A Family of Neuropeptides That Inhibit Juvenile Hormone Biosynthesis in the Cricket, Gryllus bimaculatus*

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Four nonapeptides that inhibit juvenile hormone synthesis have been isolated by four high performance liquid chromatographic steps from extracts of the brain of the field cricket, Gryllus bimaculatus. The primary structures of these peptides were assigned by Edman degradation and mass spectrometry as Gly-Trp-Gln-Asp-Leu-Asn-Gly-Gly-Trp-NH₂ (Grb-AST B1), Gly-Trp-Arg-Asp-Leu-Asn-Gly-Gly-Trp-NH₂ (Grb-AST B2), Ala-Trp-Arg-Asp-Leu-Ser-Gly-Ser-Trp-NH₂ (Grb-AST B3), and Ala-Trp-Glu-Arg-Phe-His-Gly-Ser-Trp-NH₂ (Grb-AST B4). Each of the peptides shows high sequence similarity to the locustamoinhibiting peptide (Lom-MIP), but is structurally different from all the allatostatins so far identified. The synthetic allatostatins Grb-AST B1-4 are potent inhibitors (50% inhibition at 10⁻⁶ to 7 x 10⁻⁹ M) of juvenile hormone III biosynthesis by corpora allata from 3-day-old virgin females of Gryllus bimaculatus using an in vitro bioassay. At 10⁻⁷ M, Grb-AST B1 also strongly inhibits juvenile hormone III biosynthesis by corpora allata from 2-day-old adult males and 1-day-old (males and females) and 4-day-old (females) last instar larvae of Gryllus bimaculatus. The inhibitory effect of Grb-AST B1 was also evident on corpora allata from a related species, Acheta domestica. Inhibition of juvenile hormone synthesis by Grb-AST B1-4 is reversible.

Inhibition of juvenile hormone (JH) biosynthesis by corpora allata (CA) in vitro has provided the basis for the isolation of a family of allatostatins (Tyr-Xaa-Phe-Gly-Leu-NH₂) from brain extracts of various cockroach species (Diploptera punctata, Periplaneta americana, and Blattella germanica) (1-6). In D. punctata and P. americana, molecular cloning has led to the isolation of cDNA that encodes a precursor polypeptide containing 13 and 14 potential allatostatic sequences, respectively, including those formerly identified through conventional purification techniques (7, 8). In D. punctata, the expression of the allatostatin gene in midgut endocrine cells was also demonstrated (9). In the blowfly, Calliphora vomitoria, six peptides with sequence similarity to cockroach allatostatins have been identified; these inhibit JH III biosynthesis in cockroaches, but do not inhibit synthesis of JH II bisepoxide in the fly itself (10, 11). An allatostatin that is structurally unrelated to the cockroach allatostatins has been purified and characterized from heads of pharate adults of the moth Manduca sexta (12).

Very recently, we identified two peptide inhibitors of JH III biosynthesis from extracts of the brain of the field cricket, Gryllus bimaculatus, which were designated Grb-AST A1 and Grb-AST A2, respectively (13). Each of the peptides shows C-terminal amino acid sequence similarity to cockroach allatostatins and blowfly callatostatins. In this report, we describe the isolation and primary structure of four nonapeptides from brains of Gryllus bimaculatus that are potent inhibitors of JH III synthesis in vitro by CA from virgin adult females, males, and last instar larvae of the species. These peptides are structurally different from all allatostatins so far identified, but are homologous to locustamoinhibiting peptide (Lom-MIP), a peptide that inhibits the spontaneous contractions of the hindgut and ovipositor of Locusta migratoria (14). We propose that this family of peptides represents a novel type of neurohormone that inhibits JH biosynthesis in crickets.

MATERIALS AND METHODS

Insects and Tissue Dissection

Virgin adult females and males of Gryllus bimaculatus (de Geer) (Ensifera, Gryllidae) reared at 27 °C and staged according to chronological age (15) were used in the experiments. Brains (2700) of 2-4-day-old females were dissected free of optic lobes, corpora cardiaca-CA complexes, and adhering fat body and stored in extraction medium (methanol/water/acetate acid (100:10:1, v/v/v)) at −25 °C prior to purification. Single CA of 3-day-old females were used to test chromatographic fractions for allatostatic activity and to measure dose response and reversibility of inhibition of synthetic peptides. These CA exhibit the highest and most consistent rate of JH III biosynthesis from animal to animal (13). Inhibition of JH III synthesis was also tested on CA from 2-day-old adult males and 1-day-old (males and females) and 4-day-old (females) last instar larvae of Gryllus bimaculatus and from 5-day-old adult females of the house cricket, Acheta domestica. Adult A. domestica females were removed from stock cultures on the day of emergence and maintained at 27 °C until use.

