Inhibition of vitellogenin production by allatostatin in the German cockroach

D. Martín, M.D. Piulachs, X. Bellés*

Centro de Investigación y Desarrollo (CSIC), J. Girona 18, 08034 Barcelona, Spain

Abstract

Allatostatins with a typical YXFGL-amide C-terminus constitute a neuropeptide family, which was discovered because of its inhibitory action on insect juvenile hormone synthesis. In the search for possible new functions for allatostatins we focused our attention on the fat body. Our previous studies on the cockroach *Blattella germanica* suggested the occurrence of factors terminating vitellogenesis, and the hypothesis here was that allatostatins might be one of these factors. Our experiments have shown that allatostatin impaired vitellogenin release in fat bodies incubated in vitro, and that this effect appears to be mediated by the inhibition of vitellogenin glycosylation. Fluvastatin also inhibited vitellogenin release, and mevalonolactone counteracted the inhibitory effects of allatostatin. These results suggest that allatostatin acts upon the mevalonate pathway and synthesis of dolichol, which would explain the inhibition of vitellogenin glycosylation. We finally conclude that allatostatins may effectively contribute to the termination of the vitellogenic cycle in *B. germanica*.

Keywords: Allatostatins; Fat body; Vitellogenin; Fluvastatin; Mevalonate; *Blattella germanic*

1. Introduction

Allatostatins with a typical YXFGL-amide C-terminus constitute a neuropeptide family, which was discovered because of its inhibitory action on insect juvenile hormone synthesis [1]. Several allatostatins belonging to this family and showing this biological activity have been isolated and identified in the cockroaches *Diploptera punctata* [2–5], *Periplaneta americana* [6] and *Blattella germanica* [7], and in the cricket *Gryllus bimaculatus* [8]. Furthermore, a total of 13 allatostatins have been deduced from a cloned cDNA sequence of *D. punctata* [9] and 14 have been inferred in *P. americana* following the same approach [1].

In addition, 6 neuropeptides with high sequence similarity to the cockroach and cricket allatostatins have

been identified in the blowfly *Calliphora vomitoria* [10,11]. However, these allatostatins do not act upon juvenile hormone biosynthesis in the blowfly, but are potent inhibitors of peristaltic movements of the ileum [10–12]. Similarly, allatostatins have been shown to elicit antimyotropic activity in gut tissues in *D. punctata* [13,14].

These findings suggest that YXFGL-amide allatostatins have functions other than the inhibition of juvenile hormone synthesis. This suggestion also comes from immunocytochemical and in situ hybridization studies, which have shown that allatostatin immunoreactivity is widespread in many areas of the central nervous system as well as in gut tissues [1,15]. Studies showing that allatostatins circulate in the haemolymph (*D. punctata*: [16], *B. germanica*: unpublished), also point to 'classical' hormonal functions in peripheral tissues for these peptides.

In search of possible new functions for allatostatins we focused our attention on the fat body. On the one

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* Corresponding author. Tel.: + 34 3 4006124; fax: + 34 3 2045904; e-mail: xbragr@cid.csic.es

hand, because our previous studies on the cockroach *B. germanica* suggested the occurrence of undescribed factors contributing to the termination of vitellogenesis (see below), and allatostatins might be one of these factors. On the other hand, Cusson et al. [17] reported the occurrence of proteins in the fat body of *D. punctata* which bound specifically to allatostatins, and might be involved in allatostatin recognition.

The fat body is a key organ in vitellogenesis, since it produces vitellogenin, which is released to the haemolymph, and then incorporated into developing oocytes [18]. In most insects, juvenile hormone is a major endocrine effector acting on vitellogenesis, as it induces the synthesis of vitellogenin, and in those species where vitellogenesis is cyclic, such as *B. germanica*, production of juvenile hormone is also cyclic [19]. In this cockroach the first vitellogenic cycle lasts 8 days, but whereas the juvenile hormone cycle peaks on day 6 [19] that of vitellogenin peaks on day 4 [20,21].

In addition, we have found that 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) synthase is expressed in the fat body of *B. germanica* adult females [22], and further studies have shown that the expression of this enzyme of the mevalonate pathway is cyclic, the protein and the activity levels peaking on day 4 (unpublished), concomitant with the peak of vitellogenin production [20,21]. As in many other insects, *B. germanica* vitellogenin is a glycosylated protein of the high mannose type [23]. Therefore, a plausible link between the mevalonate pathway and vitellogenesis might be the production of dolichol, which would donate the oligosaccharide residues for vitellogenin glycosylation.

