Relative Hypertrehalosaemic Activities of Naturally Occurring Neuropeptides from the AKH/RPCH Family

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Dose-response curves for the ability to increase blood carbohydrates of the cockroach are compared for various naturally occurring neuropeptides from the corpus cardiacum of several insect species. The peptides investigated are the (decapeptides) locust adipokinetic hormone I (AKH I) and stick insect hypertrehalosaemic factor II, as well as the (octapeptides) cockroach hypertrehalosaemic hormones I and II (M I and M II), adipokinetic hormone II from the migratory locust (AKH II-L), and the crustacean red pigment-concentrating hormone (RPCH). The data show clearly that M I and M II display almost identical dose-response curves with a maximal response at about 5 pmol and ED50 values (the amount of peptide which is needed to produce 50% of the hypertrehalosaemic response) of 1.9 and 1.8 pmol, respectively. The stick insect peptide is remarkably more potent (ED50 value: 0.9 pmol), but the other decapeptide, AKH I, gives only about a 70% response compared with M I and M II. As does the octapeptide RPCH. The ED50 values of those peptides are 5.9 and 4.5 pmol, respectively. Biological activity after injection of AKH II-L, which lacks a proline residue in the molecule, is only measurable at pharmacologically high doses. An attempt is made to relate the observed differences in the dose-response relationships to the amino acid sequences of the neuropeptides.

Recently, the long-known but elusive hypertrehalosaemic hormones of the American cockroach, Periplaneta americana, were isolated [1], and they proved to be the same compounds [2, 3] as the myoactive factors M I and M II isolated and sequenced from the cockroach corpora cardiaca by O’Shea and co-workers [4, 5]. In addition, the structure of cockroach neurohormone D, which increases the frequency of heart-beat, was shown to be identical to M I [6], and the structures of two peptides from cockroach corpora cardiaca by O’Shea and co-workers [4, 5]. In addition, the structure of cockroach neurohormone D, which increases the frequency of heart-beat, was shown to be identical to M I [6], and the structures of two peptides from cockroach corpora cardiaca by O’Shea and co-workers [4, 5].

The cockroach hypertrehalosaemic hormones act via the activation of adenylate cyclase and phosphorylase [8]; they are octapeptides (I: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH2; II: pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH2) [5, 7] and show close similarities in structure to the locust decapetide adipokinetic hormone I (AKH I: pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Thr-NH2) [9] and the crustacean octapeptide red pigment-concentrating hormone (RPCH: pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH2) [10]. Two other compounds, both called adipokinetic hormone II, have been isolated [11–13] and sequenced as octapeptides from the corpora cardiaca of Schistocerca species (AKH II-S: pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH2) and of Locusta migratoria (AKH II-L: pGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp-NH2) [14, 15]. Both neuropeptides resemble quite markedly the structure of RPCH. Furthermore, another member of the AKH/RPCH family has been isolated from the corpora cardiaca of the stick insect Carausius morosus [1]. This so-called stick insect factor II increases trehalose levels in the haemolymph of ligated stick insects [1, 16]. The amino acid composition data suggested that the active factor was a nonapeptide, with only one aspartate residue less than AKH I [17]. However, sequence analysis of this compound by fast atom bombardment mass spectrometry proved that it is a decapeptide with the following structure: pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH2 [18].

In view of the close similarities in structure between all the peptides mentioned above, this investigation studies in detail the hypertrehalosaemic potencies of these naturally occurring neuropeptides in the cockroach. Former work on this subject, independently performed by two research groups, revealed large discrepancies in the reported potencies for AKH I and RPCH in cockroaches [19, 20]. Furthermore, AKH II-S was also tested and shown to be less effective than RPCH or AKH I; however, it was wrongly assumed that AKH II-S had “an iden-
tical amino acid composition to RPCH" [20]. This study was therefore conducted to clarify the observed differences in action of the above-mentioned peptides. This work should also allow a better understanding of the structure-activity relationships of the AKH/RPCH family, especially when considered alongside our data on the adipokinetic potencies of these peptides in *L. migratoria* [21].

