Hyperprolinaemia caused by novel members of the adipokinetic hormone/red pigment-concentrating hormone family of peptides isolated from corpora cardiaca of onitine beetles

Gerd GÄDE

Zoology Department, University of Cape Town, Rondebosch 7700, South Africa

Two novel members of the adipokinetic hormone/red pigment-concentrating hormone family of peptides were identified in dung beetles of the genus *Onitis* using heterologous (measuring lipid and carbohydrate mobilization in locusts and cockroaches) and a homologous (measuring proline increase in the haemolymph) bioassay(s). Isolation of the peptides was achieved by single-step reverse-phase HPLC of corpora cardiaca extracts. The primary structure was elucidated by automated Edman degradation and by electrospray MS. Both peptides are blocked octapeptides containing three aromatic amino acids. Peptide 1, designated Ona-CC-I, is pGlu-Tyr-Asn-Phe-Ser-Thr-Gly-Trp-NH_2, and peptide 2, designated Ona-CC-II, is pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-Trp-NH_2. The synthetic peptides were chromatographically indistinguishable from the natural compounds. They both had a hyperprolinaemic effect in the dung beetle. Moreover, flight experiments established that proline is an important fuel to power flight metabolism in *Onitis* species. Therefore, it is concluded that these novel and unique peptides are involved in regulating proline-based flight metabolism.

INTRODUCTION

Intermediary energy metabolism in insects is regulated by small neuropeptides of the adipokinetic hormone/red pigment-concentrating hormone family (AKH/RPCH) [1,2]. The peptides consist of 8–10 amino acids, are blocked at both termini (N-terminus: pyroglutamic acid; C-terminus: carboxyamide), contain aromatic amino acids at positions 4 (mostly phenylalanine, but in one peptide tyrosine) and 8 (always tryptophan) and a glycine residue at position 9, and all but two members are not charged; furthermore, one member is glycosylated and another one has an unknown post-translational modification [3,4]. During long-distance flight in adult locusts and flight in adult tobacco hornworm moths the respective peptides control lipid mobilization [5,6], while in flies and cockroaches, carbohydrate mobilization is regulated [7,8]. The third major fuel for flight in insects, proline, has been shown to play an important role to power flight muscle metabolism in tsetse flies and the Colorado potato beetle (see [9]). Recently, other beetles, a fruit beetle [10] and a blister beetle [11], were found to oxidize proline in appreciable amounts; alanine appears to be the end product. It accumulates in the haemolymph and is conveyed to the fat body, where it participates in the resynthesis of proline. The question then arises whether mobilization of proline is also under hormonal control. Previous results gave some indication that peptides from the insect corpus cardiacum gland were involved. Injection of a crude gland extract into the Colorado beetle *in vivo* decreased the haemolymph alanine concentration, and proline synthesis was stimulated *in vitro* by gland extracts from various insect species and by synthetic adipokinetic hormone I of the migratory locust *Locusta migratoria* (*Lom*-AKH-I) [12]. In the fruit beetle, *Pachnoda sinuata*, injection of a gland extract resulted in an increase in haemolymph proline levels and a decrease in alanine levels [13].

The present study was therefore initiated to address the following questions. (1) Do dung beetles of the genus *Onitis* contain members of the AKH/RPCH family of peptides in their corpora cardiaca and are these peptides structurally different from previously characterized members? (2) Which substrate is mobilized by these peptides and is this substrate used by the beetles as fuel for flight?

MATERIALS AND METHODS

Insects

Beetles of the genus *Onitis* (*O. aygulus*, *O. caffer*, *O. alexis* and *O. pecuarius*) were adult specimens. They were caught in the wild in cattle dung pats near Franschhoek and Swellendam in the Western Cape Province of South Africa. After capture, beetles were kept in large boxes with cattle dung, in which they were held for up to three weeks in the laboratory. Corpora cardiaca were immediately dissected upon arrival in the laboratory. For heterologous bioassays (see below), 15–25-day old adult male migratory locusts, *L. migratoria*, from our own colony were used [14]. Adult male cockroaches, *Periplaneta americana*, were kept and fed as described previously [15]. Adult fruit beetles, *P. sinuata*, were taken from our own colony [10].

