A novel adipokinetic octapeptide found in the damselflies *Pseudagrion inconspicuum* and *Ischnura senegalensis*

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A member of the adipokinetic hormone family of peptides was identified in the damselflies *Pseudagrion inconspicuum* and *Ischnura senegalensis* using a heterologous (in migratory locusts and American cockroaches) and a homologous (in *P. inconspicuum*) bioassay. After isolation of the peptide by reversed-phase h.p.l.c. of corpora cardiaca, its structure was determined by automated Edman degradation and matrix-assisted laser-desorption ionization m.s. The sequence of a blocked uncharged octapeptide was established: pGlu-Val-Asn-Phe-Thr-Pro-Gly-

TrpNH₂. One corpus cardiacum of *P. inconspicuum* contains about 2.4 pmol of this novel peptide. The synthetic peptide was chromatographically indistinguishable from the natural compound, and on injection in low quantities into the damselfly *P. inconspicuum*, haemolymph levels of lipids were increased. It is concluded that the novel peptide may be involved in controlling lipid mobilization during flight. Hence the peptide is designated *Psi-AKH*, *Pseudagrion inconspicuum* adipokinetic hormone.

INTRODUCTION

Neuropeptides regulating insect energy metabolism and acting on colour change in crustaceans are structurally similar and belong to the adipokinetic hormone (AKH)/red-pigment-concentrating hormone (RPCH) family of peptides [1,2]. The peptides are 8- to 10-mers, are characterized by N- and C-blocked termini (pyroglutamate and an amide respectively), aromatic amino acids at positions 4 and 8, a glycine residue at position 9, and all but two members are not charged [1,2]. In insects, most of the peptides have been sequenced from orthopteran species or from highly evolved beetles, moths and flies [2]. Recently, we have analysed the structures of such peptides in the more primitive order of dragonflies (Odonata). It became clear that *Libellula auripennis*, a member of the most advanced family of Odonata, the Libellulidae, contains a slightly different member of the AKH/RPCH family from that in *Anax imperator*, a member of the less advanced Aeshnidae (pGlu-Val-Asn-Phe-Thr-Pro-Ser-TrpNH₂ compared with pGlu-Val-Asn-Phe-Ser-Pro-Ser-TrpNH₂) [3,4]. Both to extend the available structural data to another family of the Odonata and to provide a basis for studies into the role of neuropeptides in the regulation of energy metabolism during flight in Odonata, we have isolated and sequenced the AKH/RPCH family member of the damselflies *Pseudagrion inconspicuum* and *Ischnura senegalensis*. Both species belong to the family Coenagrionidae, suborder Zygoptera, and not to the suborder Anisoptera like *L. auripennis* and *A. imperator*. The Coenagrionidae represent the most primitive origin of all the present-day Odonata [5]. Another aim of this study was to find out whether species other than the previously studied *Carausius morosus* have a post-translationally modified AKH/RPCH family member. In *C. morosus* it had been shown that the decapeptide occurs in an unmodified and modified form; results indicate the modification to be a glycosylation [6]. Remarkably, tryptophan has been identified as the

modified residue, which means the existence of a new glycosylation site in addition to the usual N- or O-glycosylation sites at asparagine or threonine.

MATERIALS AND METHODS

Insects

All damselflies used for isolation of peptides were adult specimens of the two species *P. inconspicuum* (43.5 ± 3.5 mg, *n* = 21) and *I. senegalensis* (34.0 ± 4.5 mg, *n* = 17), belonging to the family Coenagrionidae. Insects were collected in the wild by netting on the banks of the Liesbeek River (Mowbray, Cape Town, South Africa) during the austral summers of 1992/1993 and 1993/1994. After capture, damselflies were immediately placed into small containers kept on ice. In the laboratory, the corpora cardiaca were immediately dissected as described previously [3]. For heterologous bioassays (see below), 15–25-day-old adult male migratory locusts, *Locusta migratoria*, from our own colony were used, which was maintained as outlined previously [7]. Adult male cockroaches, *Periplaneta americana*, were kept and fed as described previously [8]. For homologous bioassays (see below), adult damselflies of the species *P. inconspicuum* were used. Immediately after netting, damselflies were placed into small containers (40 ml volume) containing wet paper towel, and kept in a cooler box with ice. This procedure prevented evaporative water loss and wing flapping, thus keeping the metabolic rate low.

