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ACCELERATED COMMUNICATION

ω-Agatoxin-TK Containing D-Serine at Position 46, but Not Synthetic ω-[L-Ser^{46}]Agatoxin-TK, Exerts Blockade of P-Type Calcium Channels in Cerebellar Purkinje Neurons

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SUMMARY

ω-Agatoxin-TK (ω-Aga-TK), a 48-amino-acid peptide isolated from the venom of the funnel web spider (*Agelenopsis aperta*), is a selective and potent inhibitor of P-type calcium channels in the nervous system. We have synthesized a peptide that has the amino acid sequence identified for native ω-Aga-TK. The synthetic ω-Aga-TK, however, showed 80–90-fold less potent inhibition of P-type calcium channels, compared with native ω-Aga-TK. Enantiomer analysis of native ω-Aga-TK revealed D-serine at position 46, and synthetic ω-[D-Ser^{46}]Aga-TK had the same potency as native ω-Aga-TK for blocking P-type calcium channels in cultured cerebellar Purkinje neurons. Two peptide fragments of ω-Aga-TK, namely ω-Aga-TK(1–43) and the carboxyl-terminal peptide fragment ω-Aga-TK(44–48), did not produce any significant inhibition of P-type calcium channels or interfere with the blockade of the channels elicited by native ω-Aga-TK. Molecular dynamics calculations showed that the carboxyl-terminal, six-amino-acid peptide of ω-Aga-TK containing D-Ser^{46} assumes a different conformation than does the peptide containing L-Ser^{46}. These results suggest that the specific conformation of the carboxyl-terminal region of ω-Aga-TK, particularly the configuration of Ser^{46}, together with a β-sheet structure formed by four disulfide bonds, might be essential for blockade of P-type calcium channels.

Multiple types of voltage-dependent calcium channels in mammalian neurons play important roles in controlling various nervous functions, such as synaptic transmission, gene expression, neuronal development, and differentiation. There are four subtypes of calcium channels, namely T-type, L-type, N-type, and P-type channels, classified on the basis of their electrophysiological and pharmacological properties (1, 2). Among these, the P-type calcium channels have recently been reported to be primarily associated with neuronal transmission, through regulation of the release of excitatory amino acids and catecholamines (3–5).

A 48-amino acid peptide termed ω-Aga-IVA, which was isolated from the venom of the funnel web spider (*Agelenopsis aperta*), was found to be a potent blocker of P-type calcium channels in rat cerebellar Purkinje neurons but had no activity against T-type, L-type, or N-type channels in a variety of neurons (6, 7). ω-Aga-IVA was recently synthesized according to the peptide sequence determined for the native toxin, and the synthetic ω-Aga-IVA was found to block P-type calcium channels with the same high selectivity and potency as the native toxin (8, 9). We have isolated a homologous peptide, ω-Aga-TK, that displays potent and selective inhibition of P-type calcium channels in rat cortical neurons (10). ω-Aga-TK is 12 times more abundant in venom than ω-Aga-IVA and has 71% amino acid identity with ω-Aga-IVA (10). Adams *et al.* (11) also found this peptide, designated ω-Aga-IVB, and showed that the peptide selectively inhibits P-type calcium channels in rat Purkinje neurons.

Amino acids in the amino-terminal region of ω-Aga-TK are quite different from those of ω-Aga-IVA, whereas the two toxins have quite well conserved amino acid residues in their carboxyl-terminal regions, except for the amino acids at positions 46 and 47 (Fig. 1). An NMR analysis of ω-Aga-TK suggested that ω-Aga-TK has four disulfide bonds at the same positions as in

ABBREVIATIONS: ω-Aga-IVA, ω-agatoxin-IVA; ω-Aga-TK, ω-agatoxin-TK; DMEM, Dulbecco's modified Eagle's medium; ω-Aga-TK(L), ω-[L-Ser^{46}]agatoxin-TK; ω-Aga-TK(D), ω-[D-Ser^{46}]agatoxin-TK; MD, molecular dynamics; ω-CgTx, ω-conotoxin GVIA; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

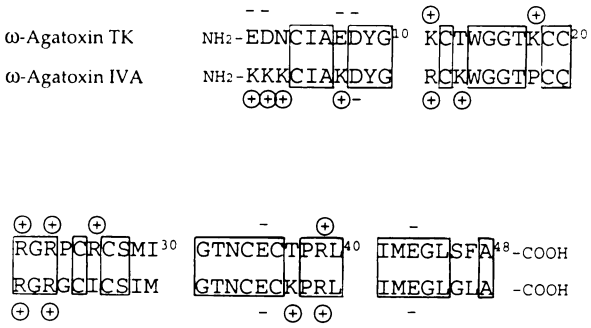

Fig. 1. Amino acid sequences of ω-Aga-TK and ω-Aga-IVA. Identical residues in the two sequences are *boxed*. + and −, Basic and acidic amino acid residues, respectively.

synthetic ω-Aga-IVA, forming a three-stranded antiparallel β-sheet structure, and that the three amino-terminal residues (Glu^1–Asn^3) and the 10 carboxyl-terminal residues (Arg^{39}–Ala^{48}) adopt random-coil conformations (11, 12). It is therefore likely that the carboxyl-terminal tails of the two toxins play a role in the inhibition of P-type calcium channels.

