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The fruitfly *Drosophila melanogaster* contains a novel charged adipokinetic-hormone-family peptide

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A member of the RPCH/AKH (red-pigment-concentrating hormone/adipokinetic hormone) family of arthropod neuropeptides was identified in the fruitfly *Drosophila melanogaster*, and its structure was determined by automated Edman degradation and m.s. using fast-atom-bombardment ionization and a tandem hybrid instrument capable of high sensitivity. The sequence of this peptide, which we call 'DAKH', is pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2 (where pGlu is pyroglutamic acid and Trp-NH_2 is tryptophan carboxyamide). H.p.l.c. analyses of extracts of the three body segments revealed that more than 80% of the peptide is contained in the thorax. Although DAKH is typical of family members in its general structure and distribution in the animal, it is unique in containing a residue which is charged under physiological conditions. The evolutionary significance of this change is considered.

INTRODUCTION

The RPCH/AKH (red-pigment-concentrating/adipokinetic hormone) family is a large group of structurally similar neuropeptides. One or two of these peptides occur in most, if not all, species of arthropods, and it is likely that this peptide family will ultimately be shown to extend to other phyla (Schooneveld *et al.*, 1987; Suzuki *et al.*, 1989). Table 1 shows the sequences that have been established for members of this family. RPCH/AKH peptides were recognized more than half a century ago, as hormones causing colour change in crustaceans and effecting energy metabolism in insects (Perkins, 1928; Steele, 1961). Recently, observations of bioactivities *in vitro* of peptide family members and localization of these peptides to neurons and nerve endings by immunohistochemistry suggest that at least some family members function as locally released modulators that influence gut, heart, skeletal-muscle and other neurons (Schooneveld *et al.*, 1983; O'Shea *et al.*, 1984; Dickinson & Marder, 1989).

The wide variety of insects provides an opportunity to observe many specific examples of how this neuropeptide family has evolved over a considerable evolutionary distance. Both to extend the available structural data to another order of insects, and to provide a basis for molecular-biological and genetic studies on an RPCH/AKH peptide in a species particularly suited for such studies, we have isolated and sequenced an RPCH/AKH-family member from the fruitfly *Drosophila melanogaster* which we have named 'DAKH' (pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2, where pGlu is pyroglutamic acid and Trp-NH_2 is tryptophan carboxyamide). We have recently learned that a peptide of identical sequence has been characterized in another dipteran, the blowfly *Phormia terraenovae* [the following paper (Gäde *et al.*, 1990)]. The structures of two other dipteran peptides have just been reported (Jaffe *et al.*, 1989).

MATERIALS AND METHODS

Animals

Approx. 15000 *Drosophila* fruitflies (strain Oregon R) grown in standard laboratory cultures were used in this study. Flies were dissected under CO_2 anaesthesia.

Peptide isolation

DAKH was extracted from thoraces dissected by hand, or from 'bodies' retained by a screen after a 'head preparation' procedure (Oliver & Phillips, 1970). Tissues were homogenized on ice in methanol/water/acetic acid (90:9:1, by vol.) (4.5 ml/3000 bodies) using a Tissuemizer (Tekmar, Cincinnati, OH, U.S.A.). The homogenate was centrifuged for 10 min at 0 °C and 10000 *g*, and the pellet was re-extracted as described above. The combined supernatants were dried in a vacuum centrifuge, and the residue was resuspended in 0.3 ml of 50 mM-ammonium acetate, adjusted to pH 4.5 with acetic acid, and centrifuged at 10000 *g* for 10 min. The supernatant was fractionated on a Sep-Pak C_{18} cartridge (Millipore), which was washed with 50 mM-ammonium acetate, pH 4.5, containing 16% (v/v) acetonitrile and eluted with the same buffer containing 35% acetonitrile. The eluate was purified by three rounds of reverse-phase h.p.l.c. The sample was first applied to an Anspec (Deerfield, IL, U.S.A.) packed Bondapac 10 μ column (300 mm × 4.6 mm) eluted at 1.5 ml/min using an LKB 2152 controller and two 2150 pumps (Pharmacia LKB, Uppsala, Sweden) with 50 mM-ammonium acetate, pH 4.5, and a gradient of acetonitrile varying from 20 to 37% (v/v) over 30 min. The effluent was monitored by fluorescence (excitation 283 nm, emission 350 nm) using a McPherson FL-750/BX fluorimeter with a xenon–mercury lamp. The second step used the same apparatus and a Regis (Morton Grove, IL, U.S.A.) 5 μ C_{18} column (25 mm × 4.6 mm) eluted isocratically at 1 ml/min with 5 mM-ammonium acetate adjusted to pH 6.6 with acetic acid and containing 20% (v/v) acetonitrile. The third step used an Applied Biosystems Aquapore RP 300 column (100 mm × 2.1 mm) and trifluoroacetic acid as described previously (Dawson *et al.*, 1989). Samples chromatographed on the Aquapore column were collected for Edman degradation and mass spectrometry. No synthetic samples of DAKH were chromatographed on any of the h.p.l.c. systems until all natural samples had been collected for analysis.

