Differential Targeting of Nicotinic Acetylcholine Receptors by Novel αA-Conotoxins*

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We describe the isolation and characterization of two peptide toxins from Conus erminsae venom targeted to nicotinic acetylcholine receptors (nAChRs). The peptide structures have been confirmed by mass spectrometry and chemical synthesis. In contrast to the 12–18 residue, 4 Cys-containing α-conotoxins, the new toxins have 30 residues and 6 Cys residues. The toxins, named αA-conotoxins AIVA and AIVB, block both Torpedo and mouse α1-containing muscle subtype nAChRs expressed in Xenopus oocytes at low nanomolar concentrations. In contrast to α-bungarotoxin, αA-IVA is inactive at α7-containing nAChRs even at micromolar concentrations. In this regard, αA-IVA is similar to the previously described α-conotoxins (e.g. α-MI and α-GI) which also selectively target α1- versus α7-containing nAChRs. However, α-MI and α-GI discriminate between the α/β versus α/γ subunit interfaces of the mouse muscle nAChR with 10,000-fold selectivity. In contrast, αA-conotoxin EIVA blocks both the α/γ site and α/δ site with equally high affinity but with distinct kinetics. The αA-conotoxins thus represent novel probes for the α/γ as well as the α/δ binding sites of the nAChR.

The carnivorous cone snails (genus Conus) comprise 500 species that specialize on a variety of prey, including fish. There are ~50–70 different fish-hunting Conus species which use venom as the primary weapon for immobilizing prey. Their venoms are extremely complex, and each Conus species has its own distinct complement of biologically active venom peptides. Nevertheless, all fish-hunting Conus venoms examined so far inhibit neuromuscular transmission. In all cases, one major molecular target involved in this inhibition is the nicotinic acetylcholine receptor (nAChR) at the neuromuscular junction. Thus, all fish-hunting cone snail venoms appear to contain a major venom peptide which, like the snake toxin α-bungarotoxin, potently inhibits the postsynaptic nAChR by competitively blocking the acetylcholine (ACh) binding site.

Despite this common mechanism of action, there are considerable differences in the structures of the nAChR-antagonist peptides in the venoms of various fish-hunting cone snails. The major family of such peptides identified so far are the α-conotoxins, which have been characterized from several species of Indo-Pacific fish-hunting cone snails (1). Recently, a peptide which is a competitive nAChR antagonist was purified and characterized from the Eastern Pacific piscivorous Conus species, Conus purpurascens (the purple cone) (2). This peptide, αA-PIVA, has a disulfide framework entirely different from all other competitive nAChR antagonists from Conus. The peptide has three disulfide bonds instead of the two usually found in α-conotoxins. Furthermore, no obvious homology is detected between α- and αA-conotoxins when their sequences are aligned. Thus, the isolation of αA-PIVA suggests that there may be two major groups of competitive nicotinic antagonists in the venom of fish-hunting cone snails, the α- and the αA-conotoxins.

Perhaps the most closely related fish-hunting Conus species to C. purpurascens is the “turtle” cone, Conus erminsae, which is found throughout the tropical Atlantic (see Fig. 1). However, the first nAChR antagonist which we purified from C. erminsae venom was not homologous to αA-PIVA, but was instead a highly divergent α-conotoxin, α-EI (3). In this report, we demonstrate that in addition to α-EI, the venom of C. erminsae contains two other nicotinic antagonists, which are αA-conotoxins by virtue of their structural homology to αA-PIVA.

The discovery of these two peptides, αA-conotoxins AIVA and EIVB, expands the membership of the αA-conotoxin family and provides initial structure-function information which will be useful for studies using this family of nicotinic antagonists. The three-dimensional structures of α-conotoxin GI (4) and Pn1A are known (5), and that of the first αA-conotoxin was recently solved.2 Thus, the α- and αA-conotoxins now provide two sets of ligands of known three-dimensional structure that can be used to probe the ligand binding sites of nAChRs in vertebrate muscle. We demonstrate here that the new αA-conotoxins have a selectivity profile which differs from both the α-conotoxins and α-bungarotoxin.

EXPERIMENTAL PROCEDURES

Materials—Crude venom was obtained from milkings of C. erminsae kept in aquaria. The venom was stored at ~70 °C until used. Trifluoroacetic acid (sequencing grade) was from Aldrich, anion exchange resin (UG grade) was from Baxter, and [125]α-BTX was from NEN Life Science Products.

