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α-Conotoxins Selectively Inhibit One of the Two Acetylcholine Binding Sites of Nicotinic Receptors

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SUMMARY

Muscle subtypes of the nicotinic acetylcholine receptor contain two acetylcholine binding sites that can be distinguished pharmacologically. The affinities of several α-conotoxins for the two acetylcholine binding sites on nicotinic receptors from BC_3H1 cells and *Torpedo* electric organ were investigated. α-Conotoxins MI, GI, and SIA each inhibited the binding of ^{125}I-α-bungarotoxin to nicotinic acetylcholine receptors on BC_3H1 cells with two distinct and independent affinities, which differed by >10,000-fold. The affinities of α-conotoxins SI and SII were significantly lower and the differences in the affinities of each of these toxins for the two sites were <400-fold. α-Conotoxins MI, GI, SIA, and SI had higher affinity for the acetylcholine binding site near the α/δ subunit interface of nicotinic receptors from BC_3H1 cells. However, when assessed using nicotinic receptors from *Torpedo* electric organ, α-conotoxin MI displayed higher affinity for the acetylcholine binding site near the α/γ subunit interface. These observations suggest that species variations in the sequences of the γ and δ subunits resulted in a dramatic reversal of the relative affinities of the α-conotoxins for each acetylcholine binding site. Some of the practical implications of these observations are discussed.

The venoms of *Conus* marine snails contain a variety of peptide neurotoxins (1, 2). Some of these peptides (the conotoxins) antagonize nicotinic acetylcholine receptors (α-conotoxins), voltage-gated sodium channels in muscle (μ-conotoxins), and neuronal voltage-gated calcium channels (ω-conotoxins). The conotoxins are cleaved from larger propeptide precursors, which presumably serve to facilitate the proper folding of the mature active toxins (3). A characteristic feature of the conotoxins is the presence of conserved cysteine residues that form distinctive disulfide frameworks. The disulfide framework varies among the different conotoxins and is responsible for maintaining a fully active tertiary structure (4–6).

α-Conotoxins are defined by their ability to inhibit the function of nicotinic acetylcholine receptors (1). The sequences and biological activities of 10 naturally occurring α-conotoxins (Fig. 1) have been published (7–13). The affinity of a given α-conotoxin depends upon the species and subtype of nicotinic receptor. For example, in a comparison of nicotinic receptors at the neuromuscular junction of frog and mouse, α-conotoxin ImI has higher affinity for frog receptors, whereas α-conotoxin GI has higher affinity for mouse receptors (7). Within a given species, however, α-conotoxin GI appears to be more active against muscle receptors and α-conotoxin ImI appears to be more active against neuronal receptors (7, 14).

In addition to species and subtype selectivities, some α-conotoxins are also able to distinguish between the two acetylcholine binding sites present on a single nicotinic receptor. Although a site preference for nicotinic receptor ligands is not unprecedented, it is remarkable that the affinities of α-conotoxin MI for the two acetylcholine binding sites have been reported to differ by 2–4 orders of magnitude (15, 16). The affinities of α-conotoxin MI for receptors composed of mouse muscle αβγ and αβδ subunits expressed in mouse fibroblasts suggest that α-conotoxin MI binds with higher affinity to the acetylcholine binding site located at the α/δ subunit interface (15). However, using nicotinic receptors from *Torpedo* electric organ, a photoaffinity-labeled derivative of α-conotoxin GIA has been shown to react preferentially with the γ subunit (11). Furthermore, α-conotoxin GI preferentially blocks covalent incorporation of a photoaffinity-labeled derivative of neurotoxin II into the γ subunit of the *Torpedo* receptor (17). Finally, *d*-tubocurarine has been shown to selectively block the interaction of α-conotoxins GI and MI with their higher affinity binding site on *Torpedo* receptors, suggesting that α-conotoxins have higher affinity for the acetylcholine binding site near the α/γ subunit inter-

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ABBREVIATIONS: α-BTX, α-bungarotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

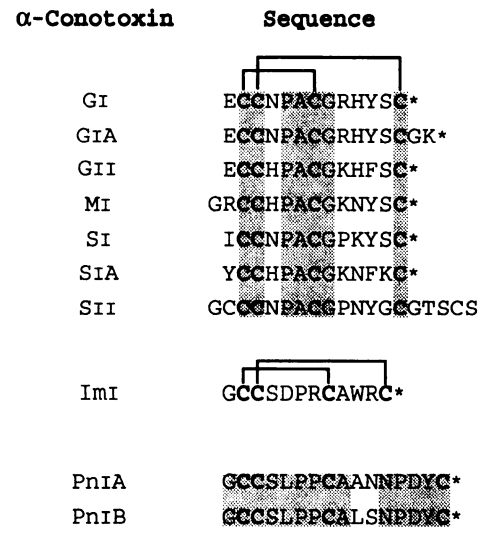

Fig. 1. Sequences of the α-conotoxins. The sequences of all 10 known α-conotoxins are shown for comparative purposes. *Solid lines*, confirmed disulfide bridges between conserved cysteine residues (*bold type*). Strictly conserved amino acid residues are *shaded*. *, Amidated carboxyl terminus.

face (16). It is not clear, then, which of the two acetylcholine binding sites has higher affinity for α-conotoxins.

