nAChRs was tested for three different ACh con-
Stoichiometry-dependent Block of α3β4 nAChRs by AuIB Isomers

The mechanism of nAChR inhibition by AuIB(globular) is consistent with non-competitive receptor antagonism. To further examine mechanism(s) of action of AuIB(globular), ACh concentration-response curves were determined in the presence of different concentrations of AuIB(globular) at 1:10 ratio injected oocytes where the ribbon isomer is inactive. Increasing concentrations of AuIB(globular) did not induce a parallel shift of the ACh concentration-response curve (see Fig. 7A) and even saturating ACh concentrations did not impair inhibition of HS α3β4 nAChRs by AuIB(globular) when it was applied at concentrations >1 μM. A similar result was obtained in 10:1 injected oocytes (data not shown). Thus, inhibition of α3β4 nAChRs by AuIB(globular) was insurmountable with increasing agonist concentration, which is characteristic of a non-competitive inhibition. Given that some antagonists of neuronal nAChRs have been shown to be voltage-dependent channel pore blockers (for review, see Ref. 21), the voltage dependence of AuIB(globular) (3 μM) inhibition of 1:10 ratio injected oocytes was examined at three different holding potentials: −120, −80, and −45 mV (Fig. 7B). Average inhibition by AuIB(globular) did not differ significantly at the different membrane potentials; 42.7 ± 7.0, 50.5 ± 4.2, and 46.8 ± 10.9% at −120, −80, −45 mV, respectively (p > 0.3 for −120 versus −80 mV and p > 0.7 for −45 versus −80 mV, unpaired t test) indicating that AuIB(globular) blockade of α3β4 nAChRs is voltage-independent. These data strongly suggest that the two isomers of α3-conotoxin AuIB inhibit α3β4 nAChRs differentially dependent on receptor subunit stoichiometry.

The Fifth Subunit in the α3β4 nAChR Pentamer Determines Binding of AuIB Isomers—Our experimental results have demonstrated that the stoichiometry of α3β4 nAChR has significant impact on its antagonist selectivity, namely AuIB(globular) versus AuIB(ribbon). To investigate the fifth subunit effect, two pentameric homology models (α3)3(β4)2 (10:1) and (α3)2(β4)3 (1:10) were constructed and assembled using different approaches to exclude the approach-dependent bias. Different receptor states were resembled by implementing the C-loop movements observed in AChBP co-crystal structures (10–13). The semi-flexible docking simulations of both AuIB(globular) and AuIB(ribbon) were performed with all α3β4 nAChRs models. Two dominant binding sites, above and below the C-loop binding pocket (α±)(β−) interface where ACh binds (supplemental Fig. S1), were identified for AuIB(globular) in both stoichiometries (Fig. 8A). This finding is in agreement with our experimental data where AuIB(globular) was shown to be a non-competitive inhibitor irrespective of α3β4 stoichiometry (see Figs. 3 and 7). In contrast, the docking simulation of AuIB(ribbon) to (αβ)3(β3) (10:1) showed only one dominant binding site at the C-loop binding pocket (Fig. 8A), suggesting a

centrations: 5 μM, 50 μM (~EC50), and 5 mM. The concentration-response curves obtained for the globular isomer exhibited a rightward shift at low toxin concentrations; however, AuIB(globular) retained full efficacy even at a saturating ACh concentration (5 mM). The maximal inhibition produced by 30 μM AuIB(globular) was 97.8 ± 0.2% at 5 μM ACh, 94.4 ± 1.1% at 50 μM ACh, and 96.1 ± 1.3% at 5 mM ACh (Fig. 6A). Halfmaximal inhibitory AuIB(globular) concentrations were 1.24, 3.0, and 5.8 μM for 5 μM, 50 μM, and 5 mM ACh, respectively (Table 3). Because AuIB(ribbon) inhibition is competitive and ACh concentration dependent in LS nAChRs, there is a possibility that in HS inhibition is masked due to lower ACh EC50 for HS; however, AuIB(ribbon) was not active in 1:10 ratio injected oocytes even at low ACh concentrations (3 μM) (Fig. 6B, inset). These data suggest that the lack of activity of AuIB(ribbon) at 1:10 nAChRs may be due to dependence of the inhibition on α3β4 nAChR stoichiometry, probably, because of a modified ACh binding site at 1:10 α3β4 nAChRs. Effects of different ACh concentrations on concentration-response curve parameters for the AuIB isomers obtained in 10:1 and 1:10 oocytes are summarized in Table 3.