Isolation of corpora cardiaca peptides

Corpora cardiaca were dissected either into distilled water or into 80 % methanol and frozen at −25 °C. Methanolic extracts

Abbreviations used: AKH, adipokinetic hormone; ES-MS, electrospray MS; Fmoc, *N*-fluoren-9-ylmethoxycarbonyl; *Lom*-AKH-I, *Locusta migratoria* adipokinetic hormone I; RPCH, red pigment-concentrating hormone; RP-HPLC, reverse-phase HPLC.

were prepared in batches of 10–20 glands as described previously [16]. Gland extracts were applied to a Nucleosil 100 C-18 column for reverse-phase HPLC (RP-HPLC), using equipment described previously [17]. Fractions displaying distinct UV and/or fluorescence peaks were collected manually and used for bioassays (see below). In subsequent runs, sufficient material from previously identified biologically active fractions was collected manually and used for the elucidation of the primary structure.

Sequence determination and MS

About 30–50 pmol of purified active material (two peaks each) from the two species *O. aygulus* and *O. pecuarius*, was enzymically deblocked by using L-pyroglutamate aminopeptidase, as described in [18], except that the enzyme was purchased from Sigma Chemical Company. Under the conditions employed, about 85 % of the peptide material was deblocked. The deblocked peptides were separated from the undigested peptides by RP-HPLC [19], manually collected and subjected to automated Edman degradation (model 477 A; Applied Biosystems, Foster City, CA, U.S.A.). The sequencer was connected to an on-line phenylthiohydantoin amino acid analyser (model 120 A; Applied Biosystems). Sequencing and phenylthiohydantoin analyses were carried out according to standard protocols. The undigested peptides of each HPLC. run (see above) were also manually collected, dried, taken up in 50 % aq. acetonitrile containing 0.05 % formic acid and used for electrospray MS (VG Quattro ES-MS, VG Organic, Altrincham, U.K.).

Synthetic peptides

Lom-AKH-I was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). The novel peptides of this study were synthesized by standard solid-phase techniques employing *N*-(fluoren-9-ylmethoxycarbonyl) (Fmoc) chemistry. The peptides were purified by RP-HPLC and their identity verified by amino acid analysis and MS.

Bioassays

The lipid-mobilizing bioassay of *L. migratoria* and the carbohydrate-mobilizing bioassay of *P. americana* were performed as outlined elsewhere [20]. Bioassays with fruit beetles and dung beetles were performed as follows: beetles were kept separately at room temperature ($\pm$22 °C) in small plastic containers the night before experimentation. Before and 120 min after injection of the appropriate test solution, a 0.5 or 1 μl haemolymph sample was taken from the neck membrane and used for the determination of total carbohydrates or lipids (as in the locust and cockroach bioassay) or for amino acid analysis (for derivatization procedure and subsequent HPLC separation; see [10]).

Flight experiments

Experiments with adult *O. pecuarius* and *O. aygulus* were conducted at dawn or at night, when the respective species started their daily flight activity behaviour [21]. Beetles were allowed to fly freely in a large room at about 20–22 °C, thus they had to produce lift and drag, and the power output of flight muscles was maximal.

Haemolymph was taken as described above from individual beetles before flight, after 1 min of flight, and 60 min after a flight of 1 min duration when the beetles had rested in their individual containers again. In another series of experiments, flight muscles were removed from resting, 1-min-flown and 1-min-flown plus 60-min-resting beetles; tissue was immediately frozen in liquid nitrogen. Preparation of perchloric acid extracts and the determination of the concentrations of glycogen and the amino acids proline and alanine, were as outlined previously [10].

RESULTS

Biological activity of beetle corpora cardiaca extract

In the first series of experiments, widely used heterologous bioassays [1] were performed to show the presence of adipokinetic/hypertrehalosaemic factors in the beetle corpora cardiaca. A crude methanolic extract of one gland of *O. aygulus* is able to elicit increases of haemolymph carbohydrates and lipids in cockroaches and locusts respectively, which were about 50 % of those caused by injection of the respective own-gland extract known to result in maximal increases (Table 1).

The concentration of total carbohydrates in the dung beetle *O. aygulus* was very low in resting animals, and the level of total lipids was also relatively low and quite variable, as depicted by the high S.D. values (Table 2). Conspecific injections of one gland-equivalent of the beetle resulted in no increases in either of the metabolites (Table 2).

A third heterologous bioassay performed was to test for increases of proline in the haemolymph of the fruit beetle, *P. sinuata* (see Introduction). Compared with control injections, one gland-equivalent of the beetle significantly increased the proline concentration and caused a decrease in the alanine level (Table 3). This result prompted us to analyse these amino acids in *O. aygulus* as well. Proline levels in the haemolymph of the beetle are about 50 % of those determined in *P. sinuata* (Table 2), but about 5-fold higher than in locusts and cockroaches [11]. Upon conspecific injections, proline levels in *O. aygulus* were significantly increased (Table 2). Thus, we had a heterologous, but also a homologous, bioassay available to monitor biological activity during purification.