Nicotinic acetylcholine receptors from the mouse muscle-derived BC_3H1 cell line and from *Torpedo* electric organ have proven to be useful model systems for investigations of receptor structure and function. Because of their high affinity, their subtype selectivity, and their relative ease of synthesis, the α-conotoxins are useful probes for studies of nicotinic receptors (11). In this study, we determined the apparent affinities of several α-conotoxins for the two acetylcholine binding sites on nicotinic receptors from intact BC_3H1 cells. We also identified the high affinity α-conotoxin binding site on nicotinic receptors from both BC_3H1 cells and *Torpedo* electric organ.

Experimental Procedures

Materials. Bipinnatin-B was purified as described previously (18). Stocks of bipinnatin-B (10 mM) in 100% dimethylsulfoxide were stored at −20° for several months without loss of biological activity. ^{125}I-α-BTX (10–20 μCi/μg) was purchased from DuPont-NEN. Metocurine (dimethyl-*d*-tubocurarine) was a gift from Lilly. Purified membranes from *Torpedo* electric organ were a gift from David A. Johnson (University of California, Riverside). α-Conotoxins ImI and SII were obtained from William R. Gray, J. Michael McIntosh, and Baldomero M. Olivera (University of Utah). α-Conotoxins PnIA and PnIB were provided by Michael Fainzilber (Free University of Amsterdam). All other materials, chemicals, and media were purchased from Fisher, Life Technologies, Sigma, and VWR.

Maintenance of BC_3H1 cells. BC_3H1 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°. Cells in growth medium were seeded into gelatin-coated, 24-well plates at a density of 8,000–12,000 cells/well and were grown as described previously (19, 20). Before each experiment, the cells were equilibrated at 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 $MgSO_4$, 0.06 mg/ml bovine serum albumin, pH 7.4), and equilibrated for 20 min in 1.0 ml of fresh assay buffer. All experiments were performed at room temperature.

Inhibition of ^{125}I-α-BTX binding to nicotinic receptors on BC_3H1 cells. The assay buffer in the multiwell plates was removed and the cells were equilibrated for 30 min with 250-μl aliquots of α-conotoxin and/or metocurine at the indicated concentrations. After this incubation, 10 μl of ^{125}I-α-BTX (10–20 nM final concentration) were added to each well and cells were allowed to incubate for 15 min. Each well was then washed twice with 2.0 ml of assay buffer to remove unbound ^{125}I-α-BTX, and the cells were resuspended with two 0.5-ml washes of 1% Triton X-100 in water. The Triton X-100 washes were collected and counted to determine the amount of bound ^{125}I-α-BTX. In some experiments, selective and irreversible inhibition of the acetylcholine binding site near the α/δ subunit interface on nicotinic receptors from BC_3H1 cells was performed using bipinnatin-B, as described previously (19). The affinities of α-conotoxins for the remaining sites were measured as described above.

All assays were performed in triplicate. Nonspecific binding of ^{125}I-α-BTX was determined using cells that were incubated with 250 μl of 100 nM α-BTX for 30 min, washed twice with 2.0 ml of assay buffer, and incubated with 260 μl of ^{125}I-α-BTX (15–20 nM) for 15 min. The total number of ^{125}I-α-BTX binding sites (determined after a 60-min incubation with ^{125}I-α-BTX) was 140 ± 10 fmol/well (12 experiments). In the absence of competing drug, approximately 64% of the total population of sites were labeled during the 15-min incubation with ^{125}I-α-BTX.

Inhibition of ^{125}I-α-BTX binding to membrane preparations of nicotinic receptors. Membranes prepared from *Torpedo* electric organ (0.33 nmol of ^{125}I-α-BTX binding sites/mg of protein) were diluted to 135 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 MI and/or metocurine. Triton X-100 was included in the assay buffer to ensure that both acetylcholine binding sites were assayed (21). Membranes prepared from BC_3H1 cells were treated in a similar manner (22). The membranes were incubated with drugs for at least 2 hr, and then 15 μl of ^{125}I-α-BTX (10–20 nM final concentration) were added. The reactions were then incubated for 30 sec and duplicate 50-μl aliquots were removed and spotted on 2.5-cm, DE81, ion exchange filters (Whatman). The aliquots were allowed to absorb to the filters for 30 sec, and the filters were then washed (for at least 10 min each time) with two 600-ml volumes of wash buffer (10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100). The filters were blotted dry between paper toweling and counted to determine the amount of bound ^{125}I-α-BTX.

