Binding of chimeric analogs of ω-conotoxin MVIIA and MVIIC to the N- and P/Q-type calcium channels

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Abstract Despite their high sequence homology, the peptide neurotoxins ω-conotoxin MVIIA and MVIIC selectively block N- and P/Q-type calcium channels, respectively. To study the recognition mechanism of calcium channel subtypes, two chimeric analogs of ω-conotoxin MVIIA and MVIIC were synthesized by exchanging their N- and C-terminal halves. Binding assay for both N- and P/Q-type calcium channels showed that amino acid residues restricted to the N-terminal half are important for the recognition of N-type channels, whereas essential residues for P/Q-type channel recognition are widely spread over the whole ω-conotoxin molecule.

Key words: ω-Conotoxin MVIIA; ω-Conotoxin MVIIC; Calcium channel; Chimeric analog

1. Introduction

Voltage-gated calcium channels play crucial roles in regulating intracellular calcium concentrations in a wide variety of cells, and are classified into several subtypes according to their electrophysiological and pharmacological properties [1–3]. Among them, N- and P/Q-type channels are essential for the regulation of neurotransmitter release from a wide variety of neurons. Various specific ligands have been used to distinguish the subtypes of calcium channels pharmacologically. ω-Conotoxins form a large family of peptide neurotoxins isolated from the venom of marine *Conus* snails. N-type calcium channels are blocked by ω-conotoxin GVIA (ωGVIA) and MVIIA (ωMVIIA), and P/Q-type channels by ω-conotoxin MVIIC (ωMVIIC) [4].

Although ωGVIA is most widely used as a standard pharmacological tool to identify the N-type channel, the amino acid sequence of ωMVIIC is much more similar to that of ωMVIIA than to that of ωGVIA. Therefore, in order to study in detail the structural differences that determine toxin selectivity for N- and P/Q-type calcium channels, ωMVIIA is the most appropriate representative of the N-type calcium channel blockers.

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Abbreviations: ωGVIA, ω-conotoxin GVIA; ωMVIIA, ω-conotoxin MVIIA; ωMVIIC, ω-conotoxin MVIIC; [^{125}I]ωGVIA, [^{125}I]ω-conotoxin GVIA; [^{125}I]ωMVIIC, [^{125}I][Nle^{12}]ω-conotoxin MVIIC; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; Fmoc, 9-fluorenylmethoxycarbonyl; MALDI-TOF-MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry

In the present study, we synthesized two chimeric analogs of ωMVIIA and ωMVIIC, namely A-C and C-A, which have the N-terminal half of ωMVIIA and the C-terminal half of ωMVIIC, and vice versa, respectively, in order to search broadly for the residues essential for the recognition of calcium channel subtypes (Fig. 1). Binding assays for both N- and P/Q-type channels suggested that the amino acid residues in the N-terminal half are important for the recognition of N-type channels, whereas essential residues for the P/Q-type recognition are widely spread over the whole ωMVIIC molecule.

2. Materials and methods

2.1. Materials

Fmoc-amino acids and other reagents used on the synthesizer were obtained from Applied Biosystems Japan (Chiba, Japan). Fmoc-NH-SAL-resin was obtained from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). Other reagents for peptide synthesis were obtained from Peptide Institute (Osaka, Japan) or Kokusan Chemical Works Ltd. (Tokyo, Japan). Lysyl endopeptidase and thermolysin were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan).

2.2. Peptide synthesis

Solid phase peptide synthesis was conducted on an Applied Biosystems 431A peptide synthesizer. Amino acid analyses were performed on a Beckman System Gold amino acid analyzer after hydrolysis in 6 M hydrochloric acid at 110°C for 24 h and derivatization by 4-dimethylaminoazobenzene-4′-sulfonyl chloride. MALDI-TOF-MS was measured on a PerSeptive Biosystems Voyager Linear mass spectrometer by using α-cyano-4-hydroxy-cinnamic acid as a matrix. Analytical HPLC was conducted on a Shimadzu LC-6A system with ODS column (4.6×250 mm). Preparative HPLC was performed with a Shimadzu LC-8A system with ODS column (20×250 mm).

