Role of Thr^{11} in the binding of ω-conotoxin MVIIC to N-type Ca^{2+} channels

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Abstract As replacement of Thr^{11} of ω-conotoxin MVIIC with Ala significantly reduced the affinity for both N- and P/Q-type calcium channels, we examined the effect of substitution at this position with other residues. Binding assays using rat cerebellar P_2 membranes showed that the affinity is in the order of Leu > Val, aminobutyric acid, Thr > Asn ≫ Ser, Ala, Asp, Phe, Tyr for N-type channels and Thr > Leu, Val, aminobutyric acid, Asn, Ser > Ala ≫ Asp, Phe, Tyr for P/Q-type channels, suggesting that aliphatic amino acids with longer side chains are favorable for block of N-type channels. The effects of substitution were examined electrophysiologically in BHK cells expressing N-type Ca^{2+} channels. Inhibition of Ba^{2+} current by the analogs did not completely correlate with binding affinity, although binding to BHK cells was comparable to rat cerebellar membranes.

Key words: ω-Conotoxin MVIIC; Ca^{2+} channel; Binding assay; Patch-clamp

1. Introduction

Voltage-sensitive Ca^{2+} channels play important roles in neuronal activity [1]. They are composed of α_1, α_2, β, γ, and δ subunits, and the major α_1 subunit forms the channel pore. At least nine types of α_1 subunit encoded by distinct genes form Ca^{2+} channels with different characteristics [2,3]. Among them, N- and P/Q-type channels are specifically expressed in brain and play a crucial role in the regulation of neurotransmitter release [4]. Various specific ligands have been used to distinguish Ca^{2+} channel subtypes pharmacologically, including ω-conotoxins isolated from the venom of marine Conus snails. ω-Conotoxin GVIA and MVIIA specifically block N-type Ca^{2+} channel activity [4]. In contrast, ω-conotoxin MVIIC preferentially inhibits the P/Q-type Ca^{2+} channel, however, it also inhibits N-type Ca^{2+} channels at about 50-fold higher concentrations [4]. MVIIC and MVIIA are about 70% identical to each other in amino acid sequence (Fig. 1). Based on the results obtained with chimeric analogs of MVIIA and MVIIC, residues in the second loop between Cys^8 and Cys^{15} have been shown to be important for binding to N-type Ca^{2+} channels [5–7]. Moreover, replacement of Thr^{11} of MVIIC by Ala resulted in a loss of affinity for N-type Ca^{2+} channels [8]. In the present study, we synthesized analogs of MVIIC in which Thr^{11} was replaced with aminobutyric acid (Abu), Asp, Asn, Leu, Phe, Ser, Thr, Tyr, or Val (Fig. 1) in an attempt to obtain a selective P/Q-type Ca^{2+} channel blocker, and tested their ability to bind to membranes from rat cerebellum or baby hamster kidney (BHK) cells expressing N-type Ca^{2+} channel, and inhibit Ba^{2+} currents in BHK cells.

2. Materials and methods

2.1. Materials

Reagents and chemicals were obtained as follows: fetal bovine serum and geneticin, Gibco-BRL, Life Technology (Rockville, MD, USA); methotrexate, Sigma (St. Louis, MO, USA); streptomycin and penicillin, Meiji Seika (Tokyo, Japan); [^{125}I]GVIA and [^{125}I]MVIIC, NEN (Boston, MA, USA); tetrodotoxin, Wako (Osaka, Japan).

2.2. Cell culture

BHK cells expressing α_{1B} and β_{1A} subunits from rabbit brain and α_2/δ subunit from rabbit skeletal muscle that formed N-type Ca^{2+} channels (BHKN101) were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, streptomycin (600 μg/ml), penicillin (30 U/ml), geneticin (600 μg/ml) and methotrexate (0.25 μM) as described [9].

2.3. Synthesis and purification of peptides

Solid phase peptide synthesis, amino acid analysis and purification were performed as described previously [5,7,8,10]. Circular dichroism spectra of all the analogs synthesized in this study were similar to that of native MVIIC.

2.4. Binding assay

The binding activity to N- or P/Q-type Ca^{2+} channels in rat cerebellar P_2 membranes was determined by competition assay with

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Abbreviations: Abu, aminobutyric acid; BHK, baby hamster kidney; GVIA, ω-conotoxin GVIA; [^{125}I]GVIA, [^{125}I]ω-conotoxin GVIA; [^{125}I]MVIIC, [^{125}I]ω-conotoxin MVIIC; MVIIA, ω-conotoxin MVIIA; MVIIC, ω-conotoxin MVIIC; 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, T11L indicates an analog in which Thr^{11} is replaced with Leu.