These data taken together suggest that the production of juvenile hormone does not modulate the cycle of vitellogenin, and that other effectors may be involved in termination of vitellogenesis. Therefore, according to the hypothesis that allatostatins might be one of these effectors, we studied the effect of one of these neuropeptides on vitellogenin production in the fat body of *B. germanica* following our previously established in vitro experimental model [21].

2. Materials and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at $30 \pm 1°C$ and 60–70% r.h. Freshly moulted virgin females were isolated and used at the appropriate physiological ages, which were assessed by measuring the basal oocyte length.

2.2. Bioactive compounds

Allatostatin 2 (Blast-2: DRLYSFGL-amide) from *B. germanica* [7] was used to inhibit vitellogenin production. It was synthesized in an Abimed AMS 422 multiple synthesizer using Fmoc chemistry. Fluvastatin, a synthetic HMGCoA reductase inhibitor that inhibits insect juvenile hormone biosynthesis [24], was from Sandoz (Sandoz code: XU 62-320). Mevalonolactone, used to restore the mevalonate pathway [25], was from Sigma.

2.3. Fat body incubations

Incubations of periovaric fat bodies were carried out in Grace's medium, with L-glutamine and without insect haemolymph (from Whittaker Bioproducts), as previously described [21]. The two periovaric fat bodies from the same female were dissected out and washed for 10 min in Ringer's saline, pre-incubated for 30 min in Grace's medium, and then transferred to 300 μl of fresh medium, where the experimental incubation was carried out (in the dark, at 30°C and with gentle shaking). Since both periovaric fat bodies are symmetrical in terms of vitellogenin production [21], one of them was used as control and the other one as treated for each experiment. Vitellogenin released to the medium and its content in the incubated periovaric fat body was measured by ELISA (see Section 2.5.) at the end of the incubation period.

2.4. Periovaric fat body processing for protein studies

After the incubation in vitro, the periovaric fat bodies were thoroughly rinsed in Ringer's solution and homogenized in Tris buffer (0.4 M NaCl, 50 mM Tris–HCl, pH 7.3, 1 mM EDTA, 1 mM PMSF) with a plastic pestle. Homogenates were centrifuged at 10 000 rev./min for 20 min, and the resulting supernatant, lying between the pellet and a lipid plug, was recovered for ELISA studies (see Section 2.5), total protein quantification according to Bradford [26], and Western blot analysis (see Section 2.6).

2.5. ELISA quantification of vitellogenin

An aliquot accounting for 400–500 ng of total soluble proteins from the extract of fat body tissues was used. It was dissolved in carbonate buffer (0.05 M, pH 9.6) and absorbed to ELISA microplates by incubation overnight at room temperature, with gentle shaking. To analyze the incubation medium, appropriate aliquots of medium were dissolved in fresh Grace's medium and absorbed to microplate wells at 4°C overnight according to Martin et al. [21]. The subsequent steps, namely incubation with antibodies and revealing procedures, were as previously described [20].

2.6. Western blot analysis of vitellogenin

SDS-PAGE and ECL Western blotting of vitellogenin was carried out as described [21]. Aliquots of two homogenized and pooled periovaric fat bodies were applied onto a 7.5% SDS-PAGE gel and protein bands were transferred to a nitrocellulose membrane. After the transfer, proteins were visualized by Ponceau S stain and the molecular weight standard proteins were located and marked. The polyclonal antibody against vitellogenin was the same as used in the ELISA, and here was applied at a dilution of 1:15 000. It binds equally well to glycosylated and non-glycosylated vitellogenin [20]. ECL Western blotting (from Amersham) was used following the manufacturer's protocol. Chemiluminescent immunoblots were exposed to X-ray film for 5 min. Bands corresponding to pre-pro-vitellogenin and pro- vitellogenin were identified according to Martin et al. [21], Purcell et al. [27] and Wojchowski et al. [23]. Four replicates were obtained for each Western blot analysis.

3. Results

3.1. Effects of allatostatin on vitellogenin production

Since vitellogenin production shows a decline after day 4 [21], and maximal concentration of haemolymph allatostatins during the first vitellogenic cycle of *B. germanica* is around 10^{-6} M (unpublished), we used periovaric fat bodies from 4-day-old females, and doses of Blast-2 comprised between 10^{-5} M and 10^{-7} M, in three different periods of incubation (3, 5 or 7 h).

Results show that inhibition of vitellogenin release to the medium (Fig. 1a) was dose- and time-dependent. However, the differences between the three doses are best observed after 7 h of incubation, when the percentages of inhibition were, on average, 73% for 10^{-5} M, 57% for 10^{-6} M and 51% for 10^{-7} M. Conversely, vitellogenin content in the incubated periovaric fat body was not significantly affected by any treatment (Fig. 1b).