All the analogs were synthesized by a similar procedure as described previously for the synthesis of ωMVIIC and its analog [5]. Briefly, linear precursors of ω-conotoxin analogs were synthesized by solid phase methodology of Fmoc chemistry. After trifluoroacetic acid cleavage, crude linear peptide was diluted to a final peptide concentration of 0.05 mM and subjected to oxidative disulfide bond formation at 4°C for 3–5 days in 1 M ammonium acetate buffer (pH 7.8) containing reduced/oxidized glutathione (molar ratio of peptide:GSH:GSSG was 1:100:10). The folding reaction was monitored by HPLC and stopped by lowering the pH of the solution to 3–4 with AcOH. The crude cyclic products were purified by successive chromatographies with Sephadex G-50F, CM-cellulose CM-52, and preparative HPLC with an ODS column. The structure and purity of synthetic peptide were confirmed by analytical HPLC, amino acid analysis, and MALDI-TOF-MS measurements.

2.3. Enzymatic digestion for the determination of disulfide bond combination

To a solution of synthetic peptide (0.4 mg) in 100 μl of 0.1 mM phosphate buffer (pH 6.5) was added a solution of lysyl endopeptidase (10 μg) in 20 μl of the same buffer. The mixture was incubated at 37°C for 1.5 h and subjected to HPLC separation and MALDI-TOF-MS measurements. The major fragment was lyophilized and dissolved into 0.4 ml of 0.1 M ammonium formate buffer (pH 6.5). To 100 μl of this

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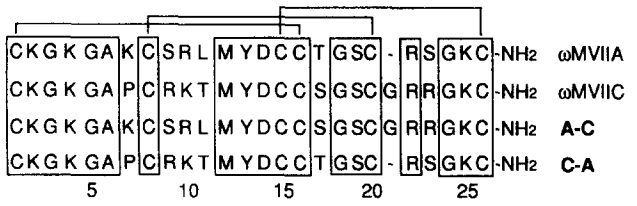

Fig. 1. Amino acid sequences and disulfide bonds of ωMVIIA, ωMVIIC, and their chimeric analogs.

solution were added a solution of thermolysin (20 μg) in 20 μl of the same buffer and 80 μl of $CaCl_2$ solution (2.5 mM in the same buffer). The mixture was incubated at 37°C for 3 h and subjected to HPLC separation and MALDI-TOF-MS measurements.

2.4. CD measurements

CD spectra were recorded on a JASCO J-600 spectropolarimeter in H_2O solution (0.01 M sodium phosphate, pH 7.0) at 20°C, using a quartz cell of 1 mm path length. The spectra are expressed as molar ellipticity [θ].

2.5. Binding assay

Rat cerebellar P2 membranes (10 μg) in 0.1 ml of 25 mM Tris, 150 mM NaCl, 0.1% bovine serum albumin adjusted to pH 7.4 with HCl (TBSA) were incubated with 0.5 nM [^{125}I]ωGVIA or [^{125}I]ωMVIIC for 1 h at 30°C. Membrane-bound radioactivity was measured after rapid filtration and washing on GF/C (Whatman) filters treated with 0.3% polyethyleneimine as described previously [6].

3. Results

3.1. Synthesis of peptides

We synthesized two chimeric analogs of ωMVIIA and ωMVIIC, namely A-C and C-A, which have the N-terminal half of ωMVIIA and the C-terminal half of ωMVIIC, and vice versa, respectively (Fig. 1). Linear precursors of A-C and C-A prepared by solid phase methodology were subjected directly to an oxidative folding reaction (disulfide bond formation), since these crude products showed high purity by HPLC analysis (Fig. 2). After 3 days reaction, the HPLC profile of the reaction solution became constant and showed one major component and several minor peaks. These major products were purified until they migrated as a single peak on an analytical HPLC.

