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IN VITRO INHIBITION OF JUVENILE HORMONE III BIOSYNTHESIS BY PRECOCENE II AND 3,4-DIHYDROPRECOCENE II ON *BLATTELLA GERMANICA*

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Abstract—*In vitro* incubations of corpora allata from 6-day old females of *B. germanica* with precocene II showed that the glands are intrinsically sensitive to the action of this allatocidin. However, irreversible cytotoxic effects on corpora allata were only obtained after 9 h of incubation at 10^{-3} M. Measurement of intraglandular juvenile hormone III and methyl farnesoate contents of treated and control corpora allata showed a depletion of juvenile hormone III contents which was parallel to the inhibition of juvenile hormone release. Conversely, methyl farnesoate levels of treated glands were not different from those found in controls which suggested that a specific inhibition of the epoxidase did occur in addition to the general inactivation of the glands. Finally, results from incubations with 3,4-dihydroprecocene II showed that this compound is also a juvenile hormone inhibitor and the effects elicited are comparable with those observed for precocene II.

Key Word Index: *Blattella germanica*, Juvenile Hormone III, corpora allata, precocenes

INTRODUCTION

In a preceding paper (Bellés *et al.*, 1985) we reported the antigonadotropic effects of topically applied precocenes on freshly ecdysed virgin females of *Blattella germanica*. Our results showed that these compounds inhibited oöcyte growth and colleterial gland development, although these effects appeared to be reversible in most cases.

In order to assess the intrinsic sensitivity of the corpora allata of *B. germanica* to precocenes, we have studied the inhibitory activity of precocene II on the *in vitro* juvenile hormone III release using the radiochemical assay developed by Pratt and Tobe (1974) and further adapted to the case of *B. germanica* by Bellés *et al.* (1987). For this purpose, we used an experimental procedure comprising a variable period of incubation in the presence of the test compound, which is preceded and followed by fixed periods of incubation in fresh medium. This method allows the study of the inhibition of juvenile hormone release and the degree of reversibility of such effect in individual assays.

In addition, complementary experiments using 3,4-dihydroprecocene II are also reported. Although this derivative lacks the double bond which has been claimed to be crucial for precocene activity (Pratt *et al.*, 1980; Soderlund *et al.*, 1980), it also induced *in vivo* antigonadotropic effects on *B. germanica* (Bellés and Messeguer, 1981).

Finally, with the aim of clarifying the relationship between biosynthesis and release of juvenile hormone (see Feyereisen, 1985) for this species within our experimental conditions, juvenile hormone III and methyl farnesoate contents of control and precocene II-treated corpora allata have also been measured.

MATERIALS AND METHODS

Insects

Adults of *B. germanica* were reared at 26(±1)°C as described elsewhere (Bellés and Piulachs, 1983). Freshly ecdysed virgin females isolated from the colony were used at appropriate ages which were rigorously determined using the basal oöcyte length as the main criterion (Bellés *et al.*, 1987).

Compounds and analytical techniques

Chemicals and solvents were all of analytical grade and used as received. All glassware was coated with 1% of 201 silicone emulsion (Siliconas Hispania, Barcelona). Juvenile hormone III was obtained from Sigma. Methyl farnesoate, precocene II, 3,4-dihydroprecocene II were prepared in our laboratory following previously reported procedures (Liedtke and Djerassi, 1972; Camps *et al.*, 1980 and Camps *et al.*, 1979, respectively). [10-^{3}H(*N*)]-juvenile hormone III (11 Ci/mmol) was obtained from New England Nuclear and [methyl-^{3}H]methionine (80–85 Ci/mmol) from Amersham.

Thin-layer chromatography analyses were performed on Merck precoated Silicagel plates (aluminium sheets, layer thickness 0.2 mm with concentrating zone). High-performance liquid chromatography (HPLC) analyses were carried out with a Waters system provided by two model 510 pumps, an automated gradient controller, a data model and

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a 481 u.v. detector. Radioactivity was measured with a LKB 1217 Rackbeta scintillation counter.

