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New physiological activities of myosuppressin, sulfakinin and NVP-like peptide in *Zophobas atratus* beetle

Pawel Marciniak · Mariola Kuczer · Grzegorz Rosinski

Abstract Three neuropeptides Zopat-MS-2 (pEDVDHV-FLRFa), Zopat-SK-1 (pETSDDYGHLRFa) and Zopat-NVPL-4trunc. (GRWGGFA), recently isolated from the neuroendocrine system of the *Zophobas atratus* beetle, were tested for their myotropic and hyperglycaemic activities in this species. These peptides exerted differentiated dose-dependent and tissue specific physiological effects. Zopat-MS-2 inhibited contractions of the isolated heart, ejaculatory duct, oviduct and hindgut of adult beetles and induced bimodal effects in the heart contractile activity of pupae in vivo. It also increased the haemolymph free sugar level in larvae of this species, apart from myotropic activity. Zopat-SK-1 showed myostimulatory action on the isolated hindgut of the adult beetles, but it decreased contractions of the heart, ejaculatory duct and oviduct. Injections of this peptide at a dose of 2 μg also caused delayed cardioinhibitory effects on the heartbeat of the pupae. Together with the ability to increase free sugar level in the haemolymph of larvae these were new physiological activities of sulfakinins in insects. Zopat-NVPL-4trunc. inhibited the muscle contractions of the two organs: hindgut and ejaculatory duct but it was inactive on the oviduct and the heart of the adult beetles. This peptide also increased free sugar level concentration in the haemolymph of *Z. atratus* larvae. These physiological actions are the first biological activities discovered for this group of the insect peptides. The present work showed pleiotropic activity of three neuropeptides and indicates that the visceral muscle contractions and the haemolymph sugar homeostasis in *Z. atratus* are regulated by complex mechanisms.

Keywords Insect neuropeptides · *Zophobas atratus* beetle · Myotropic activity

Introduction

In insects, neuropeptides are important messenger molecules that can act as neurotransmitters, neuromodulators or classical hormones (Nässel 2002). Recently, neuropeptidomes of several species have been explored and a huge number of peptide hormones have been isolated (Audsley and Weaver 2006; Clynen and Schoofs 2009; Hummon et al. 2006; Li et al. 2008; Predel et al. 2008, 2010; Verleyen et al. 2004). However, physiological actions of most of the identified peptides remain undiscovered (De Loof 2008) or have mainly been studied by usage of heterologous bioassays (Nässel 2002).

In Coleopterans, the largest insect order, only several neuropeptides have been identified (Weaver and Audsley 2008) and neuropeptidome of only one species *Tribolium castaneum* was studied (Li et al. 2008). Information on physiological actions of majority of the neuropepties in this insect order is unknown. Recently, we identified several neuropeptides from the *Zophobas atratus* adult beetle (Marciniak et al. 2010b). Here, we report new physiological activities of three peptides myosuppressin Zopat-MS-2, sulfakinin Trica-SK-1, and NVP-like peptide Trica-NVPL-4trunc. in this beetle species. To better clarify these as *Z. atratus* endogenous peptides, we rename previously

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identified peptides Trica-SK-1 and Trica-NVPL-4trunc. as Zopat-SK-1 and Zopat-NVPL-4trunc., respectively, according to recently published paper on insect neuropeptide nomenclature by Coast and Schooley (2010).

Myosuppressins are a group of FMRFamide related peptides characterized by C-terminal sequence $X_1DVX_2HX_3$-FLRFa (where $X_1 = pE$, P, T or A, $X_2 = D$, G, V and $X_3 = V$ or S). First neuropeptide from this family, leucomyosuppressin (pEDVDHVFLRFa, LMS) was isolated in 1986 from the cockroach *Leucophaea maderae* (Holman et al. 1986). Myosuppressins are widely known as inhibitors of visceral muscles, such as the foregut, hindgut, oviduct, heart and Malpighian tubules (Lange et al. 1991; Orchard et al. 2001; Skonieczna and Rosinski 2004). Few bioanalogs, such as SchistoFLRFa enhance the force of neurally evoked contractions of the skeletal muscles (Elia and Orchard 1995). Moreover, it was showed that myosuppressins inhibit the release of the adipokinetic hormones from *corpora cardiaca* (Vullings et al. 1998) and are involved in feeding regulations in insects (Audsley and Weaver 2009).

Insect sulfakinins are neuropeptides characterized by sulfated tyrosine residues, C-terminal sequence $Y(SO_3H)$GHMRFa and structural and functional similarities to vertebrate gastrin and cholecystokinin (Schoofs and Nachman 2006). The first peptide hormone of this family was isolated from *L. maderae* by its ability to stimulate the hindgut contractions (Nachman et al. 1986). Currently, sulfakinins are known mainly as the gut and heart contraction modulators in several insect species (Schoofs and Nachman 2006). They also inhibit the food uptake in locusts and cockroaches and stimulate the release of the digestive enzymes in the scallop *Pecten maximus* and insect *Rhynchophorus ferrugineus* (Nachman et al. 1997). Recent studies suggest that non-sulfated peptides can also fulfill their biological functions (Nichols et al. 2009). Non-sulfated form of sulfakinin was detected in *Z. atratus* brain (Marciniak et al. 2010b), so in this studies we report its physiological properties in the beetle.

