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Regulatory Peptides 53 (1994) 237–247

Regulatory Peptides

Allatostatic neuropeptides from the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). Identification, immunolocalization and activity

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Received 3 May 1994; revised version received and accepted 2 August 1994

Abstract

Four allatostatic neuropeptides were isolated from extracts of the brain of the cockroach *Blattella germanica*. The primary structures of these peptides were assigned as Leu-Tyr-Asp-Phe-Gly-Leu-NH_2 (BLAST-1), Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2 (BLAST-2), Ala-Gly-Ser-Asp-Gly-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2 (BLAST-3) and Ala-Pro-Ser-Ser-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2 (BLAST-4). Each of the peptides showed C-terminal amino acid sequence similarity to cockroach allatostatins and blowfly callatostatins. The four peptides inhibited in vitro juvenile hormone production by *corpora allata* from virgin females of *B. germanica*. Immunoreactivity against allatostatins was seen in the lateral neurosecretory neurons and in the axonal pathway leading to the *corpora allata*.

Keywords: Allatostatin; Juvenile hormone; *Corpora allata*

1. Introduction

Juvenile hormones (JH) play important roles in insect physiology. Among others, they promote larval features in preimaginal stages and induce vitellogenin synthesis in the adult. The finely tuned mechanism that controls JH production involves neuropeptides that either inhibit (allatostatins) or stimulate (allatotropins) the *corpora allata* (CA), i.e., the JH producing glands [1,2].

Techniques of purification and analysis have led

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SSDI 0167-0115(94)00074-3

to the identification of one allatotropin and one allatostatin in the moth *Manduca sexta* [3,4], seven allatostatins in the cockroach *Diploptera punctata* (dipstatins: DASTs) [5–8] and two allatostatins in another cockroach, *Periplaneta americana*, (peastatins: PEASTs) [9]. In the blowfly *Calliphora vomitoria* five peptides (callatostatins: CASTs, recently re-named Leu-CASTs and Met-CAST) with sequence homology to cockroach allatostatins have been identified [10,11]. Although these peptides inhibit JH biosynthesis in cockroaches, they do not inhibit the synthesis of JH bisepoxide in the fly itself. In *D. punctata*, molecular cloning has led to the isolation of a cDNA that encodes a precursor polypeptide containing 13 potential DAST sequences, including the seven formerly identified through conventional methods of analysis [12].

As in the viviparous *D. punctata* and oviparous *P. americana*, ovarian development in the oviviparous cockroach *Blattella germanica* is mainly regulated by JH. The discrete cycles of JH production that parallel the process of oocyte maturation are separated by periods of ootheca transport, during which the development of a new batch of basal oocytes remains arrested, in a state that is functionally equivalent to pregnancy [13]. A peculiar feature of *B. germanica* is that virgin females are able to produce JH through a cyclical pattern and complete oocyte maturation and oviposition in a period of time similar to that of mated females [14]. Conversely, JH production in virgin females of *P. americana* and *D. punctata* is very low and non-cyclical, whereas mating has a stimulatory effect upon CA activity in these species [15,16].

Despite these differences, the cyclical activity of the CA during oocyte maturation of *B. germanica* clearly suggests precise mechanisms of regulation most probably by means of allatostatic factors, preliminary evidence of which has been obtained from brain extracts of this cockroach [17]. Here we report on the purification, identification, immunolocalization in the CA and allatostatic activity of four neuropeptides from *B. germanica* with sequence similarity to cockroach allatostatins and blowfly callatostatins.

2. Materials and methods

2.1. Insects and tissue dissection

Virgin adult females of *B. germanica*, reared at 30°C and staged according to chronological age and basal oocyte length [13] were used in all experiments. Brains (1200) of 5- to 6-day-old females were dissected free of optic lobes and adhering fat body, and stored in 0.2 M HCl/75% ethanol at −20°C, prior to purification.

