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α-Conotoxin AuIB Selectively Blocks α3β4 Nicotinic Acetylcholine Receptors and Nicotine-Evoked Norepinephrine Release

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Neuronal nicotinic acetylcholine receptors (nAChRs) with putative α3β4-subunits have been implicated in the mediation of signaling in various systems, including ganglionic transmission peripherally and nicotine-evoked neurotransmitter release centrally. However, progress in the characterization of these receptors has been hampered by a lack of α3β4-selective ligands. In this report, we describe the purification and characterization of an α3β4 nAChR antagonist, α-conotoxin AuIB, from the venom of the "court cone," *Conus aulicus.* We also describe the total chemical synthesis of this and two related peptides that were also isolated from the venom. α-Conotoxin AuIB blocks α3β4 nAChRs expressed in *Xenopus* oocytes with an IC_{50} of 0.75 μM, a k_{on} of 1.4×10^6 min^{-1} M^{-1}, a k_{off} of 0.48 min^{-1}, and a K_d of 0.5 μM. Furthermore, α-conotoxin AuIB blocks the α3β4 receptor with >100-fold higher potency than other receptor subunit combinations, including α2β2, α2β4, α3β2, α4β2, α4β4, and α1β1γδ. Thus, AuIB is a novel, selective probe for α3β4 nAChRs. AuIB (1–5 μM) blocks 20–35% of the nicotine-stimulated norepinephrine release from rat hippocampal synaptosomes, whereas nicotine-evoked dopamine release from striatal synaptosomes is not affected. Conversely, the α3β2-specific α-conotoxin MII (100 nM) blocks 33% of striatal dopamine release but not hippocampal norepinephrine release. This suggests that in the respective systems, α3β4-containing nAChRs mediate norepinephrine release, whereas α3β2-containing receptors mediate dopamine release.

Key words: nicotinic acetylcholine receptor; α conotoxin; α3β4; norepinephrine; dopamine; hippocampus; striatum; synaptosomes; Xenopus *oocytes*

Nicotinic acetylcholine receptors (nAChRs) are widely distributed in both the PNS and CNS. In vertebrates, eight α-subunits (α2–α9) and three β-subunits (β2–β4) have been cloned. When expressed in oocytes or cell lines individually (i.e., α7, α8, and α9) or in various pairwise combinations of α- and β-subunits, they yield functional nicotinic receptors (for review, see Sargent, 1993). Furthermore, receptor combinations with up to four different subunits have been demonstrated both *in vitro* and *in vivo* (Conroy and Berg, 1995). Thus, the number of potential molecular forms of nicotinic receptors is very large. Elucidation of the precise structure and function of various neuronal nAChRs *in vivo* is particularly challenging, in large part because of the scarcity of ligands selective for specific receptor subtypes.

In an effort to improve this situation, we have been systematically screening components from the venoms of carnivorous cone snails (*Conus*) for selective nicotinic ligands. There are >500 species of these snails, and their venoms contain small disulfide-bonded peptides that target receptors and ion channels in a highly subtype-selective manner. Every venom examined thus far has its own distinct complement of nicotinic receptor antagonists. From *Conus magus*, a fish-hunting cone, we previously isolated α-conotoxin MII, specific for α3β2 nAChRs (Cartier et al., 1996a). MII has been used to pharmacologically dissect the nAChR subtypes in sympathetic and parasympathetic ganglia (Tavazoie et al., 1997; Ullian et al., 1997). From a worm-hunting cone, *Conus imperialis*, we previously characterized α-conotoxin ImI, which specifically blocks α7 homomers expressed in oocytes as well as putative α7-containing receptors in hippocampus (McIntosh et al., 1994; Johnson et al., 1995; Pereira et al., 1996). In this report we describe the first peptides isolated from the venom of the snail-eating cone *Conus aulicus* and demonstrate that one of them, α-conotoxin AuIB, selectively inhibits α3β4 nAChRs.

Presynaptic nAChRs modulate the release of several neurotransmitters in the CNS, including norepinephrine and dopamine (for review, see Wonnacott, 1997). Changes in CNS norepinephrine levels appear to be involved in mood disorders (for review, see Schatzberg and Nemeroff, 1995; Mongeau et al., 1997), and dopamine appears to play an important role in addictive and psychotic disorders (Kahn and Davis, 1995; Pontieri et al., 1996). A greater understanding of the molecular mechanisms regulating the release of these neurotransmitters may be valuable in the development of treatments for these illnesses. This report describes the effects of α-conotoxin AuIB on nicotine-evoked norepinephrine and dopamine release. These studies begin to elucidate the subunit composition of nAChRs mediating the release of these neurotransmitters.

MATERIALS AND METHODS

Peptide isolation and sequencing

Venom extraction. Crude venom from dissected ducts of *C. aulicus* (see Fig. 1) was collected in the Philippines, lyophilized, and stored at −70°C until used. Extraction procedures were conducted at 4°C. For direct screening on oocytes, lyophilized venom was dissolved in ND96 buffer to

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provide a 50 mg/ml extract. This extract was diluted to 6.5 mg/ml and applied to *Xenopus* oocytes expressing α3β4 receptors. For venom purification, 15 ml of 0.1% trifluoroacetic acid (TFA) was added to 500 mg of lyophilized venom, and extraction was performed as described previously (Cartier et al., 1996a).

RPLC purification. All reverse-phase liquid chromatography (RPLC) columns were from Rainin Instruments (Ridgefield, NJ). Crude venom extract was fractionated on a semipreparative Vydac C_{18} column (10 mm × 25 cm, 5 μm particle size, 300 Å pore size) equipped with a guard module (catalog #83-223-65). All subsequent chromatographic purifications used an analytical Vydac C_{18} column (4.6 mm × 22 cm, 5 μm particle size, 300 Å pore size). Synthetic peptide was purified on a preparative Vydac C_{18} column (22 mm × 25 cm, 10 μm particle size, 300 Å pore size). For all chromatographic gradients, buffer A was 0.1% TFA and buffer B was 0.1% TFA with either 60 or 90% acetonitrile. TFA (sequencing grade) was from Aldrich (Milwaukee, WI); acetonitrile (UV grade for semipreparative and analytical RPLC, nonspectroscopic grade for preparative RPLC) was from Baxter (Deerfield, IL).

Pyridylethylation and purification of modified peptide. Peptide from the final purification was stored in the RPLC buffer in which it eluted. A solution of this purified peptide was combined with 0.5 M Tris-base (20:1 v/v) to raise the pH to a value between 7 and 8 (as measured with pH paper). Dithiothreitol was added to a final concentration of 10 mM, the reaction vessel was flushed with argon, and the reaction was incubated at 65°C for 15 min. The solution was allowed to cool, 4-vinyl pyridine (20% in ethanol) was added (1:25, v/v), and the solution was reacted for a further 25 min at room temperature in the dark. The reaction was diluted threefold with 0.1% TFA, and the alkylated peptide was purified on an analytical C_{18} column (see above).