In the present study, we have synthesized ω-Aga-TK and examined its inhibitory effect on P-type calcium channels. We found that synthetic (all L-amino acid) ω-Aga-TK showed much less potent inhibition of P-type calcium current in rat Purkinje neurons than did native ω-Aga-TK, and we established that the presence of D-serine at position 46 in the native molecule is essential for the blockade of P-type calcium channels.

Experimental Procedures

Materials. Fetal calf serum, heat-inactivated horse serum, trypsin solution, penicillin, streptomycin, and DMEM were obtained from Life Technologies Inc. (Grand Island, NY), and insulin, sodium selenite, putrescine, biotin, progesterone, sodium pyruvate, poly-L-lysine, nifedipine, cytosine arabinofuranoside, and DNase I were from Sigma Chemical Co. (St. Louis, MO). ω-CgTx and ω-Aga-IVA were purchased from Peptide Institute Inc. (Osaka, Japan). Other reagents used were of reagent grade.

Native and synthetic ω-Aga-TK. ω-Aga-TK was purified to homogeneity from the crude venom of *A. aperta* by a two-step reverse phase HPLC procedure, as described in detail previously (10). Two stereoisomers of ω-Aga-TK containing either L- or D-serine at position 46, namely ω-Aga-TK(L) and ω-Aga-TK(D), were synthesized basically by the method described previously (8), using a peptide synthesizer (type 430A; Applied Biosystems, Foster City, CA). Details will be reported elsewhere.

Limited proteolysis. Native ω-Aga-TK and ω-Aga-TK(L) were digested with *Staphylococcus aureus* V8 protease (Miles Scientific, Naperville, IL). Each peptide (250 μg) was dissolved in 1.5 ml of 0.1 M ammonium acetate buffer, pH 4.0, and the solutions were incubated with V8 protease at a substrate/enzyme weight ratio of 1:15 or 1:30, as described in the legend to Fig. 2. The digested fragments were purified by reverse phase HPLC (LC-10A; Shimadzu, Kyoto, Japan) on a TSK ODS-120T column (4.6 mm × 150 mm; Tosoh Co., Tokyo, Japan), using a linear gradient system consisting of acetonitrile (10–50%) in 0.1% aqueous trifluoroacetic acid. The flow rate was constant at 1.2 ml/min, and eluted peptides were detected at 215 nm. The fractions corresponding to the peptide fragments and full length ω-Aga-TK were collected manually and were immediately dried with a Speed-Vac concentrator.

Peptide characterization. The chromatographic properties of synthetic ω-Aga-TK(L) and ω-Aga-TK(D) were compared with those of native ω-Aga-TK by reverse phase HPLC under acidic and neutral elution conditions. The acidic elution was performed on a TSK ODS-120T column using a linear gradient of acetonitrile (10–50%) in 0.1% aqueous trifluoroacetic acid, at a flow rate of 1.2 ml/min. The neutral elution was carried out on a Capcell Pac C_{18} column (4.6 mm × 150 mm; Shiseido Co., Tokyo, Japan) using a linear gradient of acetonitrile (10–60%) in 0.1 M dipotassium hydrogen phosphate, pH 7.0, at a flow rate of 1.0 ml/min. Peptide fragments generated by limited proteolysis were characterized with a Shimadzu PSQ-1 gas-phase protein sequencer, Beckman (Palo Alto, CA) 6300 amino acid analyzer, JEOL (Tokyo, Japan) SX-102A fast-atom bombardment mass spectrometer, and Kratos MALDI III laser desorption time-of-flight mass spectrometer (Shimadzu Europe GmbH, Duisburg, Germany). One nanomole of the peptides was hydrolyzed with 6 N HCl for 24 hr at 110°. The D- and L-amino acids in the acid hydrolysates were determined by HPLC after derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate (Fluka Chemie AG, Buchs, Switzerland), according to the method of Einarsson *et al.* (13).

MD calculations. Molecular mechanics and MD calculations were performed using the AMBER 3.0 (revision A) software package (14). The all-atom force field was employed for all standard residues. Electrostatic and van der Waals interactions were calculated between all atom pairs. The carboxyl-terminal tail structures of ω-Aga-TK(D) and ω-Aga-TK(L) were represented by the *N*-acetyl, carboxyl-terminal, six-residue peptide, and the conformational properties of the peptides were analyzed by high-temperature MD simulation at 1000°K for 100 psec. Every 50 fsec the structure was saved, and after the simulation the saved structures were minimized and classified into 36 conformation categories defined by the Cα dihedral angle of four amino acid residues at positions 43–46 or 44–47. All calculations were performed on a Silicon Graphics Indigo^2 workstation.

Cell culture. Cerebellar Purkinje neurons were prepared from Wistar rats of postnatal day 1. The cerebella were dissected in ice-cold Hanks' balanced salt solution and incubated at 37° for 15 min in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution containing 0.25% trypsin and 0.2 mg/ml DNase I. The cerebellar tissues were then dissociated to single cells by gentle trituration using a glass pipette with a fire-polished tip. The cell suspension was mixed with DMEM supplemented with 10% fetal calf serum, 10% heat-inactivated horse serum, 5 μg/ml insulin, 30 nM sodium selenite, 100 μM putrescine, 20 nM progesterone, 15 nM biotin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate, as described (15). The cell suspension was centrifuged and the resulting pellets were resuspended in the medium described above. The cerebellar cells were then pelleted again by centrifugation, suspended in the medium, and plated onto poly-L-lysine-coated coverslips. The cells were cultured in a CO_2 incubator (5% CO_2) at 37° for 1 day and the coverslips were then transferred onto a confluent glial cell layer and cultured for 2–3 weeks in DMEM containing 5% heat-inactivated horse serum, 25 mM KCl, and the same supplements as described above. The cerebellar cells were treated with 10 μM cytosine arabinofuranoside for 4 days (it was added to the culture medium 1 day after plating) to reduce the growth of contaminating non-neuronal cells. The glial cells used were obtained from postnatal day 1 rats of the Wistar strain. The cerebral cortex was dissected and triturated in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, and the glial cells were cultured in a CO_2 incubator (5% CO_2) at 37° for 2–3 weeks before use.

