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α-Conotoxin Imi Exhibits Subtype-Specific Nicotinic Acetylcholine Receptor Blockade: Preferential Inhibition of Homomeric α7 and α9 Receptors

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SUMMARY

Through a study of cloned nicotinic receptors expressed in *Xenopus* oocytes, we provide evidence that α-conotoxin Imi, a peptide marine snail toxin that induces seizures in rodents, selectively blocks subtypes of nicotinic acetylcholine receptors. α-Conotoxin Imi blocks homomeric α7 nicotinic receptors with the highest apparent affinity and homomeric α9 receptors with 8-fold lower affinity. This toxin has no effect on receptors composed of α2β2, α3β2, α4β2, α2β4, α3β4, or α4β4 subunit combinations. In contrast to α-bungarotoxin, which has high affinity for α7, α9, and α1β1γδ receptors, α-conotoxin Imi has low affinity for the muscle nAChR. Related *Conus* peptides, α-conotoxins MI and GI, exhibit a distinct specificity, strictly targeting the muscle subtype receptor but not α7 or α9 receptors. α-Conotoxins thus represent selective tools for the study of neuronal nicotinic acetylcholine receptors.

Multiple subtypes of nicotinic ACh receptors are present in the central and peripheral nervous systems (1). Nicotinic receptors have been demonstrated to play a role in normal cognition (2, 3) and have been implicated in neuropsychiatric disorders such as Alzheimer's disease (4, 5), schizophrenia (6–8), Tourette's syndrome (9, 10), and Parkinson's disease (11, 12). Progress in the functional study of these receptors, however, has been hampered by a lack of subtype-specific ligands.

A family of structurally related peptides known as α-conotoxins has been isolated from the venom of fish-hunting cone snails (13–17). The α-conotoxins are disulfide-rich peptides that are considerably smaller than the nAChR-targeted snake toxins α-bungarotoxin and α-cobratoxin (12–20 amino acids versus approximately 60–80 amino acids). The small size of the α-conotoxins has allowed chemical synthesis and structural modification (18). α-Conotoxins isolated from several fish-hunting cone snails target the muscle subtype of nicotinic receptor (19). Recent research has indicated that one of these toxins, α-conotoxin MI, is able to distinguish between two ACh-binding sites present on the mammalian muscle receptor by more than 4 orders of magnitude (20). ACh-binding site selectivity for α-conotoxins MI and GI has also been demonstrated in *Torpedo* (21). Thus, previously characterized α-conotoxins from fish-hunting snails appear to be highly selective probes for the muscle nAChR.

There are approximately 500 species of cone snails (22). In an attempt to isolate ligands with selectivity for neuronal nAChRs, we have studied venoms from a number of *Conus* species. We recently described the isolation and biochemical characterization of α-conotoxin IMI isolated from the vermivorous *C. imperialis* (23). This 12-amino acid peptide shares the conserved cysteine framework of previously isolated α-conotoxins. The intercysteine residues, however, are quite distinct, suggesting that the specificity of α-conotoxin Imi may be unique. Hypervariability in the noncysteine residues of ω-conotoxins, for example, confers specificity for different calcium channel subtypes (24). α-CTx-IMI is a potent inhibitor of the muscle nAChR in frog, whereas intraperitoneal injection of α-CTx-IMI into mice has no effect. In contrast, ICV injections of α-CTx-IMI into mice or rats produce complex seizures, suggesting that this toxin may be

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ABBREVIATIONS: ACh, acetylcholine; α-BTX, α-bungarotoxin; α-CTx-IMI, α-conotoxin IMI; α-CTx-GI, α-conotoxin GI; α-CTx-MI, α-conotoxin MI; nAChR, nicotinic acetylcholine receptor; ICV, intracerebroventricular; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

selectively targeting neuronal nAChRs in mammalian systems (23).

