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A microdialysis study of allatostatin degradation in *Blattella germanica* (L.) (Dictyoptera, Blattellidae)

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Abstract. Allatostatins with a typical C-terminal sequence YXFGL-NH_2 are insect neuropeptides with inhibitory properties upon Juvenile Hormone production in the corpora allata, vitellogenin release by the fat body, and gut and dorsal vessel motility. All these biological effects are rapidly reversible, suggesting the occurrence of effective mechanisms for inactivation of the peptides. We have studied the degradation of DRLYSFGL-NH_2 (BLAST-2), one of the allatostatins of *Blattella germanica*, in the internal milieu of adult females of this cockroach. The experimental approach combined the use of the radioiodinated derivative [^{125}I-Tyr^4]BLAST-2, microdialysis techniques and HPLC analysis with a radioisotope detector. Under these experimental conditions, the half-life of BLAST-2 in the internal milieu of the adult female of *B. germanica* was between 3 and 6 min. Such a short half-life explains the high doses of allatostatins required to obtain the expected biological effects when tested *in vivo*, and suggests that circulating allatostatins are subject to rapid rates of synthesis and degradation in order to be operative physiologically.

Key words. Allatostatin, *Blattella germanica,* cockroach, metabolism, microdialysis.

Introduction

Arthropod allatostatins with the characteristic C-terminal sequence YXFGL-NH_2 were identified by their inhibitory action on the production of Juvenile Hormone (JH) in the corpora allata of cockroaches (Pratt *et al*., 1989; Woodhead *et al*., 1989). Thereafter, orthologous peptides have been discovered in other insect orders (Stay *et al*., 1994), although the inhibition of synthesis of JH seems restricted to cockroaches (Bellés *et al*., 1994; Stay *et al*., 1994) and crickets (Lorenz *et al*., 1995); this activity is not elicited in locusts (Veelaert *et al*., 1996) and in more modified insect orders, such as dipterans (Duve *et al*., 1993). What seems to be more general is the antimyotropic action of allatostatins on gut motility, which has been described in Dictyoptera (Duve *et al*., 1995; Lange *et al*., 1995), Orthoptera (Veelaert *et al*., 1996), Diptera (Duve & Thorpe, 1994; Duve *et al*., 1996) and Lepidoptera (Duve *et al*., 1997). In the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae), four allatostatins (BLAST-1–4) have been identified from brain extracts (Bellés *et al*., 1994) and up to 13 (including BLAST-1–4) have been deduced from the allatostatin cDNA (Bellés *et al*., 1999). In this cockroach, we have reported that allatostatins inhibit production of JH in the corpora allata (Bellés *et al*., 1994), vitellogenin release from the fat body (Martín *et al*., 1996) and dorsal vessel motility (Vilaplana *et al*., 1999b).

One common characteristic to all biological effects elicited by allatostatins, in *B. germanica* and in other species, is that they are rapidly reversible, which suggests the occurrence of effective mechanisms of inactivation. The purpose of the present contribution is to report our results on the degradation

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of one of the allatostatins of *B. germanica*. A relevant antecedent of our study was published by Garside *et al*. (1997a, b) describing the metabolism of allatostatins in the haemolymph and in membrane preparations of a selection of tissues from the cockroach *Diploptera punctata* (see also Bendena *et al*., 1997). In our case, we were interested in determining the half-life of allatostatin in the internal milieu of the insect under an experimental approach as close as possible to physiological conditions. For this reason we have made use of microdialysis techniques. To the best of our knowledge, the only study to use microdialysis to investigate insect haemolymph is that of Ikemoto *et al*. (1993), where it was applied to monitor the levels of different biogenic amines.

Materials and methods

Insects

Adult females of *B. germanica* were obtained from a colony fed on dog chow and water, and reared in the dark at 30 ± 1°C and 60–70% r.h. Freshly moulted virgin females were isolated and used when they were 4-days-old, an age at which allatostatin contents in different tissues show medium levels (Vilaplana *et al*., 1999a).

