Active Site of \( \mu \)-Conotoxin GIIIA, a Peptide Blocker of Muscle Sodium Channels*

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The amino acid sequence of \( \mu \)-conotoxin GIIIA (otherwise called geographotoxin I), a peptide having 22 amino acid residues with three disulfide bridges, was modified by replacing each residue with Ala or Lys to elucidate its active center for blocking sodium channels of skeletal muscle. NMR and CD spectra were virtually identical between native and modified toxins, indicating the similarity of their conformation including disulfide bridges. The inhibitory effect of these modified peptides on twitch contractions of the rat diaphragm showed that Arg at the 13th position and the basicity of the molecule are crucial for the biological action. The segment Lys\(^1\)-Asp\(^{12}\)-Arg\(^{13}\) has been reported to be flexible (Lancelin, J.-M., Kohda, D., Tate, S., Yanagawa, Y., Abe, T., Satake, M., and Inagaki, F. (1991) Biochemistry, in press), and this may represent a clue for the subtle fit of Arg\(^{13}\) to the specific site of sodium channels. Since known ligands to sodium channels, such as tetrodotoxin, anthopleulin-A, etc., contain guanidine groups as a putative binding moiety, Arg may be a general residue for peptide toxins to interact with the receptor site on sodium channels.

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\( \mu \)-Conotoxin GIIIA (\( \mu \)-CTX)\(^1\) (which we previously called geographotoxin I) isolated from the marine snail Corus geographus is a peptide toxin composed of 22 amino acid residues with three disulfide bridges (1-3). The amino acid sequence (4, 5) and disulfide pairings (6) of the toxin were determined as shown in Fig. 1. This toxin preferentially blocks skeletal muscle sodium channels (7, 8). \( \mu \)-Conotoxin has previously been shown to discriminate between the tetrodotoxin/saxitoxin receptor sites on muscle and nerve sodium channels by means of ligand-binding methods (9) and is a promising tool to investigate sodium channels (10). In the present study, we synthesized various analogs of \( \mu \)-CTX and measured their inhibitory activity to elucidate the molecular nature of its active center. The results were also interpreted on the basis of the three-dimensional structure of \( \mu \)-CTX.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Linear precursors of the analogs were synthesized by solid phase methodology of common t-butoxycarbonyl chemistry starting from MBHA resin using a Biosearch model 9600 peptide synthesizer. After hydrogen fluoride cleavage, the crude linear peptides were extracted with 2 M AcOH and diluted to the final peptide concentration of 0.6 M. The solutions were adjusted to pH 7.8 with aqueous NH\(_4\)OH and stirred slowly at room temperature. Cyclization reaction to make disulfide bridges in the molecule was monitored by HPLC, and the crude cyclized products were purified by successive chromatographies with Sephadex G-50, carboxymethylcellulose CM-52, preparative HPLC with ODS columns, and Sephadex G-25. Structures and the purities of all the analogs were confirmed by analytical HPLC, amino acid analysis, and FAB-MS measurement.

Bioassay—The inhibitory effect of synthesized analogs on twitch contraction of the isolated rat diaphragm to direct electrical stimuli was assayed as reported previously (7). Male rats (Wistar, 200-300 g) were stunned and bled. Diaphragm muscle was excised and cut into four strips, which were electrically stimulated with 5-ms pulses at 0.1 Hz (supramaximal voltage). Twitch-tension responses were recorded isometrically. Activity of the peptides was expressed as inhibition of twitch response. The \( \mathrm{ED}_{50} \) value is a concentration of peptide necessary for 50% inhibition. Most \( \mathrm{ED}_{50} \) values are the mean of two sets of experiments except for native, \([\mathrm{Ala}^{13}]\), and \([\mathrm{Ala}^{13}]\) CTX. For a comparison of \( \mathrm{ED}_{50} \) values of these three peptides, the statistical analysis was performed by Student’s \( t \) test.

NMR and CD Measurements—NMR spectra were recorded on a Bruker AM 400 spectrometer operating at 400 MHz for proton frequency. peptides were dissolved in 90% H\(_2\)O, 10% D\(_2\)O at \( 20^\circ \)C. CD spectra were recorded on a Jasco J-600 spectro-polarimeter in H\(_2\)O solution (0.01 M sodium phosphate, pH 7.0) at \( 20^\circ \)C.

RESULTS

To identify the functional residues in \( \mu \)-CTX, each amino acid (other than Cys residue) of the peptide was replaced by Ala, a small nonpolar residue. The yields of most synthesized analogs starting from MBHA resin were 2-4%. We could not isolate \([\mathrm{Ala}^{13}]\) CTX, because the cyclization reaction did not give a major product.