Several insect peptides, such as NVP-like peptides (NVPL) have been named after their partial N-terminal sequence. The first hormones from NVPL family were found in a peptidomic study of the honey bee brain (Hummon et al. 2006). MS analysis yielded seven peptides from the NVPL peptide family in *T. castaneum* (Li et al. 2008). Recently, we found Zopat-NVPL-4trunc. in the brain, CC/CA neurosecretory complex and ventral nerve cord of *Z. atratus* (Marciniak et al. 2010b). Thus far their function and status as neuropeptides remain unclear.

In this paper, we report the first myotropic and hyperglycaemic actions of NVPL peptide in insects. Furthermore, we confirm myostimulatory activity of sulfakinins and discover its myoinhibitory and hyperglycaemic activities. Myosuppressin are proved to be myoinhibitory

peptides in beetles. In addition, the organ specific myoregulatory activity of all three hormones are compared.

Materials and methods

Insects

Zophobas atratus Fab. adults (2- to 4-week old), pupae (1-day old) and last instar larvae (just after moult, body weight 600–800 mg) were obtained from a colony maintained at the Department of Animal Physiology and Development, Adam Mickiewicz University according to the Quennedy procedure (Quennedey et al. 1995).

Peptides

Peptides were synthesized by the classical solid phase method according to the Fmoc-procedure (Fields and Noble 1990). Amino acids were assembled either on a Wang (peptide acids) or Rink amide MBHA resin (peptide amides). As a coupling reagent HBTU in the presence of HOBt was used. The N-Fmoc group was removed with 20% piperidine in *N,N*-dimethylformamide (DMF). The peptide-resin was cleaved with trifluoroacetic acid (TFA) in the presence of ethanedithiol (EDT). All peptides were purified by preparative HPLC on a Varian ProStar column—Tosoh Biosciences ODS-120T C18 (ODS 300×21.5 mm). Analytical HPLC was performed with a Thermo Separation Products with a VYDAC C_{18} column (ODS 250×4.6 mm). The molecular weights of the peptides were determined with a Bruker Daltonics microTOF-Q mass spectrometer (USA). Synthetic peptides were dissolved in saline A (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.0) for in vitro bioassays or saline B (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$) for in vivo bioassays to yield a stock solution of 1 mM and they were stored at $-30°C$. Working dilutions were made from the stock solution in appropriate saline.

In vitro heart bioassay

In the bioassay, the microdensitometric method was used to measure peptide effects on the semi isolated heartbeat frequency of the adult beetle (Rosinski and Gäde 1988). In this method many pulse applications (10 μl) of samples could be sequentially assayed in a single preparation. After the initial 15 min stabilization, the control activity of the isolated heart was recorded for 0.5 min. Next, the sample was applied and the heart activity was recorded for further 1.5 min. The activities of the tested peptides were presented as percentage changes in the control frequency of the heart contractions.

In vivo heart bioassay

The in vivo heart bioassay was conducted on 1 day old *Z. atratus* pupae by means of noninvasive optocardiographic technique of Slama and Rosinski (Slama and Rosinski 2005) as described previously (Marciniak et al. 2010a). The heart rhythm was recorded for 24 h. Sample injections were performed after 2 h of initial stabilization of pupae heartbeat rhythm. Changes in the heartbeat frequency after injection were recorded and compared to the control rhythm (physiological saline injection). To each pupae the recording started always at the same time of the day.

Ejaculatory duct, oviduct and hindgut in vitro videomicroscopy bioassays

In the bioassays, a video microscopy technique and the computer-based method of data acquisition and analysis were used to study the action of the peptides on the frequency contractions of the continuously perfused preparations of ejaculatory duct, oviduct or hindgut as described previously (Marciniak and Rosinski 2010). The superfusion system and incubation chamber were similar to those used in the heart bioassay. The activities of tested peptides were presented as percentage changes in the control frequency of the ejaculatory duct or oviduct contractions. The hindgut bioassay was performed exactly the same way as the reproductive tract bioassays.

Haemolymph sugar bioassay

Hyperglycaemic activity of the peptides was tested in the *Z. atratus* larvae according to the previously described bioassay (Rosinski and Gäde 1988). Peptides were injected in an adequate concentration range through the ventral membrane between the second and the third abdominal segments toward the head, into the larvae just after ecdysis. Control insects were injected by saline B. Haemolymph samples (2 μl) were collected just before and two hours after injections. The samples were mixed with 500 μl of 70% ethanol and shaken several minutes to obtain full extraction of free sugar. The extracts were centrifuged at $10,000 \times g$ for 4 min and the supernatants were used for free sugar concentration analysis (Dubois et al. 1956) using trehalose as a standard.

Results

Effect of peptides on the adult and pupa heartbeat

The *Z. atratus* adult heart rhythm in vitro remained regular during superfusion with saline A and showed on average

34 ± 6 beats/min. Application of two of the tested peptides Zopat-SK-1 and Zopat-MS-2 caused fast, dose-dependent and reversible decrease of the heart contractile activity (not shown). Zopat-NVPL-4trunc. showed no significant effects on the myocardium frequency contractions in the whole concentration range of the peptide tested (Fig. 1c). A comparison of the dose–response curves indicated that Zopat-SK-1 was more potent cardioinhibitory peptide than Zopat-MS-2 (Fig. 1). EC_{50} value determined for this peptide (1.1×10^{-11} M) was much lower than for myosuppressin (5.6×10^{-7} M). However, the maximal cardioinhibitory effect induced by Zopat-SK-1 (25% inhibition in comparison to saline A injection) was twofold smaller in comparison to the action of Zopat-MS-2 (55% inhibition).