2.2. Radiochemical assay for allatostatic activity

Corpora cardiaca-corpora allata complexes were incubated in 100 μl of Millipore-filtered 199 medium (Biochrom) containing L-methionine (0.1 mM), Hank's salts, Hepes buffer (20 mM) plus Ficoll (20 mg/ml), and BSA (0.1%) to which L-[*methyl*-^{3}H]methionine (Amersham) had been added to achieve a final specific activity of 7.4 GBq/mmol. Brain extracts, fractions from HPLC purification or synthetic peptides, were added to the labelled culture medium. After 2 h of incubation the medium was processed for extraction and JH III quantification as described [18].

2.3. Radioimmunoassay of allatostatins

Aliquots (2–200 μl) from HPLC fractions (see below) were monitored in two RIAs developed for the identification of the Leu- and Met-CASTs of *C. vomitoria*. The antiserum (B2–4) used to detect allatostatins with a C-terminal sequence Tyr-Xaa-Phe-Gly-Leu-NH_2 was raised against Leu-CAST 3, Ala-Asn-Arg-Tyr-Gly-Phe-Gly-Leu-NH_2. The antiserum (D1–4) used to detect allatostatins with a C-terminal sequence Tyr-Xaa-Gly-Phe-Met-NH_2 was raised against Met-CAST, Gly-Pro-Pro-Tyr-

Asp-Phe-Gly-Met-NH_2 [11,19]. The basic details of the RIA protocol were as published [20].

2.4. Purification of allatostatins

A summary of the chromatographic steps used to purify the allatostatins of *B. germanica* (BLASTs) is shown in Fig. 1. Two groups of 600 brains were homogenized and centrifuged (8000 *g*, 10 min). The supernatant obtained was diluted to 10% ethanol containing 0.1% trifluoroacetic acid (TFA), loaded onto a C_{18} Sep-Pak cartridge (Waters) and eluted with a gradient of CH_3CN with 0,1% TFA. The bulk of the allatostatic activity was found in the 17–40% CH_3CN eluate.

The 17–40% CH_3CN eluate of 1200 brain equivalents was lyophilized, dissolved in 50 μl of water with 0.1% TFA and processed in two HPLC separations carried out with a Merck-Hitachi low pressure system with automatic gradient controller (L-6200A) and UV-VIS detector (L-4200). The column used was a C_{18} LiChrospher, Merck (4 × 125 mm, 5 μm particle size). Fractions from the first HPLC profile were collected at 3 min intervals to test the biological activity in vitro. Fractions from the second HPLC profile were collected at 1 min intervals and tested by RIA.

Selected fractions containing the major amounts of immunoreactive and bioactive material were pooled and processed in a narrow-bore HPLC system (Waters 625 LC with Waters 486 Tunable Absorbance Detector) using a C_{18} Delta-Pak Waters column (150 × 2 mm, 5 μm particle size). Individual peaks were collected manually and tested by both Leu- and Met-CAST RIAs. For the final steps of purification, a Hewlett-Packard 1090 HPLC equipped with a C_{18} Vydac column (150 × 2.1 mm, 5 μm particle size) was used. Individual peaks were collected and tested in RIA. In the last step, an aliquot of each of the purified peptides was assayed by RIA and the remainder was used for sequence analysis and mass spectrometry.

2.5. Sequencing of allatostatins

The amino acid sequences of purified peptides (5–50 pmol) were determined with an automatic protein sequencer (model 475A, Applied Biosystems) equipped with an on-line HPLC system for the detection of the amino acid phenylthiohydantoin derivatives, which were separated on a C_{18}-DB column (5–7943, Supelco). All chemicals and solvents were sequence or HPLC grade (Applied Biosystems).

2.6. Mass spectrometry

An aliquot of each of the purified peptides was analyzed by matrix assisted laser desorption mass spectrometry using a BenchTOP instrument equipped with reflector (Bruker-Franzen). 50 μl (containing 5–50 pmol) of each sample were dried and redissolved in 5 μl of 0.1% TFA in 30% CH_3CN, then a 0.5 μl aliquot was mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid in the same solvent. After drying, the mixture was analyzed in the reflected mode at + 19.5/20 kV using FMRFamide (M_r = 598.8) and lymnaDFamide (M_r = 1517.6) [21] as internal standards. With this mode of operation the accuracy is better than 0.2 at M_r = 1000, wich is sufficient to distinguish between the C-terminal free acid and amidated form (ΔM_r = 1.0).