Venom Preparation—Individual milkings of C. erminsae venom (2, 3) collected from 10 snails were pooled (final volume 1.5 ml) and concentrated by lyophilization to 0.5 ml. The concentrate was mixed with 3 ml of 0.1% trifluoroacetic acid just prior to application onto an HPLC column.

Goldfish Bioassay—Purification of the paralytic toxin was monitored by injecting fractions into goldfish as described previously (3).

1 This abbreviations used are: nAChR, nicotinic acetylcholine receptor; BTX, bungarotoxin; HPLC, high performance liquid chromatography.

2 Han, K. H., Hwang, K. J., Kim, S. K., Gray, W. R., Oliveira, B. M., Rivier, J., and Shon, K. J. (1997) Biochemistry 36, 1669–1677.
HPLC Purification—The HPLC apparatus consisted of HPXL pumps and either a Dynamax model UVI or UV-DII detector (Rainin, Woburn, MA). All columns were also from Rainin. For isolation of peptide from venom and all subsequent purifications, buffer A consisted of 0.1% trifluoroacetic acid, and buffer B was 0.092% trifluoroacetic acid, 60% acetonitrile. Initial purification of αA-EIVA from milked venom was accomplished using a semipreparative C\textsubscript{18} Vydac column (10 mm × 25 cm, 5-μm particle size), with a flow rate of 5.0 ml/min. Subsequent purification steps of αA-EIVA utilized an analytical C\textsubscript{18} Microsorb or Vydac column (4.6 mm × 25 cm, 5-μm particle size) with a flow rate of 1.0 ml/min.

Sequence Analysis—Purified peptides were reduced by dithiothreitol, alkylated with 4-vinylpyridine, and HPLC-purified by previously described methods (3). Sequencing was performed by an Applied Biosystems Model 477A protein sequencer at the Protein/DNA Core Facility at the University of Utah Cancer Center.

Mass Spectrometry—Liquid secondary ionization mass spectra were measured using a Jeol HX110 (JEOL, Tokyo, Japan) double-focusing instrument under these conditions was typically better than 50 ppm.

Electrophysiology—cRNA encoding nAChR subunits was prepared and injected into Xenopus oocytes as described previously (8). Oocytes were injected 1–2 days after harvesting and used for voltage clamp recording 1–7 days after injection. "α1β1γ" and "α1β1δ" receptors were expressed by omitting cRNA for either the δ or γ subunit, respectively, from the usual α1βγδ-cRNA injection mixture used to express wild-type muscle receptors (9, 10).

Voltage clamp recording was done as described previously (8). Briefly, oocytes were clamped at −70 mV with a two-electrode system and perfused with ND96 containing 1 μM atropine (to block any endogenous muscarinic acetylcholine receptors). ND96 consists of 96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1.0 mM MgCl\textsubscript{2}, 5 mM Hepes (pH 7.1–7.5). ACh-gated currents were elicited with 1-s pulses of 10 μM ACh (for Torpedo and mouse α1β1γ and α1β1δ nAChRs), 1 μM ACh (for wild-type muscle nAChRs), 300 μM (for α4β2, α3β2, and α3δ2 nAChRs), and 1 mM ACh (for α7 nAChRs), applied at a frequency of 1/min, except for α4β2 receptors which were pulsed every 3 min to avoid desensitization. To elicit currents sufficient for recording a 10-fold higher concentration of ACh was necessary with α1β1γ receptors whereas a submaximal concentration was chosen to allow the typically large currents to be voltage-clamped. When both ACh concentrations were tested on the wild-type muscle receptors the same toxin affinity was observed (data not shown). These results were expected given the short duration of ACh exposure during test pulses and the comparatively slow dissociation rate of the toxin. The ACh applied was approximately the lowest concentration required to elicit maximal current responses at each receptor subtype. For the muscle and Torpedo receptors, however, a submaximal concentration was chosen to allow the typically large current responses to be well-resolved.

Toxins were applied in ND96 containing 1 μM atropine and 0.1 mg/ml bovine serum albumin. Exposure to each concentration of αA-EIVA or αA-EIVB was continued until the peak amplitudes of the ACh-gated currents reached a steady state. The average peak amplitude of at least four control responses recorded in the absence of toxin was used to normalize the response at each toxin concentration to obtain the "frac-
tional response." After application of toxin, the perfusion medium was switched back to ND96 with atropine. All kinetic and dose-response curves were generated using Prism software (GraphPad Software Inc., San Diego, CA). Dose-response curves were fit to the equation: fraction response = 1/[1 + ((toxin)/IC_{50})^{n_{\text{h}}}] where n_{\text{h}} is the Hill coefficient.

