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Binding of Six Chimeric Analogs of ω-Conotoxin MVIIA and MVIIC to N- and P/Q-Type Calcium Channels

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Replacement of the N-terminal half of ω-conotoxin MVIIC, a peptide blocker of P/Q-type calcium channels, with that of ω-conotoxin MVIIA significantly increased the affinity for N-type calcium channels. To identify the residues essential for subtype selectivity, we examined single reverse mutations from MVIIA-type to MVIIC-type in this chimeric analog. A reverse mutation from Lys^7 to Pro^7 decreased the affinity for both P/Q- and N-type channels, whereas that from Leu^{11} to Thr^{11} increased the affinity for P/Q-type channels and decreased the affinity for N-type channels. The roles of these two residues were confirmed by synthesizing two MVIIC analogs in which Pro^7 and Thr^{11} were replaced with Lys^7 and Leu^{11}, respectively.

***Key Words:* ω-conotoxin MVIIC; calcium channel; chimeric analog**

Voltage-gated calcium channels play crucial roles in regulating calcium signaling in a wide variety of cells, and have been 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 many neurons. Various specific peptide ligands have been used for the pharmacological dissection of calcium channel subtypes, including the ω-conotoxins isolated from the venom of marine *Conus* snails. The defining ligands for N-type calcium channels are ω-conotoxin GVIA and MVIIA, while P/Q-type channels are blocked by ω-conotoxin MVIIC (Fig. 1) (4). However, MVIIC retains weak affinity for N-type channels. We reported previously that replacement of N-terminal half of MVIIC with that of MVIIA significantly increased the affinity for N-type calcium channels (5). Because only four residues of the N-terminal half of MVIIC differ from those of MVIIA, these four residues are thought to be important for the subtype selectivity of MVIIA and MVIIC.

In the present study, we examined single reverse mutations from MVIIA-type to MVIIC-type in the chimeric analog named **AC** to identify the residues essential for subtype selectivity (Fig. 1). Among the four residues examined, residues in the 7th and 11th positions were shown to be important. The roles of these two residues were confirmed by synthesizing two MVIIC analogs in which Pro^7 and Thr^{11} of MVIIC were replaced with Lys^7 and Leu^{11} of MVIIA, respectively.

MATERIALS AND METHODS

Materials. Fmoc-amino acids, Fmoc-NH-resin, and other reagents used on a synthesizer were obtained from Perkin Elmer–Applied Biosystems (Chiba, Japan). Other reagents were obtained from Watanabe Chemical Industries Ltd. (Hiroshima, Japan), Peptide Institute (Osaka, Japan) or Kokusan Chemical Works Ltd. (Tokyo, Japan). Lysyl endopeptidase and thermolysin were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan).

Synthesis and purification of peptides. Solid phase peptide synthesis was performed on a Perkin Elmer-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 carried out with a PerSeptive Biosystems Voyager Linear DE mass spectrometer using α-cyano-4-hydroxy-cinnamic acid as a matrix. Analytical HPLC was conducted on a Shimadzu LC-6A system with an ODS column Shim-pack CLC-ODS (4.6 × 250 nm, Shimadzu). Preparative HPLC was performed on a Shimadzu LC-8A system with an ODS column Shim-pack PREP-ODS (H) (20 × 250 nm, Shimadzu).

All the analogs were synthesized by a procedure similar to that described previously for the synthesis of MVIIC and its analogs (5,

Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; GSH, reduced glutathione; GSSG, oxidized glutathione; GVIA, ω-conotoxin GVIA; HPLC, high-performance liquid chromatography; [^{125}I]GVIA, [^{125}I]-ω-conotoxin GVIA; [^{125}I]MVIIC, [^{125}I][Nle^{12}]ω-conotoxin MVIIC; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MVIIA, ω-conotoxin MVIIA; MVIIC, ω-conotoxin MVIIC; ODS, octadecylsilane. Analogs are designated by a letter and number indicating the identity and position of the substituted amino acid, followed by a letter indicating the identity of the replacement residue; for example, **K7P** indicates an analog in which Lys^7 is replaced with Pro.

