Determinants Involved in the Affinity of α-Conotoxins GI and SI for the Muscle Subtype of Nicotinic Acetylcholine Receptors†

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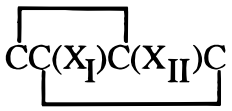
ABSTRACT: Nicotinic acetylcholine receptors from muscle contain two functionally active and pharmacologically distinct acetylcholine-binding sites located at the α/γ and α/δ subunit interfaces. The α-conotoxins are competitive antagonists of nicotinic receptors and can be highly site-selective, displaying greater than 10 000-fold differences in affinities for the two acetylcholine-binding sites on a single nicotinic receptor. The higher affinity site for α-conotoxins GI, MI, and SI is the α/δ site on mouse muscle-derived BC_3H-1 receptors. However, α-conotoxins GI and MI exhibit higher affinity for the other site (α/γ site) on nicotinic receptors from *Torpedo californica* electric organ. α-Conotoxin SI does not distinguish between the two acetylcholine-binding sites on *Torpedo* receptors. In this study, α-conotoxins [K10H]SI and [K10N]SI displayed wild-type affinity for the two acetylcholine-binding sites on BC_3H-1 receptors but a 10−20-fold decrease in apparent affinity at one of the two acetylcholine-binding sites on *Torpedo* receptors. α-Conotoxin [P9K]SI displayed a selective and dramatic increase in the apparent affinity for the α/δ site of BC_3H-1 receptors and for the α/γ site of *Torpedo* receptors. α-Conotoxin [R9A]GI displayed a reduction in affinity for both acetylcholine-binding sites on BC_3H-1 receptors, although the extent of its selectivity for the α/δ site was retained. α-Conotoxin [R9A]GI also displayed a loss of affinity for the two acetylcholine-binding sites on *Torpedo* receptors, but its site-selectivity was apparently abolished. These results indicate that positions 9 and 10 in α-conotoxins GI and SI are involved in complex species- and subunit-dependent interactions with nicotinic receptors.

Nicotinic acetylcholine receptors from *Torpedo californica* electric organ and mouse muscle-derived BC_3H-1 cells have been used as model receptor systems for understanding nicotinic receptor structure and function (*1*). The embryonic muscle subtype of the nicotinic acetylcholine receptor is a ligand-gated ion channel composed of four homologous subunits ($\alpha_2\beta\gamma\delta$) in a pentameric arrangement that spans the lipid bilayer (*2*). The two acetylcholine-binding sites located near the α/γ and α/δ subunit interfaces (the α/γ and α/δ sites) are pharmacologically distinct due to sequence differences between the γ and δ subunits (*3*, *4*). For example, a specific amino acid (γTyr^{117}) has been identified in the γ subunit of the nicotinic receptor from BC_3H-1 cells that confers higher affinity for *d*-tubocurarine (*5*). In addition, three amino acids in the δ subunit (δSer^{36}, δTyr^{113}, and δIle^{178}) have been shown to be determinants for the higher affinity of α-conotoxin MI at the α/δ site of BC_3H-1 receptors (*6*). However, in contrast to BC_3H-1 receptors, α-conotoxin MI has a higher affinity for the opposite site (the α/γ site) on nicotinic receptors from *Torpedo* electric organ (*7*, *8*). Presumably, this is because the amino acids that confer high affinity for α-conotoxin MI are present in the γ subunit and not in the δ subunit of *Torpedo* receptors.

The α-conotoxins are small peptides (<20 amino acids) that competitively inhibit the function of nicotinic acetylcholine receptors (*9*). Structurally, the α-conotoxins can be characterized by a pair of conserved disulfide bridges that are required for activity of the toxins (*10*−*12*). The conserved structure of the α-conotoxins can be schematically represented as



where disulfides are formed between the first and third cysteines and the second and fourth cysteines, and where X_I and X_{II} represent different amino acid sequences of varying lengths. The α-conotoxins can be grouped into several structural subclasses according to the number of amino acids present in X_I and X_{II}. For example, α-conotoxins GI, GIA, GII, MI, SI, SIA, and SII belong to the α3/5 subclass because each has three amino acids in X_I and five amino acids in X_{II} (*13*−*17*). α-Conotoxins EI, MII, PnIA, and PnIB belong to the α4/7 subclass, and α-conotoxin ImI belongs to the α4/3 subclass (*18*−*21*). A new structural class, the αA-conotoxins, has also been described that has a unique disulfide connectivity among six cysteines (*22*).

