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Prostaglandins: Their role in egg-laying of the cricket *Teleogryllus commodus*

(arachidonic acid/prostaglandin-synthesizing complex)

WERNER LOHER, IRAJ GANJIAN, ISAO KUBO, DAVID STANLEY-SAMUELSON, AND STEPHEN S. TOBE*

Department of Entomological Sciences, University of California, Berkeley, California 94720

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ABSTRACT **Mating of the *Teleogryllus commodus* female causes increased oviposition mediated by PGE_2, which is synthesized in the female's spermatheca from the precursor, arachidonic acid, in the presence of a PG-synthesizing complex. The latter, together with sperm, is transferred from the male to the female via a spermatophore. Only nanogram quantities of PGE_2 injected into the oviduct are necessary to simulate mating-induced egg release.**

During copulation in mammals, prostaglandins (PGs) are transferred in the seminal fluid to the female, where they elicit several physiological effects, among them the contraction of smooth uterine musculature (1). In insects, a similar mode of PG transfer has been suggested for the silkmoth (2, 3), whereas in the house cricket PG synthesis activity is associated with the reproductive tract of males and mated females, and transfer of the biosynthetic enzyme during mating has been suggested (4).

Insemination of the female of the Australian field cricket, *Teleogryllus commodus*, terminates premating behavior, which consists of strong circadian-controlled locomotion and attraction to the calling male. A successful mating also provokes increased oviposition (5). Egg-laying does not appear to be released by the mechanical stimulation resulting from mating or by the transfer of spermatophores from testectomized males. Therefore, a chemical "mating factor" has been proposed (6). This substance originates in the male's testes and during mating it is transferred to the female via a spermatophore, where it causes massive egg release.

We here report for *T. commodus* the presence of PGE_2 in the spermathecae of mated females and its virtual absence from those of virgins and from spermatophores. The precursor of PGE_2, arachidonic acid, has been demonstrated in spermathecae of virgin females. Substantial PG biosynthetic activity has been found in spermatophores and spermathecae of mated crickets. We also show that only nanogram quantities of PGE_2 are necessary to release oviposition when injected into the oviduct.

MATERIALS AND METHODS

The experimental crickets were taken from a colony that had been in the laboratory for only three generations. They were held under light–dark or dark–light conditions of 12:12 hr at 27 ± 1°C.

PGE_2 Analysis. Spermathecae were dissected from 100 virgin or mated 4- to 6-week-old females. A total of 900 spermatophores was collected from 50 males on consecutive evenings by gently squeezing the rear end, whereupon the emerging spermatophore was removed with fine forceps. The tissues were homogenized in a grinder and extracted in 80% (vol/vol) ethanol. Because the minute quantities of PGs precluded identification by means of mass spectrometry, the active material was derivatized with *p*-bromophenacyl bromide to obtain a *p*-bromophenacyl ester of PG. That compound was detected by high-pressure liquid chromatography on a reversed-phase column at a yield of 90%. Technical details are given elsewhere (7).

Determination of Polyunsaturated Fatty Acids. Spermathecae from 100 virgin 4- to 6-week-old females were removed and placed in vials containing 2 ml of chloroform/methanol (2:1, vol/vol) and hydroquinone to prevent autoxidation. The total extract was saponified by refluxing in alkaline methanol for 3 hr. After removal of nonsaponifiable lipids the fatty acids were transesterified to methyl esters by heating in acidified methanol. The methyl esters were concentrated in 30 μl of 2,2,4-trimethylpentane for chromatography. Each sample was chromatographed three times. Quantitative estimation of peak areas was done by the method of Bartlet and Smith (8). Individual peaks were identified by logarithmic plots of relative retention times and by comparison with runs of authentic standards (single and mixed 99% pure fatty acids) interspersed between experimental runs. Further technical details have been reported (9).

PG Biosynthesis. To obtain an enzyme preparation for PG biosynthesis, spermatophores were collected from 10- to 60-day-old males. Spermathecae from 15- to 25-day-old females were dissected 2 hr after insemination and their contents were emptied into portions of Tris·HCl incubation medium. Similarly, because previous investigation had shown that PG biosynthetic activity was associated only with the spermatophore contents and not with its structure, spermatophores were split in half and the contents were transferred to measured droplets of incubation fluid. [1-^{14}C]Arachidonic acid (54.6 mCi/mmol; 2.02 GBq/mmol; Radiochemical Centre, Amersham, England) was used as substrate and incubation was performed in 100 mM Tris·HCl, pH 7.8/0.1 mM hydroquinone/5 mM glutathione/2 μM hematin. [1-^{14}C]Arachidonic acid was 3.6 μM and the incubation volume was 200 μl. The apparent K_m of the PG-synthesizing complex is about 5 μM. The equivalent of contents of two spermatophores or of one spermatheca was added to each incubation tube and results of biosynthesis are expressed as pmol of PG per equivalent of spermatophore or spermatheca. All incubations were performed in the dark at 32°C.

