The α-Conotoxins GI and MI Distinguish between the Nicotinic Acetylcholine Receptor Agonist Sites while SI Does Not†

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ABSTRACT: The α-conotoxins are paralytic peptide toxins from Indo-Pacific cone snails. This paper presents a detailed analysis of how α-conotoxins inhibit [^{125}I]-α-bungarotoxin (^{125}I-BTX) equilibrium binding to the acetylcholine receptor (AChR) from electric organ of *Torpedo californica* and *Torpedo nobiliana*. All three α-conotoxins studied, SI, GI, and MI, completely inhibited ^{125}I-BTX binding with the same order of potency in both species (MI ≈ GI > SI ≈ *d*-tubocurarine). BTX−concentration curves showed that this inhibition is competitive. However, while SI appeared to bind to a homogeneous population of sites, both GI and MI displayed curare-like heterogeneous binding. Studies using partially-blocked AChR demonstrated that both GI and MI display different affinities toward the two agonist sites, much like small curariform antagonists do. The high-affinity site for these two α-conotoxins is also the high-affinity *d*-tubocurarine site, which is believed to be located at the αγ-subunit interface. The high-affinity binding of MI and GI was of the same order of magnitude as that of *d*-tubocurarine; however, their affinity for the other agonist site was somewhat greater than that of dTC, resulting in less site selectivity. Despite being homologous to GI and MI, SI did not distinguish between the two sites. A possible molecular basis for this difference is presented.

The muscle-type nicotinic acetylcholine receptor (AChR)[1] is a ligand-activated cation channel, which exists as a transmembrane pentamer formed by four homologous subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$. Each AChR molecule has two nonidentical agonist-binding sites, whose difference was first observed as nonequivalent affinity labeling (Damle & Karlin, 1978) and markedly different affinities toward small curariform antagonists (Neubig & Cohen, 1979; Sine & Taylor, 1981). These observations initially presented a paradox as studies showed that the agonist sites were formed mainly by the two identical α-subunits [for a review, see Karlin (1993)]; however, they are now known also to involve the neighboring γ- and δ-subunits, whose participation renders the sites nonidentical (Blount & Merlie, 1989; Pedersen & Cohen, 1990). High-resolution images suggest that the agonist site is a cavity which is centered within the α-subunit but has one wall formed by the neighboring subunit (Unwin, 1993).

Curare and its close structural analogues possess two cationic centers about 1 nm apart which are separated from each other by a rigid hydrophobic region (Pauling & Petcher, 1973). These molecules bind with higher affinity to the αγ site than to the αδ site (Blount & Merlie, 1989; Pederson & Cohen, 1990; Sine & Claudio, 1991). Specific AChR residues influencing the high-affinity binding have recently been identified by affinity-labeling and site-directed mutagenesis studies. These include tyrosine residues on both α (Sine et al., 1994; O'Leary et al., 1994) and γ (Sine, 1993), a tryptophan residue on γ (Chiara & Cohen, 1992; O'Leary et al., 1994), and serine and isoleucine residues on γ (Sine, 1993). Site selectivity can result when δ residues are different from the homologous γ residues which contribute to high-affinity binding (Sine, 1993).

The α-conotoxins are short peptides found in the venom of predatory Indo-Pacific cone snails (Gray et al., 1988). They paralyze vertebrate muscle via postsynaptic inhibition and were originally referred to as "alpha" because they mimicked physiologically the effects of the well-characterized snake α-neurotoxins (Olivera et al., 1985). However, α-conotoxins act faster and are more quickly reversible than the snake toxins. In this respect, they more closely resemble the small curariform antagonists, with which they share certain features (Gray et al., 1985; Pardi et al., 1989).

The main purpose of the present research was to characterize in detail the interaction of α-conotoxins with the AChR agonist-binding sites. The nature of α-conotoxin binding to AChR has not been well-studied, although IC_{50}'s for α-conotoxin inhibition of α-neurotoxin binding to AChR have been reported (McManus et al., 1981; Zafaralla et al., 1988; Ramilo et al., 1992). Measuring α-conotoxin inhibition of ^{125}I-BTX binding to *Torpedo* AChR, it was found that two α-conotoxins displayed curare-like selectivity toward the agonist sites, while a third α-conotoxin did not.

