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TWO NOVEL TACHYKININ-RELATED NEUROPEPTIDES IN THE ECHIUROID WORM, *URECHIS UNICINCTUS*

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Two novel neuropeptides, urechistachykinin I (H-Leu-Arg-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2) and urechistachykinin II (H-Ala-Ala-Gly-Met-Gly-Phe-Phe-Gly-Ala-Arg-NH_2), were isolated from the ventral nerve cords of the echiuroid worm, *Urechis unicinctus*. These peptides showed a contractile action on the inner circular body-wall muscle of the animal. Their amino acid sequences were found to be significantly homologous with those of the vertebrate and insect tachykinins. The urechistachykinins potentiated spontaneous rhythmic contractions of the cockroach hindgut.

Eledoisin and locustatachykinin I / II (Lom-TK I and II) have been isolated from the salivary glands of the cephalopod, *Eledone moschata* (1) and the ganglia of the locust, *Locusta migratoria* (2), respectively. Both these groups of substances are structurally related to the vertebrate tachykinins. Schoofs *et al.* (2) have proposed that the Lom-TKs may be a branch of the ancient superfamily of tachykinin-like peptides. The tachykinin-like immunoreactivities have been demonstrated in animals of other invertebrate phyla, Coelenterata (3), Platyhelminthes (4) and Annelida (5, 6). Thus, tachykinin-like bioactive peptides may be widely distributed in invertebrates.

Urechis unicinctus is a member of the Echiura which is closely related to the Annelida. We have previously shown that the ventral nerve cord of *U. unicinctus* possesses a number of peptides, which exhibit biological activities on the inner circular body-wall muscle of the worm (7). In previous studies, although we have determined the structures of eight purified species of neuropeptides (8, 9, 10, 11), none of them was structurally related to any vertebrate neuropeptides. In this

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Abbreviations: Uru-TK I, H-Leu-Arg-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2; Uru-TK II, H-Ala-Ala-Gly-Met-Gly-Phe-Phe-Gly-Ala-Arg-NH_2; Lom-TK I, H-Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2; Lom-TK II, H-Ala-Pro-Leu-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; TFA, trifluoroacetic acid; Hepes, N-[2-Hydroxyethyl]piperazine-N'-2-ethanesulfonic acid; Tris, tris[hydroxymethyl]aminomethane.

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experiment, we purified two novel tachykinin-related neuropeptides from the ventral nerve cords of *U. unicinctus*, and termed them urechistachykinin I and II (Uru-TK I and II).

MATERIALS AND METHODS

Purification: The ventral nerve cords of *Urechis unicinctus* were excised from 2,000 specimens and extracted with ethanol-acetic acid (96:4). The extract was condensed and subsequently allowed to pass through C-18 cartridges (Sep-pak, Waters). The retained material was eluted with 100% methanol in 0.1% TFA (pH 2.2), and the eluate was concentrated and subjected to HPLC separation. The retained material was initially applied to a C-18 reversed-phase column (Capcell Pak C_{18}, Shiseido) followed by elution with a 120-min linear gradient of 0-60% acetonitrile in 0.1% TFA (pH 2.2). Six bioactive peaks (peak 1-6) were obtained at this stage. The contractile peptides Uru-TK I and II were purified from peaks 3 and 5, which were eluted at 20 and 28% acetonitrile, respectively. Bioactivities of the fractions were assayed with the inner circular body-wall muscle of *U. unicinctus*.

The fractions of peak 3 were concentrated and applied to a cation-exchange column (TSKgel SP-5PW, Tosoh) before elution with an 80-min linear gradient of 0-0.8 M NaCl in 10 mM phosphate buffer (pH 6.8). A contractile peak was obtained with 0.3 M NaCl. At the third step, we eluted the sample via another C-18 reversed-phase column (TSKgel ODS80T_M, Tosoh) with a 50-min linear gradient of 8-18% acetonitrile in 0.1% TFA. Finally, Uru-TK I was purified by isocratic elution on the same column with 14% acetonitrile in 0.1% TFA (Fig. 1A).

