Neuronal Calcium Channel Antagonists. Discrimination between Calcium Channel Subtypes Using ω-Conotoxin from *Conus magus* Venom†

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ABSTRACT: The ω-conotoxins from the venom of fish-hunting cone snails are probably the most useful of presently available ligands for neuronal Ca channels from vertebrates. Two of these peptide toxins, ω-conotoxins MVIIA and MVIIB from the venom of *Conus magus*, were purified. The amino acid sequences show significant differences from ω-conotoxins from *Conus geographus*. Total synthesis of ω-conotoxin MVIIA was achieved, and biologically active radiolabeled toxin was produced by iodination. Although ω-conotoxins from *C. geographus* (GVIA) and *C. magus* (MVIIA) appear to compete for the same sites in mammalian brain, in amphibian brain the high-affinity binding of ω-conotoxin MVIIA has narrower specificity. In this system, it is demonstrated that a combination of two ω-conotoxins can be used for biochemically defining receptor subtypes and suggested that these correspond to subtypes of neuronal Ca^{2+} channels.

Changes in intracellular calcium ion concentration play a major role in the control of many biochemical processes. Often such changes result from transduction of a signal by proteins embedded in the cell membrane. In excitable cells electrical signals are transduced by voltage-activated Ca^{2+} channels, which allow a transient influx of the ion down a voltage and concentration gradient. It is becoming clear that a variety of such channels exist in different tissues (Hille, 1984; Hagiwara & Byerly, 1981; Tsien, 1983; Janis & Triggle, 1983; Miller, 1986).

Calcium channels from cardiac and skeletal muscle have been studied intensively with the dihydropyridine compounds (e.g., nitrendipine and nifedipine), and the channel protein from T-tubule membranes has been purified. However, the dihydropyridines do not block Ca^{2+} currents in neurons as effectively as in cardiac and skeletal muscle [for a review, see Miller (1987)]. Recently we described a new class of calcium channel antagonists, the ω-conotoxins, which were isolated from the venoms of the fish-hunting snails (Olivera et al., 1984). Five ω-conotoxins from *Conus geographus* were characterized; these are peptides of 26–29 amino acids containing two to three hydroxyproline residues (Olivera et al., 1985). We previously demonstrated (Cruz et al., 1987) that one of these, ω-conotoxin GVIA, preferentially binds neuronal Ca^{2+} channels; calcium channels of cardiac or skeletal muscle are not physiologically significant targets.

We report here the biochemical characterization of ω-conotoxins from the venom of a different fish-hunting cone, *Conus magus*. As in *C. geographus* venom, there are multiple toxin classes present in *C. magus* venom. In addition to two ω-conotoxins described below, there is an α-conotoxin (MI) homologous to the *C. geographus* α-conotoxin (GI), which blocks nicotinic acetylcholine receptors (McIntosh et al., 1982), and an excitatory peptide, κ-conotoxin (Olivera et al., 1985). The biochemical characterization of the latter is incomplete. No μ-conotoxins, which target specifically to muscle Na^+ channels (Cruz et al., 1985), have yet been found in *C. magus* venom. In addition to reporting the sequences of two *C. magus* ω-conotoxins, we detail the complete chemical synthesis of one of these using two different strategies.

Preliminary data suggested that the first ω-conotoxin purified from *C. magus*, ω-conotoxin MVIIA (ω-CmTx),[1] might have somewhat different target specificity from ω-conotoxin GVIA from *C. geographus* (ω-CgTx). We describe studies that define the specificity of ω-CmTx vis-à-vis the previously characterized ω-CgTx. These results demonstrate that combinations of different ω-conotoxin species will be powerful tools for the biochemical definition of neuronal Ca^{2+} channel subtypes.