Isolation of corpora cardiaca peptides

Corpora cardiaca were dissected into distilled water and frozen at –25 °C. Methanolic extracts were prepared in batches of 50–100 glands as described previously [9]; they were applied to a Nucleosil 100 C-18 column for reversed-phase liquid chromatography (RP-h.p.l.c.), using equipment described previously [10].

Abbreviations used: AKH, adipokinetic hormone; Ani-AKH, *Anax imperator* adipokinetic hormone; Fmoc, fluoren-9-ylmethoxycarbonyl; Lia-AKH, *Libellula auripennis* adipokinetic hormone; Lom-AKH-I, *Locusta migratoria* adipokinetic hormone I; Pea-CAH-I, *Periplaneta americana* hypertrehalosaemic hormone I; Psi-AKH, *Pseudagrion inconspicuum* adipokinetic hormone; RPCH, red-pigment-concentrating hormone; RP-h.p.l.c., reversed-phase h.p.l.c.

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Table 1 Adipokinetic and hypertrehalosaemic activity of a crude methanolic extract of corpora cardiaca from the damselfly *Pseudagrion inconspicuum*

Insects were injected as indicated. Total haemolymph lipid concentration in adult male locusts and total haemolymph carbohydrate concentration in adult male cockroaches are expressed as mg/ml of haemolymph, and results are means $\pm$ S.D. The significance of the difference between values before and after injection is indicated by † $P < 0.01$ and ‡ $P < 0.001$, using paired t tests.

Acceptor insect...	<i>Locusta migratoria</i>				<i>Periplaneta americana</i>			
		Haemolymph lipids (mg/ml)				Haemolymph carbohydrates (mg/ml)		
Treatment	<i>n</i>	0 min	90 min	Difference	<i>n</i>	0 min	120 min	Difference
Control (10 μ l of distilled water)	8	10.1 $\pm$ 1.7	11.8 $\pm$ 2.9	1.7 $\pm$ 2.1	7	17.8 $\pm$ 3.8	19.6 $\pm$ 3.9	1.8 $\pm$ 2.0
Damselfly extract (1.6 gland equivalents)	6	9.5 $\pm$ 3.0	26.8 $\pm$ 11.6	17.3 $\pm$ 9.2†	9	17.1 $\pm$ 2.5	35.8 $\pm$ 11.5	18.7 $\pm$ 9.7‡
Locust extract (0.1 gland equivalents)	8	10.4 $\pm$ 1.9	55.9 $\pm$ 4.8	45.5 $\pm$ 4.1‡	—	—	—	—
Cockroach extract (0.1 gland equivalents)	—	—	—	—	7	15.8 $\pm$ 3.0	48.1 $\pm$ 12.7	32.3 $\pm$ 11.3‡

Table 2 Conspecific adipokinetic and hypertrehalosaemic activity of a crude methanolic extract of corpora cardiaca as well as native synthetic peptide from the damselfly *Pseudagrion inconspicuum*

Adult damselflies were injected as indicated. Significant difference: † $P < 0.01$; ‡ $P < 0.001$ compared with water injection using Student's t test. Values are means $\pm$ S.D. The acceptor insect was *Pseudagrion inconspicuum*.