Nonspecific binding of ^{125}I-α-BTX was determined using membranes incubated in 135 μl of assay buffer with 100 nM α-BTX for 30 min, followed by a 30-sec incubation with ^{125}I-α-BTX. The total number of ^{125}I-α-BTX binding sites (determined after a 30-min incubation with ^{125}I-α-BTX) was 3.1 ± 0.12 nM (11 experiments). In the absence of competing drug, approximately 89% of the total population of sites were labeled in 30 sec with ^{125}I-α-BTX.

Whole-cell, voltage-clamp recording from single BC_3H1 cells. Cells in growth medium were seeded into 35-mm dishes (1700–3400 cells/cm^2) and incubated for 24 hr in a humidified atmosphere of 5% CO_2/95% air at 37°. The growth medium was replaced with reduced-serum medium (Dulbecco's modified Eagle's medium with 1.0% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin), and cells were incubated for 4 days. The reduced-serum medium was changed and the cells were used within the next four days. Whole-cell, voltage-clamp recordings from single BC_3H1 cells were obtained at 21° with a bath solution of 150 mM NaCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.4 mM KCl, 10 mM Na-HEPES, pH 7.5. The pipette solution contained 150 mM KCl, 5 mM $MgCl_2$, 2.5 mM Na_2ATP, and 10 mM K-HEPES, pH 7.2. Cells were voltage-clamped at −60 mV with an EPC-7 patch-clamp amplifier, and analog current signals (filtered at 3 or 10 kHz) were digitized at 10 Hz. Carbamylcholine and α-conotoxin MI were applied with a microsuperfusion apparatus.

Data analysis. Functions describing the competitive binding of a ligand to one site, to two homogeneous independent sites, or to a

single site with a variable Hill coefficient were fit by nonlinear regression to the inhibition of the specific binding of ^{125}I-α-BTX to nicotinic receptors. The best-fit function for each set of data was determined by performing an *F* test on the sum of squares of the residuals (23). A more complex model was accepted when a value of $p < 0.0001$ was obtained. Similar affinities were obtained whether the data were analyzed as percentage inhibition of specifically bound ^{125}I-α-BTX or as percentage inhibition of the apparent bimolecular association rate constant for ^{125}I-α-BTX (24, 25). Nonlinear regression analysis was performed on a Northgate 386 personal computer, using the software program Prism (GraphPad).

Results

The ability of several α-conotoxins to inhibit the binding of ^{125}I-α-BTX to nicotinic acetylcholine receptors from BC_3H1 cells was determined. α-Conotoxins MI, GI, and SIA each displayed two apparent affinities, which differed by >10,000-fold (Fig. 2, *upper*; Table 1). The interaction of these α-conotoxins was clearly consistent with the presence of two independent binding sites and inconsistent with the presence of a single site or negatively cooperative sites. α-Conotoxins SI and SII displayed relatively low affinities for both acetylcholine binding sites (Fig. 2, *lower*; Table 1). As with α-conotoxins MI, GI, and SIA, data on the inhibition of ^{125}I-α-BTX binding by α-conotoxin SI fit significantly better to a two-site function. The data for α-conotoxin SII fit significantly better to a function describing interaction of the toxin with a single site and a variable Hill coefficient (Table 1). However, because the highest concentration of SII (10 μM) inhibited only approximately 50% of the specifically bound ^{125}I-α-BTX, it was not possible to demonstrate that the more complex two-site model fit the data significantly better than the simpler cooperative model. Concentrations of α-conotoxin ImI up to 2 μM and of α-conotoxins PnIA and PnIB up to 5 μM did not inhibit ^{125}I-α-BTX binding to nicotinic receptors on BC_3H1 cells (Fig. 2). These results are consistent with the previously proposed species and subtype selectivities of these α-conotoxins (7, 13).

