

Prostaglandin E₂ in Previtellogenic Ovaries of the Prawn *Macrobrachium rosenbergii*: Synthesis and Effect on the Level of cAMP

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Eicosanoids are thought to play a role in the regulation of invertebrate reproduction, as they do in vertebrate systems. This was investigated using the previtellogenic ovary of the freshwater prawn *Macrobrachium rosenbergii* as a biological model. Concentrations of prostaglandin E₂ (PGE₂), assessed by means of radioimmunoassay, in the previtellogenic ovary (oocyte diameter 20–40 μm) were 32.4 ± 14.1 pg/mg ovary. Preincubation of the ovary with indomethacin (10 μM) inhibited PGE₂ synthesis by 43%. In addition, if indomethacin was added to the culture medium, cAMP levels decreased by 48%. When previtellogenic ovaries were incubated *in vitro* with PGE₂ (0.05 μg/ml medium and up), cAMP levels in the tissue homogenate sharply increased. The levels of cAMP rose most significantly (up to 10-fold) when 1–10 μg PGE₂/ml medium was applied. These results suggest that PGE₂, and possibly other prostaglandins, may play a role in the endocrine regulation of crustacean reproduction. © 1995 Academic Press, Inc.

Prostaglandins are biologically active lipids present in almost all mammalian tissues and body fluids. It is known that a major signal transduction pathway of prostaglandins involves cAMP. Prostaglandins may either stimulate or inhibit the enzyme adenylate cyclase, depending on the type of target cell. The biological action of prostaglandins has been investigated largely in terms of the physiological and clinical significance of these compounds in mammalian reproductive processes. They participate in ovarian steroidogenesis (Denning-Kendall and Wathes, 1994), in the induction of ovulation (Tsafriri *et al.*, 1972; Priddy and Kilklick, 1993), in luteolysis (Abayasekara *et al.*, 1993), and in abortion and delivery (Steyn and Pienaar, 1993; Behrens, 1995).

It has been suggested that prostaglandins may also play a role in invertebrate reproduction (reviewed by Stanley-Samuelson, 1987, 1994; Fingerhman *et al.*, 1993; De Petrocellis and Di Marzo, 1994). There is, however, only scattered evidence to support this supposition. The best known physiological role of prostaglandins in

invertebrate reproduction is their involvement in the egg-laying behavior in insects (Loher *et al.*, 1981). Prostaglandins induce spawning in two species of molluscs, the abalone *Haliotis refescens* and the mussel *Mytilus californianus* (Morse *et al.*, 1977). Prostaglandin E₂ (PGE₂) also appears to stimulate egg production in the freshwater snail *Helisoma durgii* (Kunigelis and Saleuddin, 1986).

In lower crustaceans, such as the barnacle *Balanus balanoides*, the hatching substance that acts on the musculature of mature embryos appears to be a prostaglandin-related compound (Clare *et al.*, 1982, 1985). Recently, some supporting evidence for the regulatory role of prostaglandins has been presented for decapod crustaceans. In the Florida crayfish *Procambarus paeninsulanus*, changes have been reported in ovarian prostaglandins during the vitellogenic process (Spaziani *et al.*, 1993).

The endocrine regulation of reproduction in decapod crustaceans is not fully understood. However, in recent years there has been increasing interest in the regulation of decapod repro-

duction due to the economic importance of these species and the difficulties of propagating them in captivity (Quackenbush, 1991). In the light of the above-described considerations, we investigated whether PGE₂ is involved in the early steps of crustacean gonad maturation, using the previtellogenic ovary of the freshwater prawn *Macrobrachium rosenbergii* as a biological model. We studied the presence and synthesis of PGE₂ in the ovary. Once the existence of the hormone had been established, we focused on testing its effects on cAMP levels in previtellogenic ovaries of this decapod crustacean.