In vitro *incubation methods*

In all experiments glands from 6-day old females were used. Corpora allata–corpora cardiaca complexes were incubated in Millipore-filtered TC-199 medium (0.2 ml) containing L-methionine (0.1 mM), glutamine, Hank's Salts, HEPES medium buffer (20 mM) plus Ficoll (20 mg/ml) and to which radioactive methionine (2 μCi) was added to achieve a final sp. act. of 100 mCi/mmol. Dissection and transfer of the corpora allata–corpora cardiaca complexes to the incubation medium, measurement of basal oöcytes for determination of the physiological age of each specimen, incubations and radiochemical assay for juvenile hormone III quantification were performed as previously described (Bellés *et al.*, 1987). According to the designed experimental model, after an initial 3 h period of incubation, glands were transferred to fresh medium which contained a 0.1 μl ethanolic solution of the test compound at the desired concentration and incubation was pursued for 3, 6 or 9 h. Finally a post-treatment incubation was carried out by transferring the glands to fresh medium. In all cases, control incubations with medium containing 0.1 μl of ethanol were performed.

Quantification of juvenile hormone III and methyl farnesoate intraglandular contents

Corpora allata–corpora cardiaca complexes from 6-day old females were incubated for 3 h in fresh medium followed by 3 h in medium containing either 0.1 μl of ethanol (control experiments) or 0.1 μl of a 10^{-3} M solution of precocene II in ethanol. When incubation was completed, the glands were taken out from the medium, rinsed thoroughly (3 × 100 μl of fresh medium), transferred to a tube which contained methanol (200 μl) and sonicated (2 × 30 seg at 40 W using a Labsonic 1510 probe). After centrifugation (10 min at 1500 *g*, 4°C), the supernatant was collected and the pellet was washed with methanol (200 μl) and centrifuged under the same conditions. The joined supernatants were concentrated under nitrogen to a volume of aprox. 25 μl and injected onto the HPLC column. HPLC analyses were carried out with a 30 × 0.39 cm (i.d.) column packed with Spherisorb ODS-2 (10 μ, Tracer Analitica, Barcelona). Elution conditions were set as follows: 75:25 methanol–water (1 ml/min) for 12 min. From min 12 to 15 a gradient from 75:25 to 95:5 (1.5 ml/min) was programmed and final conditions were maintained up to min 19.5. Then the flow rate was reduced at 1 ml/min to allow elution of methyl farnesoate at the same flow rate as that used for juvenile hormone III. Under these conditions, retention times for juvenile hormone III and methyl farnesoate were 11.30 and 20.24 min, respectively. Eluates corresponding to 1-min fraction were collected and radioactivity measured with the scintillation counter. The limit of detection for standard radioactive juvenile hormone III was estimated in 0.03 pmol.

RESULTS

Effect of precocene II on the juvenile hormone III release

The inhibitory effects of precocene II on the *in vitro* juvenile hormone III release are summarized in Table 1. Comparison of the values obtained at the three different incubation periods (3, 6 or 9 h) assayed with the same concentration of precocene II (10^{-3} M) indicates that the degree of inhibition depends on time of incubation. Nevertheless, the results of the post-treatment period showed that the glands incubated for 3 or 6 h with 10^{-3} M of precocene II were able to recover almost completely their normal biosynthetic capacity. Conversely, the inhibitory effects induced after 9 h of incubation appeared to be irreversible as inferred from the comparison of the release values measured in the 12–24 h period of this