Each incubation was terminated and the mixture was extracted with 600 μl of diethyl ether/methanol/0.2 M citric acid, 30:4:1 (vol/vol), at pH 3.5–4. Aliquots of the ether extracts were removed, evaporated under N_2 and applied to thin-layer chromatography plates (plastic backed, 0.2 mm thick, Merck) in diethyl ether. Authentic markers $PGF_{2\alpha}$, PGE_2, PGA_2, and

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Abbreviation: PG, prostaglandin.

* On sabbatical leave from the Dept. of Zoology, Univ. of Toronto, Toronto, Ontario, Canada.

arachidonic acid (Sigma, Upjohn) were added to the ether extracts (12.5 μg each). Chromatograms were developed in ethyl acetate/acetone/acetic acid, 90:10:1 (vol/vol) (10). Marker compounds were located by brief exposure to iodine vapor. The areas of interest on the thin-layer chromatogram were cut out and placed in scintillation vials filled with 10 ml of Aquasol (New England Nuclear). Liquid scintillation spectrometry was performed on a Beckman LS-100, using external standardization for quench correction.

PGE_2 Injections. Various concentrations of PGE_2 solutions were made by dissolving the compound in 80% ethanol, and, after volume reduction in a waterbath, diluting them with different quantities of insect Ringer's solution. For injection, a female was anesthetized in crushed ice for 5 min and then pinned to the slanted surface of a plasticine block, with the ventral side up and the ovipositor pointing to the observer. By using a calibrated glass capillary, a 1-μl droplet was delivered into the common oviduct region.

To investigate the effect of PGE_2 on egg-laying, the numbers of eggs deposited by a female before and after an injection were compared. Adult females were kept individually in vials (100 ml) containing food (lettuce and dog chow) and a 3-cm-deep layer of moist sand. The females were transferred daily to fresh vials and the old sand was dried and inspected for eggs. Because few eggs are laid during the first 8 days of adult life (6), only the eggs deposited from the 9th day to the day of injection were counted. The average daily number of eggs laid during the pretrial period was then compared with the egg number obtained during the first 24 hr after injection. For controls, females were injected with Ringer's solution, left untreated, or mated. The experimental crickets were 18 ± 6 days old at the time of treatment.

RESULTS

During mating, the male of *T. commodus* transfers a spermatophore to the mounted female and its contents are stored in her spermatheca (11). Given a suitable substrate, the female then lays many eggs within the next 2–24 hr. Injection of PGE_2 into the body cavity of virgins similarly releases oviposition within a few hours, simulating the effect of mating (12). We first determined if PGs or some components leading to their production are transferred to the female during copulation. Spermathecae from 100 mated and virgin females each were analyzed for the presence of PGE_2, after derivatization to the *p*-bromophenacyl ester, on high-pressure liquid chromatography (7). The active factor was tentatively identified as PGE_2 on the basis of having a volume retention similar to that of authentic PGE_2. The spermathecae from mated females were calculated to contain 500 pg of PGE_2 per spermatheca. In contrast, spermathecae from 100 unmated controls did not contain any detectable PGE_2, with the limit of sensitivity being 0.5 ng.

These results, while demonstrating chemically the presence of PGE_2 in the spermathecae of mated females, do not distinguish between the possibilities that PGE_2 either originated in the male and was transferred in the spermatophore or was synthesized in the female, utilizing precursors or cofactors from the male. Accordingly, spermatophores were extracted and derivatized as described and analyzed for the presence of PGE_2. Spermatophores were found to contain an average of 20 pg per spermatophore. This is far less than the 500 pg of PGE_2 present per spermatheca and suggests that the female synthesizes PGE_2 in her spermatheca by employing a factor derived from the male during mating.

In search of potential precursors of PGs, spermathecae from virgin crickets were analyzed by gas/liquid chromatography. The proportions of fatty acids are expressed in percentages of the total amount of lipids (Table 1). Sixteen fatty acids, among them seven polyunsaturated fatty acids, were found, including 2.2% $C_{20:4}$ or arachidonic acid, the precursor of PGE_2. Also present, but in lesser amounts, were precursors of PGE_1 and PGE_3, as well as very high concentrations of linoleic acid.

