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Phosphoramidon enhances allatostatin-mediated inhibition of juvenile hormone biosynthesis in the corpora allata of the cockroach, *Diploptera punctata*

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Abstract. Use of the enkephalinase inhibitor phosphoramidon in the in vitro radiochemical assay for juvenile hormone biosynthesis enhanced allatostatin-mediated inhibition of hormone production by corpora allata of the cockroach, *Diploptera punctata*. Significant increases in inhibition in day 2 virgin female CA by AST 1 (at 10^{-7}M) and AST 4 (10^{-8}-10^{-7}M) were observed in the presence of phosphoramidon (10^{-5}M or greater). No significant increases in inhibition were seen in CA from day 6 mated females with AST 4 (10^{-9}-10^{-7}M) and phosphoramidon combined. Phosphoramidon alone had no effect on JH biosynthesis. Analysis of allatostatin content of the CA, as determined by ELISA, revealed that addition of phosphoramidon to the medium increased the endogenous allatostatin content in CA of virgin and mated females. The similarity in primary structure between allatostatins and enkephalin-like peptides and their similar distribution makes it probable that phosphoramidon acts by preventing breakdown of allatostatins within the CA.
Key words. Corpora allata; juvenile hormone biosynthesis; allatostatins; phosphoramidon; enkephalinase; cockroach; *Diploptera punctata*; ELISA.

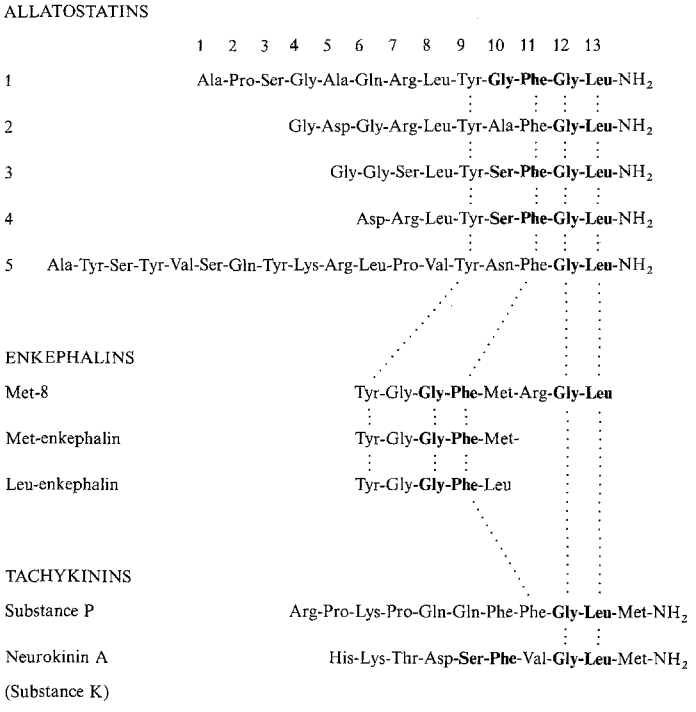
Allatostatins (ASTs), isolated from brains of the cockroach *Diploptera punctata*, are potent inhibitors of juvenile hormone (JH) biosynthesis by the corpora allata (CA) in vitro[2-5]. These peptides are probably important in the regulation of JH production by the CA and as such, may be involved in the regulation of reproduction in the adult female cockroach. AST 1 has been the most extensively studied with regard to its physiological effects. Sensitivity of CA to AST 1 varies during the first reproductive cycle of the adult female, but the greatest inhibition of in vitro rates of JH biosynthesis by AST 1 occurs in CA from day 2 virgin and day 6 mated females[3,5]. However, the inhibitory effect is neither stage- nor species-specific, and both larval CA and those of mated *Periplaneta americana* females are also inhibited to some degree[2,3].