Removal of pGlu

A 400 pmol portion of DAKH was digested with 0.4 munits

Abbreviations used: AKH, adipokinetic hormone; RPCH, red-pigment-concentrating hormone; N-DAKH, the asparagine-analogue of DAKH (pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2, where pGlu is pyroglutamic acid and Trp-NH_2 is tryptophan carboxyamide).

Table 1. RPCH/AKH family

Key to references: 1, Fernlund & Josefsson (1972); 2, Stone *et al.* (1976); 3, Siegert *et al.* (1985); 4, Gäde *et al.* (1986); 5, Gäde *et al.* (1988); 6, Scarborough *et al.* (1984); 7, Witten *et al.* (1984); 8, Gäde & Kellner (1989); 9, Hayes *et al.* (1986); 10, Gäde & Rinehart (1986); 11, Gäde & Rinehart (1987*a*); 12, Gäde & Rinehart (1987*b*); 13, Ziegler *et al.* (1985); 14, Jaffe *et al.* (1986); 15, Jaffe *et al.* (1988); 16, Jaffe *et al.* (1989); 17, Gäde *et al.* (1990).

Genus	Common name	Peptide name	Sequence	Reference(s)
Pandalus	Shrimp	RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2	1
Locusta/Schistocerca	Locust/grasshopper	AKH I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2	2
Schistocerca	Grasshopper	AKH II	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH_2	3,4
Locusta	Locust	AKH II	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp-NH_2	3,4
Romalea	Lubber grasshopper	Rc I	pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2	5
Periplaneta/Leptinotarsa	Cockroach/potato beetle	M II (C.C.II), Pea-CAH-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2	6,7,8
Periplaneta/Leptinotarsa	Cockroach/potato beetle	M II (C.C.II), Pea-CAH-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2	6,7,8
Blaberus/Nauphoeta	Cockroach	HTH	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-Thr-NH_2	9,10
Gryllus/Romalea	Cricket/lubber grasshopper	AKH-G/Ro II	pGlu-Val-Asn-Phe-Ser-Thr-Gly-Trp-NH_2	5,11
Carausius	Stick insect	HTF II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2	12
Manduca/Heliothis	Tobacco hornworm/ Corn-earworm moth	Manduca AKH	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH_2	13,14
Heliothis	Corn-earworm moth	Hez-HrTH	pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-Asn-NH_2	15
Tabanus	Horseflies	Taa-AKH	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-NH_2	16
Tabanus	Horseflies	Taa-HoTH	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-Tyr-NH_2	16
Drosophila/Phormia	Fruitfly/blowfly	DAKH	pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2	17

(approx. 2 μg of enzyme) of a crude preparation of L-pGlu-peptide hydrolase (EC 3.4.11.8) from calf liver (Boehringer Mannheim, Indianapolis, IN, U.S.A.) in 60 μl of 0.1 M-NH_4HCO_3/l mM-EDTA/l mM-dithiothreitol through which N_2 had been bubbled for 10 min before use. The digestion was carried out at 37 °C for 2 h, and the products were purified by h.p.l.c. on Aquapore RP 300 as described above. These conditions produced only 40 % conversion of the peptide, but produced no unwanted reactions, as judged by the chromatographic pattern (A_{216}).