Compounds and preparation of iodinated derivatives

The allatostatin studied here was DRLYSFGL-NH_2 (BLAST-2), which had been identified in *B. germanica* by purification of brain extracts (Bellés *et al*., 1994) and furthermore deduced from the cDNA corresponding to the precursor protein (Bellés *et al*., 1999). BLAST-2 was synthesized in an Abimed AMS 422 multiple synthesiser using Fmoc chemistry. The peptide was ^{125}I-labelled following the chloramine T method (see McConahey & Dixon, 1980). Briefly, BLAST-2 was dissolved in 0.5 M sodium phosphate (pH 7.5), and added with $Na^{125}I$ and chloramine T. The mixture was incubated at room temperature for 60 s, and the reaction was stopped with chloramine T stop buffer (2.4 mg/mL sodium metabisulfite, 10 mg/mL tyrosine, 10% glycerol, 0.1% xylene cylanol in PBS). The radioiodinated peptide, [^{125}I-Tyr^4]BLAST-2, was eluted from a C_{18} Sep-Pak (Waters, Milford, MA, U.S.A.) with methanol, 0.1% trifluoroacetic acid (TFA), and was further purified by reversed-phase HPLC under the following conditions: column, Spherisorb C_{18} (Merck, Darmstadt, Germany) (4 × 125 mm, 5 µm particle size); solvent A, H_2O with 0.2% TFA; solvent B, acetonitrile with 0.2% TFA; 10% to 50% B in 15 min at a flow rate of 0.8 mL/min; total run time 30 min. ^{125}I-Tyr was prepared similarly.

Microdialysis

The experimental specimens were injected with 2.2 µL Ringer solution containing 35 ng [^{125}I-Tyr^4]BLAST-2 (specific activity 164 000 cpm/ng, that is 574 × 10^4 cpm injected). The compound was injected with a microsyringe (Hamilton 75N, Bonaduz, Switzerland) in the membrane between the second and the third sternites. Immediately after the injection, a microdialysis probe CMA/12 (CMA Microdialysis, Stockholm, Sweden) was inserted in the hole left by the syringe needle using a guide cannula (CMA Microdialysis) (Fig. 1). This probe has a needle-like stainless steel shaft (14 × 0.64 mm o.d.) with a dialysis membrane (4 × 0.5 mm o.d.). The membrane is made of polycarbonate and its cut-off