AuIB(Globular) Inhibition of α3β4 nAChRs Is Voltage Independent and Non-competitive—AuIB(globular) inhibition of α3β4 nAChRs, in contrast to AuIB(ribbon), is not significantly impaired by subunit stoichiometry or agonist concentration.

### Table 3

Concentration-response curve parameters determined for AuIB(globular) and - (ribbon) inhibition at different α3β4 subunit ratios and ACh concentrations (n = 3)

| AuIB isomer | 10:1 | 1:10 |
|-------------|------|------|
|             | [ACh] | IC50 (μM) | nH | [ACh] | IC50 (μM) | nH |
| Globular    | 50 μM | 1.1  | 1.38 | 5 μM | 1.24 | 0.85 |
|             | 175 μM | 0.86 | 1.37 | 50 μM | 3.0 | 1.0 |
|             | 500 μM | 2.36 | 1.68 | 5 mM | 5.8 | 1.63 |
| Ribbon      | 50 μM | 0.86 | 0.94 | 3 μM | NA* | |
|             | 175 μM | ND | 50 μM | NA |
|             | 500 μM | ND | | |

*NA, not active.
ND, not determined.

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FIGURE 5. Inhibition concentration-response curves for globular and ribbon isomers of AuIB in 10:1 oocytes obtained with different concentrations of ACh used as an agonist. A, concentration-response curves for AuIB(globular) in 10:1 oocytes obtained with different concentrations of ACh. B, concentration-response curves for AuIB(globular) in 10:1 oocytes obtained with different concentrations of ACh. Note that increased agonist concentration impairs AuIB(ribbon) block, whereas inhibition by AuIB(globular) remains relatively unchanged.

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### Table 3

Concentration-response curve parameters determined for AuIB(globular) and - (ribbon) inhibition at different α3β4 subunit ratios and ACh concentrations (n = 3)

| AuIB isomer | 10:1 | 1:10 |
|-------------|------|------|
|             | [ACh] | IC50 (μM) | nH | [ACh] | IC50 (μM) | nH |
| Globular    | 50 μM | 1.1  | 1.38 | 5 μM | 1.24 | 0.85 |
|             | 175 μM | 0.86 | 1.37 | 50 μM | 3.0 | 1.0 |
|             | 500 μM | 2.36 | 1.68 | 5 mM | 5.8 | 1.63 |
| Ribbon      | 50 μM | 0.86 | 0.94 | 3 μM | NA* | |
|             | 175 μM | ND | 50 μM | NA |
|             | 500 μM | ND | | |

*NA, not active.
ND, not determined.
Stoichiometry-dependent Block of α3β4 nAChRs by AuIB Isomers

**A** Globular

![Inhibition concentration-response curves for globular and ribbon isomers of AuIB in 1:10 oocytes obtained with different concentrations of ACh used as an agonist. A. concentration-response curves for AuIB(globular) in 1:10 oocytes obtained with different concentrations of ACh. B, decreasing ACh concentration (3 μM) below ACh EC50 (22.6 μM) does not improve inhibition by AuIB(ribbon) in 1:10 oocytes. Inset, bar graph showing the absence of inhibition by 10 μM AuIB(ribbon) in 1:10 oocytes with 3 μM ACh used as agonist.](image)

**B** Ribbon

**DISCUSSION**

Here we show that the heterologous expression of different ratios of rat α3β4 nAChR subunits in X. laevis results in high and low sensitivity nAChRs reflecting different stoichiometries of α and β subunits. The globular and ribbon isomers of α-conotoxin AuIB differentially inhibit the high and low sensitive α3β4 nAChRs: AuIB(globular) inhibits both HS and LS nAChRs, whereas the ribbon isomer is active only at LS nAChRs. Furthermore, the isomers exhibit distinct inhibitory mechanisms: AuIB(ribbon) acts as a classical competitive antagonist, whereas AuIB(globular) acts as a non-competitive antagonist.

