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A NOVEL PEPTIDE FROM FUNNEL WEB SPIDER VENOM, ω-Aga-TK, SELECTIVELY BLOCKS P-TYPE CALCIUM CHANNELS

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SUMMARY: In the course of purification of ω-Aga-IVA, a specific P-type calcium channel blocker, from the venom of *Agelenopsis aperta* we discovered a novel peptide. This peptide, named ω-agatoxin Tsukuba (ω-Aga-TK), also blocked P-type channels and was twelve times more abundant in the venom than ω-Aga-IVA. ω-Aga-TK was purified to homogeneity by a two-step reverse-phase HPLC procedure. Its amino acid sequence is 71% identical to that of ω-Aga-IVA. ω-Aga-TK has a negatively charged N-terminus, whereas ω-Aga-IVA has a positively charged one. Electrophysiological data indicate that ω-Aga-TK is a potent and selective inhibitor of P-type channels.

Voltage-dependent calcium channels in neurons are very important in controlling various functions in the nervous system. There are four subtypes of calcium channels, namely T, L, N, and P-type, based on their electrophysiological and pharmacological properties (1,2). Among these four subtypes, the P-type calcium channel has been reported to be associated with the release of glutamate (3). However, the function of the P-type calcium channel in the nervous system has yet to be fully elucidated. The venom of the funnel web spider *Agelenopsis aperta* (*A. aperta*) (4,5) and the peptide ω-Aga-IVA (6,7), isolated from the venom, have been shown to block the P-type calcium channel. In the course of purification of this peptide, for a study of its effect on neurons, we discovered a novel peptide, which we have named ω-agatoxin Tsukuba (ω-Aga-TK), that also displayed a potent and selective inhibition of the P-type calcium channel. ω-Aga-TK was twelve times more abundant than ω-Aga-IVA in the venom of *A. aperta*. We report here the isolation and characterization of ω-Aga-TK.

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Materials and Methods

Purification

Frozen crude venom from the spider, *A. aperta*, was purchased from Spider Pharm (Feasterville, PA). Purification was performed by high-performance liquid chromatography (HPLC) (TSK CCPM instrument; Tosoh Co., Tokyo, Japan). In the first step, 20-70 µl of the crude venom was fractionated on a Brownlee RP-8 column (4.6 mm x 25 cm, 300 Å, 7 µm, Applied Biosystems Inc., San Jose, CA) with a linear gradient of acetonitrile from 0 to 50% in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min . Peaks were collected individually and evaporated on a speed-vac concentrator. In the second step, fraction 19 (see Fig.1A), which contained both ω-Aga-TK and ω-Aga-IVA, was applied to a Spherisorb C8 column (4.6 mm x 15 cm, 80 Å, 5 µm, GL Science, Tokyo, Japan) and eluted with a linear gradient of 2-propanol from 15 to 45% in 1% TFA at a flow rate of 1 ml/min.

Structure determination

Mass spectrometric measurement of the purified ω-Aga-TK was performed on a matrix-assisted laser desorption time-of-flight mass spectrometer (Kratos MALDI II, Shimadzu, Kyoto, Japan).
The amino acid composition of the peptide was determined by using a Beckman 6300 amino acid analyzer after acid hydrolysis at 110 °C *for 24 hr in vacuo*.

The complete amino acid sequence was determined by the combination of N-terminal amino acid sequence analysis of S-carboxyamidemethylated ω-Aga-TK, analysis of the peptide fragments generated by lysyl endopeptidase (Wako Pure Chem., Osaka, Japan), and C-terminal analysis using carboxypeptidase Y (Pierce, Rockford, IL). Reduced and S-carboxyamidemethylated ω-Aga-TK was prepared as described by Ui (8). The alkylated sample was digested with lysyl endopeptidase in 0.1 M Tris-HCl buffer, pH 8, in the presence of 4 M urea at 37 °C for 20 hr. The peptide fragments were separated on a PLRP-300 column (4.6 mm x 15 cm, Polymer Lab., London, UK) using a linear gradient of acetonitrile from 0 to 50% in 1% TFA at a flow rate of 1 ml/min. Automated Edman degradation of the peptides was performed on a Shimadzu PSQ-1 gas-phase protein sequencer (Kyoto, Japan).