Treatment	<i>n</i>	Total haemolymph [lipid] (mg/ml)	<i>n</i>	Total haemolymph [carbohydrate] (mg/ml)
Without injection	17	16.1 $\pm$ 5.8	22	6.4 $\pm$ 2.5
Control (1 μ l of distilled water)	33	14.6 $\pm$ 4.3	36	5.9 $\pm$ 2.6
Damselfly extract (1 gland equivalent)	19	25.8 $\pm$ 6.2‡	22	7.9 $\pm$ 2.5†
Active peak material from h.p.l.c. (1 gland equivalent)	11	29.4 $\pm$ 9.6‡	—	—
Control	47	11.1 $\pm$ 4.3		
Synthetic peptide				
1 pmol	36	21.2 $\pm$ 8.2‡		
2.5 pmol	34	18.1 $\pm$ 7.0‡		

In the first h.p.l.c. run, 1-min fractions were collected and used for bioassays. In later runs, only the active fractions were collected manually, and the combined purified material from a total of 350 corpora cardiaca was used for the elucidation of the primary structure by automated sequencing and m.s. and for structural confirmation by isolation on various h.p.l.c. supports.

Sequence determination and m.s.

About 100 pmol of purified active material was enzymically deblocked by using L-pyrogutamate aminopeptidase, as described in [11], except that the enzyme was purchased from Sigma Chemical Co. Under these conditions, about 70 % of the peptide was deblocked. The deblocked peptide was separated from the undigested peptide by RP-h.p.l.c. [12], manually collected, spotted on to polybrene-coated glass-fibre discs and subjected to automated Edman degradation (model 477A; Applied Biosystems, Foster City, CA, U.S.A.). The sequencer was connected to an on-line phenylthiohydantoin amino acid

analyser (model 120A; Applied Biosystems). Sequencing reagents and solvents were from Applied Biosystems. Sequencing and phenylthiohydantoin analysis were carried out according to standard protocols. Mass spectra were acquired using a matrix-assisted laser-desorption ionization spectrometer (Bruker REFLEX, Bruker-Franzen, Bremen, Germany) [13]. The acceleration voltage was set to 30 kV for the linear mode. The matrix was a saturated solution of α -cyano-4-hydroxycinnamic acid dissolved in water/acetonitrile (7:3, v/v) [14]. Sample solution (0.5 μ l, about 1 pmol) was mixed on target with matrix solution (1:1, v/v) and left to dry before introduction into the mass spectrometer. Depending on the signal-to-noise ratio, each spectrum is the average of between 50 and 200 single-shot spectra acquired in sets of five shots.

Synthetic peptides

The hypertrehalosaemic peptide from the American cockroach, *Pea*-CAH-I, and the adipokinetic peptide of the migratory locust, *Lom*-AKH-I, came from Peninsula Laboratories (Belmont, CA, U.S.A.). The adipokinetic peptides from the dragonflies *L. auripennis*, *Lia*-AKH, and *A. imperator*, *Ani*-AKH, had been custom-synthesized previously by EMBL. The novel damselfly peptide was synthesized on a multiple solid-phase peptide synthesizer (AMS 422; Abimed) employing Fmoc [N-(9-fluorenylmethoxycarbonyl)] chemistry. The peptide was purified by RP-h.p.l.c. and its identity verified by amino acid analysis and m.s.

Bioassays

The lipid-mobilization bioassay in *Locusta migratoria* and the carbohydrate-mobilization assay in *Periplaneta americana* were performed as described elsewhere [15].

Adult *P. inconspicuum*, of both sexes, were collected from the wild by netting. Two individuals were kept on ice in a 40 ml plastic container, filled with wet paper towel, to prevent wing movement and evaporative water loss. The damselfies quietened down as soon as they gripped on to the paper tissue. They were transferred to the laboratory within 2 h of capture and were left at room temperature for another 2 h in the dark before the experiment. Each individual had 1 μ l of the appropriate test solution injected into the abdomen, taking care not to puncture the gut. Haemolymph samples (0.5 μ l) were introduced into 100 μ l of H₂SO₄, at either 90 or 120 min after injection, and