Previously, the IC50 of AuIB(ribbon) at heterologously expressed α3β4 nAChRs was estimated to be ~27 μM based on two concentrations tested and assuming 100% efficacy (6). Investigation of a broader range of AuIB(ribbon) concentrations used here surprisingly revealed that the maximal inhibition observed with the ribbon isomer is limited to only 50%. Furthermore, the IC50 of 0.77 μM for inhibition of oocyte-expressed α3β4 nAChRs by AuIB(ribbon) is less than that of the globular isomer (2.48 μM) (see Table 1). We hypothesized that the partial block of α3β4 nAChRs by AuIB(ribbon) may be due to nAChR stoichiometry because α3β4 nAChR likely exists in at least two stoichiometries, differing in their fifth subunit (either α or β) as has been demonstrated previously for rat and human α4β2 nAChRs expressed in oocytes (22, 23).

The existence of distinct stoichiometries for α4β2 nAChRs heterologously expressed in Xenopus oocytes (22, 23) and HEK cells (24) has been established and indications of their presence in vivo have also been reported recently (25). The distinct stoichiometries of α4β2 nAChRs have been shown to have different sensitivities to agonists, antagonists, Zn2+ modulation, as well as Ca2+ permeability and induction by chronic nicotine
Stoichiometry-dependent Block of α3β4 nAChRs by AuIB Isomers

FIGURE 7. Inhibitory action of AuIB(globular) in 1:10 oocytes is not consistent with competitive blocking or voltage-dependent pore-blocker paradigms. A, increasing concentrations of AuIB(globular) do not induce a parallel shift of ACh concentration-response curve and inhibition is not surmountable as AuIB(globular) continues to block at saturating concentrations of ACh. B, bar graph of the inhibition by 3 μM AuIB(globular) in 1:10 oocytes activated by 50 μM ACh at different holding potentials. Note the absence of voltage dependence of AuIB block.

FIGURE 8. Molecular modeling and docking simulation of globular and ribbon AuIB to (α3)2(β4)2 and (α3)3(β4). A, the α and β subunits were colored in blue and green, respectively, with the top 20 energetically favorable docking solutions presented. Two dominant binding site clusters above and below the C-loop binding pocket of (α3)2(β4), and (α3)3(β4) were identified with globular AuIB. In contrast, ribbon AuIB was shown to dock into the C-loop binding pocket of (α3)2(β4) and the β(+1)-α(−) interface of (α3)3(β4). B, the schematic representation of (α3)2(β4) and (α3)3(β4) pentamer. The solvent accessible area (Å²) of each interface generated by Hex 5.1 (bold) and Modeler/Sculptor (italic) are indicated. The fifth α and β subunits are highlighted in blue and green, respectively.

exposure (22, 24, 26–28). A number of theoretically possible subunit combinations was narrowed down to two likely stoichiometries, (α4)2(β2)3 and (α4)3(β2)2 by using [35S]methionine labeling, reporter mutations, concatenated subunits and, ultimately, linked pentameric α4β2 constructs restricting receptor stoichiometry (23, 24, 29–31).

In experiments with altered α3β4 injection ratios, we demonstrated differential inhibitory action of the two AuIB isoforms (Figs. 2 and 3). Blockade by AuIB(ribbon) was dependent on α3β4 injection ratios such that excess α subunits increased efficacy and excess β subunits abolished inhibition completely. In contrast, concentration-response curves of AuIB(globular) were not significantly affected by altering the α3β4 ratio (Fig. 2). To our knowledge nAChR stoichiometry-restricted antagonism has not been reported previously for any nAChR antagonists including α-conotoxins and it is surprising that only one of the two AuIB isomers is stoichiometry selective.