**Materials and Methods**

*Insects*

Adult male cockroaches, *Periplaneta americana*, were supplied by Professor Dr. K. Hansen (Universität Regensburg) and kept as described previously [2].

*Preparation of peptides*

Synthetic myoactive factors, M I and M II (identical to the cockroach hypertrehalosaemic hormones I and II), AKH I and RPCH were obtained from Peninsula Laboratories (Belmont, CA, USA). Natural AKH II from *L. migratoria* corpora cardiaca and hypertrehalosaemic factor II material from stick insect corpora cardiaca were purified by reversed-phase high-performance liquid-chromatography (RP-HPLC) as outlined elsewhere [13, 17]. Storage of stock solutions of the peptides and preparation for injection into acceptor cockroaches in a 10 μl dose were as described previously [8].

*Assay for hypertrehalosaemic activity*

Handling of cockroaches and determination of haemolymph carbohydrate (total anthrone-positive material) concentrations were carried out as outlined previously [8]. The dose-response curves for the six peptides studied here were analysed using Hill Plots (see [22]) to calculate the ED50 values (the amount of peptide which is needed to produce 50% of the maximum hypertrehalosaemic response); the calculations of all ED50 values were based on a maximum response of 34 mg/ml, and it was assumed that all peptides bind to the same receptor.

*Reversed-phase HPLC*

The material of each synthetic peptide, AKH I, RPCH, M I and M II, dried by vacuum-centrifugation (Savant speed-vac) was resuspended in 80% methanol and applied to a Nucleosil C-18 column. Details of the equipment used is given elsewhere [17]. A linear gradient from 45% to 60% B in 12 min was used with a flow rate of 1 ml/min (solvent A: 0.11% trifluoroacetic acid; solvent B: 0.1% trifluoroacetic acid in 60% acetonitrile). The eluent was monitored at 210 nm at a sensitivity of 0.08 absorbance units full scale (LKB 2151 variable wavelength detector with 10 μl HPLC flow cell; 10 mm pathlength).

**Results**

1. *Separation of the synthetic peptides AKH I, RPCH, M I and M II*

The HPLC chromatogram of the separation of the commercially available synthetic neuropeptides is shown in Fig. 1. The upper graph (Fig. 1A) shows...
the trace when 80% methanol is injected; a clear baseline can be seen. The synthetic peptides are well separated from each other (Fig. 1B), although three of them, RPCH, M I and M II, are octapeptides with only little structural differences. Despite injecting similar amounts (250 pmol each), the peak heights are quite different, especially for M I and M II; however, no comparisons between peak-areas were made. In addition, some minor peaks not seen in the control run with methanol are detected, possibly due to slight contamination and/or breakdown products of the peptides.

2. Hypertrehalosaemic responses of different neuropeptides

The results of various amounts of synthetic hypertrehalosaemic hormones I and II (M I and M II) upon the levels of total haemolymph carbohydrates are shown in Fig. 2. The maximal response of approx. 30 mg/ml is elicited when about 5 pmol of M I and M II, respectively, had been injected. The dose-response curves are remarkably similar; the ED₅₀ values are 1.9 and 1.8 pmol for hormone I and II, respectively.

The data for haemolymph carbohydrate elevation in cockroaches after injection of various concentrations of stick insect hypertrehalosaemic factor II material are given in Fig. 3. The maximal response is in the range of that elicited with the cockroach hypertrehalosaemic hormones, but it is already obtained with about 4 pmol, and the ED₅₀ value is 0.9 pmol.

The hypertrehalosaemic effect of various amounts of synthetic AKH I and RPCH in the blood of cockroaches is shown in Fig. 4. The maximal response elicited by both compounds never reaches more than 21 mg/ml, even when 50 pmol are injected. Interestingly, AKH I is very potent at low doses; in fact, at very low concentrations up to 1 pmol, it is more effective than M I and M II, respectively, and this can easily be seen by the shift of the dose-response curve for AKH I to the left (Fig. 4A). The ED₅₀ values of 4.5 and 5.9 pmol for RPCH and AKH I, respectively, express the differences in this assay system between these peptides and between these and the cockroach hypertrehalosaemic hormones.