Figure 1 Isolation of damselfly adipokinetic neuropeptide

Analyses were performed on a Nucleosil 100 C-18 column eluted with a linear gradient of 0.11% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in 60% acetonitrile (solvent B). The gradient ran from 43 to 53% solvent B within 20 min at a flow rate of 1 ml/min. (a) A methanolic extract of 15 glands from *P. inconspicuus* and *I. senegalensis* was injected on to the column and the u.v. absorbance monitored at 214 nm. Fractions (1 ml each) were collected, dried *in vacuo*, dissolved in 75 μ l of distilled water, and 10 μ l was injected into at least six acceptor locusts to test for adipokinetic activity. Results are means $\pm$ S.D. of the differences between pre- and post-injection values. (b) as (a) but fluorescence was monitored at 276 nm (excitation) and 350 nm (emission). (c) A methanolic extract of 15 glands from the damselflies was co-injected with about 80 pmol of synthetic adipokinetic peptide from *A. imperator* (Ani-AKH; peak labelled 1) and about 30 pmol of synthetic adipokinetic peptide from *L. auripennis* (Lia-AKH; peak labelled 2). U.v. absorbance at 214 nm was monitored. Note: the biologically active peak from the damselflies is the peak labelled 3.

analysed for total lipid content by the sulphophosphovanillin method and for carbohydrates by the anthrone method.

Determination of blood volume

A trace amount of labelled inulin [$^3\text{H}(\text{G})$]inulin; 355.3 mCi/g; Du Pont} contained in 1 μ l of water (36487 c.p.m.) was injected into the damselflies as described above. After 20 min, a 0.5 μ l haemolymph sample was obtained and collected directly into 4.5 ml of scintillation fluid (Ultima Gold XR; Packard) in a scintillation vial and assayed for radioactivity [16].

RESULTS

Biological activity of damselfly corpora cardiaca extract

Crude methanolic extracts of damselfly (*P. inconspicuus*) corpora cardiaca were assayed for lipid- and carbohydrate-mobilizing

activity in acceptor locusts and cockroaches respectively. These widely used heterologous bioassays [1] showed the presence of adipokinetic/hypertrehalosaemic factors in the damselfly gland (Table 1). The dose used was able to elicit increases in haemolymph lipids and carbohydrates compared with control (water) injections in the respective bioassay insects. Compared with the maximal possible responses in locusts and cockroaches respectively, by injecting 0.1 gland equivalent of the corpora cardiaca of the respective species, the increase in damselfly material amounted to about 40% for lipid release and 62% for carbohydrate release. The concentration of total lipids in the haemolymph of non-injected control damselflies was about 2.5-fold higher than the total carbohydrate levels (Table 2). Conspecific injections of one gland equivalent of the damselfly resulted in a significant increase in the concentration of the haemolymph lipids compared with control injections, but carbohydrate levels were only marginally affected (Table 2).

Table 3 Amino acid sequence of damselfly neuropeptide by automated Edman degradation

The N-terminal pyroglutamic acid residue of the h.p.l.c.-purified peptide from the damselflies *Pseudagrion inconspicuum* and *Ischnura senegalensis* was cleaved off enzymically, and the deblocked peptide was analysed.

Amino acid	Recovery (pmol)
Val-1	34
Asn-2	18
Phe-3	17
Thr-4	13
Pro-5	10
Gly-6	15
Trp-7	3
— 8	—
— 9	—

Table 4 Chromatographic behaviour of natural peptide isolated from corpora cardiaca of *Pseudagrion inconspicuum* and synthetic pGlu-Val-Asn-Phe-Thr-Pro-Gly-TrpNH₂

The parameters for the Waters Nova-Pak columns were: dimensions, 3.9 mm × 150 mm; particle size, 4 µm; pore size, 6 nm (60 Å). Parameters for the Nucleosil column were 4.6 mm × 250 mm, 5 µm and 100 Å. The solvents used were as described in the legend to Figure 1. For the short columns the flow rate was 0.5 ml/min and the gradient ran from 35 to 45% B within 20 min, whereas the long column was developed at a flow rate of 1 ml/min and the gradient ran from 43 to 53% B within 20 min.