Experimental Procedures

Isolation of Natural Toxins from Venom. Methods for the extraction and isolation of peptides from *Conus* venoms have previously been described (Olivera et al., 1984; Cruz et al., 1985). Purification of the ω-conotoxins was monitored by using intracerebral bioassays in young mice, the toxins eliciting a characteristic shaking behavior. Final isolation was from HPLC on a Vydac C_{18} column (5-μm diameter, 300-Å pore size, end capped; 0.46 × 25 cm). Conotoxins MVIIA and MVIIB, which were obtained from different venom preparations, eluted at approximately 32% and 26% solvent B, respectively [solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in acetonitrile/water (60/40 v/v)].

Amino acid analysis was carried out on a Beckman Model 121 analyzer, after hydrolysis with redistilled 6 N HCl for 20 h at 105 °C under vacuum.

Sequence analysis was carried out in a Beckman Model 890D sequencer (Edman & Begg, 1967), according to methods

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[1] Abbreviations: ω-CmTx, ω-conotoxin MVIIA; ω-CgTx, ω-conotoxin GVIA; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; Boc, *tert*-butoxycarbonyl; DMF, dimethylformamide; Z, benzyloxycarbonyl; Acm, acetamidomethyl; TEAP, triethylammonium phosphate; MSA, methanesulfonic acid.

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described previously (Olivera et al., 1984; Cruz et al., 1985). Where radioactive modifications were present, small samples were taken from each cycle for counting. (1) Conotoxin MVIIA was reduced and carboxamidomethylated with [^{14}C]iodoacetamide. Two peaks were obtained upon HPLC purification of the products. Both were subjected to 30 cycles of degradation. (2) Conotoxin MVIIB and synthetic MVIIA were reduced and carboxymethylated as described for the μ-conotoxins (Cruz et al., 1985), except that dithiothreitol was used as the reducing agent. The whole reaction mixture was applied to the sequencer cup, and degradation was carried out for 30 cycles. The cup was covered by a box to exclude light.

Synthesis of ω-Conotoxin MVIIA. All *tert*-butoxycarbonyl (Boc) amino acids were of the L configuration except for Gly and were purchased from Bachem, Torrance, CA. Boc amino acids included Cys (Acm), Cys (*S*-*p*-OMeBzl), Lys (2-Cl-Z), Ser (OBzl), Thr (OBzl), Arg (tosyl), Tyr (2,6-Cl_2Bzl), and Asp (OBzl). Methionine was incorporated with an unprotected side chain.

Merrifield's solid-phase approach was used manually to prepare the protected peptide resin, applying published protocols (Merrifield, 1963; Marki et al., 1981).

In summary, *p*-methylbenzhydrylamine resin (4 g, 0.55 mequiv/g) was subjected to a deblocking cycle including treatment with TFA (60% in CH_2Cl_2; 2% ethanedithiol), Et_3N (10% in CH_2Cl_2), and CH_2Cl_2 and MeOH washes. Couplings (ca. 2 h) were mediated by dicyclohexylcarbodiimide in either CH_2Cl_2, DMF, or mixtures thereof, depending on the solubility of the respective protected amino acids. Extent of coupling was monitored by the ninhydrin test of Kaiser et al. (1970). Recouplings were performed for Ser^{22,9}, Thr^{17}, and Arg^{10}. Couplings were followed by acetylation (10% Ac_2O in CH_2Cl_2) at cycles 20, 17, 16, 10, and 9. Peptide resin (14.1 g) was obtained and cleaved batchwise in HF/anisole/ethyl methyl sulfide (20:1.5:0.5) and 15 mL/g resin at 0 °C for 1 h. The crude, partially deprotected peptide was extracted with dilute acetic acid (1%) and lyophilized (yield 2.7 g). It was then purified (2 g) by preparative HPLC on a 5 × 30 cm cartridge containing Vydac C_{18} silica (15–20 μm) as reported by Rivier et al. (1984). The purity of the isolated $(Acm)_6$ peptide (150 mg) was assessed to be greater than 95% by analytical HPLC with Vydac C_{18} columns and TEAP/CH_3CN (Rivier, 1980) or 0.1% TFA/CH_3CN (Bennet et al., 1977) solvent systems. $[\alpha]$ = −44.1 (*c* 1, 0.1% TFA). Amino acid composition analysis gave the expected ratios after acid hydrolysis (4 N MSA, 24 h under vacuum). (Acm)Cys was not detected.

