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Determination by HPLC-RIA of Immunoreactive Prostaglandin E_2 in *Blattella germanica* and *Gryllus bimaculatus*

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The application of a combined HPLC-RIA methodology to estimate immunoreactive PGE_2 levels in two insect species, namely, males of the cricket *Gryllus bimaculatus* (Orthoptera, Gryllidae) and males and females of *Blattella germanica* (Dictyoptera, Blattellidae) is reported. From the results obtained, it can be concluded that, whereas in the crickets the presence of a female does exert a stimulating effect on the PGE_2 levels of the male, in cockroaches nonsignificant differences on PGE_2 contents were observed among virgin and mated individuals under the conditions assayed, thus suggesting that the biosynthetic pattern of this prostanoid in the cricket studied cannot be extended to the case of the Dictyoptera.

Key words: HPLC-RIA of Prostaglandin E_2, *Blattella germanica* (Dictyoptera), *Gryllus bimaculatus* (Orthoptera).

Since prostaglandin (PG) synthetase activity was first demonstrated in reproductive tracts of the male house cricket *Acheta domestica* (Orthoptera, Gryllidae) (6), some prostanoids have been identified in several species of insects although quantitative estimations of *in situ* levels reported to date are rather scarce (2).

Prostaglandin analyses in insects have been essentially based on the use of high performance liquid chromatography (HPLC) (8) or radioimmunoassay (RIA) (4, 11, 17) techniques. In the first case, the quantitative methods for PG measurement

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involve complex extraction and purification steps with subsequent sophisticated derivation procedures such as those required for efficient UV detection in HPLC determinations. In the second case, the RIA affords excellent sensitivity but its specificity is usually inadequate due to cross-reaction of the antibody with a variety of related prostanoids which could also be present in the biological sample (10). It is well known that the direct RIA procedure on a biological extract can only at best give group specificity (11). However, this lack of specificity can be conveniently counterbalanced if each prostanoid is first isolated by HPLC so that the various collected eluates of interest are then more efficiently assayed by radioimmunoanalysis (9). In particular, PGE_2 can be separated from other prostanoids by HPLC techniques in a triethylamine buffer eluent system (3, 7).

The present report describes the application of this combined HPLC-RIA methodology to the determination of PGE_2 levels in the cricket *Gryllus bimaculatus* (Orthoptera, Gryllidae) and the cockroach *Blattella germanica* (Dictyoptera, Blattellidae), two insect species not previously studied in this manner.

In the case of Orthoptera, studies carried out by several authors on the species *Acheta domestica* (5, 6) and *Teleogryllus commodus* (14) have shown that during the reproductive stage, males transfer PG synthetase complex in spermathophores to females through mating, and moreover, that PGs, particularly PGE_2, exert a stimulating effect on oviposition (15). However, there are no detailed studies to date on PGE_2 levels in males. In this context, the comparison between PGE_2 levels in virgin males and males reared in the presence of females could afford valuable data to ascertain whether these particular compounds, which are closely connected to the reproductive process in this insect order, are also involved in the courtship period.

Conversely, it is also well known that reproduction in cockroaches proceeds by a completely different mechanism involving definite gonadotropic and ovipositional cycles. The mating influence on these cycles appears to be quite relative and, in the specific case of *B. germanica*, it is limited to the oöcyte growth speed (19). Accordingly, it seemed of interest to find out if potential differences in PGE_2 levels of both virgin and mated females and males of this species could be in some way correlated with their particular reproductive strategy.

Materials and Methods

Chemicals. Acetonitrile (ACN), HPLC grade, was purchased from Koch-Light Ltd. and methyl formate and petroleum ether (puriss p.a.) from Fluka AG. Triethylamine, ethanol and acetone were from Scharlau (Barcelona, Spain) and formic acid was from Merck. Gelatine, sodium azide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and sodium chloride were purchased from Merck and charcoal and Dextran T70 from Sigma. (^{3}H)-Prostaglandin E_2 (160 Ci/mmol) was from New England Nuclear. PGE_2 was a generous gift from Dr. J. E. Pike (Upjohn Co., Kalamazoo, Mich., USA) and PGE_2 antiserum (Affinity $Ka = 8.2 \times 10^{10}\ M^{-1}$, $B = 40\ \%$ calculated for 10 pg of (^{3}H) PGE_2, specific activity 160 Ci/mmol) from the Pasteur Institut.