MATERIALS AND METHODS

Animals

M. rosenbergii prawns (supplied by the aquaculture unit of Kibbutz Nir-David, Israel) were selected according to the color of the eggs carried by the females (O'Donovan *et al.*, 1984). Elimination of late-vitellogenic animals possessing mature ovaries was performed in terms of orange coloration of the ovary observed through the carapace (Sagi and Ra'anani, 1985). The gonadosomatic index (GSI = Gonad Wt./Body Wt. × 100) and oocyte diameter (O'Donovan *et al.*, 1984), for a random sample of 15 oocytes per ovary, were determined for each dissected female. The latter was measured by means of an objective micrometer.

Ovary Incubation

The prawns were dissected on ice. The ovaries were removed, separated from the oviducts, weighed, and sectioned into approximately 2-mm fragments. The fragments were incubated at 28° in Dulbecco's modified Eagle's medium (DMEM) adjusted to the osmolarity of *M. rosenbergii* (Sagi *et al.*, 1991), with slight agitation under an oxygen-enriched atmosphere.

Determination of PGE₂ Levels in the Incubated Ovary

The ovarian fragments had been incubated for 1 hr as described above, with or without indomethacin (10 μM). They were removed from the medium, which was collected with additional 10 μM of indomethacin, to prevent further synthesis, and frozen before analysis. PGE₂ in the medium was determined in duplicates by radioimmunoassay (RIA). Anti-PGE-BSA serum (BioMakor Ltd., Rehovot, Israel) reacted equally with PGE₂ and PGA₂. PGE₁, PGA₁, PGB₂, and PGB₁ also cross-reacted with the antiserum, while PGF_{2α}, PGD₂, and 6-keto-PGF_{1α} did not produce signifi-

cant cross-reactions. Thus, the assay could identify prostaglandins of the E series, both monoenoic and dienoic, and their dehydration products (PGA and PGB). However, the latter probably do not occur biologically, but are formed chemically during extraction (Campbell, 1990). The results were expressed as PGE₂ equivalents. [³H]PGE₂ (Radiochemical Centre, Amersham) had a specific activity of 160 Ci/mmol. The sensitivity of the assay was 0.15 ng/ml. The significance of differences in PGE₂ equivalents between treatments was tested by a nonparametric Kruskal-Wallis ANOVA by ranks.

Effects of PGE₂ and Indomethacin on cAMP Levels

The effects of PGE₂ (10 μg/ml) and indomethacin (10 μM) on ovarian tissue (incubated as described above with or without 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase) were determined by measuring cAMP levels in the incubated fragments. At the end of the incubation period tissue fragments were boiled for 15 min in 0.4 ml acetate buffer, 50 mM, pH 4. The fragments were homogenized, and the resulting solutions were centrifuged at 1000g for 15 min. The supernatants were collected for cAMP measurement by RIA (Frandsen and Krishna, 1976) using [¹²⁵I]cAMP (20,000 cpm/0.1 ml, Nuclear Center-Negev, Israel) and anti-cAMP antibodies (1:1000, in Tris buffer, pH 7.4, Bio Makor, Israel).

The effects of different concentrations of PGE₂ were examined by measuring cAMP levels in ovarian tissue. Incubations were conducted as described above using the following concentrations of PGE₂: 0.05, 0.5, 5, and 50 ng/ml, and 0.5, 1, 5, and 10 μg/ml. cAMP levels were determined by RIA as described above.

The significance of the differences between treatments was determined in cases of a normal distribution by one-way ANOVA followed by a LSD test, and by Kruskal-Wallis ANOVA by ranks for distributions that were not normal.

RESULTS

Properties of Previtellogenic Ovaries and Release of PGE₂ in Vitro

The previtellogenic ovaries contained oocytes averaging 20.3 ± 0.7 μm in diameter. The gonadosomatic index for nine ovaries averaged 0.39% of the body weight. The average amount of PGE₂ equivalents released from four previtellogenic ovaries into the culture medium during 1 hr of incubation was 32.4 ± 14.1 pg/mg tissue (Table 1).