A remarkable characteristic of α-conotoxins GI and MI is their 10 000-fold greater affinity for the α/δ site (compared to the α/γ site) of BC_3H-1 receptors (*6*, *8*). Previous *in situ* studies of α-conotoxin analogs were unable to observe this site-selectivity. Function-based assays of selective antagonists are limited to measuring interaction at only the higher

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affinity site because functional antagonism of nicotinic receptors is dependent on occupation of only one of the two acetylcholine-binding sites (*8*, *23*, *24*). Although not detected in function-based assays, important pharmacological differences between the two acetylcholine-binding sites on nicotinic receptors have been observed in studies characterizing the interaction of ligands at both sites (*3*, *4*, *8*, *25*−*28*).

α-Conotoxins GI and SI are very similar, differing in sequence only at the 1st, 9th, and 10th positions. Although the affinity of α-conotoxin GI for BC_3H-1 receptors is very high, the affinity of α-conotoxin SI is significantly lower (*8*, *15*). Previous *in situ* structure−activity studies have indicated that amino acids preceding the N-terminal cysteines (Cys^2 and Cys^3 in α-conotoxin GI) have relatively little effect on affinity (*12*, *29*). The amino acid sequence at positions 9 and 10 in α-conotoxin GI is Arg-His while in α-conotoxin SI it is Pro-Lys. Because the N-terminal amino acids appear to have relatively little effect on activity, it seems likely that differences in the affinities of α-conotoxins GI and SI for nicotinic receptors result from sequence differences at positions 9 and 10. Amino acid substitutions were made at positions 9 and 10 in α-conotoxin SI and at position 9 in α-conotoxin GI to investigate the effects of sequence on the interaction of the toxins with nicotinic receptors. The apparent affinity and the site-selectivity of the α-conotoxins and their analogs were determined for the two acetylcholine-binding sites on nicotinic receptors from BC_3H-1 cells and *Torpedo* electric organ.

MATERIALS AND METHODS

Materials. Purified membranes from *Torpedo californica* electric organ were a gift from David A. Johnson (University of California, Riverside). Metocurine was a gift from Lilly.

Synthesis and Purification of α*-Conotoxin Analogs.* The synthesis and extensive purification of the α-conotoxins were performed to ensure that the correct disulfide-paired isomer (containing cystines Cys^2−Cys^7 and Cys^3−Cys^{13}) was isolated. α-Conotoxins were synthesized by solid-phase methods using standard fmoc-based chemistry, as described previously (*20*). During the synthesis of the α-conotoxins, trityl-protected cysteines were incorporated at positions 2 and 7. The trityl goups can be removed by treatment with trifluoroacetic acid (TFA). However, acetamidomethyl-protected (acm) cysteines were incorporated at positions 3 and 13. The acm group is stable to acid treatment. Deprotection and cleavage of the full-length peptide from the support resin with TFA (*20*) resulted in a linear peptide with the acid-stable acm groups remaining on cysteines 3 and 13. The linear, partially deprotected peptide was purified by reversed-phase HPLC on a Vydac C_{18} column using a gradient of acetonitrile in 0.1% TFA. The purified linear peptide solution was adjusted to pH 7.5 with solid Tris and air-oxidative closure of the first disulfide (Cys^2−Cys^7) performed overnight with stirring. Monocyclic peptide was purified by HPLC on a C_{18} column using a gradient of acetonitrile in 0.1% TFA. Deprotection and oxidative closure of the second disulfide (Cys^3−Cys^{13}) were carried out by slowly adding the purified monocyclic peptide solution with rapid stirring to an equal volume of 10 mM I_2 in 20% TFA. The reaction was incubated at room temperature for an additional 5.0 min with stirring. After incubation, residual free I_2 was scavenged by adding 0.5 M ascorbic acid. The reaction was then diluted 5−10-fold with 0.1% TFA, and the bicyclic peptide was purified by HPLC on a C_{18} column using a gradient of acetonitrile in 0.1% TFA. In some instances, an additional purification was performed by reversed-phase HPLC on a C_{18} column using a gradient of acetonitrile in 0.05 M triethylamine phosphate at pH 2.25.

