Modification of Arg-13 of μ-conotoxin GIIIA with piperidinyl-Arg analogs and their relation to the inhibition of sodium channels

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Abstract **μ-Conotoxin GIIIA, a peptide toxin isolated from the marine snail *Conus geographus*, preferentially blocks skeletal muscle sodium channels in vertebrates. In this study, analogs of μ-conotoxin GIIIA in which essential Arg-13 was replaced with arginine analogs consisting of a piperidyl framework to regulate length and direction of the side chain were synthesized. Synthesized analogs exhibited similar CD and NMR spectra to that of GIIIA, suggesting a three-dimensional structure identical to that of the native toxin. The biological activities of piperidyl analogs were decreased or lost despite the small change in the side chain of Arg-13. The investigated structure–activity relationships in inhibiting electrically stimulated muscle contraction suggest that the guanidinium group at amino acid position 13 interacts best when spaced with three to four carbons and placed in a vertical direction from the peptide loop. Thus, the position of the guanidinium group at Arg-13 of GIIIA must be located in a certain range for its strong interaction with the channel protein.** **© 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: μ-Conotoxin GIIIA; Sodium channel; Twitch contraction

1. Introduction

It is well known that some cone snails produce highly strong venom to hunt [1]. μ-Conotoxin GIIIA (GIIIA) is one of the toxins isolated from *Conus geographus* [2]. Toxicity was caused by the preferential block of the skeletal muscle type sodium channels, binding to the site which overlaps the tetrodotoxin and saxitoxin binding sites [3]. GIIIA is constituted of 22 amino acid residues, compactly packed with three disulfide bridges and three hydroxyproline residues. These disulfide bridges are indispensable to inhibit sodium channels. Point mutational studies for GIIIA suggested that Arg-13 of GIIIA may be responsible for the strong binding activity [4,5]. Further, Chang et al. [6] suggested that Arg-13 interacts with Glu-758 in domain II of the rat skeletal muscle sodium channel with approx. 3 kcal/mol, and that about half was electrostatic and half non-electrostatic interaction. More or less in the potency of biological activity, other responsible residues of GIIIA have been studied on rat skeletal muscle sodium channels [7–10]. These previous analyses on the interaction of GIIIA with sodium channels have been achieved mainly by the replacement of standard amino acids with other amino acids. Accordingly, we focused here on the role of Arg-13 in the interaction with sodium channels by synthesizing analogs of GIIIA in which Arg-13 was replaced with the Arg analogs containing a piperidyl framework to regulate the direction and length of the side chain (Fig. 1). The resulting structure–activity relationship provides some precise information on the spatial placement of the Arg guanidinium group. The novel strategy, modification of the side chain of a single responsible amino acid in the peptide or protein, may be useful to define the molecular interaction between peptide ligands and its effectors.

2. Materials and methods

2.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-amino-(1-carbamimidoylpiperidin-4-yl)acetic acid (R1) [2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc)]-OH, Fmoc-2-amino-3-(1-carbamimidoylpiperidin-4-yl)propionic acid (R2) (Pmc)-OH and Fmoc-2-amino-4-(1-carbamimidoylpiperidin-4-yl)butyric acid (R3) (Pmc)-OH were purchased from RSP Amino Acid Analogues, Inc. (MA, USA). Other Fmoc-amino acid was purchased from Watanabe Chemical Industries (Hiroshima, Japan). Fmoc-NH-resin and other reagents used on a synthesizer were obtained from Perkin Elmer-Applied Biosystems (Tokyo, Japan).

2.2. Synthesis and purification of peptide

Solid phase peptide synthesis was performed on a 433A peptide synthesizer (Perkin Elmer-Applied Biosystems). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out with a Voyager DE Pro mass spectrometer (PerSeptive Biosystems, Tokyo, Japan) using α-cyano-4-hydroxycinnamic acid as a matrix. Analytical reverse phase (RP)-high performance liquid chromatography (HPLC) was conducted on a Jasco 1500 system (Jasco, Tokyo, Japan) with an ODS column Develosil ODS-MG-5 (3.0×150 mm, Nomura Chemicals, Aichi, Japan). Preparative RP-HPLC was performed on a Jasco 1500 system with an ODS column Develosil ODS-5 (20×250 mm, Nomura Chemicals). GIIIA ana-