The effects of replacement of amino acid residue in \( \mu \)-CTX with Ala on \( \mathrm{ED}_{50} \) values in the inhibition of twitch contraction of the rat diaphragm are shown in Fig. 2. With Arg, the most basic amino acid residue among the 20 standard residues in protein, was replaced, \( \mathrm{ED}_{50} \) values increased, indicating that the substitution lowered the potency of the biological action. Ala substitution at Arg\(^{13}\) significantly (\( p < 0.01 \)) reduced the potency approximately 200-fold less than the natural compound; \( \mathrm{ED}_{50} \) values for \([\mathrm{Ala}^{13}]\) CTX and the native \( \mu \)-CTX were 4.11 ± 0.59 \( \times 10^{-5} \) M \(( n = 4) \) and 1.75 ± 0.18 \( \times 10^{-7} \) M \(( n = 5) \), respectively. Substitutions at Arg\(^{13}\) and Arg\(^{12}\) also reduced the potency 25- and 5-fold. The replacement of another basic residue, Lys at position 8, 11, or 16, also raised the \( \mathrm{ED}_{50} \) value to 2-6 times that of the native toxin, while substitution at Lys\(^{8}\) did not. On the other hand, Ala substitutions at the 2nd and 12th Asp, an acidic residue, reduced
whereas replacement of other basic residues with Ala reduced bonds. This Cotton effect was absent in the spectrum of the CTX showed that the basicity of the molecule is a crucial factor for activity. Especially, replacement of Arg13 with Ala seemed that Arg is more or less replaceable with Lys, the basicity around the 13th position is suggested to be more important than those of other positions for the biological activity.

Lys is less basic than Arg. Thus, we replaced each Arg in μ-CTX with Lys to investigate the exchangeability between the two basic residues (Fig. 2). Replacement of Arg13 with Lys did not affect the activity. In contrast, ED50 values for [Lys13]μ-CTX was significantly (p < 0.01) smaller than that for μ-CTX. These results indicate that basicity of the molecule, especially around the 13th position, is important for the biological activity.

In addition, we synthesized linear peptide [Ala3,4,10,15][20-21]p-CTX, by replacing all Cys residues with Ala. This peptide at 10^{-4} M did not exhibit any significant inhibitory effect on twitch contraction (data not shown). Thus, specific conformation formed by disulfide bonds is required for the biological activity.

NMR spectra of synthetic analogs were essentially identical to that of the native μ-CTX except for the substituted residues (e.g. Fig. 3). The CD spectra of the analogs were also superimposable to that of native peptide (e.g. Fig. 4). CD spectra of both native and [Ala13]μ-CTX showed a similar negative Cotton effect around 260 nm, which reflects the disulfide bonds. This Cotton effect was absent in the spectrum of the linear analog. These results clearly show that the observed change in inhibitory activity was due to the difference in the nature of the side chains themselves rather than a conformational change (if any) associated with the replacement.

**DISCUSSION**

Present study on the structure-activity relationship for μ-CTX showed that the basicity of the molecule is a crucial factor for activity. Especially, replacement of Arg13 with Ala remarkably reduced activity compared with natural μ-CTX, whereas replacement of other basic residues with Ala reduced activity less extensively, suggesting that Arg13 is mainly responsible for the inhibitory action of μ-CTX. The specific role of the guanidino group of Arg13 was supported by the finding that [Lys13]μ-CTX showed a large loss of activity. In contrast, without significant loss of activity, Arg13 can be replaced by Lys, implying that only a positive charge is required at this position. Positively charged groups other than Arg13 may assist in positioning of Arg13 in a proper binding site of sodium channels.

Replacement of amino acid residues at position 5, 7, 8, or 9 with Ala did not significantly modify the inhibitory potency of twitch contractions. It seems that the sequence between Cys4 and Cys10 is not essential for the biological activity. However, a linear peptide, [Ala3,4,10,15,20-21]μ-CTX, possessed no inhibitory activity. Furthermore, Ala substitution at Hyp6 failed to form a proper set of disulfide bridges. Therefore, the peptide loop between Cys4 and Cys10 would be important in forming the specific conformation of the molecule for exerting the biological activity.
Within the macromolecular arrangement of sodium channels, anionic sites are detected through a number of electro-physiological experiments (11) and expected to be in the extracellular “funnel” part of the channel (12). Tetrodotoxin and saxitoxin, famous sodium channel blockers, contain guanidino group(s) which may bind to the anionic sites of the channels (13). Arg is the only amino acid residue having a guanidino group among the 20 amino acids in protein. Since study of the three-dimensional structure of μ-CTX by two-dimensional NMR (Fig. 5) suggests that Arg may be a general active site and other part(s) of the molecule, as yet undefined, may be specific for the binding to muscle sodium channels.

In the present study, we have shown a clear-cut relation between the active site and the three-dimensional structure of μ-CTX. Its molecular architecture of a rigid core and flexible side chains shares the proposed general feature in the peptide-receptor interaction (17). The structure-activity relationship of μ-CTX will provide valuable information on the structure of sodium channels and the difference between muscle and nerve subtypes of sodium channels.

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REFERENCES