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Insect neuropeptides regulating substrate mobilisation

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Insect flight muscles perform their work completely aerobically, and working flight muscles are known to be the most metabolically active tissue in nature with respect to oxygen uptake. Various substrates can be oxidised and utilised as fuels for flight. Insects such as Diptera and Hymenoptera power their flight muscles by the breakdown of carbohydrates, whereas lipids are the predominant fuel for the contracting flight muscles of Lepidoptera and Orthoptera during long-distance flight. The amino acid proline can also be used as a substrate for flight, especially in tsetse flies and beetles (Colorado potato beetle, blister beetles, certain dung beetles). Neuropeptides from the corpus cardiacum are well-known to be responsible for carbohydrate and lipid mobilisation from the fat body. In this short overview, we show that peptides belonging to the large adipokinetic hormone/red pigment-concentrating hormone family are also thought to be the chemical messengers for initiating proline homeostasis. The peptides isolated and sequenced so far from glands of beetles from the genera *Pachnoda*, *Scarabaeus* and *Onitis* all have a tyrosine residue (at position 2 or 4) and seem to be related to each other.

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Neuropeptides are the most numerous and diverse of all the known types of chemical messengers of metazoans. This is also true for insects, which constitute by far the largest group of extant animals. The existence of neuropeptides which regulate physiological, developmental and behavioural events in insects has been known for a long time, but only during the last two decades have a great number of neuropeptides been purified, isolated and their primary structures completely characterised (see Gäde 1997a; Gäde, Hoffmann & Spring 1997).

A major group of these neuropeptides is that which regulates physiological homeostasis. The first members were discovered in the 1960s in the American cockroach and in locusts where they are involved in the control of carbohydrate and lipid breakdown, respectively. We know now that these peptides are members of a large family of structurally related peptides which are found in crustaceans and insects (Gäde 1996). Such peptides became known under the acronym AKH/RPCH family peptides on the basis of the first members of this family to be fully characterised, viz. an adipokinetic hormone from locusts (Stone, Mordue, Batley & Morris 1976), now called Lom-AKH-I according to the nomenclature proposed in Raina & Gäde (1988), and a chromatotropic peptide from prawns (Fernlund & Josefsson 1972), the red pigment-concentrating hormone (code name: Pab-RPCH). The peptides are present in the neurosecretory X-organ/sinus gland complex in the eyestalks of crustaceans and in the intrinsic neurosecretory cells of the corpora cardiaca of insects. Both structures are neurohaemal organs, analogous to the vertebrate hypothalamo/hypophyseal system, and it can thus be inferred that the peptides can be released from the neurohaemal organs into the circulation and, thus, act as true hormones. However, release has been demonstrated in only a few cases, for example during flight in locusts, blowflies and the moth *Manduca sexta* (Gäde 1992).

Improvements in isolation techniques, notably high performance liquid chromatography (HPLC), and in protein chemical detection methods, including new generations of automated amino acid sequencers and mass spectrometers, have facilitated the elucidation of the primary structures of AKH/RPCH family peptides (Gäde 1996). The corpora cardiaca in insects are the source of these peptides and, compared to most other insect neuropeptides, relatively large quantities are stored in these glands. Accordingly, the corpora cardiaca are routinely dissected, extracted in 80% methanol and sufficiently purified for structural analysis by a single-step procedure on reversed phase HPLC (Gäde 1990). Common characteristics of the family are that the peptides have a length of eight to ten amino acids, are N-terminally blocked by a pyroglutamate residue, have a C-terminal amide block, have the amino acids tryptophan and glycine at positions eight and nine, are mainly uncharged and contain at least two aromatic amino acids, at position four (mostly phenylalanine, but sometimes tyrosine) and at position eight (always tryptophan) (Gäde 1996, 1997a; Gäde, Hoffmann & Spring 1997). Representative peptides have been found in most insect orders. At present, the family consists of 32 members (see above references).