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[^{125}I]GVIA or [^{125}I]MVIIC as described [11]. P_2 membranes of BHKN101 cells were prepared according to the method of cerebellar P_2 membrane preparation [12]. Briefly, cultured BHKN101 cells were harvested with cell scraper, and centrifuged at 2000 rpm for 5 min. Pellets were suspended with 10 mM Tris–HCl containing various protease inhibitors: 1 μM pepstatin A, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 mM 1,10-phenanthroline monohydrate, and 1 mM phenylmethylsulfonyl fluoride, and sonicated. After centrifugation (2000 rpm for 5 min), the supernatant was recovered and spun at $10\,000\times g$ for 15 min. Protein was assayed by the Bradford method using an IgG standard. P_2 membranes of BHKN101 cells were incubated with [^{125}I]GVIA (0.5 nM) for 1.5 h on ice or at 25°C, and membrane-bound radioactivity was measured as described [11].

2.5. Electrophysiology

Whole-cell voltage-clamp recording [13] was performed using an Axopatch 1B amplifier (Axon Instruments, Foster, CA, USA). Patch electrodes with a tip resistance of 2–5 MΩ were used. Cells were held at −70 mV, then activated by a depolarization pulse at +10 mV (150 ms) preceding hyperpolarization pulse at −90 mV (20 ms), and recorded membrane currents were stored after A/D converting with Digidata 1200 (Axon Instruments, Foster, CA, USA). Results were analyzed with pClamp version 6.0 (Axon Instruments, Foster, CA, USA). The patch pipette solution contained (in mM): CsCl 120, TEA-Cl 20, EGTA-Cs 10, ATP-Mg 3, GTP 0.2, and HEPES-Cs 10. The bath solution contained (in mM): NaCl 135, $BaCl_2$ 10, CsCl 5.5, $MgCl_2$ 2, HEPES-Na 10, and tetrodotoxin 0.001. Both solutions were adjusted at pH 7.2 by CsOH or NaOH, respectively. All experiments were performed at room temperature.

3. Results

In rat cerebellar P_2 membranes, MVIIA selectively bound to N-type Ca^{2+} channel (Fig. 2). The binding of [^{125}I]GVIA was inhibited with MVIIA at low concentrations ($IC_{50} = 4.0\times10^{-10}$ M), but the binding of [^{125}I]MVIIC was not ($IC_{50} > 10^{-6}$ M). In contrast, MVIIC competed with the binding of [^{125}I]MVIIC at low concentrations ($IC_{50} = 3.0\times10^{-9}$ M), but a 20 times higher concentration was required to compete with [^{125}I]GVIA binding ($IC_{50} = 6.5\times10^{-8}$ M), thus MVIIC displayed low specificity (Fig. 2).

Previously, we found that T11A-MVIIC lost binding activity for N-type Ca^{2+} channels ($IC_{50} > 10^{-6}$ M), and only retained weak affinity for P/Q-type Ca^{2+} channels ($IC_{50} = 1.7\times10^{-6}$ M) [8]. We therefore synthesized a series of mutants by replacing Thr^{11} with various amino acids (Fig. 2, open circles). T11L showed a 30-fold increase in affinity for N-type Ca^{2+} channels ($IC_{50} = 2.2\times10^{-9}$ M), and a 10-fold decrease in affinity for P/Q-type Ca^{2+} channel ($IC_{50} = 3.3\times10^{-8}$ M), indicating improved specificity for N-type Ca^{2+} channels. On the other hand, T11V, T11Abu, T11N, T11S had varying affinities for N-type channels, but their affinity for P/Q-type channel was almost the same as T11L. The IC_{50} values of T11V, T11Abu, and T11N for the N-type channel were 3.2×10^{-8} M, 3.7×10^{-8} M, and 1.0×10^{-6} M, respectively. T11S lost affinity for the N-type channel. T11D, T11F and T11Y displayed a significant decrease in affinity for both types of Ca^{2+} channel

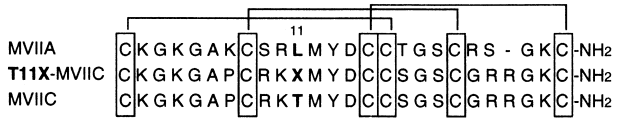

Fig. 1. Amino acid sequences and disulfide bonds of ω-conotoxin MVIIA, MVIIC and T11X-MVIIC.