Allatostatins are neuropeptides of up to 18 amino acids[2,4] and have amino acid sequences that show some similarity to the vertebrate enkephalin-related peptide Met-enkephalin-Arg[6]-Gly[7]-Leu[8] (met-8). Brains, CA and corpora cardiaca (CC) of *D. punctata* contain cells which show immunoreactivity to met-8 antiserum; these cells may contain allatostatin-like substances with cross-reactivity to the antibody[6]. Met-8 (**Tyr-Gly**-Gly-**Phe**-Met-Arg-**Gly-Leu**) has 4 or 5 amino acids in similar sequence, with deletions, to the C-terminus of allatostatin 1 (-Tyr-Gly-Phe-Gly-Leu-NH_2). Therefore, substances influencing the metabolism of met-8 or the enkephalins may be expected to exert similar effects on the metabolism of the allatostatins.

Enkephalins and related peptides are subject to enzymatic degradation by enkephalinases. Sequence similarity between the allatostatins and the enkephalins raises the possibility that they are also cleaved by enkephalinase-like enzymes that may be present in the brain, CA or CC. N-[α-L-rhamnopyranosyloxyhydroxyphosphinyl]-L-leucyl-L-tryptophan (phosphoramidon) is a specific inhibitor of enkephalinase (E.C. 3.4.24.11, endopeptidase-24.11) enzymatic activity[7]. Since the primary structures of the allatostatins and enkephalins are similar, phosphoramidon may also protect the allatostatins from degradation (table 1). It would therefore have an enhancing effect on allatostatin-mediated inhibition of juvenile hormone biosynthesis in the cockroach CA. Our experiments were designed to test this possibility and to determine if phosphoramidon could be used to improve the effectiveness of our assay for inhibition of JH biosynthesis.

In this regard, we have tested the effect of either substitutions in the sequence or truncations in the length of the

Table 1. Amino acid sequences of allatostatins and related peptides. Boldface type represents potential cleavage sites of enkephalinase. Sequence similarities are shown by the connecting lines.



allatostatins on the degree of inhibition of JH biosynthesis in the presence of phosphoramidon. Woodhead et al.[2] and Stay et al.[8] have previously tested some of these substances in the absence of phosphoramidon. Our study provides additional information on the biological activity of the AST molecule in relation to its primary structure.

Materials and methods

The in vitro radiochemical assay of Tobe and Pratt[9], as adapted by Feyereisen and Tobe[10] was used to measure JH release by the CA. CA from female *D. punctata* of known age and mating status were incubated for 3 h in medium 199 (1.3 mM Ca^{2+}, 2% Ficoll, methionine-free), in the presence of L-[methyl-^{14}C]-methionine (50 µM, spec. act. 1.48–2.03 GBq/mmol, New England Nuclear or Amersham). Since previous studies with allatostatins had shown that virgin females (day 2) produce the most consistent and least variable results[3], these were used for the majority of the experiments. Mated females (day 6) were also used for experiments with allatostatin 4, since their CA are also very sensitive at this time[3,5]. Phosphoramidon and allatostatins dissolved in H_2O were mixed prior to incubation and added to the medium at the appropriate concentrations. Following incubation, the glands were removed, the medium was extracted and the radioactivity determined by liquid scintillation spectrometry. The results represent the mean and standard error of each group. Student's t-test was used to determine the statistical significance between groups.

Allatostatin content within the glands was determined using an ELISA procedure with antibody specific to AST 1[11]. CA from virgin (day 2) and mated (day 6) females (4–5 pair per tube) were incubated for 3 h in medium 199 alone or with addition of 10^{-4}M phosphoramidon. The glands were then transferred into 50 µl of saline and the AST content determined. The incubation medium was also assayed for AST released from the CA.

Results

Enhancement of inhibition of juvenile hormone biosynthesis. Phosphoramidon, in the presence of allatostatins, increased the inhibitory effect of the peptides on in vitro JH release by the CA. Phosphoramidon alone at concentrations from 10^{-6} to 10^{-2}M had no effect on JH release in virgin females (fig. 1). Phosphoramidon at concentrations of 10^{-5}M or greater, in the presence of AST 4 (10^{-7}M), significantly enhanced inhibition of JH release in virgin females (30.7–50.9%, $p \leq 0.01$) by the allatostatin (fig. 1). At 10^{-4}M phosphoramidon, inhibition of JH biosynthesis in virgin female CA by AST 4 (10^{-8}-10^{-7}M) was also significantly enhanced (26.7–45.2%, $p \leq 0.04$), but not at 10^{-9}M AST 4 (fig. 2, top).