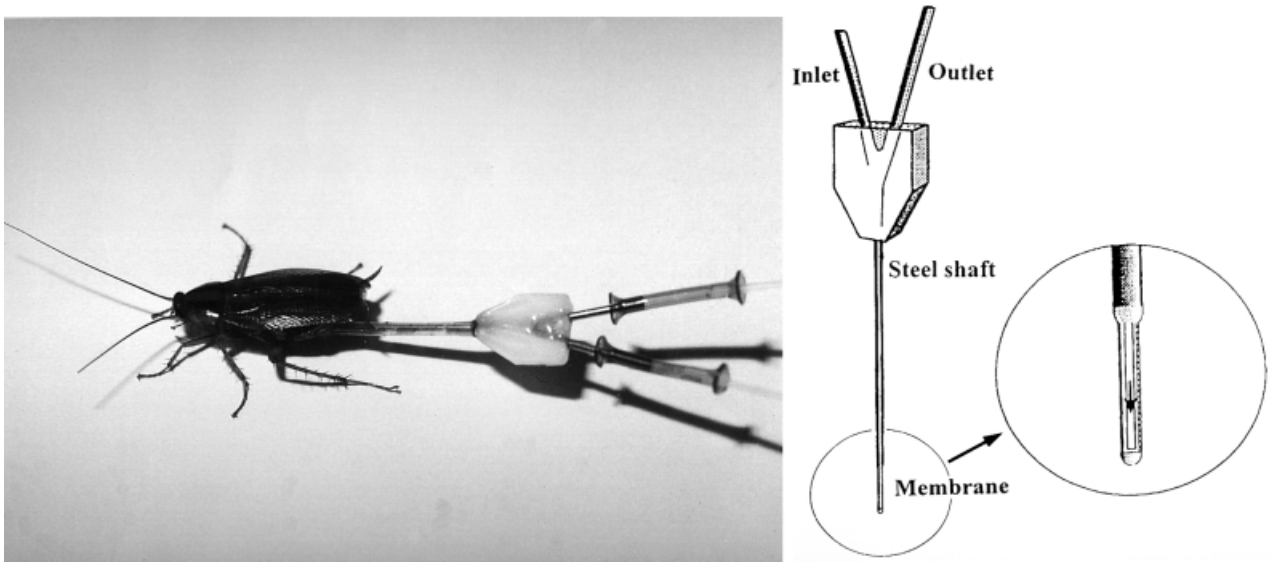

Fig. 1. A 4-day-old adult female of *Blattella germanica* with the microdialysis probe inserted in the ventral part of the abdomen, and a scheme of the microdialysis probe used. It has the following characteristics: membrane diameter, 0.24 mm; membrane length, 2 mm; stainless-steel shaft diameter, 0.38 mm; shaft length 14 mm; inlet internal volume, negligible; outlet internal volume, 1 µL.

molecular mass is 20 000 Da. The probe also has inlet and outlet ports connected, respectively, to a pump and to a sampling tube by Polythene lines (i.d. inlet 0.28 mm, outlet 0.12 mm, length maximum 5 cm for the outlet line). Microdialysis is carried out by circulating the solution from the inlet port of the probe, dialysing at the membrane region and then collecting the microdialysate from the outlet port. In our experiments, Ringer saline (0.9 g NaCl, 0.02 g KCl, 0.02 g $NaCO_3H$, 0.02 g $CaCl_2$, 100 mL H_2O; pH: 7.4) was pumped at a flow rate of 3 µL/min using a syringe pump CMA/100 (CMA Microdialysis). After 1 min of flow the microdialysate was collected every 3 min for 12 min and stored at –20°C until HPLC analysis. The total volume of every fraction (9 µL) was used for each HPLC run (see below).

HPLC analysis of the microdialysate

HPLC analysis of the microdialysate was carried out with a Waters 600 system with a radioisotope detector (Beckman 171, Palo Alto, CA, U.S.A.). The conditions were similar to those used previously to separate *B. germanica* allatostatins (Bellés *et al*., 1994; Vilaplana *et al*., 1999a). The column was a reverse phase C_{18} LiChrospher 100RP-18 (Merck) (4 × 125 mm, 5 µm particle size). As solvents we used H_2O/acetonitrile, both having 0.1% TFA (pH adjusted to 3.5 with triethylamine). The flow rate was 0.8 mL/min and the gradient was 3% acetonitrile/min. [^{125}I-Tyr^4]BLAST-2 and ^{125}I-Tyr, which in these conditions elute at 7.4 and 2.8 min, respectively, served as standards. Relative decrease of the peak corresponding to [^{125}I-Tyr^4]BLAST-2 in the chromatograms of the microdialysates obtained at different times allowed us to estimate the half-life of the peptide.

Results

In three preliminary series of experiments, microdialysates were collected every 10 min for 30 min. However, the HPLC analysis showed no trace of [^{125}I-Tyr^4]BLAST-2 in the fractions corresponding to 20–30 min, and only one series of the fraction 10–20 min showed a quantifiable peak corresponding to [^{125}I-Tyr^4]BLAST-2. Therefore, in subsequent experiments microdialysates were collected every 3 min for 12 min. Figure 2 shows the radiochromatograms corresponding to the sequence of microdialysates of a representative specimen