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Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; GIIIA, μ-conotoxin GIIIA; HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; R1, amino-(1-carbamimidoylpiperidin-4-yl)acetic acid; R2, 2-amino-3-(1-carbamimidoylpiperidin-4-yl)-propionic acid; R3, 2-amino-4-(1-carbamimidoylpiperidin-4-yl)-butyric acid; RP, reverse phase; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl. μ-Conotoxin GIIIA analogs are designated with a letter and number indicating the identity and position of the replaced residue: for example R13A indicates an analog in which Arg-13 is replaced with Ala

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logs were synthesized by a procedure similar to that described previously [4,11]. Briefly, linear precursors of GIIIA analogs were synthesized by solid phase methodology of Fmoc chemistry. Arg-13 residue was incorporated using Fmoc-R1(Pmc)-OH, Fmoc-R2(Pmc)-OH and Fmoc-R3(Pmc)-OH. After trifluoroacetic acid cleavage, crude linear peptide was diluted to the final peptide concentration of 0.05 mM and subjected to oxidative disulfide bond formation in 0.2 M ammonium acetate buffer (pH 7.8) for 1 day. 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 purity and structure of synthetic peptides were confirmed by analytical HPLC and MALDI-TOF-MS measurements, respectively.

2.3. CD and NMR measurements

All the CD spectra were measured on a JASCO J-720 spectropolarimeter (Jasco) in H_2O solution (0.01 M sodium phosphate, pH 7.0) at 20°C at peptide concentrations of 0.05 mM for 190–250 nm and 1 mM for 240–360 nm using a quartz cell of 1 mm path length. The spectra are expressed as molecular ellipticity [θ] in degree cm^2 $dmol^{-1}$. NMR spectra were recorded on a Bruker AM-400 spectrometer (Bruker, Ibaragi, Japan) operating at 400 MHz for proton frequency. Peptides were dissolved in 90% H_2O/10% ^2H_2O to approx. 2 mM.

2.4. Bioassay

Inhibitory effects of synthesized analogs on the twitch contraction of skeletal muscle to direct electrical stimuli were assayed, as reported previously [4,11,12]. Male Wistar rats (body weight 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. The activity of the peptides was expressed as percent inhibition of twitch response.

3. Results and discussion

The linear precursor peptides for R13R1-GIIIA, R13R2-GIIIA and R13R3-GIIIA were synthesized using conventional Fmoc chemistry, and cleaved from resin. Disulfide bonds were formed by air oxidation, giving crude products of GIIIA analogs with 1% overall isolation yield. The products were purified by HPLC. Analytical HPLC exhibited a single peak for each product (data not shown), suggesting high purity. The products were analyzed using MALDI-TOF-MS (R13R1-GIIIA: *m/z* 2634.0 (calcd = 2634.1), R13R2-GIIIA: *m/z* 2648.1 (calcd = 2648.2), R13R3-GIIIA: *m/z* 2662.2 (calcd = 2662.2)).

The three-dimensional structure of GIIIA analogs was confirmed by CD and ^1H NMR spectra. The CD spectra showed that all tested GIIIA analogs had a secondary structure similar to that of GIIIA (Fig. 2, left panel). The ^1H NMR spectra of these GIIIA analogs were compared to estimate the chemical shift values between the α-proton of each amino acid and the value of the random coil [13]. Each α-proton of the amino acids of the GIIIA analogs had chemical shifts similar to those of GIIIA, except for the substituted residue at position 13 (Fig. 2, right panel), supporting their structural similarity. Taken together, the data indicate that synthesized R13R1-GIIIA, R13R2-GIIIA and R13R3-GIIIA have a three-dimensional structure similar to that of native GIIIA.

The biological activities of GIIIA analogs were measured by analyzing their effects on the contractile response of the isolated rat diaphragm to direct electrical stimulation as previously described [4,11,12] (Fig. 3). The derivative of R13R1-GIIIA elicited a dose-dependent inhibition of the contractile response. R13R1-GIIIA was approx. 20 times less potent than GIIIA. The other derivatives tested, R13R2- and R13R3-GIIIA, only elicited a small contractile inhibition of less than 10% at the relatively high concentration of 3 μM, indicating a marked loss in activity (Table 1). These results suggest that R1, but not R2 and R3, is effectively substituted with Arg-13 for exerting biological action, though a little less potent than native GIIIA.