Maintenance of BC_3H*-1 Cells.* BC_3H-1 cells were maintained in growth medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. For experiments, BC_3H-1 cells in growth medium were seeded into gelatin-coated 24-well culture plates at a density of 8000−12 000 cells/well. The plated cells were then grown as previously described (*8*, *28*). Prior to each experiment, plated cells were equilibrated to room temperature for 30 min, washed once with 1.0 mL of assay buffer (140 mM KCl, 25 mM HEPES, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, and 0.06 mg/mL bovine serum albumin, pH 7.4), and equilibrated in 1.0 mL of fresh assay buffer for 20 min.

Inhibition of ^{125}*I-*α*-Bungarotoxin (*^{125}*I-*α*-BTX)*[1] *Binding to Nicotinic Receptors on* BC_3H*-1 Cells.* All experiments were performed at room temperature. Inhibition of the association of ^{125}I-α-BTX to nicotinic receptors on BC_3H-1 cells was performed as previously described (*8*). Briefly, cells were incubated for 30 min with metocurine or for 2.0 h with α-conotoxins in assay buffer. After this incubation, ^{125}I-α-BTX was added (final concentration of 20 nM) and incubated for 15 min. The cells were washed twice with assay buffer, and the bound ^{125}I-α-BTX was collected by rinsing each well with two 0.5 mL volumes of 1% Triton X-100 in water. The two Triton X-100 washes were pooled and counted to determine the amount of bound ^{125}I-α-BTX. In experiments designed to identify the higher affinity α-conotoxin-binding site, cells were incubated for 2.0 h with a concentration of α-conotoxin that selectively occupied the higher affinity α-conotoxin-binding site. Then, the apparent affinity of metocurine for the remaining available acetylcholine-binding sites was determined as described above (*8*, *20*).

Nonspecific binding of ^{125}I-α-BTX was determined from cells previously incubated for 30 min with 100 nM α-BTX. The total number of ^{125}I-α-BTX binding sites (determined after a 60 min incubation with ^{125}I-α-BTX) was 130 ± 18 fmol/well ($n = 8$). In the absence of any competing drugs, 74 ± 3.8% ($n = 8$) of the total population of binding sites were labeled by ^{125}I-α-BTX in 15 min.

Inhibition of ^{125}*I-*α*-BTX Binding to Nicotinic Receptors from Torpedo Electric Organ.* All experiments were performed at room temperature. Membranes from *Torpedo* electric organ (0.33 or 1.1 nmol of ^{125}I-α-BTX binding sites/mg of protein) were diluted to 108 μL with assay buffer (10 mM sodium phosphate, pH 7.4, 1.0 mM EDTA, 1.0 mM EGTA, 0.1% Triton X-100) containing the indicated concentrations of α-conotoxin or metocurine. Triton X-100 was included in the buffer to ensure that both acetylcholine-binding sites could be assayed with ^{125}I-α-BTX (*30*). Receptors were incubated in buffer with drug for 2.0 h, and then 12 μL of 10 nM ^{125}I-α-BTX was added to the reaction. After a 30−60 s incubation, single 50 μL aliquots of the reaction were spotted onto each of two 2.5-cm DE81 ion-

[1] Abbreviation: ^{125}I-α-BTX, ^{125}I-α-bungarotoxin.

α-Conotoxin	Sequence
GI	ECCNPACGRHYSC*
[R9A]GI	ECCNPACG**A**HYSC*
SI	ICCNPACGPKYSC*
[P9K]SI	ICCNPACG**K**KYSC*
[K10H]SI	ICCNPACGP**H**YSC*
[K10N]SI	ICCNPACGP**N**YSC*

FIGURE 1: Structure of α-conotoxins GI, SI, and their analogs. Sequence differences between the α-conotoxins and their analogs are boldfaced and underlined. Disulfide bridges between conserved cysteine residues are indicated by the solid lines. Asterisks indicate an amidated carboxyl terminus.

exchange filters (Whatman) and allowed to absorb for 30 s. The filters were washed for at least 10 min in each of two 600−800 mL stirred baths of wash buffer (10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100). The filters were blotted between paper toweling and counted to determine the amount of bound ^{125}I-α-BTX. To identify the higher affinity α-conotoxin-binding site, receptors were incubated for 2.0 h with a concentration of α-conotoxin to selectively occupy the higher affinity α-conotoxin-binding site. Then, the apparent affinity of metocurine for the remaining available acetylcholine-binding sites was determined as described above.