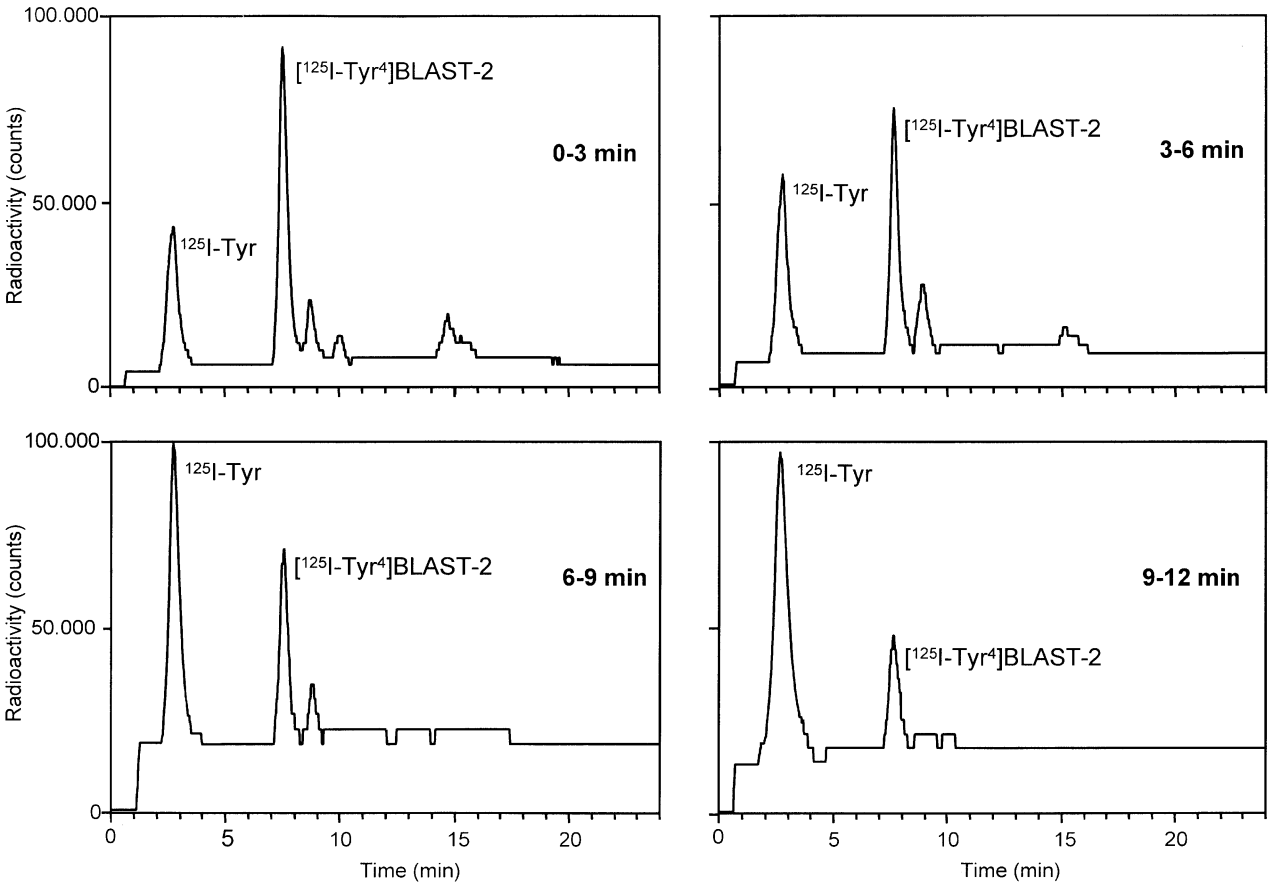

Fig. 2. Radiochromatograms corresponding to the sequence of microdialysates of a representative specimen of 4-day-old adult female of *Blattella germanica* injected with 35 ng of [^{125}I-Tyr^4]BLAST-2.

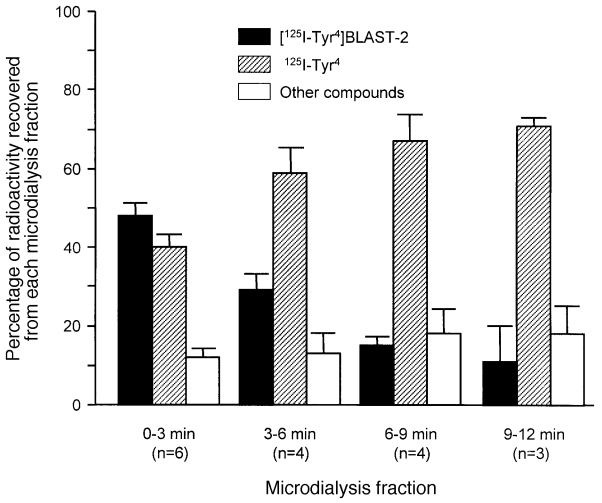

Fig. 3. Percentage of [^{125}I-Tyr^4]BLAST-2, ^{125}I-Tyr and other secondary radioactive compounds in the radiochromatograms corresponding to the sequence of microdialysates obtained from 4-day-old adult females of *Blattella germanica* injected with 35 ng of [^{125}I-Tyr^4]BLAST-2. Values correspond to the percentage of total radioactivity measured in the microdyalisate, and are expressed as the mean ± SEM.