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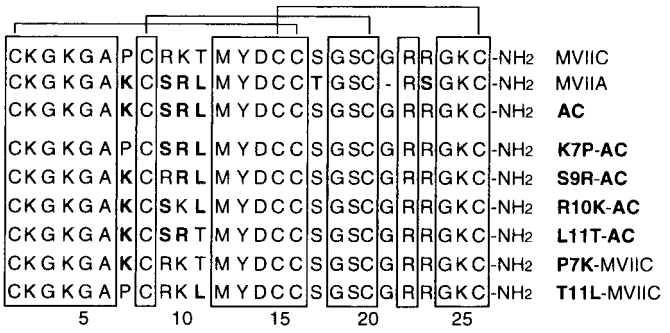

FIG. 1. Amino acid sequences and disulfide bonds of ω-conotoxin MVIIC, MVIIA, and their chimeric analogs. Amino acid residues originating from MVIIA are shown in bold.

6). Briefly, linear precursors of MVIIC analogs were synthesized by solid phase methodology of Fmoc chemistry. After trifluoroacetic acid cleavage, crude linear peptide was diluted to the final 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 chromatography on Sephadex G-50F, CM-cellulose CM-52, and preparative HPLC with an ODS column. The structure and purity of synthetic peptides were confirmed by analytical HPLC, amino acid analysis, and MALDI-TOF-MS measurements.

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 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.

CD measurements. CD spectra were recorded on a JASCO J-600 spectropolarometer 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].

Binding assay. Rat cerebellar P_2 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]GVIA or [^{125}I]MVIIC 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 (7).

RESULTS AND DISCUSSION

All the linear precursors of chimeric analogs were successfully assembled by solid phase methodology. Air oxidation of the crude linear precursors of most analogs afforded peptides with proper disulfide pairings as the major products, with good overall isolation yields. According to the method described for a chimeric analog **AC** (5), we successively digested the analogs with lysyl endopeptidase and thermolysin. All the analogs gave the same digestion products with Cys[1]-Cys[4]/Cys[3]-Cys[6] combination as in MVIIC (data not shown), indicating that they have the same disulfide bond pairings as native MVIIC (5).

CD spectra of all the analogs were similar to that of MVIIC with positive Cotton Effects around 230 nm and negative ones around 203 nm, suggesting that the conformations of the analogs are similar to that of MVIIC. Certain CD spectra are illustrated in Fig. 2 as examples. The three dimensional structures of MVIIA (8, 9) and MVIIC (9, 10) 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 dominant role in determining toxin selectivity.

The ability of the analogs to inhibit binding of [^{125}I]GVIA and [^{125}I]MVIIC to rat cerebellar P_2 membranes was compared to displacement by MVIIA and MVIIC. The IC_{50} values of the analogs are summarized in Fig. 3 together with those of MVIIA, MVIIC, and a chimeric analog **AC.** As shown in Fig. 3, it is clear that MVIIA is highly selective for N-type channels, whereas MVIIC is not completely selective for P/Q-type channels. Replacement of Lys[7] of **AC** with Pro[7] decreased the affinity for both P/Q- and N-type channels (**K7P-AC**). Replacements of Ser[9] or Arg[10] of **AC** with Arg[9] or Lys[10], respectively, did not affect the affinity (**S9R-AC** and **R10K-AC**). On the other hand, replacement of Leu[11] with Thr[11] increased the affinity for P/Q-type channels and decreased the affinity for N-type channels (**L11T-AC**). These results suggest that the basic residue Lys at the 7th position is important for the binding to both N- and P/Q-type calcium channels and that the hydrophobic residue Leu at the 11th position is favorable for N-type selectivity.

To confirm the role of the residues at the 7th and 11th positions, we synthesized two MVIIC analogs in