Sequence analysis. Sequencing was performed with Edman chemistry on an Applied Biosystems 477A Protein Sequencer at the Protein/DNA Core Facility at the University of Utah Cancer Center. Mass spectrometry was performed as described previously (Martinez et al., 1995).

Peptide synthesis

Linear peptide. The linear peptides were built on Rink amide resin by Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) procedures with 2-(1H-benzotriole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate coupling, using an ABI model 431A peptide synthesizer. Side-chain protection of non-Cys residues was in the form of t-butyl (Asp, Ser, Tyr, Thr) and trityl (Asn). Orthogonal protection was used on cysteines: Cys3 and Cys16 were protected as the stable Cys(S-acetamidomethyl), whereas Cys2 and Cys8 were protected as the acid-labile Cys(S-trityl). After assembly of the resin-bound peptide, the terminal Fmoc group was removed *in situ* by treatment with 20% piperidine in *N*-methylpyrrolidone. Linear peptide amide was cleaved from resin by treatment with TFA/H_2O/ethanedithiol/phenol/thioanisole (90:5:2.5:7.5:5 by volume) (1 ml/50 mg resin) for 1.5 hr at 20°C. This procedure simultaneously cleaved peptide from the resin and deprotected Cys(S-trityl) and non-Cys residue side chains, but not Cys(S-acetamidomethyl). Released peptide was precipitated by filtering the reaction mixture into methyl-t-butyl ether (MTBE) that had been cooled to −10°C. The cleavage reaction vessel was rinsed with 100% TFA, and this rinse was also filtered into the MTBE solution. The MTBE solution was centrifuged to pellet the precipitate. The pellet was washed twice by suspension in ~30 ml of chilled MTBE. Pelleted peptide was dissolved in ~20 ml of 0.1% TFA in 60% acetonitrile by gentle swirling (to avoid foaming). The linear peptide solution was diluted 20-fold with 0.1% TFA and purified by RPLC on the preparative C_{18} Vydac column with a 20–50% buffer B gradient over 30 min. Flow rate was 20 ml/min. This gradient was also used for all subsequent preparative RPLC purifications of the synthetic peptide.

Peptide cyclization. To form a disulfide bridge between Cys2 and Cys8 (i.e., the first and third cysteines), the major linear peptide fraction obtained by preparative RPLC (see above) was diluted to 500 ml with H_2O, and solid Tris base was added to raise the pH to ~7.5. The solution was placed in a 2 l flask and stirred vigorously at room temperature for 2–5 d until the reaction was judged to be complete by analysis of peak shape and retention time of sample subjected to analytical RPLC. The pH of the solution was decreased to a value of 2–3 by the addition of TFA. The monocyclic peptide was then purified by RPLC. Simultaneous removal of the S-acetamidomethyl groups and closure of the second disulfide bridge (Cys3–Cys16, i.e., the second and fourth cysteines) was performed by iodine oxidation. The monocyclic peptide in RPLC eluent was dripped into an equal volume of rapidly stirred solution of iodine (10 mM) in H_20/TFA/acetonitrile (78:2:20 by volume) over a period of 1 min at room temperature. The reaction was allowed to proceed for another 10 min and was terminated by the addition of ascorbic acid sufficient to cause the solution to clear. The solution was diluted 20-fold with 0.1% TFA and the bicyclic peptide purified by RPLC.

Co-elution studies. Comparison of natural and synthetic α-conotoxins AuIA, -B, and -C was performed by RPLC. Native and synthetic peptides were chromatographed individually, and subsequently equal amounts of each peptide were co-injected onto the RPLC column. Peptide was eluted with a linear positive gradient of 0.6% acetonitrile/min.

Electrophysiology

RNA preparation. cDNA clones encoding nAChR subunits were provided by S. Heinemann and D. Johnson (Salk Institute, San Diego, CA). cRNA was transcribed using RiboMAX large-scale RNA production systems (Promega, Madison, WI). Diguanosine triphosphate (Sigma, St. Louis, MO) was used for synthesis of capped cRNA transcripts according to the protocol of the manufacturer. Plasmid constructs of nAChR subunits from mouse (α1, β1, γ, and δ) and from rat (α2, α3, α4, α7, β2, and β4) were used as described (Cartier et al., 1996a) .

Voltage-clamp recording. Oocytes were harvested and injected with cRNA encoding nAChR subunits as described previously (Cartier et al., 1996a). The oocyte recording chamber consisting of a cylindrical well (~4 mm diameter × 2 mm deep; ~30 μl) fabricated from Sylgard; it was gravity-perfused either with ND96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.1–7.5) or with ND96 containing 1 μM atropine (ND96A) at a rate of ~1 ml/min. All toxin solutions also contained 0.1 mg/ml bovine serum albumin (BSA) to reduce nonspecific adsorption of peptide. The perfusion medium could be switched to one containing peptide or ACh by use of a distributor valve (SmartValve, Cavro Scientific Instruments, Sunnyvale, CA) and a series of three-way solenoid valves (model 161T031, Neptune Research, Northboro, MA). For toxin concentrations >10 μM, a 300 μl recording chamber was used with a perfusion flow rate of ~5 ml/min. Toxin was pre-applied for 5 min in a static bath (to conserve material). All recordings were made at room temperature (~22°C). ACh-gated currents were obtained with a two-electrode voltage-clamp amplifier (model OC-725B, Warner Instrument, Hamden, CT). Glass microelectrodes, pulled from fiber-filled borosilicate capillaries (1 mm outer diameter × 0.75 mm inner diameter) (WPI, Sarasota, FL) and filled with 3 M KCl, served as voltage and current electrodes. Resistances for voltage and current electrodes were 0.5–3 MΩ and 0.5–2 MΩ, respectively. The membrane potential was clamped at −70 mV, and the current signal, recorded through virtual ground, was low-pass-filtered (5 Hz cut-off) and digitized at a sampling frequency of 20 Hz. The solenoid perfusion valves were controlled with solid-state relays (model ODC5 in a PB16HC digital I/O backplane; Opto 22, Temecula, CA). A Lab-LC or Lab-NB board (National Instruments, Austin, TX) in a Macintosh computer (Quadra 630 or IIcx) was used for A/D conversion and digital control of solenoid valves. The computer communicated with the distributor valve via its serial port. Data acquisition, measurement of peak responses, and control of the distributor and solenoid valves were automated by a homemade virtual instrument constructed with the graphical programming language LabVIEW (National Instruments).