Peptide purification

The UV-elution profile of methanolic extracts of corpora cardiaca from *O. pecuarius* and *O. caffer* on an analytical RP-HPLC column are shown in Figures 1(A) and 1(B). There are only a few absorbance peaks present at 214 nm. In both species there was a pair of peaks at about 13 min, which we expected to represent the biologically active peptides. Under the conditions employed, however, these peaks were not well-resolved, therefore the material from *O. pecuarius* was run at a different gradient (Figure 2A) and, additionally, fluorescence targeting for the presence of Trp, which is conserved in the AKH/RPCH family of peptides, was monitored (Figure 2B). The UV-profile shows three distinct peaks (Figure 2A, numbered 0, 1 and 2) which all corresponded to peaks having a fluorescence signal as well (Figure 2B). Peaks 1 and 2, eluting at about 23 and 24 min, were much better separated than before and could be hand-collected separately without contamination from each other. Exactly the same pattern of elution occurred when gland material from *O. aygulus* and *O. alexis* was processed in the latter HPLC system (Figures 2C and 2D). The material of peaks 1 and 2 from corpora cardiaca of *O. aygulus* was shown to be active in elevating haemolymph proline concentration in *P. sinuata* (Table 3) and in *O. aygulus* (Table 4), but only peak 1 resulted in a slight but significant hyperlipaemic response in locusts (Table 1).

Peptide characterization, synthesis and structural conformation

Purification of sufficient peak 1 and 2 material from glands of *O. aygulus* and *O. pecuarius*, enzymic deblocking of the N-terminal

Table 1 Adipokinetic and hypertrehalosaemic activity of a crude methanolic extract of corpora cardiaca from the dung beetle *O. aygulus* and of selected peaks after HPLC

Insects were injected as indicated. Total haemolymph 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 ± S.D. The significance of the differences between values before and after injection is indicated by †$P < 0.02$, ††$P < 0.001$ and †††$P < 0.002$, using paired *t* tests.

	Locusta migratoria Haemolymph lipids (mg/ml)				Periplaneta americana Haemolymph carbohydrates (mg/ml)			
Treatment	*n*	0 min	90 min	Difference	*n*	0 min	120 min	Difference
Control (10 μl of distilled water)	8	10.4 ± 4.0	7.6 ± 1.9	−2.8 ± 2.8	8	14.5 ± 5.4	14.4 ± 6.1	−0.1 ± 1.6
Beetle extract (1 gland-equivalent)	10	10.6 ± 3.0	25.9 ± 11.5	15.3 ± 10.1††	6	18.2 ± 4.6	25.9 ± 5.5	7.7 ± 5.5†
Locust extract (0.1 gland-equivalent)	9	11.0 ± 3.1	38.9 ± 7.7	27.9 ± 6.2††	–	–	–	–
Cockroach extract (0.1 gland-equivalent)	–	–	–	–	6	12.8 ± 2.1	28.0 ± 10.6	15.2 ± 9.1†††
Peak 1 material from HPLC (1 gland-equivalent)	6	11.5 ± 2.7	15.2 ± 4.2	3.7 ± 1.7††	ß	ß	ß	ß
Peak 2 material from HPLC (1 gland-equivalent)	6	12.1 ± 2.8	12.3 ± 2.9	0.2 ± 0.6	–	–	–	–

Table 2 Conspecific assay for adipokinetic, hypertrehalosaemic and hyperprolinaemic activity of a crude methanolic extract of corpora cardiaca from *O. aygulus*

Adult dung beetles, *O. aygulus*, were injected as indicated, and haemolymph concentrations of total lipids and total carbohydrates (both expressed as mg/ml) as well as of proline and alanine (expressed as μmol/ml) were determined. Values are means ± S.D. Significant difference: †$P < 0.05$ compared with water injections using Student's *t* test.

	Treatment	
Haemolymph metabolite	Control (10 μl of distilled water)	Beetle extract (1 gland equivalent)
Lipids (mg/ml)		
n	10	7
0 min	8.5 ± 7.5	5.3 ± 3.9
120 min	6.7 ± 6.3	5.1 ± 2.6
Difference	−1.8 ± 1.6	−0.2 ± 1.8
Carbohydrates (mg/ml)		
n	10	7
0 min	2.1 ± 0.8	1.6 ± 0.7
120 min	2.4 ± 0.9	1.8 ± 0.5
Difference	0.3 ± 0.7	0.2 ± 0.4
Proline (μmol/ml)		
n	10	7
0 min	48.4 ± 9.6	39.0 ± 13.2
120 min	50.8 ± 11.0	48.7 ± 17.4
Difference	2.4 ± 7.3	9.7 ± 6.4†
Alanine (μmol/ml)		
n	10	7
0 min	4.3 ± 1.4	5.1 ± 1.6
120 min	2.6 ± 1.4	1.7 ± 2.1
Difference	−1.7 ± 1.9	−3.4 ± 1.6