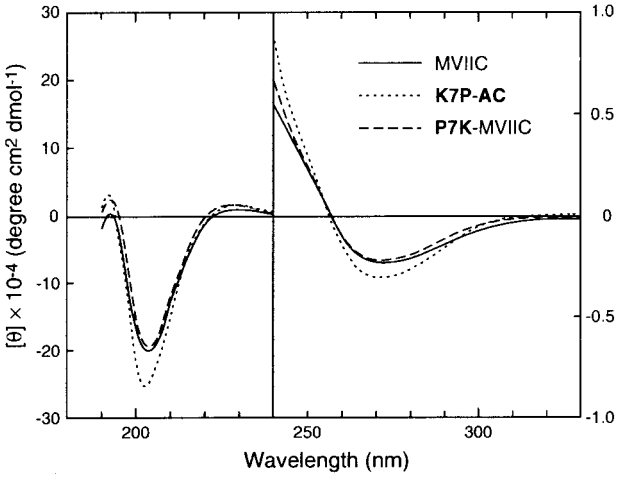

FIG. 2. CD spectra of MVIIC and its analogs in H_2O solution (0.01 M sodium phosphate, pH 7.0) at 20°C.

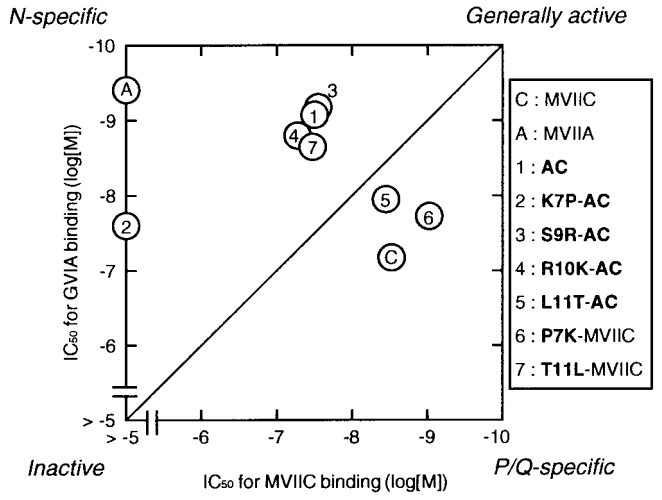

FIG. 3. Inhibition (IC_{50}) of [^{125}I]GVIA or [^{125}I]MVIIC binding to rat cerebellar P_2 membranes by chimeric analogs. Letters A and C in circles indicate the IC_{50} of native MVIIA and MVIIC, respectively.

which Pro^{7} and Thr^{11} were replaced with Lys^{7} and Leu^{11}, respectively. Replacement of Pro^{7} of MVIIC with Lys^{7} increased the affinity for both N- and P/Q-type channels as expected (**P7K**-MVIIC), although the effect was not so significant as observed in **K7P-AC** (Fig. 3). Replacement of Thr^{11} of MVIIC with Leu^{11} increased the affinity for N-type channels and decreased the affinity for P/Q-type channels (**T11L**-MVIIC). This effect was almost the opposite to that observed when Leu^{11} of **AC** was replaced with Thr^{11} (**L11T-AC**), demonstrating that the hydrophobic residue Leu at the 11th position is favorable for N-type selectivity.

Previously, we have shown that Tyr^{13} is essential for the activity of GVIA (12), MVIIA (13), and MVIIC (6), suggesting that Tyr^{13} is a common binding motif in ω-conotoxins irrespective of the calcium channel subtypes that they target. In the present study, it is suggested that the residue at the 11th position is important for the subtype selectivity of MVIIA and MVIIC. Therefore, it will be interesting to further explore the role of Thr^{11} in MVIIC, since the N-type specific peptides MVIIA and GVIA have Leu and Thr residues at the 11th position, respectively.

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 homologous domains, 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 ion selectivity. The α_1 subunits of N- and P/Q-type calcium channels have been cloned and designated as α_{1B} and α_{1A}, respectively, according to the nomenclature of voltage-gated calcium channels (14). The amino acid sequences of H5 segments are almost identical between N- and P/Q-type calcium channels (15, 16). Cloning, mutagenesis and expression of α_1 subunits has shown that the most dramatic effects on the interaction between GVIA and the N-type calcium channel involved at a single cluster of residues in the large extracellular loop between IIIS5 and IIIH5, consistent with a direct pore-blocking mechanism (17). A combination of mutational studies on both ion channels and their specific blockers should provide an insight into the architecture of the outer vestibules of the channel pores. In combination with a comparison of the amino acid sequences of calcium channel H5 segments, studies on the blocking mechanism of ω-conotoxins may enable the design and synthesis of novel blockers with appropriate specificities.

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