Table I gives the data for carbohydrate elevation in cockroaches after injection of various quantities of locust AKH II. Up to about 5 pmol there is no sig-
significant hypertrehalosaemic response to this compound, whereas with high doses up to 50 pmol a small response is seen but it never approaches that to the cockroach hypertrehalosaemic hormones.

Figure 4. The effects of increasing doses of synthetic AKH I (A) and RPCH (B) on carbohydrate mobilisation in P. americana. Points and vertical bars represent the mean ± S.D. for at least 5 observations.

Table I. Increase in total haemolymph carbohydrate concentration in adult cockroaches upon injection of various amounts of locust adipokinetic hormone II (AKH II-L). The values shown are the mean ± S.D. between control values (0 min) and 120 min post-injection.

<table>
<thead>
<tr>
<th>Treatment (10 μl injected)</th>
<th>n</th>
<th>Increase in blood carbohydrates [mg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (bidistilled water)</td>
<td>5</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>AKH II-L [pmol]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>7</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>1.0</td>
<td>5</td>
<td>3.2 ± 2.0</td>
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<tr>
<td>5.0</td>
<td>4</td>
<td>4.4 ± 2.0</td>
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<tr>
<td>10.0</td>
<td>5</td>
<td>12.8 ± 3.2</td>
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<tr>
<td>20.0</td>
<td>7</td>
<td>9.5 ± 5.4</td>
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<tr>
<td>30.0</td>
<td>5</td>
<td>11.0 ± 3.9</td>
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<tr>
<td>50.0</td>
<td>3</td>
<td>15.9 ± 1.9</td>
</tr>
</tbody>
</table>

**Discussion**

There is a special relationship between the structure of a hormone and its biological activity; the biological activity of a peptide hormone results from the recognition of a particular structure by a specific membrane-bound receptor. Therefore, studies on the structure-activity relationship of closely related peptide hormones should help to elucidate the special structural features of the peptide molecule which are requisite for such recognition. One strategy for such investigations is the preparation of synthetic analogues of the peptide under study (see, for example, [23]). However, in our first attempt to gain some insight into the structure-activity relationships of the cockroach hypertrehalosaemic hormones, we analysed compounds provided by nature. Thus, we tested naturally occurring peptides belonging to the AKH/RPCH family.

For the first time, we determined complete dose-response curves for each of the cockroach hypertrehalosaemic hormones, M I and M II. These octa-peptides show considerable homology to each other: the C-terminal sequence is identical until the Pro residue at position 6 (counted from the N-terminal), while homologous exchanges have taken place at position 5 (Ser versus Thr) and 2 (Val versus Leu), and the Asn residue at position 3 of hormone I is replaced by Thr. Our studies indicate that these replacements apparently do not influence receptor-binding. Both peptides show almost identical dose-response curves, and the calculated ED50 values are almost identical: about 1.9 pmol of each hormone is needed to produce 50% of the maximal hypertrehalosaemic response. In contrast, the locust adipokinetic hormone (AKH I) had a ED50 value of 1 in its own (adipokinetic) locust assay system [21]. Such an ED50 value was calculated in this study for the stick insect factor on its ability to cause hypertrehalosaemia in cockroaches. Thus, this compound, recently identified as a decapeptide [18] rather than as a nonapeptide as thought previously [17], is more potent than the endogenous cockroach hormones. This result is somewhat surprising and difficult to explain in relation to the structure of the stick insect peptide. It can be argued that in this peptide, which has the first eight residues in common with M II, the two additional C-terminal amino acids beyond Trp (Gly and Thr) have a positive effect on the receptor-binding. However, this reasoning is not substantiated.
by our results with AKH I, the other decapeptide, on carbohydrate mobilisation in the cockroach. The dose-response curve show that although AKH I is very active at low doses, even at high doses up to 50 pmol the response never reaches that produced by M I, M II or the stick insect peptide. The only structural difference, however, between the latter peptide and AKH I is a Thr residue at position 3 instead of an Asn residue (in AKH I), but the cockroach hormone II contains also an Asn residue at position 3 without having less activity than hormone I. Thus, an exchange at this position seems not to be critical to receptor-binding. An understanding of the significance of the difference of the activity of the decapeptides (AKH I versus stick insect factor) must await studies on the receptors with labelled compounds.