Stoichiometry of α3β4 nAChRs—In contrast to α4β2 nAChRs where any variation from the 1:1 ratio decreased ACh-evoked currents (22), we observed increased current amplitude with the 1:1 ratio group and a decrease in current amplitude in the 10:1 group (see Fig. 4). ACh concentration-response curves in 10:1 and 1:10 injections groups revealed the presence of low (LS, EC50 = 177 μM) and high (HS, EC50 = 22.6 μM) sensitivity α3β4 nAChRs, respectively (Fig. 4B and Table 2). The different current amplitudes observed in 10:1, 1:1, and 1:10 groups could be explained by the different ACh EC50 for each group. In contrast to that reported previously for α4β2 nAChRs (22), the ACh concentration-response curve obtained for (1:1) α3β4 nAChRs appeared monophasic and was best fit with one component. This difference may be explained by a less pronounced difference in ACh sensitivities for the different stoichiometries in α3β4 versus α4β2 receptors. The 101−EC50 was 7.8 times for α3β4 versus 30−100 times for α4β2 (22, 24), which would mask the presence of distinct α3β4 stoichiometries differing slightly in ACh sensitivity and give a monophasic appearance of the 1:1 ACh concentration-response curve. Another finding, not observed for α4β2 nAChRs, was the difference in Hill slopes of 10:1 versus 1:10 α3β4 nAChRs; a steeper slope in
Stoichiometry-dependent Block of α3β4 nAChRs by AuIB Isomers

10:1 receptors, indicative of positive cooperativity, implies that the LS α3β4 nAChRs operate over a narrower range of agonist concentrations (see Fig. 4B). Furthermore, the Hill slopes of ~2 in LS versus that of ~1 in HS α3β4 nAChRs suggest a difference in agonist binding cooperativity and/or binding-gating coupling mechanisms at the two α3β4 nAChR heteropentamers.

To date, there has been no comprehensive study of α3β4 nAChR stoichiometry with varied α3:β4 subunit ratios. It has been reported previously that α3β4 nAChRs expressed in oocytes exhibit different single channel conductances (32), which may be attributed to distinct receptor stoichiometries. Previous studies of α3β4β3 nAChRs have suggested that the subunit stoichiometry of the receptor is confined to 2α:3β proportion and the (α3)2(β4)3 stoichiometry has been postulated for the α3β4 receptor (33). Subsequently a pentameric α3β4 nAChR was constructed from linked α3/β4 subunits resulting in an (α3)3(β4)2 stoichiometry with properties resembling those of non-linked α3β4 nAChR (34). The alternative (α3)2(β4)3 construct was not investigated. Interestingly, a small leftward shift of the ACh concentration-response curve was observed for the linked α3β4 nAChRs compared with non-linked receptors, which may be attributed to the presence of LS (α3)3(β4)2 receptors in the population of non-linked α3β4 nAChRs injected with the 1:1 α:β RNA ratio. Therefore, the differential inhibition of α3β4 nAChRs by the two AuIB isomers, different amplitudes of ACh-induced currents, and distinct ACh concentration-response curves obtained for the three α3:β4 injection groups, indicate the existence of alternative α3β4 nAChR stoichiometries. Based on our observations for the α3β4 nAChRs, we show that the phenomenon of alternative nAChR stoichiometries is not limited to α3β2 subtype only. We predict that multiple subunit stoichiometries may be common for all heteromeric nAChRs.

**Competitive versus Non-competitive Antagonism and Implications for SAR** — The effect of agonist (ACh) concentration on concentration-response curves obtained for inhibition α3β4 nAChRs by the two AuIB isomers suggested different inhibitory mechanisms for the globular and ribbon AuIB isoforms (Figs. 6 and 7). In the case of LS α3β4 nAChR, increasing the ACh concentration led to markedly reduced inhibition by AuIB(ribbon), whereas inhibition by AuIB(globular) did not change significantly. Higher ACh concentrations at HS α3β4 nAChRs produced a slight shift of concentration-response curve for AuIB(globular) inhibition at low toxin concentrations without affecting maximal inhibition, suggesting both competitive and non-competitive action of AuIB(globular) at HS with low and high affinity, respectively. AuIB(ribbon) was not active at HS α3β4 nAChRs over a range of ACh concentrations suggesting that AuIB(ribbon) inhibition is α3β4 stoichiometry-dependent, presumably due to differences in ACh binding site properties between the two stoichiometries.