pyroglutamatyl residue and subsequent isolation of the des-pGlu-peptides via RP-HPLC, resulted in homogeneous material for automated Edman degradation sequencing. In both species peptide 1 had the sequence Tyr-Asn-Phe-Ser-Thr-Gly-Trp, and peptide 2 was Phe-Asn-Tyr-Ser-Pro-Asp-Trp (Table 5). The two novel octapeptides with an N-terminal pGlu and a C-terminal Trp-NH_2 were synthesized. The synthetic peptides were eluted at essentially the same retention times on various chromatographic supports (Novapak C-8 and C-18; Nucleosil C-18; μBondapak Phenyl) as the native peptides isolated from gland extract (results not shown). Co-injection of active-peak fractions and synthetic peptides always resulted in co-elution (results not shown). Further confirmation that the correct structures were assigned came from mass spectrometric data. The positive ion mass spectrum showed clear peaks at m/z 1006 and 1022 for peptide 1 and at m/z 1060 and 1076 for peptide 2, corresponding to the sodium $[M + Na]^+$ and potassium $[M + K]^+$ cationized molecules respectively. From the spectra it is clear that the molecular mass of peptide 1 is 983 and of peptide 2 is 1037. Thus, the combined data identify the novel peptides of onitine beetle as: peptide 1, pGlu-Tyr-Asn-Phe-Ser-Thr-Gly-Trp-NH_2; and peptide 2, pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-Trp-NH_2.

Biological parameters

When both (synthetic) peptides were injected at a low dose of 5 pmol each into resting *O. aygulus*, a significant increase in the concentration of proline in the haemolymph was measured (Table 4).

To demonstrate which substrate *Onitis* beetles use during flight, individuals of *O. pecuarius* were allowed to fly freely for 1 min, and haemolymph and flight muscle substrates were determined. The concentrations of total lipids and carbohydrate in the haemolymph were low and no changes occurred upon flight or 1 h rest after flight (Figure 3A). In contrast, the haemolymph proline level was high in resting beetles and was significantly decreased (by 40 %) during flight, returning to the pre-flight concentration 60 min after flight. Alanine concentration increased significantly during flight and was back to resting levels after 1 h (Figure 3A). Similarly, glycogen concentration in flight muscles was low and not affected by flight (Figure 3B), whereas proline and alanine concentrations in flight muscles significantly decreased and increased respectively during flight, and were back to pre-flight levels upon recovery after flight (Figure 3B).

Table 3 Hyperprolinaemic activity of a crude methanolic extract of corpora cardiaca from *O. aygulusas* as well as selected fractions after HPLC

Adult male fruit beetles, *P. sinuata*, were injected as indicated, and haemolymph concentrations of proline and alanine were determined. Values are means ± S.D and are expressed as μmol/ml of haemolymph. Significant difference: †$P < 0.05$; ††$P < 0.001$ compared with water injection using Student's *t* test.

Treatment	Haemolymph proline (μmol/ml)				Haemolymph alanine (μmol/ml)			
	n	0 min	90 min	Difference	*n*	0 min	90 min	Difference
Control (10 μl of distilled water)	5	99.0 ± 14.7	98.2 ± 19.1	−0.8 ± 13.2	5	8.7 ± 2.8	8.9 ± 2.4	0.2 ± 2.5
Beetle extract (1 gland-equivalent)	11	86.1 ± 11.9	96.6 ± 11.5	10.5 ± 4.7†	11	8.6 ± 2.9	1.9 ± 1.8	−6.7 ± 2.5††
Control (10 μl of distilled water)	5	101 ± 16.6	99.4 ± 17.0	−2.0 ± 4.4	5	4.8 ± 2.9	3.7 ± 2.4	−1.1 ± 1.8
Peak 1 material from HPLC (1 gland-equivalent)	10	104.5 ± 11.9	117.6 ± 15.1	13.1 ± 4.8††	10	5.0 ± 3.2	1.1 ± 0.5	−3.9 ± 3.1
Peak 2 material from HPLC (1 gland-equivalent)	10	101.6 ± 23.7	108.9 ± 22.2	7.3 ± 3.8††	10	3.9 ± 2.9	0.8 ± 0.6	−3.1 ± 2.6