EXPERIMENTAL PROCEDURES

Materials. Three synthetic α-conotoxins were used in this study: SI, originally isolated from *Conus striatus* (Zafaralla et al., 1988); GI, originally from *Conus geographus* (Cruz et al., 1978); and MI, originally from *Conus magus* (McIntosh et al., 1982). Synthetic α-conotoxins (97−99% pure

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; BTX, α-bungarotoxin; ^{125}I-BTX, [^{125}I]Tyr^{54}-α-bungarotoxin; dTC, *d*-tubocurarine; IC_{50}, concentration of inhibitor producing half-maximal inhibition; n_H, Hill coefficient; *C*, coefficient of determination.

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by HPLC), as well as dTC and unlabeled BTX, were from Sigma Chemical Co. ^{125}I-BTX was from DuPont−New England Nuclear. Frozen electric organ from *Torpedo nobiliana* (Biofish Assoc.) or *Torpedo californica* (Pacific Biomarine Lab) was maintained at −50 to −80 °C prior to use.

Preparation and Characterization of AChR-Rich Membranes. AChR-rich membranes were prepared from the total membrane fraction of homogenized electric organ by ultracentrifugation as described by Lindstrom et al. (1980) and modified by Szczawinska et al. (1992). The AChR-rich membranes were recovered from the interphase between 32% (w/w) and 36% sucrose, pelleted by ultracentrifugation, and resuspended in buffer A (50 mM NaCl, 10 mM $NaHPO_4$, and 1 mM EDTA at pH 7.4). Protease inhibitors were present at all times during the preparation of membranes. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Total AChR was determined from ^{125}I-BTX concentration curves as described below. The specific activity of the AChR-rich membranes ranged from 0.5 to 1.5 nmol of ^{125}I-BTX-binding sites per milligram of protein.

^{125}I-BTX Equilibrium Binding Assay. A modification of the filtration method of Schmidt and Raftery (1973) was used to measure ^{125}I-BTX specifically bound to AChR. Membranes were diluted 1:200 (v/v) in solubilizing buffer B [buffer A plus 0.1% (v/v) Triton X-100 at pH 7.4] and preincubated for 30 min at room temperature with or without inhibitors as indicated. ^{125}I-BTX in buffer B was then added and the sample incubated for 60 min. Under these conditions, ^{125}I-BTX binding to detergent-solubilized receptor reached a maximum within 20−30 min and did not increase over a 24-h period of incubation. The final receptor concentration was about 2−3 nM. Replicate 100 μL aliquots of each sample were filtered over vacuum (2−3 in. Hg) through 3-fold 1.3 cm DEAE-cellulose disks (Whatman) prewashed with buffer B. The filters were then quickly washed with 1 mL of buffer B and counted in a Beckman 5500 γ counter. The amount of specifically bound ^{125}I-BTX was calculated by subtracting the counts in samples preincubated with 0.63 μM unlabeled BTX.

BTX−concentration curves were used to quantify the total AChR concentration and determine the BTX dissociation constant (K_D) for each membrane preparation. For these curves, the final BTX concentration ranged from 1 nM to 1 μM. At BTX concentrations greater than 100 nM, ^{125}I-BTX was diluted 1:4 with unlabeled BTX. The ^{125}I-BTX−concentration curve data fit the equation for a homogeneous population of noninteracting binding sites (eq 1a below, without inhibitor) with all AChR preparations used in these studies from both *T. nobiliana* (for example, see control curve in Figure 4) and *T. californica* (not shown). Therefore, ^{125}I-BTX equilibrium binding does not display selectivity or cooperativity within the sensitivity of this assay and can be used in radioligand displacement studies to characterize the mechanism of inhibitor binding.