3.2. Disulfide bond combination

According to the method as described for ωMVIIC by Kubo et al. [7], we successively digested ωMVIIC, A-C, and C-A with lysyl endopeptidase and thermolysin. First digestion with lysyl endopeptidase gave a single major component with MH^+ 2618 for ωMVIIC, 2346 for A-C, and 2505 for C-A, indicating that the C-termini of Lys residues were selectively hydrolyzed. After a second digestion with thermolysin, HPLC of the reaction solution showed one major peak and a few minor peaks (Fig. 3). Compounds from peaks 1A–3A showed the same MH^+ 869 by MALDI-TOF-MS analysis and those from peaks 1B, 2B, and 3B gave 1537, 1270, and 1424, respectively. The structures of these fragments are summarized in Fig. 4. Isocratic elution on reversed phase HPLC of 1A–3A showed the same retention time both in co-injection and individual injection (data not shown), indicating that A-C and C-A have the same disulfide bond combination as that of native ωMVIIC.

3.3. CD spectra

CD spectra of A-C and C-A were measured and compared to those of ωMVIIA and ωMVIIC (Fig. 5) [8]. CD spectral profiles seemed to be related to the number of amino acid residues. Both 25-mer peptides, ωMVIIA and C-A, showed similar CD profiles to each other with positive Cotton effects around 245 nm and negative ones around 205 nm. In contrast, peptides with 26 residues, ωMVIIC and A-C, showed positive Cotton effects around 230 nm and negative ones around 203 nm. These results suggest that the chain length between fifth and sixth Cys residues is reflected in the difference in CD spectra.

3.4. Biological activity

Dose-inhibition curves of chimeric analogs on the binding of [^{125}I]ωGVIA and [^{125}I]ωMVIIC to rat cerebellar P2 membranes were compared to those of ωMVIIA and ωMVIIC (Fig. 6). Analog A-C showed high affinity (IC_{50}: 8.2×10^{-10} M) for the N-type calcium channel comparable to that of ωMVIIA (IC_{50}: 4.0×10^{-10} M), while C-A showed very low affinity (IC_{50}: 8.2×10^{-8} M) like ωMVIIC (IC_{50}: 6.5×10^{-8} M) (Fig. 6A). Therefore, the N-terminal half of ωMVIIA

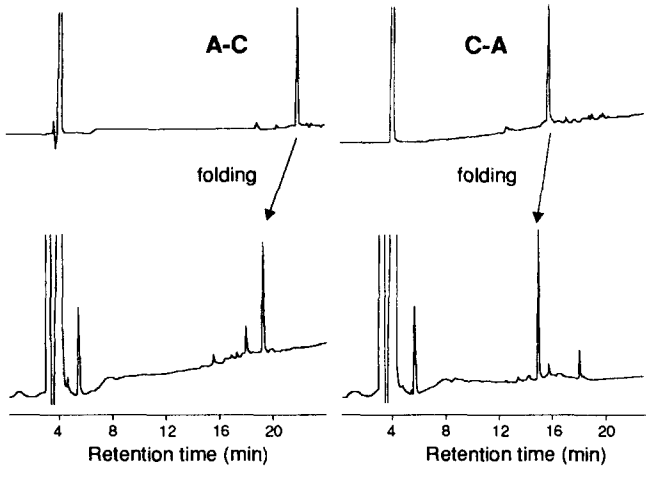

Fig. 2. HPLC profiles of the oxidative folding reaction of chimeric analogs. The upper parts show the profiles of crude linear precursors, and the lower parts show those of the reaction mixture after 3 days. Large peaks at around 4 min are due to the solvent, GSH, or GSSG. Column: Shim-pack CLC-ODS (4.6×250 mm, Shimadzu). Solvent: linear gradient from 5% to 35% CH_3CN in 0.1% TFA for 30 min. Flow rate: 1 ml/min. Monitoring: absorbance at 230 nm (intensity is not scaled).

seemed to contain all the residues essential for high affinity recognition of the N-type calcium channel. For the P/Q-type channel, both A-C (IC_{50}: 3.2×10^{-8} M) and C-A (IC_{50}: 3.3×10^{-7} M) showed weaker affinity than ωMVIIC (IC_{50}: 3.0×10^{-9} M) (Fig. 6B), suggesting that the essential residues for the interaction with P/Q-type channel are spread over the whole ωMVIIC molecule.