Fuels for flight and fuel-mobilising neuropeptides

Although AKH/RPCH family peptides have quite a number of diverse functions in insects, we will discuss in this brief review only the 'classical' roles of fuel mobilisation during flight.

Many insects are well known for their impressive flight performance. This may be intermittent in nature, as in cockroaches and flies, or of long duration, as in migrating locusts, butterflies and moths. Flight muscles have a high energy demand for their contraction and are known to be metabolically the most active tissues in nature, elevating resting oxygen consumption up to 100-fold during flight (Sacktor 1975). Further, the relatively small fuel depots in flight muscles and haemolymph must be replenished by mobilising fuel stores from the fat body. Peptides of the AKH/RPCH family are thought to be responsible for these events. We will briefly mention the well-known functions in flies/cockroaches and

locusts/moths, and then explain in a bit more detail our recent results on proline as fuel for flight and its possible regulation by neuropeptides of the AKH/RPCH family.

1. Carbohydrates as fuel

In those insects which are known to use carbohydrates as fuel for flight (cockroaches and flies) endogenous peptides of the AKH/RPCH family have been identified in their corpora cardiaca; it has been shown that they stimulate fat body glycogenolysis (activation of glycogen phosphorylase) and make the resulting products available as precursors for the biosynthesis of haemolymph carbohydrate, which is mainly trehalose (see Keeley, Bradfield, Sowa, Lee & Lu 1994). The peptides characterised from various fly and cockroach species are shown in Table 1. It is noteworthy that both horsefly peptides (Taa-AKH and Taa-HoTH, see Table 1) were originally assayed in male face flies, *Musca autumnalis* (Jaffe, Raina, Riley, Fraser, Nachman, Vogel, Zhang & Hayes 1989). Both peptides were later shown to induce hyperglycaemic responses in the tabanid *Tabanus lineolata*, but only Taa-HoTH had an adipokinetic effect in *T. lineolata*; no hypotrehalosaemic response could be shown (Woodring & Leprince 1992).

The sequence information from the cockroach peptides has been utilised to construct possible phylogenetic trees (Gäde 1989, 1995). Recently, the structural data on the hypertrehalosaemic peptides from the woodroach *Cryptocercus punctulatus* and the cockroach *Therea petiveriana* helped to support the previous morpho-anatomical data placing the woodroach inside the cockroach subfamily Polyphaginae (Gäde, Grandcolas & Kellner 1997).

2. Lipids as fuel

Although lipids are the main fuels for long-distance flight in locusts, carbohydrates are used during the initial phase of flight and still contribute substantially during the later phase (see Beenakkers, van der Horst & van Marrewijk 1985). Similar patterns for the use of fuels during flight have been found in the brown locust, *Locustana pardalina* (Gäde, unpublished), and in the pyrgomorphid grasshopper, *Phymateus morbillosus* (Gäde, Kellner & Rinehart 1996). Mobilisation of both substrates is controlled by peptides of the AKH/RPCH family, which stimulate either glycogen phosphorylase or a lipase (not convincingly shown yet) in the fat body. After a lipase is activated, triacylglycerols are broken down to monoacylglycerols, which are subsequently re-acylated to form stereospecific sn-1,2-diacylglycerols, and these are released from the fat body into the haemolymph (see Beenakkers, van der Horst & van Marrewijk 1985). There, AKH/RPCH family peptides are responsible for an overall increase in the lipid-carrying capacity. For this, the predominant species of lipoprotein in resting locust haemolymph, high-density lipophorin, is loaded with the lipids released from the fat body and simultaneously associates with an apoprotein, apolipophorin III. The overall result is the creation of a larger but less dense particle, low-density lipophorin (see Kanost,

Table 1 Primary structures of peptides of the AKH/RPCH family from insects with carbohydrate-based flight metabolism (flies and cockroaches)