Insects. Experimental specimens of *Gryllus bimaculatus* were taken from a colony reared at 26 ± 1°C and 18 h of photophase. Virgin males were segregated in small groups (3-6 specimens) shortly after the adult ecdysis and single couples were formed with freshly ecdysed adults. Both groups were maintained under the same conditions as the general colony and specimens were sacrificed on day 8 of adult age.

Colonies of *B. germanica* were reared as previously described (1). Freshly ecdysed adults were sexually segregated until day 6 of adult age. Mated specimens were obtained by forming single couples of the above age. Both specimens were killed 2 hours after the mating and in all cases it could be previously observed that the spermatophore had been ejected by the female. Virgin specimens of both sexes were sacrificed also on day 6 of adult age.

All experimental specimens were killed by freezing at −20° and were maintained at this temperature for the extraction process.

Extraction of PGs. Homogenization of insects (10 individuals for *B. germanica* and 2 individuals for *G. bimaculatus*) was carried out in cold acetone (2 × 7 ml) by the use of a Polytron set (Ystral, GmbH, Gottingen, West Germany). After centrifugation (18,500 *g*) at 4°C during 10 min, the supernatants were collected and evaporated to dryness in a rotary film evaporator. Finally, the residue was taken in 10 ml 0.04 M formic acid: triethylamine buffer (TEA-F) (pH 3.15) and passed through a reverse phase octadecylsilica C_{18} cartridge (Waters Assoc., Milford, Mass., USA). The dry residue was additionally washed with 10 ml of buffer containing 10 % of ethanol which were also passed through the cartridge. Elution of non-polar lipids with petroleum ether (20 ml) was followed by 20 ml of methyl formate to elute the prostaglandins. The residue obtained after evaporation of the eluate to dryness under a helium stream was resuspended in 150 µl of TEA-F/ACN (69:31) at pH 3.15 and injected onto the HPLC column.

HPLC separations. HPLC analyses were carried out in 30 cm × 3.9 mm µ-Bondapak reverse phase columns (Waters Assoc.) eluted isocratically with the mixture TEA-F/ACN (69:31), according to the method described by DESIDERIO *et al.* (3). The instrumentation consisted of two Kontron pumps model 414 (Kontron AG, Zurich, Switzerland), controlled by a solvent programmer model Series 2000. Biological samples were injected through a Reodyne 7125 injector. Eluent fractions, collected at the appropriate retention time for PGE_2 were lyophilized prior to the RIA determinations. The absolute retention time of PGE_2 had been established before-hand by injection of $(^3H)PGE_2$, previously purified by HPLC. The addition of unlabeled PGE_2 standard was avoided in order to prevent memory effects on the HPLC system. The isocratic elution resulted in excellent reproducibility of retention times which in turn facilitated the blind collection of the PG eluate fractions.

RIA analyses. After redissolving the lyophilized eluate in 400 µl of phosphate buffer (pH 7.40), RIA determinations were carried out in triplicate using a rabbit PGE_2 antiserum and following the recommended specifications of the Pasteur Institut. The amount of added $(^3H)PGE_2$ was approximately 5.7 nCi (≃ 12 pg).

Results and Discussion

The procedure applied to the insect extracts involves selective retention on a reversed phase Sep Pak C_{18} cartridge followed by clean-up of neutral lipids by extraction with petroleum ether and subsequent elution of retained PGs into methyl formate according to a modification of Powell's method (18). Recovery calculated by addition of $(^3H)PGE_2$ prior to the homogenization step was 85.5 % ± 7.8 % (n = 20). Intraassay reproducibility was 7.2 % (n = 10) whereas the equivalent interassay value was 12.6 % (n = 8). In order to minimize the losses due to the adsorption of PGE_2 on the residue obtained from the acetone homogenates, an additional washing with ethanolic buffer solution (10 % ethanol) was carried out. Pros-

Table I. *Immunoreactive PGE_2 levels (mean ± S.D.) in males of* Gryllus bimaculatus.

