The importance of C-terminal residues of vertebrate and invertebrate tachykinins for their contractile activities in gut tissues

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Abstract The C-terminal residues of mammalian tachykinins and urechistachykinins (Uru-TKs), tachykinin-related peptides of echiuroid worm origin, were substituted for each other. Their contractile effects were assayed on the cockroach hindgut and the guinea pig ileum. [Met[10]] substitution of Uru-TKs caused a 1000 times lower activity on the hindgut, but a 1000 times higher activity on the ileum. In contrast, [Arg[11]]substance P (SP) was 100 times more and 400 times less potent than SP on the hindgut and ileum, respectively. A SP antagonist blocked these Uru-TK activities on the hindgut. These results demonstrated that the C-terminal Met-NH$_2$ is necessary for ileum contraction and the Arg-NH$_2$ is required for hindgut contraction, which was caused by binding to the cockroach's neurokinin-like receptor.
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Key words: Invertebrate tachykinin; Urechistachykinin; C-terminal residue; Cockroach hindgut; Guinea pig ileum

1. Introduction

Since Schoofs et al. proposed in 1990 that locustatachykinins (Lom-TKs) I and II might be members of a branch of the ancient superfamily of tachykinin-related peptides, many tachykinin-related peptides to date have been isolated from the nervous system and the intestinal organs of invertebrates, such as arthropods [1,2,5–10], echiuroids [3] and mollusks [4]. All of the invertebrate tachykinin-related peptides studied have Arg-NH$_2$ in common at the C-terminal position instead of Met-NH$_2$, which is in vertebrate tachykinins. These peptides increase the amplitude and frequency of spontaneous contractions and tonus of hindgut muscle in cockroaches [10]. We isolated two tachykinin-related peptides, urechistachykinins I and II (Uru-TKs I and II), from the ventral nerve cords of the echiuroid worm *Urechis unitinctus* as contractile substances of the inner circular body-wall muscle [3]. Uru-TKs elicited excitatory effects on spontaneous contractions of the cockroach *Periplaneta americana* hindgut similar to those of Lom-TKs on the cockroach *Leucophaea maderae* hindgut. [Met[10]]Uru-TKs were synthesized by a substitution of C-terminal Arg for the Met residue. These peptides contracted the guinea pig ileum (GPI) as well as mammalian tachykinins such as substance P (SP), neurokinins A and B (NKA and NKB), where-

as the C-terminal Arg to Met substitution resulted in loss of activity on the cockroach hindgut [3]. Although the cockroach hindgut assay was heterologous with respect to the Uru-TKs and both the Uru-TKs and SP were somewhat alien to the cockroach hindgut, the hindgut and the ileum were employed as each representative tissue of invertebrates and vertebrates to study the importance of C-terminal residues of Uru-TKs and mammalian tachykinins. Structure-activity studies have been done on the *L. maderae* hindgut to determine the core sequence of Lom-TK I essential for receptor binding and activity using N-terminal truncated peptides [11]. In this report, we present the effects of the C-terminal Arg to Met or Met to Arg substitution of Uru-TKs or mammalian tachykinins in the cockroach hindgut and the GPI assay.

2. Materials and methods

2.1. Animals

American cockroaches *P. americana* were kept in stock colonies maintained at 25°C. For biological assays, adult male cockroaches were taken from the colonies. Male guinea pigs (300–500 g body weight) were maintained at 22°C with a 12 h light/dark cycle. The animals had free access to both food and water.

2.2. Peptide synthesis

Analogs of Uru-TKs and mammalian tachykinins were synthesized by a solid-phase peptide synthesizer (PE Biosystems Japan Model 433A, Tokyo, Japan) using the FastMoc method. A SP antagonist, [D-Pro[4], D-Trp[7,9]]SP (4-11), D-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH$_2$ [12] and its C-terminal Arg analog were also synthesized. The peptides and analogs are shown in Fig. 1.

2.3. Bioassays

An adult male *P. americana* was pinned down on a paraffin-filled petri dish on its back after its legs and wings were removed. Both sides of the abdominal segment were incized from the subgenital plate to the first abdominal segment and the ventral abdominal sclerites were removed. The Malpighian tubules and adipose tissues were cut from the surface of the hindgut, and the hindgut was isolated. Both ends of the tissue were tied with cotton threads. One thread was connected to the bottom of an aerated chamber and the other to a displacement transducer. After spontaneous contractions of the tissue had stabilized, the peptides were applied to the chamber by dripping in the stock solutions. Displacements of the tissue lengths, after amplification, were recorded on a pen recorder. The physiological saline used for the hindgut was of the following composition: 154 mM NaCl, 13 mM KCl, 1 mM CaCl$_2$, 11 mM glucose and 10 mM HEPES-NaOH, pH 6.9.