Mated females (day 6) showed differences from 29.9–67.4% in AST 4-mediated inhibition (10^{-9}-10^{-7}M) with the addition of 10^{-4}M phosphoramidon, but the increases were not statistically significant, as a consequence of the large variability in rates of JH biosynthesis observed at this age (fig. 2, bottom). Phosphoramidon alone (10^{-4}M) also enhanced the inhibition (35%), but again the differences were not significant. Treatment of

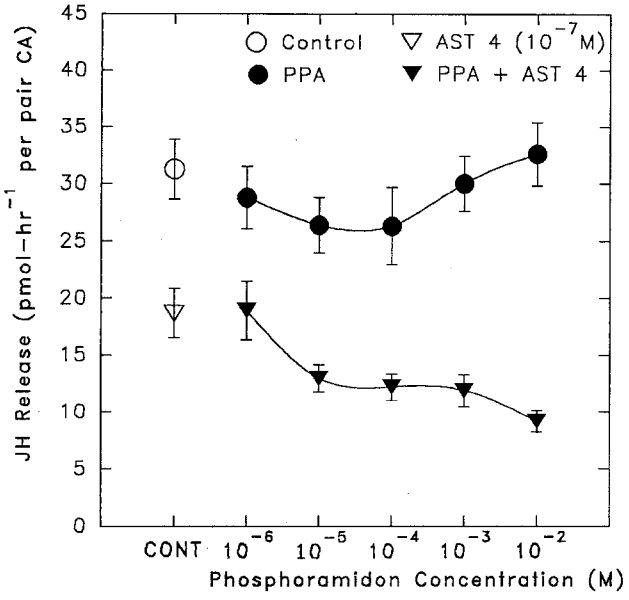


Figure 1. Effect of phosphoramidon (PPA) on JH release by CA of day 2 virgin *D. punctata*. Phosphoramidon at concentrations from 10^{-6}-10^{-2}M did not significantly affect release relative to the untreated controls. However, phosphoramidon at 10^{-5}M or greater significantly enhanced ($p \leq 0.01$) the inhibitory effect of AST 4 (10^{-7}M). Each point represents the mean ± SEM ($n \geq 19$).