Removal of Acm Protecting Groups and Oxidation. $(Acm)_6$-ω-conotoxin (30 mg) was dissolved in distilled H_2O (1 mL), the pH was adjusted to 4.0 with AcOH, and mercuric acetate (30 mg) was added; the pH was readjusted to 4.0. The solution was allowed to stand for 10 h at room temperature before β-mercaptoethanol (100 μL) was added. The precipitate was eliminated by centrifugation, and the supernatant was chromatographed on Sephadex G-10 (1.5 × 15 cm column) with 50% AcOH at a flow rate approximately 1 mL/min.

Excluded peptide was brought to 30 mL with water, and the pH was adjusted to 8.2 with NH_4OH. The solution was allowed to stand, with slow stirring, in an open beaker for 24 h, at which time the Ellman test (Ellman, 1959) was negative for sulfhydryl content. The solution was loaded batchwise ($^1/_3$ total) (see Figure 1A and legend) onto a semipreparative HPLC column. The main fraction from three consecutive runs was collected, diluted with water (1/1), and desalted by using the same column and the volatile 0.1% TFA/CH_3CN buffer (not shown). The main peak fraction was collected and lyophilized to yield pure toxin (3.2 mg).

Alternative Synthesis Strategy. In an attempt to streamline the synthesis of MVIIA, a second strategy was also found to be successful. The amino acids were identical with those in the first attempt with the exception of cysteine (*S*-*p*-OMeBzl) and tyrosine (2-Br-Z). Stepwise buildup of the peptide on *p*-methylbenzhydrylamine (4.1 g, 0.43 mequiv/g) was done automatically on a Beckman 990B synthesizer with 1.25 equiv of protected amino acid/g of resin. Trifluoroacetic acid (60% in CH_2Cl_2) was used for deblocking and diisopropylethylamine (10% in CH_2Cl_2) for neutralization; resin washing was accomplished by application of 2-propanol (1% ethanedithiol) after TFA treatment and by application of methanol or dichloromethane at other steps. Couplings (45–120 min) were mediated by diisopropylcarbodiimide in either dichloromethane or dimethylformamide, depending upon the solubility of the respective amino acid derivatives. Recouplings were automatically performed at Cys^{20} and Arg^{10}. A final peptide resin (9.6 g) was obtained. The peptide was released from the peptide resin (6.0 g) as the COOH-terminal amide by treatment with distilled anhydrous HF (100 mL in the presence of *p*-cresol, 12 g) and 6 mL of ethyl methyl sulfide at 0 °C for 30 min. After removal of the HF under reduced pressure, the resin was washed portionwise with 300 mL of diethyl ether. The peptide was quickly extracted from the resin and further diluted to 3.5 L with chilled distilled water. The solution was adjusted to pH 7 with NH_4OH and allowed to slowly stir and air oxidize at 4 °C for 2 days. The peptide solution, which initially gave a positive Ellman test with 5,5′-dithiobis(2-nitrobenzoic acid), at this point showed no evidence of free sulfhydryl groups. After acidification of the solution to pH 5 with acetic acid, the peptide was concentrated by passage through a column containing approximately 75 mL of Bio-Rad Bio-Rex-70 cation-exchange resin (H^+ form). After the solution was extensively washed with distilled H_2O (300 mL) and 5% aqueous acetic acid (300 mL), the peptide was removed from the resin by addition of 50% aqueous acetic acid (300 mL). The peptide solution was concentrated to a pale oil on a rotary evaporator and immediately taken up in distilled H_2O, shell frozen, and lyophilized to a white powder (yield 1170 mg). The crude peptide was purified by preparative HPLC techniques previously described; the gradient of acetonitrile applied to the preparative cartridge in TEAP, pH 2.25, was 0 to 15% in 1 h, with a flow rate of 100 mL/min. Analysis of the generated fractions was achieved by using gradient conditions (7.5% to 28.5% acetonitrile in 0.1% trifluoroacetic acid) on a 5-μm Vydac C_{18} column. Desalting was carried out with an acetonitrile gradient from 0 to 30% in 0.1% trifluoroacetic acid in 40 min. Highly purified fractions were pooled and lyophilized, yielding conotoxin MVIIA as the trifluoroacetate (18.0 mg).