TABLE I
PROPERTIES OF *M. rosenbergii* PREVITELLOGENIC OVARIES AND THEIR *IN VITRO* RELEASE OF PGE₂ EQUIVALENTS DURING 1 HR OF INCUBATION

Oocyte diameter (Mean $\mu\text{m} \pm \text{SEM}$)	GSI (Mean % $\pm \text{SEM}$)	PGE ₂ equivalents release (pg/mg ovary)		
		(Mean $\pm \text{SEM}$)	(Minimum)	(Maximum)
20.3 \pm 0.7 ($n = 9 \times 15$) ^a	0.39 \pm 0.05 ($n = 9$)	32.4 \pm 14.1 ($n = 11$) ^b	7.0	171.1

^a Sample represents nine ovaries, 15 oocytes per ovary.

^b Sample represents 11 cultured fragments from four ovaries.

Effect of Indomethacin on PGE₂ Synthesis by Incubated Previtellogenic Ovaries

Indomethacin (10 μM), a prostaglandin synthase inhibitor, inhibited PGE₂ synthesis in incubated ovarian fragments (Fig. 1) by 43%. During 1 hr of incubation control fragments released 20.1 \pm 2.8 pg PGE₂ equivalents/mg, while in the presence of indomethacin this value was reduced significantly (Kruskal–Wallis: $P \leq 0.052$) to an average of 11.5 \pm 2.2 pg PGE₂ equivalents/mg.

Effect of Indomethacin on cAMP Levels in Incubated Previtellogenic Ovaries

Incubated ovarian fragments produced cAMP levels of 1.25 \pm 0.14 ng/mg ovary, which were, as expected, augmented by IBMX (Kruskal–Wallis, $P \leq 0.004$). Indomethacin caused a reduction in cAMP levels in the incubated ovarian fragments (Fig. 2). The levels of cAMP were

significantly reduced by over 48% in the presence of indomethacin ($P \leq 0.009$). In the presence of IBMX, indomethacin caused a significant reduction of over 39% ($P \leq 0.047$).

Effect of PGE₂ on cAMP Levels in Incubated Previtellogenic Ovaries

In Fig. 3 it can be seen that PGE₂ caused a significant increase in the level of ovarian cAMP (ANOVA: $P \leq 0.0001$): a 5.5-fold increase, from 0.648 \pm 0.096 to 3.534 \pm 0.504 ng/mg was detected. In the presence of IBMX, PGE₂ caused a less pronounced but still significant (ANOVA: $P \leq 0.029$) 1.7-fold increase (from 2.886 \pm 0.744 to 4.827 \pm 0.881).

Dose–response experiments revealed a typical S-shaped curve for cAMP production (Fig.

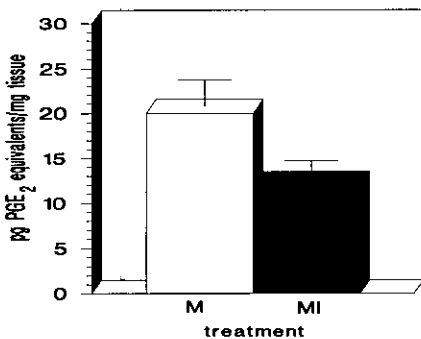


FIG. 1. Effect of indomethacin on PGE₂ synthesis in incubated previtellogenic *M. rosenbergii* ovaries. M, culture medium ($n = 8$); Mi, culture medium with 10 μM indomethacin ($n = 6$). Error bars represent SEM.

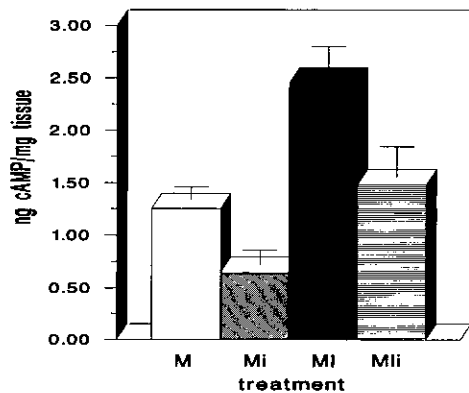


FIG. 2. Effect of indomethacin on cAMP levels in incubated previtellogenic *M. rosenbergii* ovaries. M, culture medium ($n = 8$); Mi, culture medium with 10 μM indomethacin ($n = 8$); MI, culture medium with IBMX ($n = 9$); Mli, culture medium with IBMX and indomethacin ($n = 9$). Error bars represent SEM.