Electrophysiological recording. Whole-cell membrane currents were recorded by a perforated patch technique using the ionophore nystatin (Wako Pure Chemicals, Osaka, Japan), as described by Horn and Marty (16). The pipette solution contained 130 mM CsCl and 5 mM HEPES, and the pH was adjusted to 7.2 with CsOH. The glass patch electrode had a tip resistance of 4–8 MΩ. Cerebellar Purkinje neurons cultured on a coverslip were mounted in a recording chamber (0.3 ml) and were superfused with Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM D-glucose, 10 mM HEPES; the pH was adjusted to 7.4 with NaOH) at a flow rate of 3 ml/min. After whole-cell currents reached a steady state, the bathing solution

was replaced with an external solution for recording of calcium currents. The external solution contained 145 mM tetraethylammonium chloride, 5 mM $CaCl_2$, 24 mM D-glucose, and 10 mM HEPES, and the pH was adjusted to 7.4 with tetraethylammonium hydroxide. Calcium channel currents filtered at 1 kHz were recorded with a patch-clamp amplifier (Axopatch-1D; Axon Instruments, Foster City, CA). The calcium currents were elicited by application of 100–200-msec pulses to −10 mV from a holding potential of −80 mV every 15 or 20 sec. Control experiments showed that the calcium currents did not exhibit a significant run-down phenomenon during recordings. When the calcium currents showed a steady level for at least 1 min, the external solution was changed to a solution containing a test compound. Data were digitized and analyzed using pCLAMP software (Axon Instruments). Calcium currents were corrected for leak and capacitative currents by subtraction of an appropriately scaled current elicited by a $P/5$-mV hyperpolarization. All experiments were carried out at 23–25°. Values are expressed as means ± standard errors.

Results

Structural difference between native and synthetic ω-Aga-TK. We have synthesized ω-Aga-TK(L), composed of all L-amino acids, and compared it with native ω-Aga-TK. The native ω-Aga-TK molecule isolated from the spider venom had chromatographic properties distinct from those of synthetic ω-Aga-TK(L). Reverse phase HPLC analysis showed that the native peptide was eluted more slowly than the synthetic peptide under neutral conditions [ω-Aga-TK(L), 15.6 min; native ω-Aga-TK, 17.4 min], although the native and synthetic peptides had very similar retention times under acidic conditions [ω-Aga-TK(L), 39.4 min; native ω-Aga-TK, 39.8 min]. After reduction of the disulfide bonds with dithiothreitol, native ω-Aga-TK still had a longer retention time, compared with synthetic ω-Aga-TK(L). Because native ω-Aga-TK had a molecular weight (MH^+ of 5274) identical to that of synthetic ω-Aga-TK(L), as determined by laser desorption mass spectrometry, the most probable explanation for these results was the presence of D-amino acid(s) in native ω-Aga-TK.

Limited proteolysis of ω-Aga-TK. The HPLC profiles of native ω-Aga-TK and synthetic ω-Aga-TK(L) after limited proteolysis with *S. aureus* V8 protease are shown in Fig. 2. To inhibit the possible rearrangements of disulfide bridges in the peptide fragments, the proteolysis and subsequent HPLC purification were performed under acidic conditions (17). Two peptide fragments were generated from native and synthetic ω-Aga-TK(L), and these peptides were characterized by automated Edman degradation, amino acid analysis, and mass spectrometry. The results indicated that the V8 protease cleaved only the carboxyl-terminal Glu^{43}-Gly^{44} bond of both native and synthetic ω-Aga-TK(L), and the fragments were identified as residues 1–43 [ω-Aga-TK(1–43)] and a carboxyl-terminal pentapeptide [ω-Aga-TK(44–48)], as designated in Fig. 2. ω-Aga-TK(1–43) derived from the native and synthetic peptides had the same retention times by HPLC analysis and the same molecular weight (MH^+ of 4779) by laser desorption mass spectrometry. In addition, Edman degradation of both peptides revealed identical amino-terminal sequences starting with Glu^1. These data clearly indicate that no bond other than the Glu^{43}-Gly^{44} bond was cleaved and that the ω-Aga-TK(1–43) fragments from native and synthetic ω-Aga-TK had the same primary structure. In contrast, in HPLC analysis ω-Aga-TK(44–48) derived from the native peptide was eluted slightly faster than that derived from synthetic ω-Aga-TK(L). No difference in amino acid sequence or molecular weight (MH^+ of 495) was found between the ω-Aga-TK(44–48) fragments from the native and synthetic peptides. However, the native ω-Aga-TK was more resistant to V8 proteolysis than was synthetic ω-Aga-TK(L); when the peptides were incubated at an enzyme/substrate weight ratio of 1:15 at 37° for 16 hr, 78% of synthetic ω-Aga-TK(L) was digested, whereas only 28% of native ω-Aga-TK was hydrolyzed. These results strongly suggested that the conformations around the Glu^{43}-Gly^{44} bond might be different in native ω-Aga-TK and synthetic ω-Aga-TK(L).