Iodination of Toxin. ^{125}I-Labeled toxin, as well as unlabeled mono- and diiodinated ω-conotoxin MVIIA, was prepared and purified by HPLC essentially as described for ω-conotoxin GVIA (Cruz & Olivera, 1986).

Preparation of Synaptosomal Membranes. The preparation of synaptosomal membranes was carried out as previously described (Cruz & Olivera, 1986; Rivier et al., 1987).

Biological Assays. The activity of ω-conotoxin was monitored by intracerebral injection of mice as described in Olivera et al. (1984).

Results

Amino Acid Analysis. Purified ω-conotoxin MVIIA, prepared as described under Experimental Procedures, gave the

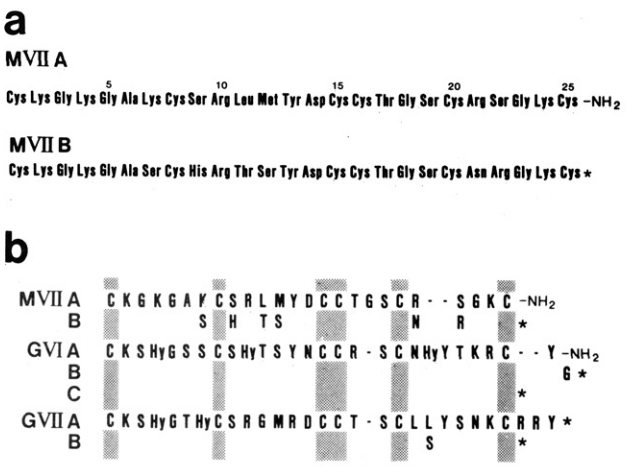

FIGURE 1: Sequences of ω-conotoxin. (a) Sequence of ω-conotoxin MVIIA (top) and ω-conotoxin MVIIB (bottom). The C-terminal end of ω-conotoxin MVIIA is amidated; in ω-conotoxin MVIIB this has not been directly established. By homology with all other ω-conotoxins, we infer that it is likely to be blocked. (b) Comparison of all presently known ω-conotoxin sequences from fish-hunting cones. Asterisks denote C-termini likely to be amidated but not directly proven.

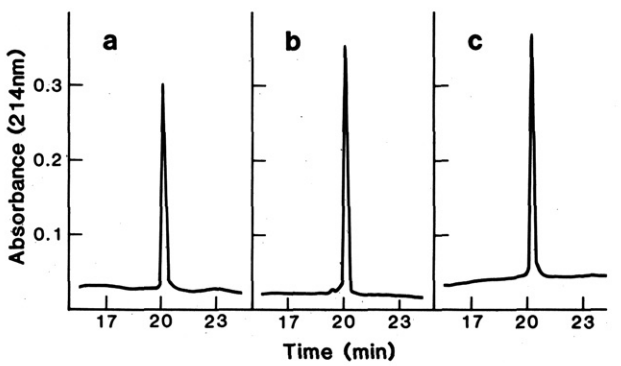

FIGURE 2: HPLC analysis of synthetic and natural ω-conotoxin MVIIA. (a) Synthetic ω-conotoxin MVIIA (1.2 μg), synthesized as described under Experimental Procedures, as well as (b) the natural toxin (1.2 μg) and (c) an approximate equimolar mixture of the two (0.6 μg each) were run on reversed-phase HPLC with a Vydac C_{18} column as described under Experimental Procedures. The solvent used was 0.1% TFA, containing acetonitrile. The gradient program was to run 9% acetonitrile for 2 min, a gradient from 9% to 18% acetonitrile for 15 min, and a continuous elution with 18% acetonitrile for an additional 10 min. In all cases, the toxin eluted approximately 3.2 min after application of the 18% acetonitrile.