A male guinea pig was anesthetized with intraperitoneally administration of sodium pentobarbital and the segment of the small intestine in the ileal region was removed. After the surgical excision, the animal was killed by administration of a lethal dose of sodium pentobarbital. The isolated ileum was steeped in oxygenated (95% O$_2$-5% CO$_2$) Tyrode's medium and was gently stirred to remove its luminal contents and remnants of the anesthetic. One end of the ileum (a 4–5 cm length) was fastened with a small clip to an experimental chamber containing Tyrode's medium and the other end was attached to a displacement transducer. The medium was oxygenated and kept at

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37°C during the experiments. The ileum displacement was recorded in the same way as the hindgut contraction.

3. Results and discussion

The contractile effects of Uru-TKs, mammalian tachykinins and their analogs were illustrated in Figs. 1 and 2. Dose-response curves were shown in Fig. 3. Uru-TKs I and II contracted the *P. americana* hindgut at the approximate threshold concentrations of 10^{-9} and 10^{-7} M, respectively (Fig. 3a). SP had a myotropic effect in *P. americana* [13]. Actually, SP showed a small effect on the hindgut at 10^{-6} M (Fig. 1) and was a weak agonist on the hindgut (Fig. 3b). Both NKA and NKB were almost inactive (Fig. 3b). Estimated by the approximate thresholds, substitution of the C-terminal Arg residue of Uru-TKs for the Met residue diminished the activity to 1/1000 the original activity (Fig. 3a). In contrast, Arg substitutions at the C-termini of SP (Fig. 1) and NKA significantly increased the activity (Fig. 3b). [Arg^{11}]SP contracted the hindgut in concentrations as low as 10^{-8} M. [Arg^{10}]NKA clearly contracted the hindgut at 10^{-6} M, but [Arg^{10}]NKB showed weak contraction even at 10^{-5} M (Fig. 3b). The enhancement of the activity by Arg substitution was estimated at 10–100-fold based on the approximate threshold concentrations. It is noteworthy that the maximal response of [Arg^{11}]SP observed at 10^{-6} M was three times higher than that of SP in the hindgut assay (Fig. 3b).

