Iodo-α-Conotoxin MI Selectively Binds the α/δ Subunit Interface of Muscle Nicotinic Acetylcholine Receptors†

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ABSTRACT: The embryonic mouse muscle nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel formed by α1, β1, δ, and γ subunits. The receptor contains two ligand binding sites at α/δ and α/γ subunit interfaces. [^{3}H]Curare preferentially binds the α/γ interface. We describe the synthesis and properties of a high-affinity iodinated ligand that selectively binds the α/δ interface. An analogue of α-conotoxin MI was synthesized with an iodine attached to Tyr-12 (iodo-α-MI). The analogue potently blocks the fetal mouse muscle subtype of nAChR expressed in *Xenopus* oocytes. It failed, however, to block α3β4, α4β2, or α7 nAChRs. Iodo-α-MI potently blocks the α1β1δ but not the α1β1γ subunit combination expressed in *Xenopus* oocytes indicating selectivity for the α/δ subunit interface. α-Conotoxin MI was subsequently radioiodinated, and its properties were further evaluated. Saturation experiments indicate that radioiodinated α-conotoxin MI binds to TE671 cell homogenates with a Hill slope of 0.95 ± 0.0094. Kinetic studies indicate that the binding of [^{125}I]α-conotoxin MI is reversible (k_{off} = 0.084 ± 0.0045 min^{-1}); k_{on} is 8.5 × 10^7 min^{-1} M^{-1}. The calculated k_d is 0.98 nM. This potency is ∼20-fold higher than the unmodified α-MI peptide. Unlike [^{125}I]α-bungarotoxin, [^{125}I]α-conotoxin MI binding to TE671 cell homogenates is fully displaceable by the small molecule antagonist *d*-tubocurarine.

Nicotinic acetylcholine receptors (nAChRs)[1] in skeletal muscle are heteropentameric ligand-gated ion channels formed by four subunits, α1β1γδ. Small molecule toxin antagonists of the nAChR have been isolated from a number of natural sources including plants, corals, and gastropods. Often, these toxins serve to discourage consumption by predators. *d*-Tubocurarine from the *Chondodendron tomentosum* bush, dihydro-β-erythroidine derived from the seeds of the trees and shrubs of the genus *Erythrine*, methyllycaconitine from the seeds of *Delphinium brownii* (the larkspur plant), and the lophotoxins from the soft corals of *Lophogorgia* and *Pseudopterogorgia* are prominent examples. In addition, more than 90 nAChR-targeted toxins have been isolated from the venoms of over 30 species of land and sea snakes of the Elapidae and Hydrophidae families. Both the small molecule and peptide toxins have played a pivotal role in defining the structure and function of nicotinic receptors (for reviews, see refs *1*−*3*). One molecule of acetylcholine must bind to each of two separate sites on the muscle nicotinic receptor to enable channel opening. These binding sites are nonequivalent and are located at the α/δ and α/γ subunit interfaces in fetal muscle and α/δ and α/ε subunit interfaces in adult muscle (*4*). Whereas the snake toxin α-bungarotoxin (α-BTX) binds with high affinity to both binding interfaces, curariform antagonists bind with 1−2 orders-of-magnitude higher affinity to the α/γ site than to the α/δ site of the mouse muscle nAChR (*5*). The waglerins from the pit viper *Trimeresurus wagleri* have a 2000-fold preference for α/ε over the α/γ and α/δ interfaces, and the α-neurotoxin from *Naja mossambica mossambica* has 10 000-fold selectivity for α/γ and α/δ interfaces over α/ε (*6*, *7*).

Cone snails produce a family of peptides known as α-conotoxins that are part of a venom strategy to immobilize prey. In contrast to the above toxins, several α-conotoxins from piscivorous *Conus* have 10 000-fold preference for the α/δ versus the α/γ subunit interface of the fetal mouse muscle receptor (*8*, *9*). Studies utilizing site-directed mutagenesis and chimeric receptor subunits have identified three specific amino acid differences in the δ and γ subunits that account for the differential α-conotoxin MI (α-MI) affinity (*10*). Additionally, α-conotoxins MI and GI preferentially target muscle vs neuronal subtypes of nicotinic receptors (*11*).

[^{125}I]α-BTX is widely used to nonselectively label the α/δ and α/γ subunit interfaces of muscle nAChRs. [^{3}H]*d*-Tubocurarine, a ligand that has 1−2 orders-of-magnitude selectivity for the α1/γ subunit interface, has been used for photoaffinity labeling of nAChRs (*12*, *13*). In this report, we describe the synthesis and properties of an iodinated ligand with high potency and selectivity for the α/δ interface of nAChRs of embryonic mouse muscle. Although addition of an iodine atom into a small peptide often compromises activity, addition at Tyr-12 of α-MI significantly *increases* activity.

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[1] Abbreviations: α-BTX, α-bungarotoxin; [^{125}I]α-BTX, [^{125}I]α-bungarotoxin; α-MI, α-conotoxin MI; [^{125}I]α-MI, [^{125}I]α-conotoxin MI; ACh, acetylcholine; BSA, bovine serum albumin; DMEM, Dulbecco's modified eagle's medium; IB, incubation buffer; nAChR, nicotinic acetylcholine receptor; RPLC, reverse-phase liquid chromatography; WB, wash buffer.