composition $Lys_{4.38(4)}$, $Arg_{1.68(2)}$, $Asp_{1.06(1)}$, $Thr_{0.95(1)}$, $Ser_{2.42(3)}$, $Cys_{4.65(6)}$, $Gly_{3.99(4)}$, $Ala_{0.93(1)}$, $Met_{0.96(1)}$, $Leu_{1.06(1)}$, $Tyr_{0.96(1)}$, where numbers in parentheses indicate the number of residues found by sequence analysis. Values for Lys and Arg suggested possible microheterogeneity, but none was found during sequencing. Insufficient MVIIB was obtained to carry out this analysis.

Sequencer Analysis of ω-Conotoxin MVIIA. Upon reduction and carboxamidomethylation of homogeneous ω-conotoxin MVIIA, two peaks were obtained after HPLC purification. Both peaks of carboxamidomethylated toxin gave the same results, a likely explanation being that the earlier eluting material contained methionine sulfoxide. A single clear sequence (Figure 1) was obtained through cycle 25, after which all amino acids decreased. All assigned Cys residues were confirmed by detection of the [^{14}C]carboxamidomethyl label in the appropriate fractions (1, 8, 15, 16, 20, 25).

Carboxy Terminus of ω-Conotoxin MVIIA. A nominal molecular weight of 2637 was assigned on the basis of fast atom bombardment mass spectrometry, confirming that the sequence shown in Figure 1 represents the entire molecule and indicating that the carboxy terminus is amidated and all cysteines are present as disulfides. These conclusions are fully supported by the comparisons between natural and synthetic peptides reported below.

Sequencer Analysis of ω-Conotoxin MVIIB. A single run on carboxymethylated material gave the sequence shown in Figure 1. The peptide is clearly homologous with MVIIA (19 of 25 residues identical) and with the ω-conotoxins from *C. geographus* (Olivera et al., 1984). Earlier elution of MVIIB than of MVIIA in reversed-phase HPLC is consistent with replacement of the hydrophobic Leu and Met by Thr and Ser, respectively.

Characterization of Synthetic ω-Conotoxin MVIIA. The chemical synthesis of ω-conotoxin MVIIA was carried out as described under Experimental Procedures. The final products from two different synthetic strategies were compared to each other and with authentic natural toxin by analytical HPLC. Synthetic product eluted as a sharp peak (Figure 2a), and purity was judged to be greater than 98%. The synthetic toxin was identical with the natural toxin by all criteria used: HPLC elution times (Figure 2b,c), amino acid sequencing, and biological activity upon intracerebral injection into mice (results not shown). FAB mass spectrometric analysis gave the expected molecular ion.

A number of unsuccessful attempts at synthesis were made before the successful route was found. Deprotection of the Acm group using excess iodine in 95% acetic acid (10 °C, 10 h) yielded oxidized ω-conotoxin MVIIA (with methionine sulfoxide in position 12). Removal of the Acm group using silver nitrate rather than mercuric acetate did not yield the expected deprotected peptide. Although the conditions for synthesis have not been studied systematically, the successful procedure described above starting with 150 mg of the $(Acm)_6$ peptide by Merrifield synthesis resulted in an 11% yield of authentic ω-conotoxin. A similar overall yield was obtained when the alternative Cys (*S-p*-OMeBzl) protection strategy was used.

Iodination of the Synthetic Peptide. The synthetic ω-conotoxin MVIIA was iodinated with Iodogen as described above. Reaction products were analyzed and purified by HPLC and tested for biological activity according to methods described previously (Cruz & Olivera, 1986). Two major modified peaks eluted 4.2 and 7.0 min later than the unmodified toxin on reversed-phase HPLC. Pronase–trypsin digestion of the peak eluting closer to unmodified toxin yielded monoiodotyrosine, while the second modified toxin yielded diiodotyrosine. Since ω-conotoxin MVIIA has only one tyrosine, we suggest that the two new major toxin peaks are the [$^{125}I_1$-Tyr^{13}]-ω-conotoxin MVIIA and [$^{125}I_2$-Tyr^{13}]-ω-conotoxin MVIIA, respectively.

