Peptides 69 (2015) 127–132

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

The activity of the nonsulfated sulfakinin Zopat-SK-1 in the neck-ligated larvae of the beetle *Zophobas atratus*

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ARTICLE INFO

Article history:
Received 13 February 2015
Received in revised form 21 April 2015
Accepted 25 April 2015
Available online 7 May 2015

Keywords:
Nonsulfated sulfakinin
Zopat-SK-1
Fat body
Zophobas atratus
Insect
Ligation

ABSTRACT

Insect sulfakinins (SKs) are multifunctional neuropeptides structurally and functionally homologous to the mammalian gastrin/cholecystokinin (CCK). It has been proposed that SKs play a role in modulating energy management in insects by interacting with adipokinetic hormone (AKH), the principle hormone controlling insect intermediary metabolism. To exclude head factors (including AKH) that influence the activity of the nonsulfated sulfakinin Zopat-SK-1 in the larvae of the beetle *Zophobas atratus*, ligature and *in vitro* bioassays were used. Our study showed that in the neck-ligated larvae, Zopat-SK-1 evoked a much more pronounced glycogenolytic effect in fat body tissue and a significantly higher hypertrahelosemic effect in hemolymph than in larvae without ligation. We found that the concentration of the sugar trehalose increased under hormonal treatment but no changes in glucose levels were observed. Under *in vitro* conditions, the maximal glycogenolytic effect of Zopat-SK-1 in fat body was observed at 10 pmol of hormone. Ligature and *in vitro* bioassays indicated that Zopat-SK-1 activity in the *Z. atratus* larvae is modulated by head signals and/or factors from the gastrointestinal tract. Our data indicate the existence of a brain–gastrointestinal axis that has a role in controlling of energy (carbohydrate) metabolism in the insect body. Moreover, these results, together with immunological evidence of a cholecystokinin-like (sulfakinin) receptor in the *Z. atratus* fat body, help us to better understand the SK signaling pathways and its physiological role in insect biology.

Introduction

Sulfakinins (SKs) are insect neuropeptides structurally and functionally related to the mammalian gastrin/cholecystokinin (CCK) neuropeptides [4]. SKs are widely distributed in a variety of insect species including cockroaches [29], locusts [31], flies [7,9], crickets [21] and beetles [19,30]. Most sulfakinins possess a sulfated tyrosyl residue in a core sequence. The sulfated forms of sulfakinins are involved in the regulation of various physiological processes, including food intake control [17,21,31,34], digestive enzyme release [11,22] and carbohydrate ingestion [5]. The sulfakinins with nonsulfated tyrosine (nsSKs) have been shown to have myotropic activity in *Drosophila melanogaster* gut [23] and various visceral muscles of the *Zophobas atratus* beetle [19]. A recent study has demonstrated that nsSKs are engaged in the regulation of carbohydrate and lipid metabolism in the fat body of larva and pupa of the *Z. atratus* beetle [26], suggesting the presence of SK receptors in this tissue. Sulfakinin receptors (SKR) are G-protein coupled receptors (GPCRs) and crucial components in sulfakinin signaling pathways. Sulfakinin receptors have been functionally characterized in *D. melanogaster* [2,14] and *Tribolium castaneum* [37]. An expression profile of SKR in *T. castaneum* shows that SKR transcripts are expressed throughout all developmental stages (larva, pupa and adult). The highest expression of SKR genes has been found in the larva stage [34]. However, prominent expression of SKR genes has also been detected in the insect head and, to a much lower extent, in other tissues, such as the decapitated body and gut [34]. Recently, Zels et al. have reported a distribution profile of SKR transcript, with the highest expression in the brain [37]. SKR transcript has been also detected in peripheral tissues, such as the fat body and in the testis and gut. Thus, distribution patterns may indicate a pleiotropic role for SKs in signaling pathways involved in the brain–intestinal functional axis.

The presence of *skr* transcript [37] and the activity of nsSKs [26] in the insect fat body suggest the involvement of SKs in energy processes that take place in this trophic organ. The regulation of energy metabolism in the fat body is mainly mediated by adipokinetic hormone (AKH), which is synthesized and stored in the glandular lobes

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http://dx.doi.org/10.1016/j.peptides.2015.04.023
0196-9781/

of the *corpora cardiaca*. Thus, it has been suggested that by interacting with AKH, SKs play a role in the regulation of energy store mobilization. The antagonistic action of SKs and AKH in energy metabolism was previously observed in the cockroach *Periplaneta americana* [33] and the beetle *T. castaneum* [37].

Because SKR is found in the larva stage with a high expression in the fat body and AKH and SK possibly collaborate in insect energy management, we injected a synthetic analog of the nonsulfated sulfakinin Zopat-SK-1 (pETSDDYGHLRFa, the only SK so far identified in *Z. atratus* brain [18] to neck-ligated beetle larvae). Next, we determined the Zopat-SK-1 activity in relation to carbohydrate metabolism by measuring fat body glycogen levels and hemolymph free sugar levels. Moreover, to exclude factors from the gastrointestinal tract, which can influence SK activity, the glycogenolytic capacity of Zopat-SK-1 in fat body tissue in *in vitro* conditions was also analyzed. To confirm the presence of sulfakinin receptors at the protein level in the fat body, an immunological detection method was applied.