To apply a pulse of ACh to the oocyte, the perfusion fluid was switched to one containing ACh for 1 sec. This was automatically done at intervals of 1–5 min. The shortest time interval was chosen such that reproducible control responses were obtained with no observable desensitization. This time interval depended on the nAChR subtype being tested. The concentration of ACh was 1 μM for test of α1β1δγ, 1 mM for α7, and 300 μM for all other nAChRs. The ACh was diluted in ND96A for tests of all except α7, in which case the diluent was ND96. For control responses, the ACh pulse was preceded by perfusion with ND96 (for α7) or ND96A (all others). No atropine was used with oocytes expressing α7, because it has been demonstrated to be an antagonist of these receptors (Gerzanich et al., 1994). For responses in toxin (test responses), the perfusion solution was switched to one containing toxin while maintaining the same interval of ACh pulses. Toxin was continuously perfused until responses reached a steady state. All ACh pulses contained no toxin, for it was assumed that little, if any, bound toxin would have washed away in the brief time (<2 sec) it takes for the responses to peak. Oocytes were exposed to static solutions of toxin in two special cases to conserve material: during toxin purification and when concentrations of >10 μM toxin were used.

Data analysis. The average peak amplitude of three control responses just preceding exposure to toxin were used to normalize the amplitude of

each test response to obtain "% response" or "% 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 equation: % response = $100/\{1 + ([\text{toxin}]/IC_{50})^{nH}\}$, where n_H is the Hill coefficient. Data fits were performed with Prism software (GraphPad Software, San Diego, CA) running on an Apple Power Macintosh.

Nicotine-stimulated neurotransmitter release

Materials. [^{3}H]-dopamine (dihydroxyphenyl-ethylamine, 3,4 [7-^{3}H]-) (~30 Ci/mmol) and [^{3}H]-norepinephrine (norepinephrine, levo-[ring-2,5,6-^{3}H]-) (~42 Ci/mmol) were purchased from DuPont NEN (Boston, MA) (NET-131 and NET-678, respectively). These were distributed into 5 and 14.1 μCi aliquots, respectively, and stored under argon at −80°C. (−)-Nicotine hydrogen tartrate was from Sigma. Pargyline HCl and mecamylamine HCl were from Research Biochemicals International (Natick, MA). On the day of use, all drugs were prepared in superfusion buffer (SB) consisting of 128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.6 mM $MgSO_4$, 25 mM HEPES, 10 mM D-glucose, 1 mM L-ascorbic acid, 0.1 mM pargyline, 0.1 mg/ml BSA, with the pH adjusted to 7.5 with NaOH. α-Conotoxin MII was synthesized as described previously (Cartier et al., 1996a).

Animals. Male Sprague–Dawley rats, weighing 200–400 gm, were maintained on a 12 hr light/dark cycle. Rats were drug-naive and housed three per cage, and food and water were available *ad libitum*.

Synaptosomal preparation and [^{3}H] radioligand loading. Synaptosomes were prepared as described previously (Kulak et al., 1997). A crude P2 synaptosomal fraction was resuspended in SB (0.5 ml/100 mg wet tissue weight) containing 0.12 μM [^{3}H]-dopamine for striatal tissue or 0.2 μM [^{3}H]-norepinephrine for hippocampal tissue and incubated at 37°C for 10 min. The loaded synaptosomes were centrifuged at 1000 × *g* for 5 min at room temperature (24°C), and the pellet was gently resuspended in 2.0 ml of SB. The high [K^+]-stimulated release solution was SB in which the [K^+] was elevated to 22.4 mM and [Na^+] was decreased to 108 mM.

Superfusion. The assay system was as described previously (Kulak et al., 1997). Briefly, the system had 12 identical channels connected to a pump that continuously pulled the superfusate through individual filter holders containing the synaptosomes at a rate of 0.5 ml/min. Teflon tetrafluoroethylene tubing and Teflon-coated parts were used upstream of the synaptosomes to avoid plasticizers such as Tinuvin 770 (a common light and UV radiation stabilizer used in a wide range of plastics), which are known to block neuronal nAChRs (Papke et al., 1994).

After a preliminary superfusion period of 13 min (for assays containing α-conotoxin AuIB) or 31 min (for all other toxins), a 1 min (0.5 ml) pulse of synaptosomal buffer with or without agonist was delivered simultaneously to all channels by switching on 12 three-way solenoid valves. Nicotine concentration was 3 μM in dopamine-release experiments and 100 μM in norepinephrine-release experiments. Fractions (2 min each) per channel were collected in polypropylene minivials containing 4.0 ml of scintillation fluid. After the collection period, the filters holding the synaptosomes were removed to determine the residual radioactivity. A liquid scintillation counter (Beckman LS9800, 57.2% efficiency) was used to determine tritium levels.

Data analysis. It has previously been shown that tritium released by nAChR agonists or by depolarizing concentrations of KCl is directly proportional to total radioligand released (Rapier et al., 1988). Thus levels of tritium released are assumed to correspond directly to amounts of radioligand released.

Release is calculated as (dpm in the peak fractions − baseline)/baseline, where baseline is the average dpm of two prerelease and two postrelease fractions. Release is expressed as a percentage of agonist-stimulated release under control conditions. Agonist-stimulated release with superfusate containing different α-conotoxin concentrations was compared with those of controls without toxin and analyzed for statistically significant mean differences using a *t* test with SPSS software (SPSS, Chicago, IL).

RESULTS

Isolation and biochemical characterization of AuI α-conotoxins

Venom extract from the snail-eating mollusk *C. aulicus* (Fig. 1*A*) was applied at a concentration of 6.5 mg/ml to voltage-clamped *Xenopus* oocytes expressing α3β4 receptors; this concentration blocked 99% of the ACh-gated current. This initial encouraging result provided incentive to purify *C. aulicus* venom as shown in Figure 1*B*. The strategy basically entailed repeated RPLC purifications of venom fractions. To test for activity, fractions were lyophilized, resuspended in ND96 buffer, and assessed for inhibition of ACh-gated current in *Xenopus* oocytes expressing α3β4 nAChRs as described in Materials and Methods. After the initial semipreparative RPLC, two consecutive fractions (0.1% of each) totally inhibited α3β4 receptors in the oocyte assay. These active fractions were further purified by applying small portions (e.g., 16%) of the active fractions to an analytical RPLC column.