Curve-fitting of Toxic Kinetics—It is assumed that the toxin (T) reversibly binds to its receptor site (S) according to the scheme,

\[ T + S \rightleftharpoons TS \]

\[ k_{\text{on}} \]

\[ k_{\text{off}} \]

with forward and reverse rate constants \( k_{\text{on}} \) and \( k_{\text{off}} \) respectively. It is also assumed that the amount of toxin greatly exceeds the number of sites, and thus the forward reaction follows pseudo-first order kinetics. We define \( p(t) \) as the probability that a site is occupied by toxin at time \( t \) and \( q(t) \) as the probability that the site is unoccupied; so \( q(t) = (1 - p(t)) \). If the system is initially at equilibrium, then when the concentration of toxin is perturbed to a new concentration \( [T] \), the toxin occupancy of the site will relax to a new equilibrium value exponentially with a rate constant \( k = (k_{\text{on}}[T] + k_{\text{off}}) \) (see e.g. Ref. 6). Thus, \( p(t) \) will follow a first course from its initial value \( p(0) \) to its new equilibrium value \( p(\infty) \) according to the equation

\[ p(t) = (p(0) - p(\infty))e^{-kt} + p(\infty) \quad (Eq. 1) \]

which rearranged yields

\[ p(t) = p(0)e^{-kt} + p(\infty)(1 - e^{-kt}) \quad (Eq. 2) \]

Note that since \( p(0) \) and \( p(\infty) \) are equilibrium values before and after the toxin concentration was perturbed (say from \([T]_{0}\) to \([T]\)), their values are determined by the well-known relationship described by a rectangular hyperbola or binding isotherm, that is

\[ p(0) = \frac{1}{1 + \frac{k_{\text{off}}}{k_{\text{on}}[T]}} \quad (Eq. 3) \]

and

\[ p(\infty) = \frac{1}{1 + \frac{k_{\text{off}}}{k_{\text{on}}[T]}} \quad (Eq. 4) \]

It is also assumed that each receptor has \( N \) independent toxin-binding sites, and that occupancy of a single site is sufficient to inactivate the receptor. Then the probability that a receptor would be given by the probability that none of the sites on the receptor is occupied, and this in turn is given by the product of the probabilities of each site being unoccupied; in other words, the fraction of receptors that are active, \( FA(t) \), is given by \( q(t) \) if the binding sites are kinetically equivalent. If sites are not equivalent, \( FA(t) \) is the product of probabilities

\[ FA(t) = \prod_{i=1}^{N} q_i(t) \quad (Eq. 5) \]

where \( q_i \) represents the probability that the \( i \)th site is unoccupied. Substituting the relationship in Equation 2 for \( (1 - p(t)) \) gives the following term for \( q(t) \)

\[ q(t) = (1 - p(0)e^{-kt} - p(\infty)(1 - e^{-kt})) \quad (Eq. 6) \]

which rearranged yields

\[ q(t) = (1 + [p(\infty) - p(0)]e^{-kt} - p(\infty)) \quad (Eq. 7) \]

When \( k, p(0), \) and \( p(\infty) \) are replaced with their expanded versions given above, this yields

\[ q(t) = \left( 1 + \frac{1}{k_{\text{on}}[T]} - \frac{1}{k_{\text{off}}[T]} \right) e^{-kt} + \frac{1}{k_{\text{off}}[T]} \quad (Eq. 8) \]

Thus, for nAChRs with two nonequivalent binding sites, where occupation of either site is sufficient to block receptor function, the fraction of active receptors is given by

\[ FA(t) = \prod_{i=1}^{N} q_i(t) = q(t) \]

\[ (Eq. 8) \]

with \( q(t) \) specified by Equation 8 using the respective \( k_{\text{on}} \) and \( k_{\text{off}} \) for each site.