Nonspecific binding of ^{125}I-α-BTX was determined from membranes previously incubated for 30 min with 100 nM α-BTX. The total concentration of ^{125}I-α-BTX binding sites (determined after a 60 min incubation with ^{125}I-α-BTX) was $(2.8 \pm 0.11) \times 10^{-10}$ M ($n = 6$). In the absence of any competing drugs, $45 \pm 5.3\%$ ($n = 6$) of the total population of binding sites were labeled by ^{125}I-α-BTX in 1 min.

Data Analysis. Functions describing competitive binding of a ligand to either a single site or two independent sites were fit by nonlinear regression to the inhibition of ^{125}I-α-BTX binding to nicotinic receptors. A two-site competition model was accepted over a single-site model only when a statistical comparison between the two models gave a value of $p < 0.05$ (*31*). All averaged IC_{50} values reported with an associated SEM included data from three to five independent experiments. All curves that were fit significantly better to a two-site competition model contained the two sites in approximately equal abundance. Nonlinear regression was performed on a Northgate 386 personal computer using Prism (GraphPad).

RESULTS

The apparent affinities of α-conotoxins SI, [P9K]SI, [K10H]SI, and [K10N]SI (Figure 1) were determined for nicotinic acetylcholine receptors from mouse muscle-derived BC_3H-1 cells. α-Conotoxin SI displayed two affinities for BC_3H-1 muscle receptors that differed by 220-fold (Figure 2, upper panel, Table 1). These results are similar to previous observations (*8*). The apparent affinities of α-conotoxins [K10H]SI and [K10N]SI for the two acetylcholine-binding sites on BC_3H-1 receptors were not substantially different from those of α-conotoxin SI. In contrast, α-conotoxin [P9K]SI displayed a profound increase in affinity (870-fold) for only one of the two acetylcholine-binding sites, resulting in IC_{50} values for the two acetylcholine-binding sites that differed by 47 000-fold (Figure 2, upper panel, Table 1). The IC_{50}'s of α-conotoxin [P9K]SI for BC_3H-1 receptors were similar in magnitude to those of α-conotoxins GI and MI for the same receptors (*8*, *32*).

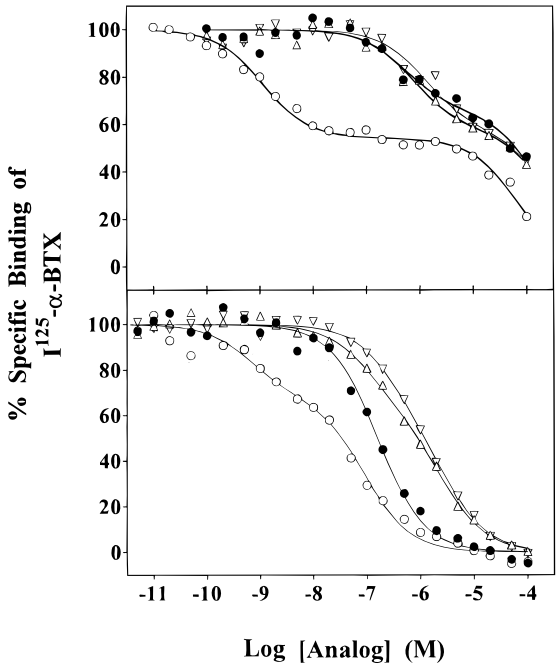

FIGURE 2: Inhibition of ^{125}I-α-BTX binding to nicotinic receptors by α-conotoxin SI and its analogs. Mouse muscle receptors on BC_3H-1 cells (upper panel) and receptors from *Torpedo* electric organ (lower panel) were incubated with increasing concentrations of α-conotoxins SI (●), [P9K]SI (○), [K10H]SI (△), or [K10N]SI (▽). The data shown for each curve are an average of 3−4 experiments. Error bars have been omitted for clarity. Lines through the data were calculated from the competition model providing the best fit to the data as determined by a statistical comparison of functions.