Our previous results concerning the effects of modification at Arg-13 of GIIIA on biological activity [5] are also summarized in Table 1 and Fig. 3. The replacement of Arg-13 with

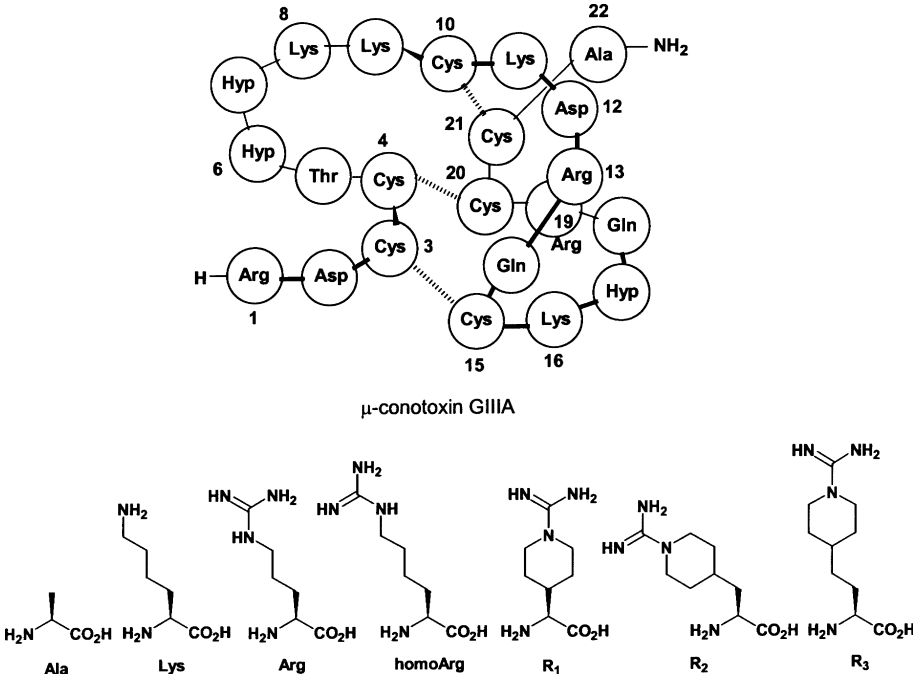

Fig. 1. The structure of μ-conotoxin GIIIA (upper) and amino acids introduced to the Arg-13 position (bottom). Dotted lines are disulfide bonds. The numbers indicate the amino acid order from the N-terminal region.

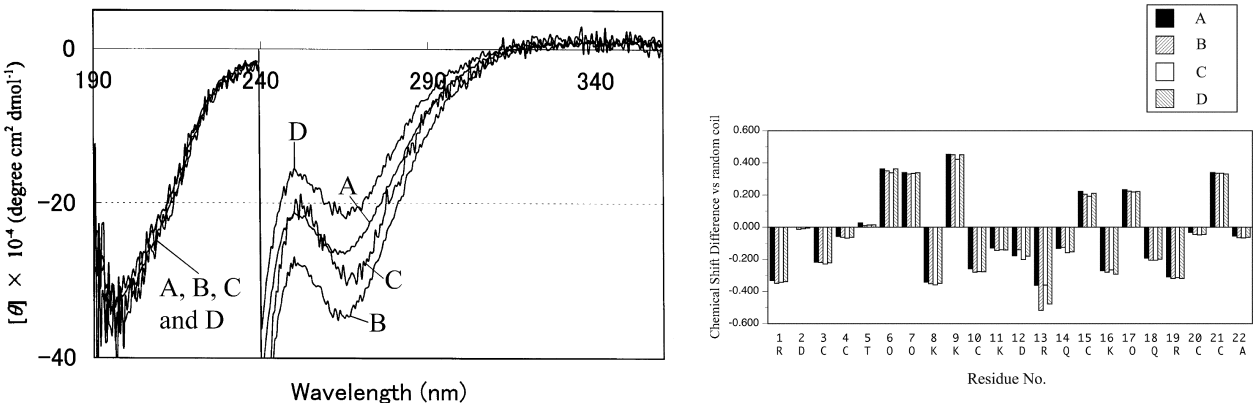

Fig. 2. CD spectra of GIIIA and its analogs (left) and NMR chemical shift difference between the α-protons of GIIIA analog residues and the random coil value (right). A: GIIIA; B: R13R1-GIIIA; C: R13R2-GIIIA; D: R13R3-GIIIA.