2.7. Synthetic allatostatins

Synthesis of peptides identified from *B. germanica* was carried out in an Abimed AMS 422 multiple synthesizer, using Fmoc chemistry. After cleavage, the identity and purity (ca. 95%) of each peptide was assessed by amino acid analysis and HPLC, respectively. DAST-7 from *D. punctata* (Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2) and an analogue of one of the *B. germanica* peptides (BLAST-2 in free acid form) were also synthesized following the same methodology.

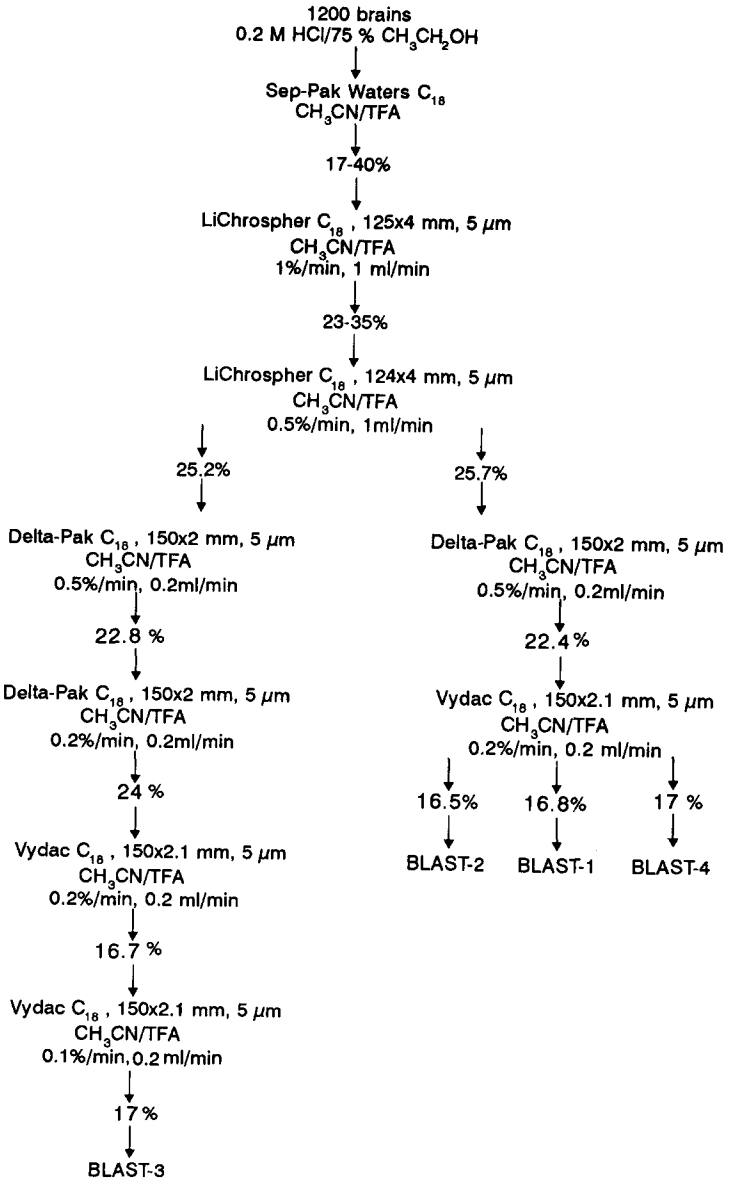

Fig. 1. Flow diagram summarizing the chromatographic steps used to purify the allatostatins identified in *Blattella germanica* (BLASTs). The percentages quoted between steps refer to the percentages of acetonitrile at which the active fractions eluted. Activity was determined either in bioassay for the inhibition of JH synthesis or by RIA for Leu- or Met-callatostatin. Trifluoroacetic acid (TFA) as counter-ion was used at 0.1%.

2.8. Immunocytochemistry

Brain-corpora cardiaca-corpora allata complexes from 6 day-old female virgins were fixed in Bouin's fluid, embedded in paraffin and sectioned at 8 μm. Sections were processed for immunocytochemistry according to previously published methods [11] with an antiserum against Leu-CAST, B2–4, used at a concentration of 1:1000.