Radiochemical Assay for Allatostatic Activity

Juvenile hormone III biosynthesis in vitro was determined by the radiochemical assay described previously (16-18), with some modifications. The radiolabeled precursor for JH III was L-[(methyl-¹⁴C)methionine (Amersham Buchler, Braunschweig, Germany; final specific activity of 1.11–1.30 × 10⁸ Bq/mmol; final concentration of L-methionine of 0.24–0.28 mM). After a 1.5-h preincubation in medium without [¹⁴C]methionine, single CA were incubated for 2 h in 20 μl of medium TC199 (Sigma M7653 (Deisenhofen, Germany); with Hanks’ salts and sodium bicarbonate, without L-glutamine; buffered with 25 mM HEPES; supplemented with CaCl₂, to a final concentration of 3 mM; fortified with 1% Ficol 400 (Pharmacal, Uppsala, Sweden); sterilized by suction through a 0.2-μm filter; adjusted to pH 7.0; containing 12,500-18,000 Bq of L-[(methyl-¹⁴C)methionine) to establish rates of JH synthesis by untreated glands; subsequently, CA were transferred to medium with the extract/synthetic peptide to be tested for a second 2-h period. The percentage change in rates of synthesis was calculated. Following incubation, the medium was processed for extraction and JH III quantification as described (18).

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The abbreviations used are: JH, juvenile hormone; CA, corpora allata; HPLC, high performance liquid chromatography; FMOC, N-(9-fluorenylmethoxycarbonyl; AKH, adipokinetic hormone.
Brain Extracts and Prepurification by Solid-phase Extraction

A total of 54 batches, each batch representing 50 brains, was extracted as described previously (13). The extracts were subsequently prepurified on Sep-Pak cartridges (13). Allatostatic material eluted in the 40% CH$_3$CN fraction. A total of 13 of these Sep-Pak separations were required to process the entire material.

Chromatography

HPLC separations were carried out on a Shimadzu HPLC system (13). The eluted material was detected at 215 nm.

First HPLC Run—The conditions were as follows: column: LiChroCART 125-4 Superspher 100 RP-18 with LiChroCART 4-4 guard column (same material; Merck, Darmstadt, Germany); solvent A: 0.115% trifluoroacetic acid in water; solvent B: 0.1% trifluoroacetic acid in CH$_3$CN; gradient: 0–5 min of 0% solvent B, 5–8 min of 0–20% solvent B (linear gradient), and 8–51 min of 20–33% solvent B (linear gradient, 0.3%/min) followed by a 10-min wash at 100% solvent B; and flow rate: 1 ml/min. Samples (500–600 brain equivalents in 1 ml of 0.1% trifluoroacetic acid) were loaded onto the column and collected in 1-min fractions. Five of these highly reproducible first HPLC runs were required to process all of the material (2700 brain equivalents). Fractions 13–60 were assayed for allatostatic activity (20 brain equivalents). The fractions eluting at 19 and 20 min (corresponding to 25.7–26.0% CH$_3$CN) showed the highest allatostatic activity and led to the isolation of Grb-AST A1 and A2 (13). In this paper, we report the further purification of fractions 23 and 24 (corresponding to 26.9–27.2% CH$_3$CN), which also showed consistent allatostatic activity.