Automated amino acid sequencing

The peak of deblocked peptide was eluted earlier than starting material on reverse-phase h.p.l.c. and is completely resolved from it. The deblocked peptide was collected directly on to a glass-fibre filter, and subjected to automated Edman degradation in an Applied Biosystems (Foster City, CA, U.S.A.) model-470A amino acid sequencer with an on-line model-120A amino acid phenylthiohydantoin derivative analyser using the manufacturer's chemicals and programming.

Peptide synthesis

DAKH and the asparagine-7 analogue of DAKH (N-DAKH) were synthesized by using an Applied Biosystems model-430A peptide synthesizer employing t-butoxycarbonyl amino acids. Cleavage from the resin and side-chain deprotection were performed with HF. The products were more than 95 % pure as judged by h.p.l.c. The identities of the products were confirmed by fast-atom-bombardment m.s. using a VG Masslab (Manchester, U.K.) model-30-250 quadrupole mass spectrometer. The amino acid composition of the synthetic peptides and their concentration in solutions used for bioassays were verified with a Pico-Tag System (Waters Division of Millipore, Milford, MA, U.S.A.).

M.s.

Conventional and tandem m.s. analyses of the *Drosophila* peptide were performed using a VG ZAB SEQ hybrid instrument (VG Analytical, Manchester, U.K.) of *BE/qQ* geometry (where B = magnetic sector, E = electric sector, q = radio-frequency-only quadrupole-collision region and Q = quadrupole mass filter). Peptide samples were analysed by fast-atom bombardment using a saddle field atom gun (Ion Tech, Teddington, Middx., U.K.); the primary beam was neutral xenon at an energy of approx. 8 keV. The liquid matrix consisted of equal parts of 2,2′-dithiodiethanol and thioglycerol and was applied to the sample probe; 3–4 μl of a methanolic solution of the peptide was subsequently added. All spectra were acquired by using a VG 11-250 data system. Conventional mass spectra were recorded by scanning the magnetic field from m/z 1500 to m/z 100 at 5 s/decade. Calibration of magnet scanning was made using CsI. During tandem m.s. analyses, precursor ions were selected at 1000 resolution using *BE* and subjected to collision with argon at an estimated pressure of 0.02 Pa (2×10^{-4} mbar) in the RF-only quadrupole (q). The collision energy in the laboratory frame of reference was 9 eV. Product-ion scans were recorded by scanning Q; the scan time was 10 s for the mass range 50–1000. The resolution of Q was 2 mass units. Acquisition was in the 'multi-channel analyser' mode with accumulation of five to ten scans to optimize signal-to-noise ratios. Mass calibration of Q was made using product ions of m/z 912 derived from CsI and was checked by analysis of the product ions of the $[M+H]^+$ ion of substance P (fragment 2–11) (Gaskell & Reilly, 1988). The product ion spectra reported here were subjected to post-acquisition smoothing, using one pass of a conventional five-point moving-average routine.

Bioassays

Grasshoppers (*Schistocerca nitans*) were obtained from our laboratory culture. The assay of the extensor-tibialis muscle activity, which simultaneously indicates contraction of a skeletal muscle and stimulation of the rate of an accessory heart, was performed as described previously (O'Shea *et al.*, 1984). The lipid-mobilization assay was performed as described by Stone & Mordue (1980), except that the colour reagents described by Zollner & Kirsch (1962) were used. The second haemolymph samples were taken at 1.5 h after injection.

RESULTS

Purification

Pilot purifications were done with 50–100 thoraces, which were chosen on the assumption that the foregut, and particularly the ring gland, would be rich sources of the peptide. Members of the RPCH/AKH family stimulate both activities of the extensor-tibialis assay (O'Shea *et al.*, 1984), so fractions collected from the first h.p.l.c. step (Fig. 1) were tested with this assay, and material associated with the expected activity was purified further as described above. At each subsequent step in the purification, the bioactivity was co-eluted with the material that gave rise to most of the fluorescence.