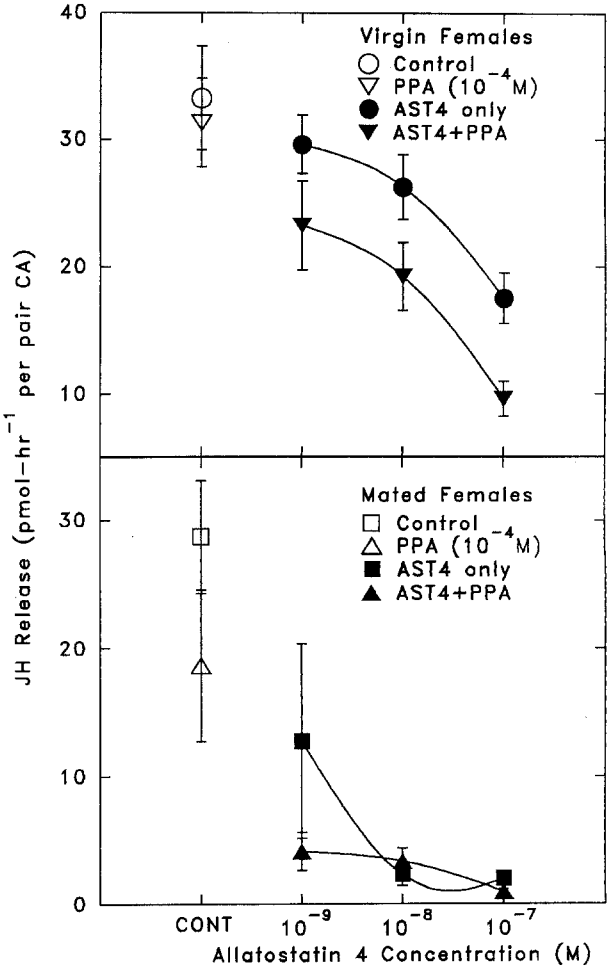


Figure 2. Inhibition of JH release by allatostatin 4 in CA of day 2 virgin (top) and day 6 mated (bottom) *D. punctata* with or without phosphoramidon (PPA, 10^{-4}M). Significant enhancement of inhibition in virgin female CA at 10^{-8} and 10^{-7}M was observed in the presence of phosphoramidon ($p \leq 0.04$). No significant increases in AST 4-mediated inhibition were seen in virgin female CA at 10^{-9}M or in mated female CA at 10^{-9}-10^{-7}M with phosphoramidon added. Phosphoramidon itself did not increase inhibition in both mated or virgin animals. Each point represents the mean ± SEM ($n = 10$).

day 2 virgin female CA with AST 1 (fig. 3), a more potent peptide inhibitor of JH biosynthesis, at a concentration of 10^{-7}M, also revealed a significant increase in inhibition (27.9%, $p = 0.01$) in the presence of 10^{-4}M phosphoramidon. However, no significant increases in inhibition were observed following treatment with 10^{-9} or 10^{-8}M AST 1 with phosphoramidon.

Effect of phosphoramidon on the AST content of the CC-CA. In vitro incubation of CC-CA from day 2 virgin and day 6 mated females with 10^{-4}M phosphoramidon increased the endogenous content of ASTs as determined by ELISA (table 2). The AST content within the glands of mated females treated with phosphoramidon in the medium was 0.31 and 0.29 ng/pair vs 0.22 and 0.18 ng/pair without phosphoramidon, in two separate trials. Similarly, the AST content of day 2 virgin CC-CA was dramatically enhanced by phosphoramidon treatment: 0.38 and 0.36 ng/pair relative to control values of 0.24 and 0.21 ng/pair in two separate trials. The release of AST from the CA into the control and phosphoramidon-treated medium was also assayed, but the quantity released was below the limits of detection of the assay.

Discussion

Enkephalins and related peptides are subject to enzymatic degradation by enkephalinases. Enkephalinase (E.C. 3.4.24.11, endopeptidase-24.11) hydrolyses enkephalins and related peptides[12] (refer to table 1) at the Gly^3-Phe^4 bond and tachykinins, e.g. substance P, at Gly^9-Leu^{10}. Allatostatin 1 contains both bonds in its C-terminal sequence (-Gly^{10}-Phe^{11}-Gly^{12}-Leu-^{13}-NH_2). In allatostatins 2, 3 and 4, Ala or Ser are substituted for Gly at the 7, 6 and 5 positions (4th residue from the carboxyl terminus). Martins et al.[13] found hydrolysis of neurokinin-A by endopeptidase-24.11 at the Ser^5-Phe^6 and Gly^8-Leu^9 bonds. AST 3 and 4 share a similar sequence at the C-terminus (-Ser-Phe-Gly-Leu-NH_2).

The sequence similarity to the enkephalins and the other peptides noted above and the co-occurrence of allatostatin immunoreactivity with met-8 immunoreactivity[6] makes it probable that allatostatins are also susceptible to attack by enkephalinase-like enzymes which may be present in the brain, CA or CC. Phosphoramidon is able to enhance allatostatin-mediated inhibition of juvenile hormone biosynthesis by cockroach CA. It therefore appears that such enzymes are present within the CA and are inhibited by phosphoramidon. This suggestion is also