Both cardioinhibitory peptides were also tested for their activity in vivo on the 1-day-old *Z. atratus* pupae. Heart rhythm of pupae was manifested by the regular alternations of the forward orientated (anterograde), the backward orientated (retrograde) cardiac pulsations together with diastasis as described previously (Marciniak et al. 2010a). The heartbeat frequency in control insects showed on average 30 beats/min in anterograde phase and 12 beats/min in retrograde phase. Zopat-SK-1 retained cardioinhibitory properties in vivo (Fig. 2b). Injection 2 μg of the peptide induced a delayed negative chronotropic effect in the both phases of the heart contractile activity in comparison to injection of saline B, which caused no effect (Fig. 2a). The cardioinhibitory effect started 8 h after the peptide injection and it intensified to 18 h after injection. Cardioinhibitory in vitro peptide Zopat-MS-2 in vivo showed bimodal actions on the heart contractile activity of pupae (Fig. 2c). Injection of 2 μg of this peptide caused the positive chronotropic effect in the anterograde phase of the heart contractions. Maximal cardiostimulatory effect of the peptide was observed 10 h after injection. The heartbeat contraction rhythm in this phase remained higher even 20 h after peptide injection. An opposite cardioinhibitory effect Zopat-MS-2 showed in the retrograde phase. It gradually decreased the myocardium contractions from 6 h after injection and after 20 h the contraction frequency was still lower than the control frequency. Lack of a clear statistically significant differences in comparison to saline injections were indicated due to large fluctuations of the pupal heartbeat rate.

Myoregulatory activity of peptides on the reproductive organs

The ejaculatory duct of the *Z. atratus* adult male during perfusion with saline A showed irregular contractions with the frequency on average 4 ± 2 contractions/min. All three peptides tested caused mainly myoinhibitory reversible effects on the ejaculatory duct contractions (Fig. 3a–c).
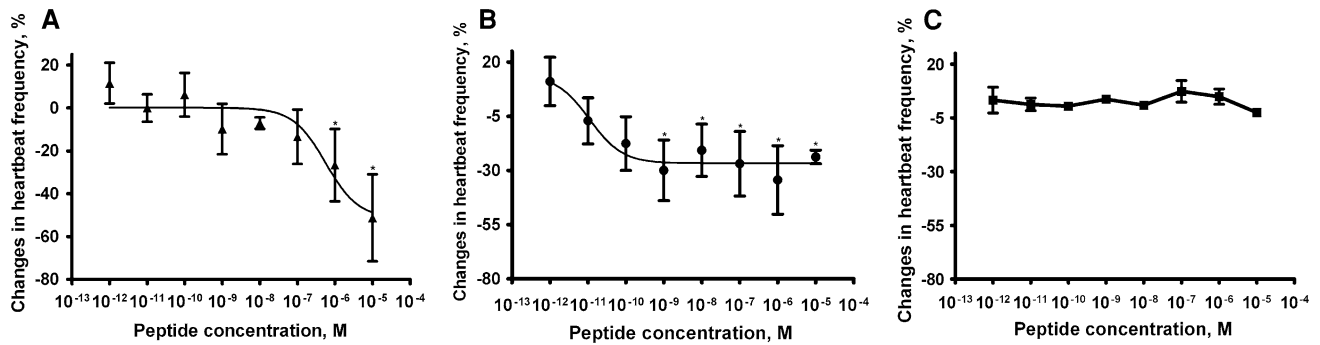
Fig. 1 Changes compared to control in the heartbeat frequency of adult beetles after application of Zopat-MS-2 (**a**), Zopat-SK-1 (**b**) and Zopat-NVPL-4trunc. (**c**). Mean ± SEM are given from at least eight determinations. Statistically significant differences ($P \leq 0.05$) in heartbeat frequency from control (saline A application) are indicated by *asterisks* (*t* student test)
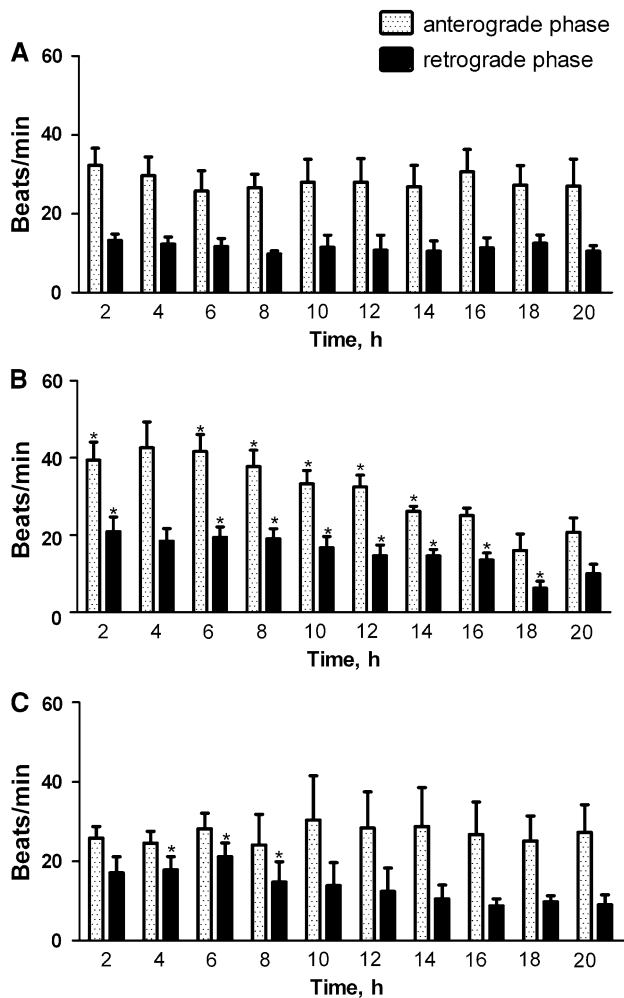