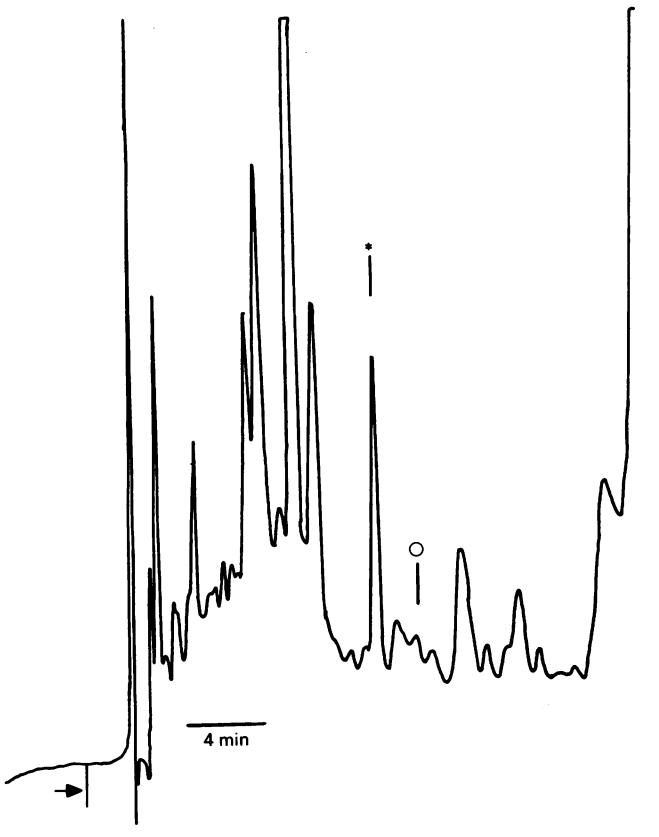

Fig. 1. First h.p.l.c. step

The elution profile was monitored by fluorescence as described in the Materials and methods section. The arrow indicates the time of injection; the bar shows an interval of 4 min. The gradient was started with the injection and reached the detector at 4 min. The asterisk (*) indicates the peak associated with bioactivity (DAKH), the 'O' symbol indicates the elution time of N-DAKH.

Edman degradation

All known members of this peptide family have an *N*-terminal pGlu residue. The isolated material was therefore treated with pyroglutamyl-peptide hydrolase and the expected conversion into a compound which was eluted earlier on reverse-phase chromatography was observed. Automated Edman degradation of 150 pmol of this material (estimated by area of the A_{216} peak compared with peaks of known amounts of synthetic peptide) gave the following results (pmol): Leu, 58; Thr, 33; Phe, 30; Ser, 10; Pro, 18; Asp, 10; Pro, 4; Trp, 0.7. On the basis of the results of Edman degradation and the structure of other family members, the peptide pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2 was synthesized and purified by h.p.l.c. on the RP 300 column, as was the natural compound.

M.s.