Code name	Species	Sequence	Reference
Taa-HoTH	*Tabanus atratus*	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-Tyr amide	Jaffe *et al.* 1989
Taa-AKH	*Tabanus atratus*	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp amide	Jaffe *et al.* 1989
	Phormia terraenovae		
Phl-HrTH	*Drosophila melanogaster*	pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp amide	Gäde *et al.* 1990 Schaffer *et al.* 1990
Bld-HrTH	*Blaberus discoidalis*	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-Thr amide	Hayes et al. 1986
	Nauphoeta cinerea		Gäde & Rinehart 1986
	Leucophaea maderae		Gäde & Rinehart 1990
	Gromphadorhina portentosa		Gäde & Rinehart 1990
	Blattella germanica		Gäde & Rinehart 1990
			Veenstra & Camps 1990
Pea-CAH-I	*Periplaneta americana*	pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp amide	Witten *et al.* 1984;
			Bauman & Penzlin 1984;
			Scarborough *et al.* 1984;
			Siegert & Morduc 1986
	Blatta orientalis		Gäde & Rinehart 1990
Tem-HrTH	*Polyphaga aegyptiaca*	pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp amide	Gäde & Kellner 1992
	Cryptocercus punctualus		Gäde *et al.* 1997b
	Therea petiveriana		Gäde *et al.* 1997b
Pea-CAH-II	*Periplaneta americana*	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp amide	Witten *et al.* 1984;
			Scarborough *et al.* 1984
			Siegert & Morduc 1986
	Blatta orientalis		Gäde & Rinehart 1990
Poa-HrTH	*Polyphaga aegyptiaca*	pGlu-Ile-Thr-Phe-Thr-Pro-Asn-Trp amide	Gäde & Kellner 1992

Kawooya, Law, Ryan, van Heusden & Ziegler 1990). Direct action of AKH/RPCH family peptides on the utilisation of fuel at the flight muscles has been previously discussed as well (Goldsworthy 1983, 1990).

Similar results have been corroborated for the moth, *Manduca sexta*, which uses lipids as the main fuel for flight muscle contraction (Ziegler 1995). The peptides characterised from various locusts/grasshoppers and from moths are shown in Table 2. Clearly, peptides of the locusts/grasshoppers are very closely related.

3. Proline as fuel

The amino acid proline can also be used as a substrate for flight. There are, however, major differences between insect species in the quantitative participation of proline in flight metabolism. Only a little proline is metabolised during the onset of flight in the blowfly *Phormia regina*, to provide tricarboxylic acid intermediates necessary for maximal oxidation of pyruvate ('sparker function'; see Sacktor & Childress 1967). In the tsetse fly *Glossina morsitans*, however, proline is present in impressively high concentration in the flight muscles and is thought to be the exclusive fuel during flight (Bursell 1981). Proline is only partially oxidised and the alanine formed is transported to the fat body for re-synthesis of proline (Bursell 1981). In certain beetles varying degrees of oxidation of carbohydrates and proline have been found. In the blister beetle *Decapotoma lunata*, for example, proline is an important substrate for flight, although its role is secondary to that of carbohydrates (Auerswald & Gäde 1995). In the Colorado potato beetle *Leptinotarsa decemlineata*, as well as in the African fruit beetle *Pachnoda sinuata*, proline is the major flight substrate and carbohydrates play only a minor role (Weeda, de Kort & Beenakkers 1979; Zebe & Gäde 1993; Lopata & Gäde 1994). Recently, it was shown that proline is the exclusive substrate for endothermic warm-up during flight preparation in *P. sinuata* (Auerswald, Schneider & Gäde 1998). Furthermore, the same authors found that proline levels in haemolymph and flight muscles were diminished by about 50% of the pre-flight level during 30 s of lift-producing flight (simulating free flight very closely), whereas such changes were much more protracted (about 30 min) when tethered flight was monitored. The importance of proline for the life style of *P. sinuata* is demonstrated by two additional observations. First, the proline concentration in the haemolymph follows a circadian rhythm with peak levels occurring in the early morning, coincident with the first flight activity during the day (Auerswald 1997). Second, during starvation for up to one month, carbohydrate reserves in haemolymph and flight muscles are almost completely diminished. Proline, however, is still present in both compartments in high concentrations; beetles are able to fly after such a period of starvation and then use exclusively proline as fuel (Auerswald 1997). Proline seems to be the only substrate oxidised during flight in two other genera of scarab beetles, dung beetles of the genera *Onitis* and *Scarabaeus*, since carbohydrate levels are very low (Gäde 1997b,c).

