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Inhibition of insect juvenile hormone synthesis by phorbol 12-myristate 13-acetate

René Feyereisen and Dan E. Farnsworth

Departments of Entomology and Agricultural Chemistry, Oregon State University, Corvallis, OR 97331, USA

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The synthesis of insect juvenile hormone III (JH III) by isolated corpora allata of the cockroach *Diploptera punctata* incubated in vitro is inhibited by phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate and 1-oleyl-2-acetylglycerol. 4α-Phorbol 12,13-didecanoate and diolein are inactive. The inhibitory effect of phorbol 12-myristate 13-acetate is fully reversed by 2*E*,6*E*-farnesol or by 2*E*,6*E*-farnesoic acid. It is highest in corpora allata that are past their peak in secretory activity or that have been inhibited by injections of 20-hydroxyecdysone. This effect of phorbol esters implicates protein kinase C in the regulation of insect corpus allatum activity.

Juvenile hormone synthesis; Phorbol ester; Insect reproduction; *(Diploptera punctata)*

1. INTRODUCTION

The sesquiterpenoid juvenile hormone III (JH III) controls vitellogenesis in adult female cockroaches. The synthesis of JH III by the corpora allata is regulated by stimulatory and inhibitory factors [1]. The chemical identity and cellular transduction mechanisms of these external regulatory factors are largely unknown. In the viviparous cockroach, *Diploptera punctata*, an allatostatin originating in the brain reaches the corpora allata via nervous connections where it can be released experimentally in response to high-K^+ treatment [2]. This inhibitory peptide may utilize cAMP as second messenger, because it causes an elevation in cAMP levels in the corpora allata [2] and its effects can be mimicked by forskolin [3]. In addition to cAMP [3,4], calcium has been implicated as an intracellular regulator of JH III synthesis in *D. punctata* [5].

Correspondence address: R. Feyereisen, Departments of Entomology and Agricultural Chemistry, Oregon State University, Corvallis, OR 97331, USA

It is likely that several second messenger systems interact to account for the complex and precise regulation of JH III synthesis during reproduction. We therefore decided to explore the effects of phorbol 12-myristate 13-acetate (PMA) which is a phorbol ester activator of protein kinase C, an enzyme that is normally activated by diacylglycerol liberated by the receptor-mediated hydrolysis of inositol phospholipids [6,7]. We report here that PMA is a potent inhibitor of JH III synthesis, and that the changing pattern of sensitivity of the corpora allata to PMA suggests a role for protein kinase C in the decline of JH III synthesis that occurs at the end of oocyte development.

2. MATERIALS AND METHODS

2.1. *Insects*

Adult female *D. punctata* were reared as in [4]. Except when noted otherwise, all insects were taken 9 days after adult emergence and mating, i.e. shortly after oviposition of the first batch of eggs in the brood sac. Injections of 20-hydroxyecdysone were performed as described [8].

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2.2. *JH synthesis assay*

Isolated corpora allata were incubated in medium 199 (Gibco) with Hank's salts, L-glutamine, 25 mM Hepes at pH 7.2, 2% Ficoll (Sigma), and 50 μM L-methionine provided as [*methyl*-^{3}H]methionine (200 mCi/mmol; New England Nuclear). Rates of JH III release by glands from split pairs (control vs experimental) were measured after 3 h incubations by an isooctane partition assay as described [9]. Control and experimental assays with phorbol esters and lipids all contained 1% dimethyl sulfoxide.

2.3. *Chemicals*

All chemicals were from Sigma except 20-hydroxyecdysone, farnesol and farnesoic acid which were from Simes (Milan, Italy), Aldrich and Zoecon, respectively.

3. RESULTS

The phorbol esters PMA and phorbol 12,13-dibutyrate were tested on corpora allata from day 9 mated female *D. punctata*. Both compounds caused a dose-dependent inhibition of JH III synthesis (fig.1), with PMA being the most potent phorbol ester. Half-maximal effect was achieved at less than 1 nM PMA, and 85% inhibition of JH III synthesis was the maximal effect achieved at PMA concentrations ranging between 10 nM and 1 μM. The time course for the inhibition of JH III synthesis by 10 nM PMA showed that the rate of JH III synthesis declined after 30 min and was maximal after 2 h. 4α-Phorbol didecanoate which is inactive as a protein kinase C activator in other systems [7] did not affect JH III synthesis when tested at concentrations ranging from 10 nM to 10 μM. The synthetic diacylglycerol 1-oleyl-2-acetylglycerol inhibited JH III synthesis at 0.1 mM (6.0 ± 0.7 pmol JH III/h per gland, 38% inhibition), whereas diolein was inactive.