3.2. Western blotting analysis of vitellogenin

In order to study the effect of Blast-2 on the vitellogenin pattern in the incubated periovaric fat body, SDS-PAGE and ECL western blot analysis were used. Therefore, periovaric fat bodies which had been incubated for 1, 3, 5 and 7 h in control medium or in the presence of 10^{-5} M of Blast-2 were compared. SDS-PAGE showed that protein pattern was qualitatively similar in treated tissues and controls (not shown). However, the ECL western blot (Fig. 2) showed that the vitellogenin pattern was slightly different in treated

tissues and in controls. In both cases the antibody revealed the band of 215 kDa corresponding to pre-pro-vitellogenin, and that of 240 kDa corresponding to the glycosylated form (pro-vitellogenin), but the latter dissapeared progressively during incubation in treated fat bodies, and was practically undetectable after 7 h of incubation (Fig. 2).

3.3. Inhibition of vitellogenin production by fluvastatin

ECL western blot results suggested that Blast-2 may inhibit glycosylation of vitellogenin, thus preventing its release from the fat body. This led us to postulate that Blast-2 could act on the mevalonate pathway, inhibiting the synthesis of dolichol, an intermediate necessary for vitellogenin glycosylation.

Following this reasoning, and in order to afford additional evidence in support of a functional link between the mevalonate pathway and vitellogenin production, we tested the effect of fluvastatin on the periovaric fat body incubated in vitro for 3 h. Results (Fig. 3) showed that fluvastatin inhibited the release of vitellogenin to the medium. An average inhibition of 45–50% was obtained at concentrations of 10^{-6}–10^{-5} M. However, no effect was observed in vitellogenin content in the incubated fat body (data not shown).

3.4. Effect of mevalonolactone

The results of the above experiments suggested that Blast-2 inhibition of vitellogenin release might be due to an inhibitory action on the mevalonate pathway. According to this hypothesis, we tested whether mevalonolactone would correct the inhibitory effects induced by Blast-2. Therefore, we incubated periovaric fat bodies from 4-day-old females for 5 h in medium treated with the most effective dose of Blast-2 (10^{-5} M) plus mevalonolactone at a dose of 5×10^{-3} M. The dose of mevalonolactone was chosen according to the results describing the restoring action of this compound on the synthesis of juvenile hormone in corpora allata of *B. germanica* treated with an HMGCoA reductase inhibitor [25]. The results (Fig. 4) showed that this treatment was able to restore the levels of vitellogenin release found in controls.

4. Discussion

The results have shown that Blast-2 inhibits vitellogenin release in vitro by periovaric fat bodies from 4-day-old females of *B. germanica*, an age which precedes the decline of vitellogenin production in the reproductive cycle of this species [20,21].