At the second step of Uru-TK II purification, peak 5 was applied to an anion-exchange column (TSKgel DEAE-5PW, Tosoh) and eluted with a 40-min linear gradient of 0-0.4 M NaCl in 10 mM Tris buffer (pH 9.5). Contractile activities, however, were found in the flowthrough fractions. These fractions were concentrated, applied to a C-18 reversed-phase column (TSKgel ODS80T_M) and eluted with a 50-min linear gradient of 15-25% acetonitrile in 0.1% TFA. The active material eluted at 18-21% acetonitrile was then applied to a cation-exchange column (TSKgel SP-5PW) before eluting with a 60-min linear gradient of 0-0.6 M NaCl in 10 mM phosphate buffer (pH 6.8). After repeating the purification procedures twice with the C-18 reversed-phase column (TSKgel ODS80T_M), the active peak was subjected to a final HPLC purification via the same column. The column was eluted isocratically with 19.5% acetonitrile in 0.1% TFA (Fig. 1B).

Structure determination: Each of the purified substances was subjected to amino acid sequence analysis by the automated Edman degradation method with a gas-phase sequencer (Shimazu PSQ-1 for Uru-TK I; Applied Biosystems 477A coupled with Applied Biosystems 120A for Uru-TK II), followed by quantitative amino acid analysis (Tosoh CCPM for Uru-TK I; Hitachi L-8500 for Uru-TK II) and determination with a fast atom bombardment mass spectrometry (FAB-MS; JEOL JMS HX-100). These two peptides were synthesized by a solid-phase peptide synthesizer (Applied Biosystems 430A) using the FastMoc method. Their HPLC characteristics and bioactivities on the body-wall muscle of *U. unicinctus* were compared with those of native peptides.

Bioassay and pharmacology: After each HPLC purification step, biological activities of the fractions were examined on the isolated small bundle (1.5 mm width and 20 mm in length) of the inner circular body-wall muscle of *U. unicinctus*. Methods for dissecting and recording the tension of the muscle have been described previously (12). Pharmacological actions of the Uru-TKs were examined on the hindgut of the American cockroach, *Periplaneta americana*. The hindgut was cut at both ends and suspended isotonically in a 2-ml aerated organ bath. The Uru-TKs were applied to the muscle by dripping the stock solution in the bath. The tension of the hindgut was recorded through an isotonic muscle transducer. Other methods for recording the hindgut tension were the similar to those used for the muscle of *U. unicinctus*. Artificial seawater (ASW) used for the body-wall muscle of *U. unicinctus* was of the following composition: 445 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$ and 10 mM Tris-HCL; pH 7.8. However, physiological saline used for *P. americana* was of a different composition: 154 mM NaCl, 13 mM KCl, 1 mM $CaCl_2$, 11 mM glucose and 10 mM Hepes-NaOH; pH 6.9.

RESULTS AND DISCUSSION

As shown in Fig. 1, each single peak of the two Uru-TKs obtained at the final purification steps showed contractile activities on the body-wall muscle of *U. unicinctus*. Quantitative amino

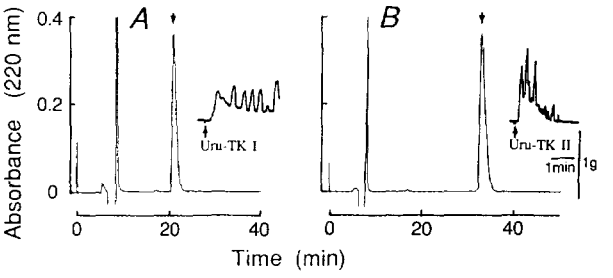

Fig. 1. HPLC profiles of the final purification procedure of urechistachykinins, Uru-TK I (A) and II (B), and bioactivities of the purified peptides. The column (C-18 reserved-phase column, TSKgel ODS80T$_M$) was eluted with 14% (A) and 19.5% (B) acetonitrile in 0.1% TFA (pH 2.2). Fractions of the absorbance peaks indicated by the downward arrows were those that elicited contractile activities on the inner circular body-wall muscle of *U. unicinctus* (insets). Uru-TKs applied to the muscle (upward arrows) corresponded to 80 nerve cords/ml ASW.