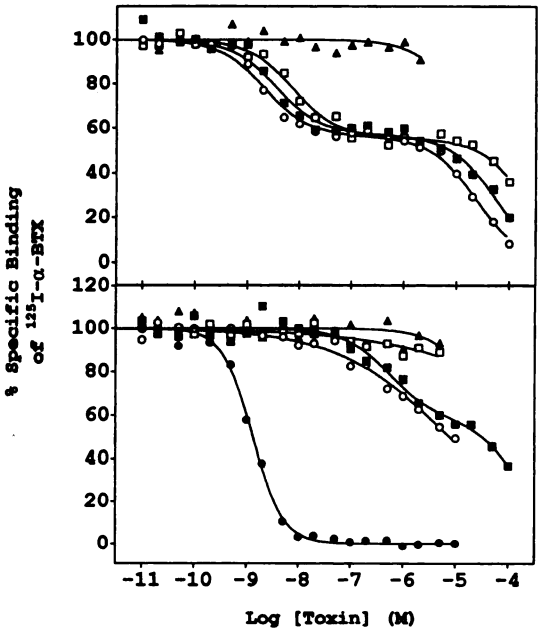

Fig. 2. Inhibition by the α-conotoxins of the binding of ^{125}I-α-BTX to nicotinic receptors on BC_3H1 cells. *Upper*, ○, α-conotoxin MI; ■, α-conotoxin GI; □, α-conotoxin SIA; ▲, α-conotoxin ImI. *Lower*, ■, α-conotoxin SI; ○, α-conotoxin SII; □, α-conotoxin PnIA; ▲, α-conotoxin PnIB. ●, Inhibition by α-cobratoxin of the binding of ^{125}I-α-BTX to nicotinic receptors is shown to demonstrate 100% occupation of both ^{125}I-α-BTX binding sites. The data shown are representative of at least three independent experiments for each toxin. *Lines* through the data are for the best-fit equation, as determined by a statistical comparison of functions.

α-Conotoxin MI displayed an 890-fold difference in apparent affinities for the two acetylcholine binding sites on nicotinic acetylcholine receptors from *Torpedo* electric organ (Fig. 3; Table 1). Despite the disparate receptor sources, the IC_{50} values of α-conotoxin MI for the two acetylcholine binding sites on receptors from *Torpedo* electric organ appeared to be relatively similar to those for the two sites on receptors from BC_3H1 cells (Table 1).

The known site selectivity of two different receptor antagonists was exploited to identify the higher affinity α-conotoxin binding site. Bipinnatin-B is a cyclic diterpene neurotoxin that irreversibly inhibits the two acetylcholine binding sites on nicotinic receptors on BC_3H1 cells at different rates (19). Bipinnatin-B has been shown to interact preferentially with the acetylcholine binding site near the α/δ subunit interface of mouse muscle receptors, and it can be used to selectively and irreversibly block the α/δ site while leaving the α/γ site available for interaction with a second ligand (19, 26). Nicotinic receptors on BC_3H1 cells were incubated with bipinnatin-B to irreversibly inhibit 58 ± 1.2% (11 experiments) of the total available acetylcholine binding sites, and the affinities of α-conotoxins MI, GI, and SIA for the remaining α/γ sites were determined. α-Conotoxins MI, GI, and SIA displayed only low affinity for the remaining α/γ sites (Fig. 4). Selective irreversible inhibition of the α/δ site with bipinnatin-B did not significantly alter the affinities of the α-conotoxins for their lower affinity site.

Metocurine and *d*-tubocurarine are reversible nicotinic receptor antagonists that have higher affinity for the acetylcholine binding site near the α/γ subunit interface of both BC_3H1 cell and *Torpedo* nicotinic receptors (26–30). The affinity of metocurine for nicotinic receptors on BC_3H1 cells was determined in the absence and presence of a concentration of α-conotoxins MI, GI, SIA, or SI sufficient to block >93% of the higher affinity α-conotoxin binding site and <10% of the lower affinity site. In the absence of the α-conotoxins, metocurine inhibited the binding of ^{125}I-α-BTX to receptors on BC_3H1 cells with two distinct and independent affinities, which differed by >40-fold (Fig. 5, *upper*; Table 2). However, in the presence of the α-conotoxins, metocurine inhibited the binding of ^{125}I-α-BTX with a single affinity, similar to the IC_{50} for its higher affinity binding site (Fig. 5, *upper*; Table 2). These results indicate that, with respect to nicotinic receptors from BC_3H1 cells, α-conotoxins MI, GI, SIA, and SI have higher affinity for the acetylcholine binding site that has low affinity for metocurine. Similar results were obtained with BC_3H1 cell membranes solubilized in 0.1% Triton X-100 (data not shown).