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EXPERIMENTAL PROCEDURES

Drugs and Reagents. [^{125}I]Na (approximately 2180 Ci/mM) was obtained from New England Nuclear Research Products (Boston, MA). Drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO).

Synthesis of Iodo-Tyr-α-MI. The peptide was built on a RINK amide resin by Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) procedures using 2-(1*H*-benzotriole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate coupling. Amino acid derivatives were from Advanced ChemTech (Louisville, KY). Fmoc-iodo-3-L-tyrosine was used to incorporate an iodotyrosine in place of Tyr-12 (found in native α-MI). Side chain protection for other non-cysteine residues was in the form of *tert*-butyl (serine), trityl (asparagine, histidine), Boc (lysine), and Pbf (arginine). Cysteine residues were protected in pairs with either *S*-trityl or *S*-acetamidomethyl groups. A two-step oxidation protocol was used to selectively fold the peptide into the correct disulfide configuration as previously described for α-conotoxin PnIA (*14*). Briefly, orthogonal protection was used on cysteines, that is, Cys-4 and Cys-14 were protected as the stable Cys(*S*-acetamidomethyl), while Cys-3 and Cys-8 were protected as the acid-labile Cys(*S*-trityl). After cleavage from the resin, linear peptide was cyclized to form a disulfide bridge between Cys-3 and Cys-8 (i.e., the first and third cysteines) using ferricyanide. After purification by reverse-phase liquid chromatography (RPLC), the monocyclic peptide was oxidized to the bicyclic peptide by treatment with iodine, which simultaneously removed the S-acetamidomethyl groups and closed the second disulfide bridge. The bicyclic peptide was purified by RPLC.

Synthesis of [^{125}I]-Labeled α-MI. Twenty-five microliters of a 1 μM solution of α-MI was added to 40 μL of 0.3 M NH_4Ac, pH 5.3. Ten millicuries of [^{125}I]Na, volume ~22 μL, was added. The iodination reaction was initiated by the addition of 40 μL of freshly prepared 0.4 mM chloromine-T and reacted at room temperature (~22 °C) for 10 min. The reaction was terminated by the addition of 65 μL of 0.5 M ascorbic acid. Trifluoroacetic acid (0.8 mL, 0.1%) was added to lower the pH of the reaction mix prior to RPLC. Mono- and diiodinated peptides were separated from unmodified peptide by RPLC using an analytical VYDAC C_{18} column. Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.09% trifluoroacetic acid, 60% acetonitrile. Loading loop size was 5 mL. The gradient was 10−50% buffer B over 40 min, flow rate was 1 mL/min, and absorbance was monitored at 220 nm. Fractions containing peptide were collected in polypropylene tubes containing 10 μL of 20 mg/mL lysozyme to decrease radioligand binding to the tubes. Carrier-free iodine-125 (Perkin-Elmer, radionucludic purity 99.9%) was utilized for radiolabeling. The specific activity of the radiolabeled peptide was assumed to be that of the radionuclide, that is, ~2180 Ci/mmol. The peptide was diluted to a concentration of 50 nM in incubation buffer (IB), which consisted of NaCl 115 mM, KCl 5 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 1.3 mM, NaN_3 1.5 mM, TRIS−HCl 33 mM, pH 7.4., and BSA 1 mg/mL. [^{125}I]α-MI was kept in solution at 4 °C and could be used for >4 months without notable difference in its binding properties.

Preparation of Cell Membranes. TE671 cells (RD cell line) was from American Type Culture Collection (Manassas, VI) (catalog no. ATCC CRL-8805). Dulbecco's modified eagle's medium (DMEM; catalog no. 30-2002) and fetal bovine serum (catalog no. 30-2021) were also from ATCC. Culture medium was 90% DMEM, 10% fetal bovine serum. Cells were incubated at 37 °C in the presence of 5% CO_2. Culture medium was replaced every 2−3 days. Tissue culture was performed in Falcon tissue culture dishes (100 mm × 20 mm) treated by vacuum gas plasma. Membranes for binding assay were prepared by scraping the tissue culture cells into a 50 mL Sarstedt tube and centrifuging at 2000 rpm at room temperature for 10 min. The supernatant was discarded, and approximately 1 mL of 5 mM TRIS, pH 7.4, was added to the pellet. The cells were homogenized using a Polytron at setting 6 for 10−20 s. Membranes were then centrifuged at 27 000*g* at 4 °C for 10 min. The supernatant was discarded, and the pellet was vortexed with 30 mL of incubation buffer and recentrifuged as above. The resulting pellet was resuspended in incubation buffer at 200 μL per original confluent plate of cells. Five microliters of this homogenate was used in each binding assay and contained approximately 50 μg of protein. Thus, from one confluent plate approximately 40 binding reactions could be carried out. Membranes were stored at 4 °C. Membranes stored under these conditions were usable for several months.