Table 1. Influence of incubation time with precocene II (P II) or 3,4-dihydroprecocene II (DHP II) on the juvenile hormone III (JH III) release *in vitro* by corpora allata from 6-day old females of *Blattella germanica*

			pmol HJ/period × pair CA				
Treatment	*N*	BO	0–3 h	3–6 h	6–9 h	9–12 h	12–24 h
Untreated	26	1.77 ± 0.03	7.95 ± 0.93	8.22 ± 0.81	8.37 ± 0.51	7.11 ± 0.30	14.52 ± 1.44
0.05% Ethanol (3 × 3 h)	37	1.76 ± 0.03	8.19 ± 0.93	**8.31 ± 0.69**	**8.79 ± 0.57**	**8.49 ± 0.69**$^{(0)}$	12.48 ± 1.92
10^{-3} M P II (1 × 3 h)	12	1.76 ± 0.03	6.96 ± 1.35	**2.49 ± 0.42**$^{(3)}$	5.67 ± 0.54$^{(0)}$	—	—
10^{-3} M P II (2 × 3 h)	8	1.83 ± 0.05	6.36 ± 0.99	**2.97 ± 0.84**	**1.92 ± 0.54**$^{(3)}$	4.92 ± 0.72$^{(2)}$	—
10^{-3} M P II (3 × 3 h)	12	1.81 ± 0.04	7.35 ± 1.59	**3.36 ± 0.57**	**2.55 ± 0.42**	**1.14 ± 0.30**$^{(3)}$	0.36 ± 0.12
10^{-4} M PII (3 × 3 h)	9	1.87 ± 0.04	9.81 ± 1.44	**7.65 ± 0.90**	**9.60 ± 1.14**	**7.68 ± 0.96**$^{(0)}$	15.48 ± 2.88
10^{-3} M DHP II (2 × 3 h)	5	1.79 ± 0.03	6.51 ± 0.75	**4.41 ± 0.24**	**2.70 ± 0.21**$^{(2)}$	7.68 ± 0.51$^{(1)}$	—
10^{-3} M DHP II (3 × 3 h)	13	1.80 ± 0.02	7.53 ± 1.05	**4.47 ± 0.45**	**3.00 ± 0.33**	**3.15 ± 0.15**$^{(3)}$	6.96 ± 1.32
10^{-4} M DHP II (3 × 3 h)	10	1.84 ± 0.03	7.50 ± 0.57	**5.34 ± 0.39**	**3.84 ± 0.45**	**3.96 ± 0.60**$^{(3)}$	3.60 ± 0.96
10^{-5} M DHP II (3 × 3 h)	7	1.76 ± 0.04	6.93 ± 0.96	**8.49 ± 1.47**	**7.41 ± 1.05**	**6.21 ± 0.87**$^{(0)}$	11.40 ± 0.96

Absolute values for each period are given as $\bar{X} \pm$ SEM and those corresponding to the incubation periods with the test compound are indicated in boldface. Results of the *t*-test (from the comparison of the values resulting after the treatment with those obtained in the pre-treatment period of the same experiment) are summarized with the following subindexes: 0 (N.S.); 1 ($P < 0.05$); 2 ($P < 0.01$); 3 ($P < 0.001$). BO: Length (mm) of basal oöcyte of the corpora allata donors.

experiment with those obtained in the same period for controls incubated with ethanol.

On the other hand, when concentration of precocene II was lowered to 10^{-4} M, incubations during a 9 h period did not induce inhibition effects.

Effect of 3,4-dihydroprecocene II on the juvenile hormone III release

The effects induced by 3,4-dihydroprecocene II are also depicted in Table 1. At 10^{-3} M the inhibition in this case was also dependent on the incubation period with the compound (6 or 9 h). However, the degree of inhibition elicited by this derivative was lower than that induced by precocene II (58 and 84% of inhibition as average, respectively, after 9 h of incubation). In addition, the results obtained in the post-treatment period of incubation showed the recovery of the biosynthetic capacity for the glands incubated for 6 h, whereas the recovery was partial and quite variable for those incubated for 9 h.

On the other hand, when concentration of 3,4-dihydroprecocene II was lowered to 10^{-4} M, the inhibitory activity attained after the 9-h incubation period was comparable to that obtained at 10^{-3} M under the same conditions. Nevertheless, at 10^{-5} M the compound did not elicit any significative inhibition.