The synthetic, monoiodinated ω-CmTx peptide has 30–65% of the biological activity of the unmodified peptide, depending on the precise assay condition used. In contrast, the diiodinated derivative had lost all detectable activity in the standard "shaker" assay. The minimum doses that elicited symptoms in 9–11-g mice in this assay were 12, 36, and >130 pmol of the unmodifed and monoiodo- and diiodotyrosine derivatives of the toxin, respectively.

Binding Studies Using ^{125}I-Labeled ω-CmTx. We have determined whether the ^{125}I-labeled peptides bind specifically to receptor sites on neuronal tissue. Specific binding of the [^{125}I]monoiodotyrosine derivative of ω-CmTx was demonstrated with chick brain membranes (Table I); the filter binding assay described previously was used (Cruz & Olivera,

Table I: Reversibility of Binding of [$^{125}I_1$-Tyr^{13}]-ω-conotoxin MVIIA to Chick Brain Synaptosomes[a]

labeled toxin	specific binding (cpm) ω-CgTx	ω-CmTx
20-min incubation	16590	13150
20-min incubation, 25-min chase		
no unlabeled toxin added	13590	12470
excess unlabeled toxin added	14980	530
^{125}I displaced by unlabeled toxin (%)	<10	>95

[a] Reaction mixtures containing 1.0 mg/mL chick brain synaptosomal membrane protein and either ^{125}I-ω-CgTx or ^{125}I-ω-CgTx (4 nM), specific activity 1.6×10^{-8} cpm/nmol, were incubated for 20 min as described under Experimental Procedures. Aliquots (0.5 mL) were mixed either with an equal volume of 1 μM unlabeled ω-CgTx or ω-CmTx or with an equal volume of buffer with no unlabeled toxin and incubated for an additional 25 min. Control incubations were preincubated with 1 μM unlabeled toxin before labeled toxin was added. Aliquots (0.1 mL) were filtered and counted.

1986). The binding is eliminated if the synaptosomes are preincubated with an excess of either unlabeled ω-CmTx or ω-CgTx. In contrast, the diiodinated derivative of ω-CmTx, which showed greatly reduced biological potency, did not show significant specific binding when the filter binding assay was used (results not shown). Since the filter binding assay requires high affinity between the ligand and the receptor, the diiodinated derivative presumably has such a reduced affinity for the receptor that the receptor–toxin complex cannot be detected by filter binding.

Although binding of the monoiodinated ω-CmTx can be detected by the filter binding assay, dissociation of the labeled toxin from the specific receptor site still proceeds at a measurable rate. Iodinated ω-CmTx can be completely displaced from receptor sites by unlabeled toxin within 25 min (Table I). Thus, the off-time of iodinated *C. magus* from receptor sites in chick brain synaptosomes is significantly faster than for the previously characterized iodinated toxin from *C. geographus*, [$^{125}I_1$-Tyr^{22}]-ω-conotoxin GVIA (Cruz et al., 1987).

Binding Specificity of ω-Conotoxins: Overlap of Receptor Sites in Mammalian Brain. When mice are injected intraperitoneally, neither ω-CgTx nor ω-CmTx has any obvious effects. When these toxins are directly introduced into the central nervous system, both cause characteristic "shaker" symptoms (Olivera et al., 1985).

A comparison between ω-CgTx and ω-CmTx binding was carried out on mammalian brain synaptosomal membranes. There was direct equivalence of the toxins (ω-CgTx and ω-CmTx). Unlabeled ω-CmTx and ω-CgTx appear to displace radiolabeled ligand ($^{125}I_1$-ω-CgTx) from bovine brain sites to the same extent (Figure 3). If labeled ω-CmTx is used as the ligand, both toxins also displace the radiolabel to the same extent (results not shown). These binding-competition experiments suggest that ω-CgTx and ω-CmTx bind to, and compete for, equivalent high-affinity sites in mammalian brain.