Binding Assays. Aliquots of TE671 cell homogenates equivalent to approximately 50 μg of protein were added to tubes containing incubation buffer (described above). A protease inhibitor cocktail (Sigma P 2714) was added with the final concentration of inhibitors as follows: AEBSF 500 μM, EDTA 250 μM, bestatin 32.5 μM, E-64 0.35 μM, leupeptin 0.25 μM, aprotinin 0.075 μM. [^{125}I]α-MI was added at the indicated concentrations and incubated at room temperature (approximately 22 °C) for the indicated times. In all cases, the assay volumes were maintained at 0.2 mL. Nonspecific binding was determined with either 10 μM α-conotoxin GI or 300 μM *d*-tubocurarine. Specific binding was defined as the difference between total binding and nonspecific binding. Concentrations of radioligands were verified by counting the contents of the incubation tube during the incubation period. Radioligand bound to tissue was consistently <5% of the total radioligand present. For saturation binding experiments, the free ligand concentration was assumed to be equal to the total ligand concentration. Incubations were terminated by vacuum filtration through Whatman GF/C filters using a Brandel cell harvester. Filters were prewet with 0.5% polyethylenimine for 15 min prior to filtration to reduce binding to the filter. Filters were washed three times with 1 mL aliquots of wash buffer (identical in composition to incubation buffer except that BSA was 0.1 mg/mL). Filters were counted in a γ-counter. Saturation and competition binding data were analyzed by nonlinear regression analysis using Prism software (GraphPad Software Inc., San Diego, CA).

RNA Preparation. cDNA clones encoding rodent nAChR subunits were provided by S. Heinemann (Salk Institute, San Diego, CA). Neuronal nAChR clones were from rat, and muscle nAChR clones were from mouse (*15*).

Xenopus Oocyte Electrophysiology. Preparation and injection of oocytes were performed as previously described (*15*) with the exception that amikacin 100 μg/mL was added to the oocyte culture media to eliminate the presence of a bacteria resistant to penicillin G, streptomycin, and genta-