Juvenile hormone III and methyl farnesoate contents in precocene-treated corpora allata

The results obtained on the influence of precocene II treatment on the intraglandular content of juvenile hormone III and methyl farnesoate are shown in Table 2. Concerning the pre-treatment period it should be noted that the strict selection of experimental animals using the basal oöcyte criteria, resulted in juvenile hormone release values which were not statistically different for glands subsequently used as controls or in the incubations with precocene II. It can also be observed that intraglandular contents of juvenile hormone III are clearly lower in the case of precocene II-treated corpora allata. Conversely, values of methyl farnesoate are not different in controls and in glands incubated with the test compound.

On the other hand, in control glands, juvenile hormone III contents in corpora allata are relatively high in comparison with release values, but the relationship between both parameters is not very different from those found in other Dictyoptera, as, for example, *Diploptera punctata* (Tobe and Stay, 1977) or *Periplaneta americana* (Weaver *et al.*, 1975). In addition, when juvenile hormone III release was plotted against juvenile hormone III synthesis (i.e. intraglandular content plus released hormone, see Tobe and Stay, 1977), the same linear correlation was observed either in controls or in precocene II incubated corpora allata (Fig. 1). This result indicates that precocene treatment did not affect the juvenile hormone release mechanisms. Moreover, the overall values can be fitted into the function $y = 0.914x + 0.008$ ($r = 0.998$), in which the ordinate at the origin is close to 0, thus suggesting that there is not intraglandular accumulation of juvenile hormone.

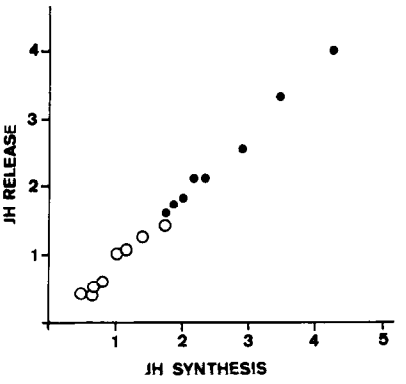

Fig. 1. Relationship between the rate of juvenile hormone III (JH III) synthesis and the rate of JH III release (pmol/h × pair corpora allata) by individual pairs of control (points) and precocene-incubated corpora allata (circles) from 6-day old females of *Blattella germanica*.

DISCUSSION

From the comparison of the results obtained with the incubations with precocene II and 3,4-dihydroprecocene II, it can be concluded that at 10^{-3} M and 3 or 6 h of incubation, precocene II shows a higher inhibitory activity on juvenile hormone III release (64 and 70% of inhibition as average, respectively) than 3,4-dihydroprecocene II for 6 h of incubation (58%, of inhibition as average). The use of the same dose through 9 h of incubation induced the highest inhibitory effects in the case of precocene II (84% as average), which were irreversible, while 3,4-dihydroprecocene II elicited a less pronounced (58% on average) and partially reversible inhibitory activity.

The incomplete recovery of the biosynthetic capability in glands treated with 3,4-dihydroprecocene II (10^{-3} M, 3 × 3 h) suggests the possible induction of cytotoxic effects on the corpora allata in a similar

Table 2. *In vitro* juvenile hormone (JH) III release by corpora allata (CA) from 6-day old females of *Blattella germanica* incubated with precocene II (P II) for 3 h and intraglandular contents of juvenile hormone III (JH III) and methyl farnesoate (MF)

			JH III release (pmol/h × pair CA)		Intraglandular content (pmol)	
Treatment	*N*	BO*	0–3 h	3–6 h	JH III	MF
10^{-3} M P II	8	1.81 ± 0.04	2.61 ± 0.33	**0.92 ± 0.13**	0.25 ± 0.06	0.05 ± 0.009
0.05% Ethanol	8	1.77 ± 0.05	2.45 ± 0.25	**2.42 ± 0.27**	0.60 ± 0.09	0.04 ± 0.008

*BO: Length (mm) of basal oöcyte of the corpora allata donors.