To identify the higher affinity α-conotoxin binding site on nicotinic receptors from *Torpedo* electric organ, the affinity of metocurine was determined in the absence and presence of α-conotoxin MI. In the absence of α-conotoxin MI, metocurine inhibited the binding of ^{125}I-α-BTX to receptors from *Torpedo* electric organ with two affinities, which differed by >70-fold (Fig. 5, *lower*). However, in the presence of a concentration of

TABLE 1

Affinities of α-conotoxins for nicotinic acetylcholine receptors

Conotoxin	Receptor source	IC_{50_1}	IC_{50_2}	High affinity sites	IC_{50_2}/IC_{50_1}
		nM	μM	%	
MI	*Torpedo*	2.6 ± 1.0	2.3 ± 0.7	45 ± 0.02	880
MI	BC_3H1	1.5 ± 0.24	22 ± 1.1	44 ± 1.5	15,000
GI	BC_3H1	4.9 ± 1.9	58 ± 12	41 ± 2.8	12,000
SIA	BC_3H1	7.7 ± 0.14	200 ± 8.6	41 ± 2.0	26,000
SI	BC_3H1	680 ± 160	220 ± 45	41 ± 1.3	320

Conotoxin	Receptor source	IC_{50}	Hill coefficient
		μM	
SII	BC_3H1	18 ± 6.6	0.44 ± 0.03
ImI	BC_3H1	>2.0	ND[a]
PnIA	BC_3H1	>5.0	ND
PnIB	BC_3H1	>5.0	ND

[a] ND, for α-conotoxins ImI, PnIA, and PnIB, a Hill coefficient could not be determined.

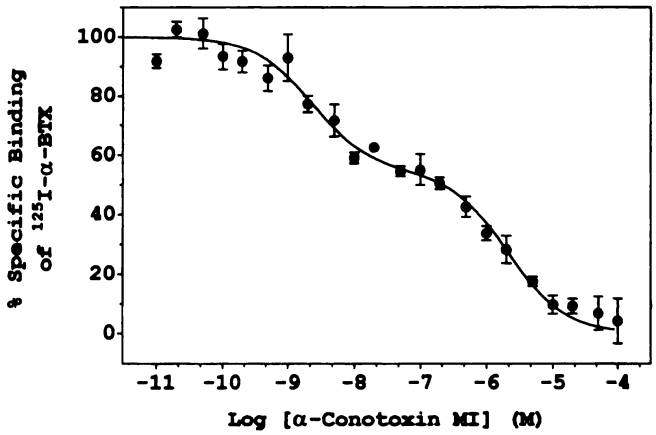

Fig. 3. Inhibition by α-conotoxin MI of the binding of ^{125}I-α-BTX to nicotinic receptors from *Torpedo* electric organ. The data points represent the mean and standard error of four independent experiments. The *line* through the data is the best-fit equation, as determined by a statistical comparison of functions.

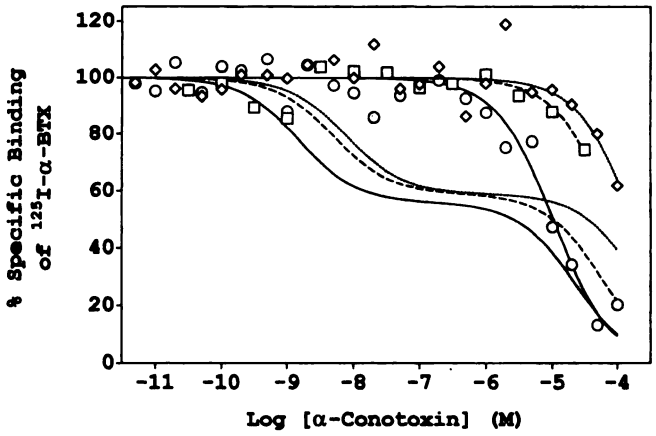

Fig. 4. Inhibition by the α-conotoxins of the binding of ^{125}I-α-BTX to nicotinic receptors on BC_3H1 cells after selective irreversible inhibition of the α/δ sites by preincubation with bipinnatin-B. Inhibition of specifically bound ^{125}I-α-BTX by α-conotoxins MI (—○—), GI (– –□– –), and SIA (···◇···) after preincubation of nicotinic receptors with bipinnatin-B is shown. For comparison, values from Table 1 were used to generate dose-response curves for the inhibition of specifically bound ^{125}I-α-BTX by α-conotoxins MI (——), GI (– – –), and SIA (· · · ·) in the absence of treatment with bipinnatin-B. The data shown are representative of two to five experiments for each α-conotoxin. *Lines* through the data are for the best-fit equation (single-site model), as determined by a statistical comparison of functions.