In the present study, we used cloned neuronal and muscle nAChR subunits heterologously expressed in *Xenopus* oocytes to test the effect of α-CTx-Imi. We present evidence that this toxin preferentially blocks certain nonmuscle nAChR subtypes. In addition, we show that α-conotoxins Mi and Gi selectively block only α1-containing muscle nAChRs.

Experimental Procedures

Materials. α-Conotoxin Imi was synthesized as previously described (23). Synthetic α-conotoxin Gi was a gift from W. R. Gray (Department of Biology, University of Utah, Salt Lake City, UT). Synthetic α-conotoxin Mi was a gift from R. A. Myers. ACh was obtained from Sigma Chemical Co. (St. Louis, MO), and α-BTX was obtained from Research Biochemicals, Inc. (Natick, MA).

Bioassay. ICV injections into adult Sprague-Dawley rats were performed as previously described (23). Briefly, toxins were dissolved in 150 mM NaCl, and 3 μl of toxin or control solution was injected via a stereotaxically placed cannula into the lateral ventricle. Cannula position was confirmed histologically. If no behavioral effects of the injected toxin were observed, a subsequent injection was made with a higher dose of toxin.

Oocyte expression. cRNA was transcribed *in vitro* as previously described (25) with SP6, T3, and T7 polymerases (Stratagene, La Jolla, CA). Plasmid constructs of mouse and rat nAChR subunits were as previously described: α1, β1, γ, δ (26); α2 (27); α3 (28); α4 (29); α7 [1]; α9 (30); β2 (31); and β4 (32).

Oocytes were injected after harvesting, and recordings were made 1–7 days after injection. Fifty nanoliters containing 5 ng cRNA of each subunit were injected into oocytes, except for muscle subunits, when 0.5–0.05 ng of each subunit was used. The physiological recording was carried out in the two-electrode voltage-clamp configuration with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Electrodes were fabricated from Dagan FLG 15 glass capillary tubes. Both the voltage and current electrode contained 3 M KCl. The resistance of voltage electrodes was 4–10 MΩ and of current electrodes was 0.5–1.5 MΩ. Oocytes were perfused within a 0.5-ml Lucite chamber with frog Ringer's solution (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, and 1.8 mM $CaCl_2$, pH 7.4) without atropine; atropine has been demonstrated to act as an antagonist at α7 and α9 homomeric receptors (30, 33). Noninjected oocytes did not respond to ACh test applications. ACh (control) or ACh plus toxin was applied in 1.8-ml aliquots at a flow rate of 5.5 ml/min, generating an agonist "pulse" of approximately 20 sec. The concentrations of ACh that were used were 300 μM for oocytes expressing α2β2, α3β2, α4β2, α2β4, α3β4, or α4β4; 500 μM for α7; 100 μM for α9; and 500 nM for the muscle subtype. The ACh applied was approximately the lowest concentration required to elicit maximal current responses at each receptor subtype. For the muscle receptor, however, a submaximal concentration was chosen to allow the typically large current responses to be voltage clamped. Toxins were perfused into the chamber, and flow was stopped over the oocyte for a 5- or 20-min toxin preincubation period before ACh-plus-toxin application. For the α-conotoxins, a preincubation period of 5 min was sufficient for maximal blockade; for α-BTX, a 20-min period was required for maximal blockade. Control preincubation periods with frog Ringer's did not attenuate ACh-induced currents. Current responses were recorded with a Gould (Cleveland, OH) chart recorder. Peak current responses were also recorded directly from the amplifier digital display. Recordings were made at a holding potential of −60 mV. Current responses were in the range of 0–2.0 μA. Data represent the mean ± standard error of three to five oocytes for each experimental condition. Data were normalized to the initial responses of each oocyte to a control application of ACh. Curve fits were performed with SigmaPlot software using the following equation:

$$\% \text{ Response} = [100/(1 + \text{concentration}/IC_{50})^{n_H}]$$

where n_H is the Hill coefficient.

[1] J. Boulter, unpublished observations.