Note that when the initial concentration of toxin is zero (i.e. \([T]_0 = 0\)), then Equation 8 reduces to

\[ q(t) = \left( 1 - \frac{1}{1 + \frac{k_{\text{off}}}{k_{\text{on}}[T]}} \right) e^{-kt} \quad (Eq. 10) \]

Conversely, when the toxin is washed out (i.e. \([T] = 0\)) following equilibration with a toxin concentration of \([T]_0\), Equation 8 reduces to

\[ q(t) = \left( 1 - \frac{1}{1 + \frac{k_{\text{off}}}{k_{\text{on}}[T]}} \right) e^{-kt} \quad (Eq. 11) \]

Our experiments monitored \( FA(t) \) with pulses of ACh that produced brief responses (time to peak ~1 s). Thus, the receptors are exposed to ACh only for a relatively short time compared with the off-kinetics of the toxin (\(k_{\text{off}} \sim 1\) min), and ACh has only minimal opportunity to displace toxin from the receptors. We therefore assumed that the pulses of ACh did not perturb the binding of toxin to receptor. To simplify the curve-fitting of toxin wash-out, the binding isotherm \( p(0) \), used to describe the initial conditions in Equation 11, was replaced by a variable, \( 1 - y_{\text{min}} \), and final conditions were described by a second variable, \( y_{\text{max}} \), yielding the equation \( y = y_{\text{max}} - (1 - y_{\text{min}})e^{-kt} \). The values for \( k_{\text{off}} \) were then used in Equation 10 to fit toxin wash-in curves and calculate \( k_{\text{on}} \) values.

Inhibition of \( 125\text{I} - \alpha\text{-BTX} \) Binding to Nicotinic Receptors on Intact \( B_{C_3}H-1 \) Cells—Binding methods were as described previously (3). Briefly, \( B_{C_3}H-1 \) cells were incubated in 250 \( \mu \)l of assay buffer (140 mM KCl, 25 mM HEPES, 5.4 mM NaCl, 1.8 mM CaCl\(_2\), 1.7 mM MgSO\(_4\), 0.06 mg/ml bovine serum albumin, pH 7.4) with or without \( \alpha\text{-conotoxin EIVA}. 125\text{I} - \alpha\text{-BTX} (10 \mu \text{f}, final concentration 20 nM) was then added, and the reaction was allowed to incubate for an additional 15 min. Cells were then washed twice with 2.0 ml of assay buffer to remove unbound ligands, and receptor-bound 125\text{I}-\alpha-BTX was removed from the wells with two 0.5-ml washes of 1% Triton X-100 in water and counted in a \( \gamma \) counter. Nonspecific binding was determined with cells previously exposed to 100 nM \( \alpha\text{-BTX} \) for 30 min. The total density of \( 125\text{I}-\alpha\text{-BTX} \) binding sites was determined from a 60-min incubation in the absence of competing drug. Approximately 60–70% of the total specific \( 125\text{I}-\alpha\text{-BTX} \) binding sites were labeled by 125\text{I}-\alpha-BTX during the 15 min of the assays. All assays were performed at room temperature in triplicate.

RESULTS

Purification—Crude venom from \( C. \text{ermineus} \) obtained by milking snails was fractionated by HPLC. The elution pattern of two of the peptides paralytic to fish are shown in Fig. 2. These venom fractions were subfractionated until the peptides were purified to homogeneity. Disulfide bridges were reduced, and cysteines were alkylated prior to sequencing. Their amino acid sequences are as follows.

\[
\begin{align*}
\text{GCCGYPONAACRDOCCSVGRROYCDROSNG} & \quad (A) \\
\text{GCCGYPYONACRDOCCSVGRROYCDROSNG} & \quad (B)
\end{align*}
\]

SEQUENCE 1

These sequences were confirmed by mass spectrometry (MH\(^+\) = 3095.2 and 3099.1); these values are consistent with the peptides amided at their C termini and with all of the Cys residues in disulfide linkages (calculated mass 3095.2 and 3099.2).

Chemical Synthesis of the Purified Peptides—Based on the putative sequences, the two peptides were synthesized by solid-state methods as described under “Experimental Procedures.” The linear peptides were oxidized to form disulfide linkages. When air oxidation was used, the material corresponding to native peptide was not formed in high yield (data not shown). However, when glutathione oxidation was performed the major products corresponded to natural peptides by the criteria de-
0.092% trifluoroacetic acid, 60% acetonitrile. Milked venom (see text)
consistently with the predicted sequences (MH$^+$
experiments were carried out with cloned skeletal muscle
the functional effects of these peptides, electrophysiological
differences obtained when the peptides were tested on oocytes ex-
with nearly identical IC$^{50}$ values. Given these results and the
potent inhibitors of ACh-gated currents under these conditions,
which has been shown to be an antagonist of the nAChR at the

200 ng of resin typically yielded 5–10 mg of biologically active peptide product.
When co-injected on HPLC the purified, chemically synthesized peptides co-eluted with the corresponding native peptide, as shown in Fig. 3, C and D. Mass spectrometric analysis showed that the molecular masses of synthetic peptides are consistent with the predicted sequences (MH$^+$ = 3095.2 and 3099.2; calculated mass 3095.19 and 3099.18). Furthermore, the paralytic activity of the synthetic peptides was qualitatively the same as that of the native peptides when injected into goldfish (data not shown). Thus, under glutathione oxidation conditions these peptides appear to form the disulfide linkages necessary to confer biological activity. Due to the limited availability of natural peptides, all subsequent experiments were performed with synthetic material.