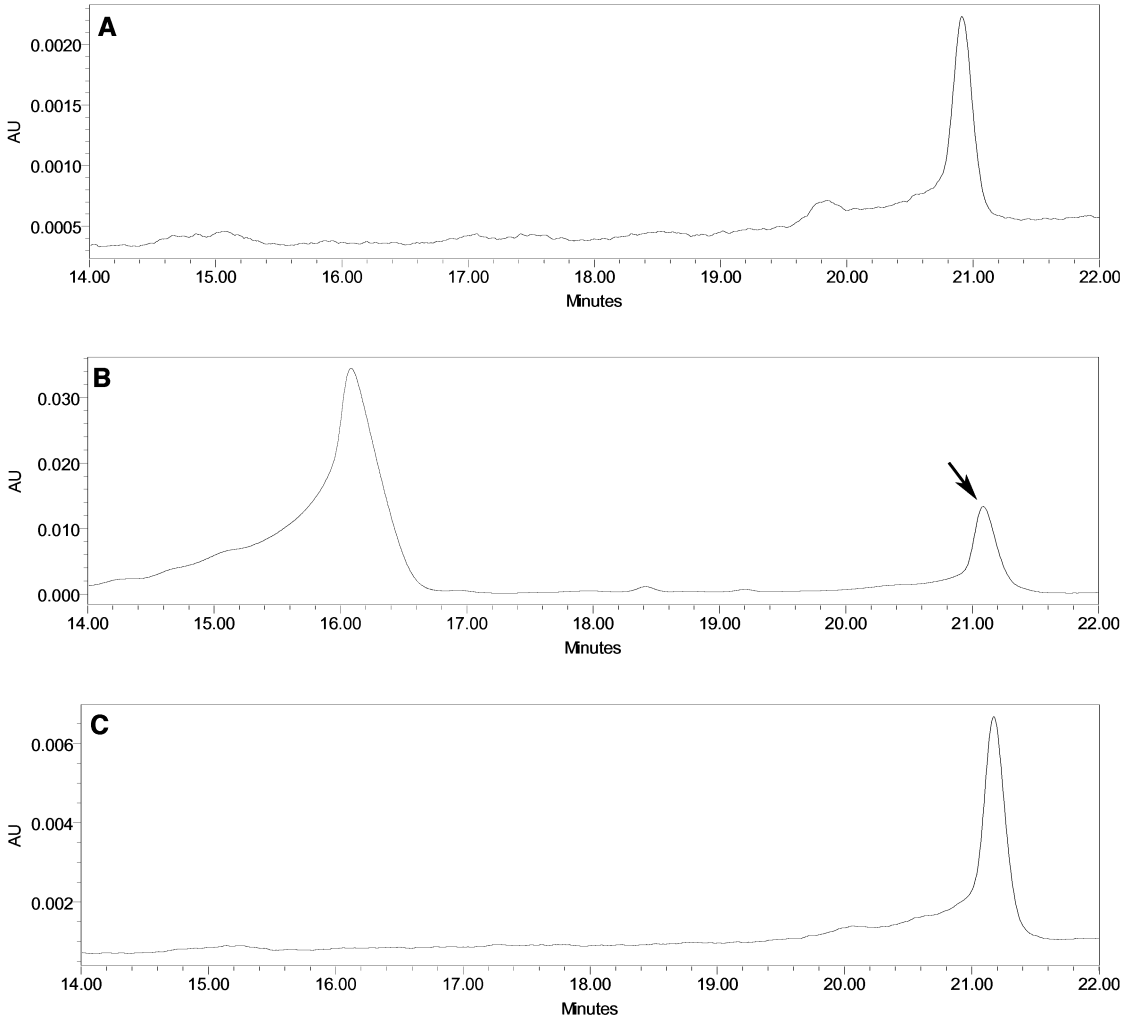

FIGURE 1: Panel A shows RPLC of iodo-α-MI produced by solid-phase synthesis utilizing a Vydac C_{18} column. The gradient was 10−50% buffer B over 40 min. Buffer A = 0.1% trifluoroacetic acid; buffer B = 0.09% trifluoroacetic acid/60% acetonitrile. Flow rate was 1 mL/min. Absorbance was monitored at 280 nm. Panel B shows RPLC purification of iodo-α-MI. α-MI was synthesized and subsequently iodinated. Monoiodo-α-MI was separated from reactants and other products by RPLC with conditions described in panel A. Monoiodo-α-MI (arrow) is well separated from unreacted α-MI (broad peak at ∼16 min). Panel C shows coelution of 250 pmol each of the materials eluting at ∼21 min shown in panels A and B.

mycin. Oocyte recordings were as previously described (*14*, *16*). The concentration of ACh was 3 μm for $\alpha1\beta1\delta\gamma$, $\alpha1\beta1\gamma$, and $\alpha1\beta1\delta$, 1 mM for α7, and 100 μM for $\alpha3\beta4$ and $\alpha4\beta2$ nAChRs. Toxins were perfusion applied until block of ACh-induced current reached equilibration.

RESULTS

Chemical Synthesis. The sequence of native α-MI is GRCCHPACGKNYSC* (*, amidated C-terminus). Solid-phase chemical synthesis of nonradioactive iodo-Tyr-α-MI was undertaken to provide an abundant supply of this peptide for subsequent electrophysiological studies. Fmoc-iodotyrosine was utilized. Cysteine groups on the peptide were protected in pairs to form disulfide bonds as Cys-3−Cys-8 and Cys-4−Cys-14. The major product had the correct mass (calculated MH^+ 1619.46, observed 1619.59), indicating that the unprotected side chain of the iodotyrosine did not substantially contribute to side products. A broad skewed RPLC peak of α-MI is characteristic of this peptide and is the result of slow equilibration between two conformations (*17*). A skewed peak shape is also present in monoiodo-α-MI (Figure 1). Material isolated from the middle third of a typical skewed peak was rerun under the same RPLC conditions. The complete original profile was regenerated (data not shown). In addition, a similar broad peak was seen on a separate RPLC column that gave sharp peaks with other conotoxins. Thus, like native α-MI, iodo-α-MI appears to slowly equilibrate between conformations.

Synthesis of [^{125}I]α-MI. Tyr-12 of α-MI was directly iodinated using NaI. Chloramine-T was used to generate iodine monochloride, which reacts with the ortho position of tyrosine through an electrophilic attack. Excess peptide over iodide was used in the reaction mixture to limit iodination primarily to the monoiodo derivative. We initially utilized nonradioactive iodine to verify the procedure. Iodinated product was purified by RPLC (Figure 1). Monoiodination was confirmed with mass spectrometry (calculated MH^+ 1619.46, observed 1619.47). Chemical sequencing of the peptide confirmed iodination of tyrosine and not histidine. Monoiodo-Tyr-α-MI produced by Fmoc synthesis was compared to monoiodo-Tyr-α-MI produced by iodination of α-MI. The two peptides had similar elution times. When mixed together, the two peptides coeluted confirming identity (Figure 1).