It became apparent during the purification that there were multiple active fractions. Some of these fractions (Fig. 1*B*, *AuIA* and *AuIC*) were difficult to resolve chromatographically. The active components were purified by RPLC using the gradient system described in Figure 1*B* (*panels 2* and *3*). To obtain a homogeneous product by RPLC it was necessary that the presumed impurities in the leading and tailing edges of the peak be excluded from the recovered peptide sample. Activity of all fractions on α3β4 receptors was verified using the oocyte assay. Repeated RPLC and collection of these peak fractions eventually resulted in pure material. To obtain AuIA and AuIC separately, material was collected from the left half (AuIA) and far right half (AuIC) of a coalesced peptide absorbance (Fig. 1*B*, *panel 3*). Ultimately three active peptides were isolated and designated as fractions A, B, and C. However, because of the large number of peptides eluting in this portion of the chromatogram, it is possible that there were additional α3β4-active peptides that were not recovered during this process.

The disulfide bonds of the purified peptides were reduced, the cysteines alkylated, and the peptides sequenced as described in Materials and Methods. Three sequences were obtained as shown in Table 1. Liquid secondary ion mass spectrometry was performed on each peptide and indicated that Cys residues are present as disulfides and that the C-terminal α-carboxyl group in each peptide is amidated. Monoisotopic MH^+ mass calculated for each peptide is 1725.6, 1572.5, and 1667.6 Da for α-conotoxins AuIA, B, and C, respectively. The observed masses were 1725.6, 1572.5, and 1667.6 Da, respectively. The sequences were further verified by total chemical synthesis as described below. The three sequences are clearly homologs of each other and resemble previously isolated α-conotoxins in their Cys spacing, particularly α-conotoxins MII, PnIA, and PnIB, which also target neuronal nAChRs. The AuI peptides, however, differ substantially in non-Cys amino acids (Table 1). As will be shown below, the AuIB peptide selectively targets the α3β4 receptor, and it is likely that these non-Cys amino acids confer this unique specificity.

Chemical synthesis

Solid-phase chemical synthesis of the AuI α-conopeptides was achieved by methods similar to those used to synthesize α-conotoxin MII. For all α-conotoxins characterized to date, the disulfide bonding pattern has been conserved and is first Cys-third Cys, second Cys-fourth Cys. To synthesize the AuI peptides, we assumed this conservation of disulfide bonds and protected Cys groups in pairs to direct the disulfide formation. Acid-labile protecting groups were removed from the first and third cysteines during the cleavage reaction, which released the linear peptide. The first disulfide bridge was closed via air oxidation, and monocyclic peptide was purified by RPLC. The acid-stable protecting groups were subsequently removed from the second and fourth cysteines, and the second disulfide bridge was

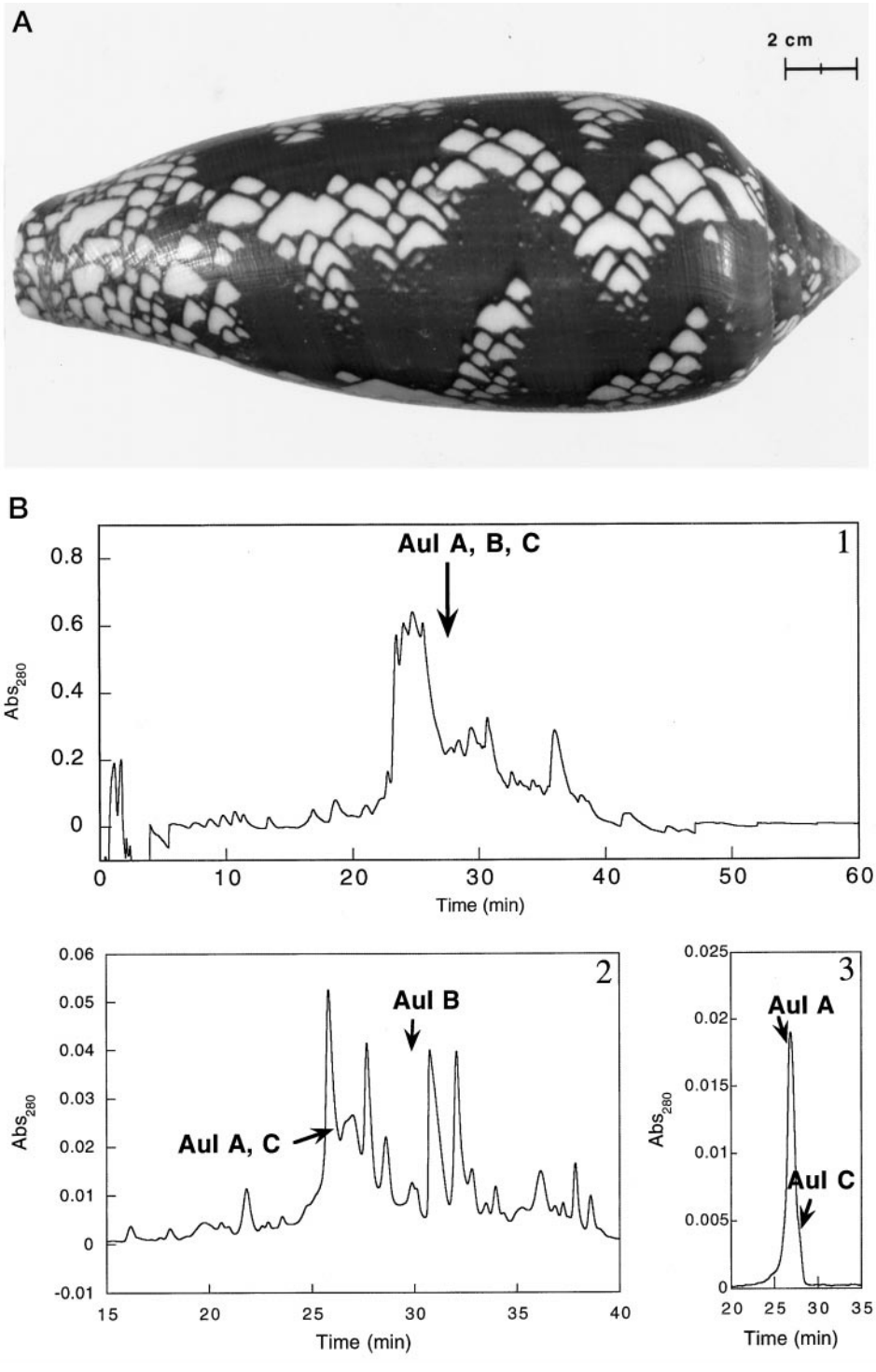


Figure 1. *A*, *C. aulicus*. Cone snails are venomous marine predators. *C. aulicus* is found in coral reefs and sand substrates in the Indo-Pacific (except Hawaii) and hunts primarily gastropods but also small fish. For the first time, nicotinic antagonists were isolated from its venom. *B*, Purification of AuI α-conotoxins by RPLC. *Panel 1*, Filtrate of venom extract was loaded onto a semi-preparative Vydac C_{18} column with 100% buffer A and eluted with a gradient of 5–65% buffer B per hour. Flow rate was 5 ml/min. *Panel 2*, Sixteen percent of the material eluting in the position indicated by the *arrow* in *panel 1* was diluted with 2 vol of 0.1% TFA and repurified on an analytical Vydac C_{18} column, using a flow rate of 1 ml/min. The gradient was 25–30% buffer B for 5 min and then 30–55% buffer B for 50 min. *Panel 3*, Fractions indicated in *panel 2* were rechromatographed as described to obtain the final purified products. Although AuIB is well separated, AuIA and AuIC nearly co-elute. A 5-ml-sample loading loop was used in all chromatography. Buffer A = 0.1% TFA; buffer B = 0.1% TFA, 90% acetonitrile (*panel 1*) and 0.1% TFA, 60% acetonitrile for all other purifications steps. Absorbance was monitored at 280 nm.

closed by rapid iodine oxidation. The final bicyclic peptide was purified by RPLC.