4. Discussion

For the structure-activity relationship study of peptides with multiple disulfide bonds, it is essential to confirm that the analogs have the same disulfide pairings as those of native peptide. Kubo et al. determined the mode of disulfide pairings of native ωMVIIC by comparing its enzymatic digestion products with two synthetic model peptides [7]. Although we did not synthesize these model peptides, comparison of enzymatic digestion products of the analogs to that of ωMVIIC clearly showed that these peptides were identical to each other. During second digestion with thermolysin, we unexpectedly observed a cleavage of the Arg^{10}-Leu^{11} bond in A-C as assigned from peak 2B with MH^+ 1270. Fortunately, this cleavage did not affect the identification of the mode of disulfide parings.

In addition to the confirmation of conserved disulfide parings, CD spectra were measured to prove that the analogs have similar overall conformation to those of native ω-conotoxins. Recently, we reported that the common profiles observed in the CD spectra of ωMVIIC and ωMVIIA reflected their conformational similarity such as triple-stranded antiparallel β-sheet stabilized by three intramolecular disulfide bonds [8]. The similarity of CD spectra of A-C and C-A to those of ωMVIIC and ωMVIIA, respectively, suggested that their overall conformations were not significantly affected by amino acid substitutions. It is interesting that the chain length between the fifth and sixth Cys residues seems to reflect the difference in CD spectra. The fifth and sixth Cys residues are located in the second and the third β-strand, respectively, and the residues between the fifth and sixth Cys residues connect these two β-strands. The length of this hinge region may not affect the relative orientation of two β-strands but may affect the CD spectral profiles.

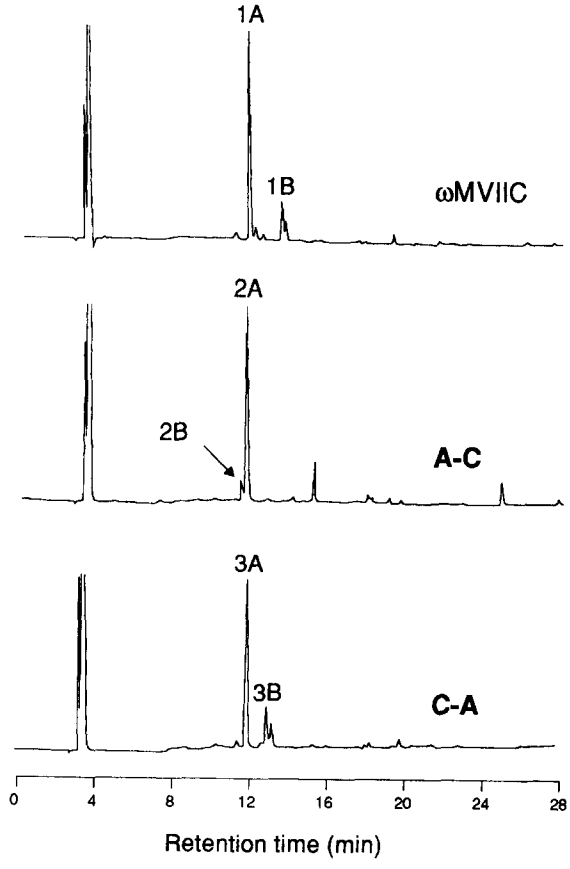

Fig. 3. HPLC profiles of the reaction solution of ωMVIIC, A-C, and C-A treated successively with lysyl endopeptidase and thermolysin. Analysis was carried out in the same condition as described in the legend for Fig. 2 except that the linear gradient was from 1% to 31% CH_3CN in 0.1% TFA for 30 min.