A total of 300 pmol of the natural DAKH was used in the analysis. Fig. 2(*a*) shows the spectrum obtained by conventional fast-atom-bombardment m.s. analysis of 15% of this sample of DAKH. An ion was observed at m/z 975 with a signal/background ratio of $\sim 2:1$. The ion at m/z 997 is attributable to the same analyte in the $[M+Na]^+$, rather than the $[M+H]^+$ form. No structural information is discernible from the conventional spectrum. The precursor ion at m/z 975 was selected for tandem m.s. analysis using the hybrid instrument. Fig. 2(*b*) shows the spectrum of product ions formed by spontaneous and collision-induced dissociation of the selected precursor. Approx. 50% of the sample (150 pmol) was used for this analysis. The fragmentation pattern observed agrees closely with that obtained during equivalent analysis of synthetic DAKH (Fig. 2*c*). The product-ion spectra provide extensive sequence information, and include both *C*-terminal and *N*-terminal fragments (Fig. 3). The product ion at m/z 790 (observed with both the synthetic and natural peptides) may correspond to a *C*-terminal rearrangement ion. The prominent peak at m/z 958 likely reflects the loss of NH_3 from the *C*-terminal amide. The very close correspondence between the spectra obtained during analysis of the synthetic and natural peptides are fully consistent with identity of the two compounds. The ambiguity with respect to identification of leucine and isoleucine should be noted, but Edman degradation clearly identified leucine in DAKH. Minor product ions which appear in the spectrum of the natural peptide, but not in the synthetic peptide, are attributable to contributions to the precursor ion signal at m/z 975 by contaminants from the *Drosophila* extract and reflect the very small quantities of the analyte that were available. The results confirm the presence of pyroglutamic acid, and tryptophanamide and exclude the presence of other amino acids or modifications. The identity of the natural and synthetic peptides was further confirmed by the demonstration of co-elution under all the h.p.l.c. conditions used in the purification.

Recovery controls

DAKH, unlike all other sequenced members of this peptide family, contains a charged residue, aspartic acid, and it was conceivable that this was generated from asparagine during the isolation. Therefore the asparagine-7 analogue of DAKH (N-DAKH) was synthesized and shown to be well resolved from DAKH in the first step of h.p.l.c. (Fig. 1). Just before homogenization, N-DAKH was added to a thorax preparation in amounts comparable with those observed for DAKH, and 60% was recovered. By contrast, there is relatively little fluorescent material in this position in unspiked preparations. When 10-fold more N-DAKH was used to spike a preparation, there was no increase in the yield of DAKH.