Fig. 2 The heartbeat contraction rhythm in anterograde and retrograde phases of 1-day old pupae of *Z. atratus* up to 20 h after injection of saline B (**a**), Zopat-SK-1 (**b**) and Zopat-MS-2 (**c**). Mean ± SEM are given from at least eight determinations. Statistically significant differences ($P \leq 0.05$) from control (saline B injection) are indicated by *asterisks* (*t* student test)

As on the heart, application of Zopat-MS-2 caused inhibition of the ejaculatory duct contractions in majority of the tested concentration range (Fig. 3a). Maximal myoinhibitory effect of peptide (85–90% inhibition) was observed in concentration 10^{-5} M where it caused short arrests in contraction activity of this organ. More potent in inhibition of the ejaculatory duct contractions was Zopat-SK-1 (Fig. 3b). Sulfakinin caused inhibition in almost whole tested concentration range (10^{-12}–10^{-5} M), however, it induced the strongest effect (80% of inhibition) in 10^{-10} M whereas the weakest effect in 10^{-5} M. Calculated EC_{50} values for all three peptides tested (Table 1) indicated that the most potent inhibitory peptide on the ejaculatory duct of *Z. atratus* was Zopat-NVPL-4trunc. In low concentration range (10^{-12}–10^{-10} M) it caused no significant effects. In higher concentrations (10^{-7}–10^{-5}) it inhibited contractions of the ejaculatory duct by 50–60% (Fig. 3c). The EC_{50} value for this peptide (7.6×10^{-10} M) was only a slightly lower than for Zopat-SK-1 (8.4×10^{-9} M).

The oviduct of *Z. atratus* adult female showed irregular contraction frequency with a level 14 ± 7 contractions/min during superfusion with saline A. Application of the tested peptides caused fast, dose-dependent and reversible effects on the oviduct contraction frequency.

Zopat-MS-2 was a weak inhibitor of the oviduct contractions. The peptide caused the strongest effect (inhibition by 20%) when it was applied in pharmacological concentration 10^{-5} M (Fig. 4a). The most potent myoinhibitory peptide which was tested on the oviduct was SK-1. It caused inhibition in almost all concentration range (10^{-12}–10^{-7} M). The strongest myoinhibitory effect was observed in concentration 10^{-10} M, and the weakest in 10^{-5} M (Fig. 4b). Similar to Zopat-MS-2 Zopat-NVPL-4trunc. was also weak inhibitor of the oviduct contractions (Fig. 4c). No significant changes in contractions frequency were observed in the tested concentration except 10^{-5} M, however, the effect was very weak (10% inhibition).
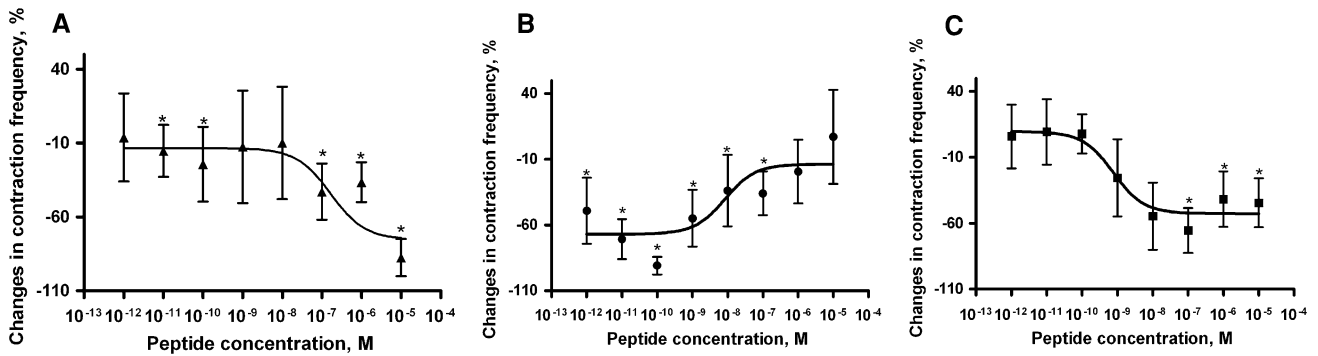
Fig. 3 Changes compared to control in the ejaculatory duct contractions frequency of Z. atratus adult males after application of Zopat-MS-2 (**a**), Zopat-SK-1 (**b**) and Zopat-NVPL-4trunc. (**c**). Mean ± SEM are given from at least eight determinations. Statistically significant differences ($P \leq 0.05$) from control (saline A) are indicated by *asterisks* (*t* student test)