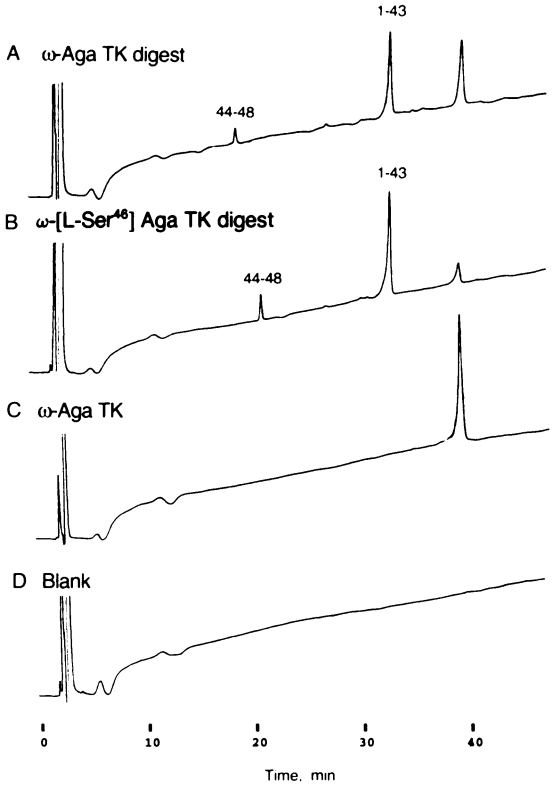

Fig. 2. HPLC profiles of native and synthetic ω-Aga-TK(L) after limited proteolysis by *S. aureus* V8 protease. A, The digest of native ω-Aga-TK incubated at an enzyme/substrate weight ratio of 1:15 for 40 hr at 37°. B, The digest of synthetic ω-Aga-TK(L) incubated at an enzyme/substrate weight ratio of 1:30 for 16 hr at 37°. C, Native ω-Aga-TK without any treatment. D, Digestion blank incubated under the same conditions as in A, in the absence of ω-Aga-TK.

D-Serine residue in native ω-Aga-TK. Table 1 shows the results of amino acid analysis of D- and L-enantiomers present in the peptide fragments of native ω-Aga-TK and synthetic ω-Aga-TK(L). The results clearly demonstrate that ω-Aga-TK(44–48) contains one mol of D-serine in the molecule. On the other hand, ω-Aga-TK(1–43) was found to consist of only L-amino acids, because the data for the ω-Aga-TK(1–43) fragments derived from native ω-Aga-TK and ω-Aga-TK(L) were basically identical. Under the conditions used, amino acids were approximately 10% racemized during acid hydrolysis, which is in good agreement with the report by Hayashi and Sasagawa (18). On the basis of these results, it was concluded that ω-Aga-TK purified from the venom of *A. aperta* contains D-serine at position 46. This was confirmed by synthesis of ω-Aga-TK(D), followed by comparison of the chromatographic and pharmacological properties of the natural and synthetic peptides. ω-Aga-TK(D) exhibited HPLC properties identical to those of

TABLE 1

Amino acid composition of the peptide fragments ω-Aga-TK(1–43) and ω-Aga-TK(44–48) derived from native and synthetic ω-Aga-TK(L)

Amino acid values are given as molar ratios. Theoretical compositions based on the amino acid sequence are given in parentheses. Tryptophan and cysteine were not determined.

Amino acid	Native ω-Aga-TK(44–48)		Native ω-Aga-TK(1–43)		Synthetic ω-Aga-TK(1–43)	
	D-form	L-form	D-form	L-form	D-form	L-form
			mol			
Asp	0	0	0.5	3.2 (4)	0.5	3.3
Thr	0	0	0	3.2 (4)	0	3.4
Ser	0.8	0 (1)	0	0.9 (1)	0	0.8
Glu	0	0.1	0.2	3.6 (4)	0.2	3.6
Pro	0	0	0	2.0 (2)	0	2.2
Ala	0	1.0 (1)	0.1	1.1 (1)	0	1.0
Val	0	0	0.1	0.1	0	0
Met	0	0	0.1	1.5 (2)	0.1	1.6
Ile	0	0	0.2	2.8 (3)	0.1	3.2
Leu	0	1.0 (1)	0.1	1.0 (1)	0.1	1.1
Tyr	0	0	0.1	0.5 (1)	0.1	0.4
Phe	0	1.0 (1)	0.1	0	0	0
His	0	0	0.1	0	0.1	0
Lys	0	0	0.3	2.3 (2)	0.3	2.0
Arg	0	0	0.3	3.5 (4)	0.3	3.6

native ω-Aga-TK [the retention time of ω-Aga-TK(D) under neutral elution conditions was 17.4 min], and synthetic ω-Aga-TK(D) had the same pharmacological potency as did native ω-Aga-TK, as described below.