α-Conotoxin binding and dTC binding were studied by measuring displacement of ^{125}I-BTX binding to the AChR at equilibrium. For the BTX−concentration curves, the final conotoxin concentration, when present, was held constant (SI, 7.90 μM; GI, 1.85 μM; MI, 1.88 μM). For the inhibitor−concentration curves, the final ^{125}I-BTX concentration was held constant at approximately 10 nM while the inhibitor concentration, when present, was varied. In order to measure conotoxin interaction mainly with the low-affinity dTC site, *T. nobiliana* AChR was preincubated 30 min in buffer B in the absence or presence of 10 μM dTC and varying amounts of conotoxin. ^{125}I-BTX was then added and the mixture incubated for 60 min. Control samples contained neither dTC nor conotoxin. In order to measure conotoxin interaction mainly with the high-affinity dTC site, membrane-bound *T. nobiliana* AChR was preincubated for 2 h in buffer A containing 10 μM dTC in the presence or absence of 10 nM unlabeled BTX. Membranes were pelleted by ultracentrifugation and, after removing most of the dTC and unbound BTX, solubilized in buffer B and then incubated 60 min with 10 nM ^{125}I-BTX and varying amounts of conotoxin. Control membranes were preincubated only with dTC and contained no conotoxin.

Data Analysis. Curve-fitting was done on data pooled from a minimum of three experiments. PSI-Plot software (Poly Software International) employing the Marquardt−Levenburg nonlinear curve-fitting method was used in all data fittings. Goodness-of-fit was estimated from the coefficient of determination: $C = [\sum(x_i - \mu)^2 - \sum(x_i - y_i)^2]/\sum(x_i - \mu)^2$ where x_i is the observed data, μ is the mean of the observed data, y_i is the calculated value based on best-fit parameters, and $\sum$ indicates the sum of n observations. The closer C is to 1, the better is the fit.

Several different model equations were used to fit the binding data. Symbols used in these equations are as follows: RT, concentration of ^{125}I-BTX−AChR complex in the presence of inhibitor; RT_c, concentration of ^{125}I-BTX−AChR complex in the absence of inhibitor (control); T, free ^{125}I-BTX concentration in the presence of inhibitor; T_c, free ^{125}I-BTX concentration in the absence of inhibitor; I, J, free inhibitor concentration; K_D, measured dissociation constant for ^{125}I-BTX; K_I, K_J, dissociation constant for the inhibitor; R_t, total concentration of ^{125}I-BTX-binding sites.

(I) Competitive Inhibition. (1) In the "one-site" model, both ^{125}I-BTX-binding sites on the AChR molecule display the same affinity for a single inhibitor I.

$$RT = R_tT/[T + K_D(1 + I/K_I)] \quad (1a)$$

$$RT/RT_c = [T(T_c + K_D)]/\{T_c[T + K_D(1 + I/K_I)]\} \quad (1b)$$

(2) In the "two-site" model, the two ^{125}I-BTX-binding sites have different affinities for either a single competitive inhibitor I

$$RT = 0.5R_tT/[T + K_D(1 + I/K_{I1})] + 0.5R_tT/[T + K_D(1 + I/K_{I2})] \quad (2a)$$

$$RT/RT_c = [0.5T(T_c + K_D)]/\{T_c[T + K_D(1 + I/K_{I1})]\} + [0.5T(T_c + K_D)]/\{T_c[T + K_D(1 + I/K_{I2})]\} \quad (2b)$$

or two different competitive inhibitors I and J

$$RT/RT_c = [0.5T(T_c + K_D)]/\{T_c[T + K_D(1 + I/K_{I1} + J/K_{J1})]\} + [0.5T(T_c + K_D)]/\{T_c[T + K_D(1 + I/K_{I2} + J/K_{J2})]\} \quad (2c)$$

(II) Noncompetitive Inhibition.