Table 1 EC_{50} values calculated for the effects of peptides on the contraction frequency of Z. atratus visceral muscles

Tested organ	Peptide		
	Zopat-MS-2	Zopat-SK-1	Zopat-NVPL-4trunc.
Heart	↓ 5.6×10^{-7} M	↓ 1.1×10^{-11} M	–
Ejaculatory duct	↓ 1.7×10^{-7} M	↓ 8.4×10^{-9} M	↓ 7.6×10^{-10} M
Oviduct	↓ 0.6 M	↓ 2.5×10^{-7} M	↓ 0.2 M
Hindgut	↓ 9.4×10^{-8} M	↑ 2.7×10^{-9} M	↓ 8.3×10^{-8} M

↑ stimulation

↓ inhibition

Effects of peptides on the hindgut contractions

The hindgut of the Z. atratus adult, superfused with physiological saline A showed irregular contractions activity with a level 5 ± 4 contractions per minute. The tested peptides caused differentiated myotropic effects on the hindgut.

Zopat-MS-2 induced myoinhibitory effect in concentration range of 10^{-6}–10^{-5} M. Its application decreased the hindgut contractions by 50%. In low concentrations 10^{-12}–10^{-8} M the hindgut contractions rhythm remained unchanged (Fig. 5a). Only one tested peptide Zopat-SK-1 increased the hindgut contractions frequency (Fig. 5b). This peptide stimulated contractions in almost whole tested concentration range (10^{-12}–10^{-5} M). The strongest effect (almost 100% stimulation) caused application of low physiological concentrations of Zopat-SK-1 (10^{-12}–10^{-9} M), whereas, its concentrations from 10^{-8} to 10^{-5} M stimulated hindgut by 20% when compared to control application of saline B. However, these results were not
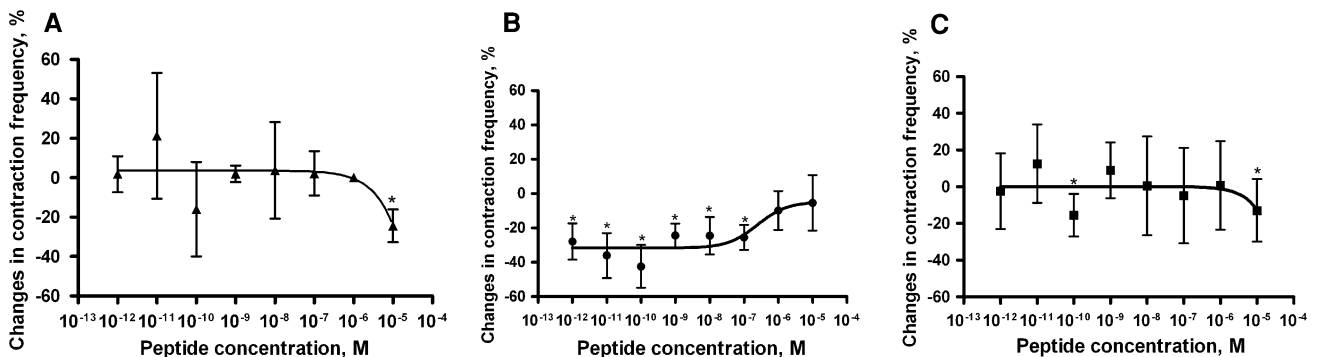

Fig. 4 Changes compared to control in the oviduct contractions frequency of Z. atratus adult females after application of Zopat-MS-2 (**a**), Zopat-SK-1 (**b**) and Zopat-NVPL-4trunc. (**c**). Mean ± SEM are given from at least eight determinations. Statistically significant differences ($P \leq 0.05$) from control (saline A) are indicated by *asterisks* (*t* student test)
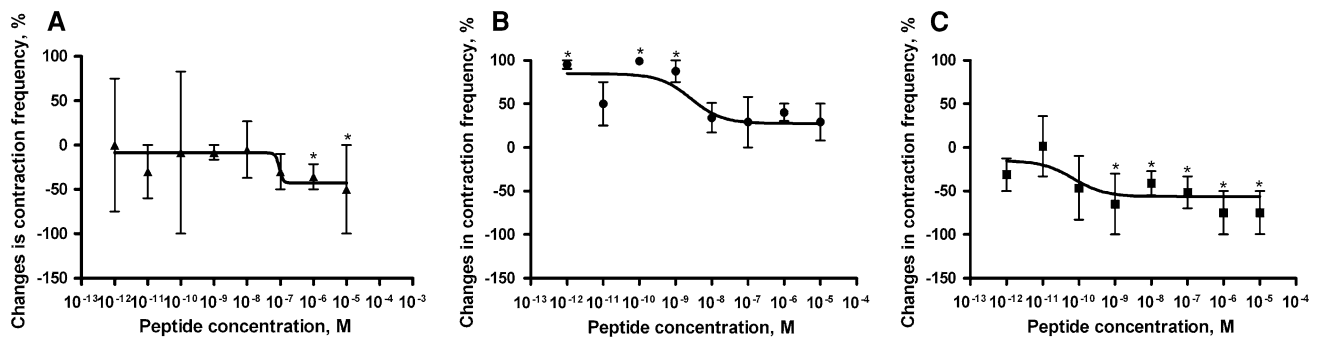
Fig. 5 Changes compared to control in the hindgut contraction frequency of *Z. atratus* adults after application of Zopat-MS-2 (**a**), Zopat-SK-1 (**b**) and Zopat-NVPL-4trunc. (**c**). Mean ± SEM are given from at least eight determinations. Statistically significant differences ($P \leq 0.05$) from control (saline A) are indicated by *asterisks* (*t* student test)

statistically significant. Similar effect to myosuppressin was produced by Zopat-NVPL-4trunc. This peptide also inhibited the gut contractions with a level 50% (Fig. 5c). However, threshold concentration for observable effects was lower (10^{-8} M) than in Zopat-MS-2 (10^{-7} M). In concentration range of 10^{-8}–10^{-5} M the inhibitory effects remained on the same level (50% inhibition).