Cells

Effects of ω-Aga-TK on calcium channels were examined in rat cerebral cortical neurons, baby hamster kidney (BHK) cells expressing L-type calcium channels, chick sympathetic ganglion cells and rat dorsal root ganglion (DRG) cells. Cortical neurons obtained from 17-day-old rat fetuses were cultured for 14-17 days and were used to study P-type calcium channel current. BHK cells were cotransfected with four expression plasmids containing α1 (9), α2 (9), β or γ subunit cDNAs. The skeletal muscle β (10) and γ (11) subunit cDNAs were prepared by using the polymerase chain reaction (PCR). BHKC12 cells were isolated by screening G418 and MTX-resistant clones and by DHP binding experiments. Chick sympathetic ganglion cells isolated from 10-day-old chick embryos were cultured for 7 to 14 days and were used to study N-type calcium channel current. DRG cells obtained from 17-day-old rat fetuses were cultured for 7 to 14 days and used to examine T-type calcium channel current.

Electrical measurements

Experiments were performed in a whole cell patch clamp mode as described by Hamill et al (12). Barium was used to measure calcium channel current. The barium currents were recorded by a

patch clamp amplifier Axopatch-1D (Axon Instrument, Foster City, CA). The glass patch electrode with a tip resistance of 5 to 10 MΩ contained an internal solution of (in mM): CsCl 130, $MgCl_2$ 1, Na_2ATP 5, EGTA 5, HEPES 5, and the pH was adjusted to 7.2 by CsOH. The external barium solution contained in mM: tetraethylammonium chloride (TEA) 145, $BaCl_2$ 5, D-glucose 24, HEPES 10, and the pH was adjusted to 7.4 by TEAOH. All experiments were carried out at 23-25 °C. Nifedipine (Sigma, St. Louis, MO) (13), ω-conotoxin GVIA (ω-CgTx) (Peptide Institute, Osaka, Japan) (14), ω-Aga-IVA (Peptide Institute, Osaka, Japan) (6,7), and amiloride (Sigma, St. Louis, MO) (15) were used to block L-type, N-type, P-type and T-type calcium channels, respectively.

Results and Discussion

Purification and characterization of ω-Aga-TK

ω-Aga-TK was purified to homogeneity from the venom of *A. aperta* by the two-step reverse-phase HPLC procedure. Fig.1A shows the HPLC profile of 20 µl of the crude venom. Known agatoxins were assigned by direct N-terminal sequence analysis of the HPLC fractions. In this initial separation, ω-Aga-TK co-eluted with ω-Aga-IVA in fraction 19. This fraction was further purified by HPLC as shown in Fig.1B which was almost identical to the chromatograms obtained by more than 10 experiments using 2 lots of the venom. ω-Aga-TK, eluted as the largest peak, was clearly separated from ω-Aga-IVA and ω-Aga-IIA. The homogeneity of the purified ω-Aga-TK was confirmed by the observation of a single N-terminal sequence upon Edman degradation and a single peak upon isocratic reverse-phase HPLC. Additionally, matrix-assisted laser desorption mass spectrometry of ω-Aga-TK revealed a single component of 5265 Da (accuracy; ±0.1%). Purification from a total of 300 µl of the crude venom resulted in the following yields: ω-Aga-TK, 1.2 mg; ω-Aga-IVA, 0.1 mg. The yields were estimated by amino acid analysis of the corresponding fractions, and by the amino acid composition of ω-Aga-TK (described below) and ω-Aga-IVA (6).

Fig.2 summarizes the amino acid sequence data. The direct N-terminal amino acid sequencing of S-carboxyamidemethylated ω-Aga-TK allowed the assignment of residues 1-35. The remainder of the sequence was obtained by analysis of the four peptides (K1-K4) generated by lysyl endopeptidase. Additionally, the C-terminal amino acid sequence of ω-Aga-TK was confirmed by carboxypeptidase Y digestion. These data show that ω-Aga-TK contains 48 amino acid residues including eight cysteines and one tryptophan, which is