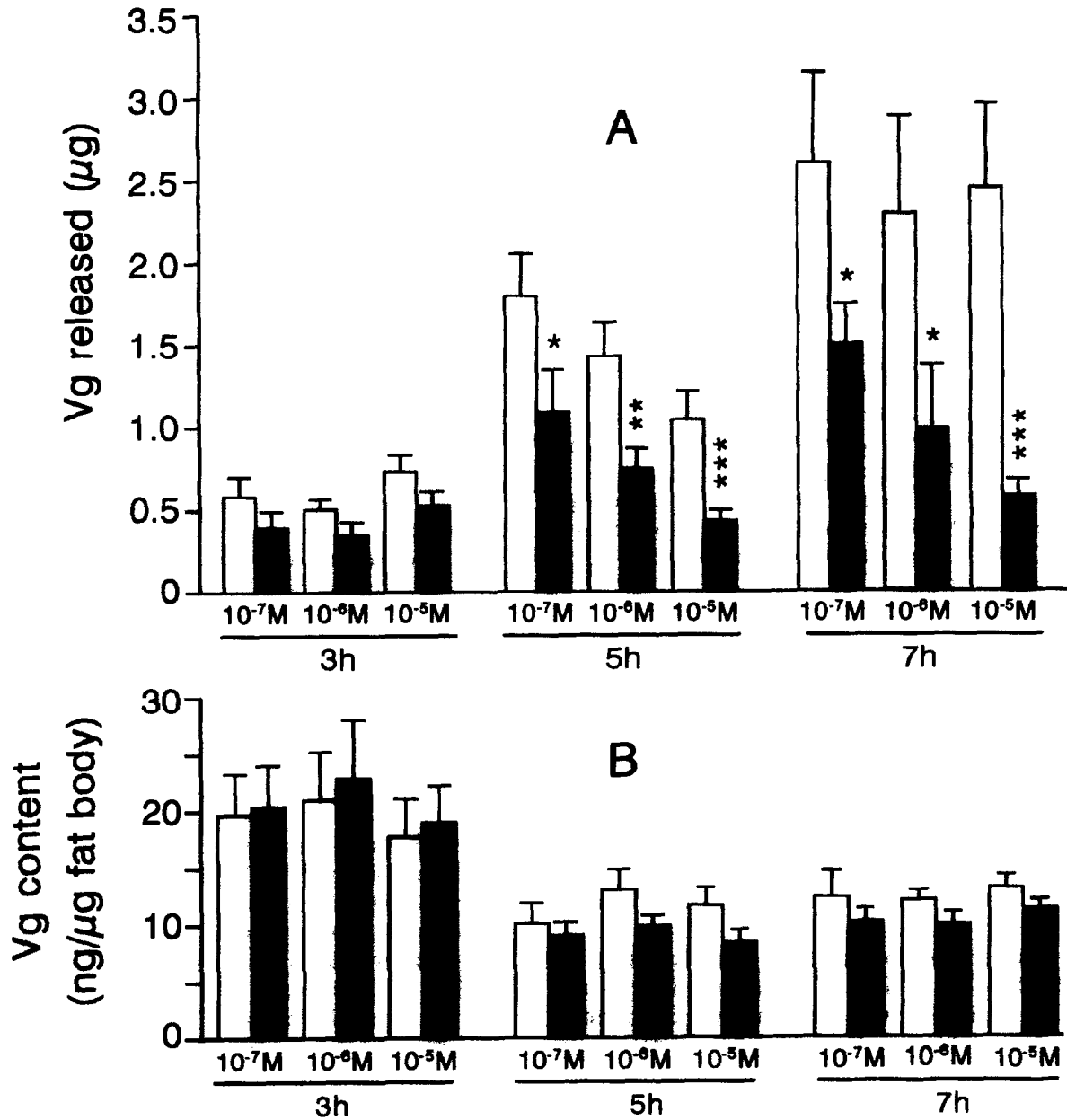Furthermore, results from ECL western blot analysis showed that intracellular glycosylated vitellogenin (pro-

Fig. 1. (A) Vitellogenin (Vg) released by periovaric fat bodies from 4-day-old females of *B. germanica* incubated for 3, 5 or 7 h in the presence of Blast-2 (10^{-5}, 10^{-6} and 10^{-7} M). (B) Vg content in the periovaric fat bodies described in (A). White columns, controls; black columns, treated. All values are expressed as the mean ± SEM ($n = 7-9$). Significance of differences (*t*-test) between treated and the respective controls are represented by asterisks (*$P \leq 0.1$; **$P \leq 0.05$; ***$P \leq 0.005$).

vitellogenin) disappeared progressively during incubation in treated fat bodies, thus suggesting that Blast-2 may have inhibited glycosylation. Since non-glycosylated vitellogenin (pre-pro-vitellogenin) cannot be exported from the fat body, this would explain the inhibition of vitellogenin release described above. In the context of this hypothesis, the fact that intracellular accumulation of pre-pro-vitellogenin was not apparent in Blast-2-treated fat bodies suggests that inhibition of release inhibited, in turn, the synthesis of this precursor. Concerning the ECL western blot analysis of control

fat bodies, it is also worth noting that the decrease in intensity of the pro-vitellogenin band in the 3 h lane corresponds with the drop in vitellogenin titre observed within the first 3 h of incubation in vitro [21].

The above results led us to hypothesize that Blast-2 might inhibit the synthesis of dolichol, an intermediate necessary to vitellogenin glycosylation, and in this line we tested the effect of fluvastatin on the periovaric fat body incubated in vitro. Results showed that this HMGCoA reductase inhibitor, which inhibits insect juvenile hormone biosynthesis [24], reduced the release
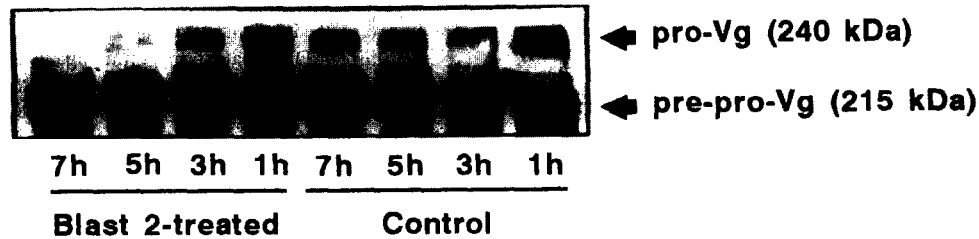
Fig. 2. Immunodetection on ECL western-blots of pre-provitellogenin (pre-pro-Vg, 215 kDa), and pro-vitellogenin (pro-Vg, 240 kDa) in periovaric fat body from 4-day-old-females of B. germanica incubated for 1, 3, 5 and 7 h in control medium or in medium added with 10^{-5} M of Blast-2.