Results

Bioassay. We previously reported that ICV injections of α-CTx-Imi produce seizures similar to those seen with α-BTX (23). We have more quantitatively compared the relative potencies of the two toxins *in vivo*. ICV injections of varying doses of the two toxins were administered to Sprague-Dawley rats, and behavioral effects were monitored for 90 min. Symptoms elicited by α-CTx-Imi and α-BTX were similar and included ataxic gait, outstretched hindlimbs, eye blinking, head weaving, and clonic limb movements. These seizures, which were produced by both α-CTx-Imi and α-BTX, were complex, repetitive, and, at higher doses, lethal. Results are summarized in Table 1.

Physiology. We have previously shown using intracellular recordings from *Rana pipiens* that α-CTx-Imi blocks the postsynaptic nAChR in frog neuromuscular junction (23). In the present study, we directly examined the effects of this toxin on mammalian nAChRs. Nicotinic receptors composed of various functional receptor subunit combinations were expressed in *Xenopus* oocytes so we could assess the subtype specificity of this toxin. A summary of the data is presented in Table 2. α-CTx-Imi was found to block α7 homomeric receptors (Fig. 1), and it did so with 230-fold higher apparent affinity than blockade of α1β1γδ muscle-type receptors (Fig. 2). α-CTx-Imi had no detectable activity on α2β2, α3β2, α4β2, α2β4, α3β4, or α4β4 receptor subunit combinations at concentrations as high as 5 μm. As shown in Fig. 3, homomeric α7 nAChRs were antagonized by both α-CTx-Imi (IC_{50} = 0.22 μM, n_H = 0.89) and α-BTX (IC_{50} = 0.52 nM, n_H = 1.9). The effect of α-CTx-Imi on α7 homomeric receptors was reversible within 5 min (Fig. 1), whereas the α-BTX antagonism remained nearly complete after a 10-min wash. The difference in Hill coefficients for the two toxins at α7 nAChR raises an interesting but speculative possibility that two α-BTX molecules are required to block the receptor, whereas one α-CTx-Imi molecule is sufficient. However, because α-BTX essentially acts irreversibly, it is also possible that a slower apparent rate of association at low α-BTX concentrations has

TABLE 1

Behavioral activity of α-CTx-Imi and α-BTX

Toxins were injected ICV into the lateral ventricle of Sprague-Dawley rats, and the animals were observed for 90 min for seizures. α-BTX was approximately 4-fold more potent than α-CTx-Imi in producing similar behavioral effects. The number of rats affected indicates the number of rats exhibiting seizures divided by the total number of rats injected at that toxin dose.

Toxin	Amount	No. of rats affected
	nmol	
α-BTX	4	2/3
α-BTX	2	0/3
α-BTX	1	0/3
α-CTx-Imi	20	4/5
α-CTx-Imi	16	3/3
α-CTx-Imi	10	0/6
α-CTx-Imi	8	0/5

TABLE 2

Summary of α-conotoxin and α-BTX antagonism of cloned mammalian nicotinic receptors heterologously expressed in *Xenopus* oocytes

Toxins were assayed on oocytes expressing the indicated nAChR subunits for ability to block ACh-induced current. Numbers represent IC_{50} values in nM. NE indicates no effect at toxin concentrations to 5 μM (to 10 μM for α7 and α9); NT, not tested.

	α2β2	α3β2	α4β2	α2β4	α3β4	α4β4	α7	α1β1γδ	α9
α-CTx-GI	NE	NE	NE	NE	NE	NE	NE	20	NE
α-CTx-MI	NE	NE	NE	NE	NE	NE	NE	12	NE
α-CTx-ImI	NE	NE	NE	NE	NE	NE	220	51,000	1800
α-BTX	NT	NT	NT	NT	NT	NT	0.52	4.9	4.0