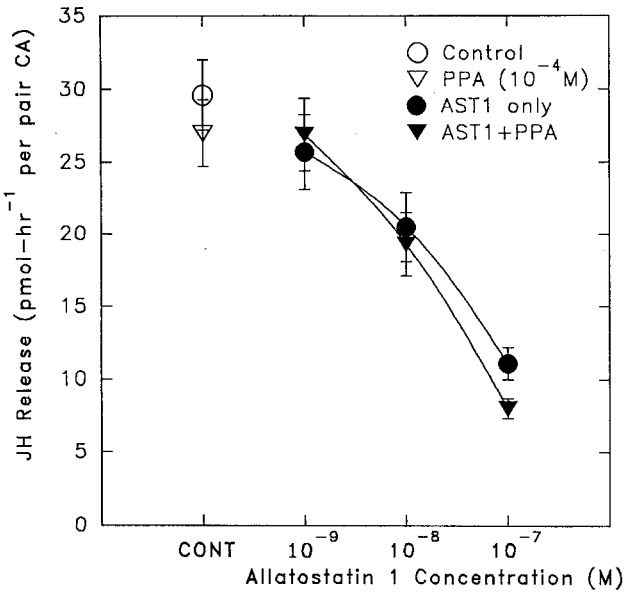


Figure. 3 Inhibition of JH biosynthesis by allatostatin 1 in CA of day 2 virgin *D. punctata* with or without phosphoramidon (PPA, 10^{-4}M). Significant enhancement of inhibition was observed only at 10^{-7}M ($p = 0.01$). Each point represents the mean ± SEM ($n \geq 17$).

Table 2. AST content of the corpora allata of female *D. punctata* as determined by ELISA. Corpora allata were incubated with or without phosphoramidon (100 μM) for 6 h in medium 199 and the AST content of CA and that released into the medium was determined.

	AST content (ng/pair)	
	Control	Phosphoramidon-treated
Mated day 6		
Trial 1	0.22 (4)*	0.31 (4)
Trial 2	0.18 (5)	0.29 (5)
Virgin day 2		
Trial 1	0.24 (5)	0.38 (5)
Trial 2	0.21 (5)	0.36 (5)

Note: Measurements of AST release into the medium were below the lower limit of the standard curve. *n in parentheses = number of pairs of CA per group.

supported by the results of the ELISA that show an increase in AST content in glands incubated in medium containing phosphoramidon (table 2).

Phosphoramidon may be used to improve the sensitivity of assays for AST-like peptides that inhibit JH biosynthesis because it may prevent the breakdown of the peptides by enkephalinase-like enzymes. Addition of phosphoramidon enhanced the inhibitory effect of AST 4 in virgin females to a level approximately the same as AST 1 (compare fig. 2, top vs fig. 3, 10^{-7}M). However, AST 1 is less affected by addition of phosphoramidon, possibly due to its longer length; the longer length of AST 1 may prevent the enkephalinase from attacking it effectively. Interestingly, AST 5 is longer than AST 1, with a slightly different C-terminal sequence (-**Tyr**-Asn-**Phe-Gly-Leu**-NH_2), and exhibits a level of inhibition twice that of AST 1[4]. AST 5 in the presence of phosphoramidon was not tested in these experiments.

Phosphoramidon itself does not appear to enhance inhibition of JH biosynthesis by the endogenous allatostatins present within the CA. This is surprising since phosphoramidon treatment should elevate the endogenous reserve of allatostatins. In day 6 mated females, there was an apparent increase (35%) in inhibition with phosphoramidon alone, but because rates of JH biosynthesis in animals of this age show high variability (JH biosynthesis is declining rapidly at this time), the results are not significant. Virgin females also showed a negligible increase in inhibition with addition of phosphoramidon. However, the amount of allatostatin present may be insufficient to effect significant inhibition over the 3 h incubation period. ELISA studies indicate that the content of allatostatin in brains of virgin and young mated females is low compared to that of day 5 mated females[11]. Inhibition of JH biosynthesis may require constant release of allatostatin from the brain into the CA by way of nerves or haemolymph. Allatostatin-immunoreactive material has been detected in *D. punctata* haemolymph using ELISA[11].

Routine use of phosphoramidon (10^{-4}M) in assays testing the effects of substitutions or truncation of the AST molecule on JH biosynthesis have now been instituted (X. C. Guan, T. Hayes and S. S. Tobe, unpublished). These experiments, used in conjunction with the ELISA, will further our understanding of the biological activity of the allatostatins and related peptides with respect to their primary structure as well as mechanisms of degradation of these important peptides.

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