Second HPLC Run—The conditions were as follows: column: 250 x 4.6-mm Shiseido CAPCELL PAK C$_18$ SG 300 with 10 x 4.6-mm guard column (same material; Grom, Herrenberg-Kayh, Germany); solvent A: 0.13% heptafluorobutyric acid in water; solvent B: 0.13% heptafluorobutyric acid in CH$_3$CN; gradient: 0–2 min of 5% solvent B and 2–52 min of 5–60% solvent B (linear gradient, 1.1%/min) followed by a 7-min wash at 100% solvent B; and flow rate: 1 ml/min. The pooled fractions 23 and 24 from the first HPLC runs (2400 brain equivalents) were dried down to 500 µl, diluted with 500 µl of 0.13% heptafluorobutyric acid in 10% CH$_3$CN, and loaded onto the column. Peaks were collected manually into reaction tubes and tested for allatostatic activity (33.3 brain
Table I

Sequence, molecular size, content per brain, and IC_{50} values (concentration required for 50% inhibition of JH III biosynthesis) of several peptides

| Peptide     | Sequence             | [M + H]^+ found by mass spectrometry | Calculated [M + H]^+ for amidated peptide | Calculated peptide content | IC_{50} |
|-------------|----------------------|-------------------------------------|------------------------------------------|---------------------------|---------|
|             |                      | pmol/brain                          | brain                                    |                           | M       |
| Grb-AST A1  | AQQHQSFGL-NH$_2$     | 1049.8                              | 1050.16                                  | 0.18                      | 4 × 10^{-9} |
| Grb-AST A2  | AGGRQYGFL-NH$_2$     | 1025.20                             | 1025.16                                  | 0.09                      | 8 × 10^{-9} |
| Grb-AST B1  | GWQDLNGGW-NH$_2$     | 1031.39                             | 1032.10                                  | 0.73                      | 7 × 10^{-8} |
| Grb-AST B2  | GWRDLNGGW-NH$_2$     | 1059.28                             | 1060.16                                  | 0.19                      | 2 × 10^{-8} |
| Grb-AST B3  | AWRDLGGGW-NH$_2$     | 1045.80                             | 1047.16                                  | 0.17                      | 7 × 10^{-8} |
| Grb-AST B4  | AWERFHSNGW-NH$_2$    | 1174.05                             | 1175.30                                  | 0.14                      | 1 × 10^{-8} |
| Lom-MIP     | AMQDLASGW-NH$_2$     | 1060.21                             | ~10^{-4}                                  |                           |         |
| Grb-AKH     | pEVNFSGTW-NH$_2$     | 920.98                               | Not reached                              |                           |         |

Allatostatic Neuropeptides in Crickets

Data for Grb-AST A1 and A2 are taken from Ref. 13.

Computer Sequence Analysis

Computer sequence analysis was carried out performing a blitz E-mail search at EMBL and a tblastn search at the NCBI Blast network server. SwissProt release 30 (December 1994), EMBL release 41 (December 1994), and GenBank release 88 (April 1995) were searched for similarity to peptides Grb-AST B1-4 using program default parameters.

RESULTS

HPLC Separation of Allatostatic Neuropeptides—Bioactive material from the first HPLC runs (pooled fractions 23 and 24) eluted from the second HPLC system between 30 and 40 min (Fig. 1). Four fractions, eluting at 32.90 min (41.2% CH$_3$CN; fraction B1), 34.75 min (43.2% CH$_3$CN; fraction B2), 35.01 min (43.5% CH$_3$CN; fraction B3), and 36.28 min (44.9% CH$_3$CN; fraction B4), showed allatostatic activity. In the third HPLC system, each fraction yielded one major peak with allatostatic activity (fraction B1: 22.40 min (30.3% CH$_3$CN); fraction B2: 21.62 min (29.3% CH$_3$CN); fraction B3: 22.35 min (30.1% CH$_3$CN); and fraction B4: 23.08 min (31.0% CH$_3$CN)). Active fractions from the third HPLC run eluted in the final HPLC system as single peaks (Fig. 1) at 25.07 min (37.7% CH$_3$CN; fraction B1), 24.45 min (37.0% CH$_3$CN; fraction B2), 24.43 min (37.0% CH$_3$CN; fraction B3), and 23.98 min (36.5% CH$_3$CN; fraction B4).