Binding Specificity of ω-Conotoxins: Evidence for Two Distinct Amphibian Sites. In amphibians there is a striking contrast after intraperitoneal injection of ω-CgTx and ω-CmTx: the former causes paralysis and death while the latter has no obvious gross effects (Olivera et al., 1985). Given the difference in biological activity, it was of interest to compare binding specificity of the two toxins in this system and to compare the results to those obtained for mammalian sites.

The data are shown in Figure 4. Binding-competition studies reveal that there is one subset of sites that can be displaced by unlabeled ω-CgTx, but not by unlabeled ω-CmTx, and a second subset that can be displaced by both toxins. The

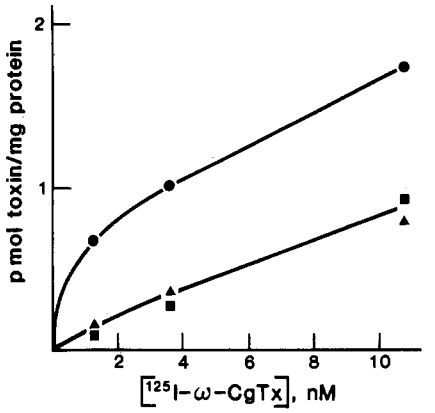

FIGURE 3: Competition of ω-CmTx with ^{125}I-labeled ω-CgTx for calf brain receptor sites. Filter binding assays were carried out as described under Experimental Procedures and in the legend to Figure 4, except that ^{125}I-ω-CgTx was the labeled toxin probe. Circles show counts retained on a filter without preincubation with unlabeled toxins; triangles show data points where the cow brain synaptosomes were first preincubated with 0.5 μM ω-CmTx; squares show points preincubated with 0.5 μM unlabeled ω-CgTx. The specific activity of the ^{125}I-ω-CgTx used in this experiment was 5.5×10^7 cpm/nmol. The data points are from a single but typical experiment.

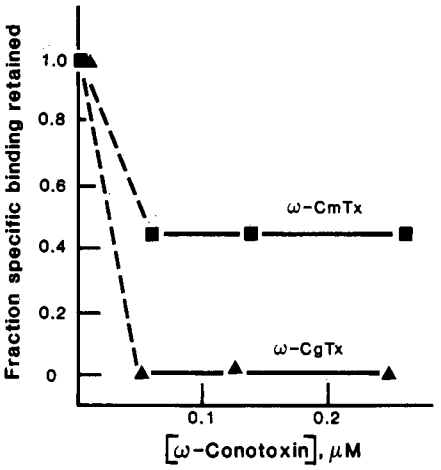

FIGURE 4: Competition between ω-conotoxins for receptor sites in frog brain. ^{125}I-Labeled ω-CgTx was used for binding. Synaptosomal preparations were from frog brain (Experimental Procedures), and the ability of an excess of unlabeled ω-CmTx and ω-CgTx to compete for specific binding sites was measured. The specific activity of the ^{125}I-ω-CgTx used in this experiment was 8.9×10^7 cpm/nmol; the concentration was 1.13 nM. The amount of specific binding in the absence of any unlabeled toxin is normalized to 1.0; the level of binding when the synaptosomes are preincubated with 0.125 μM ω-CgTx is designated the nonspecific binding, and this is subtracted from all values. Squares show experiments where ω-CmTx is used to compete with the ^{125}I-labeled toxin; triangles show the points where ω-CgTx was used.

particular experimental points in Figure 4 are typical of several experiments that have been carried out; although there is some variation in the proportion of the two binding subtypes from one synaptosome preparation to the next, the minority subtype is at least 40% of the total.

These results define two types of ω-conotoxin receptor sites in amphibian brain. The binding results are consistent with the more pronounced toxic activity of ω-CgTx, compared to ω-CmTx, and suggest that a target inhibited by ω-CgTx but not by ω-CmTx is essential for viability; blocking this target causes paralysis and death.