To show that the inhibition of JH III synthesis was not a non-specific toxic effect on the corpus allatum cells, glands were treated with 10 nM PMA in the presence of 0.2 mM 2*E*,6*E*-farnesol or 36 μM 2*E*,6*E*-farnesoic acid, two precursors of JH III which are known to stimulate the synthesis of JH III by serving as exogenous substrates in the biosynthetic pathway [1]. Table 1 shows that 10 nM PMA was not able to inhibit the activity of corpora allata incubated in the presence of either farnesol or farnesoic acid. PMA therefore did not inhibit biosynthetic steps between farnesol and JH III or the cellular availability of NAD^+, *S*-adenosylmethionine or NADPH, which are required cosubstrates for farnesol utilization. This strongly suggests that the target of the inhibition by PMA in spontaneously active glands is situated before the utilization of farnesol (presumably by farnesol dehydrogenase [11]).

PMA (0.1 nM–10 μM) was tested on corpora allata which are at the peak of their cycle of activity (day 5 mated females) to determine whether the sensitivity of the glands to the phorbol ester was

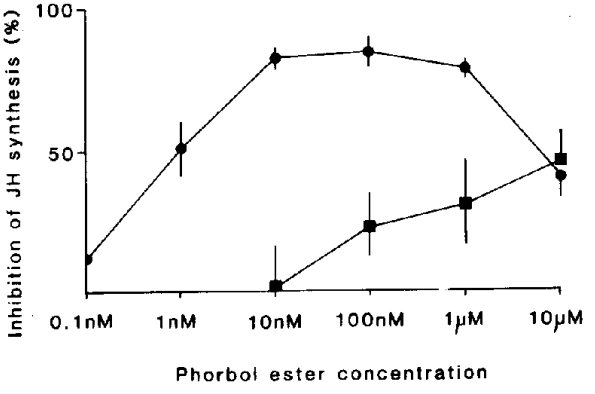

Fig.1. Effects of PMA (●) and phorbol 12,13-dibutyrate (■) on JH III synthesis by corpora allata from day 9 mated females of *D. punctata*. Each point is the mean ± SE of 7–14 assays.

Table 1

Effect of PMA on control (spontaneously active) and precursor-stimulated corpora allata from day 9 mated females

	JH III release rate (pmol/h per gland)
Control	10.0 ± 1.1
10 nM PMA	2.8 ± 0.4
0.2 mM farnesol	31.3 ± 2.3
0.2 mM farnesol + 10 nM PMA	31.3 ± 2.2
36 μM farnesoic acid	34.3 ± 2.1
36 μM farnesoic acid + 10 nM PMA	29.9 ± 1.8

Values are means ± SE of 8–17 assays

constant or dependent on the physiological state of the glands. Significant inhibition by PMA was observed at 10 nM, but the level of inhibition (26%) was considerably lower than that seen in day 9 mated females. The level of inhibition did not increase significantly at concentrations up to 10 μM. Thus, the potency of PMA was identical in corpora allata from day 5 and day 9 mated females, but the maximal effect was much lower in the active glands. To test the physiological importance of this finding further, we tested PMA on corpora allata from mated females at various stages of the gonotrophic cycle. Fig.2 shows that two distinct periods of sensitivity to PMA were observed. The first period, between day 0 (adult emergence and mating) and day 5 (the peak in secretory activity of the corpora allata), was characterized by a low sensitivity to PMA (20–40% inhibition). The second, starting on day 6 (when JH III secretory activity precipitously declines) and lasting at least until the middle of pregnancy, was characterized by a much higher sensitivity to PMA. Detailed monitoring of the physiological age of the insects used in these experiments showed that the switch from low to high sensitivity to PMA was not gradual between day 5 and 6, but was achieved abruptly in all insects with oocytes longer than 1.52 mm.