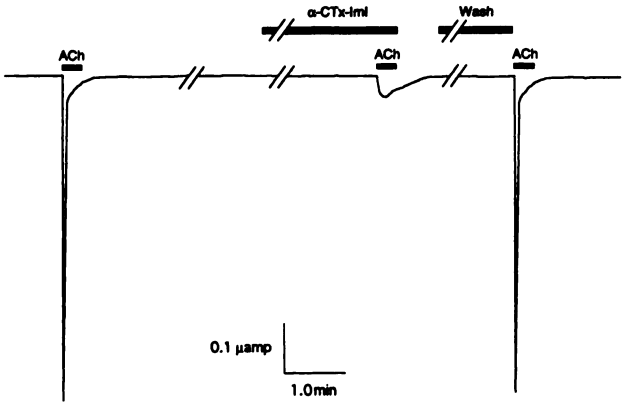


Fig. 1. α-CTx-ImI blocks α7 homo-oligomers expressed in *Xenopus* oocytes. Bath application of 2.5 μM α-CTx-ImI for 5 min to an oocyte expressing homomeric α7 nAChRs resulted in 94% inhibition of the current response elicited with 500 μM ACh. The ACh-induced current recovered almost completely after a 5-min washout of toxin.

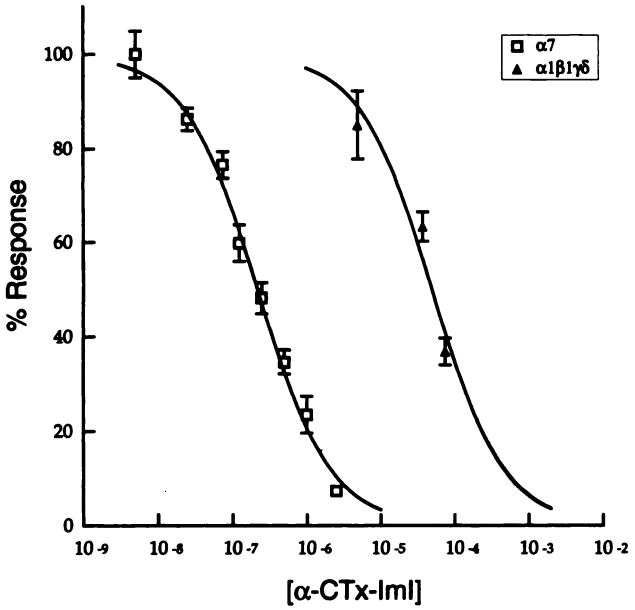


Fig. 2. α-CTx-ImI discriminates between α7 and α1β1γδ receptors. Oocytes expressing homomeric α7 and α1β1γδ muscle nAChRs were preincubated with α-CTx-ImI and then exposed to ACh plus toxin. The IC_{50} for α-CTx-ImI antagonism of homomeric α7receptors was 220 nm (n_H = 0.89) versus 51 μM (n_H = 1.3) for the α1β1γδ muscle receptor subtype. *Error bars*, mean ± standard error for three to five oocytes for each toxin concentration.

artificially produced the apparent difference. α-Conotoxins MI and GI had no detectable effect on this receptor subtype at concentrations as high as 10 μM.