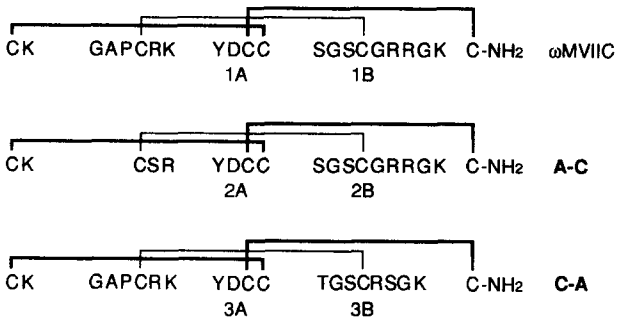

Fig. 4. Structures of enzymatic digestion products of ωMVIIC, A-C, and C-A.

The three-dimensional structures of ωGVIA [9–12], ωMVIIA [13–15], and ωMVIIC [16,17] have been determined by NMR analysis. Despite differences in primary amino acid sequences, the polypeptide chain framework is conserved in all of the ω-conotoxins. Thus the nature of the amino acid side chains may have a leading role in determining the toxin selectivity. In the present study, we have found that the essential residues are largely different for selective binding to N- and P/Q-type calcium channels.

Previously, we showed that Tyr^{13} is essential for the activity of ωGVIA and that Lys^2 is the second important residue based on the results of systematic single Ala substitution [18,19]. Because replacement of other residues by Ala did not affect the binding, we proposed a two-point binding model between ωGVIA and the N-type calcium channel [19]. In the case of ωMVIIA, replacement of Tyr^{13} by Ala also resulted in a significant loss of affinity, whereas substitution of Lys^2 did not affect the binding [20]. Loss of the basic side chain of Lys^2 may be compensated by other basic residues such as Lys^7 [13]. Nadasdi et al. also reported that the replacement of Tyr^{13} in ωMVIIA by Phe reduced the affinity to 0.5% of native ωMVIIA and that the elimination of some positive charges also affected the binding [21]. These previous observations agree with the present results demonstrating that a limited number of residues are important for the binding of ωMVIIA to N-type calcium channels. Although the sequence of chimeric analog A-C is almost the same as that of ωMVIIC except for four residues in the N-terminal half (Fig. 1), its affinity for N-type channels is almost the same as that of ωMVIIA and 100 times higher than that of ωMVIIC. This result indicates that some of these four residues, which are located around the Tyr^{13} residue in a three-dimensional structure, are essential for the recognition of N-type calcium chan-

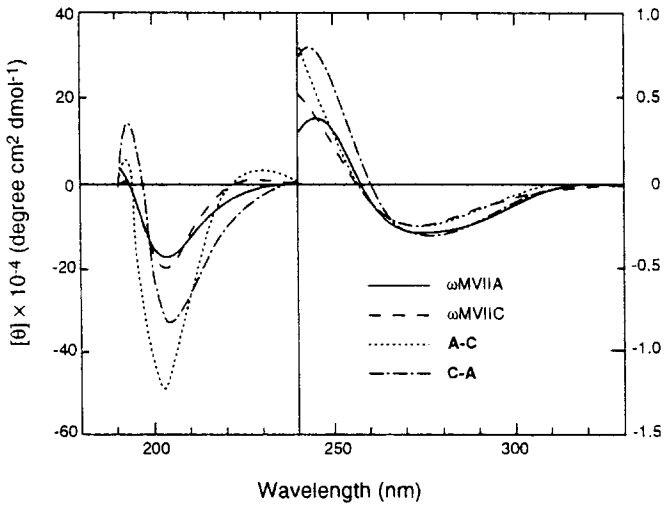

Fig. 5. CD spectra of ωMVIIA, ωMVIIC, and their chimeric analogs in H_2O solution (0.01 M sodium phosphate, pH 7.0) at 20°C.

nels. We are currently studying the mechanism of selective binding to N-type channels by replacing these four residues in several combinations.