Hyperglycaemic activity of peptides

The experiments demonstrated hyperglycaemic activity of peptides. Injections of 1, 2 and 4 µg of all three peptides tested increased the haemolymph sugar level in freshly moulted *Z. atratus* larvae. As the phenol–sulphuric acid method for carbohydrates determinations does not give any information on the nature of the free sugars in the haemolymph, we identified sugars qualitatively by thin-layer chromatography. The predominant haemolymph sugar in *Z. atratus* larvae was the disaccharide trehalose (>90% of total sugars) accompanied by very small amounts of glucose, fructose and maltose (data not shown). Therefore, we use the more general therm "hyperglycaemia" instead of "hypertrehalosaemia" in this context, since only total haemolymph free sugars were determined.

The strongest hyperglycaemic effects were caused by Zopat-SK-1. Injection of 4 µg of this peptide increased haemolymph sugar level by 150% (Fig. 6). A similar effect occurred after injection of 2 µg, however, it was much weaker (increased by 50–60%). Two other tested peptides Zopat-NVPL-4trunc. and Zopat-MS-2 increased the sugar level by 100%. Hyperglycaemic effect of Zopat-MS-2 remained still after injection of 2 µg with a level of 75%, whereas injections of 1 and 2 µg of Zopat-NVPL-4trunc. caused no effects. A similarly 1 µg of Zopat-MS-2 caused no effect in the haemolymph sugar concentrations (Fig. 6). However, these effects were not statistically significant.
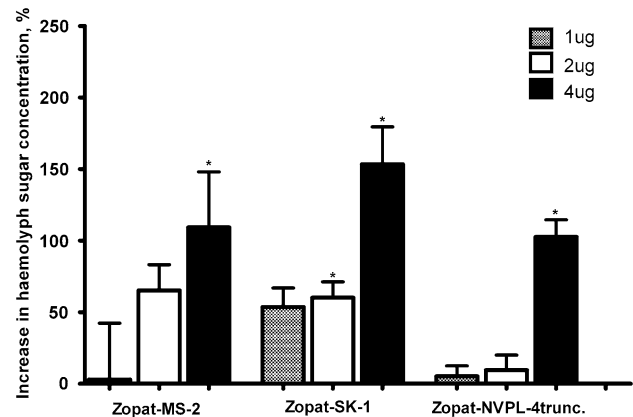

Fig. 6 Changes in haemolymph free sugar concentration in the larvae injected with the different doses of Zopat-MS-2, Zopat-SK-1, Zopat-NVPL-4trunc. Columns represent mean ± SEM from at least five determinations. Statistically significant differences ($P \leq 0.05$) from control (saline B injection) are indicated by *asterisks* (*t* student test)

Discussion

In this study we examined myotropic and hyperglycaemic activities of three peptides Zopat-MS-2, Zopat-SK-1 and Zopat-NVPL-4trunc. isolated recently from neuroendocrine system of the *Z. atratus* beetle. Previous studies on the different physiological effects caused by hormones in this beetle were performed only in heterologous bioassays by usage of peptides from other insect species (Marciniak and Rosinski 2010; Skonieczna and Rosinski 2004; Sliwowska et al. 2001). Three tested neuropeptides caused differentiated, dose-dependent and organ specific physiological activity. A few newly discovered myotropic and glycaemic activities were new physiological properties of myosuppressins, sulfakinins and NVPL peptides in insects.

Effects of Zopat-MS-2

The decapeptide Zopat-MS-2 is a member of myosuppressin family, which belongs to a large, structurally

diverse group of peptides related to FMRFamide (FaRPs) with pleiotropic activity (Nässel 2002). The beetle *Z. atratus* is a second insect, after *L. migratoria*, with two identified myosuppressins LMS (pEDVDHVFLRFa) and Zopat-MS-2 (pEDVEHVFLRFa) (Marciniak et al. 2010b). Thus far, the most ubiquitous peptide from this group was leucomyosuppressin (LMS) (Orchard and Lange 2006). The physiological activity of LMS and other FaRPs was studied in several insects, including beetles, with different myotropic and metabotropic bioassays (Orchard et al. 2001; Wasielewski and Skonieczna 2008).