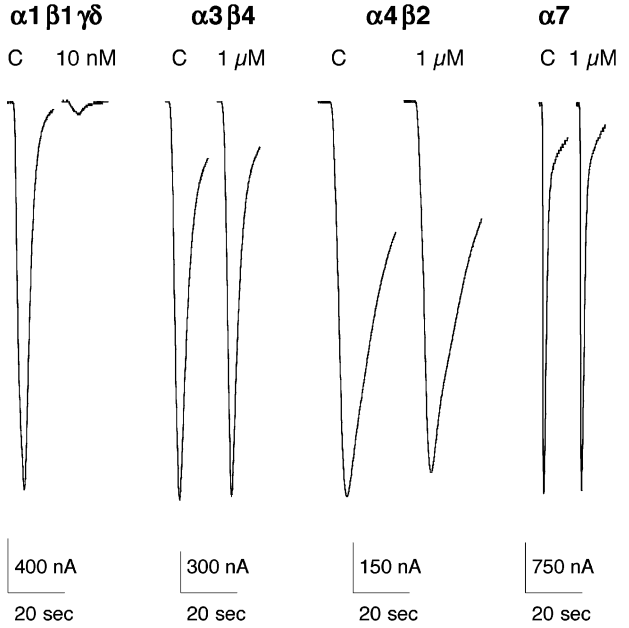

FIGURE 2: Subtype selectivity of iodo-α-MI. Iodo-α-MI was applied to *Xenopus* oocytes heterologously expressing the indicated receptor subtypes. Oocytes were voltage-clamped at −70 mV, and the response to a 1-s pulse of acetylcholine was recorded. Toxin was perfusion applied at 10 nM (α1β1$\gamma\delta$) and 1 μM (α3β4, α4β2, and α7) until no further block was observed. Experiments were performed on three oocytes for each subtype. Percent response (±SEM) compared to control was 4.9% ± 0.85% for α1β1$\gamma\delta$, 97% ± 2.2% for α3β4, 86% ± 6.7% for α4β2, and 111% ± 3.8% for α7. SEM stands for standard error of the mean. C indicates control response.

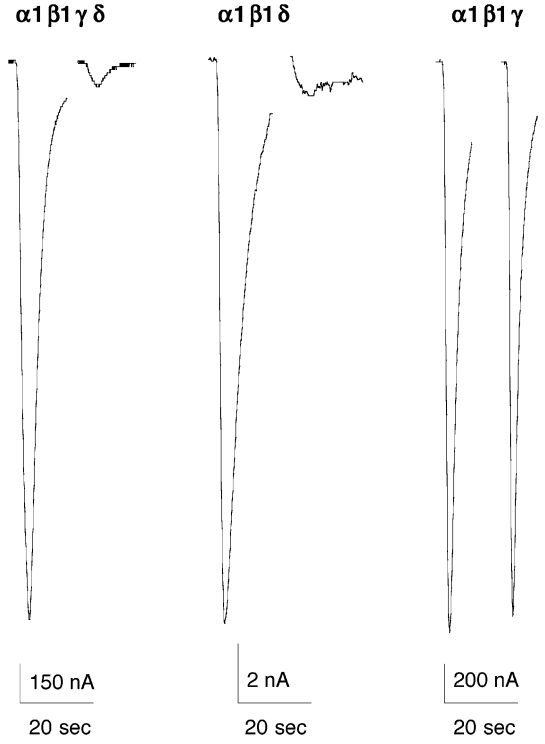

FIGURE 3: Selectivity of iodo-α-MI for the α1/δ subunit interface. Iodo-α-MI, 10 nM, was applied to α1β1$\gamma\delta$ (wild-type), and γ-less (α1β1δ) and δ-less (α1β1γ) subunit combinations heterologously expressed in oocytes as described in Experimental Procedures and Figure 2. The first trace in each series is a control response to acetylcholine. The second trace is the response to acetylcholine in the presence of toxin. Iodo-α-MI blocked 95.1% ± 0.86% in wild-type, 92.5% ± 4.2% in γ-less and 6.00% ± 4.6% in δ-less receptors.

In our experience, some iodinated peptides are unstable with lyophilization. We therefore chose to dilute the RPLC fraction containing [^{125}I]α-MI with incubation buffer and store it in this form. Our experience with other conotoxins indicates that storage at more dilute concentration also helps to preserve peptide integrity. Under the described storage conditions (see Experimental Procedures), [^{125}I]α-MI appeared quite stable and was successfully used for two half-lives of the radionuclide. We did not test the peptide beyond this time frame.

Subtype Selectivity. Monoiodo-α-MI (nonradioactive) was tested on receptor subtypes expressed in *Xenopus* oocytes. On the muscle subtype, 10 nM peptide blocked >95% of the acetylcholine-induced current. In contrast, 1 μM peptide had little or no effect on the major neuronal subtypes α3β4, α4β2, and α7 (Figure 2). The subunit interface selectivity of iodo-α-MI was further assessed using oocytes expressing muscle receptors lacking either the δ or γ subunits. The peptide potently blocks the α1β1δ but not the α1β1γ subunit combination (Figure 3).

Binding Constants. [^{125}I]α-MI binding was evaluated using cell homogenates from TE671 cells. TE671 cells express human muscle-type nAChRs containing α1, β1, γ, and δ subunits (*18*, *19*). Results are from three separate experiments with triplicate data points in each experiment. As shown in Figure 4, specific binding represented 70−88% of total binding. Binding of [^{125}I]α-MI over concentrations from 20 pM to 5 nM had a Hill coefficient of 0.95 ± 0.009 with an apparent dissociation constant (k_d) of 4.1 ± 0.71 nM and B_{max} of 220 ± 23 fmol/mg of protein. Values with standard errors were derived from three independent experiments with one experiment shown in Figure 4. At the highest concentration, 5 nM, the receptor population is slightly more than half occupied. Under these conditions, the data do not rule out the possibility that [^{125}I]α-MI binds to more than one site on the human muscle receptor.