2.9. Alignment and comparison of peptide sequences

Allatostatins isolated from *B. germanica* were compared with those from other species using the PileUp program from the Sequence Analysis Software Package (Genetics Computer Group) [22]. The program produces a multiple sequence alignment using progressive, pairwise alignments. It uses an UPGMA algorithm and generates a dendrogram depicting the clustering relationships used to produce the alignment, which reflects the structural relationships and degree of similarity between the sequences.

3. Results

3.1. Allatostatic activity of brain extracts in relation to age

Prior to accumulation of brains for peptide isolation, we studied the allatostatic activity of brain extracts from virgin females of *B. germanica* of different ages within the first gonadotrophic cycle. For these purposes, brain extracts were prepurified by Sep-pak and the 17–40% CH_3CN eluate was used (see below). Results (Fig. 2) indicated that brains from 5- to 6-day-old females exhibited the highest allatostatic activity. Therefore, brains of these two age groups were collected for purification purposes.

3.2. Identification of allatostatins

The separation of BLAST-1, -2 and -4 in the last purification step is illustrated in Fig. 3. The unequivocally determined sequences of these peptides as well as that of BLAST-3 purified from an adjoining fraction (not shown) are given in Fig. 4. We assume that all four peptides are amidated at the C-terminus since non-amidated allatostatins are not detected by the RIAs used ([10], unpublished results). Furthermore, the relative molecular mass (M_r) obtained for three of the peptides (Fig. 4) matched the expected values when measured with an accuracy that allows for the detection of the amide as opposed to the free acid. We were not able to obtain a mass spectrum of BLAST-1.

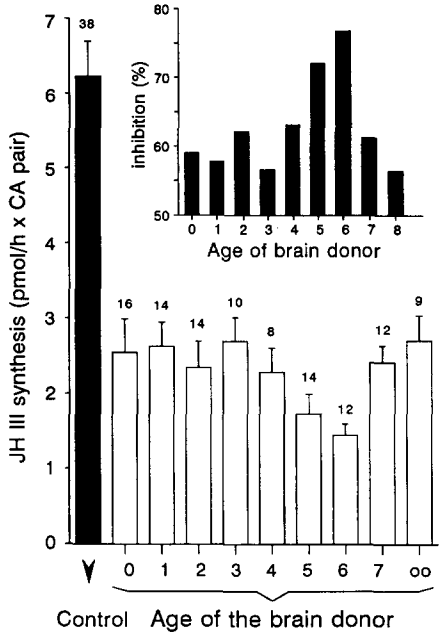

Fig. 2. Allatostatic activity of brain extracts from virgin females of *Blattella germanica* of different ages within the first gonadotrophic cycle. Experiments were carried out on CA from 6-day-old virgin females and the dose used was 2 brain equivalents in all experiments. The lower graph indicates the values of JH synthesis (mean ± S.E.M., number of replicates at the top of each bar). The upper graph summarizes the percentage of inhibition. oo: 8-day-old females in the first day of ootheca transport.