α-conotoxin MI sufficient to block >97% of its higher affinity sites and <5% of its lower affinity sites, metocurine inhibited the binding of ^{125}I-α-BTX with a single affinity, similar to the IC_{50} for its lower affinity binding site (Fig. 5, *lower*; Table 2). In contrast to BC_3H1 cells, these results indicate that, with respect to receptors from *Torpedo* electric organ, α-conotoxin MI has higher affinity for the same acetylcholine binding site that has higher affinity for metocurine. Similar results were obtained with *d*-tubocurarine (data not shown).

The functional consequences of occupying only one acetylcholine binding site on the receptor with α-conotoxin was investigated. Whole-cell, voltage-clamp recordings of nicotinic acetylcholine receptors were obtained from single BC_3H1 cells in the absence and presence of α-conotoxin MI. The concentration of α-conotoxin MI (500 nM) was chosen such that >99% of its higher affinity binding sites were occupied by toxin, whereas <3% of the lower affinity sites were occupied. Cells were exposed to 10 μM carbamylcholine for 10-sec intervals before and during application of α-conotoxin MI (Fig. 6, *upper*). By itself, α-conotoxin MI did not elicit an inward current response. However, within 60 sec complete inhibition of the carbamylcholine-induced inward current was observed. These results demonstrate that occupation of only one of the two acetylcholine binding sites by α-conotoxin MI was sufficient to block >98% of receptor function. To investigate the rate of association of α-conotoxin MI with its higher affinity binding site, BC_3H1 cells were perfused with a lower concentration of carbamylcholine (5 μM) for 60 sec, to obtain a steady state level of receptor activation. α-Conotoxin MI (500 nM) was then added in the continued presence of carbamylcholine (Fig. 6, *lower*). Within 60 sec, >98% of the carbamylcholine-induced response was blocked by α-conotoxin MI. A first-order exponential decay function was fit to the data, revealing an apparent first-order association rate constant of 0.18 ± 0.01 sec^{-1} (four experiments) for association of α-conotoxin MI with its higher affinity site.

Discussion

α-Conotoxins MI, GI, and SIA each demonstrated a >10,000-fold difference in IC_{50} values for the two acetylcholine binding sites on nicotinic receptors from BC_3H1 cells. The two IC_{50} values of α-conotoxin MI for receptors from

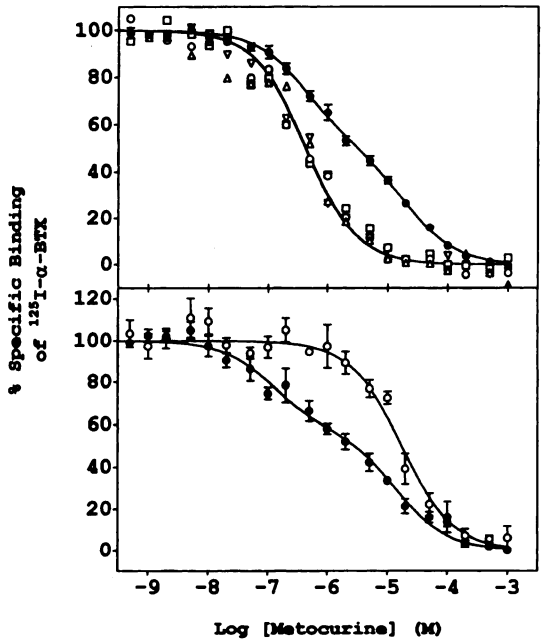

Fig. 5. Inhibition by metocurine of the binding of ^{125}I-α-BTX to nicotinic receptors, in the absence and presence of the α-conotoxins. *Upper*, the affinity of metocurine for nicotinic receptors on BC_3H1 cells was determined in the absence (●) and presence of 0.10 μM α-conotoxin MI (○), 0.32 μM α-conotoxin GI (□), 1.0 μM α-conotoxin SIA (▽), or 10 μM α-conotoxin SI (△). In the absence of metocurine, the α-conotoxins inhibited 40.1 ± 2.5% (four experiments) of the total population of ^{125}I-α-BTX binding sites. The data for metocurine alone represent the mean and standard error of seven independent experiments. The data for metocurine in the presence of the α-conotoxins are from individual experiments. *Lower*, the affinity of metocurine for nicotinic receptors from *Torpedo* electric organ was determined in the absence (●) and presence of 0.10 μM α-conotoxin MI (○). In the absence of metocurine, α-conotoxin MI inhibited 51.6 ± 1.5% (three experiments) of the total population of ^{125}I-α-BTX binding sites. The data for each curve represent the mean and standard error of at least three independent experiments. *Lines* through the data are for the best-fit equation, as determined by a statistical comparison of functions.