Table 1: Affinities of α-Conotoxins GI, SI, and Their Analogs for the Two Acetylcholine-Binding Sites on Nicotinic Acetylcholine Receptors

α-conotoxin	receptor source	IC^1_{50} (μM)	IC^2_{50} (μM)	IC^2_{50}/IC^1_{50}
SI	BC_3H-1	1.3 ± 0.43	290 ± 110	220
[P9K]SI	BC_3H-1	0.0015 ± 0.0006	70 ± 1.2	47 000
[K10H]SI	BC_3H-1	0.75 ± 0.10	320 ± 81	430
[K10N]SI	BC_3H-1	2.1 ± 0.97	420 ± 89	200
GI	BC_3H-1	0.0013 ± 0.0003	60 ± 1.0	46 000
[R9A]GI	BC_3H-1	0.060 ± 0.013	≥1000	>10 000
SI	*Torpedo*	0.17 ± 0.037	0.17 ± 0.037	(1.0)
[P9K]SI	*Torpedo*	0.00088 ± 0.00004	0.097 ± 0.007	110
[K10H]SI	*Torpedo*	0.097 ± 0.062	2.0 ± 0.32	21
[K10N]SI	*Torpedo*	0.52 ± 0.1	5.3 ± 0.94	10
GI	*Torpedo*	0.0045 ± 0.0013	0.087 ± 0.024	19
[R9A]GI	*Torpedo*	2.2 ± 0.27	2.2 ± 0.27	(1.0)

The apparent affinities of α-conotoxins SI, [P9K]SI, [K10H]SI, and [K10N]SI were also determined for nicotinic receptors from *Torpedo californica* electric organ. α-Conotoxin SI displayed a single affinity for *Torpedo* receptors (Figure 2, lower panel, Table 1). These results are consistent with previous observations (*7*). α-Conotoxins [K10H]SI and [K10N]SI displayed an approximately 10-fold decrease in affinity at only one of the two acetylcholine-binding sites, resulting in a slight increase in selectivity (Figure 2 lower panel, Table 1). However, as with BC_3H-1 receptors, α-conotoxin [P9K]SI displayed a large increase in affinity (190-fold) for only one of the two acetylcholine-binding sites on *Torpedo* receptors, resulting in two IC_{50} values that

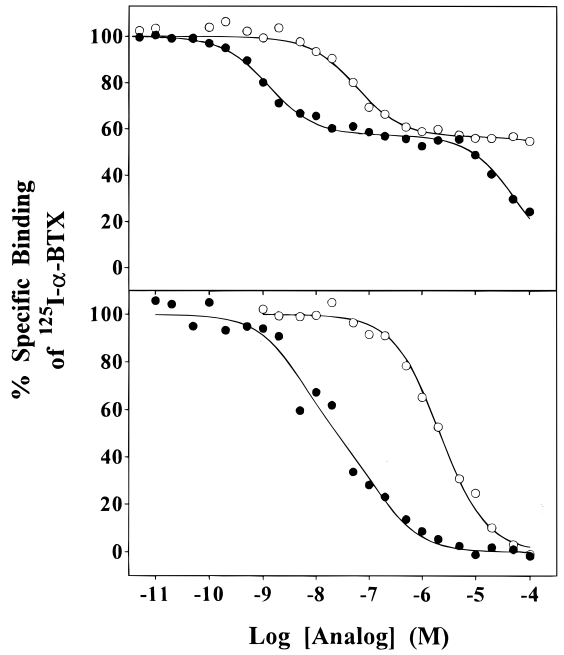

FIGURE 3: Inhibition of ^{125}I-α-BTX binding to nicotinic receptors by α-conotoxins GI and [R9A]GI. Mouse muscle receptors on BC_3H-1 cells (upper panel) and receptors from *Torpedo* electric organ (lower panel) were incubated with increasing concentrations of α-conotoxins GI (●), and [R9A]GI (○). The data shown for each curve are an average of 3−4 experiments. Error bars have been omitted for clarity. Lines through the data were calculated from the competition model providing the best fit to the data as determined by a statistical comparison of functions.