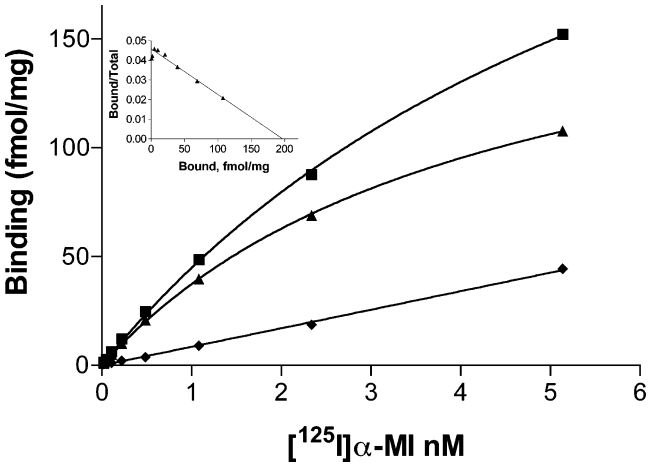

FIGURE 4: Saturation binding of [^{125}I]α-MI to TE671 membrane homogenates. Saturation plot of specific binding of [^{125}I]α-MI from 20 pM to 5 nM. Incubation time with radioligand was 180 min. Solid square represents total binding; solid triangle represents specific binding; solid diamond represents nonspecific binding. The inset shows a Scatchard-like plot of specific binding data. Data shown are from a single experiment.

Kinetic Constants. Association of [^{125}I]α-MI was assessed by incubating membranes with peptide from 3 to 180 min

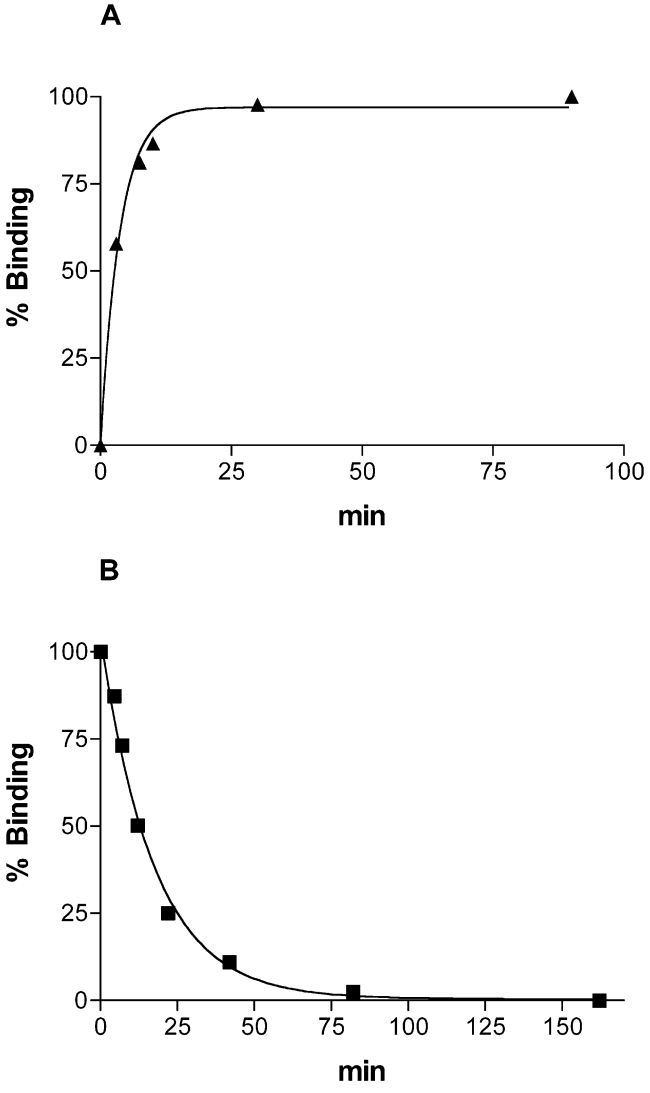

FIGURE 5: Kinetics of [^{125}I]α-MI binding. In panel A, 2.0 nM [^{125}I]α-MI was added to approximately 50 μg of TE671 cell homogenates. The reaction was terminated by filtration at time points indicated. $k_{obs} = 0.2545$ min^{-1}. In panel B, binding of 2.0 nM α-MI was allowed to reach equilibrium (60 min incubation) after which a saturating concentration (10 μM) of α-conotoxin GI was added. The reaction was terminated by rapid filtration at the indicated time points.

(Figure 5A). At 2.0 nM concentration, equilibrium was reached in less than 60 min. Dissociation was then measured by adding an excess (10 μM) of α-conotoxin GI to prevent any reassociation of [^{125}I]α-MI to the receptor (Figure 5B). The dissociation constant, k_{off}, was 0.0839 ± 0.0045 min^{-1}. k_{on} calculated from $(k_{obs} - k_{off})/f$ (where f = the free radioligand concentration estimated to be 2.0 nM) $= 8.53 \times 10^7$ min^{-1} M^{-1}. $k_d = k_{off}/k_{on} = 0.983 \times 10^{-9}$ M. Kinetic values were derived from three independent experiments with triplicate data points within each individual experiment.