Amino Acid Sequence Analysis—Aliquots from HPLC fractions B1-4 were subjected to automated Edman degradation (fraction B1: 210 pmol/286 brain equivalents; fraction B2: 103 pmol/551 brain equivalents; fraction B3: 125 pmol/740 brain equivalents; and fraction B4: 86 pmol/604 brain equivalents). Sequence analyses indicated four nonapeptides. The primary structures were elucidated for fraction B1 as Gly-Trp-Gly-Asn-Gly-Gly-Gly-Trp, for fraction B2 as Gly-Gly-Trp-Arg-Asp-Leu-Asn-Gly-Gly-Trp, for fraction B3 as Ala-Arg-Asp-Leu-Ser-Gly-Gly-Trp, and for fraction B4 as Ala-Trp-Glu-Arg-Phe-His-Gly-Ser-Trp (Table I). Mass spectral analysis indicated that the C terminus was amidated in all the peptides. Values for mass ([M + H]^+) determined by spectrometry match precisely the calculated molecular sizes (Table I). Peptide contents per brain were calculated from the data of the sequence analysis (loss of material during chromatographic steps not taken into account; Table I).

Peptide Synthesis

Peptide synthesis was performed on a Model 9050 peptide synthesizer (Milligen, Eschborn, Germany) using Fmoc/HOBt chemistry. Peptides were synthesized in the amide form using an Fmoc-peptide amide linker polyethylene glycol-polyoxyethylene resin (Milligen). Synthetic peptides were purified after cleavage from the resin by reversed-phase HPLC. Confirmation of the correct sequence of synthetic peptides was attained by mass analysis and coelution with the native peptides.
Grb-AST B1–4 are shown in Fig. 2. Fifty percent inhibition of JH III synthesis was achieved with 7 × 10^−8 M Grb-AST B1, 2 × 10^−8 M Grb-AST B2, 7 × 10^−9 M Grb-AST B3, and 1 × 10^−8 M Grb-AST B4 (Table I).

A computer search for similar peptides revealed sequence similarities with locust amyo-inhibiting peptide (Lom-MIP) (Table I) (14). Sequence similarity between Grb-AST B1 and Lom-MIP, however, showed a maximal inhibitory activity on JH III biosynthesis of crickets that was significantly lower than that of Grb-AST B1–4 (compare values for 50% inhibition; Fig. 2 and Table I). On the other hand, half-maximal inhibition (which is different from 50% inhibition since the glands are not inhibited up to 100%) is reached at 5 × 10^−8 M. This is only slightly higher than the respective doses of Grb-AST B1–4 required for half-maximal inhibition. This means that Lom-MIP and Grb-AST B1–4 may bind equally effectively to the cricket allatostatin receptor, but that Lom-MIP causes a weaker response. The adipokinetic peptide of G. bimaculatus (Grb-AKH) (20), which shares the two C-terminal amino acids with Grb-AST B1–3 (Table I), showed ~30% inhibition at 10^−5 M, the highest concentration soluble in the incubation medium (data not shown).

Reversibility of Inhibition—After 1 h of incubation in medium without [14C]methionine, single CA were transferred to radioactive medium and incubated for 2 h to establish rates of synthesis by untreated glands; then CA were transferred to medium containing 10^−7 M allatostatic peptides B1–4 and incubated for another 2 h. Finally, CA were transferred to medium without addition of peptides and incubated again for 2 h. Glands recovered completely from inhibited rates (Fig. 3).

Sex and Species Specificity—Grb-AST B1 was tested on single CA from 2-day-old adult males of G. bimaculatus at concentrations ranging from 10^−8 to 10^−6 M (Fig. 4). Fifty percent inhibition was achieved with 4 × 10^−8 M Grb-AST B1. Grb-AST B1 was also tested on 1-day-old male and female last instar larvae. At a concentration of 10^−5 M, Grb-AST B1 inhibited JH III biosynthesis by CA from these larvae by 55.1 ± 7.7% (mean ± S.E.; Table II). No sex differences could be found. When tested on single CA from 4-day-old female last instar larvae, 10^−7 M Grb-AST B1 reduced JH III biosynthesis by 43.3 ± 12.8% (Table II). Grb-AST B1 (10^−7 M) also inhibited JH III synthesis by CA from 5-day-old adult female house crickets, A. domesticus, by 70.3 ± 2.6% (Table II).