It was suggested (Weeda 1981) that proline metabolism in the Colorado beetle is under hormonal control and AKH/RPCH family peptides were the likely candidates, but no endogenous peptide from the Colorado beetle was known at that time. It was shown that injection of crude corpora cardiaca extract decreased haemolymph alanine concentration in the beetle *in vivo*. Furthermore, proline synthesis was stimulated *in vitro* by corpus cardiacum extracts from various insects as well as by synthetic Lom-AKH-I (Weeda 1981). In 1989 Gäde & Kellner reported the primary structures of the endogenous AKH/RPCH family peptides from the Colorado potato beetle, which were identical to those purified earlier from the American cockroach (see Tables 1 and 3). However, no rigorous tests have been carried out to prove that those peptides are responsible for proline synthesis. In the tsetse fly, attempts have been made to show hormonal regulation of lipolysis and proline synthesis, but no structural information on such factors is available yet (Pimley & Langley 1982; Pimley 1984).

In beetles of the genera *Scarabaeus* and *Onitis*, respectively, two octapeptides each (one common to both groups) have been identified and fully characterised from the corpus

Table 2 Primary structures of peptides of the AKH/RPCH family from insects with lipid-based flight metabolism (moths and locusts/grasshoppers)

Code name	Species	Sequence	Reference
Hez-HrTH	*Heliothis zea*	pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-Asn amide	Jaffe *et al.* 1988
Mas-AKH	*Manduca sexta*	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly amide	Ziegler *et al.* 1985
	Heliothis zea		Jaffe *et al.* 1986
	Bombyx mori		Ishibashi *et al.* 1992
Lom-AKH I	*Locusta migratoria*	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	Stone *et al.* 1976; Siegert *et al.* 1985
	Schistocerca gregaria		Stone *et al.* 1976
Phm-AKH	*Phymateus morbillosus*	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Ser amide	Gäde *et al.* 1996
Phl-CC	*Phymateus leprosus*	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Ser amide	Gäde & Kellner 1995
Lom-AKH II	*Locusta migratoria*	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp amide	Siegert *et al.* 1985; Gäde *et al.* 1986
Scg-AKH-II	*Schistocerca gregaria*	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp amide	Siegert *et al.* 1985; Gäde *et al.* 1986
	Schistocerca nitans		Gäde *et al.* 1986
	Phymateus morbillosus		Gäde *et al.* 1996
	Phymateus leprosus		Gäde & Kellner 1995
Lom-AKH-III	*Locusta migratoria*	pGlu-Leu-Asn-Phe-Thr-Pro-Trp-Trp amide	Oudejans *et al.* 1991

Table 3 Primary structures of peptides of the AKH/RPCH family from insects with proline-based flight metabolism (beetles)

Code name	Species	Sequence	Reference
Mem-CC	*Melolontha melolontha*	pGlu-Leu-Asn-Tyr-Ser-Pro-Asp-Trp amide	Gäde 1991
	Geotrupes stercorosus		Gäde 1991
	Pachnoda marginata		Gäde *et al.* 1992
	Pachnoda sinuata		Gäde *et al.* 1992
Scd-CC-I	*Scarabaeus deludens*	pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-Trp amide	Gäde 1997b
	Garreta nitens		Gäde 1997b
	Onitis aygulus		Gäde 1997c
	Onitis pecuarius		Gäde 1997c
Scd-CC-II	*Scarabaeus deludens*	pGlu-Phe-Asn-Tyr-Ser-Pro-Val-Trp amide	Gäde 1997b
	Garreta nitens		Gäde 1997b
Ona-CC-I	*Onitis aygulus*	pGlu-Tyr-Asn-Phe-Ser-Thr-Gly-Trp amide	Gäde 1997c
	Onitis pecuarius		Gäde 1997c
Pea-CAH-I	*Leptinotarsa decemlineata*	pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp amide	Gäde & Kellner 1989
Pea-CAH-II	*Leptinotarsa decemlineata*	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp amide	Gäde & Kellner 1989