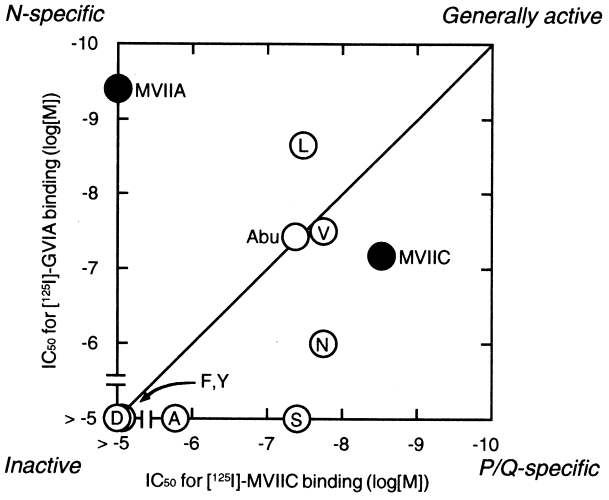

Fig. 2. Inhibition by MVIIC analogs of [^{125}I]GVIA or [^{125}I]MVIIC binding to rat cerebellar P_2 membrane fraction. Closed circles indicate the IC_{50} value of native MVIIA and MVIIC, respectively. Open circles indicate the IC_{50} values of Thr^{11} mutants of MVIIC. The substituted amino acids are indicated in the circles.

($IC_{50} > 10^{-6}$ M). These results further indicate that the amino acid at the 11th position is important for the selective binding to N- and P/Q-type Ca^{2+} channel. T11S showed marked decrease in affinity for N-type Ca^{2+} channel, but retained affinity for P/Q-type Ca^{2+} channels. Thus, we considered T11S to be an analog of MVIIC that is selective for P/Q-type Ca^{2+} channels.

Because binding assays do not directly reflect channel blockade, we used the patch-clamp technique to examine the ability of analogs to inhibit Ba^{2+} currents in BHKN101 cells expressing N-type Ca^{2+} channel genes. Surprisingly however, as shown in Fig. 3, there was essentially no difference in the inhibition of Ba^{2+} currents in BHKN101 cells between MVIIA, MVIIC and T11S, with IC_{50} values of 5.7×10^{-9}

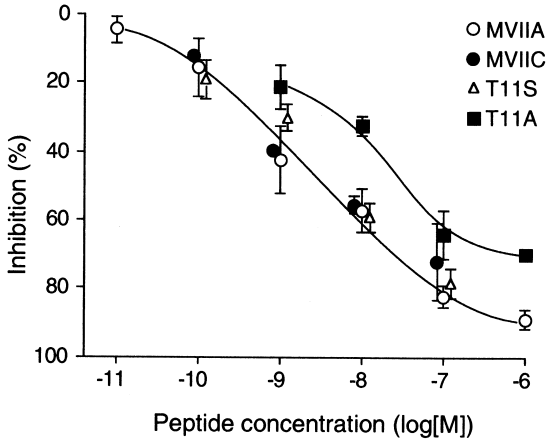

Fig. 3. Inhibition by MVIIA, MVIIC and their analogs, T11S and T11A of Ba^{2+} currents in BHKN101 cells. All experiments were performed in the whole-cell patch configuration. Cells were held at −70 mV, then activated by a depolarizing pulse to +10 mV (150 ms) preceding a hyperpolarizing pulse to −90 mV (20 ms). Conotoxins were applied to the bath. Results are expressed as means ± S.E.M. The inhibition by T11A at 10^{-8} M was the only significant difference from other conotoxins at the same concentration using Student's t test for unpaired data ($P < 0.05$).