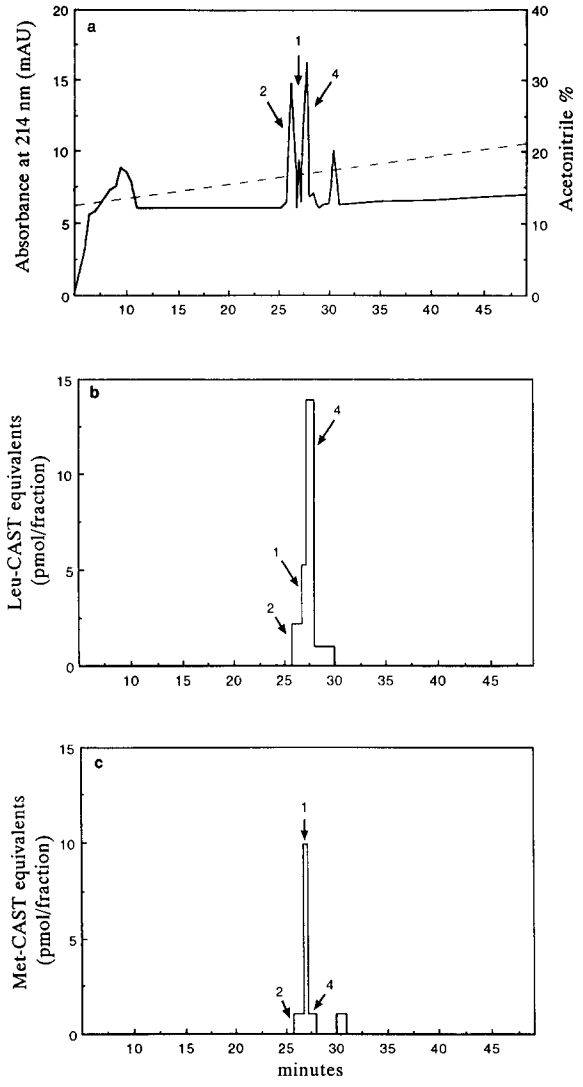

Fig. 3. (a) Profile corresponding to the last Vydac C_{18} HPLC step leading to the isolation of BLASTs 1, 2 and 4 (see flow diagram of Fig. 1). The discontinuous line represents the acetonitrile gradient. mAU: absorbance milliunits. (b) Results of Leu-CAST RIA. (c) Results of Met-CAST RIA. The immunoreactive compound eluting at 30 min was not identified.

3.3. Structural comparison with other allatostatins

The allatostatins identified so far in *B. germanica* are very similar to those found in the cockroaches *D. punctata* (DASTs 1–13) (see [12] for peptide numeration) and *P. americana* (PEASTs 1–2) [9], and to the callatostatins isolated from the blowfly *C. vomitoria* (Leu-CASTs 1–4 and Met-CAST) [10,11]. The dendrogram depicting the structural relationships between all these allatostatins (Fig. 5) shows that those of *B. germanica* have high sequence similarity with *D. punctata* DASTs. Two peptides are common to both species: BLAST-2, which is identical to DAST-5, and BLAST-1, which has the same structure as DAST-1, the last being inferred from cDNA sequence [12] but not yet isolated from *D. punctata* tissues. When allatostatins of *B. germanica* are compared with those of *P. americana* and *C. vomitoria*, the sequence similarity is somewhat lower. In any case, the dendrogram shows that the clustering relationships are not phylogenetically congruent, since the sequences corresponding to the different species and orders appear largely intermingled with each other.

3.4. Immunocytochemistry

Discrete Leu-CAST immunoreactive neurones and axonal pathways were detected in the brain and the rest of the CNS. Because of the sequence similarity of the BLASTs with Leu-CAST-3 from which peptide the C-terminal specific antisera were produced, it is clear that the immunoreactivity represents the sites of the allatostatins of *B. germanica*. Important in the context of the present study was the presence of 3 to 4 pairs of lateral neurosecretory neurones on either side of the brain with axons in the *nervi corporis allati II* leading to the CA (Fig. 6). The immunoreactive material was particularly noticeable in the axon leading into the centre of the CA, whereas peripheral immunoreactivity was less prominent or absent.

CODE	AMINOACID SEQUENCE	M_r measured	M_r calculated
BLAST-1	Leu-Tyr-Asp-Phe-Gly-Leu-NH_2	ND	725.8
BLAST-2	Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2	968.9	969.1
BLAST-3	Ala-Gly-Ser-Asp-Gly-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2	1241.3	1241.4
BLAST-4	Ala-Pro-Ser-Ser-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2	1365.5	1365.5

Fig. 4. Amino acid sequences of the allatostatins of *Blattella germanica* isolated from brain extracts.