Column material	Retention time (min)		
	Natural peptide	Synthetic peptide	Natural and synthetic peptide co-injected
Nucleosil C-18	11.5	11.3	11.3
Nova-Pak C-18	11.0	11.1	11.1
Nova-Pak C-8	11.1	11.3	11.1
Nova-Pak Phenyl	13.4	13.3	13.3

Peptide purification

A methanolic extract from a mixture of corpora cardiaca from the damselfly species *P. inconspicuum* (about 85% of the sample) and *I. senegalensis* was fractionated on an analytical RP-h.p.l.c. column in a gradient mode with trifluoroacetic acid as ion-pairing reagent (Figure 1). Under the conditions employed, there were only a few distinct absorbance peaks at 214 nm (Figure 1a). When each 1 min fraction was tested for adipokinetic activity in locusts, only fractions 13 and 14 were active (see histogram in Figure 1a). This activity corresponds to the u.v. absorbance peak at 12.7 min (peak 3) which also showed a clear fluorescence signal (Figure 1b), indicating that the compound contains a tryptophan or tyrosine residue. No corresponding fluorescence was observed for the u.v. peak at 16.3 min, thus it contains no tryptophan or tyrosine residue. It was also not active in the locust bioassay; this is in agreement with the lack of a tryptophan residue, as all AKH/RPCH family members contain such a residue, which is important for biological activity [1]. A portion of the peak fraction material eluted at 12.7 min, representing one damselfly gland equivalent, was also injected into the damselfly *P. inconspicuum*. The concentration of haemolymph lipids increased significantly when compared with control injections

(Table 2). In subsequent experiments on the analytical column, it was shown that, for corpora cardiaca extracts prepared from either *P. inconspicuum* or *I. senegalensis*, the peak associated with bioactivity had the same retention time (results not shown). Comparison of the retention time of the active material from damselflies with those of the two known peptides, sequenced previously from the dragonflies *L. auripennis* [3] and *A. imperator* [4], revealed that no co-elution occurred (Figure 1c). Both peptides were eluted earlier, at 10.8 (*Ani*-AKH) and 11.9 min (*Lia*-AKH). We therefore concluded that the damselflies contain a novel AKH-family peptide.

Peptide characterization and quantification per gland

All known members of the AKH family have an N-terminal pyroglutamyl residue [1]. A sample of isolated active material from 60 corpora cardiaca was therefore enzymically deblocked, and the unblocked material, which was eluted about 6.5 min earlier on RP-h.p.l.c. (results not shown), was sequenced. As depicted in Table 3, automated Edman degradation of about 70 pmol resulted in the sequence Val-Asn-Phe-Thr-Pro-Gly-Trp. The octapeptide amide comprising pyroglutamic acid and the above amino acid sequence was synthesized. The synthetic peptide was eluted at essentially the same retention time on various chromatographic supports as the active material isolated from a crude extract of *P. inconspicuum* corpora cardiaca (Table 4). Co-injection of active-peak fraction and synthetic peptide resulted in co-elution on all the column materials examined (Table 4). Further structural information was obtained by m.s. The main ion was observed at *m/z* 952 which was attributed to the [M + Na]⁺ form of the peptide. The above sequence plus the N-terminal pGlu residue and the C-terminal amide was calculated to have a molecular mass of 929 Da. These results then identify the novel damselfly neuropeptide as having the sequence pGlu-Val-Asn-Phe-Thr-Pro-Gly-Trp-NH₂.

By comparing the peak area of known amounts of the synthetic peptide with those of three samples of glands of *P. inconspicuum*, it was calculated that one corpus cardiacum contains 2.4 pmol of the peptide.

Haemolymph volume

The haemolymph volume of *P. inconspicuum* was determined to be 6.5 ± 1.4 µl (males, *n* = 7) and 7.7 ± 1.8 µl (females, *n* = 5). There was no significant difference between the sexes. Blood volume of *I. senegalensis* was determined to be 5.4 ± 2.5 µl (*n* = 9; six males and three females).

Bioactivity of the novel peptide

The effect of the novel (synthetic) peptide was assayed in two doses on the concentration of haemolymph lipids in *P. inconspicuum* (Table 2). Both concentrations used produced a significant adipokinetic effect when compared with the water-injected controls.