In the GPI assay, the effects of substitution of the C-terminal residues were exactly the opposite. Though Uru-TKs had almost no effect on GPI even at 10^{-5} M (Fig. 2), their [Met^{10}] analogs showed potent contractions at 10^{-9}–10^{-8} M or higher concentrations (Fig. 3c). [Arg^{11}]SP caused potent contractions on GPI at 10^{-6} M (Fig. 2), but the EC$_{50}$ was 1/400 that of SP (Fig. 3d). [Arg^{10}]NKA and [Arg^{10}]NKB showed contractions at 10^{-5} M, but the EC$_{50}$ values were 100 and 10 times higher than those of NKA and NKB, respectively (Fig. 3d). The Met substitution enhanced the activities of Uru-TKs at more than 1000 times, whereas, the Arg substitution decreased those of SP, NKA, and NKB to 1/10–1/400 the original activity in the
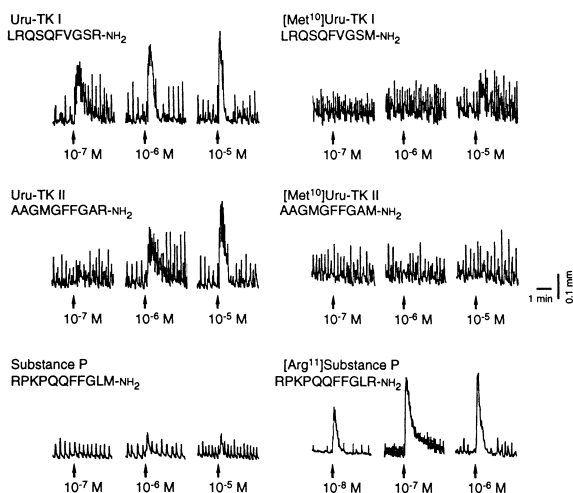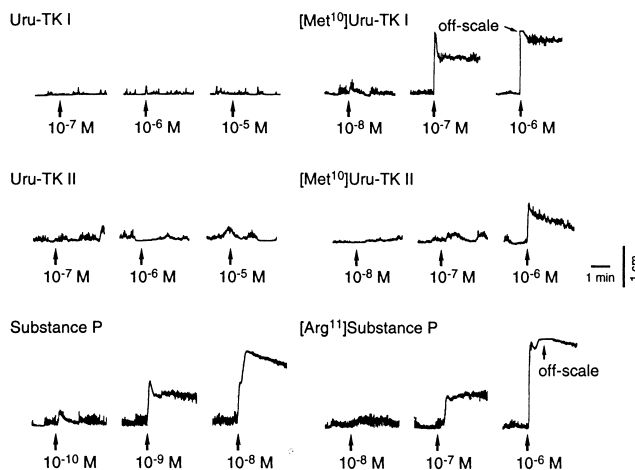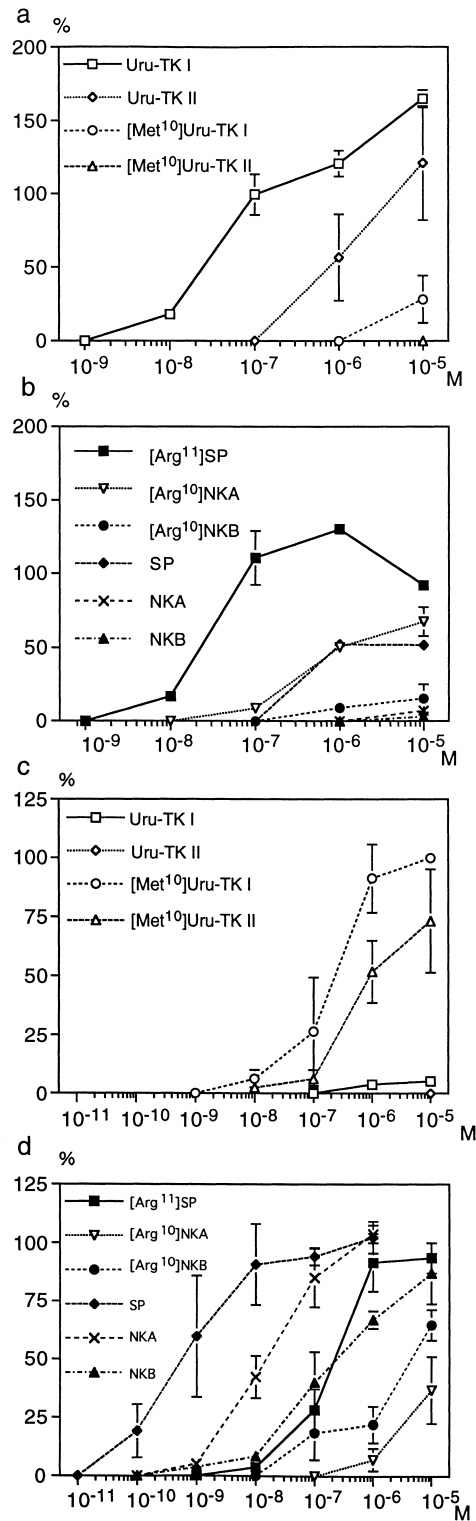

Fig. 2. Decrease in the potency of SP and increase in those of Uru-TKs in the GPI assay due to the C-terminal Met to Arg substitution. Uru-TK I was inactive even at 10^{-5} M, but [Met^{10}]Uru-TK I applied at 10^{-6} M caused off-scale contractile effects on the GPI.

GPI assay. These findings demonstrated that the C-terminal amino acid residue is most important for the contractile response of the ileum or the hindgut, i.e. the Met residue is necessary for tonic contractions on the ileum and the Arg residue is required for contractile effects on the hindgut.

NK receptors are G-protein-coupled and have seven common hydrophobic transmembrane α-helical regions, connected by extracellular and intracellular loops. Our results demonstrate that the C-terminal substituted peptides may be useful tools to study interaction of the tachykinin C-terminal residue and the NK receptors.