Mechanism of Action of the Purified Peptides—The peptide sequences have considerable homology with that of αA-PIVA, which has been shown to be an antagonist of the nAChR at the neuromuscular junction. Given the strong homology between the peptides from C. purpureascens and C. ermineus, our initial hypothesis was that the C. ermineus peptides cause paralysis because they are also nAChR antagonists. To directly assess the functional effects of these peptides, electrophysiological experiments were carried out with cloned skeletal muscle nAChRs expressed in Xenopus oocytes. Fig. 4A shows the results obtained when the peptides were tested on oocytes expressing Torpedo nAChRs. It is clear that both peptides are potent inhibitors of ACh-gated currents under these conditions, with nearly identical IC$^{50}$ values. Given these results and the structural homology to αA-PIVA, we designate these peptides as αA-conotoxins EIVA and EIVB.

α-MI is typical of many α-conotoxins from Indo-Pacific fish-hunting Conus venoms in that it prefers the αβ over the αγ interface of the mouse muscle nAChR (11, 12) and the αγ over the αβ (11–13) interface in the Torpedo electric organ nAChR. We compared the affinity of αA-EIVA for each interface by using receptors expressed in oocytes in which only cRNA for the

mouse muscle α1, β1, δ or α1, β1, γ subunits was injected, forcing the formation of receptors with only αβ or αγ interfaces, respectively (9, 10). As a control, 10 nm α-bungarotoxin was tested on these receptors in duplicate experiments. α-Bungarotoxin blocked ~85% of the α1β1δ receptor response and ~92% of the α1β1γ receptor response after 15 min of exposure to toxin (data not shown). Fig. 4B shows the potency of αA-
EIVA when tested on α1β1γ nAChRs, termed wild-type receptors, and on the subunit-deficient variants of this receptor. These results differ markedly from those previously shown for α-MI, which prefers α1β1γ receptors with a discrimination index of 10$^4$ (14). The IC$^{50}$ values of αA-EIVA for wild-type, α1β1δ, and α1β1γ receptors are almost identical. Thus, it appears that both the α1δ and α1γ interfaces are high affinity targets for αA-EIVA.

Toxin Kinetics—The wild-type mouse muscle nAChR is known to contain ACh binding sites at the α1δ and α1γ subunit interfaces (15). Both of which must be occupied by agonist to activate the receptor. Receptors containing only α1β1δ or α1β1γ subunits have also been shown to contain two agonist binding sites which behave pharmacologically as a single class

FIG. 2. Purification of αA-conotoxins EIVA and EIVB from C. ermineus venom. Buffer A = 0.1% trifluoroacetic acid; buffer B = 0.092% trifluoroacetic acid, 60% acetonitrile. Milked venom (see text) was applied to a semipreparative Vydac column. The gradient program was 0–15% B for 5 min, 15–40% B for 74 min, 40–75% B for 3 min, followed by 75–100% B for 14 min; flow rate was 5 μl/min. The major components of the peptide absorbances indicated by closed arrows correspond to α-conotoxins EIVA and EIVB, respectively (eluting at 31.1 min and 39.6 min). These materials were purified to homogeneity using the semipreparative Vydac column described above with a gradient program beginning at 5–30% B for 75 min. Each of the major peaks was then subsequently run on an analytic Microsorb C18 column and eluted with a gradient program of 4–30% B for 75 min and flow rate of 1 ml/min (data not shown). The peptide absorbance indicated by the open arrow at 51.7 min corresponds to α-conotoxin EI, the isolation of which has been described previously (3).

FIG. 3. Chemical synthesis of αA-EIVA and αA-EIVB. Disulfide bonds were formed in linear peptides by oxidation with glutathione (1 mM reduced, 0.5 mM oxidized, pH 7.5) for 24 h, yielding mixtures of isomers as shown by HPLC chromatograms in A for αA-EIVA and in B for αA-EIVB. The major peak in each reaction mixture was purified to homogeneity and found to co-elute using HPLC when co-injected with the natural peptides as shown in C for αA-EIVA and in D for αA-EIVB. HPLC-run conditions are detailed under Peptide Synthesis under “Experimental Procedures.” Absorbance was measured at 220 nm.