DISCUSSION

Peptide structure and evolution of the AKH/RPCH family

We have fully elucidated the primary structure of a novel peptide from the corpora cardiaca of two damselfly species (family Coenagrionidae). It is a typical member of the AKH/RPCH family of peptides by the criteria given in the Introduction. Although the original sequence data were obtained from a mixture of corpora cardiaca of the two species, it was clearly

shown using RP-h.p.l.c. that active material from both species was eluted at identical retention times. No second, thus also no glycosylated, peptide with adipokinetic activity was found in the damselflies. This is in agreement with our previous results on other Odonata families [3,4], although a few other insect orders contain two AKH/RPCH family members in their glands. Many neuropeptides are amidated at the C-terminus, probably for protection against carboxypeptidases in the blood. Good evidence for this structural modification is provided by m.s. We and others have previously used fast atom bombardment m.s. or tandem fast atom bombardment m.s. in a number of geometrical combinations, but always needed at least 50 pmol to obtain reasonable spectra [17]. In contrast, to obtain an accurate mass with the new generation of laser-desorption instruments only about 1 pmol is necessary.

Most amino acid substitutions in the AKH/RPCH family can be explained, on consideration of the genetic code, by point mutations [3]. The situation is quite complex, however, at position 7, where the five amino acid residues (Ser, Gly, Asn, Asp, Trp) observed to occur in AKH/RPCH peptides cannot be explained by single-step point mutations between each other. Two possible routes with either Ser⁷ or Gly⁷ as the ancestral amino acid at this position were recently proposed [2], but the latter variant was favoured because Gly⁷ is also found in Crustacea (RPCH). The novel peptide supports this view, because it is found in members of the family Coenagrionidae, which are generally considered to be ancestors of other Odonata [5].

Physiological implications

There are some indications that energy for flight in damselflies is provided by oxidation of fats and that the novel peptide may be involved in regulation of lipid homeostasis. First, the total lipid concentration is higher than that of the carbohydrates in the blood and in the same range as in the libellulid *L. auripennis* [3], but not as high as in the aeshnid *A. imperator* [4]. The concentration of metabolites possibly reflects flight behaviour. *A. imperator* is continuously on the wing, and this appears to be associated with a much higher concentration of haemolymph lipid. This is in contrast with typical libellulids and members of the Zygoptera (such as *P. inconspicuum*) who perch much more often [18] and seem to have a correspondingly lower concentration of lipid in the blood. There is good evidence that lipid is a major fuel for flight in libellulid and aeshnid dragonflies. Fatty acids are oxidized at a high rate by flight muscle preparations or the isolated mitochondria of the libellulid *Pantala flavescens* [19] and of the aeshnid *A. imperator* [20] respectively. In addition, after forced flight of 2 h, diacylglycerols in the flight muscles of *P. flavescens* were significantly reduced and the non-esterified fatty acid content significantly increased [21]. Moreover, in *A. imperator*, flight muscles are characterized by high activity of a key enzyme of β -oxidation, β -hydroxyacyl-CoA dehydrogenase [20]. Secondly, injection of crude corpus cardiacum extract of the damselfly as well as the novel synthetic peptide elicited a significant lipid increase in the haemolymph.

The concentration of the injected synthetic peptide could be calculated because the haemolymph volume (7 μ l) had been determined. Injection of 1 pmol of the novel peptide, which was enough to elicit a maximum response (Table 2), corresponds to a haemolymph concentration of 0.14 μ M. This is in the same order of magnitude as the EC_{max} value of the hypertrehalosaemic response of the endogenous peptide (*Melolontha melolontha* corpus cardiacum peptide) in the beetle, *Pachnoda sinuata* [22], but 3–10-fold higher than the EC_{max} values for lipid mobilization of the conspecific peptides in the cricket, *Acheta domesticus* [23], or in the tobacco hornworm moth, *Manduca sexta* [24], and about 100-fold higher than the response of *Locusta migratoria* to its own peptide [23]. Because injection of low doses of the novel synthetic peptide (smaller quantities than is found of the endogenous peptide in the corpora cardiaca) elicited a significant increase in blood lipids and thus appears to be involved in lipid homeostasis, the peptide is designated *Psi-AKH*, *Pseudagrion inconspicuum* adipokinetic hormone.

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