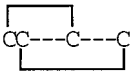
The proper synthesis of each peptide was confirmed by liquid secondary ionization/mass spectrometry. The observed masses for α-AuIA, -B, and -C were 1725.6, 1572.5, and 1667.7 Da, in good agreement with the calculated values. Proper synthesis was further verified by mixing equal portions of synthetic and native peptides and fractionating the mixture by RPLC. In each case, the native and synthetic peptides co-eluted (data not shown).

Kinetics and selectivity of nAChR block

The peptides were tested for their ability to block ACh-induced currents in *Xenopus* oocytes expressing α3β4 nAChRs. The intent of the studies was to assess the effect of the peptide on the nicotinic receptors in their resting state. For this reason we preincubated the preparation with toxin until equilibrium *for toxin* was reached. Thus, peptide was perfused onto the oocyte and the response to a 1 sec pulse of ACh was assessed every 1–5

Table 1. Sequences of α-conotoxins

α-CTx	Sequence	Species	Prey	Reference
AuIA	GCCSYPPCFATNSDYC*	*C. aulicus*	Mollusc	This report
AuIB	GCCSYPPCFATNPDC*	*C. aulicuc*	Mollusc	This report
AuIC	GCCSYPPCFATNSGYC*	*C. aulicuc*	Mollusc	This report
MII	GCCSNPVCHLEHSNLC*	*C. magus*	Fish	Cartier et al., 1996
ImI	GCCSDPRCAWRC*	*C. imperialis*	Worm	McIntosh et al., 1994
PnIA	GCCSLPPCAANNPDYC*	*C. pennaceus*	Mollusc	Fainzilber et al., 1994
PnIB	GCCSLPPCALSNPDYC*	*C. pennaceus*	Mollusc	Fainzilber et al., 1994
EI	RDOCCYHPTCNMSNPQIC*	*C. ermineus*	Fish	Martinez et al., 1995
MI	GRCCHPACGKNYSC*	*C. magus*	Fish	McIntosh et al., 1982

Disulfide bonding: 

An asterisk indicates that the COOH-terminal α-carboxyl group is amidated.
The *Conus* species from which each toxin was isolated and the prey of that species are indicated.

min. Toxin application was continued until no further changes in the responses to ACh were observed (i.e., equilibrium with toxin was achieved). It was assumed that little if any toxin dissociates during ACh application for two reasons. (1) The application of ACh is brief (1 sec) compared with the off-rate of the toxin (see below). (2) In our recording chamber the bolus of ACh does not project directly at the oocyte but rather enters tangentially, 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). Thus the reduction in toxin bathing the oocyte during the ACh pulse is minimized. Under these conditions (pre-equilibration with toxin) the relative attenuation of the peak amplitude of the response to ACh is assumed to be a direct measure of the fraction of receptors blocked by toxin and to be relatively independent of the ACh concentration in the pulse. Indeed, in support of this assumption, α-conotoxin AuIB (3 μM) blocked 83.9 ± 1.8% of the response when a 300 μM ACh bolus was used (n = 10) and blocked 81.7 ± 3.3% of the response when the bolus was 60 μM ACh (n = 3).

Preliminary tests indicated that α-conotoxins AuIA, AuIB, and AuIC each preferentially blocked the α3β4 receptor versus other receptor subunit combinations expressed in oocytes (data not shown). When tested at a peptide concentration of 3 μM α-conotoxin, AuIB blocked a greater fraction of the ACh response of α3β4 receptors than did either α-conotoxin AuIA or AuIC (Fig. 2*A*). Thus, AuIB was selected for particular scrutiny. Block of the ACh response by α-conotoxin AuIB is fully reversible, as shown in Figure 2*B*.

Previous studies of α-conotoxins have determined that these peptides bind at the ligand binding interface of α- and non-α-subunits of nAChRs (Sine et al., 1995). Kinetics of the block of α3β4 receptors by α-conotoxin AuIB were determined by perfusing toxin at a concentration below the IC_{50}, so the fraction of receptors blocked by toxin is dominated by singly occupied receptors, i.e., receptors with only one of the two putative ACh binding sites (at α3β4 interfaces) occupied by toxin. Development of block of the ACh response during toxin wash-in and recovery from block after toxin washout were measured. An individual experiment is shown in Figure 3. The results of six experiments were averaged to determine the rate constants k_{on} = $1.4 \times 10^6 \pm 0.3 \times 10^6$ min^{-1} M^{-1}, and k_{off} = 0.48 ± 0.06 min^{-1}.

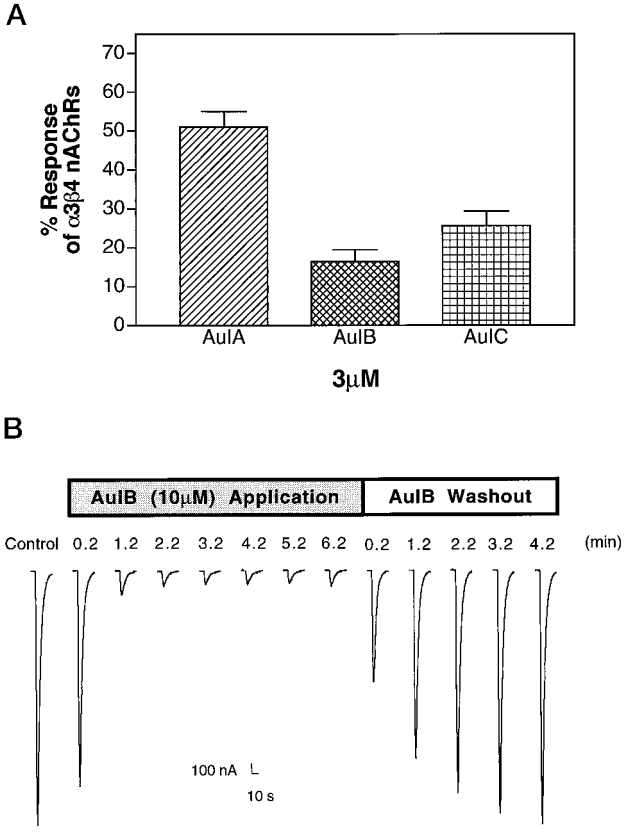


Figure 2. *A*, α-Conotoxin AuIA, AuIB, and AuIC block ACh responses in oocytes expressing α3β4 nAChRs. *Xenopus* oocytes expressing α3β4 nAChRs were voltage-clamped, and the responses to 1 sec pulses of ACh were monitored before exposure to toxin and during equilibrium exposure to 3 μM α-conotoxins. Note that the block by α-conotoxin AuIB is the greatest of the three peptides. *B*, AuIB (10 μM) reversibly blocks 95% of the ACh response. Peptide application and washout are indicated by the *bars*.