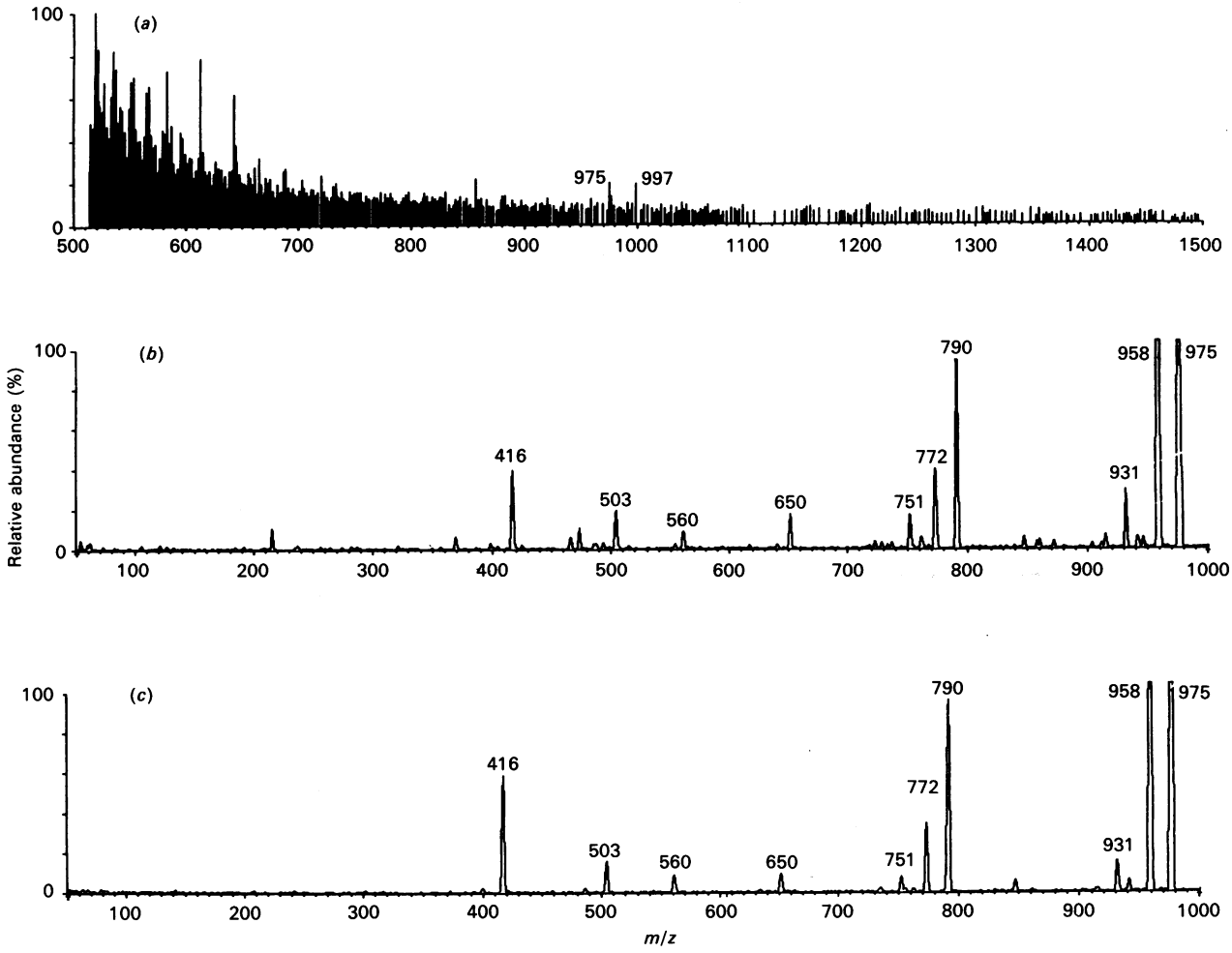

Fig. 2. M.s. of DAKH

(*a*) Conventional fast-atom-bombardment m.s. analysis of DAKH purified from *Drosophila* (corresponding to 15% of the total available sample). (*b*) Spectrum of product ions formed by low-energy collisional activation of ions of *m/z* 975 obtained by fast atom bombardment (50% of the available sample). (*c*) Spectrum of product ions from synthetic DAKH (pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2).

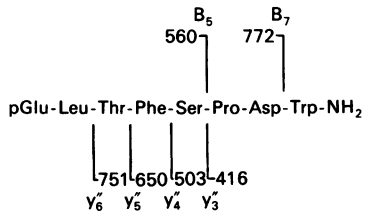

Fig. 3. DAKH fragmentation scheme

Fragment ions observed during tandem m.s. analyses of the natural and synthetic peptides after low-energy collisional activation of ions of *m/z* 975. The nomenclature of Roepstorff & Fohlman (1984) is used. Ions of *m/z* 790 (Fig. 2) are presently unassigned.

Peptide localization

The yield of DAKH from thoraces, corrected for recovery, suggests that each thorax contains about 100 fmol of DAKH. Yields from 'body preparations' were somewhat lower than from thoraces alone. When dissected head and abdomen preparations were subjected to the same purification scheme, no

Table 2. Lipid-mobilizing activity of DAKH and N-DAKH

Compounds were tested as described in the Materials and methods section. A unit of activity is defined as the increase in lipid in μg/h per μl of haemolymph. All doses of N-DAKH and AKH shown in the Table below caused a significant ($P < 0.05$) (difference) in increase in haemolymph lipid compared with saline-injection controls as judged by a *t* or *t'* test. The highest dose of DAKH gives a response not significantly different ($0.1 > P > 0.05$) from that produced by 10 pmol of AKH, which causes a maximal response. Results are means ± S.E.M.

Compound	Dose (pmol)	Number tested	Activity
Saline	–	14	−0.4 ± 1.2
AKH	10	12	8.9 ± 3.1
N-DAKH	10	7	1.2 ± 2.1
	30	8	2.8 ± 1.9
	50	8	6.3 ± 2.1
DAKH	250	8	2.9 ± 1.1
	500	12	5.6 ± 3.4
	1000	8	7.1 ± 3.5

DAKH was detected in the first h.p.l.c. step, although as little as 10% of the thorax content could have been detected. Thus the great majority of DAKH is contained in the thorax. This is not surprising, since in the American cockroach (*Periplaneta americana*), for example, the foregut and particularly the ring-gland homologue are rich sources, whereas the hindgut and the brain contain relatively small amounts (O'Shea *et al.*, 1984).