As shown in Fig. 4, α1β1γδ muscle subtype receptors were

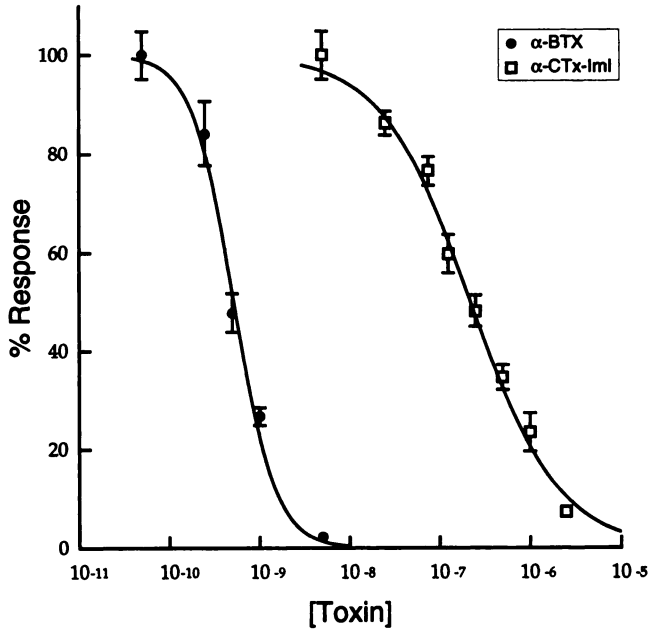


Fig. 3. Comparison of α-CTx-ImI and α-BTX on homomeric α7 nAChRs. Oocytes were preincubated with toxin for 5 (α-CTx-ImI) or 20 min (α-BTX) and then exposed to ACh plus toxin. For α-CTx-ImI, the IC_{50} is 220 nM (n_H = 0.89). For α-BTX, the IC_{50} is 0.52 nM (n_H = 1.9). *Error bars*, mean ± standard error for three to five oocytes for each toxin concentration.

potently blocked by α-BTX (IC_{50} = 4.9 nM, n_H = 1.1), α-CTx-MI (IC_{50} = 12 nM, n_H = 1.3), and α-CTx-GI (IC_{50} = 20 nM, n_H = 1.0). α-CTx-ImI blocked muscle receptors with an apparent affinity more than 3 orders of magnitude lower (IC_{50} = 51 μM, n_H = 0.91). Again, the α-BTX blockade remained nearly complete after a 10-min wash, and the α-CTx-ImI block was reversed fully with a 5-min wash. Receptors composed of α2β2, α3β2, α4β2, α2β4, α3β4, and α4β4 receptor subunit combinations were not detectably blocked by any of the conotoxins at concentrations as high as 5 μM (Table 2) and have been reported to be insensitive to α-BTX (25, 27, 34).

We also tested the effects of α-CTx-ImI and α-BTX on homomeric channels of the recently isolated α9 nAChR (30). Table 2 and Fig. 5 show that homomeric α9 receptors were blocked much less potently by α-CTx-ImI (IC_{50} = 1.8 μM, n_H = 0.98) than by α-BTX (IC_{50} = 4.0 nM, n_H = 1.1). The blockade induced by either toxin was reversed after a 5-min wash. Therefore, both toxins exhibit approximately 1 order of magnitude higher apparent affinity for α7 homomeric receptors than for α9 homomeric receptors. α-CTx-GI and α-CTx-MI had no detectable effect on homomeric α9 receptors at concentrations as high as 10 μM.

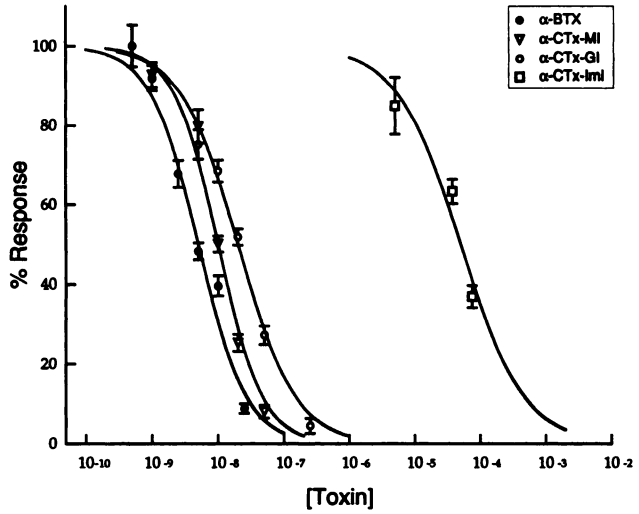


Fig. 4. α-CTx-ImI has little activity on muscle nAChRs. α-CTx-ImI and α-BTX were tested on oocytes expressing $\alpha1\beta1\gamma\delta$ muscle subtype nAChRs. In contrast to α-BTX (IC_{50} = 4.9 nM, n_H,= 1.1) and two previously isolated α-conotoxins, MI and GI (IC_{50} = 12 nM, n_H = 1.3; and IC_{50} = 20 nM, n_H = 1.0, respectively), α-CTx-ImI has weak activity at the muscle receptor subtype (IC_{50} = 51 μM, n_H = 0.91). *Error bars*, mean ± standard error for three to five oocytes for each toxin concentration.