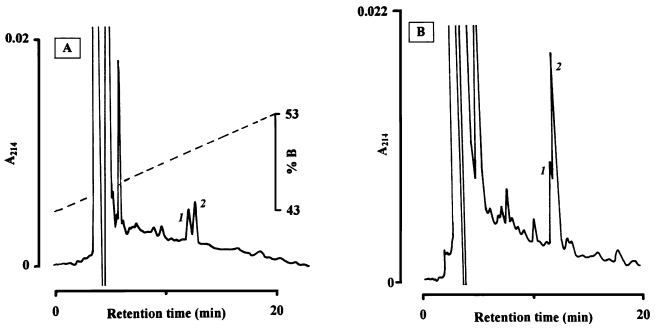

Figure 1 Isolation of neuropeptides of corpora cardiaca from *Onitis* species

Analyses were performed on a Nucleosil 100 C-18 column eluted with a linear gradient of 0.11% (v/v) trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid (v/v) in 60% (v/v) acetonitrile (solvent B). The gradient ran from 43 to 53% B within 20 min at a flow rate of 1 ml/min. (**A**) A methanolic extract of three glands from *O. pecuarius* was injected and the UV absorbance was monitored at 214 nm. (**B**) A methanolic extract of ten glands from *O. caffer* was injected; otherwise as in (**A**).

In *O. aygulus*, significant changes in the concentration of the amino acids in the haemolymph after 1 min of flight occurred as well: pre-flight levels were 49.7 ± 7.1 (proline) and 4.1 ± 2.0 (alanine), whereas these values were 29.3 ± 4.6 and 14.8 ± 4.9 after flight, and 44.5 ± 4.9 and 3.0 ± 2.3 after 60 min of recovery from flight (all values μmol/ml, means ± S.D. for $n = 5$; $P < 0.001$, flight versus resting, for both substrates).

DISCUSSION

Chemical characterization

The primary structures of two peptides from the corpora cardiaca of onitine beetles have been fully determined. Although sequenced from only two species, HPLC behaviour suggests that all *Onitis* species investigated produce the same two peptides. According to the criteria given in the Introduction, both peptides are novel members of the AKH/RPCH family. They are unique members of this family in the sense that they are the first to contain three aromatic residues. An aromatic residue, tyrosine in the case of peptide 1 and phenylalanine in peptide 2, has never been shown to occur at position 2; previously, mostly leucine or valine, or in two cases isoleucine, have been sequenced at this position (see Table 6) [1,2]. Indeed, leucine bioanalogues of each of the novel peptides have previously been shown to exist. Peptide 1 is identical (except for the tyrosine/leucine exchange at position 2) with a peptide code-named *Scg*-AKH-II (see Table 6), which is found as one of two peptides in the desert locust, *Schistocerca gregaria* [22,23], the two pyrgomorphid grasshoppers, *Phymateus leprosus* and *P. morbillosus* [24,25], and certain long-horned grasshoppers [14]. The leucine bioanalogue of peptide 2 is code-named *Mem*-CC and is found in melolonthine [19] and cetonine beetles [26]. These beetles, as the genus *Onitis*, all belong to the superfamily Scarabaeoidea. Considering the genetic code, the amino acid substitution leucine to phenylalanine can be explained by single-step point mutation, whereas leucine to tyrosine cannot. Tyrosine, however, could have evolved via a phenyl ancestor. This suggests that peptide 2, containing phenylalanine at position 2, is the evolutionarily older peptide of the two, and that peptide 1 evolved later by gene duplication and mutations. Both peptides are also remarkable in another way: peptide 2 is one of the three charged members of the AKH/RPCH family containing an aspartic acid residue at position 7, and peptide 1 is one of the six members missing a proline residue at position 6.

Biological characterization

The crude corpus cardiacum extract had various biological activities in heterologous tests. This aided monitoring of the peptides in question during purification. Surprisingly, when the HPLC-separated peak fractions were tested for hyperlipaemia, only the one containing peptide 1 had biological activity; the material from peptide 2 did not (Table 1). Even 500 pmol of synthetic peptide 2 did not elicit lipid release reliably in locusts (results not shown). Knowing the structures of both peptides allows an interpretation of these results. Peptide 1 is similar to *Scg*-AKH-II (Tyr^2 versus Leu^2), which has good activity in *L. migratoria*, albeit that the maximal effective dose is 10-fold higher than for *Lom*-AKH-I [27]. Peptide 2, however, is a bioanalogue of *Mem*-CC, which has very little activity in the