TABLE 2

Affinity of metocurine for nicotinic acetylcholine receptors in the absence and presence of α-conotoxins

Receptor source	α-Conotoxin	IC_{50_1}	IC_{50_2}	High affinity sites
		μM	μM	%
BC_3H1	None	0.51 ± 0.16	22 ± 3.6	50 ± 4.3
BC_3H1	GI	0.46	NA[a]	100
BC_3H1	MI	0.44	NA	100
BC_3H1	SI	0.43	NA	100
BC_3H1	SIA	0.44	NA	100
Torpedo	None	0.26 ± 0.18	19 ± 4.1	44 ± 8.8
Torpedo	MI	NA	17 ± 1.2	0

[a] NA, a second affinity was not applicable when the results fit better to a single-site model.

BC_3H1 cells were similar to the affinities determined for mouse muscle αβδ and αβγ receptor variants (15). α-Conotoxins SI and SII are similar to α-conotoxins MI, GI, and SIA in primary sequence and secondary structure, but α-conotoxins SI and SII displayed micromolar affinities for both acetylcholine binding sites. In addition, the IC_{50} values of α-conotoxins SI and SII for the two acetylcholine binding sites differed by <400-fold. The IC_{50} values of α-conotoxins SI and SII are consistent with the observation that these peptides are substantially less toxic than α-conotoxins MI and GI after interperitoneal injection into mice (10). The reduced toxicity of both α-conotoxins SI and SII appears to result from a significantly lower affinity for only one of the two acetylcholine binding sites. The loss of high affinity for the receptor may be due to the replacement of lysine or arginine in α-conotoxins MI, GI, and SIA with proline in α-conotoxins SI and SII.

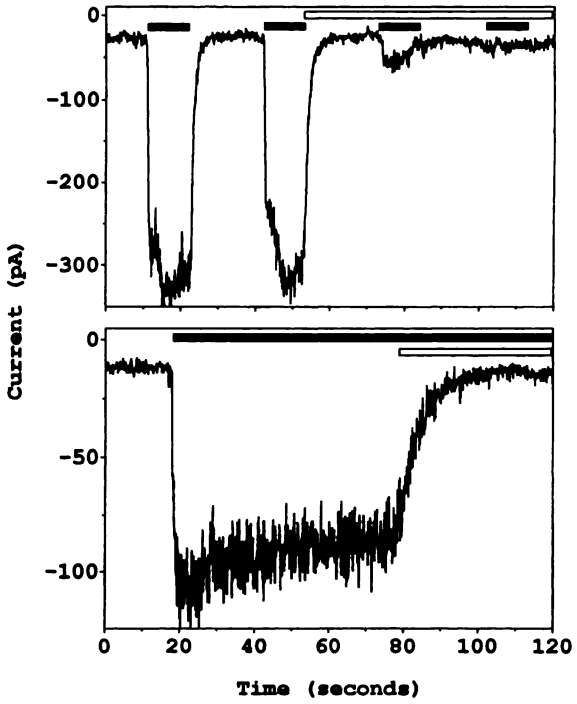

Fig. 6. Whole-cell, voltage-clamp recording from single BC_3H1 cells in the absence and presence of α-conotoxin MI. *Upper*, the response of a single BC_3H1 cell to 10-sec applications of 10 μM carbamylcholine in the absence (*black bars*) or presence (*white bar*) of 500 nM α-conotoxin MI is shown. *Lower*, the response of a single BC_3H1 cell to 5 μM carbamylcholine was recorded (*black bar*) and then 500 nM α-conotoxin MI was applied (*white bar*). Each panel is representative of at least four independent experiments.

α-Conotoxins MI, GI, and SIA displayed only low affinity for nicotinic receptors on BC_3H1 cells after selective irreversible inhibition of the α/δ site by bipinnatin-B, suggesting that these α-conotoxins and bipinnatin-B have higher affinity for the same acetylcholine binding site. However, in the presence of α-conotoxin MI, GI, SIA, or SI, metocurine displayed only high affinity for the receptor, suggesting that the α-conotoxins and metocurine have high affinity for different acetylcholine binding sites. Because bipinnatin-B selectively inhibits the α/δ site and metocurine selectively inhibits the α/γ site, these results indicate that the α-conotoxins have higher affinity for the acetylcholine binding site near the α/δ subunit interface on nicotinic receptors from BC_3H1 cells. This conclusion is consistent with the observation that α-conotoxin MI has a higher affinity for receptors composed of mouse muscle αβδ subunits than for receptors composed of αβγ subunits (15).

