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Identification of a tachykinin-related peptide with orexigenic properties in the German cockroach

Núria Pascual [a], José L. Maestro [a,*], Cristina Chiva [b], David Andreu [b], Xavier Bellés [a,**]

[a] Department of Physiology and Molecular Biodiversity, Institut de Biologia Molecular de Barcelona (CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

[b] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Doctor Aiguader 80, 08003 Barcelona, Spain

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ABSTRACT

A number of evidences suggest that tachykinin-related peptides (TRPs) of insects can stimulate food consumption after being released from the midgut to the hemolymph. The idea of the present work has been to test this hypothesis in the anautogenous cockroach *Blattella germanica*. First, we have identified the peptide LemTRP-1 (APSGFLGVR-NH_2) from brain extracts, by means of an ELISA developed with a polyclonal antibody against this peptide. ELISA studies have also shown that, whereas brain LemTRP-1 levels were fairly constant, midgut levels increase to a maximum on day 3 after adult emergence, falling thereafter until the end of the gonadotrophic cycle. Interestingly, maximum values of food consumption are concomitant with the decrease of LemTRP-1 immunoreactivity in the midgut. Furthermore, starvation decreases LemTRP-1 immunoreactivity in midgut, whereas in the hemolymph it increases. Finally, injection of synthetic LemTRP-1 to adult females significantly stimulates food consumption. The whole observations suggest that LemTRP-1 is released from the midgut to the hemolymph when sustained food consumption is required to maintain vitellogenesis at the highest levels, and that LemTRP-1 in the hemolymph stimulates food consumption in these days.

1. Introduction

Tachykinin-related peptides (TRPs) constitute a family of invertebrate peptides with a characteristic carboxy-terminal sequence FX_1GX_2R-NH_2. The name comes from their relative sequence similarity with vertebrate tachykinins, which show a conserved C-terminal sequence FXGLM-NH_2. In addition, TRPs and vertebrate tachykinins share other characteristics, as their occurrence in both nervous system and gut tissues, and their stimulatory activity of gut musculature contractions [20].

The first peptides belonging to the TRP family (Lom-TK I and II) were purified from brain-corpora cardiaca-corpora allata-suboesophageal ganglion extracts of the locust, *Locusta migratoria*, by monitoring their myotropic activity on cockroach hindgut [21]. Since then, peptides belonging to TRP family have been identified in insects belonging to Orthoptera, Diptera, Dictyoptera and Hymenoptera orders [20,23]. Furthermore, cDNAs encoding TRP precursors have been cloned and sequenced in the fruit fly *Drosophila melanogaster* [22], the mosquito *Anopheles gambiae* [18], the honeybee *Apis mellifera* [23] and the cockroaches *Leucophaea maderae* and *Periplaneta americana* [17]. In all cases, the cDNA sequence confirmed the identity of the previously reported peptides.

Immunocytochemical studies in cockroaches have revealed the occurrence of TRP immunoreactivity in interneurons of the

* *Corresponding author*. Tel.: +34 934006135; fax: +34 932045904.
** *Corresponding author*. Tel.: +34 934006124; fax: +34 932045904.
E-mail addresses: jmgagr@ibmb.csic.es (J.L. Maestro), xbragr@cid.csic.es (X. Bellés).
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central nervous system, in the stomatogastric nervous system, in processes to the corpora cardiaca glandular lobe, in nerves innervating different gut areas and in midgut endocrine cells [10,13]. This wide distribution has been observed in all the species tested [14], and suggests that TRPs have multiple functions. Indeed, a remarkable variety of activities has been reported for these peptides in insects, including stimulation of visceral and skeletal muscle contractions, induction of adipokinetic hormone release by corpora cardiaca, neuronal depolarization, stimulation of urine production, and induction of pheromone biosynthesis [6,14,20].

Furthermore, it has been demonstrated that the midgut of *L. maderae* and *L. migratoria* incubated in vitro release TRPs in response to an increase of K^+ levels in the bathing solution [25]. In *L. migratoria*, starvation increases the concentration of TRP-immunoreactive material in the hemolymph, concomitantly with a decrease in the midgut, which suggested that TRPs are released as hormones from the midgut, and that this release could be linked to the nutritional status [25]. These results make TRPs good candidates for being tested as orexigenic factors, under the hypothesis that its release in starved specimens might stimulate food consumption.