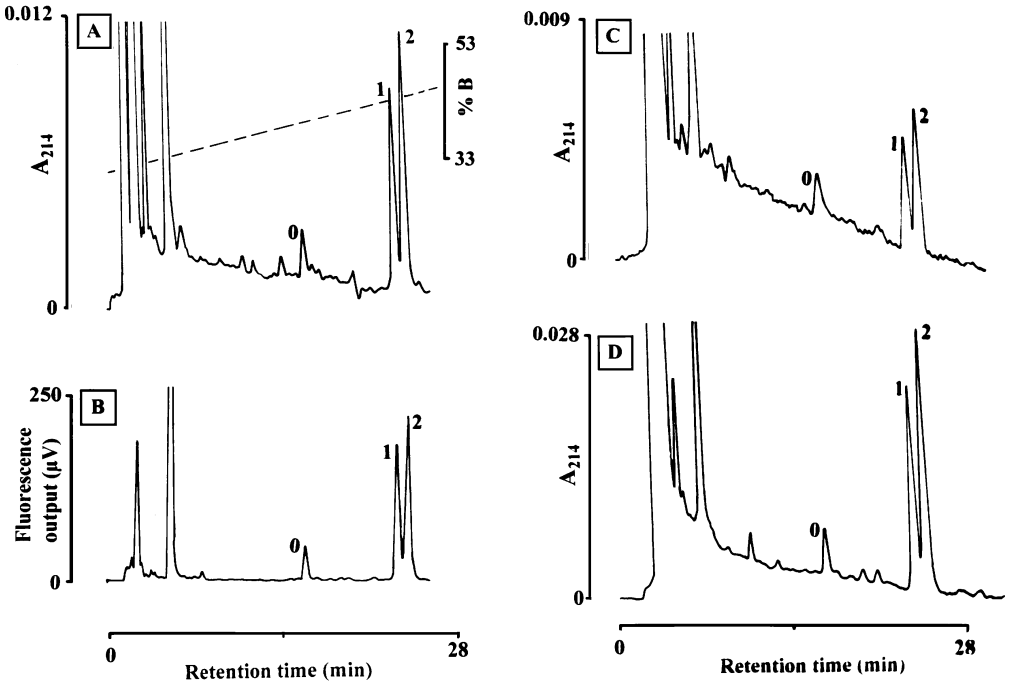

Figure 2 Isolation of *Onitis* neuropeptides

Analyses were performed as described in the legend to Figure 1, except that the gradient ran from 33 to 53% solvent B within 40 min and, that in (**B**) fluorescence was monitored at 276 nm (excitation) and 350 nm (emission). (**A**) A methanolic extract of five glands from *O. pecuarius* was injected and UV absorbance was monitored. (**B**) as (**A**) but fluorescence was monitored. (**C**) A methanolic extract of seven glands from *O. alexis* was injected and UV absorbance was monitored. (**D**) A methanolic extract of ten glands from *O. aygulus* was injected and UV absorbance was monitored. Note: material from similar runs of peaks numbered 0, 1 and 2 was collected, dried *in vacuo,* dissolved in distilled water and used for bioassays. The results are given in Tables 1, 3 and 4.

Table 4 Conspecific hyperprolinaemic activity of HPLC fractions as well as synthetic peptides in *O. aygulus*

Adult dung beetles, *O. aygulus*, were injected as indicated. The change of metabolite concentration (in μmol/ml) for alanine and proline in the haemolymph is given between the pre- and post-injection value. Values are means $\pm$ S.D. Significant difference: †$P < 0.05$, ††$P < 0.01$ compared with water injections using Student's t test; n values apply to both amino acids.

Treatment	n	Change (μmol/ml) Proline	Alanine
Control (10 μl of distilled water)	10	2.4 ± 7.3	-1.7 ± 1.9
Peak 0 material from HPLC (1 gland-equivalent)	8	4.7 ± 7.9	-1.0 ± 1.6
Peak 1 material from HPLC (1 gland-equivalent)	8	14.2 ± 6.1†	-2.6 ± 1.7
Peak 2 material from HPLC (1 gland-equivalent)	6	13.4 ± 3.6†	-2.2 ± 1.7
Ona-CC-I (5 pmol)	10	11.8 ± 7.2††	-2.2 ± 0.8
Ona-CC-II (5 pmol)	6	13.2 ± 4.1†	-2.3 ± 1.0

Table 5 Amino acid sequences of peptides 1 and 2 from corpora cardiaca of *O. aygulus* and *O. pecuarius* by automated Edman degradation

The N-terminal pyroglutamyl residue of the HPLC-purified peptides was cleaved off enzymically, and the deblocked peptides were analysed. * Note: the presence of Trp is also indicated by the fluorescence profile in HPLC.