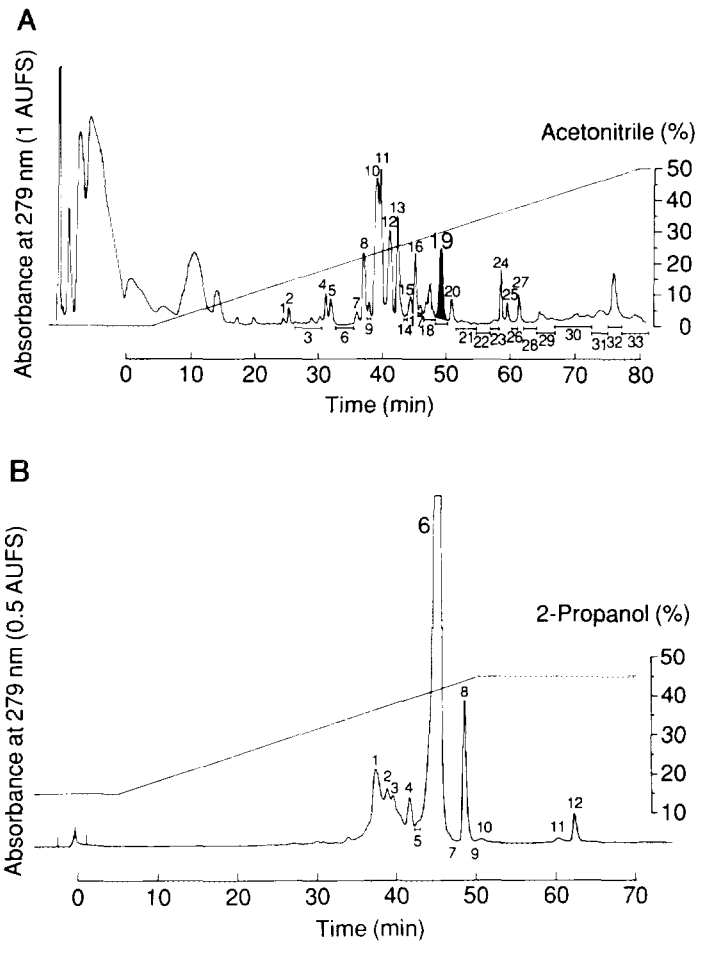

Fig.1. Isolation of ω-Aga-TK from crude *A. aperta* venom by two-step reverse-phase HPLC.
A,a HPLC profile of 20 µl of the venom.
B,a HPLC profile of fraction 19 in A obtained from 150 µl of the venom. Fractions 1, 6, and 8 contain ω-Aga-IIA, ω-Aga-TK,and ω-Aga-IVA, respectively.

consistent with the amino acid composition, ultraviolet absorbance spectrum, and mass spectrum of intact ω-Aga-TK.

The amino acid sequence of ω-Aga-TK was compared with known sequences in the protein sequence data base. Surprisingly, no homologous proteins were found apart from ω-Aga-IVA. ω-Aga-TK showed 71% identity with ω-Aga-IVA, as illustrated in Fig.3. Interestingly, ω-Aga-TK has a negatively charged N-terminus, whereas ω-Aga-IVA has a positively charged one.

Specificity of ω-Aga-TK for P-type calcium channels

The depression of calcium channel current by ω-Aga-TK was saturated at 1 µM (data not shown). In rat cortical neurons, the

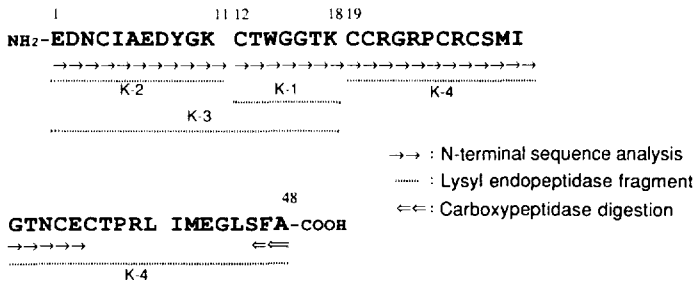

Fig.2. Summary of the proof of sequence of ω-Aga-TK.