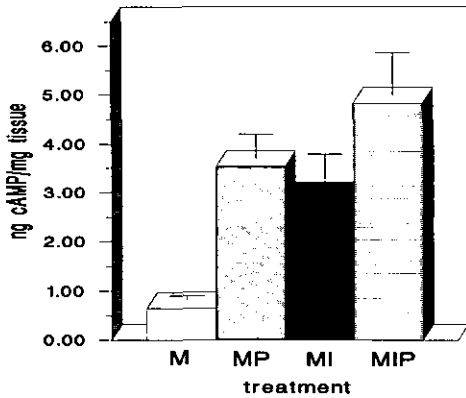


FIG. 3. Effect of PGE₂ on cAMP levels in incubated previtellogenic *M. rosenbergii* ovaries, M, culture medium (*n* = 39); MP, culture medium with 10 μg/ml PGE₂ (*n* = 41); MI, culture medium with IBMX (*n* = 30); MIP, culture medium with IBMX and PGE₂ (*n* = 26). Error bars represent SEM.

of PGE₂ cAMP levels rose some 10-fold over control.

DISCUSSION

Many substances known to be active as hormones in vertebrates have also been shown to play regulatory roles in invertebrates, suggesting conservation in evolution (Fingerman *et al.*, 1993). However, it has not been established whether the regulatory role of such hormones—including prostaglandins, which were first discovered in mammals—is similar in the different phyla. The role of prostaglandins in the regulation of vertebrate reproduction is well known. It has recently been postulated that these compounds may have a similar action in invertebrates, since they have been isolated from the reproductive organs of a number of invertebrate species (Stanley-Samuels, 1987, 1994; Fingerman *et al.*, 1993). Our findings of PGE₂ in the previtellogenic ovary of *M. rosenbergii* are in keeping with the evidence for bioconversion of arachidonic acid into prostaglandins in cray-

4). For concentrations of PGE₂ up to 5 ng/ml cAMP production was not significantly different from the basal ovarian activity. However, when 0.05 μg/ml of PGE₂ was applied, a sharp rise of the curve was evident. The curve reached its plateau near 1 μg/ml of PGE₂, which persisted up to 10 μg/ml. At these concentrations

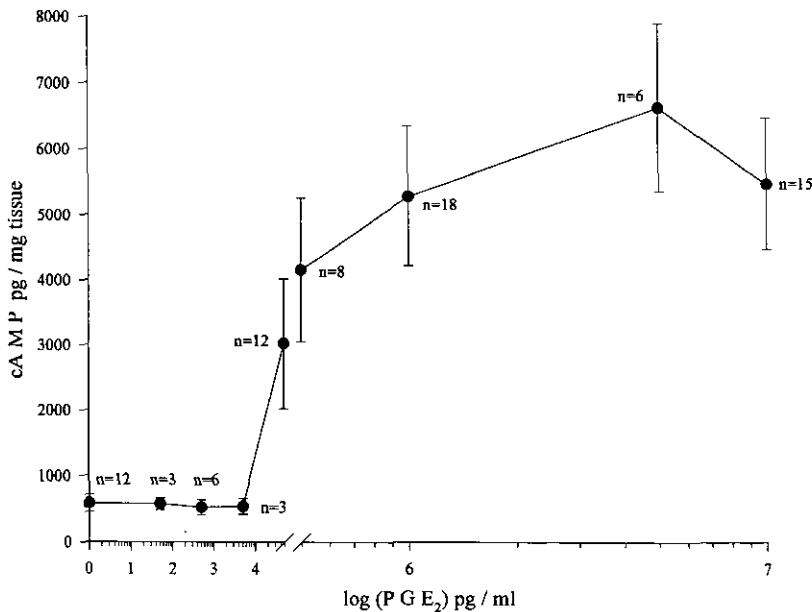


FIG. 4. Effects of different concentrations of PGE₂ on cAMP levels in incubated previtellogenic *M. rosenbergii* ovarian tissue. Errors bars represent SEM.