acid analyses of the Uru-TKs revealed the following amino acid compositions: $Glx_{2.5}$, $Ser_{1.9}$, $Gly_{1.0}$, $Arg_{2.2}$, $Val_{1.0}$, $Leu_{1.0}$, $Phe_{1.0}$ (Uru-TK I, normalized on Phe=1.0) and $Gly_{3.0}$, $Ala_{2.9}$, $Met_{0.8}$, $Phe_{2.0}$, $Arg_{1.0}$ (Uru-TK II, normalized on Phe=2.0). The determined sequences and detected amounts (picomoles) of each amino acid in the amino acid sequence analyses were as follows: Leu_{348}-Arg_{55}-Gln_{274}-Ser_{51}-Gln_{234}-Phe_{264}-Val_{204}-Gly_{141}-Ser_{33}-Arg_{61} (Uru-TK I) and Ala_{699}-Ala_{655}-Gly_{380}-Met_{378}-Gly_{271}-Phe_{179}-Phe_{174}-Gly_{140}-Ala_{172}-Arg_{91} (Uru-TK II). Molecular ion peaks in the FAB-MS spectrum of Uru-TK I and Uru-TK II were displayed at 1176.7 m/z $(M+H)^+$ and 983.3 m/z $(M+H)^+$, respectively. Based on these results, the structures of Uru-TK I and II were H-Leu-Arg-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2 and H-Ala-Ala-Gly-Met-Gly-Phe-Phe-Gly-Ala-Arg-NH_2, respectively. These peptides were thus synthesized, and chemical and pharmacological properties of the synthetic Uru-TKs were compared with those of native peptides. Both the synthetic and native peptides indicated identical behaviors on the reversed-phase and cation-exchange HPLC (Fig. 2). The synthetic peptides demonstrated contractile activities on the body-wall muscle

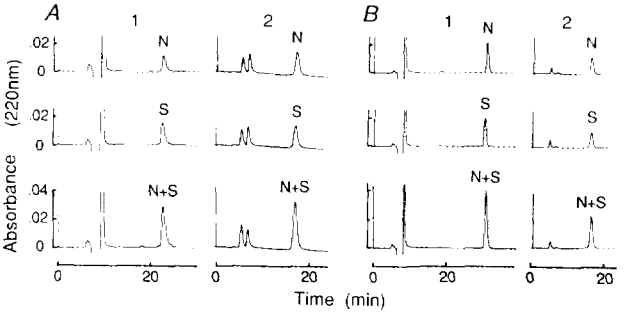

Fig. 2. Comparison between HPLC profiles of the native (N) and synthetic (S) Uru-TKs. A1: Uru-TK I was injected to a C-18 reversed-phase column (TSKgel ODS80T$_M$) and eluted with 14% acetonitrile in 0.1% TFA (pH 2.2). A2: Uru-TK I was injected to a cation-exchange column (TSKgel SP-5PW) and eluted with 0.3 M NaCl in 10 mM phosphate buffer (pH 7.0). B1: Uru-TK II was injected to the reversed-phase column and eluted with 18% acetonitrile in 0.1% TFA (pH 2.2). B2: Uru-TK II was applied to the cation exchange column and eluted with 0.2 M NaCl in 10 mM phosphate buffer (pH 7.0). N+S, represents a mixture of native and synthetic peptides.

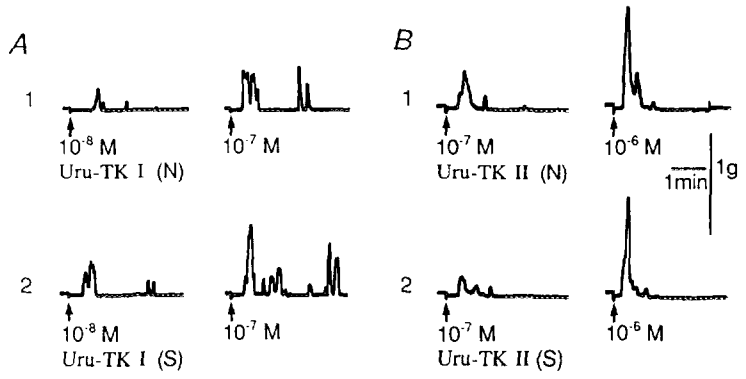

Fig. 3. Comparison between bioactivities of the native (N) and synthetic (S) Uru-TKs on the inner circular body-wall muscle of *U. unicinctus*. Concentrations of the native peptides were estimated from the results of amino acid analyses.

of *U. unicinctus* in a manner similar to the native peptides (Fig. 3). The threshold concentrations of Uru-TK-I and II for contractions of the muscle were found to be approximately 10^{-9} M and 10^{-8} M, respectively.