The K_d values determined in individual experiments from k_{off}/k_{on} were averaged to determine a K_d of 0.5 ± 0.14 μM.

α-conotoxin AuIB was tested on several nAChR subunit combinations to assess its selectivity. Although many peptide antag-

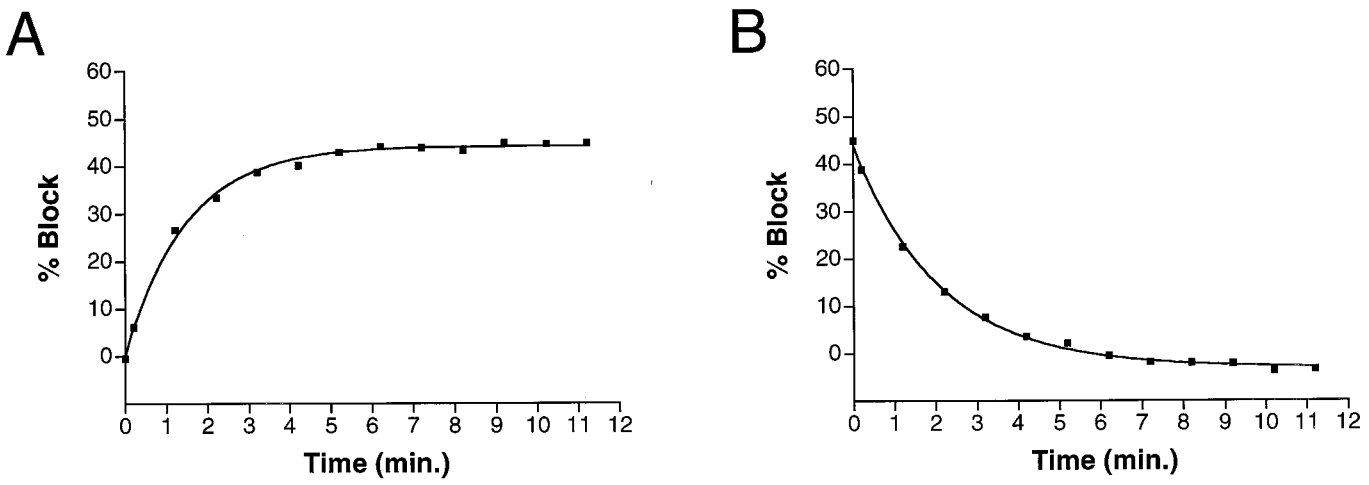


Figure 3. Kinetics of block by α-conotoxin AuIB. *A*, α-Conotoxin AuIB (300 nM) was perfused onto an oocyte expressing α3β4 receptors while the responses to 1 sec applications of ACh were measured. *B*, After maximal block was achieved α-conotoxin AuIB was washed out. *Solid lines* are single exponential curves that best fit the data.

onists of nAChRs block the skeletal muscle subtype (α1β1δγ) of receptor, α-conotoxin AuIB (3 μM) failed to do so (Fig. 4*A*). Further tests were performed with various neuronal nAChR subunit combinations. Dose–response studies indicated that AuIB has an IC_{50} of 0.75 μM for α3β4 nAChRs (Fig. 4*B*). This value is in good agreement with the K_d (0.5 μM) derived from kinetic studies described above. In contrast, α-conotoxin AuIB (3 μM) had little if any effect on the heteromeric receptor combinations α2β2, α2β4, α3β2, α4β2, α4β4, and α1β1γδ (data not shown). To further quantitate the magnitude of selectivity of α-conotoxin AuIB, we tested it at 75 μM, 100-fold the IC_{50} for the α3β4 nAChR. As shown in Figure 4*B*, α-conotoxin AuIB is at least 100 times more potent on α3β4 than on all other α/β-subunit receptor combinations tested. In contrast, α-conotoxin AuIB (3 μM) blocked a substantial portion (34 ± 5%) of the ACh response on α7 nAChRs. This indicates that the peptide is less able to discriminate between α3β4 and α7 (homomeric) receptors than between α3β4 and other non-α7-containing heteromeric receptors.

Norepinephrine release

Presynaptic nicotinic receptors are known to be involved in the release of various neurotransmitters, including norepinephrine and dopamine. The effects of α-conotoxin AuIB and other α-conotoxins were assessed in this regard. Nicotine-stimulated norepinephrine release was analyzed using synaptosomes from rat hippocampus; dopamine release was assayed in synaptosomes from rat striatum. As shown in Figure 5, AuIB (1 and 5 μM), blocked a portion (one-fifth and one-third, respectively) of nicotine-stimulated norepinephrine release but was ineffective on dopamine release. The converse result was obtained with the α3β2 selective α-conotoxin MII. MII (100 nM) blocked nicotine-stimulated dopamine release by one-third but had no effect on nicotine-evoked norepinephrine release. We did not test higher concentrations of AuIB because of the limited supply of peptide.

Specificity and effects

The specificity of α-conotoxin AuIB's block of norepinephrine release was further assessed by testing its effects on norepinephrine release stimulated by high $[K^+]$ (22.4 mM). Concentrations of AuIB that significantly block nicotine-stimulated norepinephrine release had no effect on high $[K^+]$-stimulated norepinephrine release (response in 1 μM AuIB = 92.2 ± 3.7%, $p = 0.5$; response in 5 μM AuIB = 99.0 ± 4.6%, $p = 0.95$). The experiment was performed three times with three to five replicates per experiment.