Electrophysiological properties of native and synthetic ω-Aga-TK. The effect of synthetic ω-Aga-TK(L) on P-type calcium channels in cultured rat cerebellar Purkinje neurons was examined. The Purkinje neurons were identified based on their characteristic morphology, immunochemical staining for calbindin D-28k, and their electrophysiological properties, which were similar to those of acutely isolated Purkinje neurons (6, 7). A combination of subtype-specific blockers of calcium channels, namely nifedipine at 3 μM (L-type) and ω-CgTx at 3 μM (N-type), reduced the calcium currents by 17.9 ± 1.2% (n = 70), and the remaining currents were completely abolished by further addition of ω-Aga-IVA at 100 nM (data not shown). These results indicate that the cultured Purkinje neurons have ω-Aga-IVA-sensitive P-type calcium channels as a major component of high-threshold calcium channels. The calcium currents that were insensitive to nifedipine and ω-CgTx were also completely inhibited by native ω-Aga-TK at 200 nM (Fig. 3, a and b). ω-Aga-TK(L) at 200 nM did not show any significant inhibition of P-type calcium current, although ω-Aga-TK(L) at 500 nM inhibited the P-type current by approximately 45% (Fig. 3c). The P-type calcium current was reduced by 70% by ω-Aga-TK(L) at 1 μM, and the remaining currents were completely inhibited by addition of native ω-Aga-TK at 300 nM (Fig. 3d).

Effects of ω-Aga-TK fragment peptides. We have also examined the effects on the P-type calcium current in Purkinje neurons of peptide fragments generated by limited proteolysis of native ω-Aga-TK with V8 protease. Neither ω-Aga-TK(1–43) nor ω-Aga-TK(44–48) at 300 nM inhibited the P-type calcium current, whereas the remaining currents were completely abolished by additional treatment with 300 nM ω-Aga-TK (Fig. 3, e and f). Neither of the fragment peptides at 1 μM showed significant inhibition of P-type calcium current (data not shown). In addition, a combination of ω-Aga-TK(1–43) and ω-Aga-TK(44–48), both at 300 nM, did not cause significant inhibition of the calcium current, and the remaining currents were completely blocked by addition of native ω-Aga-TK at 300 nM (Fig. 3g).

Blockade of P-type current by synthetic ω-Aga-TK(D). The effect of synthetic ω-Aga-TK(D) on P-type calcium current was examined. ω-Aga-TK(D) at 200 nM completely blocked the P-type calcium current within 3 min (Fig. 3h). The time course for depression of the P-type current by ω-Aga-TK(D) was similar to that for native ω-Aga-TK. Concentration-response curves for the inhibition of P-type calcium current by native ω-Aga-TK and ω-Aga-TK(D) were superimposable (Fig. 4), whereas the inhibitory activity of ω-Aga-TK(L) was very much lower than that of native ω-Aga-TK. The IC_{50} values for the blockade of P-type current by native ω-Aga-TK, ω-Aga-TK(D), and ω-Aga-TK(L) were 13, 15, and 1190 nM, respectively, indicating that ω-Aga-TK(L) is an 80–90-fold less potent blocker of P-type calcium channels than is native ω-Aga-TK and ω-Aga-TK(D).

Effects of ω-Aga-TK(L) and fragment peptides on blockade of P-type channels by ω-Aga-TK. We examined whether ω-Aga-TK(L) or the digested peptide fragments of ω-Aga-TK interfere with the blockade of P-type calcium current by native ω-Aga-TK. As shown in Fig. 5, neither ω-Aga-TK(L) nor the fragment peptides at 300 nM affected the inhibition of P-type current by native ω-Aga-TK (300 nM), and the rate of current depression was similar to that produced by native ω-Aga-TK alone. The fragment peptides at 1 μM did not affect the blockade of P-type calcium channels by native ω-Aga-TK at 300 nM (data not shown).

Conformation of carboxyl-terminal peptides. The most stable conformations of the carboxyl-terminal, six-residue peptides of ω-Aga-TK(D) and ω-Aga-TK(L) were calculated after MD simulations. The difference in the conformations predicted for ω-Aga-TK(D) and ω-Aga-TK(L) is illustrated in Fig. 6. These molecular models are stabilized by several hydrogen bonds, including that between the carboxylate of the side-chain of Glu^{43} and the hydroxyl hydrogen of Ser^{46}. Backbone structures between Glu^{43} and Ser^{46} are quite similar in the two peptides, but the backbones from Ser^{46} to Ala^{48} extend in opposite directions because of the difference in chirality of the Ser^{46} residue.