Displacement of [^{125}I]α-MI by Other Antagonists. α-BTX has approximately equal affinity for the α/δ and α/γ subunit interface binding sites in fetal mouse muscle, as does αA-conotoxin EIVA (*20*). α-Conotoxin GI, in contrast, selectively binds the α/δ subunit interface with >10 000-fold selectivity (*9*). In fetal human muscle present in TE671 cells, α-BTX has approximately the same affinity for α/δ and α/γ subunit interfaces (*21*), but α-conotoxin GI and αA-conotoxin EIVA have not been examined for α/γ vs α/δ

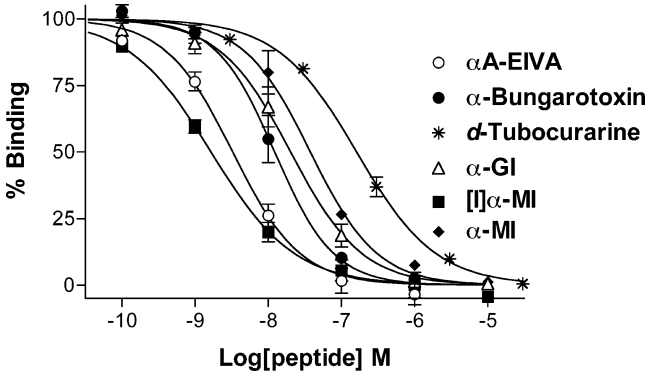

FIGURE 6: Competition binding dose response curves. Toxin (when present) was added to membranes 30 min before addition of radioligand. [^{125}I]α-MI (0.5 nM) was then added with or without the indicated antagonists, and the mixture was incubated for 60 min. Measurements were done in triplicate and the experiments repeated three times. IC_{50}'s and Hill slopes are shown in Table 1.

selectivity. Assuming the α-conotoxin GI is selective for α/γ in human fetal muscle as it is in mouse and αA-conotoxin EIVA is nonselective for human α/γ vs α/δ, then the following prediction can be made. If [^{125}I]α-MI were binding both the α/δ and α/γ subunit interfaces in fetal mouse muscle, both α-BTX and αA-conotoxin EIVA would be expected to displace approximately twice as much of the radiolabel as α-conotoxin GI. In fact, these three antagonists at saturating concentrations each displace the same amount of [^{125}I]α-MI binding. Taking into account the above assumptions, this is consistent with [^{125}I]α-MI selectively binding a single interface (Figure 6). In addition, the potency at which α-conotoxin GI displaces [^{125}I]α-MI from human fetal receptor is consistent with GI's known potency at the mouse α/δ subunit interface but not the α/γ subunit interface. It is of note that not all of the specific [^{125}I]α-BTX binding to TE671 cells is blocked by small nicotinic ligands such as *d*-tubocurarine or can be immunoprecipitated with antibodies that recognize *Torpedo* electroplax nAChRs (*18*, *22*−*24*). These curare-insensitive [^{125}I]α-BTX binding sites may represent incompletely assembled α1 subunits (*23*). In contrast, [^{125}I]α-MI is fully displaced by *d*-tubocurarine (Figure 6). This suggests that unlike [^{125}I]α-BTX, [^{125}I]α-MI may only bind fully assembled receptors.

DISCUSSION

In this study, we have selectively iodinated Tyr-12 of α-MI. We demonstrate that it effectively antagonizes the muscle nicotinic receptor in functional assays and retains its selectivity for the muscle receptor. In addition, we have synthesized a radioiodinated version of the toxin and describe its properties.

In contrast to snake polypeptide toxins, which have 60−80 amino acids, the α-conotoxins are small with approximately 13−15 amino acids. The introduction of a bulky side chain such as an iodine atom into a small peptide may considerably reduce the potency of the peptide. However, we have previously performed an alanine walk to identify residues in α-MI that contribute to the selective interaction with the α/δ site. Analysis of structure/activity relationships indicates that increasing the size of the hydrophobic side chain of the residue in position 12 decreased the toxin dissociation rate (*16*).

Table 1: Competition by Nicotinic Agonists for Binding Sites in TE671 Cell Homogenates Labeled with 0.5 nM [^{125}I]α-MI[a]

compound	IC_{50} (nM)	CI	n_H
α-bungarotoxin	12.3	9.43−16.1	1.08
α-conotoxin GI	20.3	15.3−27.1	0.893
αA-conotoxin EIVA	3.28	2.42−4.44	0.944
[I]α-conotoxin MI	1.66	1.30−2.13	0.763
α-conotoxin MI	37.8	29.3−48.8	0.955
d-tubocurarine	162	130−202	0.802

[a] IC_{50}'s and Hill coefficients were determined by nonlinear regression analysis. Values are the mean of three independent experiments. CI represents the 95% confidence interval; n_H represents thee Hill slope.