Table 1. Proportions of fatty acids as percentage of total fatty acids in the spermathecae of virgin females

Fatty acid	%
$C_{14:0}$ (myristic)	2.1
$C_{15:0}$	0.4
$C_{16:0}$ (palmitic)	17.4
$C_{16:1}$ (palmitoleic)	4.4
$C_{18:0}$ (stearic)	10.1
$C_{18:1}$ (oleic)	20.7
$C_{18:2}$ (linoleic)	*36.9*
$C_{18:3}$ (α-linolenic)	0.4
$C_{18:3}$ (γ-linolenic)	2.0
Unknown	0.3
Unknown	0.2
$C_{20:3}$	*0.6*
$C_{20:4}$ (arachidonic)	*2.2*
Unknown	0.4
$C_{20:5}$	*0.3*
$C_{22:4}$	1.5

The next step in our investigation was to demonstrate that the postulated "mating factor" (6) transferred to the female via a spermatophore is involved in the biosynthesis of PGs. Because previous work on the house cricket had reported "PG-synthetase" activity from several reproductive tissues (13, 14) we examined the PG biosynthetic capacity of two structures: the spermatophore and the female spermatheca. Their contents were incubated with [1-^{14}C]arachidonic acid as substrate and the results are summarized as follows: Spermatophore contents produced PGE_2 at about twice the rate of $PGF_{2\alpha}$ (Table 2). PG-synthesizing activity could not be dissociated from sperm by centrifugation, sonication, or homogenization; apparently the components are in close physical contact. Table 2 furnishes the evidence that enzymatic activity is transferred during mating to the female's spermatheca. Dissection of spermathecae from females 2 hr after insemination and analysis of their contents revealed substantial biosynthetic activity for both PGE_2 and $PGF_{2\alpha}$, whereas spermathecae from virgins had negligible activity.

Previous experiments, in which PGE_2 injected into the body cavity caused oviposition in virgin females, may be a simulation of natural events, whereby PGs produced in the spermatheca travel down the spermathecal canal into the common oviduct and stimulate oviposition. However, hemocoelic injections always had been given in pharmacological doses ranging between 1 and 100 μg of PG (3, 4, 12). Therefore, in three experiments

Table 2. Prostaglandin biosynthesis by spermatophore contents and by spermathecal contents of mated and virgin females

Exp.	Structure	PG synthesis, pmol hr^{-1} per spermatophore or spermatheca equivalent		*n*
		PGE_2	$PGF_{2\alpha}$	
1	Spermatophore	24.7 ± 9.1	12.3 ± 5.0	23
2	Spermatheca			
	Mated*	34.8 ± 16.2	13.0 ± 7.0	9
	Virgin	<1.0	<0.4	4

Results are mean ± SD.
* Spermathecae were dissected out 2 hr after insemination.

Table 3. Relative increase in egg deposition on the first day after PGE_2 injection

Treatment	Relative increase* and (*n*)		
	Exp. 1	Exp. 2	Exp. 3
2 ng of PGE_2	12.70[a] (8)	14.49[a] (9)	10.46[b] (6)
200 ng of PGE_2	14.08[a] (9)	20.67[a] (7)	9.69[b] (12)
Ringer's solution	1.59[b] (6)	5.83[b] (8)	2.02[c] (9)
Untreated		4.30[b] (8)	
Mated			41.04[a] (8)

Means in each column with the same superscript letter are not significantly different according to Duncan's multiple range test at $P = 0.05$.

* The ratio of the number of eggs laid during the first 24 hr after treatment to the average number of eggs per day before treatment.

the dosage was reduced and applied to locations where, according to the above suggestion, PGE_2 should normally occur. Doses of 2 or 200 ng of PGE_2 in Ringer's solution were injected into the common oviduct region, approximately at the level of the opening of the spermathecal duct. Table 3 shows the ratio of the number of eggs laid during the first 24 hr after treatment to the average number of eggs per day laid before the application. In all three experiments, the injection of PGE_2 resulted in a large increase of the number of eggs laid. A statistical comparison between PGE_2-injected groups and controls clearly shows that the two doses of PGE_2 caused significantly more egg release than Ringer's solution alone or no treatment at all. Yet when females were mated the number of eggs laid was far greater than after PGE_2 application; mated females deposited an average of 328 eggs within the first 24 hr after insemination, whereas females injected with 2 or 200 ng of PGE_2 laid only 86 and 100 eggs per female, respectively. However, these amounts were still significantly above the 21 eggs per female released after injection of Ringer's solution.