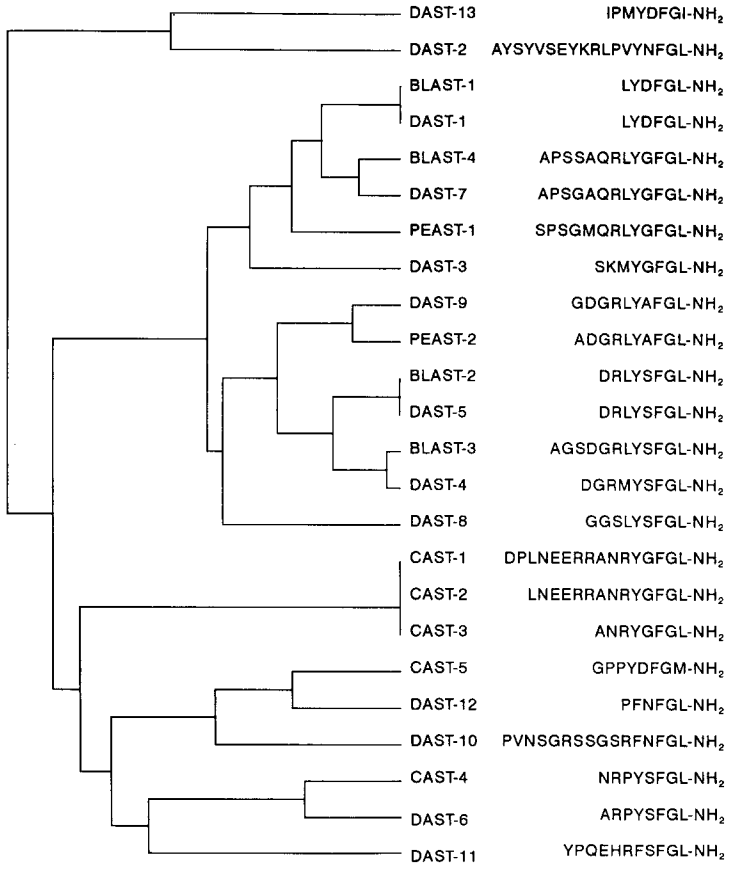

Fig. 5. Dendrogram showing the structural relationships between the allatostatins of *Diploptera punctata*: DASTs 1–13 [12], *Calliphora vomitoria*: CASTs 1–4 (Leu-CASTs) and CAST-5 (Met-CAST) [10,11], *Periplaneta americana*: PEASTs 1–2 [9] and *Blattella germanica*: BLASTs 1–4 (present work).

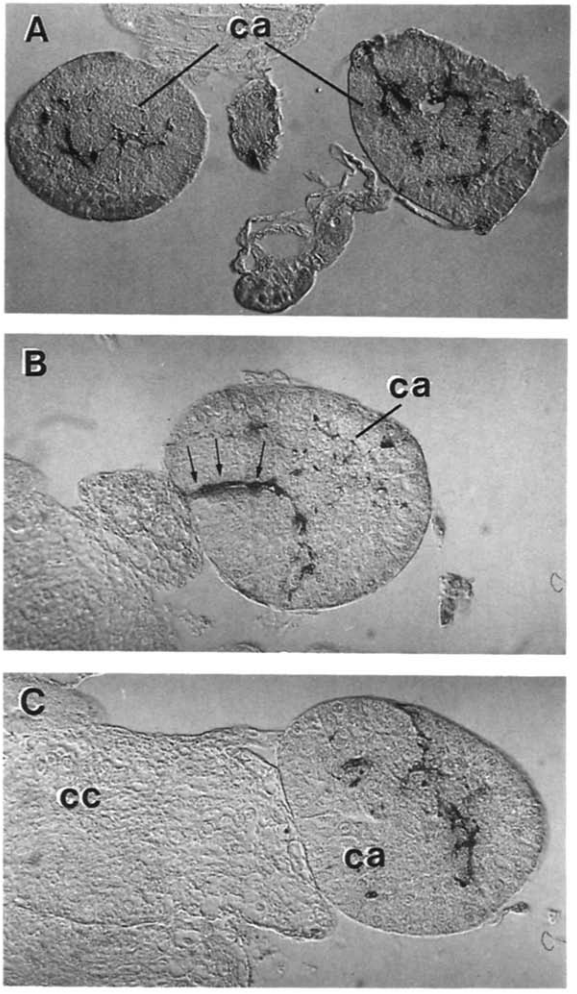

Fig. 6. Paraffin sections of the retrocerebral complex of *Blattella germanica*, immunostained with the peroxidase-antiperoxidase technique using a Leu-CAST-3 antiserum. (A) Frontal section of the pair of *corpora allata* (ca) showing immunoreactivity in axons and arborizations between glandular cells, in particular in the centre of the gland (× 500). (B) Longitudinal section of a *corpus allatum* (ca) showing immunoreactivity in the *nervi corporis allati II* axon entering the gland and varicosities between the glandular cells (× 650). (C) Longitudinal section of areas of the *corpora cardiaca* (cc) and *corpora allata* (ca) showing immunoreactivity in *corpus allatum* but not in the *corpus cardiacum* (× 650).