A good model to test this hypothesis would be the German cockroach, *Blattella germanica*, given that it is anautogenous and show a well-defined feeding cycle paralleling that of vitellogenesis [16]. The feeding cycle suggests that food intake is finely regulated, and possible regulatory mechanisms have been already reported. Thus, the peptide perisulfakinin has been identified as a putative satiety factor [8], and a number of YXFGL-NH_2 allatostatins, W^2W^9-amide myoinhibitory peptides and leucomyosuppressin have been shown to inhibit food intake in this cockroach [1–3]. Nevertheless, no information about factors stimulating food intake has been reported.

The aim of the present work has been to identify TRPs in *B. germanica*, and to study whether they may play a stimulatory role in the regulation of food intake. The reference peptide to search native TRPs in *B. germanica* was LemTRP-1 (APSGFLGVR-NH_2), which had been already identified in the cockroaches *L. maderae* and *P. americana* [11,12,17].

2. Materials and methods

2.1. Insect rearing

Adult females of *B. germanica* (L.) were obtained from a colony reared on dog chow and water, at 30 ± 1 °C and 60–70% r.h. Freshly moulted adult virgin females were isolated and used at the appropriate physiological ages within the first gonadotrophic cycle. Physiological age was assessed by measuring the basal oocyte length [4]. For starvation experiments, animals were supplied only with water since the imaginal moult.

2.2. Synthesis of peptides and conjugates

Peptides LemTRP-1: APSGFLGVR-NH_2, LemTRP-2: APEESPK-RAPSGFLGVR-NH_2, LemTRP-4: APSGFMGMR-NH_2 and LemTRP-5: APAMGFQGVR-NH_2 [11] were synthesized using standard Fmoc solid phase methods [5]. The identity and purity (ca. 90%) of each peptide were assessed by amino acid analysis, matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra and HPLC. To raise antibodies against LemTRP-1, the peptide was synthesized with its N-terminus extended by two residues of 2-aminohexanoic acid and conjugated to keyhole-limpet-hemocyanin (KLH) (Sigma, St. Louis, MO, USA) according to [19]. For ELISA plate coating, LemTRP-1 was conjugated to bovine serum albumin (BSA) (Sigma), using glutaraldehyde [24]. The conjugates were dialyzed, lyophilized and stored at −20 °C.

2.3. Antibody production and titer test

Three male white New Zealand rabbits were used to raise antibodies using LemTRP-1-KLH as immunogen. Rabbits were injected subcutaneously with 100 μg of peptide, in the conjugated form, diluted in 500 μl of water emulsified with 500 μl of Freud's complete adjuvant (Sigma) on days 0 and 7, and with incomplete adjuvant on day 14. Blood samples were obtained on day 21. Rabbits were boosted again once a month using the same dose and incomplete adjuvant, and serum was obtained 1 week after each booster injection, during 6 months. Serum was added with 0.1% thimerosal (Serva, Heidelberg, Germany) and stored at −20 °C. The titer of serum from each rabbit was determined by measuring the binding of serial dilutions to microtiter plates coated with 1 μg/ml of LemTRP1-BSA. A two-dimensional titration protocol was used for the screening and determination of the optimum concentration of both coating antigens and antisera to be used later in the competitive experiments [7].

2.4. ELISA method

LemTRP-1-BSA at a concentration of 0.15 μg/ml in 0.1 M carbonate–bicarbonate buffer (pH 9.6) was used for coating polystyrene 96 wells microtiter plates (Nunc Maxisorp, Roskilde, Denmark), in a volume of 100 μl/well, and incubated overnight at 4 °C. The plates were washed five times with PBST buffer (0.2 M, phosphate-buffered saline solution containing 0.05% Tween 20, pH 7.4). The plates were blocked with 1% polyvinylpyrrolidone (Sigma) in PBST buffer. After 1 h, plates were washed again as described above. The immunological reaction was initiated by adding dilutions of the samples or standard peptide analyte in PBST buffer (from 10^{-6} M to 10^{-10} M) in volume of 50 μl/well followed by 50 μl/well of the antibody previously diluted 1/30,000 in PBST buffer (final dilution in the well: 1/60,000). After incubation at room temperature for 2 h, the plates were washed as described above, and 100 μl/well of a 1/6000 diluted goat antirabbit IgG peroxidase conjugated (Sigma) solution were added. After 1 h incubation and a washing step, 100 μl/well of substrate solution were added and incubated in the dark with gentle shaking. Substrate solution was prepared with 12.5 ml of citrate buffer (pH 5), 200 μl of 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide and 50 μl of 1% H_2O_2. Reaction was stopped by adding 50 μl/well of 2N H_2SO_4. Absorbance was read at 450 nm with a Titertek Multiscan Plus MKII spectrophotometer (Labsystems, Helsinki, Finland). The calibration curves were analyzed using a four parameter logistic equation.