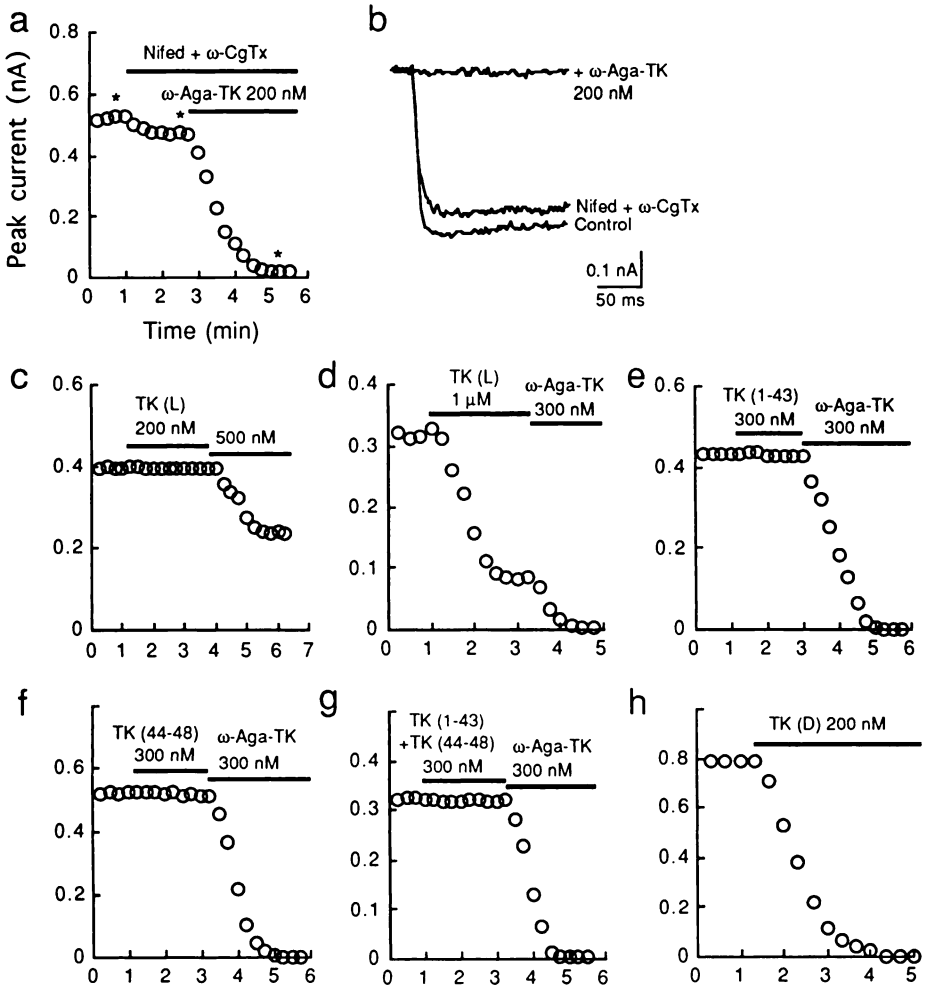

Fig. 3. Effects of native ω-Aga-TK, synthetic ω-Aga-TK(L), ω-Aga-TK(D), and the peptide fragments of ω-Aga-TK on P-type calcium current in cerebellar Purkinje neurons. The calcium currents were elicited by step pulses from −80 mV to −10 mV every 15 sec and the amplitude of peak currents was plotted against time. a, Inhibitory effect of native ω-Aga-TK, in the presence of nifedipine (*Nifed*) (3 μM) and ω-CgTx (3 μM), on high-threshold calcium currents. b, Current traces at the times shown by *asterisks in a*. c and d, Effects of synthetic ω-Aga-TK(L) on P-type calcium current. e and f, Effects of the peptide fragments of native ω-Aga-TK, namely ω-Aga-TK(1–43) and ω-Aga-TK(44–48), on P-type calcium current. g, Effects of a combination of ω-Aga-TK(1–43) and ω-Aga-TK(44–48) on P-type calcium current. h, Inhibitory effect of synthetic ω-Aga-TK(D) on P-type calcium current.

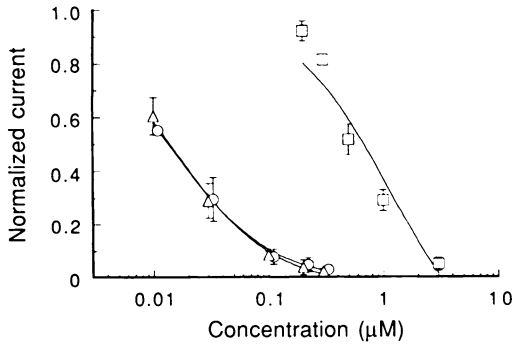

Fig. 4. Concentration-response curves for blockade of P-type calcium current in cerebellar Purkinje neurons by native ω-Aga-TK, synthetic ω-Aga-TK(L), and ω-Aga-TK(D). Experiments were performed in the presence of nifedipine and ω-CgTx, both at 3 μM. The amplitudes of peak current after addition of native ω-Aga-TK (○), ω-Aga-TK(L) (□), or ω-Aga-TK(D) (△) were normalized to the values obtained before addition of ω-Aga-TK. Each *point* represents the mean ± standard error of two to 16 measurements. The curves were fitted by the equation $I = 1 - I_{max}(1 + IC_{50}/[\text{agatoxin}])$, where I_{max} and I are the normalized amplitudes of calcium current in the absence and presence of agatoxins, respectively, and [agatoxin] is the concentration of native or synthetic ω-Aga-TK.

Discussion

ω-Aga-TK and ω-Aga-IVA isolated from the funnel web spider *A. aperta* are homologous peptides, composed of 48 amino acids, that selectively inhibit P-type calcium channels in the nervous system (7, 10, 11). ω-Aga-IVA was previously synthesized by Nishio *et al.* (8) and synthetic ω-Aga-IVA has activity indistinguishable from that of native ω-Aga-IVA against the P-type calcium channels in cerebellar Purkinje neurons (8, 9). We have synthesized ω-Aga-TK(L), composed of all L-amino acids, by a similar method, based on the proven amino acid sequence of native ω-Aga-TK isolated from the crude venom. The potency of synthetic ω-Aga-TK(L), however, was 80–90-fold less than that of native ω-Aga-TK and HPLC analysis showed different elution behavior for synthetic ω-Aga-TK(L) and the native peptide. These results raised the possibility that synthetic ω-Aga-TK(L) contains inappropriate disulfide bonds, introduced through oxidation. However, this was considered unlikely, because the estimated positions of the four disulfide bonds in synthetic ω-Aga-IVA oxidized by the same method as in the case of ω-Aga-TK(L) are in good agreement with the positions of the bonds in native ω-Aga-TK, as suggested by Adams *et al.* (11) on the basis of NMR analysis. Moreover, after dithiothreitol treatment the reduced peptide of synthetic ω-Aga-TK(L) had a different retention time by HPLC analysis, compared with that of reduced ω-Aga-TK, although both peptides had the same molecular weight, as shown by mass spectrometry. These results strongly suggested that native ω-Aga-TK contained D-amino acids. An enantiomer analysis clearly demonstrated that native ω-Aga-TK contains D-serine at position 46 (Table 1). Finally, it was demonstrated that synthetic