We also assessed the effects of other α-conotoxins on nicotine-stimulated norepinephrine release. Neither α-conotoxin ImI [α7-selective; Johnson et al. (1995)] nor α-conotoxin MI [α1-selective; McIntosh et al. (1982); Johnson et al. (1995)] had any effect on release (Fig. 5). We have shown previously that α-conotoxins ImI and MI are without effect on nicotine-stimulated dopamine release (Kulak et al., 1997). Thus, of the four structurally and functionally related peptides tested, only α-conotoxin AuIB blocks nicotine-stimulated norepinephrine release, and only α-conotoxin MII blocks nicotine-stimulated dopamine release.

DISCUSSION

We used oocytes expressing rat α3β4 nAChRs as an assay to guide the purification of components in the venom of *C. aulicus* that block nicotinic receptors. These studies have resulted in the isolation of three peptides designated α-conotoxins AuIA, -B, and -C. The structures of these homologous disulfide-rich peptides have been confirmed by mass spectrometry and total chemical synthesis. We have characterized one of these peptides, α-conotoxins AuIB, in detail and observed that although it is structurally similar to previously isolated α-conotoxins, its pharmacological profile is unique (Table 2). We used nicotinic receptors expressed in oocytes to demonstrate that α-conotoxin AuIB produces dose-dependent selective block of the α3β4-subunit combination. To our knowledge, this is the first report of nicotinic antagonists that can selectively block α3β4 receptors. The selectivity for α3β4 versus α3β2 receptors is particularly remarkable given AuIB's structural similarity to α-conotoxin MII, which potently and selectively blocks α3β2 nAChRs (Cartier et al., 1996a).

α3 and β4 subunits are distributed throughout both the PNS and CNS. How often these subunits combine to form a receptor is unknown. α3β4-like receptors have been reported to be present in trigeminal sensory, superior cervical ganglion, and habenula neu-

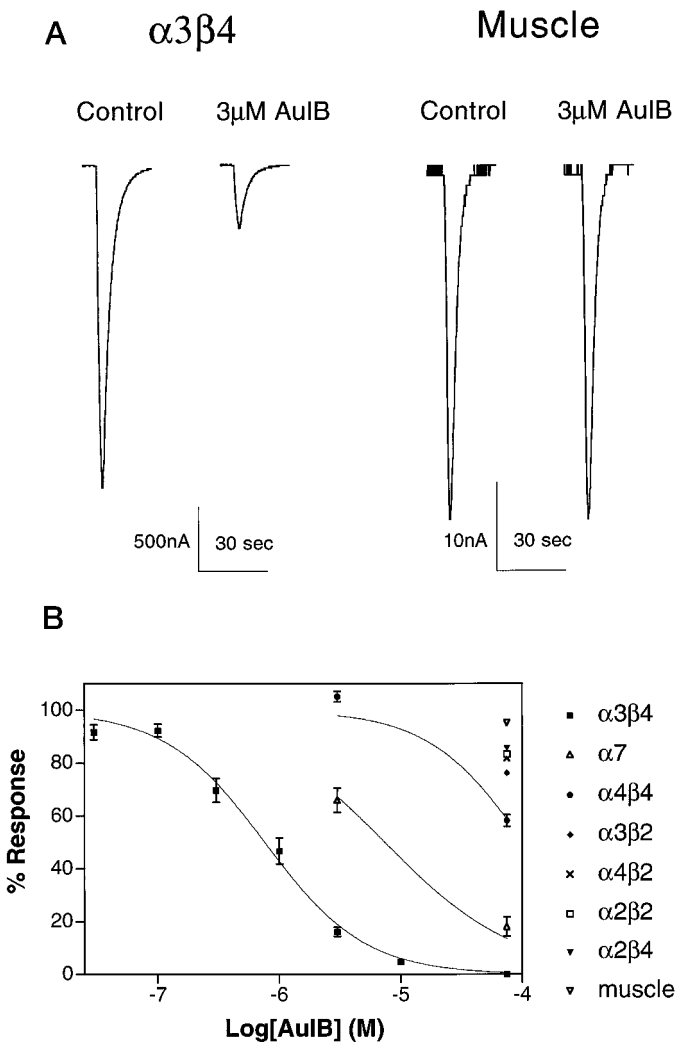


Figure 4. Selectivity of α-conotoxin AuIB. *A*, AuIB blocks α3β4 but not muscle nAChR. The peptide AuIB (3 μM) blocks 84 ± 2% of the ACh response of α3β4 receptors ($n = 10$). In contrast, it fails to inhibit the α1β1γδ (*muscle*) receptor. *B*, α-conotoxin AuIB preferentially blocks α3β4 versus other nAChR subunit combinations. The dose–response curve shows that α-conotoxin AuIB blocks α3β4 receptors with an IC_{50} of 0.75 μM, (n_H is 1.05). By comparison, AuIB is ~10-fold (α7) and >100-fold less potent on other nAChR subtypes. Each data point represents the average of three to five oocytes. Error bars are SEM. SEM is not shown for the *top right* five data points for figure clarity, but is less than 5% of the mean in each of these cases.

rons and have been implicated in CNS neurotransmitter release (Clarke and Reuben, 1996; Flores et al., 1996; Zoli et al., 1998).

In the present study, we demonstrate that α-conotoxin AuIB discriminates among heterologously expressed receptors in *Xenopus* oocytes. There is evidence, however, that α3β4 receptors expressed in oocytes differ from α3β4 receptors expressed in cultured mammalian cell lines as well as α3β4-like native receptors present in the superior cervical ganglion. Differences among these receptors include dissimilarities in channel conductance and kinetics as well as relative sensitivity to nicotinic agonists (Lewis et al., 1997; Sivilotti et al., 1997). Reasons for these differences are unknown. Possibilities include differences in post-translational modifications, variations in folding or assembly of subunits, or differences in subunit stoichiometry. Although differences in sensitivity to antagonists among these receptors have not been reported, caution should be exercised in extrapolating our results to native receptor subtypes. Nevertheless, α-conotoxin AuIB recently has been shown to discriminate among native nAChRs. α-Conotoxin AuIB (1 μM) blocks ~75% of the (putative α3β4-subunit-containing) nAChR response in habenula neurons (Lester et al., 1998). In contrast, 5 μM α-conotoxin AuIB fails to inhibit κ-bungarotoxin and α-conotoxin MII-sensitive (putative α3β2-subunit-containing) nAChRs that underlie spontaneous waves in retinal ganglion cells (Penn et al., 1998). In addition, we demonstrate in the present study that α-conotoxin AuIB distinguishes between nAChRs that mediate nicotine-evoked norepinephrine versus dopamine release. Thus α-conotoxin AuIB clearly is able to discriminate among different subtypes of native receptors and may be used in combination with other selective ligands to "fingerprint" receptor subtypes.