On the other hand, results with chimeric analogs suggested that essential residues for the P/Q-type channel recognition are widely spread over the ωMVIIC molecule. Previously we reported that the replacement of Tyr^{13} in ωMVIIC by Ala significantly reduced the affinity, suggesting that Tyr^{13} is a common binding motif in ω-conotoxins irrespective of the calcium channel subtypes that they target [5]. In order to identify the residues essential for subtype recognition, systematic Ala scanning for ωMVIIC is also currently in progress.

Voltage-gated calcium channels are complex membrane proteins consisting of multiple subunits [1–3]. A central channel pore is formed by an α_1 subunit that has four motifs, I–IV, each having six transmembrane segments, S1–S6. A pore-lining segment H5 between segments 5 (S5) and 6 (S6) is thought to be essential for the ion selectivity. The α_1 subunits of N- and P/Q-type calcium channels have been cloned and designated α_{1B} and α_{1A}, respectively, according to the nomenclature of voltage-gated calcium channels [22]. The amino acid sequences of H5 segments are almost identical between N- and P/Q-type calcium channels [23,24]. Cloning, mutagenesis and expression of α_1 subunits showed that the most dramatic effects on the interaction between ωGVIA and N-type calcium channel involved a single cluster of residues in the large putative extracellular loop between IIIS5 and IIIH5, consistent with a direct pore-blocking mechanism [25]. A combination of mutational studies on both ion channels and their specific blockers will provide knowledge of the architecture of the outer vestibules of the channel pores. The selectivity of ωMVIIC to the P/Q-type channels is not complete, since it still retains weak affinity with the N-type calcium channel (Fig. 6A). Furthermore specific blockers are not yet available for T-type calcium channels or channels coded by the class E (α_{1E}) genes. In combination with the comparison of amino acid sequences of H5 segments of calcium channels, study of the blocking mechanism of ω-conotoxins may enable the design and synthesis of novel blockers with appropriate specificities.

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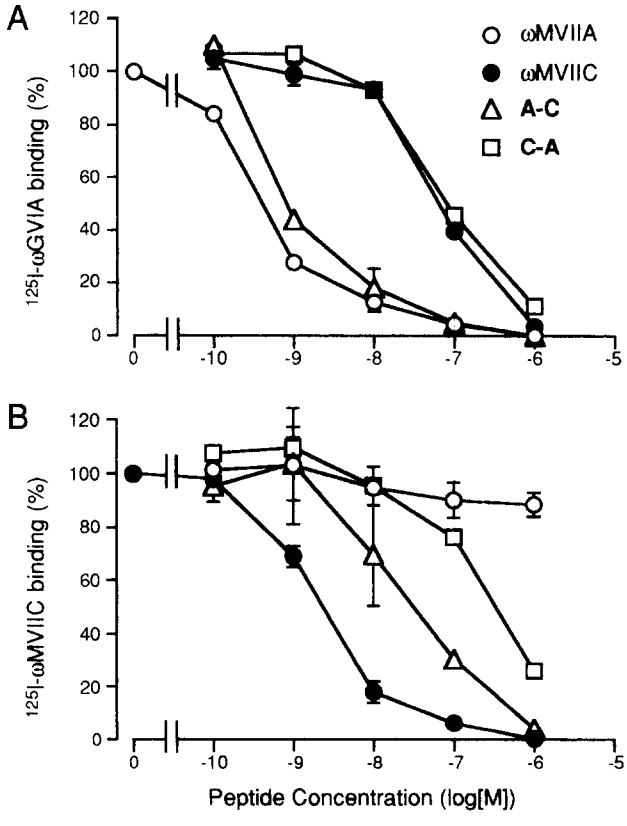

Fig. 6. Inhibition of [^{125}I]ωGVIA (A) or [^{125}I]ωMVIIC (B) binding to rat cerebellar P2 membranes by ωMVIIA, ωMVIIC, and their chimeric analogs.

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