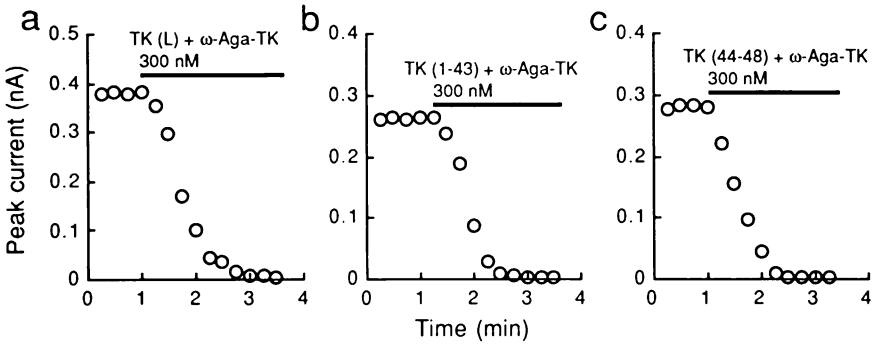

Fig. 5. Blockade of P-type calcium current by native ω-Aga-TK in the presence of ω-Aga-TK(L) or peptide fragments of native ω-Aga-TK. Effects of synthetic ω-Aga-TK(L) (a), ω-Aga-TK(1–43) (b), and ω-Aga-TK(44–48) (c) on the blockade of P-type calcium current by native ω-Aga-TK at 300 nM are shown. Native ω-Aga-TK and one of the aforementioned peptides were added to the bath solution simultaneously. All experiments were performed in the presence of nifedipine (3 μM) and ω-CgTx (3 μM), and the peptides were added to the bath solution after the calcium current had been stably inhibited by nifedipine and ω-CgTx.

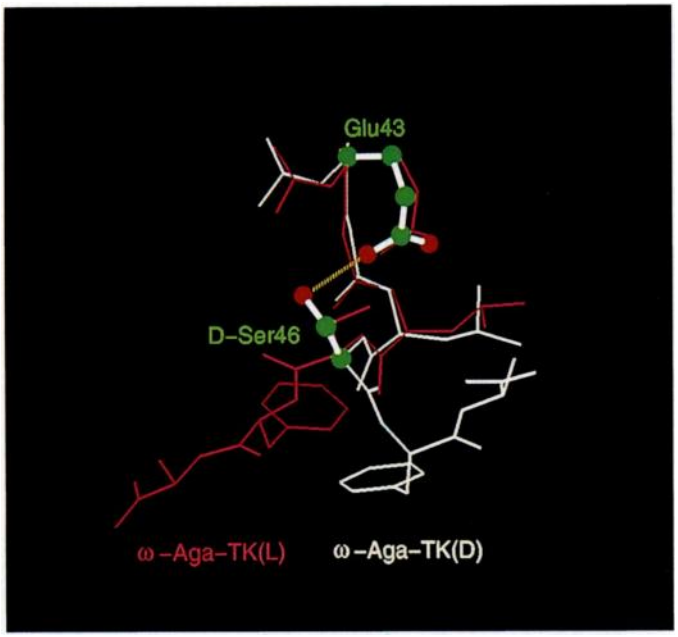

Fig. 6. Conformations predicted for the carboxyl-terminal, six-amino acid peptides. The carboxyl-terminal, six-amino acid peptides of ω-Aga-TK(L) and ω-Aga-TK(D) are superimposed. Glu^{43} and D-Ser^{46} residues are emphasized with a ball-and-stick model. *Green atoms*, carbon atoms; *red atoms*, oxygen atoms. *Yellow dashed line*, an important hydrogen bond.

ω-Aga-TK(D) has full inhibitory activity against P-type calcium channels in cerebellar Purkinje neurons. ω-Aga-TK is the first D-amino acid-containing peptide to have been isolated from a spider venom, although a few peptides are known to contain D-amino acid residues, such as frog skin dermorphins (19), deltorphins (20), a peptide that interacts with an adenosine receptor (21), and peptides isolated from an African giant snail (22).

ω-Aga-TK contains two serine residues, at positions 28 and 46, of which only Ser^{46} appeared to be in the D-configuration. The pathway of insertion of the D-serine residue into ω-Aga-TK is not clear. There are two possibilities. First, D-serine in place of L-serine might be inserted into the peptide at the translational level. If this is the case, it would be difficult to explain why only one serine residue of the two is translated in the D-configuration in a single molecule. The second possibility is that a L-serine residue in the nascent peptide might be converted to D-serine as a post-translational modification. In the case of the heptapeptide dermorphine, which was isolated from frog skin, it has been suggested from an analysis of cDNA of the precursor molecule of delmorphine that the D-alanine residue of the peptide is generated from L-alanine after translation (23). If a similar post-translational modification occurs in nascent ω-Aga-TK molecules, it is possible that the unidentified racemase or isomerase that converts L-Ser^{46} to D-Ser^{46} cannot convert L-Ser^{28} to D-Ser^{28} because of steric hindrance exerted by amino acids surrounding L-Ser^{28}.