Occupation of one acetylcholine binding site by α-conotoxin and the other site by an agonist could theoretically result in activation of the receptor. For example, fetal-type nicotinic receptors are partially activated when one acetylcholine binding site is occupied by *d*-tubocurarine and the other is occupied by acetylcholine (31, 32). However, >98% of the

carbamylcholine-induced inward current response of nicotinic receptors was blocked by a concentration of α-conotoxin MI that occupied at least 99% of its higher affinity sites and <3% of its lower affinity sites. These results are consistent with previous observations of nicotinic receptor antagonism. For example, there is a nonlinear reduction in the functional response of receptors after irreversible occupation of an increasing proportion of acetylcholine binding sites with either α-cobratoxin or lophotoxin, and the functional response of receptors from BC_3H1 cells is inhibited by metocurine with an apparent affinity similar to the IC_{50} for its higher affinity binding site (33–35).

The unique affinities of metocurine for the two acetylcholine binding sites have been shown to be due to the interaction of metocurine with specific amino acids in the γ and δ subunits (30). It therefore seems likely that the selectivity of the α-conotoxins for the two acetylcholine binding sites on a single receptor is also due to differences in the amino acid sequences of the γ and δ subunits. However, because the extent of sequence identity between the γ and δ subunits is relatively low, it is not possible to identify by visual inspection which amino acids may contribute to the differences in affinities of the α-conotoxins. It is interesting to note that the affinity of α-conotoxin SI for the α/γ site is similar to the affinities of α-conotoxins MI, GI, and SIA for the same site. This observation suggests that specific contacts responsible for the high affinity of the α-conotoxins for the α/δ site are not present at the α/γ site.

The difference in the affinities of α-conotoxin MI for the two acetylcholine binding sites (IC_{50_2}/IC_{50_1}) is reasonably maintained in receptors from both BC_3H1 cells and *Torpedo* electric organ (15,000-fold and 890-fold differences, respectively). This was not unexpected, given the overall homology of receptor subunits between the two species. However, in the presence of α-conotoxin MI, metocurine displayed only low affinity for *Torpedo* receptors. Thus, in contrast to receptors from BC_3H1 cells, α-conotoxin MI displayed higher affinity for the acetylcholine binding site near the α/γ subunit interface in *Torpedo* receptors. This conclusion is consistent with previous observations of the interaction of α-conotoxins with nicotinic receptors from *Torpedo* electric organ (11, 16, 17).

It is striking that, between receptors from BC_3H1 cells and *Torpedo* electric organ, the affinity of α-conotoxin MI for the α/δ site actually decreased 1500-fold, whereas the affinity for the α/γ site increased 8500-fold. These results represent profound species-specific changes in the affinities of the two acetylcholine binding sites for the α-conotoxins. It is likely that the change in the identity of the higher affinity α-conotoxin binding site between BC_3H1 cell receptors and *Torpedo* electric organ receptors is due to species-specific differences in the amino acid sequences of both the γ and δ subunits. The remarkable differences in the affinities of α-conotoxin MI for the α/δ and α/γ sites between BC_3H1 cell and *Torpedo* nicotinic receptors suggest that caution should be exercised when α-conotoxins are used to pharmacologically define nicotinic receptor subtypes in different species.

Some of the practical implications of site-selective nicotinic receptor antagonists have been discussed previously (35–38). For example, the potentiated antagonism that has been observed clinically for some pairs of nondepolarizing neuromuscular blocking agents may result from the combined use of two different antagonists acting selectively at different acetylcholine binding sites (36, 37). The species-dependent switch in the higher affinity α-conotoxin MI binding site observed in this study suggests that structural alterations of α-conotoxin MI could dramatically reverse its affinities for the two acetylcholine binding sites on a single receptor. However, structural alterations that result in a switch or reversal in the identity of the higher affinity site might not be readily apparent in function-based assays. This is because, for site-selective antagonists, functional antagonism (K_{ant}) is related only to antagonist occupation of the higher affinity site on the receptor, regardless of the identity of that site. For example, when $K_A \ll K_B$, $K_{ant} = K_A$ and, when $K_B \ll K_A$, $K_{ant} = K_B$ (35, 38). In addition, structural modification of a high affinity, nonselective antagonist ($K_A = K_B$) could selectively reduce the affinity for only one site ($K_A \ll K_B$), whereas a different structural modification of the same drug could selectively reduce the affinity for the other site ($K_B \ll K_A$). Function-based receptor assays would reveal relatively little decrease (2.4-fold) in the apparent affinity of each structural analog, compared with the parent compound because, when $K_A = K_B$, $K_{ant} = K_A/2.4 = K_B/2.4$ (35, 38). A more direct measure of antagonist affinity for each site and the identification of the higher affinity site are required for correct interpretation of the effects of antagonists. Otherwise, one might incorrectly conclude that structural alterations do not significantly affect antagonist affinity, compromising the utility of structure-activity results as a basis for rational drug development.

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