FIG. 4. Peptide Synthesis—The natural peptides were purified to homogeneity. The cleavage of 100 mg of resin typically yielded 5–10 mg of biologically active peptide product.
with respect to competitive antagonist binding (10). To calculate the rate constants \( k_{on} \) and \( k_{off} \) for the interaction of \( \alpha\alpha\)-EIVA with \( \alpha_1\beta_1\delta \) and \( \alpha_1\beta_1\gamma \) receptors, we used the model with two equivalent competitive antagonist binding sites (at the two \( \alpha_1\delta \) or two \( \alpha_1\gamma \) interfaces) in which binding to either site is sufficient to inhibit receptor function (10, 16). Since the wild-type receptor contains both an \( \alpha_1\delta \) and an \( \alpha_1\gamma \) toxin binding site, the receptor should exhibit kinetics consistent with both sites. In fact, this is what was observed. Fig. 5 shows the time course of \( \alpha\alpha\)-EIVA block and recovery from block on \( \alpha_1\beta_1\delta \), \( \alpha_1\beta_1\gamma \), and wild-type mouse muscle nAChRs. The parameters \( k_{on}, k_{off} \), and \( K_d \) for these receptors are summarized in Table I. As shown in Fig. 5, use of the kinetic values calculated from \( \alpha_1\beta_1\delta \) and \( \alpha_1\beta_1\gamma \) receptors provides a good description of block and recovery from block by \( \alpha\alpha\)-EIVA on the wild-type receptor.

The observed rates of both block and recovery from block by toxin were more rapid for \( \alpha_1\beta_1\delta \) versus \( \alpha_1\beta_1\gamma \) receptors. For the wild-type receptor, the observed rate of toxin block was relatively fast, like that of the \( \alpha_1\beta_1\delta \) receptor. In contrast, the rate of recovery from toxin block for wild-type receptor was slower, comparable to that observed for the \( \alpha_1\beta_1\gamma \) receptor. Thus, the kinetics of block of wild-type receptor appear to be dictated primarily by the faster toxin association rate of the \( \alpha_1\delta \) site, while the rate of recovery of receptor function following toxin wash-out is primarily influenced by the slower dissociation rate of the \( \alpha_1\gamma \) site. This is the expected result if binding of toxin to a single agonist site is sufficient to block receptor function.

To investigate further the target specificity of \( \alpha\alpha\)-EIVA, we tested its activity on the \( \alpha_7 \) subtype of neuronal nAChRs. No detectable block of ACh-gated currents was observed in \( \alpha_7 \)-expressing oocytes perfused with 1 \( \mu \)M \( \alpha\alpha\)-EIVA or upon exposure to 60 \( \mu \)M toxin in a static bath (data not shown). We also tested \( \alpha\alpha\)-EIVA on oocytes expressing \( \alpha_4\beta_2, \alpha_3\beta_2, \) or \( \alpha_3\beta_4 \) nAChRs. 1 \( \mu \)M toxin (applied by perfusion) blocked \( \alpha_4\beta_2 \) receptors ~25% but failed to block \( \alpha_3\beta_2 \) or \( \alpha_3\beta_4 \) nAChRs (\( n = 3 \)).

To assess the mechanism of \( \alpha\alpha\)-EIVA action, binding experiments with radiolabeled \( \alpha \)-bungarotoxin and muscle nAChR-expressing BC3H-1 cells were carried out. The results of these experiments are shown in Fig. 6. In this assay, the \( \alpha\alpha\)-EIVA displaced all specific binding of \( ^{125}\text{I-}\alpha \)-bungarotoxin to BC3H-1 cells, consistent with the toxin being a competitive antagonist of the nAChR. In addition, the data are best fit by a dose-response curve for a single-site model. This is consistent with the oocyte studies indicating that \( \alpha\alpha\)-EIVA has nearly equal affinity for both the \( \alpha_\gamma \) and \( \alpha_\delta \) sites.
TABLE I

| Subunits | Calculated IC50 (nM) | Predicted IC50 (nM) | Observed IC50 (nM) |
|----------|---------------------|---------------------|-------------------|
| α1β1δ   | 3.37                | 37                  | 15                |
| α1β1γ   | 0.94                | 32                  | 13                |

α The calculated IC50 is based on the calculated dissociation rate constant (koff)