$$RT = R_tT/[(T + K_D)(1 + I/K_I)] \quad (3)$$

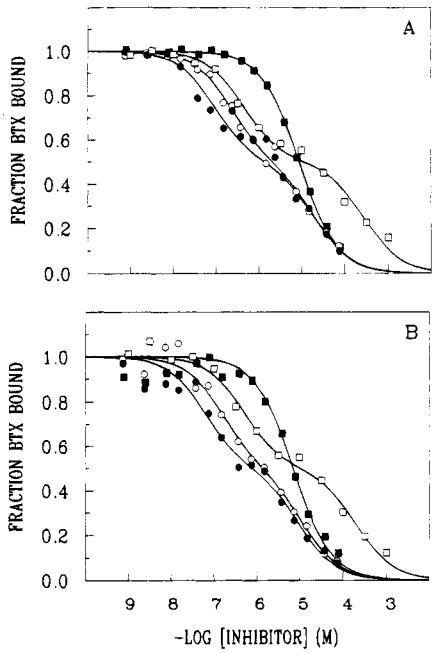

FIGURE 1: α-Conotoxin inhibition of ^{125}I-BTX binding to AChR from electric organ of (A) *T. californica* and (B) *T. nobiliana*. Detergent-solubilized AChR was incubated with 10 nM ^{125}I-BTX and the indicated concentration of inhibitor for 60 min to reach equilibrium. Control samples contained no inhibitor. The specific ^{125}I-BTX bound was determined using a filtration assay, and the fraction bound (experimental over control) was calculated. Inhibitors were MI (filled circles), GI (open circles), SI (filled squares), and dTC (open squares). Each point represents the mean of three to six experiments. The standard deviation was less than 10% of the mean in all cases. Data for SI were fit to the "one-site" model (eq 1b) while data for other inhibitors were fit to the "two-site" model (eq 2b) to generate the curves shown.

To simplify fitting, the total inhibitor concentration was used to approximate the free inhibitor concentration in all equations; the total ^{125}I-BTX concentration was substituted for the free ^{125}I-BTX concentration in the inhibitor−concentration curves. For the BTX−concentration curves, the free BTX concentration was calculated as the difference between the total measured BTX and bound BTX concentrations.

To derive the IC_{50}'s and Hill coefficients for inhibitor displacement of ^{125}I-BTX binding, experimental data between 10% and 90% inhibition were fit to the linear Hill equation:

$$\log[(RT_c/RT) - 1] = n_H \log I + n_H \log IC_{50} \quad (4)$$

RESULTS

α-Conotoxin Binding to the AChR. All three α-conotoxins inhibited ^{125}I-BTX binding to the AChR from both *T. californica* and *T. nobiliana* electric organs (Figure 1). This inhibition was concentration-dependent and complete in the high micromolar range. Inhibition by dTC is shown for comparison. The IC_{50}'s and Hill coefficients are summarized in Table 1. The order of potency for both species was MI ≈ GI > SI ≈ dTC.

SI appeared to bind to a homogeneous population of noninteracting sites. Its inhibitor−concentration curve appeared monophasic with a Hill coefficient close to 1. The one-site model (eq 1b) fit the experimental data well (*C*: 0.996 *californica*, 0.877 *nobiliana*), generating the inhibition constants shown in Table 2. Using the two-site model (eq 2b), the data gave a nearly equivalent fit (*C*: 0.997 *californica*, 0.878 *nobiliana*), but the two inhibition constants generated differed only by a factor of 3. Since this difference is not significant within the sensitivity of the assay, only the one-site inhibition constants for SI are shown in the table.

Table 1: IC_{50}'s and Hill Coefficients for Inhibition of ^{125}I-α-BTX Equilibrium Binding to *Torpedo* AChR[a]

	IC_{50} (μM ± 1 SD)		n_H (±1 SD)	
	T. californica	*T. nobiliana*	*T. californica*	*T. nobiliana*
SI	9.12 ± 0.02	7.41 ± 0.08	0.95 ± 0.02	0.94 ± 0.08
GI	1.99 ± 0.02	1.20 ± 0.01	0.49 ± 0.02	0.53 ± 0.02
MI	1.35 ± 0.02	0.71 ± 0.02	0.42 ± 0.03	0.49 ± 0.03
dTC	10.47 ± 0.10	9.55 ± 0.14	0.34 ± 0.01	0.38 ± 0.02

[a] Parameters were determined by fitting data between 10% and 90% inhibition from Figure 1 to the linear Hill equation (eq 4).

In contrast to SI, both GI and MI produced biphasic inhibitor−concentration curves with Hill coefficients much less than 1, indicating heterogeneous interaction with their binding sites. The data fit the two-site model (*C*: GI, 0.966 *californica*, 0.947 *nobiliana*; MI, 0.931 *californica*, 0.920 *nobiliana*) better than the one-site model (*C*: GI, 0.823 *californica*, 0.860 *nobiliana*; MI, 0.673 *californica*, 0.755 *nobiliana*) and generated the inhibition constants shown in Table 2. In this respect, GI and MI binding resembled dTC binding, whose parameters are shown for comparison.

α-Conotoxin Binding to the Low-Affinity dTC Site. In the presence of 10 μM dTC, over 90% of the high-affinity dTC sites but fewer than 10% of the low-affinity sites were blocked, as determined from eq 2b using parameters for dTC from Table 2. Under these conditions, α-conotoxin was competing with ^{125}I-BTX mainly for the low-affinity dTC site.