α-MI contains two potential iodination sites at His-5 and Tyr-12. Selective iodination of the tyrosine residue was achieved by using lower pH, which protonates the imidazole ring of histidine, thereby inhibiting iodination at this residue. Although the diiodo product is preferred, use of excess cold toxin results in primarily monoiodo product that was efficiently purified away from both unreactive and diiodo product; diiodo-α-MI elutes approximately 4 min after moniodo-α-MI (data not shown) under conditions described in Figure 1. Monoiodination of tyrosine and noniodination of histidine was confirmed by mass spectrometry and chemical sequencing.

Kinetic studies of radiolabeled α-MI indicate that it reversibly binds the receptor. The off-rate is approximately 30 times faster than that of [^{125}I]α-BTX on human fetal muscle in TE671 cells (*21*). Although for purposes of this study we deliberately chose to synthesize a reversible ligand, structure/function studies suggest that diiodination of Tyr-12 may produce a radioligand with significantly slower reversibility (*16*).

Using cloned receptors expressed in oocytes, we demonstrate that iodinated α-MI selectively blocks muscle vs neuronal subtypes of the nAChR. Using mouse $\alpha\beta\delta$ and $\alpha\beta\gamma$ triplet receptors expressed in oocytes, we further demonstrate that iodo-MI selectively blocks the δ-containing receptor. Competition binding assays also suggest that the ligand may selectively bind the α/δ subunit interface in human fetal muscle similar to previous studies showing selectivity for mouse α/δ vs α/γ dimers expressed in HEK cells (*25*). α-Conotoxins GI and EIVA may differ in their respective affinities for α/δ vs α/γ subunit interfaces in human vs mouse muscle. However, interpretation of the selectivity of [^{125}I]α-MI in fetal human muscle is dependent on data from previous studies that examined the selectivity of α-conotoxins GI and EIVA in fetal mouse muscle. Therefore, data from the present study do not exclude the possibility that [^{125}I]α-conotoxin MI may bind to more than one site on the human receptor.

Double mutant cycle analysis between α-MI and the α/δ subunit interface of the nAChR indicate a hydrophobic interaction between toxin and receptor. Pro-6, Ala-7, Gly-9, and Tyr-12 of the toxin appear to create a hydrophobic surface that interacts with the α/δ binding site (*26*). Mutation of Tyr-12 to a hydrophilic threonine residue decreases α-MI affinity 10 000-fold, whereas mutation to a phenylalanine or tryptophan maintains high affinity (*26*, *27*). In contrast, we demonstrate that monoiodination of Tyr-12 leads to an ~20-fold increase in potency (Figure 6, Table 1). The solution structure of α-MI has been solved by NMR (*28*).

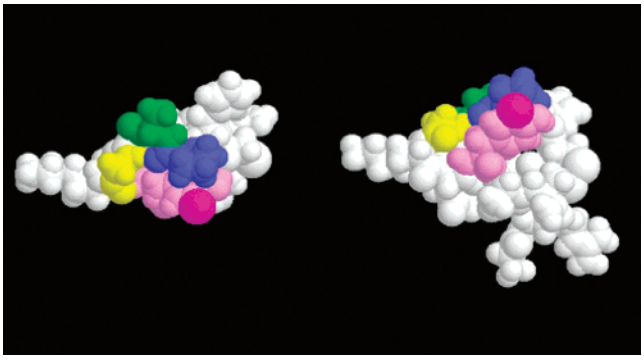

FIGURE 7: Three-dimensional structure of [I]α-MI. Two views of the toxin are shown. One is a 90° rotation of the other. The solution structure of α-MI was determined by NMR (*28*). Bioactive residues determined by mutant cycle analysis (*26*) are shown in color: blue, proline; green, alanine; yellow, glycine; light magenta, tyrosine. Iodine is magenta. The model assumes that steric hindrance forces the iodine attached to Tyr-12 to face outward.

Tyr-12 is at the end of a type I β-turn formed by residues -Gly-Lys-Asn-Tyr-. Double mutant cycle analysis indicates that Tyr-12 strongly interacts with the three major selectivity determinants present on the δ-subunit, that is, Ser-36, Tyr-113, and Ile-178, with little or no interaction with the α subunit. In contrast, Ala-7 shows strongest coupling to Tyr-198 of the α subunit. This suggest that α-MI orients in the binding site with Tyr-12 toward the δ-subunit and Ala-7 toward the α subunit (*26*). Steric considerations suggest that the iodine attached to Tyr-12 of α-MI faces outward from the peptide extending its hydrophobic, fingerlike reach (Figure 7). As indicated by a later elution time on RPLC (Figure 1), addition of iodine to α-MI increases the hydrophobicity of the peptide consistent with this orientation. Addition of this hydrophobic iodine moiety to Tyr-12 may strengthen postulated hydrophobic interactions with key residues (Ser-36, Tyr-113, and Ile-178) in loops I, III, and IV of the nAChR δ subunit. The high potency and selectivity of iodo-α-MI suggests that it will be a useful ligand for molecular studies of the α/δ ligand binding interface of the muscle nAChR.

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