3.5. Allatostatic activity

Preliminary experiments testing DAST-7 on CA from 3- 4- 5- and 6-day-old virgin females of *B. germanica* (results not shown) indicated that the later age was the most sensitive to allatostatic action. Assays on previtellogenic females were not attempted since the low rates synthesized at these ages in control specimens are already close to the limit of detection of radiochemical assay. Therefore, the four synthetic newly-identified BLASTs were assayed for allatostatic activity on CA from 6-day-old virgin females of *B. germanica*. Results (Fig. 7) show that all BLASTs elicited similar inhibitory activity upon JH synthesis, at a range of doses between 10^{-5} and 10^{-8} M. A comparative study of DAST-7 indicated that this compound inhibited JH synthesis at a similar level. The activity of BLAST-2 in free acid form was much lower when compared with the amidated form.

4. Discussion

Purification of brain extracts from the cockroach *B. germanica* has led to the isolation and identification of four allatostatic neuropeptides (BLASTs). None of these peptides shares sequence similarity with the allatostatin isolated from the lepidopteran *M. sexta* [4]. Conversely, they are very similar to the allatostatins found in the cockroaches *D. punctata* [12] and *P. americana* [9], and to the callatostatins identified in the blowfly *C. vomitoria* [10]. It is clear that the allatostatins of *B. germanica* have high sequence similarity with those of *D. punctata*. In addition to the two peptides common to both species (BLAST-1 = DAST-1 and BLAST-2 = DAST-5), BLAST-4 is almost identical to DAST-7, except for the Ser in position 4 from N-terminus instead of Gly. BLAST-3 has almost the same C-terminal nonapeptide sequence as DAST-4 but with Leu in position 6 from C-terminus instead of Met. The allatostatins of *B. germanica* also show similarities with the cal-

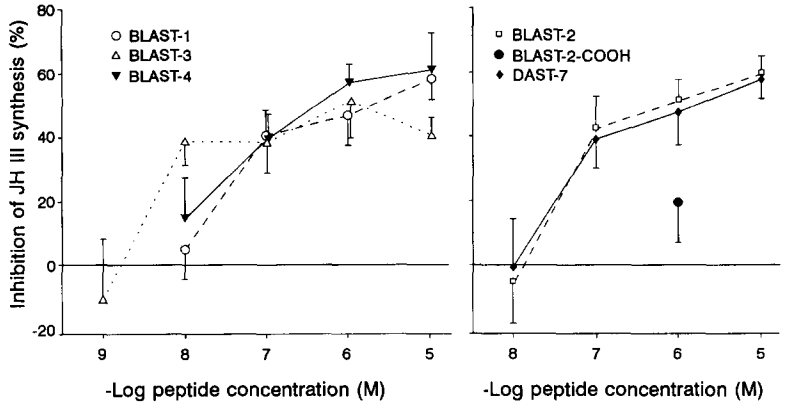

Fig. 7. Dose-responses for inhibition of JH III production by synthetic allatostatins of *Blattella germanica* (BLASTs), compared with allatostatin 7 of *Diploptera punctata* (DAST 7) and BLAST-2 in free acid form (BLAST-2-COOH). Experiments were carried out on *corpora allata* from 6-day-old virgin females of *B. germanica*. Results are expressed as the mean ± S.E.M. ($n = 8–13$).

latostatins of *C. vomitoria*. The C-terminal pentapeptide sequence of BLAST-1 when compared with Met-CAST, differs only in having Leu-NH_2 at the C-terminus, instead of Met-NH_2, whereas Asp in position 4 from C-terminus is a feature common to both. This similarity most probably accounts for the positive immunoreactivity of HPLC fractions containing BLAST-1 when assayed with Met-CAST RIA. BLASTs 2, 3 and 4 have the C-terminal pentapeptide sequence YXFGL-NH_2 (X = S or G). The residues Ser or Gly are both detected in the Leu-CAST RIA although not in the Met-CAST RIA.