In all myotropic bioassays, myosuppressin 2 showed inhibition of the frequency contraction of visceral muscles. It was the most effective on the hindgut (ED$_{50}$ = 9.4 × 10^{-8} M). MS-2 was less effective on the ejaculatory duct (ED$_{50}$ = 1.7 × 10^{-7} M), heart (ED$_{50}$ = 5.6 × 10^{-7} M) and oviduct (ED$_{50}$ = 0.6 M). In comparison to LMS, which caused inhibition of cockroach *L. maderae* oviduct contractions in concentrations 10^{-10}–10^{-7} M (Cook and Wagner 1991) and locust *L. migratoria* oviduct contractions with a threshold 10^{-7} M (Peeff et al. 1993), Zopat-MS-2 is a weaker inhibitor of the oviduct contractions. Interestingly, FMRFa prompted contrary myotropic effect on the *Z. atratus* beetle oviduct. It stimulated the contraction frequency of the isolated oviduct and ejaculatory duct of adult beetles (Marciniak and Rosinski 2010). In comparison to FMRFa this difference is probably caused by the replacement M/L in C-terminal sequence of myosuppressin (-FLRFa). The C-terminal fragment -FLRFa is a core sequence essential for myosuppressin activity (Orchard et al. 2001).

Zopat-MS-2 only partially retained its cardioinhibitory activity in in vivo studies. It inhibited the frequency of contractions in the retrograde phase and stimulated in the anterograde phase of the heart activity. This effect was different from the one produced by LMS. Injection of LMS into pupae of *Z. atratus* caused no changes in the frequency contractions but significantly reduced duration of both the anterograde and retrograde phases (Skonieczna 2006). This shows that both peptides LMS and Zopat-MS-2, despite the fact that they belong to the same neuropeptide family, act with different manner in the regulation of the heart activity. The possible reason for this is minor structural difference in amino acid composition of peptides. Zopat-MS-2 differs from leucomyosuppressin only by a conservative substitution of glutamic acid (E) for aspartic acid (D) at position 4 (Marciniak et al. 2010b). Interestingly in in vivo studies on the heart of *Z. atratus* both peptides were cardioinhibitory, however, LMS was a stronger inhibitor than Zopat-MS-2. The threshold concentration for cardioinhibitory effect of LMS was at 10^{-9} M (Skonieczna and Rosinski 2004) whereas for Zopat-MS-2 at 10^{-7} M. The maximal effect for LMS was also stronger than for Zopat-MS-2 (Skonieczna and Rosinski 2004).

Myosuppressin peptides inhibited contractions of different parts of alimentary tract in insects (Fuse and Orchard 1998). Previous studies showed that myosuppressin isolated from *S. gregaria* Scg-MS (PDVDHFLRFa) prompted bimodal effects in the contraction frequency of the *Z. atratus* larvae hindgut. It stimulated contractions at 10^{-7} M and inhibited the contraction frequency in pharmacological concentrations range of 10^{-5}–10^{-3} M (Skonieczna 2006). Myosuppressin 2 tested in our studies also decreased the contraction frequency. The inhibitory activity on the alimentary tract movement of *Z. atratus* and other insects demonstrates that peptides from this family are food uptake regulators (Audsley and Weaver 2009). Together with myoinhibitory action on the gut Zopat-MS-2 caused also hyperglycaemic activity. Similar effects showed LMS in *T. molitor* and *Z. atratus* larvae (Skonieczna 2006; Wasielewski and Skonieczna 2008). Possible mechanism of hyperglycaemic activity is stimulation of trehalose synthesis in fat body and/or an increase release of it into the haemolymph. This also suggests glycogenolitic activity of LMS in the fat body tissue of *Z. atratus* (Skonieczna 2006).

Myoregulatory and hyperglycaemic activities of sulfakinin

Thus far, sulfakinins were showed as peptides with pleiotropic activity in hemi- and holometabolic insects (Schoofs and Nachman 2006). The main physiological functions of these peptides were connected with the feeding regulation (Audsley and Weaver 2009) and modulation of the cardiac pulsation in cockroaches and flies (Nichols et al. 2002). Myotropic properties of sulfakinins were studied mainly by the usage of the gut and heart bioassays (Schoofs and Nachman 2006). They were shown to stimulate gut contractions of cockroach *L. maderae* (Nachman et al. 1986) and *D. melanogaster* (Nichols 2007), however, in some flies *C. vomitoria* and *Phormia regina* they caused no effects (Duve et al. 1994; Haselton et al. 2006). Sulfakinin found in *Z. atratus* showed the pleiotropic activity—myotropic and glycaemic. In the myotropic actions we showed tissue specific effect of the peptide. In *Z. atratus,* SK-1 was a strong stimulator of the hindgut muscle contractions, but it inhibits also the frequency of contractions of the oviduct, ejaculatory duct and heart. These are the new myotropic activities of sulfakinins in insects. In *Z. atratus*, similarly to *D. melanogaster* (Nichols 2007), the active form was nonsulfated peptide found in the neuroendocrine system of this beetle. The bioassays performed in this study showed that sulfakinins are not only involved in feeding regulation but also in the reproduction and haemolymph circulation.