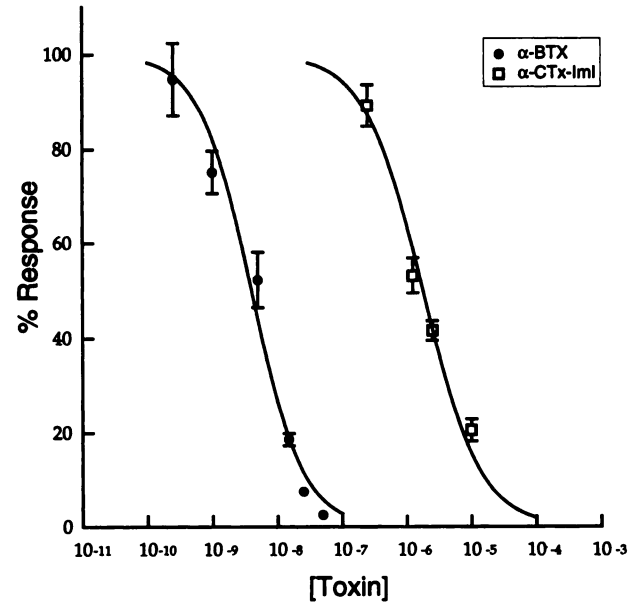


Fig. 5. Activity of α-CTx-ImI and α-BTX on homomeric α9 nAChRs. Both α-CTx-ImI and α-BTX are able to block homomeric α9 nAChRs (IC_{50} = 1.8 μM, n_H = 0.98; and IC_{50} = 4.0 nM, n_H = 1.1, respectively). The potency of both toxins is approximately 8-fold less on homomeric α9 receptors than their respective potencies on α7 nAChRs. *Error bars*, mean ± standard error for three to five oocytes for each toxin concentration.

Discussion

α-Conotoxins, although structurally related by their cysteine frameworks, are hypervariable in their intercysteine residues (Table 3). α-CTx-ImI differs from α-conotoxins GI and MI in 7 of 12 residues. The present study demonstrates that these differences in primary structure have significant functional consequences in conferring nicotinic receptor subtype specificity to each toxin.

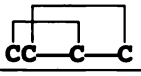
Examination of the behavioral effects of centrally versus peripherally injected α-conotoxins aids in predicting their subtype specificity. Peripheral injection of α-BTX in rodents produces paralysis, and ICV injection produces complex seizures. ICV injection of α-CTx-ImI produces seizures that are strikingly similar to those produced by injections of α-BTX. This observation led us to speculate that α-CTx-ImI might be acting with analogous specificity to α-BTX in the central nervous system. These seizures, which were originally described in the initial purification and characterization of α-CTx-ImI (23), have not been previously described in relation to an α-BTX-induced behavior. Because α-BTX has been demonstrated to block α7-containing nAChRs with high affinity (33, 35), cloned nicotinic receptors were expressed in *Xenopus* oocytes to evaluate subtype specificity of α-conotoxins. In this heterologous expression system, α-CTx-ImI blocks homomeric α7 receptors with higher apparent affinity (IC_{50} = 0.22 μM) than $\alpha1\beta1\gamma\delta$ muscle-type receptors. Based on IC_{50} ratios, α-CTx-ImI distinguishes between α7 receptors and muscle-type receptors by approximately 230-fold (Table 2). Thus, α-CTx-ImI exhibits greater selectivity for α7 receptors than that exhibited by α-BTX (IC_{50} ratio of only 10). This greater selectivity is consistent with the observation that α-CTx-ImI does not produce paralysis when injected peripherally in rodents. Because both α-CTx-ImI and α-BTX elicit very similar behaviors after ICV injection and because both preferentially target α7 receptors, it is probable that blockade of this receptor subtype is responsible for the generation of the toxin-induced seizures. Although both toxins also block homomeric α9 receptors, the limited anatomic expression of this receptor in the CNS (30) makes it a less likely candidate for direct involvement in the induced seizures. Based on the present study, we cannot exclude the possibility that α-CTx-ImI acts at other receptors to produce the observed seizure activity.