Without conotoxin or at very low conotoxin concentrations, 10 μM dTC reduced total ^{125}I-BTX binding to approximately 50% of control, as expected (Figure 2). Low concentrations of GI or MI now appeared to be ineffective as inhibitors in comparison with what was observed in the absence of dTC. At higher conotoxin concentrations, the inhibition curves approximated the shape expected for binding to a single site with the Hill coefficients increasing toward 1 (GI from 0.53 ± 0.02 to 0.83 ± 0.09; MI from 0.49 ± 0.03 to 0.71 ± 0.08). The IC_{50}'s also increased (GI from 1.20 ± 0.01 to 10.7 ± 0.1 μM; MI from 0.71 ± 0.02 to 27.5 ± 0.4 μM). Using the parameters from Table 2 for conotoxin and dTC, the two-site model with two site-selective competitive inhibitors (eq 2c) fit the data well (*C*: GI, 0.919; MI, 0.817) (Figure 2, solid lines). These fits assumed that the high-affinity dTC site was also the high-affinity conotoxin site; if the opposite assumption is made, the resulting curves did not fit the data (Figure 2, dotted lines).

α-Conotoxin MI Binding to the High-Affinity dTC Site. In these experiments, the high-affinity dTC site was protected with dTC while unlabeled BTX was allowed to bind to the unprotected low-affinity site. The dTC and unbound BTX were then removed by centrifugation, leaving most of the low-affinity sites occupied with BTX, whose binding is practically irreversible under these conditions. Over 80% of the unoccupied sites were high-affinity dTC sites while fewer than 20% were low-affinity as determined from eq 2b.

Without MI or at very low MI concentrations, the total ^{125}I-BTX binding to BTX-pretreated AChR was approxi-

Table 2: Inhibition Constants for ^{125}I-α-BTX Binding[a]

	K_{I1} (μM ± 1 SD)		K_{I2} (μM ± 1 SD)		K_{I2}/K_{I1}	
	T. californica	*T. nobiliana*	*T. californica*	*T. nobiliana*	*T. californica*	*T. nobiliana*
SI			1.55 ± 0.04	1.62 ± 0.17		
GI	0.039 ± 0.004	0.037 ± 0.006	3.33 ± 0.41	2.67 ± 0.46	85	72
MI	0.016 ± 0.002	0.015 ± 0.003	3.24 ± 0.55	2.25 ± 0.41	202	150
dTC	0.076 ± 0.008	0.120 ± 0.019	45.69 ± 4.88	45.58 ± 8.06	601	380

[a] Parameters were determined by fitting data in Figure 1 either to the "one-site" model (eq 1b) in the case of SI or to the "two-site" model (eq 2b). The single K_I's for SI are listed in the K_{I2} columns because of their closer proximity to these values.

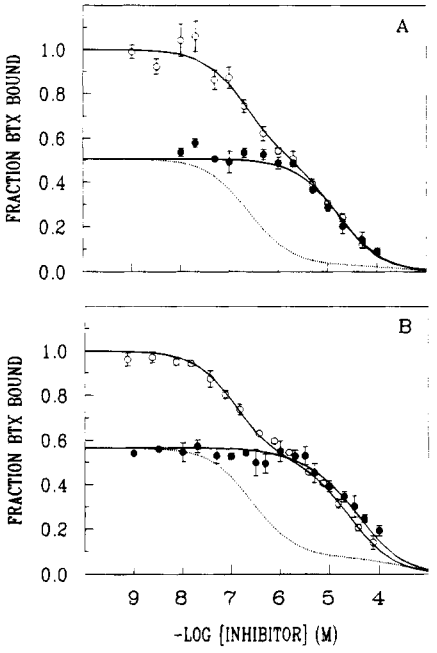

FIGURE 2: α-Conotoxin GI and MI binding to the low-affinity dTC site. AChR from *T. nobiliana* electric organ was incubated for 60 min with 10 nM ^{125}I-BTX and the indicated concentration of either (A) GI or (B) MI in the presence (filled circles) or absence (open circles) of 10 μM dTC. Control samples contained neither conotoxin nor dTC. The fraction of ^{125}I-BTX bound was determined as described in Figure 1. Each point represents the mean ± 1 standard deviation from three experiments. Curves were generated by the "two-site" model either with one selective inhibitor (eq 2b, samples without dTC) or with two selective inhibitors (eq 2c, samples with dTC) using parameters from Table 2 for dTC, GI, and MI. For samples containing dTC, the binding sites of conotoxin and dTC were assigned to correspond either high-to-high (solid lines) or high-to-low (dotted lines).