A premature decline in JH III synthesis can be forced in normal females by injections of 20-hydroxyecdysone [8], and ecdysteroids can mimick the effect of the ovarian inhibitory factor that turns down the activity of the corpora allata

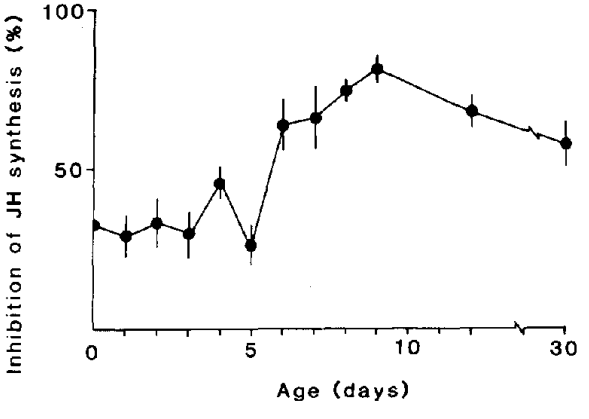

Fig.2. Effect of 10 nM PMA on JH III synthesis by corpora allata from mated females during a gonotrophic cycle. Each point is the mean ± SE of 8–17 assays.

Table 2

Effect of 20-hydroxyecdysone treatment on the sensitivity of corpora allata to PMA

	JH III release rate (pmol/h per gland)	Inhibition by 10 nM PMA (%)
Controls (n = 24)	37.1 ± 3.9	36.9 ± 4.1
Treated[a] (n = 24)	21.4 ± 2.1[b]	52.1 ± 5.2[c]

[a] 5 μg 20-hydroxyecdysone injected on day 3 and day 4. Glands were assayed on day 5. Values are means ± SE
[b] $p < 0.001$ vs controls
[c] $p < 0.015$ vs controls

[8,12]. Because the increase in sensitivity to PMA corresponded temporally with the inhibition of JH III synthesis by mature ovaries, we therefore investigated whether 20-hydroxyecdysone would cause a premature increase in PMA sensitivity of the corpora allata. Mated females from day 3 were injected with 5 μg 20-hydroxyecdysone, and the treatment was repeated on day 4. On day 5, the corpora allata were dissected and split between right and left glands. One member of the pair was assayed for spontaneous activity and the other for activity in the presence of 10 nM PMA. The 20-hydroxyecdysone treatment was effective in reducing JH III synthesis and oocyte growth, as reported previously [8]. Table 2 shows that corpora allata from 20-hydroxyecdysone-treated insects were more sensitive to PMA than the glands from control insects. This suggested that the switch in sensitivity of the glands to PMA is not merely related to the age of the glands, but reflects a physiological event, i.e. a decline in gland activity.

4. DISCUSSION

Hydrolysis of phosphatidylinositol 4,5-bisphosphate stimulated by a G-protein-coupled receptor activation generates two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. Diacylglycerol and Ca^{2+} activate protein kinase C which is responsible for specific protein phosphorylations [6,7,10]. Phorbol esters, potent in vivo and in vitro activators of protein kinase C, have become powerful tools for studying the role

of protein kinase C [7,10]. The inhibition of JH III synthesis by phorbol esters and by 1-oleyl-2-acetylglycerol (which activates protein kinase C without interaction with cell surface receptors [13]) is thus consistent with a postulated role of protein kinase C in the regulation of corpus allatum activity. The inhibition of JH III synthesis in *D. punctata* by the calcium ionophore A23187 [5] is also consistent with such a role. The potent activity of PMA, the lesser potency of phorbol 12,13-dibutyrate and the lack of activity of 4α-phorbol didecanoate observed here are in accordance with results obtained in other systems [7,10] including invertebrate endocrine systems [14]. The effect observed here is an inhibition of activity, whereas most responses to protein kinase C activation ultimately lead to the stimulation of some biological process [7].

The ligand responsible for the postulated receptor-mediated activation of polyphosphoinositide phosphodiesterase by a G-protein is still unknown, and it is desirable to explore the involvement of inositol phospholipids in corpus allatum regulation. The changing sensitivity of the corpora allata to PMA occurs precisely when the corpora allata rapidly decrease in activity in response to an ovarian inhibitory factor [12]. The identity of this factor, and the mechanism by which sensitivity to PMA is changed in the corpora allata (increase in protein kinase C levels, or appearance of an appropriate target for phosphorylation, [15]) will now be studied.

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