Similar to the tachykinin family peptides, both the *Urechis* peptides illustrated a Gly and a Phe residue at the third and fifth positions from their amidated C-termini, respectively (Table 1). Uru-TKs were more homologous to the insect tachykinins (Lom-TKs) compared with the vertebrate tachykinins and eledoisin. Forty to fifty percent sequence homology was depicted between the Uru-TKs and Lom-TKs.

Lom-TKs have stimulated an increase in spontaneous contractions of the cockroach (*Leucophaea maderae*) hindgut (2). In our present experiments, Uru-TKs elicited a similar excitatory effect on spontaneous contractions of the American cockroach (*P. americana*) hindgut (Fig. 4). Thus, it is concluded that the Uru-TKs and the Lom-TKs are closely related in their structures and bioactivities. They are convergent members of a novel family of invertebrate neuropeptides with an evolutional relation to the vertebrate tachykinins.

All the vertebrate tachykinins and eledoisin have -Met-NH_2 at their C-termini, whereas the Lom-TKs and Uru-TKs have -Arg-NH_2 at a similar position. Vertebrate tachykinins and eledoisin

Table 1. Structures of urechistachykinins, locustatachykinins, eledoisin and some vertebrate tachykinins

Phyla / Class		Structure
Echiura	Uru-TK I	Leu-Arg-Gln-Ser-Gln-Phe -Val-Gly-Ser -Arg-NH_2
	Uru-TK II	Ala-Ala-Gly-Met-Gly-Phe-Phe-Gly-Ala -Arg-NH_2
Insecta	Lom-TK I	Gly-Pro-Ser -Gly-Phe-Tyr-Gly-Val -Arg-NH_2
	Lom-TK II	Ala-Pro-Leu-Ser -Gly-Phe-Tyr-Gly-Va l-Arg-NH_2
Mollusca	Eledoisin	pGlu-Pro-Ser -Lys-Asp-Ala-Phe- Ile -Gly-Leu-Met-NH_2
Mammalia	Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
	Neurokinin A	His-Lys-Thr -Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

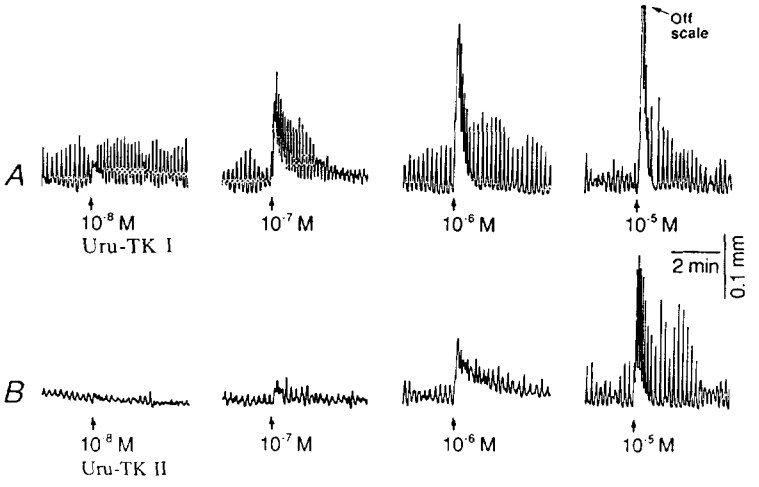

Fig. 4. Effects of Uru-TK-I (A) and -II (B) on spontaneous contractions of the isolated cockroach (*P. americana*) hindgut.

demonstrated an excitatory action on the guinea-pig ileum, whereas Uru-TKs and Lom-TKs did not show any effects on the muscle. However, the vertebrate tachykinins and eledoisin did not show any effects on spontaneous contractions of the cockroach hindgut. As such, the biological activities of these peptides are very much dependent on whether the C-terminal amino acid is Arg or Met. In fact, Uru-TKs analogues with -Met-NH_2 (instead of Arg-NH_2) at their C-termini elicited potent excitatory effects on the guinea-pig ileum without any action on the cockroach hindgut (data not shown). Studies on the structure-activity relationship of these peptides on the guinea-pig ileum and cockroach hindgut are now in progress at our laboratories.

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