Discussion

The ω-conotoxins are a novel class of peptides that block voltage-sensitive Ca^{2+} channels in neurons. The first ω-co-

notoxins were isolated from the venom of *C. geographus* (Olivera et al., 1984, 1985). In this paper, we describe a biochemical characterization of two ω-conotoxins from *C. magus* venom. A summary of all the relevant ω-conotoxin sequences is given in Figure 1. The complete chemical synthesis of the major ω-conotoxin from *C. magus* venom, MVIIA, is detailed above.

Although *C. magus* ω-conotoxins are homologous to the corresponding peptides from *C. geographus*, there are some consistent species differences. First, all *C. geographus* ω-conotoxins have two or three residues of hydroxyproline, which is absent from the two *C. magus* ω-conotoxins. It is notable that positively charged residues occupy the corresponding positions in *C. magus*. Second, there are size differences in two of the segments between homologous Cys residues. Despite these changes, however, the peptides almost certainly share a common mode of activity in blocking neuronal voltage-activated Ca^{2+} channels.

In several vertebrate classes the two types of ω-conotoxins appear to overlap completely in their biological and biochemical properties. In mammals neither toxin shows an overt action on the neuromuscular system, but both produce "shaking" syndrome upon intracerebral injection; fish are paralyzed and killed by either type of toxin; voltage-activated uptake of $^{45}Ca^{2+}$ by synaptosomes from chick brain is blocked by both types (Rivier et al., 1987; A. Azimi-Zonooz, unpublished results). In amphibians, however, clear-cut differences between the two types of toxin led us to postulate the existence of distinct classes of neuronal Ca^{2+} channels: ω-CgTx blocked transmission at the frog neuromuscular junction, while ω-CmTx did not. Likewise, intraperitoneally injected nanomolar quantities of the *C. geographus* toxin into frogs cause paralysis and death, while comparable amounts of the *C. magus* toxin show no gross effects.

Availability of both synthetic toxins, and of homogeneous radioligands, has now enabled us to investigate directly their binding to synaptosomes from avian (chick), mammalian (bovine), and amphibian (frog) brain. Both radioligands bound to chick synaptosomes, but the relative lability of the binding of the *C. magus* derivative made this system less desirable for competitive binding studies (Table I). In bovine brain the two types of toxin were found to overlap in their high-affinity binding sites (Figure 3). In sharp contrast, two distinct types of high-affinity sites were found in frog brain synaptosomes: binding assays reveal one subset of sites bound by both toxins and a second subset that only ω-CgTx binds with high affinity. This indicates the presence of two biochemically distinguishable Ca channel subtypes in this neuronal tissue.

The above results with frog and mammalian systems indicate that a set of critical neuronal Ca^{2+} channels in presynaptic termini is resistant to the action of ω-CmTx. Presumably, a complete inhibition of neurotransmitter release should be lethal. Our biochemical and biological results can be understood in terms of two major types of Ca^{2+} channels that are closely related but have undergone phylogenetic divergence in relation to sites that bind the two types of ω-conotoxin. Since both ω-CmTx and ω-CgTx are lethal to fish by intraperitoneal injection, it is presumed that the channel critical for acetylcholine release at the neuromuscular junction is blocked by either toxin. Progressive phylogenetic change in this critical type of channel has rendered higher vertebrates less vulnerable to intraperitoneal injection of one class or both classes of toxin. In the central nervous system it may be that the "noncritical" Ca^{2+} channel is involved in the release of inhibitory neurotransmitters, so that blockade by ω-conotoxins leads to the shaking syndrome seen in mice.

It should be noted that different subtypes of neuronal Ca^{2+} channels have been postulated on the basis of electrophysiological data [Nowycky et al., 1985; for a review, see Miller (1987)]. Their relationship with the biochemically distinguished classes remains to be elucidated.

The availability of different ω-conotoxins that can differentially bind Ca channel subtypes opens up the possibility not only for labeling and differentiating neuronal voltage-sensitive Ca^{2+} channels but also for using these peptides for the efficient separation and purification of different subtypes.

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