amplitude of calcium channel current was reduced by 40% of the control value by nifedipine (3 μM) plus ω-CgTx (3 μM). ω-Aga-TK (1 μM) further depressed the current by 45% (Fig.4A). Similar results were obtained with ω-Aga-IVA (Fig.4B). Moreover, after application of a combination of nifedipine (3 μM), ω-CgTx (3 μM) and ω-Aga-IVA (1 μM) to cortical neurons, ω-Aga-TK (1 μM) had no effect on the remaining current (Fig.4C). These results indicate that calcium channel current blocked by ω-Aga-TK in the presence of nifedipine and ω-CgTx in cortical neurons is identical with that blocked by ω-Aga-IVA. In BHKC12 cells, ω-Aga-TK (1 μM) did not affect the L-type current at all, whereas L-type current was completely blocked by 1 μM nifedipine (Fig.4D). This result shows that L-type channels are insensitive to ω-Aga-TK. Fig.4E shows the effect of ω-Aga-TK on N-type current. Chick sympathetic neurons are reported to have L-type and N-type channels (16); therefore, nifedipine was included in the bath solution throughout the experiment to block L-type current. ω-Aga-TK had no effect on calcium channel current in the presence of nifedipine in sympathetic neurons, whereas ω-CgTx inhibited this current completely. This result shows that N-type channels are also resistant to ω-Aga-TK. T-type current was elicited by a step

Fig.3. Sequence homologies between ω-Aga-TK and ω-Aga-IVA. Identical residues in the two sequences are boxed. Acidic and basic amino acid residues are indicated as - and +, respectively.

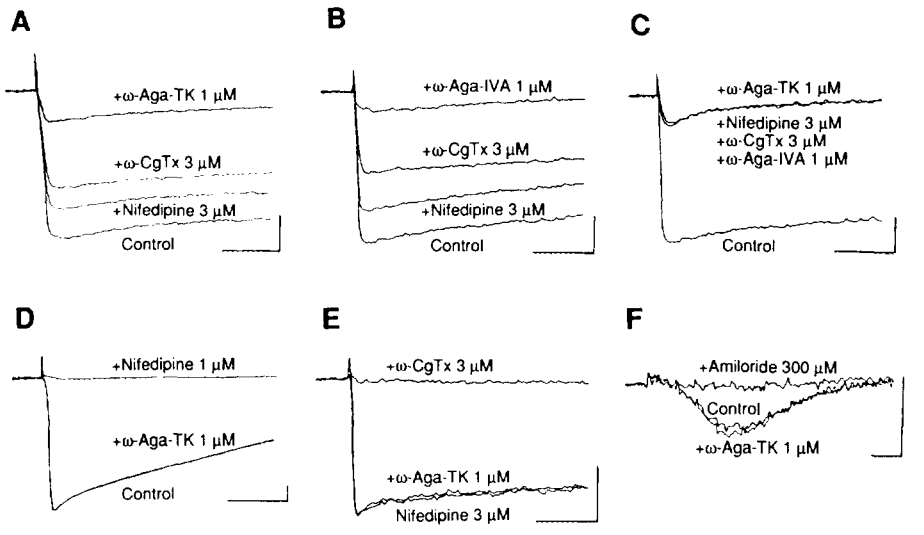

Fig. 4. Specificity of ω-Aga-TK for P-type calcium channels. Downward deflection of current traces shows inward calcium channel current in each panel. (A) ω-Aga-TK and (B) ω-Aga-IVA inhibited the nifedipine- and ω-CgTx-resistant calcium currents in rat cortical neurons. (C) Treatment with ω-Aga-TK in the presence of nifedipine, ω-CgTx, and ω-Aga-IVA had no further effect. (D) L-type current expressed in a BHKC12 cell was unaffected by ω-Aga-TK but was completely blocked by nifedipine. (E) Calcium channel current in a chick sympathetic neuron in the presence of nifedipine was insensitive to ω-Aga-TK but was abolished by ω-CgTx. (F) T-type current in a rat DRG neuron was not inhibited by ω-Aga-TK, but was blocked by amiloride. Currents were elicited by a step pulse from -80 mV to -10 mV for (A)-(E), and from -100 mV to -70 mV for (F). Horizontal and vertical scale bars for each trace indicate 50 msec and 200 pA, respectively.

pulse to -70 mV from a holding potential of -100 mV in rat DRG neurons. ω-Aga-TK did not affect the T-type current, which could be suppressed by 300 μM amiloride. These results suggest that ω-Aga-TK blocks the P-type calcium channel but is without effect on L-type, N-type and T-type calcium channels. The potency of ω-Aga-TK to block P-type calcium channels in cortical neurons is similar to that of ω-Aga-IVA. This newly discovered peptide, ω-Aga-TK, should be useful for investigating the role of P-type calcium channels in the peripheral and central nervous systems. Detailed comparison of the pharmacological properties of ω-Aga-TK and ω-Aga-IVA would also be of interest because of the difference of N-terminal charges between the two peptides.

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