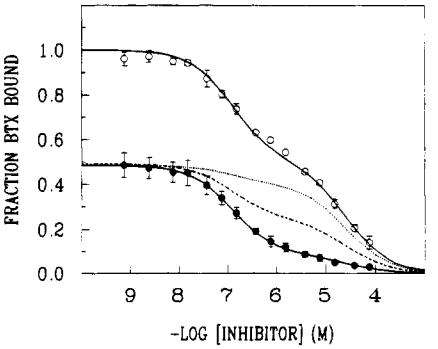

FIGURE 3: α-Conotoxin MI binding to the high-affinity dTC site. Membrane-bound AChR from *T. nobiliana* electric organ was preincubated for 2 h with 10 μM dTC and 10 nM unlabeled BTX (filled circles) or dTC alone (open circles), washed by centrifugation, and then incubated in solubilizing buffer for 60 min with 10 nM ^{125}I-BTX and the indicated concentration of MI. Control samples were pretreated with dTC alone and contained no MI. The fraction of ^{125}I-BTX bound was determined as described in Figure 1. Each point represents the mean ± 1 standard deviation from three experiments. Curves were generated by the "two-site" model (eq 2b) using parameters from Table 2 for MI. For BTX-pretreated samples, the unoccupied high-affinity dTC sites were assigned to correspond either to high-affinity MI sites (solid line), to low-affinity MI sites (dotted line), or to both (dashed line).

mately 50% of control, as expected (Figure 3). The inhibition curve of MI now shifted to the left of control with the IC_{50} decreasing from 1.82 ± 0.02 to 0.21 ± 0.01 μM. The Hill coefficient increased from 0.44 ± 0.02 in controls to 0.79 ± 0.08. The two-site model still fit the data well (*C*: 0.935) after correcting for the fraction of each site unoccupied after BTX pretreatment (Figure 3, solid line). This fit assumes that the high-affinity dTC site is also the high-affinity site for MI; if the opposite assumption is made, the resulting curve did not fit the data (Figure 3, dotted line).

Effect of α-Conotoxins on BTX−Concentration Curves. All three α-conotoxins shifted the BTX−concentration curve to the right of control, as expected (Figure 4). In all three cases, this inhibition was overcome at high BTX concentrations, indicating competitive mechanisms. The experimental data for SI more closely fit the model for competitive inhibition at a single site (eq 1a) (*C*: 0.969; Figure 4A, solid line) than the noncompetitive inhibition model (eq 1c) (*C*: 0.769; Figure 4A, dotted line). In the presence of either GI or MI, the data most closely fit the two-site model for competitive inhibition (eq 2a) (*C*: GI, 0.966; MI, 0.844; Figure 4B,C, dashed lines) compared with the models for competitive inhibition at a single site (*C*: GI, 0.811; MI, 0.268; Figure 4B,C, solid lines) and for noncompetitive inhibition (*C*: GI, 0.891; MI, 0.774; Figure 4B,C, dotted lines). These results are consistent with the findings of the α-conotoxin−concentration curves described above.

DISCUSSION

Relative Potencies of the α-Conotoxins. This report is the first detailed analysis of α-conotoxin inhibition of ^{125}I-BTX binding to AChR. The order of conotoxin potencies was the same in the two species studied, and the IC_{50}'s were of similar magnitudes. Furthermore, curare-like heterogeneity of GI and MI binding, described for the first time by this lab (Hann et al., 1993), was seen with both species.