of vitellogenin. This result is also interesting from a more general point of view, since it provides new and consistent evidence on a functional link between the mevalonate pathway and vitellogenesis.

Also under the hypothesis that the action of Blast-2 on vitellogenin release might be due to an inhibitory action on the mevalonate pathway, we showed that mevalonolactone, added at a usual dose for reversibility tests in B. germanica [25], was able to restore the normal levels of vitellogenin release in Blast-2-treated periovaric fat bodies.

These results taken together led us to postulate that allatostatins contribute to the termination of the vitellogenic cycle in the cockroach B. germanica. The fact that allatostatins circulate in the haemolymph ([16], unpublished), and the occurrence of proteins apparently involved in allatostatin recognition in insect fat body [17], are in agreement with this hypothesis. The relatively modest inhibitory activity observed in the experiments suggest that this system does not act alone, but in conjunction with others in the second half of the vitellogenic cycle. The study of these other systems is currently in progress in our laboratory.

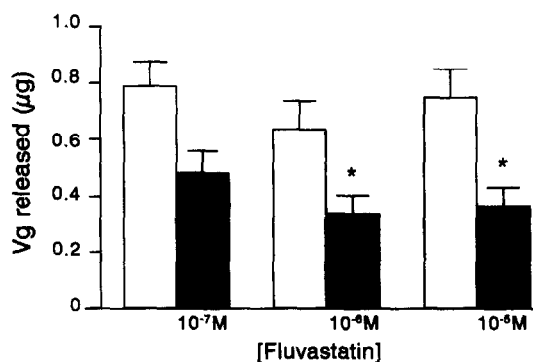The mechanism of action of allatostatin on the mevalonate pathway remains an open question. It seems, however, that the peptide acts on some biosynthetic step prior to mevalonate formation, as mevalonolactone was shown to counteract the inhibitory action of Blast-2. Indeed, the mechanism of action might be the same as that operating in the inhibition of juvenile hormone in the corpora allata, which remains unsolved. If this were true, then the fat body could become a better model than the corpora allata to study the action of allatostatins upon the mevalonate pathway, since its larger tissue mass would facilitate the experimental work in many ways, for example in the analysis at molecular level.

Acknowledgements

Fig. 3. Effect of different doses of fluvastatin (10^{-5}, 10^{-6} and 10^{-7} M) on vitellogenin (Vg) release by periovaric fat bodies from 4-day-old females of B. germanica incubated in vitro for 3 h. White columns: control, black columns: treated. All values are expressed as the mean $\pm$ SEM ($n = 6-8$). The asterisk indicates that the differences between treated and the respective controls are significant (t-test, $P \leq 0.05$).
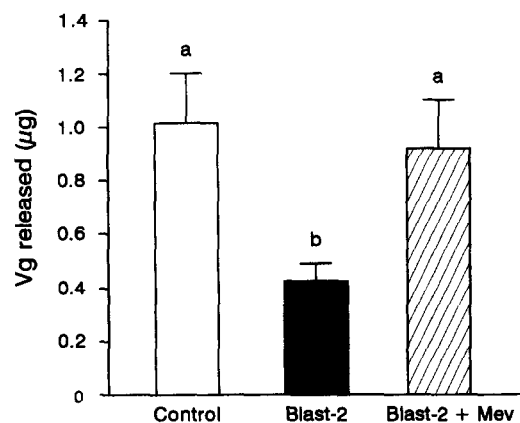

Fig. 4. Counteracting effect of mevalonolactone (Mev) upon the inhibitory effects of Blast-2 on vitellogenin (Vg) release. Periovaric fat bodies from 4-day-old females of B. germanica were incubated for 5 h in control medium (white column), in medium added with 10^{-5} M of Blast-2 (black column) or in medium added with 10^{-5} M of Blast-2 plus mevalonolactone (5×10^{-3}M) (striped column). All values are expressed as the mean $\pm$ SEM ($n = 7$). Different letters above the column indicate significant differences (t-test, $P \leq 0.005$).

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