DISCUSSION

The search for neuropeptides with allatostatic activity in G. bimaculatus has led to the isolation of four nonapeptides with sequence similarity at the C terminus (Gly-Xaa-Trp-NH2; Xaa = Gly in B1–3 and Ser in B4) and a common amino acid at position 2 (Trp). In addition, three of the four peptides (B1–3) have in common amino acids at positions 4 and 5 (Asp-Leu). These peptides have been designated G. bimaculatus allatostatic neuropeptides B1–4 (Grb-AST B1–4) in accordance with their biological activity and with the widely accepted nomenclature for invertebrate neuropeptides (21). The B designation...
has been used because another class of allatostatic neuropeptides has been recently presented in G. bimaculatus, Grb-AST A1 and A2 (13), which are members of the Tyr-Xaa-Phe-Gly-Leu-NH₂ allatostatin family first found in D. punctata (1).

The similarity of these peptides to those of the locust L. migratoria is often very inconsistent, which makes these CA unsuitable for testing allatostatic activity. The occurrence of Tyr-Xaa-Phe-Gly-Leu-NH₂ allatostatin-like peptides in cockroaches might indicate the importance of this residue with regard to the biological activity of these peptides. The information about the bioactivity therefore might be encoded not only in the C-terminal region, as shown for the Tyr-Xaa-Phe-Gly-Leu-NH₂ allatostatin family (3, 24).

One may ask whether Grb-AST B1–4 can be termed “alla-
ostatins” since the first isolation of a member of this peptide family (Lom-MIP) used a bioassay based on myoinhibiting activity of the HPLC fractions (14). In crickets, the allatostatic activity is the only effect demonstrated so far; therefore, the term allatostatin seems to be justified.

Grb-AST B1–4 proved to be neither sex/stage- nor species-
specific. Although extracted only from brains of adult females, they inhibit JH III biosynthesis by CA from adult males and last instar larval males and females as well. The allatostatic activity of the B allatostatins was also shown in a closely related cricket species, the house cricket, A. domesticus. Inter-
specific allatostatic activity was also shown for the Tyr-Xaa-
Phe-Gly-Leu-NH₂ allatostatin family (25, 26).

The existence of two types of allatostatic neuropeptides in G. bimaculatus makes it more difficult to understand their modes of function. As yet, nothing is known about changes in brain and hemolymph titer of allatostatic neuropeptides, e.g. during reproductive period of G. bimaculatus. Isolation and quantification of allatostatic receptors will be needed, the levels of which may be largely attributed to changes in CA sensitivity for allatoregulating peptides (8). The occurrence of multiple allatostatin species might suggest the existence of individual receptors for each species of molecule. It remains to be determined, however, whether each allatostatin species is associated with a different receptor. It even remains to be determined whether allatostatins are the principal regulators of JH biosynthesis in vivo. The immunocytochemical localization of Tyr-Xaa-Phe-Gly-Leu-NH₂ allatostatin-like peptides in the central nervous system as well as in midgut cells of many invertebrates (8, 27) suggests that allatostatins are multifunc-
tional neuropeptides, even though inhibition of JH biosynthesis by CA in vitro was the only bioassay utilized in their isolation. Sequence homology, as found between Lom-MIP and the family of allatostatic neuropeptides presented in this paper, may indicate a myomodulatory role of these peptides, as was also discussed for the Tyr-Xaa-Phe-Gly-Leu-NH₂ allatostatin family (28, 29).

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2 M. W. Lorenz, R. Kellner, and K. H. Hoffmann, unpublished observation.

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**TABLE II**

Rates of JH III biosynthesis by untreated single CA and degree of inhibition of JH III biosynthesis by Grb-AST B1 at a concentration of $10^{-7}$ M.

| CA taken from | Rate of JH III biosynthesis by untreated glands | Inhibition of JH III biosynthesis at $10^{-7}$ M Grb-AST B1 | n |
|---------------|-----------------------------------------------|------------------------------------------------------------|---|
| G. bimaculatus |                                              |                                                           |   |
| 1-Day last instar larvae (males and females) | $8.15 \pm 0.66$ | $55.1 \pm 7.7$ | 8 |
| 4-Day last instar larvae (females) | $1.97 \pm 0.46$ | $43.3 \pm 12.8$ | 8 |
| A. domesticus |                                              |                                                           |   |
| 5-Day adult virgin females | $12.98 \pm 2.60$ | $70.3 \pm 2.6$ | 15 |

Data are means ± S.E. For experimental details see "Materials and Methods."
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