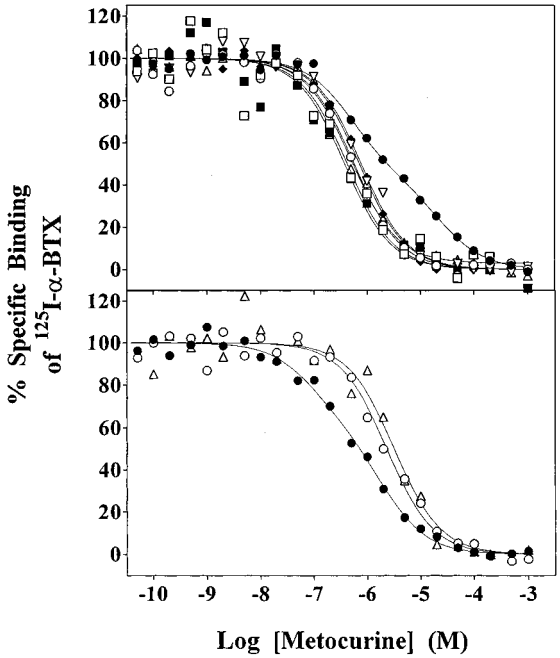

FIGURE 4: Inhibition of ^{125}I-α-BTX binding to nicotinic receptors by metocurine in the absence and presence of α-conotoxins. (Upper panel) Inhibition of ^{125}I-α-BTX binding to mouse muscle receptors by metocurine in the absence (●) and presence of α-conotoxins SI (□), [P9K]SI (○), [K10H]SI (△), [K10N]SI (▽), GI (■), or [R9A]-GI (◆). (Lower panel) Inhibition of ^{125}I-α-BTX binding to *Torpedo* receptors by metocurine in the absence (●) and presence of α-conotoxin [P9K]SI (○) or GI (△). The data for metocurine in the absence of competing α-conotoxin represent an average of 3−4 independent experiments. Error bars have been omitted for clarity. Lines through the data were calculated from the competition model providing the best fit to the data as determined by a statistical comparison of functions.

differed by 110-fold (Figure 2, lower panel, Table 1). The IC_{50}'s of α-conotoxin [P9K]SI were similar in magnitude to those of α-conotoxin GI and MI for *Torpedo* receptors (*7*, *8*).

The apparent affinities of α-conotoxins GI and [R9A]GI (Figure 1) were determined for nicotinic receptors on BC_3H-1 cells. α-Conotoxin GI displayed two affinities for the two acetylcholine-binding sites on BC_3H-1 receptors that differed in magnitude by 46 000-fold (Figure 3, upper panel, Table 1). These results are similar to previous observations (*8*). α-Conotoxin [R9A]GI displayed reduced affinities for both acetylcholine-binding sites (Figure 3, upper panel, Table 1). The IC_{50} for the second site could not be determined under the conditions of the experiment but was clearly greater than 1 mM. Thus, α-conotoxin [R9A]GI maintained a profound selectivity (>10 000-fold) for one of the two acetylcholine-binding sites on BC_3H-1 receptors (Figure 3, upper panel).

α-Conotoxin GI displayed two apparent affinities for the acetylcholine-binding sites of *Torpedo* nicotinic receptors that differed by 19-fold (Figure 3, lower panel, Table 1). These results are similar to previous observations (*7*). However, α-conotoxin [R9A]GI displayed a single, lower affinity for the two acetylcholine-binding sites of *Torpedo* receptors (Figure 3, lower panel, Table 1). In light of its reduced affinities for BC_3H-1 receptors, it is not surprising that α-conotoxin [R9A]GI displayed lower affinity for *Torpedo* receptors. However, it is interesting to note that α-conotoxin [R9A]GI did not display a site preference for *Torpedo* nicotinic receptors.

An additional selective antagonist (metocurine) was used to determine the identity of the higher affinity binding site for the α-conotoxins. Metocurine is a reversible curariform nicotinic receptor antagonist that has higher affinity for the acetylcholine-binding site near the α/γ subunit interface in receptors from both BC_3H-1 and *Torpedo* electric organ (*3*, *4*, *25*, *33*). Metocurine displayed two apparent affinities for BC_3H-1 receptors that differed by 46-fold (Figure 4, upper panel, Table 2). The site selectivity exhibited by the α-conotoxins can be exploited to preferentially block their higher affinity binding site in order to determine the affinity of metocurine at the other, unoccupied binding site. In the presence of α-conotoxins SI (20 μM), [P9K]SI (100 nM), [K10H]SI (20 μM), or [K10N]SI (20 μM), virtually all of the high-affinity α-conotoxin-binding sites were occupied. Under these conditions, metocurine displayed only a single high affinity for the remaining unoccupied acetylcholine-binding site on BC_3H-1 receptors (Figure 4, upper panel,