	Peptide 1 recovery (pmol)			Peptide 2 recovery (pmol)	
Amino acid	*O. aygulus*	*O. pecuarius*	Amino acid	*O. aygulus*	*O. pecuarius*
Tyr^1	8.9	20.1	Phe^1	22.3	30.2
Asn^2	6.2	13.5	Asn^2	13.2	22.4
Phe^3	5.0	19.7	Tyr^3	15.7	26.5
Ser^4	3.5	10.7	Ser^4	7.4	12.2
Thr^5	2.6	13.5	Pro^5	5.5	12.0
Gly^6	4.3	12.9	Asp^6	4.3	8.7
Trp^7	Trace*	4.2	Trp^7	0.8	3.2
$-^8$	–	–	$-^8$	–	–

locust [28]. In fact, it was shown that the tyrosine at position 4 reduces hyperlipaemic activity substantially, and this is maximized when an additional charged amino acid, such as aspartic acid at position 7, is introduced [28]. Onitine beetles are known to rapidly exploit the dung of herbivores for feeding and breeding purposes and have to do so by flying to the dung pats [21]. From the flight experiments with *Onitis* it became clear that carbohydrate and lipid metabolism are of minor importance, if any, in providing energy during flight. Proline, however, appears to be the major flight substrate, as shown recently to a varying degree in other beetles [10]. In our laboratory experiment beetles flew freely for 1 min, but longer times are necessary in the wild to reach the herbivore dung. Thus, proline stored in fat body-cells has to be mobilized at a constant rate for some time and, after flight, to be resynthesized from the end product of partial proline oxidation, namely alanine [29]. It is very likely that the two

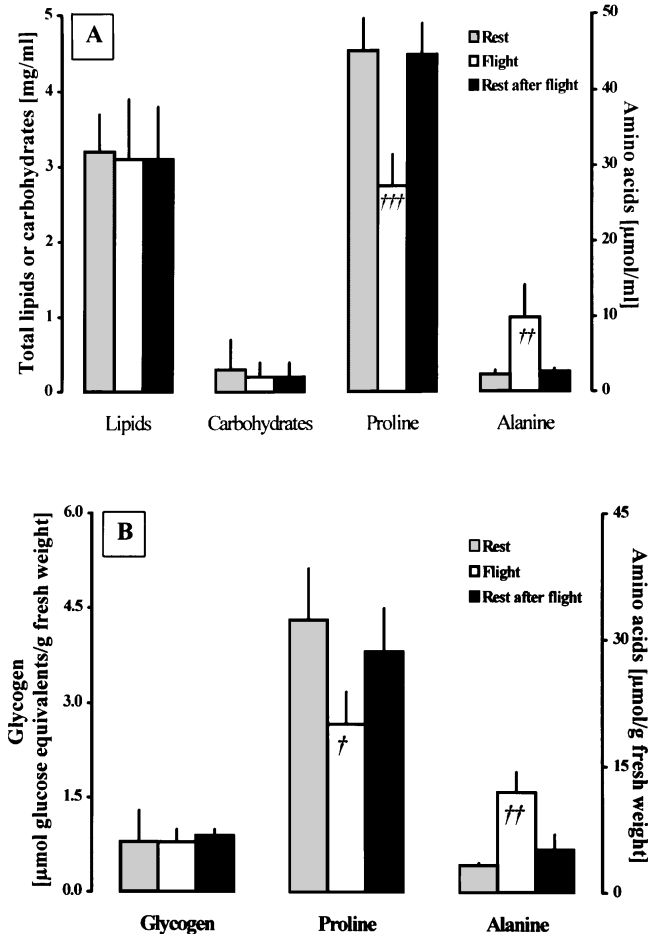

Figure 3 Changes of selected substrates during flight in *O. pecuarius*

(**A**) Concentrations of total lipids, carbohydrates and of the amino acids proline and alanine in the haemolymph are given as means ± S.D. during rest, after 1 min of flight and after 60 min of rest after flight. (**B**) Concentrations of glycogen, proline and alanine in flight muscles are given as means ± S.D. Significant difference: †$P < 0.05$, ††$P < 0.005$, †††$P < 0.001$, flight, or rest after flight, compared with resting groups using Student's *t* test.