The order of potency observed in the present report (MI ≈ GI > SI ≈ dTC) agrees generally with earlier reports, which showed that GI and MI have similar physiological potencies (Gray et al., 1983; Hashimoto et al., 1985; Zafaralla et al., 1988; Marshall & Harvey, 1990) and that both are more potent than the small curariform antagonists (McManus et al., 1981; Blount et al., 1992; Hashimoto et al., 1985; Marshall & Harvey, 1990). The only biological study directly comparing SI with MI and GI showed SI to be much less effective than MI and GI in mice (Zafaralla et al., 1988),

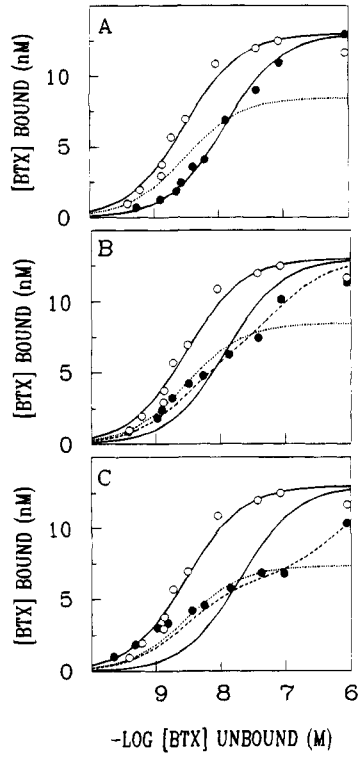

FIGURE 4: Effect of α-conotoxins on BTX−concentration curves. Detergent-solubilized AChR from *T. nobiliana* electric organ was incubated for 60 min with ^{125}I-BTX either alone (control; open circles) or in the presence (filled circles) of (A) SI, (B) GI, or (C) MI. Total specific BTX bound was determined. Each point represents the mean from at least three experiments. Curves show the best possible fit of experimental data to the "one-site" competitive inhibition model (eq 1a, solid lines), the "two-site" competitive inhibition model for GI and MI (eq 2a, dashed lines), and the noncompetitive inhibition model (eq 3, dotted lines).

which is consistent with the present study; however, that same report showed that MI and SI had similar potencies in fish.

There have been only a few reports on α-conotoxins inhibiting the binding of the AChR-specific ligand BTX. An equimolar mixture of GI and GII completely inhibited ^{125}I-BTX binding to mouse muscle, being several times more potent than dTC in this action (McManus et al., 1981). This was similar to what was seen with GI on *Torpedo* AChR in the present report (Table 1). There are only a few previous reports on α-conotoxin inhibiting BTX binding to AChR from *Torpedo* electric organ. Using a nonradioisotopic method, a potency order of GI ≈ MI > SI was reported (Stiles, 1993) with IC_{50}'s of about 2 μM for GI and MI and about 5 μM for SI. These findings agree reasonably well with those in the present report. In an earlier report, MI, GI, and SI gave IC_{50}'s of 0.3, 0.55, and 0.42 μM, respectively (Zafaralla et al., 1988); however, this same lab later reported an IC_{50} of 1.0 μM for SI (Ramilo et al., 1992). The somewhat lower IC_{50} values in these last two reports were probably due to usage of short incubation times. If ^{125}I-BTX binding is not at equilibrium, then lower IC_{50}'s are to be expected. For example, using an initial toxin-binding rate assay, this lab reported IC_{50}'s of 0.19 μM for MI and 0.29 μM for GI (Hann et al., 1993).

Nature of α-Conotoxin Interaction with the AChR. α-Conotoxin inhibition of BTX binding to *Torpedo* AChR is clearly competitive, in agreement with conclusions inferred from earlier physiological and binding studies [for a review, see Gray et al. (1988)]. Since this inhibition is complete at high concentrations, α-conotoxins must interact with both agonist-binding sites on the AChR. SI binds with the same affinity to the two sites.

By analogy with dTC, the most likely reason for the observed heterogeneous binding of GI and MI is that these peptides distinguish between the two agonist sites. However, there are three other possible mechanisms which could produce the heterogeneous binding of GI and MI and need to be ruled out. First, the two binding sites on each AChR could be initially equivalent toward GI and MI, but binding to one of the sites decreases the affinity of the other site (i.e., negative cooperativity). Second, the two binding sites on any given AChR could have equivalent affinities for each peptide, but there exist two different AChR subtypes which display different affinities. Third, each peptide could exist in solution in two conformations which display different affinities for each agonist site.