Equally difficult to interpret are the results with the prawn octapeptide RPCH, which, as AKH I, elicited no full hypertrehalosaemic response even when 50 pmol were injected into the cockroach. Structurally, there are differences between RPCH and the cockroach hormone II at position 3 (Asn versus Thr), 5 (Ser versus Thr) and 7 (Gly versus Asn). The exchanges at position 3 and 5 cannot be relevant for the receptor binding, since these changes are also present in the cockroach hormone I. This leaves the Gly residue at position 7 the determining factor for the reduced activity. To test this hypothesis, a synthetic analogue such as Gly\(^7\)-M II should be prepared in the near future. On the other hand it should be stated that we found almost identical dose-response curves for M I, M II and RPCH for the effect on lipid release in locusts [21].

One structural feature of the peptide molecule to bind to the cockroach fat body receptor seems to be clear from our study. This is the importance of the Pro residue. All the peptides tested contain this residue at position 6 except the AKH II-L. It, apparently, shows no real binding, since no response is found at doses up to 5 pmol and higher doses (up to 50 pmol) cause only a weak response which may be interpreted as a pharmacological effect. In locusts, it was suggested that the Pro residue is essential for the binding to the "adipokinetic" receptor [21, 23, 24]; the dose-(adipokinetic)response curves for AKH II-L showed that it is almost as active as AKH I at low doses, but even at very high doses the response reached only about 65% of that of AKH I [21].

Is there a possibility that contaminations of the synthetic or HPLC-purified peptides used in this study may be responsible for the above mentioned results? It is obvious that it is very difficult to refute this possibility unequivocally. The HPLC-chromatogram of the synthetic peptides, however, show quite clearly that only very minor contamination (breakdown products?) is present. HPLC analyses of the single peptides (results not shown) revealed that a minor impurity was associated with AKH I as found previously [1], but this was less than 1%. In our opinion, this cannot account for any of the effects observed above.

Finally, we should briefly compare the present results for AKH I, RPCH and AKH II with those of former studies. As already mentioned, there are some anomalies about the reported effects of AKH I and RPCH on carbohydrate release in the cockroach [19, 20]. Mordue and Stone [19] found that RPCH was more potent than AKH I in elevating sugar levels in the cockroach; with about 50 pmol of RPCH they got an increase of about 23 mg carbohydrate/ml haemolymph, whereas 150 pmol of AKH I elevated the haemolymph carbohydrates by about 11 mg/ml. In our assays, elevations in haemolymph carbohydrates of the same order required 10 pmol of RPCH and about 1 pmol of AKH I. It is difficult to compare our data exactly with those of van Norstrand et al. [20] because these authors assayed haemolymph carbohydrates after a shorter time (1 h), and expressed their data as percentage change in total carbohydrates without giving any data on the actual amount of blood carbohydrates in control animals. Nevertheless, they found a 100% change of blood carbohydrates (which in our cockroaches would mean an increase of between 14 and 18 mg/ml [8]) with about 3 pmol of AKH I and RPCH. These values are in the order as those in the present study. In contrast to our results with AKH II, they show already significant carbohydrate elevation in response to 1 and 2.5 pmol [20]. One explanation for this discrepancy may be the use of AKH II from Schistocerca gregaria in their study, while we used AKH II purified from the corpora cardiaca of Locusta migratoria. As stated above, the compounds differ in the amino acid residue at position 6 (Thr versus Ala), but both lack the Pro residue [14, 15]. In agreement, both studies ([20], this paper) found AKH II, at least at low doses, less active than AKH I.

Thus, we have shown in the present investigation that cockroach hypertrehalosaemic hormones I and
II have almost identical biological activity over the range of the dose-response curve, whereas stick insect factor II is surprisingly more potent, although another decapeptide, AKH I, is much less capable of causing hypertrehalosaemia as the cockroach hormones. Equally potent as AKH I is the prawn octapeptide RPCH. The locust octapeptide AKH II, although structurally similar to RPCH, is only active at pharmacologically high doses; this may be due to the lack of a Pro residue in this molecule.

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