α-Conotoxin MII has been used previously to study the role of α3β2-like receptors in nicotine-stimulated dopamine release (Kulak et al., 1997; Grady et al., 1997; Kaiser et al., 1998). In this report the newly isolated α-conotoxin AuIB was used to investigate a role for α3β4-like nAChRs in nicotine-stimulated neurotransmitter release. With respect to α3-containing receptors, the selectivity of α-conotoxin MII is opposite that of α-conotoxin AuIB, with MII being highly selective for the α3β2 receptor subtype and AuIB preferring α3β4. When nicotine-stimulated dopamine and norepinephrine release were investigated using synaptosomal preparations, the effects of MII and AuIB were found to be complementary. MII blocked a fraction of nicotine-evoked dopamine release but not norepinephrine release, whereas AuIB blocked a fraction of norepinephrine but not dopamine release. Previous studies had suggested that the subtype(s) of nicotinic receptors that modulates dopamine versus norepinephrine release might be different (Sacaan et al., 1995; Clarke and Reuben, 1996). Our results with α-conotoxins AuIB and MII strongly support these earlier suggestions and provide evidence for the identity of the specific nAChR subtypes likely to be involved.

The subunit composition of the nAChR(s) that modulates norepinephrine release is unknown. The native receptor may be a heteromer with two or more distinct subunits. Previous investigators have generally agreed that an α3-subunit is likely to be present but have differed as to which β-subunit(s) is present. In hippocampal slices it has been reported that an α3β2-like receptor is responsible (Sershen et al., 1997), whereas in synaptosomes it has been reported that the pharmacological profile most closely resembles that of α3β4-like receptors (Clarke and Reuben, 1996). These differences, however, may be attributable to the types of preparations (i.e., slices vs synaptosomes) being used. The magnitude of norepinephrine release in slices is severalfold larger and tetrodotoxin sensitive (Sacaan et al., 1995), compared with release in synaptosomes that is tetrodotoxin insensitive (Clarke and Reuben, 1996). Therefore, it is conceivable that the large response seen in slices is attributable primarily to distal α3β2-like receptors that indirectly stimulate norepinephrine release, whereas the receptors present at synaptic terminals are primarily α3β4-like, the latter consistent with the effects of α-conotoxins AuIB and MII on nicotine-evoked norepinephrine release from hippocampal synaptosomes.

Although the results with AuIB suggest the presence of an α3/β4-subunit interface in the nicotinic receptor that mediates norepinephrine release, it remains to be determined whether the receptor target is purely α3β4 or composed of additional subunits as well. In general it is believed that each nicotinic receptor

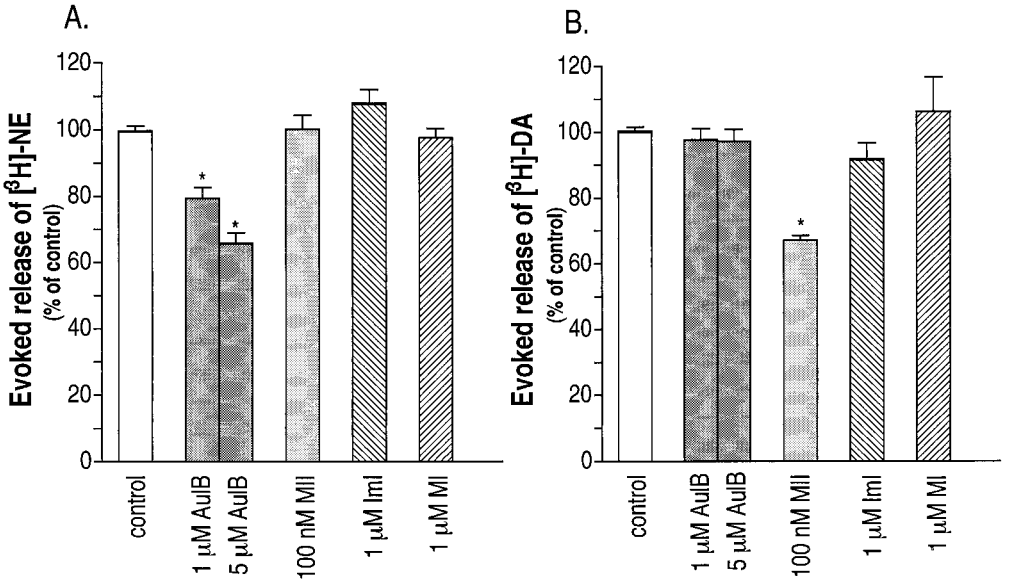


Figure 5. AuIB blocks nicotine-stimulated norepinephrine, but not dopamine, release. *A*, AuIB blocks norepinephrine release from rat hippocampal synaptosomes. In contrast, α-CTx MII (α3β2-selective), α-CTx ImI (α7-selective), and α-CTx MI (α1βγδ-selective) all fail to block release. *B,* Conversely, α-CTx MII, but not α-CTx AuIB, α-CTx ImI, or α-CTx MI, blocks dopamine release from rat striatal synaptosomes. $^{*}p \leq 0.001$. Data are from 3–10 experiments with three to six replicates within each experiment. Norepinephrine release: 1 μM AuIB, $p = 0.001$; 5 μM AuIB, $p < 0.001$; MII, $p = 0.75$; ImI, $p = 0.64$; MI, $p = 0.5$. Dopamine release: 1 μM AuIB, $p = 0.93$; 5 μM AuIB, $p = 0.68$; MII, $p < 0.001$; ImI, $p = 0.24$; MI, $p = 0.85$.

Table 2. Selectivity of α-conotoxins for mammalian nAChRs expressed in *Xenopus* oocytes

α-Conotoxin	nAChR	Ref
AuIB	α3β4	This report
ImI	α7	Johnson et al., 1995
MII	α3β2	Cartier et al., 1996
MI	α1β1γδ	Johnson et al., 1995

requires the binding of two molecules of acetylcholine for activation; therefore, the binding of one agonist site by AuIB should be sufficient to block receptor function. This scenario in fact has been demonstrated with other α-conotoxins and other nicotinic receptors (Martinez et al., 1995; Cartier et al., 1996b). Thus, it is not known what subunits in addition to the α3/β4-subunit interface are present in the AuIB-sensitive receptors. However, an α3β2-subunit interface appears to be absent, because α-conotoxin MII fails to block nicotine-evoked norepinephrine release. Pharmacological definition of the remaining subunits of these synaptic receptors may require additional antagonists with novel subunit specificity.

The isolation of α-conotoxin AuIB provides further incentive to screen *Conus* venoms for additional selective nicotinic receptor antagonists. The underlying reason for the extreme diversity of nicotinic-targeted peptides in these venoms is unknown. However, the broad diversity of prey types (five different phyla) of the cone snails and the likely high degree of heterogeneity of nicotinic receptor subtypes present in their prey (and potential predators) could provide the rationale for this diversity.

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