Schild plot analysis showed that increasing AuIB(globular) concentration did not produce a parallel shift of the ACh concentration-response curves and inhibition was clearly not mountable. Inhibition of HS α3β4 nAChRs by AuIB(globular) was not voltage dependent indicating that the globular isomer is not an open channel blocker (Fig. 7). These findings for AuIB-globular (globular) are consistent with non-competitive receptor antagonism.

To date, relatively few conotoxins acting as non-competitive nAChR antagonists have been reported. ψ-Conotoxins, a class of non-competitive nAChR antagonists having six cysteine connectivity (35). ψ-Conotoxins are generally considered to be competitive nAChRs based on binding studies for a few of them (e.g. Ref. 36). However, α-conotoxin ImII blocks α7 nAChRs (37, 38) and Torpedo californica nAChRs in a non-competitive manner (39), despite differing from ImI (a known competitive blocker) by only three residues. Interestingly, a large dimeric conotoxin VXXXB belonging to a novel class α-v-conotoxins was also shown to inhibit α7 and α3β2 nAChRs non-competitively (40).

There is mounting evidence showing that nicotinic agents target various sites on the nAChR besides the classical agonist binding site (e.g. Ref. 41). Mutations in the α(+)β(−) subunit interface outside the ACh-binding site were reported to affect binding of MII, PnLA, and GID 4/7 α-conotoxins to α3β2 nAChRs (42). Non-canonical allosteric sites of nAChRs have been studied for positive allosteric modulators of nAChRs. Positive allosteric modulators galanthamine and morantel have been shown to bind at the non-canonical β(+)α(−) interface, which is absent in homomeric receptors (41, 43). The α4/α4 interface in (α4)3(β2)2 nAChRs has been shown to account for stoichiometry-dependent Zn2+ potentiation (26). In the present study, non-competitive action of AuIB(globular) is unlikely to be at the unique α3/α3 interface of (α3)3(β4)2 because this isomer non-competitively inhibited both receptor stoichiometries. This result suggests the presence of a non-competitive site at both α3β4 stoichiometries, which was corroborated by our molecular docking study, which showed that AuIB(globular) likely binds outside the orthosteric site at the α3(+)/β4(−) interface of both stoichiometries (Fig. 8). Similarly, VXXXB is thought to inhibit nAChR allosterically by binding outside of the ACh-binding pocket in the α(+)β(−) interface to stabilize the cleft in a conformation, which does not support agonist binding (40). The allosteric site at the β(+)α(−) interface might be limited to positive modulation exclusively. AuIB(ribbon) docking results, which show binding within the classical ACh binding site of LS α3β4 nAChRs only, are in agreement with the experimental data. Stoichiometry-dependent block by AuIB(ribbon) also implies that the ACh binding site is affected by nature of the fifth subunit in the α3β4 nAChR pentamer and calculations of solvent-accessible areas of α3(+)/β4(−) interfaces for (α3)3(β4)2 and (α3)2(β4)3 nAChRs suggest a slightly larger binding pocket at HS α3β4 nAChR, which may underlie the loss of activity at this stoichiometry.

Taken together, our results are consistent with AuIB(globular) acting primarily at a non-competitive site of both α3β4 nAChR stoichiometries, whereas AuIB(ribbon) binds to the classical ACh binding site of the LS nAChR only. The α-conotoxin AuIB disulfide isoforms exhibit strikingly divergent inhibitory mechanisms of action and are potentially valuable tools for further dissecting ligand-receptor interactions and in the design of novel conopeptides with improved selectivity profile.
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