There is some evidence that the peptides MI and GI can exist as two different conformers in nonaqueous solutions (Gray et al., 1983; Kobayashi et al., 1989). However, if different conformers were producing the heterogeneity, then even with half the sites blocked, competition with ^{125}I-BTX should still result in shallow inhibitor concentration curves (for example, see the dashed line in Figure 3), with the IC_{50}'s and Hill coefficients unchanged. Since this was not observed, the possibility that different conformers of GI or MI cause their heterogeneous binding can be ruled out.

A similar argument rules out the possibility that agonist sites on different receptor subtypes display different affinities for GI and MI. Although *Torpedo* AChR is known to undergo a variety of covalent modifications which could produce subtypes with different ligand affinities, this mechanism also predicts that even after the agonist sites are partially blocked, regardless of which site is blocked, these conotoxins would still produce shallow inhibitor−concentration curves. This was not observed.

If negative cooperativity were occurring, the IC_{50} for conotoxin inhibition of BTX binding should decrease when approximately 50% of the agonist sites are blocked, regardless of which site is blocked, since the putative conformational change from high to low conotoxin affinity could no longer occur. In fact, an increased IC_{50} is observed in the experiments involving reversible block of the dTC high-affinity site with dTC (Figure 2). This observation rules out negative cooperativity as a possible explanation of heterogeneous binding.

Therefore, the α-conotoxins GI and MI actually distinguish between the two AChR agonist-binding sites, much like small curariform antagonists do. The experiments using partially-blocked AChR also show clearly that the high-affinity binding site for GI and MI is the same as the high-affinity binding site for dTC, which is at the αγ-subunit interface (Pedersen & Cohen, 1990). GI and MI both bind to this site with about 2 orders of magnitude higher affinity than to the αδ site. Their selectivity between the two sites is somewhat less than that of dTC, mainly because of dTC's relatively weaker affinity for the αδ site.

The high-affinity binding of MI and GI to the αγ site explains recently-reported AChR-labeling studies. ^{125}I-MI labeled the *Torpedo* γ-subunit much more than either the

α-subunit or the δ-subunit via a short-chain bifunctional cross-linking reagent (Myers et al., 1991). Similar results were obtained using a ^{125}I-photoaffinity label attached to α-conotoxin GIA, which is identical to GI except for having two additional amino acids on the C-terminus.

The three-dimensional structure of GI closely fits the criteria for a curariform antagonist (Kobayashi et al., 1989; Pardi et al., 1989), with the two cationic centers supposedly formed by the N-terminal amino group and a positively-charged arginine residue at position 9, which appears to be at the tip of a sharp turn. The present observations, that GI is selective toward the AChR agonist sites and that this selectivity matches that of dTC, lend support to the proposed structural similarity of GI and the curariform antagonists.

The sequence of SI, which fails to bind the αγ site with high affinity, differs from that of GI and MI at only three or four positions:

	1	2	3	4	5	6	7	8	9	10	11	12	13	
SI:	H_2N--	I--	C--	C--	N--	P--	A--	C--	G--	P--	K--	Y--	S--	C--$CONH_2$
GI:	H_2N--	E--	C--	C--	N--	P--	A--	C--	G--	R--	H--	Y--	S--	C--$CONH_2$
MI:	H_2N--	G--	R--	C--	C--	H--	P--	A--	C--	G--	K--	N--	Y--	S--C--$CONH_2$

All three peptides have first-to-third and second-to-fourth cysteine disulfide bridges. A preliminary report described the three-dimensional structure of SI as having the same general structural elements as GI, but with a tighter turn at residue 9 (Myers et al., 1993). If this is true, then the most likely cause of SI's lack of high-affinity binding at the αγ site is the displacement of the positively charged side chain which is found at position 9 in the aligned sequences of GI and MI. Our lab is now studying a series of synthetic α-conotoxin analogues to test this hypothesis and identify the structural determinants for agonist site selectivity.

ADDED IN PROOF

After this paper was revised, the authors obtained copies of two relevant articles: In the first (Utkin et al., 1994), it was reported that α-conotoxin GI binds to the *Torpedo californica* AChR αγ site with higher affinity than to the αδ site, in agreement with the findings in this paper. In the second (Kreienkamp et al., 1994), it was reported that α-conotoxin MI binds to the curare low-affinity (αδ) site of mouse AChR with much higher affinity than to the curare high-affinity (αγ) site.

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