Materials and methods

Insects

Larvae of the *Z. atratus* beetle were reared under laboratory conditions at a temperature of 28 °C and relative humidity of 65–70%. Food, including lettuce, carrots, powdered milk and eggs, and water were provided *ad libitum*. For our experiments, we chose larvae only immediately after molting. These larvae were ligated with cotton thread for 24 h. After ligation larvae were divided into 2 groups. The first group of control insects received 4 μl injections of Ringer's solution (RS 274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$, 5 mM glucose and 5 mM HEPES, pH 7.0). The second group of insects received injections of 10 pmol or 20 pmol of Zopat-SK-1 in 4 μl of RS. The dose of 20 pmol of Zopat-SK-1 (previously determined [26]) evoked the maximal hyperglycemic effect in larval hemolymph *in vivo* 2 h after only one hormone injection. Samples (fat bodies and hemolymph) were collected 2 h or 24 h after the hormone or RS injections. In our *in vitro* experiments, a left part of insect fat body was incubated with 5, 10, 20 or 30 pmol of hormone in 0.4 ml of RS. Control sample – the similar piece of right part of insect fat body was incubated only in RS. Zopat-SK-1 was produced by Fmoc solid-phase synthesis [8].

Determination of glycogen content in the fat body

Isolation of glycogen was carried out by hydrolysis of tissue in 30% KOH for 15 min at 90 °C according to the Van Handel procedure [28]. After tissue lysis, a saturated solution of Na_2SO_4 and 70% ethanol was added to precipitate the glycogen. Next, the samples were centrifuged at 10,000 × *g* for 5 min and washed twice with 70% ethanol. The pellet was dissolved in water and shook for 10 min at 80 °C. This solution was used to determine the glycogen content using a 5% phenol–sulfuric acid method [6]. Oyster glycogen was used as a standard.

Determination of total free sugars of hemolymph

The hemolymph (2 μl) was transferred into 500 μl of 70% ethanol and shaken for several minutes to fully extract the free sugars. The samples were centrifuged at 10,000 × *g* for 4 min and the supernatant was collected to determine the free sugar content by a colorimetric phenol–sulfuric acid method [6]. Trehalose was used as a standard.

Qualitative and quantitative determination of free sugars in hemolymph

Hemolymph (5 μl from one insect) from larvae was collected in 70% ethanol and kept at 4 °C. The samples were centrifuged (5 min, 10,000 × *g*) and the supernatant was used for sugar analysis by reverse phase high performance liquid chromatography (RP-HPLC). Separation was performed using a Dionex Ultimate 3000 chromatographic system comprised of a dual pump programmable solvent module and a Corona Charged Aerosol Detector (CAD). Supernatants were analyzed on a APS-2 Hypersil 5 μl column (150 × 4.6 mm, Thermo Scientific). The samples were eluted with an isocratic gradient of 80% acetonitrile with a flow rate 1.5 ml/min for 15 min at 40 °C. Trehalose, glucose and glycerol (Merck, Germany) were used as standards.

Immunodetection of cholecystokinin-like receptor

The fat body tissue from control larvae was collected and homogenized in ice-cold Ringer's solution with a Teflon glass homogenizer and centrifuged at 1000 × *g* for 5 min. The protein concentration in the supernatant was determined using Bradford's procedure [1]. Next, protein fractions were resuspended in sample buffer. SDS-PAGE electrophoresis (with a 14% polyacrylamide gel) and Western blotting were carried out as described previously [25]. 80 μg of each protein fraction were loaded on the gel. After electrotransfer, blots were blocked with 5% BSA in Tris-buffered saline with 0.1% Tween (TBS-T) for 60 min and probed overnight at 4 °C with goat polyclonal primary antibodies raised against: (1) human CCK-AR (Santa Cruz Biotechnology) at dilution 1:500 and (2) human CCK-AR and in the presence of blocking peptide (Santa Cruz Biotechnology) at dilution 1:250. Antibodies against CCK-AR is an affinity purified goat polyclonal antibody raised against a peptide mapping at the N-terminus of CCK-AR of human origin. Next, the blots were washed with TBS-T and incubated with donkey anti-goat horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at dilution of 1:20000 for 1 h at room temperature. Protein bands were visualized using the Amersham ECL system.

Statistical analysis

All data are presented as the mean values ± S.D. of the indicated number of replicates (*n*). The statistical significance of differences between the mean values of the control and experimental groups of insects was determined by using *t-test*. The statistical analysis was performed using the Graph Pad Prism software. Differences were considered statistically significant if $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results

The influence of Zopat-SK-1 on the fat body glycogen content and hemolymph free sugar levels in the neck-ligated larvae of Z. atratus

Larvae were ligated around the neck behind CC and CA and 24 h later injected with 10 or 20 pmol of Zopat-SK-1. At the same time, insects without ligation were treated with the same doses of the hormone. Both the ligated and non-ligated control insects were injected with Ringer's solution. As is shown in Fig. 1, changes in glycogen content after application of 10 pmol of Zopat-SK-1 were similar in the fat body of the neck-ligated and non-ligated larvae. In contrast, after injection of 20 pmol of hormone, glycogen concentration in the fat body of ligated larvae was significantly greater