Table 6 Primary structures of a few peptides of the AKH/RPCH family

Species	Peptide name	Sequence
Locusta migratoria	Lom-AKH-I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2
Schistocerca gregaria	Scg-AKH-II	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH_2
Gryllus bimaculatus	Grb-AKH	pGlu-Val-Asn-Phe-Ser-Thr-Gly-Trp-NH_2
Onitis aygulus	Ona-CC-I	pGlu-Tyr-Asn-Phe-Ser-Thr-Gly-Trp-NH_2
Melolontha melolontha	Mem-CC	pGlu-Leu-Asn-Tyr-Ser-Pro-Asp-Trp-NH_2
Onitis aygulus	Ona-CC-II	pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-Trp-NH_2

Received 28 May 1996/19 August 1996; accepted 6 September 1996

corpora cardiaca neuropeptides characterized in the present study are responsible for this stimulation. The utilization of proline during flight, in conjunction with the action of low doses of injected synthetic neuropeptides into resting beetles, which increase the proline concentration in the haemolymph, strongly supports the view of a hormonal role for the peptides. Because of this action, the peptides could possibly be called hyperprolinaemic hormones, but until we have analysed the proline metabolism in more detail we prefer to designate the code-names *Ona*-CC-I and II (*O. aygulus* corpus cardiacum peptide I and II).

The author thanks M. P.-E. Janssens and L. Auerswald for excellent technical help, Dr. R. Kellner (University of Mainz, Germany) for sequencing and peptide synthesis, Dr. T. van der Merwe (University of Stellenbosch, South Africa) for MS, and the Foundation for Research Development (Pretoria, South Africa) and the University of Cape Town (staff award) for financial support.

REFERENCES

1 Gäde, G. (1990) J. Insect Physiol. **36**, 1–12
2 Gäde, G. (1996) Z. Naturforsch., in the press
3 Gäde, G., Reynolds, S. E. and Beeching, J. R. (1994) in Perspectives in Comparative Endocrinology (Davey, K. G., Peter, R. E. and Tobe, S. S., eds.), pp. 119–128, National Research Council of Canada, Ottawa
4 Gäde, G. (1997) in Progress in the Chemistry of Organic Natural Products (Hertz, W., Kirby, G. W. and Tamm, C., eds.), Springer Verlag, Wien, in the press
5 Goldsworthy, G. J. (1990) in Biology of Grasshoppers (Chapman, R. F. and Joern, A., eds.), pp. 205–225, Wiley & Sons, New York
6 Ziegler, R. and Schulz, M. (1986) J. Insect Physiol. **32**, 903–908
7 Wilps, H. and Gäde, G. (1990) J. Insect Physiol. **36**, 441–449
8 van Marrewijk, W. J. A., van den Broek, A. T. M. and Beenakkers, A. M. T. (1989) Comp. Biochem. Physiol. **94B**, 165–169
9 Gäde, G. (1992) Zool. Jb. Physiol. **96**, 211–225
10 Zebe, E. and Gäde, G. (1993) J. Comp. Physiol. B **163**, 107–112
11 Auerswald, L. and Gäde, G. (1995) J. Exp. Biol. **198**, 1423–1431
12 Weeda, E. (1981) J. Insect Physiol. **27**, 411–417
13 Lopata, A. and Gäde, G. (1994) J. Insect Physiol. **40**, 53–62
14 Gäde, G. (1992) Biol. Chem. Hoppe-Seyler **373**, 1169–1178
15 Gäde, G. (1991) J. Insect Physiol. **37**, 483–487
16 Gäde, G., Goldsworthy, G. J., Kegel, G. and Keller, R. (1984) Hoppe-Seyler's Z. Physiol. Chem. **365**, 393–398
17 Gäde, G. (1985) Biol. Chem. Hoppe-Seyler **366**, 195–199
18 Gäde, G., Hilbich, C., Beyreuther, K. and Rinehart, K. L. (1988) Peptides **9**, 681–688
19 Gäde, G. (1991) Biochem. J. **275**, 671–677
20 Gäde, G. (1980) J. Insect Physiol. **26**, 351–360
21 Caveney, S., Scholtz, C. H. and McIntyre, P. (1995) Oecologia **103**, 444–452
22 Siegert, K., Morgan, P. and Mordue, W. (1985) Biol. Chem. Hoppe-Seyler **366**, 723–727
23 Gäde, G., Goldsworthy, G. J., Schaffer, M. H., Cook, J. C. and Rinehart, Jr., K. L. (1986) Biochem. Biophys. Res. Commun. **134**, 723–730
24 Gäde, G. and Kellner, R. (1995) Regul. Pept. **57**, 247–252
25 Gäde, G., Kellner, R. and Rinehart, K. L. (1996) Physiol. Entomol., in the press
26 Gäde, G., Lopata, A., Kellner, R. and Rinehart, K. L. (1992) Biol. Chem. Hoppe–Seyler **373**, 133–142
27 Gäde, G. (1990) Physiol. Entomol. **15**, 209–316
28 Gäde, G. (1993) J. Insect Physiol. **39**, 375–383
29 Bursell, E. (1981) in Energy Metabolism in Insects (Downer, R. G. H., ed.), pp. 135–154, Plenum Press, New York