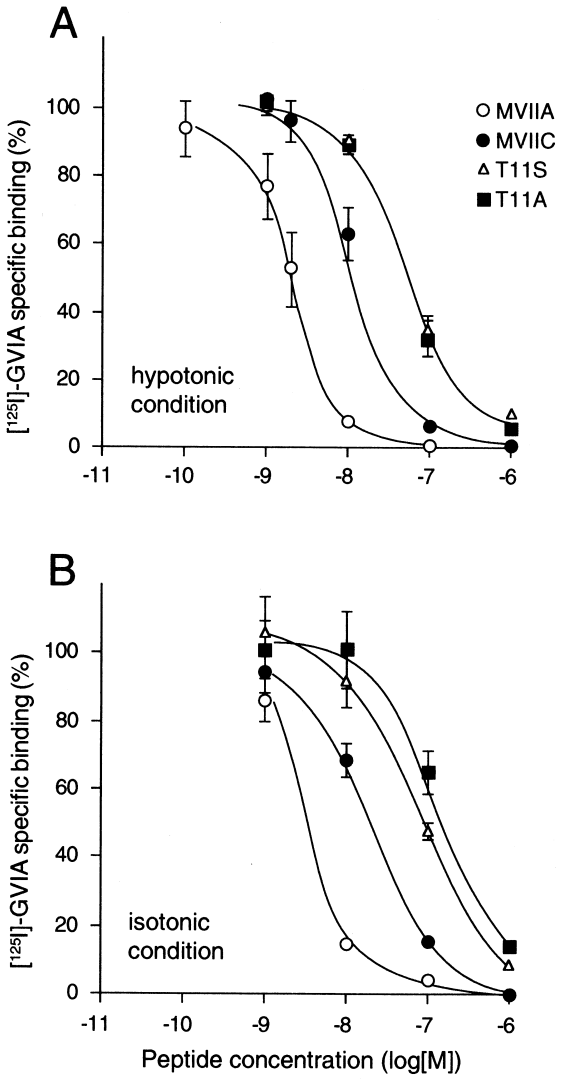

Fig. 4. Inhibition by MVIIA, MVIIC and their analogs, T11S and T11A of [^{125}I]GVIA binding to BHKN101 cell membranes. Symbols were the same as Fig. 3. A: Experiments were done in hypotonic conditions (10 mM Tris–HCl) on ice. B: Experiments were done in isotonic conditions (bath solution of whole-cell patches) at room temperature. Results are expressed as means ± S.E.M.

M, 6.9×10^{-9} M, and 7.1×10^{-9} M, respectively. T11A, which lost binding activity for cerebellar P_2 membranes, still showed significant inhibitory action with only a slight decrease in activity ($IC_{50} = 6.0\times10^{-8}$ M).

To study whether the difference came from cell preparations, binding assays were performed with BHKN101 cells, and results compared with those obtained with cerebellar P_2 membranes. Two experimental conditions were examined; (1) identical conditions to binding assays with cerebellar P_2 membranes and (2) identical conditions to patch-clamp experiments with BHKN101 cells. However, there was no significant difference of the affinity of MVIIA, MVIIC and their analogs in these conditions, except that T11A showed slightly decreased affinity in the bath solution of electrophysiological experiments (Fig. 4). In BHKN101 cells with hypotonic conditions (10 mM Tris–HCl), the rank order of competition of MVIIA, MVIIC and T11S with [^{125}I]GVIA was the same as in cerebellar P_2 membranes, but the differences were smaller. The IC_{50} value of MVIIA (2.1×10^{-9} M) was higher than in cerebellar P_2 membranes, whereas the values of MVIIC (2.4×10^{-8} M) and T11S (7.5×10^{-8} M) were decreased.

4. Discussion

Previously, we reported that Thr^{11}, Lys^{2} and Tyr^{13} of MVIIC are essential for binding to the N-type Ca^{2+} channel [5,7,8]. Among these three residues, only the amino acid at the 11th position is not conserved between MVIIA and MVIIC, and the binding activity of T11A-GVIA to the N-type Ca^{2+} channel was retained [14,15]. So, we hypothesized that Thr^{11} is an important site for MVIIC binding to Ca^{2+} channel. The results in this study are consistent with this supposition. The affinity of T11L for N-type Ca^{2+} channel was up to 1000-fold higher than T11S in rat cerebellar P_2 membranes, whereas the affinities for P/Q-type Ca^{2+} channel were the same. With analogs containing non-polar amino acids, the affinities for N-type Ca^{2+} channel were related to methylene length (L > V = Abu > A). On the other hand, addition of a hydroxyl group resulted in an increase in affinity for P/Q-type Ca^{2+} channel with the same length of side chains (T > Abu, S > A). Hydroxyl groups are known to be important for the selective binding to Ca^{2+} channels, and in particular the hydroxyl group of Tyr^{13} is essential for the activity of ω-conotoxins [10,14–17]. In contrast the basic amino acid, arginine, is essential for interaction of μ-conotoxins with Na^{+} channels [18–20]. Binding of T11S to the N-type Ca^{2+} channel was extremely weak, while low affinity binding to P/Q-type Ca^{2+} channel was conserved. Therefore the modification of other residues, in addition to the T11S mutation, will be required to design a more potent P/Q-type Ca^{2+} channel-specific analog.

In our previous investigations, we used binding assays to determine the activity of ω-conotoxins [5,7,8,11,14,16]. In this study, we measured Ba^{2+} currents in BHKN101 cells expressing the class B α_1 subunit of the N-type Ca^{2+} channel using the patch-clamp technique, revealing very similar dose-dependent inhibition by MVIIA, MVIIC and T11S. It was not however possible to examine the activity of ω-conotoxins on P/Q-type Ca^{2+} channels using BHK cells expressing the class A α_1 subunit [21], as these cells displayed very low sensitivity to both MVIIA and MVIIC (data not shown).