Cardioinhibitory effect caused by Zopat-SK-1 on the *Z. atratus* heart was observed in the threshold concentration of 10^{-11} M and was different from actions on the heart of

other insect species. For example, sulfakinins in *P. americana* showed cardiostimulation in the threshold concentration of 10^{-11} M (Predel et al. 1999). In *D. melanogaster,* drosulfakinins were cardiostimulatory in all tested developmental stages (larva, pupa, adult) (Nichols et al. 2009). In this studies Nichols et al. proved, that nonsulfated peptides nsDSKI (FDDYGHMRFa) and nsDSKII (GGDDQFDDYGHMRFa) also maintain cardioactivity and tyrosine sulfation is not crucial for binding of the peptides with receptor in myocardium. nsDSKI increased the heart contractions rate by 25% in larvae whereas nsDSKII increased the contractions rhythm by 38% in larvae and by 65% in adults (Nichols et al. 2009). All nonsulfated peptides were less cardiostimulatory than their sulfated forms. Our results obtained in this study are similar. Nonsulfated Zopat-SK-1 also maintains its physiological activity, however, we observed cardioinhibitory rather than cardiostimulatory effect. No data are avalible on activity of sulfated peptides in *Z. atratus*. Cardioinhibitory activity of SK-1 was also observed in pupae in in vivo bioassay. Inhibition was observed up to 18 h after peptide injection. It proves that sulfakinin are involved in regulation of haemolymph circulation in *Z. atratus*. In contrast to *D. melanogaster* non sulfated peptides affect also the pupal heart.

No data are available on involvement of sulfakinins in the reproductive organ motility. In this work we showed for the first time myoinhibitory properties of sulfakinins on the female (oviduct) and male (ejaculatory duct) muscles of the reproductive organs in insects. It is surprising that for the oviduct the strongest inhibitory effect of Zopat-SK-1 was observed in the lowest concentration of the peptide. Similar effect was observed on the heart and hindgut. The reason for this is unclear. Maybe higher concentrations of sulfakinins caused the receptor desensitization. Thus far, no information are accessible for the biology of sulfakinin receptor in *Z. atratus*. Further, detailed studies are needed to explain this effects. Inhibition of the oviduct muscle contractions may block oocyte movement through this organ and affect reproduction. Similarly a decrease of the ejaculatory duct contractions may arrest sperm transfer to the female and result also in reproduction disorders.

The another new physiological activity showed in this work was hyperglycaemic action of Zopat-SK-1 in larvae of *Z. atratus*. This effect was lower than caused by endogenic adipokinetic/hipertrehalosaemic peptide Tenmo-AKH (pELNFSPNWa) in adults of *Zophobas atratus* (Gäde and Rosinski 1990). However, previous studies showed that hyperglycaemic response in beetles was stage dependent. In *Tenebrio molitor*, the effects were more potent in larvae when compared to adults after the same peptide dose injections (Gäde and Rosinski 1990). Clearly, further studies are needed to unravel the hyperglycaemic mechanism of action for sulfakinins in beetles.

New physiological activities of Zopat-NVPL-4trunc.

We showed first physiological activities of NVPL peptide in insects. Thus far role of this peptide group as neurotransmitters, neuromodulators or classical hormones remain unknown. To date, bioanalogs of NVPL peptides have been found only in *A. mellifera* (Hummon et al. 2006), *T. castaneum* (Li et al. 2008) and recently in *Z. atratus* (Marciniak et al. 2010b).

Identified in *Z. atratus* heptapeptide Zopat-NVPL-4trunc. inhibited significantly muscle contractions of two visceral organs, the ejaculatory duct and hindgut of *Z. atratus* adults. Effects of neuropeptides on the insects ejaculatory duct were studied to a limited extent. Rankin et al. (2009) showed that proctolin stimulated ejaculatory duct contractions of *E. annulipes* at low concentrations range (10^{-9}–10^{-7} M) whereas at high concentrations (10^{-5} M) caused no significant effect. Proctolin stimulated also the ejaculatory duct muscle contractions of *Z. atratus* and *T. molitor* beetles (Marciniak and Rosinski 2010) and induced contractions of the male accessory gland of *G. bimaculatus* (Kimura et al. 1989). Peptides with myoinhibitory activity on the ejaculatory duct, but only at a higher concentration range, were allatostatin AST A in *E. annulipes* (Rankin et al. 2009) and FMRFamide in beetles *T. molitor* and *Z. atratus* (Marciniak and Rosinski 2010). In the *Z. atratus* males, peptide NVPL-4trunc. inhibited the ejaculatory duct contractions by 50% in concentration range 10^{-9}–10^{-5} M. It is the most active from thus far tested on the ejaculatory duct insects neuropeptides. Zopat-NVPL-4trunc. also decreased the contraction frequency of *Z. atratus* adults hindgut. This peptide caused the biggest inhibition of the gut contractions (50%) from all of three tested peptides. Apart from myotropic activities it also induced hyperglycaemic effects after injection into larvae of *Z. atratus*. However, the hyperglycaemic response caused by Zopat-NVPL-4trunc. was weaker in comparison to the effect produced by Zopat-MS-2 and Zopat-SK-1. In order to elucidate the mechanism of action of Zopat-NVPL-4trunc. further experiments are required.

In conclusion, we tested three peptides isolated from *Z. atratus* in myotropic and glycaemic bioassays. We confirm that myosuppressins have not only myoinhibitory properties on the visceral muscle contractions but also possess the hyperglycaemic activity. Zopat-SK-1 was a stimulator of the hindgut contractions and also inhibited the heart, ejaculatory duct and oviduct contractions. These are new physiological activities of sulfakinins in insects together with ability to the increase of the haemolymph free sugar level. All three physiological actions of Zopat-NVPL-4trunc. are new activities for this group of peptides. NVPL-4trunc. inhibited the hindgut and ejaculatory duct

contractions and showed the hyperglycaemic properties. Our comparative studies showed that all three tested peptides posses pleiotropic activities in regulation of the visceral organ muscle contractions and the free sugar level in the haemolymph.

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