cardiacum (Gäde 1997b,c; see Table 3). Conspecific injections of synthetic peptide material in low concentration elicited in all cases significant proline increases in the haemolymph (Gäde 1997b,c). This hyperprolinaemic effect, in conjunction with the demonstrated utilisation of proline during flight in these beetles (see above), strongly supports the idea of a hormonal function for the peptides. Very similar results have been obtained for the African fruit beetle *P. sinuata*. Hyperprolinaemia as well as a decrease of the alanine concentration in the haemolymph upon injection of synthetic Mem-CC (see Table 3) was shown (Auerswald 1997). Furthermore, it was demonstrated that this response is dose- and time-dependent.

In addition, the injection of closely related bioanalogues of Mem-CC revealed that the receptor in the fruit beetle does not recognise peptides with tryptophan, serine or valine at position 7, but tolerates changes from aspartate (as in the endogenous Mem-CC) to asparagine or glycine (Auerswald 1997). Somewhat in contrast to these observations are earlier results which suggest that position 4 is most important for receptor recognition for the mobilisation of carbohydrates from the fat body of the same beetle (Gäde, Lopata, Kellner & Rinehart 1992). Taking these different results into account, it seems likely that two different receptors for Mem-CC mediate the mobilisation of carbohydrates and the stimulation of proline synthesis in the fat body of *P. sinuata*.

A series of incubation experiments with fat body pieces of *P. sinuata*, using possible precursors for proline synthesis, revealed the following results (Auerswald 1997). Palmitic acid and oleic acid were found to be the predominant fatty acids in the triacylglycerols of the fat body. Radiolabelled palmitate could easily be incorporated into the triacylglycerol fraction of the fat body *in vitro*. When such pre-incubated fat body pieces were transferred into a medium containing alanine, the radiolabel appeared in the synthesised proline. These results clearly identified the triacylglycerol stores of the fat body as the source of the carbon units which are consumed during partial oxidation of proline in the flight muscles. Alanine and palmitate (as one of a range of possible fatty acids) serve as the main precursors for the synthesis of proline. While alanine serves a shuttle/acceptor function and provides a C_3 body and an amino group, fatty acids supply the C_2 unit in the form of acetyl-CoA via the β-oxidation pathway. The *in vitro* production of proline is equimolar to the disappearance of alanine from the incubation medium, suggesting that synthesis of proline follows the equation:

$$\text{alanine} + \text{acetyl - CoA} + \text{NADH} + \text{H}^+ \text{ (proline} + 2\ \text{H}_2\text{O} + \text{NAD}^+ + \text{CoA}$$

Alanine alone was able to stimulate proline synthesis *in vitro*, while carbon units of palmitate were only incorporated into proline in the presence of alanine in the medium. These results suggest that the action of Mem-CC on proline synthesis is very likely on the lipid breakdown in the fat body rather than on the provision of alanine. While the availability of alanine depends on endothermic warm-up or flight activity, only small amounts of free fatty acids are present in the fat body cells and therefore have to be provided by lipolysis of triacylglycerides.

Conclusion

It appears that proline metabolism during and after flight in certain insects, mainly beetles, is also controlled by members of the AKH/RPCH family of peptides as shown before for carbohydrate and lipid metabolism in other insects. The possibility that these neuropeptides activate the lipid breakdown in the fat body of proline-using insects supports the view that the oxidation of proline by the flight muscles can be seen as a special case of lipid-based flight metabolism (see Bursell 1981). However, details of the possible activation site and the transduction of the signal from the receptor to this site still have to be unraveled. In addition, the release of AKH peptides into the haemolymph upon the stimulus of flight activity has yet to be demonstrated in those insects. These remaining questions provide an interesting challenge for future research.

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