Ala (R13A-GIIIA) markedly reduced the potency of action to less than 1/300 compared with that of native GIIIA. When amino acids at positions 12–13 or 13–14 were shuffled (D12R-R13D-GIIIA or R13Q-Q14R-GIIIA), the potency of action was markedly attenuated. On the other hand, the replacement of Arg-13 with another basic amino acid of Lys (R13K-GIIIA) elicited a moderate reduction of approximately one tenth in the activity. Further, the replacement with homoArg (R13homoArg-GIIIA), having the aliphatic side chain of Arg elongated by one carbon from the peptide loop, did not significantly alter the potency of action. Consequently, the exact placement of a basic amino acid, preferentially Arg, at position 13 in GIIIA will be pivotal for the biological action. In addition, the number of carbon atoms in the aliphatic side chain protruding from the peptide loop may be variable at least in the range of 3–4 (three for Arg and four for Lys and homoArg) for exerting activity.

In the present experiments, a modification of Arg-13 with charged groups of R2 and R3 reduced the activity less than the replacement with Ala (a non-charged residue). Presumably, modification with a piperidine structure causes steric interference with the binding to sodium channels. Thus, the marked loss of activity in R13R2- and R13R3-GIIIA analogs may be attributed to not only loss of interaction but also addition of a steric repulsive force.

Incorporation of piperidine into the side chain at position 13 of GIIIA, in the present experiments, can provide more information on the chemical structure around Arg-13 for biological activity. For the R1 molecule, which has three carbon atoms between the peptide loop and nitrogen, the rotation of the C–C or C–N bond does not change the spatial position of the guanidinium group that is located nearly in a vertical direction from the peptide loop, while R2 has four carbon atoms in which three carbons are in piperidine, and the guanidinium group probably inclines by 60–70° from the vertical line with the peptide loop. In molecules of Arg, homoArg and Lys that possess three to four carbon atoms in the side chain to nitrogen, the guanidinium group or amino group can be placed in a nearly vertical direction from the peptide loop due to the rotation of the responsible C–C bond. When comparing the biological activity among the tested compounds having three to four carbon atoms in the side chain, only the substitute for R2 lost its activity, while the others retained it. The loss of activity of R13R2-GIIIA may be due to the fact that the inclination of the guanidinium group from the vertical axis in R2 interferes with the access of the guanidinium group to the receptive site of the channel protein, or brings about the neutralization of the basic charge of the guanidinium group due to interaction with the neighboring Asp-12. Although the replacement with R3 elicited a loss in activity, the substituent R3 has five carbon atoms (three in piperidine) from the peptide loop and the guanidinium group can be placed in a nearly vertical direction from the peptide loop. The length of the side chain from the peptide loop, and the guanidinium group in R3 may be too far to exert biological action. Rather, the guanidinium group may be better located in a vertical position and be separated by three to four aliphatic carbon atoms from

Table 1
Inhibitory activity of GIIIA and its analogs on skeletal muscle contraction to electric stimulation

Compound	Inhibitory activity IC_{50} (μM)	IC_{50}/IC_{50} (native GIIIA)
GIIIA	0.10	1.0
R13A-GIIIA[a]	42	420
R13K-GIIIA[a]	1.12	11.2
R13homoArg-GIIIA[a]	0.2	2
D12R-R13D-GIIIA[a]	> 30	> 300
R13Q-Q14R-GIIIA[a]	> 30	> 300
R13R1-GIIIA	1.64	16.4
R13R2-GIIIA	> 3.00	> 30
R13R3-GIIIA	> 3.00	> 30

[a]From [5].

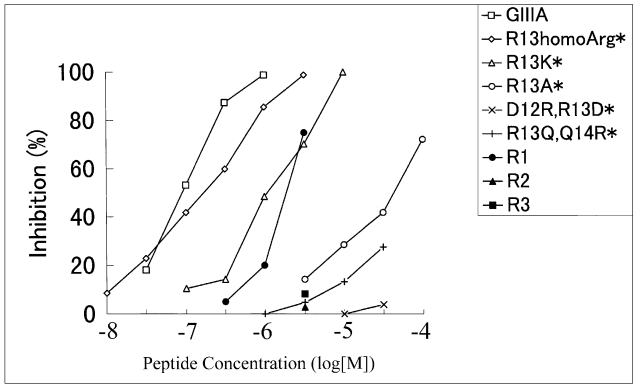

Fig. 3. Dose–inhibition curves for GIIIA and its analogs in twitch responses of isolated rat diaphragm to electrical stimuli. Ordinate represents the percent value of inhibition of contractile response. The data with asterisks are from [5].

the peptide loop of GIIIA for proper interaction with sodium channels.

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