All values are expressed as $\bar{X} \pm$ SEM and those corresponding to juvenile hormone release during the incubation period with precocene or with ethanol are indicated in boldface.

manner to that elicited by precocene II on *B. germanica* (Piulachs and Bellés, 1985) and other Dictyoptera, like *Diploptera punctata* (Feyereisen *et al.*, 1981; see also Bowers, 1985, for review). In this context, preliminary observations on the ultrastructure of the glands used in the present experiments seem to confirm this cytotoxic action (Piulachs, Cassier and Bellés, unpublished).

The question arises whether 3,4-dihydroprecocene II could be bioactivated within the corpora allata to give a cytotoxic intermediate following a similar pattern to that postulated for precocenes. In this sense, results from different metabolism studies (Pratt *et al.*, 1980; Soderlund *et al.*, 1980; Hamnett and Pratt, 1983) have suggested that corpora allata monooxigenases convert precocenes into a reactive intermediate, generally formulated as a 3,4-epoxide, which would be the responsible for the observed cytotoxic effects.

In the case of 3,4-dihydroprecocene II, two different hypotheses might be anticipated. First, that bioactivation of 3,4-dihydroprecocene II would transform this compound into precocene II which would then exert the expected cytotoxic activity. However, this hypothesis is not plausible since desaturase activity has never been reported in the corpora allata.

The other alternative for explaining the cytotoxicity of 3,4-dihydroprecocene II takes into account the direct bioactivation of the compound by the same monooxigenase system of corpora allata. In fact, 3,4-dihydroprecocene II contains an activated benzylic position which can be oxidized (e.g. hydroxylated). Dehydration of this 3,4-dihydroprecocene II hydroxylated intermediate into precocene II and further recycle of the bioactivation process of precocene II appears unfeasible. Therefore, we consider that the cytotoxic effect could be initiated by radical species involved in the above oxidation. In addition, it is also possible that a process derived from second phase metabolism (see Caldwell, 1980), could give rise to intermediates capable of suffering covalent binding with nucleophilic sites of macromolecules, analogously to the pathway postulated for bioactivated precocenes.

On the other hand, results on intraglandular contents of juvenile hormone III indicated that there is not hormonal accumulation neither in corpora allata controls nor in corpora allata incubated with precocene. Depletion of juvenile hormone contents in treated corpora allata parallels the inhibitory effect measured as juvenile hormone release. Conversely, methyl farnesoate contents seem to be unaffected by precocene II. These facts suggest a possible inhibitory action of precocene II on juvenile hormone biosynthesis at level of the methyl farnesoate epoxidation. Since precocene II is presumed to undergo biotransformation into reactive intermediates—the putative allatocidins—through the action of the same corpora allata monooxigenase which epoxidizes methyl farnesoate to give juvenile hormone III, a competitive action of precocene II for these enzymes could be postulated which would result in a diminution in terms of juvenile hormone synthesis and a maintenance of methyl farnesoate levels, as observed in our experiments.

As a more general conclusion of the results herein described, a dual action of precocenes in corpora allata of *B. germanica* adult females could be inferred. First it can be postulated that, possibly as a consequence of some sort of bioactivation, precocenes exert an inhibition of juvenile hormone biosynthesis. However, this inhibition only leads to irreversible effects under very severe treatments (i.e. incubations at 10^{-3} M during 9 h). Concomitantly, it seems that a specific inhibition of the epoxidation of methyl farnesoate does also take place, analogously to those observed for conventional inhibitors of this biosynthetic step (Staal, 1986). This mechanism of action would be reversible and could be induced and evidenced through short-term incubations in the presence of precocenes. On the other hand, this specific inhibition would lead to the accumulation of intraglandular methyl farnesoate, with an overall effect which would counterbalance the expected depletion for this compound produced by the prime inhibition mechanism.

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