TABLE II

| Subunit | a1/β | a1/γ | α7 |
|---------|------|------|----|
| α-MI    | +    | -    | -  |
| α-A-EIVA| +    | +    | +  |
| α-Bungarotoxin | + | + | + |

DISCUSSION

The data presented above demonstrate the presence of two peptides from the venom of C. ermineus, α-A-EIVA and α-A-EIVB, which are competitive antagonists of nAChRs. α-A-Conotoxin EIVA was examined in detail and has several notable functional features. First, unlike α-bungarotoxin, which has subnanomolar affinity for α7 nAChRs (17, 18), α-A-EIVA does not block these receptors even at micromolar concentrations. In this regard, α-A-EIVA behaves like α-conotoxins MI and GI, which potently target α1-containing, but not α7, nAChRs (18).

Thus, Conus has evolved two independent structures that discriminate between α1 versus α7 receptors. However, like α-bungarotoxin, α-A-EIVA has high affinity for both the αδ and αγ sites of the muscle subtype of nAChR as shown by both electrophysiological and radioligand binding experiments (although the peptide association and dissociation rates differ for each site). In contrast, α-conotoxins MI and GI are highly selective for the mouse muscle αδ versus αγ site (104-fold discrimination). These divergent specificities are summarized in Table II. The structural basis for these differences in ligand affinity is presently under investigation. In addition, a structural determination of α-A-EIVA is almost complete.

Since et al. (14) have identified three critical residues in the δ nAChR subunit (Ser-36, Tyr-113, and Ile-178) which confer upon α-MI high αδ versus αγ affinity (14). Initially residues in the homologous positions of the γ subunit were mutated to contain these amino acids, mutant α1β1γ receptors had a high affinity for α-MI. The results of the present study suggest that α-A-EIVA recognizes at least a partially different set of receptor residues from those recognized by α-MI, since α-A-EIVA has a high affinity for both the αδ and αγ sites. Although it is possible that all of the contact residues for α-A-EIVA binding are in the α subunit, it appears from the differences in kinetics of toxin binding to α1β1β1α1β1γ receptors that the δ and γ subunits also influence binding.

α-Bungarotoxin binds with high affinity and slow reversibility to both αδ and αγ sites in the mouse muscle nAChR (19, 20). Our experiments with radiolabeled α-bungarotoxin indicate that α-A-EIVA can block all specific binding of α-bungarotoxin to BC3H-1 cells in a concentration-dependent manner. The resulting dose-response curve is best fit by a single-site model. These results are consistent with those from oocyte experiments which indicate that α-A-EIVA binds with nearly equal affinity at both an αδ and αγ site. However, α-A-EIVA blocked α-bungarotoxin binding to mouse BC3H-1 cells with an IC50 that was approximately 10-fold higher than that observed for α-A-EIVA block of muscle mouse nAChRs expressed in oocytes. This difference is partly explained by the fact that α-A-EIVA must occupy two binding sites on each receptor to completely block α-bungarotoxin binding, while block of functional response can occur by occupation of either site alone. This should result in a 2.4-fold difference between the functional block described by the IC50 in oocyte experiments and the IC50 observed by competition binding (see legend to Table I). Further differences in IC50 values between the experiments may be due to kinetic factors. In oocyte experiments, the binding equilibrium of α-A-EIVA should not be affected appreciably by the brief applications (1-s pulses) of ACh due to the comparatively slow koff of the toxin. In contrast, α-A-EIVA has a much faster koff than α-bungarotoxin. During the 15-min co-incubation of these toxins in the binding experiments, occupation of free binding sites by the essentially irreversible binding of α-bungarotoxin would be expected to shift the resultant dose-response curve of α-A-EIVA toward a higher apparent IC50.

α-A-EIVA and α-A-EIVB have similar sequences and may represent polymorphism in the C. ermineus population. Although these peptides are specifically targeted to the skeletal muscle subtype of the nAChR, they are broadly active in vertebrate systems; we demonstrated directly that they are antagonists when tested against skeletal muscle nAChRs in both elasmobranchs (Torpedo electricus) and mammals (mouse).

In all fish-hunting cone snails, there appears to be at least one major peptide which is a competitive antagonist of skeletal muscle nAChRs; a summary of all such peptides from piscivorous Conus venoms which have been described thus far are shown in Table III. It is clear from the table that these peptides fall into two quite distinct families, the α-conotoxins and the α-A-conotoxins. Before this report, only one α-A-conotoxin had been described, α-A-PVIA from C. purpurascens (2). The two peptides described here clearly exhibit homology to α-A-PVIA, although they diverge substantially in sequence. As we noted previously, C. purpurascens and C. ermineus are the only fish-hunting cone snails known outside the Indo-Pacific region, and it is likely that they have long been isolated geographically from the major series of Indo-Pacific fish-hunting Conus. In all of the Indo-Pacific piscivorous species, an α-conotoxin is the major skeletal muscle nAChR antagonist.