Bioactivity

The lack of charged residues in all previously described family members suggests that there may be evolutionary pressure against such structures in these genera. One might expect the charged DAKH to be relatively inactive in the previously studied genera, and this was tested in the specific case of *Schistocerca nitans*. In the extensor-tibialis assay DAKH is 100-fold less active than the native AKH. On the other hand, the uncharged N-DAKH is only 5–10-fold less active. Table 2 shows that similar relative potencies are found with the lipid-mobilization assay.

DISCUSSION

M.s.

The value of m.s. in the characterization of peptides is becoming increasingly clear, especially with respect to its ability to identify modifications which go undetected by chemical-sequencing techniques. The chief disadvantage of m.s. has been a requirement for relatively large amounts of material compared, for example, with automated Edman degradation, but tandem mass spectrometers can be used to address this problem. A variety of instrumental approaches are possible. A substantial body of literature has developed concerning four-sector instruments (e.g. Blemann & Scoble, 1987) and triple quadrupoles (Hunt *et al.*, 1986), but the literature on hybrid sector/quadrupole instruments (Gaskell & Reilly, 1988) remains sparse. In the present study a hybrid instrument was used to obtain a diagnostic spectrum of daughter ions derived from the protonated peptide parent ($[M+H]^+$). Only about 150 pmol of peptide was required for the tandem m.s. analysis, despite the division of parent-ion current between $[M+H]^+$ and $[M+Na]^+$ ions. The analysis was facilitated by the availability of synthetic material; nevertheless, the clarity of the sequence data obtained confirms the utility of hybrid tandem m.s. in peptide analyses of this type.

RPCH–AKH-family evolution

Before the present study, one of the notable features of the RPCH/AKH peptides was the total absence of charged residues. This is in spite of the fact that there is considerable variability in several positions, including the position 7, which is occupied by asparagine, serine, alanine or glycine in different family members. The change from asparagine to aspartic acid observed in two dipteran species requires only a single base change and is one of the most commonly observed mutations (Dayhoff *et al.*, 1978), but it has not occurred in any of the other known RPCH/AKH-family members. The 100-fold decrease in potency of DAKH on two bioassays shows that a strong functional pressure against aspartic acid exists in *S. nitans*, since if *S. nitans* contained the same level of DAKH as AKH in its corpora cardiaca (the neurohaemal glands which release the hormone), it would have to release virtually all of its store to gain a maximal hyperlipaemic effect rather than the 1% required for AKH. There are several possible causes of the reduced potency of DAKH, including diminished diffusion to the receptor and accelerated destruction of the peptide by proteinases. However, the leg bioassay shows no evidence of slow onset or short duration of response to DAKH, and lipid-mobilization assays show similar potency when the response is measured after 1 and 1.5 h. It is tempting to speculate that the low potency of the *Drosophila* peptide in *S. nitans* reflects an unfavourable interaction of the charged aspartic acid with the grasshopper receptor, and that the structure of DAKH reflects a change in the *Drosophila* receptor(s) which accommodates this charge.

It remains to be established what functions DAKH serves in *Drosophila*. The first suggestion comes from work in *Phormia terraenovae*, which demonstrates that the same peptide causes a marked increase in haemolymph carbohydrate in this dipteran [the accompanying paper (Gäde *et al.*, 1990)]. Future work on the detailed anatomical distribution, molecular biology, and genetics of DAKH should help clarify its role in *Drosophila*.

Although only one family member was identified, the presence of other family members has not been excluded by the present study. Indeed, many other insects have been shown to contain two peptides, and two uncharged dipteran RPCH/AKH peptides have been isolated from a mixture of species in the family Tabanidae (Jaffe *et al.* 1989). If one of these or other, uncharged, family members were present in *Drosophila*, there would be the interesting possibility of multiple, selective receptors for structurally distinct peptides, as is thought to be the case for the opiod peptides in mammals (Lynch & Snyder, 1986).

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