Fly NK-like receptors have been cloned and characterized in *Drosophilla* DTKR [14] and NKD [15] and in a stable fly, *Stomoxys calcitrans* STKR [16]. These receptors share 38–48% identity to mammalian NK receptors within the transmembrane regions. *Xenopus* oocytes, which express DTKR cDNA, show selective responses to SP at 10^{-6} M or higher concentrations [14]. The effective concentration seems to be almost the same as that of SP on the cockroach hindgut. If [Arg^{11}]SP is assayed in this experiment, the effective concentration would be significantly lower. Additionally, the presence of a positive charge at the C-terminus may offer some information about binding sites in the DTKR. NKD-expressed mouse NIH-3T3 cells were responsive to Lom-TK II at concentrations as low as 3.2×10^{-10} M, whereas Lom-TK I was ineffective on the cells at 10^{-6} M, suggesting the possibility of another related receptor, specific for Lom-TK I [15]. The effect of Lom-TK II could be blocked by a broad-spectrum SP antagonist, spantide, D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH$_2$ [15]. A *Leucophaea* tachykinin-related peptide, LemTRP 1, was isolated as a contractile substance of the cockroach *L. maderae* hindgut [7]. The response of this peptide was also blocked by spantide [11]. These results suggested that LemTRP 1 acts on receptors that are similar to both fly NK-like receptors and NK receptors. In this report, Uru-TKs and Arg-substituted analogs caused contraction in the *P. americana* hindgut. Are these activities attributed to the interaction between these peptides and *Periplaneta* NK-like receptors? To discuss this issue, we employed another SP antagonist [D-Pro^4, D-Trp^{7,9}]SP (4-11). The effects of the C-

Fig. 1. Effects of Uru-TKs I and II and their [Met^{10}] analogs, SP and its [Arg^{11}] analog on spontaneous contractions of isolated hindgut of the cockroach *P. americana*. Peptides were applied at the times indicated by arrowheads.

Fig. 3. Comparison of dose-responses of Uru-TKs, SP, NKA and NKB and their analogs in the *P. americana* hindgut (a and b) and GPI assays (c and d). Taking the maximal contractions caused by 10^{-7} M Uru-TK I and 10^{-6} M SP as 100% in the hindgut and the GPI assays, respectively, each response was calculated. Each point represents 3–5 replicates ± S.E.M. (a) Dose-response curves of Uru-TKs and their [Met^{10}] analogs in the *P. americana* hindgut assay. (b) Dose-response curves of SP, NKA and NKB and their C-terminal Arg-substituted analogs in the *P. americana* hindgut assay. (c) Dose-response curves of Uru-TKs and their [Met^{10}] analogs in the GPI assay. (d) Dose-response curves of SP, NKA, NKB and their C-terminal Arg-substituted analogs in the GPI assay.
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suggested that Uru-TK I also acts on *P. americana* NK-like receptors although they have not been cloned yet.

Tachykinin-like peptides might be divided into two groups: the vertebrate type (Met-tachykinin) and the invertebrate type (Arg-tachykinin), based on their C-terminal residues [4,17]. Further understanding of the characteristics of invertebrate Art-tachykinins will be provided by cloning the precursor proteins of the peptides, by cloning invertebrate NK-like receptors, and then screening native ligands.

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terminal Arg analog [D-Pro^4, D-Trp^{7,9}, Arg^{11}]SP (4-11) were also tested based on the idea of a significant increase in the potency of the Art-substituted analogs. As shown in Fig. 4, both antagonists at 10^{-5} M completely blocked the contractile effect caused by Uru-TK I at 10^{-7} M and nearly 50% of the effect induced at 10^{-6} M. Although the Met to Arg substitution did not influence the antagonistic effects, these results
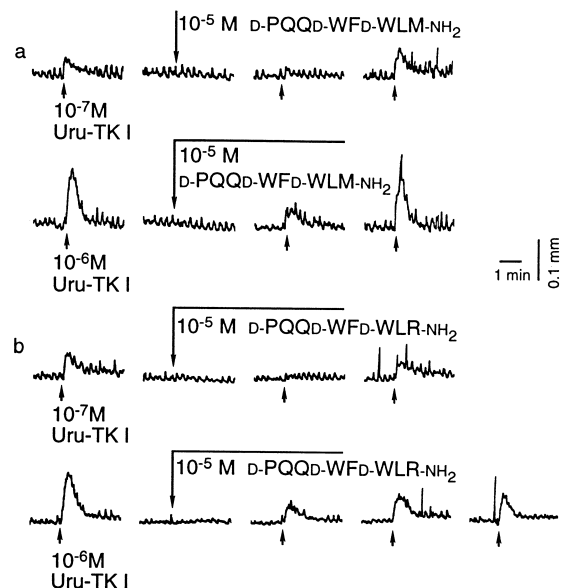
Fig. 4. (a) Blocking of Uru-TK I activities in the *P. americana* hindgut assay by an antagonist of SP. (b) The C-terminal Met of the antagonist was replaced by Arg and the effect was tested.

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