ω-Aga-TK retains 71% amino acid sequence homology to ω-Aga-IVA, and both are selective inhibitors of P-type calcium channels with very similar potencies for the blockade of P-type calcium current in cultured cerebral cortical neurons and cerebellar Purkinje neurons (10, 11). It has been suggested that the two peptides have four disulfide bonds at the same positions, namely Cys^{4}-Cys^{20}, Cys^{12}-Cys^{25}, Cys^{19}-Cys^{36}, and Cys^{27}-Cys^{34}, which form an antiparallel β-sheet structure (11, 12), and that the two peptides have a flexible tail in the carboxyl-terminal region (11, 12), in which the amino acid sequences are well conserved, although this is not the case in the amino-terminal region (Fig. 1). Nishio *et al.* (8) found that amino-terminally truncated ω-Aga-IVA(4–48) showed the same inhibitory activity as did intact ω-Aga-IVA. It is therefore likely that the intramolecular moieties contributing to the inhibitory action against calcium channels are common structures in the two peptides, and the most probable candidates are the rigid β-sheet structure and the flexible tail. We compared the inhibitory potencies of two ω-Aga-TK fragments, namely ω-Aga-TK(1–43) and ω-Aga-TK(44–48), obtained by limited proteolysis of native ω-Aga-TK with V8 protease. Neither of these fragments showed significant inhibition of P-type calcium channels, suggesting that the two fragments might block the calcium channels only when they are held in a specific conformation in a single molecule.

Yu *et al.* (12) proposed that positively charged amino acid residues present on the surface of the β-sheet structure might interact with negatively charged moieties of the calcium channels and that the carboxyl-terminal flexible tail might interfere with the passage of calcium ions through channel pores. We have examined whether the fragment peptides of ω-Aga-TK, namely ω-Aga-TK(1–43) and ω-Aga-TK(44–48), and a much less potent peptide, ω-Aga-TK(L), affect the inhibitory activity of native ω-Aga-TK. Neither ω-Aga-TK(L) nor the peptide fragments affected the blockade of P-type calcium channels at concentrations equimolar to that of native ω-Aga-TK or greater (Fig. 5). It is therefore unlikely that the β-sheet structure alone can form the binding domain of ω-Aga-TK for the P-type calcium channel molecules.

The inhibition curve for ω-Aga-TK(L) against P-type calcium channel activity is shifted to the right by almost two orders of magnitude, compared with that for ω-Aga-TK(D),

suggesting that ω-Aga-TK(L) has 80–90-fold lower affinity for P-type calcium channels than does ω-Aga-TK(D). If the two peptides share a common binding site on the P-type channel molecules, then the reduced affinity of ω-Aga-TK(L) for the binding site of the channel molecules might be caused only by the difference in the carboxyl-terminal conformation. However, a carboxyl-terminal pentapeptide containing D-Ser[46], namely ω-Aga-TK(44–48), did not produce any significant inhibition of P-type calcium channels, so it is unlikely that the carboxyl-terminal region alone interferes with the passage of calcium ions through channel molecules. In addition, ω-Aga-IVA containing a modified Trp[14] residue lacked inhibitory activity against P-type calcium channels (8). Therefore, the antiparallel β-sheet structure may also be essential for the blockade of P-type calcium channels. We propose that ω-Aga-TK forms a high affinity binding domain only when the peptide molecule adopts a specific conformation involving the β-sheet structure and the carboxyl-terminal region containing D-Ser[46] and that ω-Aga-TK(L) is unable to form such a conformation, so that it has only a low affinity binding domain for the calcium channels. The finding that ω-Aga-TK(L) did not inhibit the blockade of P-type calcium current by ω-Aga-TK(D) may support this hypothesis, which can be further tested by binding studies with labeled ω-Aga-TK.

The influence of the configuration of Ser[46] on the carboxyl-terminal conformation of ω-Aga-TK was examined by digestion experiments with V8 protease. The protease cleaved the peptide bond between Glu[43] and Gly[44], indicating that the carboxyl-terminal region of ω-Aga-TK is relatively freely accessible to the enzyme. However, the Glu[43]-Gly[44] peptide bond of ω-Aga-TK(L) was more susceptible to cleavage by the protease than was that of ω-Aga-TK(D), strongly suggesting that the molecular shapes in the peptide region surrounding the Ser[46] residue are rather different in ω-Aga-TK(L) and ω-Aga-TK(D). A theoretical conformational analysis of the six-amino acid peptides in the carboxyl-terminal region of ω-Aga-TK(D) and ω-Aga-TK(L) clearly showed that the two peptides adopt different conformations, with backbone structures extending in opposite directions (Fig. 6).

The amino acids at positions 46 and 47 of ω-Aga-IVA are Gly[46] and Leu[47], respectively, replacing D-Ser[46] and Phe[47] of ω-Aga-TK, although other amino acids in the carboxyl-terminal region are the same in the two peptides. The effect of substitution of amino acids at position 47 on inhibitory activity has not yet been elucidated. The substitution of amino acids in the carboxyl-terminal region of ω-Aga-TK would provide a clue to assess the interaction between inhibitors and P-type calcium channels. For instance, it is very likely that inhibitory activity would be retained after substitution of D-Ser[46] in ω-Aga-TK with glycine, which is present as Gly[46] in the ω-Aga-IVA molecule. It will also be of interest to examine the effects of sequential truncation of the carboxyl-terminal region of ω-Aga-TK on the inhibitory activity.

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