following this protocol. The most striking regularity in the sequence of events is the relative decrease of [^{125}I-Tyr^4]BLAST-2 and the parallel increase of ^{125}I-Tyr within the 12 min elapsed during the experiment. The other peaks appearing in the chromatograms would probably correspond to transient catabolites of [^{125}I-Tyr^4]BLAST-2, but at the times studied they represented only a small percentage of the overall radioactivity.

Proportions between [^{125}I-Tyr^4]BLAST-2, ^{125}I-Tyr and the other secondary radioactive compounds in the radiochromatograms of the microdialysates for all replicates are summarized in Fig. 3. It is clear that [^{125}I-Tyr^4]BLAST-2 decreases in relation to the increase of ^{125}I-Tyr, while the amount corresponding to other compounds remains approximately stable. From these data it can be estimated that the half-life of [^{125}I-Tyr^4]BLAST-2 under our experimental conditions is between 3 and 6 min.

Discussion

There have been a number of reports in the literature on the metabolism of insect peptides in the circulation approaching the physiological conditions. The most usual approach is to inject a known amount of radiolabelled peptide and then monitoring the radioactive metabolites by chromatographic techniques. This was the system used, for example, by Skinner *et al*. (1987) or Rayne & O'Shea (1992) to study the metabolism of adipokinetic and hyperglycemic/cardioacceleratory peptides in the cockroach *Periplaneta americana* and the locust *Schistocerca gregaria*, respectively. In other cases, especially sensitive detection techniques have been developed to detect cold metabolites, like the on-line microbore reversed-phase liquid chromatography coupled to electrospray ionization mass spectrometry, used by Li *et al*. (1997) to study the metabolism of *Manduca sexta* diuretic hormone *in vitro*. The aim of the present microdialysis strategy was to approach still more the physiological conditions *in vivo*. Applied to the case of the YXFGL-NH_2 allatostatins, it allowed to estimate that the half-life of one of the allatostatins of *B. germanica* (BLAST-2: DRLYSFGL-NH_2) in the internal milieu of the adult female is remarkably short, between 3 and 6 min.

This short half-life contrasts with those measured by Garside *et al*. (1997a) in diluted haemolymph of *D. punctata* using the allatostatins Dip-AST 7 (APSGAQRLYGFGL-NH_2), Dip-AST 9 (GDGRLYAFGL-NH_2) and Dip-AST 5 (DRLYSFGL-NH_2), the latter having exactly the same sequence as the BLAST 2 studied by us in *B. germanica*. According to Garside *et al*. (1997a), incubation of Dip-AST 7, 9 and 5, at a concentration of 5 μM, with *D. punctata* haemolymph diluted 100 times with saline (0.9% NaCl), gave as respective half-lives: 22, 22 and 374 min. When [^{3}H-Tyr]Dip-AST 5 was incubated at a concentration of 4 nM in diluted haemolymph, the half-life estimated was 97 min.

The degradation of Dip-AST 5 was studied further in membrane preparations of midgut, hindgut, brain or corpora allata of *D. punctata* by the same research team (Garside *et al*., 1997b). At a concentration of 6 μM, the half-life of Dip-AST 5 varied from 25 min in the case of brain preparations to 53 min in the case of midgut preparations. Values in the same order of magnitude were found when using a concentration of 4 nM and [^{3}H-Tyr]Dip-AST 5. The shortest half-life (19 min) was found by incubating Dip-AST 5 at 6 μM with intact corpora allata (Garside *et al*., 1997b).