fish ovaries reported by Spaziani *et al.* (1993). It appears that the hormone is synthesized in the previtellogenic ovary as is suggested by the inhibition of its release by indomethacin. Indomethacin is an effective inhibitor of mammalian cyclooxygenase, and at 10 μM is expected to cause near complete inhibition of this enzyme. The fact that in our system the inhibition was relatively modest may be explained by the low prostaglandin concentrations in some of the experiments with indomethacin (that fell below the limits of quantification of the assay). This did not allow precise assessment of the magnitude of inhibition. Alternatively, it may suggest a different isosyme in *M. rosenbergii*.

The results of our experiments with indomethacin and PGE_2 suggest that in *M. rosenbergii* prostaglandins, particularly PGE_2 , may play a role in the regulation of the ovary at the previtellogenic stage. This is shown by changes in the levels of cAMP, known to be the second messenger in the signal transduction pathway of various processes controlled by prostaglandins. However, these effects were achieved at a relatively high concentration (10 $\mu\text{g}/\text{ml}$ medium) that has been used for different prostaglandins in mammalian *in vitro* experiments with ovaries (Marsh, 1971), liver (Chayoth *et al.*, 1973), and thyroid (Zor *et al.*, 1969). In our dose-response experiments we found that the most effective concentrations of PGE_2 ranged between 1 and 10 $\mu\text{g}/\text{ml}$ (Fig. 4). Therefore, lower concentrations of PGE_2 may cause the same effects on cAMP production. However, the concentrations released by the *M. rosenbergii* ovary (up to 171 pg/mg tissue) are much lower than the effective doses suggested by our dose-response curve. The efficiency of the *in vitro* response to hormone depends upon its diffusion rate from the surrounding medium and its losses. Thus, higher hormone concentrations may be required to elicit noticeable effect in cultured tissue. In the whole organism prostaglandins may be released in close proximity to their target cells (by paracrine or autocrine mechanisms), thus their effective concentrations need not be so high as applied *in vitro*. Another possible explanation lies in the fact that we cannot be sure that the

measurements of PGE_2 released in the culture medium indeed represent the physiological level of this compound in the ovary of *M. rosenbergii*. In mammals the predominant natural product is either PGE_1 or PGE_2 , depending on the species, but since our detection of the presumed PGE_2 depends on immune recognition, the predominant natural prostaglandin in *M. rosenbergii* ovary may not be authentic PGE_2 , but a closely related compound. Definite identification must await more strict chemical analysis, e.g., by GC-MS.

In the Florida crayfish *P. paeninsulanus*, *in vitro* incubation of ovarian homogenate with [^3H]arachidonic acid resulted in production of [^3H] PGE_2 and $\text{PGF}_{2\alpha}$, which could be blocked with indomethacin (Spaziani *et al.*, 1993). There was a significantly higher yield of PGE_2 and $\text{PGF}_{2\alpha}$ synthesis in the later vitellogenic stages compared to early stages. One possible explanation for these findings may involve the induction of ovulation by prostaglandins in the crayfish. This supposition is supported by unpublished observations (Hinsch, in Spaziani *et al.*, 1993) of a 67-fold increase in peroxidase activity in the spider crab *Libinia emarginata* (Crustacea, Decapoda) prior to ovulation, suggesting an effect of prostaglandins on ovarian smooth muscle. On the other hand, our results show that PGE_2 is synthesized by and exerts an influence on the previtellogenic *M. rosenbergii* ovary (later vitellogenic stages have not yet been examined), in which the main components are oocytes, germ cells, and follicular cells (O'Donovan *et al.*, 1984).

A number of hormones are thought to play a role in the endocrine regulation of crustacean gonadal development (recently reviewed by Quackenbush, 1991). The results of the present study suggest a possible regulatory role for prostaglandins in previtellogenic ovaries of the freshwater prawn *M. rosenbergii*. Since the changes in cAMP levels induced by PGE_2 have been clearly demonstrated in the present study, further investigation is necessary in order to clarify the particular physiological processes regulated by PGE_2 through the production of cAMP.

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