C. ermineus is unusual in being the only Conus venom found

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8 K.-H. Han, K. J. Hwang, S. K. Kim, W. R. Gray, B. M. Olivera, J. Rivier, and K. J. Shon, unpublished results.

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FIG. 6. Inhibition of 125I-α-BTX binding to nicotinic receptors on BC3H-1 cells by α-A-EIVA. The data shown are the mean ± S.E. of four independent experiments. The IC50 and nH were found to be 150 ± 10 nM and 0.85 ± 0.02, respectively. The data are fit best by a single-site competition model with a variable Hill coefficient (solid line) as determined by statistical comparison between a single-site and a two-site competition model (p < 0.001).
New αA-Conotoxins and Nicotinic Acetylcholine Receptors

Table III

Competitive skeletal muscle nAChR antagonists from venoms of fish-hunting Conus

| Sequence | Conus species | Ref. |
|----------|---------------|------|
| αA-EIVA  | GCGSYPQNAACGCCCKWROQYCRDSGQ<sup>a</sup> | C. ermineus | This work |
| αA-EIVB  | GCGSPYQNAACGCCCKWROQYCRDSGQ<sup>a</sup> | C. ermineus | This work |
| αA-PIVA  | GCGSYPQNAACGCCCKWROQYCRDSGQ<sup>a</sup> | C. purpurascens | (2) |
| α-MI     | GRCCHPAGKNSC<sup>a</sup> | C. magus | (22, 23) |
| α-GI     | ECPNAPAGRHSYSC<sup>a</sup> | C. geographus | (24) |
| α-GHA    | ECPNAPAGRHSYSC<sup>a</sup> | C. geographus | (24) |
| α-GH     | ECPNAPAGKFSYSC<sup>a</sup> | C. striatus | (25) |
| α-SIA    | YCPHAPGKNFQ<sup>a</sup> | C. striatus | (26) |
| α-SI     | ICNPAGKPYSC<sup>a</sup> | C. striatus | (27) |
| α-SII    | GCPNAPACPNYGCYSGS<sup>b</sup> | C. ermineus | (3) |
| α-EI     | ROCQHYFCTCNMSNPQIC<sup>a</sup> |  |

<sup>a</sup> Amidated C terminus; O, hydroxyproline.

<sup>b</sup> Free C terminus.

so far to contain both a major α-conotoxin as well as αA-conotoxins. The α-conotoxin described from C. ermineus, α-EI, is also divergent from those found in piacivorous Indo-Pacific species. However, it remains puzzling why this Conus species has both classes of competitive antagonists. At this time, we cannot distinguish between several hypotheses. Individual specimens of C. ermineus may express only the α- or only the αA-conotoxins at any one time, such as a seasonal variation in expression, or there may be polymorphism in the population. Since only pooled venom from many snails has been analyzed so far, we cannot eliminate these possibilities directly. Alternatively, it is possible that the α- and αA-peptides have intrinsically different functional targets, and that within an individual snail venom, both classes of peptides are expressed. For example, if the peptides have different efficacies at different types of least neuromuscular junctions or if the different fish prey of C. ermineus diverge significantly in their neuromuscular junction nAChRs, then it may be advantageous for the predator to have both conotoxin classes in its venom.

Conus species produce a remarkable variety of nicotinic receptor antagonists. The α-conotoxins with a "4/7" spacing (containing sequences of 4 and 7 consecutive non-cysteine amino acids, i.e., CC-----C-----C) are widely found in worm- and snail-hunting Conus species as well as some fish-hunting species (see Table III and Refs. 8 and 21).<sup>4</sup> These toxins target muscle nAChRs as well as neuronal subtypes and may represent the ancestral stem group of nAChR antagonists. The smaller peptides with "3/5" spacing, produced by Indo-Pacific species, and the larger αA-conotoxins may represent separate evolutionary lineages for targeting vertebrate muscle nAChRs.

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<sup>4</sup> R. Jacobsen, D. Yoshikami, M. Ellison, J. Martinez, W. R. Gray, G. E. Cartier, K.-J. Shon, D. R. Groebe, S. N. Abramson, B. M. Olivera, and J. M. McIntosh, unpublished data.