From the studies in *D. punctata* it may be concluded that Dip-AST 5 is the most stable of all allatostatins investigated, and that it is degraded faster by membrane-bound peptidases than by soluble peptidases. In addition, the study of the metabolites showed that the metabolic pathway is different in different tissues. In the haemolymph the first metabolite detected was the amidated fragment Dip-AST 5^{3-8} (Garside *et al*., 1997a), whereas in the membrane preparations it was the non-amidated fragment Dip-AST 5^{1-7} (Garside *et al*., 1997b; see also Bendena *et al*., 1997).

In our case, we injected a dose of 35 ng of [^{125}I-Tyr^4]BLAST-2, which diluted in the ≈ 30 μL of haemolymph measured in 4-day-old adult females (Romañá *et al*., 1995), gives an approximate concentration of exogenous peptide of 1 ng/μL (that is, ≈ 1 μM). This is about two orders of magnitude higher than the concentration of allatostatin immunoreactive material measured by HPLC-ELISA in the haemolymph of an adult female of *B. germanica* (8 pg/μL: Vilaplana *et al*., 1999a).

It is obvious that the half-life found for Dip-AST 5 (= BLAST-2) in any of the tissues studied in *D. punctata* is longer than that described herein for *B. germanica*. In part, the longer half-life estimated in *D. punctata* would be attributable to the *in vitro* approach followed, especially in the case of the haemolymph, which was diluted 100 times in saline (Garside *et al*., 1997a). However, the different results seem mainly due to the fact that in our experiments the degradation resulted

from the integrated action of the different peptidases occurring in the insect, soluble and membrane-bound, and including all kind of tissues. Although our study was not focused to identify the metabolic pathway, it seems obvious that [^{125}I-Tyr^4]BLAST-2 was not directly cleaved to ^{125}I-Tyr, and the peaks corresponding to secondary radioactive compounds in the radiochromatograms of the microdialysates would probably represent transient intermediate metabolites. In any case, the peptide would have been inactivated when ^{125}I-Tyr^4 is released, given that SFGL-NH_2 does not exert allatostatic activity at physiological concentrations. This has been shown by Hayes *et al*. (1994) in *D. punctata*, where replacement of the native L-Tyr^4 with D-Tyr^4 or by Ala reduced dramatically the allatostatic potency. In *B. germanica*, even the pentapeptide YSFGL-NH_2 does not inhibit synthesis of JH *in vitro* at a dose of 10^{-6}M (authors' unpublished results).

The results obtained following the microdialysis approach would reflect the physiological situation, and help to explain the high doses of allatostatins required *in vivo* to obtain the expected biological effects. Early reports indicated that inhibition of production of JH required either repeated application (≈3 µg of APSGAQRLYGFGL-NH_2, or AYSYVSEYKRLPVYNFGL-NH_2 or GPPYSFGM-NH_2, twice a day for 3 days in *D. punctata*: Woodhead *et al*., 1993) or a rather high single dose (100 µg of SPSGMQRLYGFGL-NH_2 in *P. americana*: Weaver *et al*., 1994). In *B. germanica*, we have used a single dose of 50 µg of BLAST-2, or the same dose of a pseudopeptide analogue of BLAST-2 with a methyleneamino Ψ[CH_2NH] peptide bond surrogate between residues Leu^3 and Tyr^4, to moderately inhibit synthesis of JH and vitellogenin production *in vivo* (Piulachs *et al*., 1997).

Finally, another conclusion suggested by our results relates to the physiological significance of circulating allatostatins. In *B. germanica*, the immunocytochemical study of Maestro *et al*. (1998) revealed the possibility of neurohaemal release of the peptides, and this was followed by determination of peptide levels in the haemolymph by Vilaplana *et al*. (1999a). Equivalent data have been reported for *D. punctata* (Woodhead *et al*., 1993; Yu *et al*., 1993). Taken together, these results suggest possible biological action(s) at distant targets, which is in agreement with the inhibitory activity of YXFGL-NH_2 allatostatins on vitellogenin release in the fat body reported by Martín *et al*. (1996), or the modulatory action on cardiac rhythm described by Vilaplana *et al*. (1999b). However, the short half-life argues against such action(s), unless the rate of synthesis is sufficiently high. Expression studies are currently in progress in our laboratory to elucidate the rates of allatostatin gene transcription and translation in *B. germanica*.

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