It is unknown where in the cricket female the spermathecal PGs act to trigger oviposition. The spermatheca and its PG biosynthetic activity appear to be necessary for daily moderately increased egg-laying rates following the big release during the first day after mating. This is shown in the following preliminary experiment: 11 females whose premating oviposition rate was 3.0 ± 0.6 eggs per day per female were inseminated and within 24 hr they laid 129.4 ± 17.3 eggs per female. Then the spermathecae were removed and the females were given further opportunity to lay eggs. The subsequent daily oviposition rate dropped drastically and approached the premating level. Injection of 50 μg of PGE_2 into the hemocoel of two of these females on the 6th day after the operation reversed the effect, resulting in the laying of 183 and 203 eggs within the next 24 hr.

DISCUSSION

The identification of PGE_2 in the spermathecae of mated females and of its precursor arachidonic acid in those of virgins, the transfer of a PG-synthesizing complex via a spermatophore from the male to the spermatheca of the female as indicated by PG biosynthetic activity in the spermatophore and the female's storage organ, and the minute quantities of PGE_2 necessary for oviposition provide the evidence for the role of PGs in egg-laying of *T. commodus*. We have hereby demonstrated all components involved in the biosynthesis of PGE_2 in an insect reproductive system, consisting of the substrate (precursor arachidonic acid), enzyme complex, and product. Enzyme transfer, earlier suggested for the house cricket (4), contrasts with the method employed in mammals and particularly in humans, where the ejaculate itself contains at least 13 PGs (1). Crickets also appear to differ in this respect from silkmoths, in which PGE_2 and $PGF_{2\alpha}$ apparently are transferred during copulation (2, 3). It is likely that in the spermatheca of *T. commodus* still other PGs are synthesized and function in egg release, as suggested by the presence of precursors for PGE_1 and PGE_3. The higher number of eggs laid after a mating when compared to the lower egg yield after PGE_2 injection could be due to the prolonged action of a mixture of PGs, whereas PGE_2 injected only once may be rapidly metabolized. Accordingly, it would not be surprising if a single injection failed to duplicate the natural daily production of PGs. Recently, arachidonic acid has been found in the reproductive tract of the male house cricket and an unidentified field cricket species (15), but its role remains to be determined. The same applies for linoleic acid, which is the most prevalent of the fatty acids in the spermatheca (36.9% of the total). Although this compound serves in mammals as the parent polyunsaturate of several C_{20} fatty acids—i.e., precursors for PGs (16)—only circumstantial evidence is available to suggest that similar conversions to arachidonic acid (9, 17) and other unsaturated fatty acids occur in insects, and hence its involvement in PG biosynthesis is unclear.

Our preliminary investigations to further characterize biochemically the enzymatic activity (unpublished data) suggest an enzyme complex similar to the equivalent enzyme system in mammals (18). To date, it has been impossible to separate the cricket's enzyme complex from the sperm mass, suggesting close physical proximity between the spermatozoa and the enzymes.

A comparison of PG biosynthesis by contents of spermatophores and spermathecae when incubated with [1-^{14}C]arachidonic acid indicates no statistical difference in their rates of synthesis of PGE_2 and $PGF_{2\alpha}$. However, in the case of PGE_2, individual values obtained from spermathecae vary considerably. The lower rates of biosynthesis are probably the result of only partial sperm/enzyme transfer during mating. Indeed, spermatophores have been observed to empty incompletely during the insemination process.

Minute concentrations of PGE_2 applied to the oviduct region of gravid virgin females elicited egg release, whereas the same quantities injected into the hemocoel did not, and pharmacological doses were required to obtain the effect. In the house cricket, then, hemocoelic application of 100 μg of PGE_1 or PGE_2 caused substantial oviposition, but the same amounts of $PGF_{2\alpha}$ were hardly effective (4).

Where and how PGs act in stimulating oviposition is still an open question. One possibility is a direct action on the ovarian musculature that forces the accumulated eggs out of the oviduct, thereby triggering egg-laying behavior. This is reminiscent of the action of PGs on smooth muscles of the mammalian uterus, with the notable difference that insect muscles, including those of the oviduct, consist entirely of striated muscular tissue. Another method would involve the neuroendocrine system: PGs may either directly release neurosecretory material (19, 20) or generate yet another factor that acts on the brain, triggering not only egg release but also oviposition behavior (21–23).

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