Table 2: Affinities of Metocurine for the Two Acetylcholine-Binding Sites on Nicotinic Acetylcholine Receptors in the Absence and Presence of α-Conotoxins GI, SI, and Their Analogs

receptor source	α-conotoxin	IC^1_{50} (μM)	IC^2_{50} (μM)
BC_3H-1	none	0.61 ± 0.17	28 ± 0.4
BC_3H-1	SI	0.43	na[a]
BC_3H-1	[P9K]SI	0.57	na
BC_3H-1	[K10H]SI	0.56	na
BC_3H-1	[K10N]SI	0.78	na
BC_3H-1	GI	0.38	na
BC_3H-1	[R9A]GI	0.72	na
Torpedo	none	0.084 ± 0.0091	1.6 ± 0.6
Torpedo	[P9K]SI	na	2.3
Torpedo	GI	na	3.2

[a] A second affinity was not applicable (na) when the results fit better to a single-site model.

Table 2). Similarly, in the presence of α-conotoxins GI (20 nM) or [R9A]GI (20 μM), metocurine displayed only a single high affinity for the remaining, unoccupied acetylcholine-binding site on BC_3H-1 receptors (Figure 4, upper panel, Table 2). Thus, all of these α-conotoxins preferentially occupied the lower affinity metocurine binding site (the α/δ site) of BC_3H-1 receptors, leaving the higher affinity metocurine-binding site (the α/γ site) unoccupied.

Metocurine also displayed two apparent affinities for *Torpedo* nicotinic receptors that differed by 19-fold (Figure 4, lower panel, Table 2). However, in the presence of α-conotoxins [P9K]SI (20 nM) or GI (20 nM), metocurine displayed only a single low affinity for the remaining unoccupied acetylcholine-binding site on *Torpedo* receptors (Figure 4, lower panel; Table 2). These results demonstrate that α-conotoxins [P9K]SI and [R9A]GI preferentially occupied the higher affinity metocurine-binding site (the α/γ site) of *Torpedo* receptors, leaving the lower affinity metocurine-binding site (the α/δ site) unoccupied. The site preference of [P9K]SI is identical to that of α-conotoxins GI and MI for the acetylcholine-binding sites on *Torpedo* receptors (*7*, *8*, *33*). Because of the lessor extent of the site selectivity of α-conotoxins [K10H]SI, [K10N]SI, and [R9A]GI, it was not possible to reliably determine the site preference of these analogs for the two acetylcholine-binding sites on *Torpedo* receptors.

DISCUSSION

The development of clinically and experimentally useful drugs depends in part on determining the structural features of a ligand that contribute to affinity and specificity. For example, the investigation of a series of *d*-tubocurarine derivatives identified three positions where chemical substitutions affect the affinities for the two acetylcholine-binding sites on *Torpedo* receptors (*35*). Similarly, sequence differences among the α-conotoxins influence their affinities for the two acetylcholine-binding sites on nicotinic receptors (*7*, *8*, *20*). The crystal structure of α-conotoxin GI shows that amino acids Glu^1, Pro^5, and Arg^9 occupy the vertices of a triangular toxin conformation (*36*). The side chains of Arg^9 and His^{10} project prominently away from the structure. Several hydrogen bonds as well as the disulfide bonds between Cys^2/Cys^7 and Cys^3/Cys^{13} stabilize the N-terminal structure of α-conotoxin GI. Because of this stability, it is unlikely that introduction of Pro at position 9 would significantly alter the conformation of the N-terminal region of α-conotoxin GI. In addition, the torsion angles ϕ and ψ for Arg^9 are $-63.1°$ and $-14.2°$, respectively (*36*). The value of ϕ for an isolated proline residue in a polypeptide chain ($-60°$) is similar to ϕ for Arg^9 in α-conotoxin GI (*37*). However, in L-proline, two conformational energy minima for ψ occur near $-55°$ and $-145°$ (*37*). Thus, the increased negative rotation about ψ needed to accommodate Pro at position 9 would likely propagate structural alterations along the succeeding peptide chain. Indeed, comparisons of the ^{1}H-NMR structures of α-conotoxins SI and [P9K]SI indicate that significant structural differences occur only in the peptide chain following position 9 (D. Christiansen, personal communication).