Immunocytochemical studies show that the allatostatins of *B. germanica* are localized in neurosecretory cells whose axons lead to the CA, and all four peptides inhibit in vitro the synthesis of JH by CA from 6-day-old virgin females at concentrations ranging from 10^{-5} to 10^{-8} M. Our preliminary data had shown that CA from this age were the most sensitive to allatostatic action, despite the fact that 6-day-old virgin females are those which produce maximal rates of JH within the first gonadotrophic cycle [13]. This contrasts with results reported for mated females of *D. punctata*, where sensitivity to allatostatins is inversely proportional to the activity of the CA, and maximal allatostatic activity is found at ages when JH synthesis is declining [23,24]. Conversely, when DAST-7 is tested on CA from virgin females of *P. americana*, maximal inhibition is found at the stage of maximal production of JH [25], which is in agreement with the results obtained in *B. germanica*.

It is worth noting, however, that effective doses required to inhibit JH in *B. germanica* are at least 2 orders of magnitude higher than those described for *D. punctata* [5–7] or *P. americana* [9]. The changes of sensitivity of CA to allatostatin action during the gonadotrophic cycle in *D. punctata* [23,24] and the changes of allatostatin titre in the CA (see below), suggest that one of the functions of these peptides could be the maintenance of low rates of JH production during virginity. Nevertheless, *B. germanica* is able to produce a cycle of JH production and oocyte maturation in the virgin state, which may account for the observed differences of sensitivity to allatostatins.

Another peculiarity of the allatostatic activity in *B. germanica* emerges from the results obtained with brain extracts of different ages. In these assays maximal activity was found in brains from 6-day-old fe-

males. In *D. punctata*, ELISA measurements of the contents of DAST-7 in brain extracts from virgin females were relatively low and constant, whereas in mated females DAST-7 content was maximal just at the beginning of postvitellogenesis, when JH biosynthesis rapidly declines, and minimal when JH biosynthesis is maximal [26]. Again, the particular reproductive physiology of *B. germanica* may explain these differences.

It seems clear that the structural similarities between the allatostatins of dictyopterans and dipterans, two orders which are found almost at the opposite extremes of the evolutionary range in pterygote insects [27], have phylogenetic importance, and suggest that the selection of this peptide family was an early event in the evolution of the insect class, or may even date from before. Furthermore, the comparison of peptide sequences available in *D. punctata*, *C. vomitoria*, *P. americana* and *B. germanica* using a conventional algorithm leads to a dendrogram where the sequences corresponding to the different species and orders appear largely intermingled with each other. This suggests that intraspecific diversification of allatostatins could have occurred earlier than the splitting of the groups to which the species belong.

A largely open question regarding the allatostatins relates to their functions. In cockroaches, it seems clear that they inhibit JH biosynthesis, and in *D. punctata* DAST-7 has also been shown to elicit antimyotropic activity in gut tissues [28]. In *C. vomitoria* CASTs have been shown to have an inhibitory effect on the contraction of the muscles of the ileum at concentrations as low as 10^{-16} M [11] but they do not inhibit JH biosynthesis [10]. It remains to be explained, however, why allatostatin immunoreactive cells occur in many different areas of the CNS [10,11,29,30], where they can have nothing to do either with the CA or with the gut. The widespread presence of allatostatins in the CNS could suggest a neuromodulatory role for these peptides. This role could be more primitive than either myoinhibition or the inhibition of JH production, since allatostatin ubiquity in the CNS is a feature common to cockroaches and flies.

Acknowledgements

This study was supported by the DGICYT, Spain (project No PB92–0026, 'Studies on the reproductive biology in cockroaches') (X.B.), the Agricultural and Food Research Council of Great Britain (AG68/022) (A.T.), and the Danish Biotechnology Program for Signal Peptides (A.H.J.). J.L.M. is in receipt of a pre-doctoral fellowship from the Spanish Ministry of Education and Science.

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