	Virgin ♂ (n = 3)	♂♂ + ♀ * (n = 4)
pg/g of insect	53.7 ± 14.8	120.3 ± 15.2
pg/4 insects	184.0 ± 62.3	407.2 ± 63.0

Males reared in the presence of a female.

taglandin losses associated to the petroleum ether phase were negligible (3.2 % ± 2.9 %, n = 20). Total recovery including the reverse phase HPLC step corresponds to 55.9 % ± 6.5 % (n = 8).

As demonstrated by RIA dilution assays, the previous purification of the biological extracts is very convenient prior the RIA quantification of immunoreactive-PGE_2 (ir-PGE_2). The parallelism between the standard calibration and dilution RIA curves confirms that the HPLC step must be carried out to compensate for the lack of specificity of the radioimmunological technique. The combined HPLC-RIA method herein described has been successfully applied to the determination of ir-PGE_2 levels (tables I and II) in two different insect species such as *B. germanica* and *G. bimaculatus*. As far as we know, this is the first reported determination of ir-PGE_2 in these insects.

Regarding *G. bimaculatus*, the results depicted in table I show that ir-PGE_2 levels are higher in males reared in the presence of females. In concordance with the pattern of PG biosynthesis in the well-studied cases of *Teleogryllus commodus* (15) and *Acheta domestica* (4) a transference of PG synthetase from male to female through mating can be assumed. Accordingly, it seems reasonable to presume that the presence of the female could stimulate the production of PG synthetase in her partner, thus explaining the higher levels of ir-PGE_2 found in males of *G. bimaculatus*. In fact, it is known that the female of this species exerts strong physiological interactions on the male as it has been demonstrated that, in addition to the usual tactile stimulus, a sexual pheromone produced by the female is involved in courtship behaviour (12). Either the tactile or olfactory stimuli or both concomitantly could trigger a mechanism leading to the activation or increased synthesis of the enzymes involved in the PG synthetase complex. Moreover, the presence of a female has been observed to increase the frequency of the production of spermatophores by the male, as it occurs in other species of crickets, like *A. domestica* (13) and *T. commodus* (16).

In the case of *B. germanica*, the results obtained in the four groups (table II) do not show significant differences thus indicating that mating *per se* does not appear to exert any influence on ir-PGE_2 levels in either sex. It is worthwhile to point out that the reproductive strategy in this species is essentially different from that of crickets. Oöcyte development and oviposition in *B. germanica* follow definite cycles and mating has only a slight influence on the duration of these cycles (19). Thus, it is not surprising that the biosynthetic pattern of ir-PGE_2 reported for crickets, where the participation of both sexes is necessary and mating becomes a prerequisite for oviposi-

Table II. *Immunoreactive PGE_2 levels (mean ± S.D.) in adults of* Blattella germanica.

	Virgin ♂♂ (n = 6)	Mated ♂♂ (n = 7)	Virgin ♀♀ (n = 8)	Mated ♀♀ (n = 7)
pg/g of insect	62.9 ± 29.7	57.9 ± 20.5	48.5 ± 23.7	37.7 ± 22.2
pg/10 insects	29.8 ± 14.4	22.6 ± 10.7	40.7 ± 19.2	36.4 ± 22.0

tion, is not directly applicable to the case of *B. germanica.*

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Resumen

Se describe la aplicación de una técnica combinada de HPLC-RIA para la estimación de niveles de PGE_2 inmunorreactiva en machos de *Gryllus bimaculatus* y en adultos de ambos sexos de *Blattella germanica.* En los grillos, la presencia de la hembra ejerce un efecto estimulante sobre sus niveles de PGE_2, mientras que en las cucarachas no se detectan diferencias significativas entre individuos vírgenes y apareados. Estos resultados sugieren que el modelo biosintético postulado para el citado prostanoide en *Gryllidae,* que asume una transferencia de Pg-sintetasa del macho a la hembra a través de la cópula, no es aplicable al caso de *Blattella.*

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