α-Conotoxins GI and MI tolerate a variety of side chain substitutions at position 10 without significant effects on their affinity for nicotinic receptors (*11*, *12*, *29*). Thus, it is unlikely that substitutions made at position 10 alter the backbone structure of the α-conotoxins. In a consistent manner, neither α-conotoxins [K10H]SI nor [K10N]SI differed significantly from α-conotoxin SI in their apparent affinities and site-selectivities for BC_3H-1 receptors. These results further indicate that an interaction does not exist between the side chain at position 10 in the α-conotoxins and BC_3H-1 receptors. However, both α-conotoxins [K10H]SI and [K10N]SI displayed an approximate 10-fold decrease in apparent affinity for one of the two acetylcholine-binding sites on nicotinic receptors from *Torpedo* electric organ. In contrast to BC_3H-1 receptors, the selective effect on affinity at one acetylcholine-binding site on *Torpedo* receptors suggests that the side chain at position 10 in α-conotoxins K[10H]SI and [K10N]SI interacts with either the *Torpedo* δ subunit or the *Torpedo* γ subunit.

Substitution of Lys for Pro at position 9 in α-conotoxin SI resulted in an 870-fold increase in apparent affinity for the α/δ site of BC_3H-1 receptors, and a 190-fold increase in apparent affinity for the α/γ site of *Torpedo* receptors. However, only a modest increase (1.8−4.1-fold) in apparent affinity was observed at the lower affinity sites of the two receptor subtypes. The substitution of Lys for Pro^9 significantly alters the backbone structure in α-conotoxin SI near position 9, presumably resulting in a conformation more like that of α-conotoxin GI. The dramatic increase in affinity of α-conotoxin [P9K]SI for only one acetylcholine-binding site on both BC_3H-1 and *Torpedo* receptors indicates that structural differences between α-conotoxins SI and [P9K]SI are localized to the region around position 9 and that this region is oriented toward the γ and δ subunits of the nicotinic receptor, making subunit-dependent contacts.

Substitution of Ala for Arg at position 9 in α-conotoxin GI resulted in at least a 46-fold loss in apparent affinity at both acetylcholine-binding sites on BC_3H-1 receptors while retaining a greater than 10 000-fold selectivity for the α/δ site. The apparent affinities of α-conotoxin [R9A]GI for the γ and δ sites of *Torpedo* receptors were reduced 490-fold and 25-fold, respectively (relative to that of α-conotoxin GI), resulting in identical IC_{50}'s for the two acetylcholine-binding sites on *Torpedo* receptors. In addition to the loss of a positive charge, α-conotoxin [R9A]GI lacks the aliphatic chain of Arg^9. It has been observed that substitution of uncharged norleucine [$R = -(CH_2)_3CH_3$] for Lys at the comparable position in α-conotoxin MI reduces the affinity by only 3.3-fold for nicotinic receptors at the neuromuscular junction in mice (*29*). Therefore, it is not unreasonable to presume that the aliphatic side chain of Arg^9 contributes more to the affinity of α-conotoxin GI for the α/δ site of mouse receptors than does the positive charge. The results obtained with [R9A]GI reinforce the supposition that the region around position 9 in the α-conotoxins is oriented toward the δ and γ subunits of the nicotinic receptor.

Three amino acids in the δ subunit of BC_3H-1 receptors (δSer^{36}, δTyr^{113}, and δIle^{178}) have been shown to contribute significantly to receptor affinity for α-conotoxin MI. These amino acids are not present at the comparable positions in the γ subunit, and introduction of these residues into the γ subunit results in an affinity for α-conotoxin MI similar to that of the wild-type α/δ site (*6*). Presumably, these same amino acids determine the higher affinity of α-conotoxin GI for the α/δ site of BC_3H-1 receptors as well. However, α-conotoxins GI and MI display a higher affinity for the

α/γ site in *Torpedo* receptors (*7*, *8*, *33*). By simple analogy with BC_3H-1 receptors, the higher affinity of α-conotoxin MI for the α/γ site of *Torpedo* receptors is determined by γLys[34], γTyr[111], and γHis[172], which are lacking at the comparable positions in the *Torpedo* δ subunit. The observations that δTyr[113] in BC_3H-1 receptors contributes significantly to affinity for α-conotoxins and that α-conotoxin [R9A]GI exhibited lower affinity for the α/δ site make it tempting to infer that a direct interaction exists between the receptor δTyr[113] and the toxin Arg[9]. Although a direct interaction may exist, α-conotoxin [R9A]GI also displayed a loss of affinity at the α/γ site of BC_3H-1 receptors at least comparable to the loss of affinity observed at the α/δ site. Thus, it is likely that Arg[9] makes critical contacts at other points on the receptor as well, quite possibly on the α subunits.

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