As the electrophysiological data were inconsistent with the results of binding assays on cerebellar P_2 membranes, binding assays were performed using BHKN101 cell membranes. The rank order of affinity of the analogs was the same as that observed for cerebellar P_2 membranes, but the differences in affinity were less marked. The affinity of MVIIC was 11-fold lower than MVIIA and 3-fold higher than T11S in BHKN101 cells, whereas in cerebellar P_2 membranes affinities differed by up to 100-fold. Moreover, when binding assays were carried out with a buffer identical to the bath solution used in the patch-clamp experiments, the results were not markedly different, except that the IC_{50} value of T11A showed a slight rightward shift (Fig. 4B).

Two aspects of our data display discrepancies that merit further discussion. Firstly differences in the affinities of ω-conotoxin analogs for binding to native N-type channels in cerebellar membranes versus heterologously expressed N-type channels in BHK cells. Native tissues express splice variants of Ca^{2+} channel α_{1B} subunit [2], which can also associate with different β and α_2/δ subunits, and this variability may lead to

differences in pharmacological sensitivity. Satin et al. showed that MVIIA-sensitive Ca^{2+} currents in HIT-T15 cells were MVIIC-insensitive [22]. In contrast Sanger et al. reported Ba^{2+} currents carried by rat N-type Ca^{2+} channel in SCG cells and HEK 293 cells that were blocked to similar extents by MVIIA or MVIIC, although MVIIA inhibited electrical contraction of smooth muscle more potently than MVIIC [23]. Differences in subunit composition may thus underlie the discrepancies in binding activity between cerebellar P_2 membranes and BHK cells expressing specific α_{1B}, α_2/δ and β_1 subunits. In brain, region-specific expression of Ca^{2+} channels with different sensitivity to ω-conotoxins may occur, and it may thus be possible to design region-specific antagonists.

Secondly electrophysiological recording indicated very similar IC_{50} values for MVIIA, MVIIC and T11S (Fig. 3) whereas binding assays revealed clear differences in affinity. For example MVIIC inhibited Ba^{2+} currents by about 40% at low concentrations (10^{-9} M), but did not significantly displace [^{125}I]GVIA binding at the same concentration (Figs. 2 and 4B). This finding is consistent with suggestions that N-type Ca^{2+} channels contain inhibitory binding sites for MVIIC which are distinct from GVIA/MVIIA binding sites [24]. However attempts to directly measure the binding of [^{125}I]MVIIC to BHKN101 cells were not successful (data not shown). Alternatively it is possible that discrepancies between patch-clamp and binding data may be related to differences in membrane potential. For electrophysiological recording drugs are applied to cells that are voltage-clamped to −70 mV, while binding assays with broken cell membrane preparations are performed at 0 mV. Voltage-gated ion channels undergo conformational changes in response to variations in membrane potential which may in turn modulate ω-conotoxin binding affinity. It is thus possible that differences in the affinities of the analogs only become apparent at depolarized membrane potentials.

Discussion in the preceding paragraphs is based on the assumption that the discrepancy in the results comes from the different nature of channels in native (but broken) membranes, and a stable cell line heterologously expressing channels. We assume that this is the most probable explanation and that BHKN101 cells are not an ideal system for this type of study. However we should consider another possibility. It is likely that T11S displays high affinity, but dissociates much more rapidly from N-type Ca^{2+} channels than wild-type toxin. The electrophysiological data would thus reflect the high affinity, but since the radioligand used in the binding assays, [^{125}I]GVIA, dissociates extremely slowly from its binding site, the binding assay may have primarily tracked rapid dissociation of the T11S analog. If this explanation is correct, T11S is not selective for P/Q-type Ca^{2+} channels under equilibrium conditions, but it could be a useful reagent since it constitutes a readily reversible N-type channel antagonist. Further evaluation of the binding kinetics would be necessary to conclusively answer this question.

In summary, on the basis of binding assays with cerebellar P_2 membranes, a P/Q-type Ca^{2+} channel-selective analog of MVIIC (T11S) was produced by replacing Thr^{11} with Ser. Affinity may yet be improved by combining other substitutions with T11S. As we recently developed combinatorial methods for the synthesis of conotoxins [12], a variety of analogs can now be synthesized concurrently. Although the lack [^{125}I]GVIA displacement indicated that T11S did not interact with N-type channels, patch-clamp studies revealed significant N-type channel blocking activity. These findings illustrate the limitations of the design and evaluation of selective channel antagonists on the basis of binding assays alone.

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