If blockade of α7 receptors by α-CTx-ImI is responsible for the seizures, then the *in vivo* behavioral data are not consistent with the oocyte expression data for the relative amounts of α-BTX and α-CTx-ImI needed to elicit *in vivo* symptoms versus α7 receptor blockade, respectively. As shown in Table 1, α-BTX is approximately 4-fold more potent in the behavioral assay than α-CTx-ImI. However, α-BTX is 420-fold more potent than α-CTx-ImI in the oocyte assay (Table 2). Because α-BTX is a larger protein (molecular mass, ~8000 Da) than α-CTx-ImI (molecular mass, 1352 Da), the discrepancy in the assays could be due to pharmacokinetic differences between the toxins. Also, α-BTX in the oocyte experiments essentially acts irreversibly. Thus, the relative potencies of the toxins in the oocytes and bioassays may not be strictly comparable. A more intriguing possibility is that the pharmacology of the *in vivo* α7 receptors differs from that of the α7 homo-oligomers expressed in oocytes with respect to affinity of α-CTx-ImI. Several lines of evidence suggest that at least some native α7-containing receptors are not homomeric (36–39). It is therefore possible that the native receptor subtype whose blockade by α-CTx-ImI produces seizures is an α7-containing heteromer. If this hypothesis is correct, then this α7-containing heteromer would be more equally blocked by α-CTx-ImI and α-BTX than α7 homomeric receptors expressed in *Xenopus* oocytes. It will be of significant interest to physiologically measure the affinity of α-CTx-ImI on *in vivo* α7-containing receptors.

TABLE 3

α-Conotoxin primary structure and disulfide bonding pattern

Peptide sequences are represented by standard one-letter abbreviations. * Indicates the α-carboxyl group is known to be amidated. Common disulfide bond configuration for all α-conotoxins is indicated.

α-CTx	Sequence	Source	Reference
ImI	GCCSDPRC AWR C*	*C. imperialis*	23
GI	ECC NPACGRHYSC*	*C. geographus*	13
MI	GRCC HPACGKNYSC*	*C. magus*	14
Disulfide bonding pattern:			

Although α-CTx-ImI and α-BTX elicit seizures when injected centrally, α-conotoxins MI and GI do not elicit behavioral effects when injected ICV. These observations are again consistent with the oocyte expression data. Both α-CTx-MI and α-CTx-GI block the muscle nicotinic receptor subtype with high apparent affinity (Table 2 and Fig. 4) and are without detectable effect on the other subunit combinations assayed. Thus, α-conotoxins MI and GI are more selective for the $\alpha 1\beta 1\gamma\delta$ muscle subtype receptor than α-BTX (which also blocks homomeric α7 and α9 receptors).

In summary, we have shown that α-CTx-ImI blocks homomeric α7 nicotinic receptors expressed in *Xenopus* oocytes. Although α-CTx-ImI blocks α7 receptors with lower apparent affinity than α-BTX, it is more selective in its blockade of α7 receptors versus muscle subtype receptors. In contrast, α-conotoxins MI and GI block only muscle subtype receptors. Beause α-conotoxins are small, rigid peptides, two-dimensional nuclear magnetic resonance imaging can be used to assess their structure. The solution structure of α-CTx-GI has previously been reported (40), and it will be of interest to compare the structure of α7-targeting α-CTx-ImI with those of the α1-targeting α-conotoxins. Such structure-function studies may assist in the design of peptides of higher affinity and even greater subtype specificity.

Acknowledgments

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