Inhibition of ovarian steroidogenesis by cyclic GMP in a fly

G Manière, E Vanhems, F Gautron and J-P Delbecque

Université Bordeaux I, Laboratoire de Neuroendocrinologie des Insectes, Avenue des Facultés, F-33405 Talence Cedex, France

(Requests for offprints should be addressed to J-P Delbecque who is now at Université Bordeaux I, Laboratoire de Neurobiologie des Réseaux, CNRS UMR 5816, Avenue des Facultés, F-33405 Talence Cedex, France; Email: jp.delbecque@lnr.u-bordeaux.fr)

(G Manière is now at CNRS, Institut de Neurobiologie Alfred Fessard, UPR 2216, Neurobiologie Génétique et Intégrative, Equipe de Génétique Moléculaire des Rythmes Circadiens, Avenue de la Terrasse, F-91198 Gif sur Yvette Cedex, France)

(F Gautron is now at Université Bordeaux I, Laboratoire des Facteurs de Croissance et de la Différenciation Cellulaire, INSERM EPI-0113, Avenue des Facultés, F-33405 Talence Cedex, France)

Abstract

Previous investigations in the female blowfly *Phormia regina* have shown that 3-isobutyl-1-methylxanthine (IBMX), a broad spectrum inhibitor of phosphodiesterases (PDEs), fails to mimic the steroidogenic effects of cAMP on ovaries, although it efficiently increases the concentrations of this second messenger. In this study, experiments carried out to clear up this contradiction demonstrated that IBMX, besides its effect on cAMP, also increased cGMP concentrations in blowfly ovary and that these two cyclic nucleotides controlled ovarian steroidogenesis antagonistically. In particular, a selective inhibitor of cGMP-specific PDEs, unlike IBMX, had a very strong negative effect on ovarian steroidogenesis. Moreover, a cGMP analog was able to inhibit steroid biosynthesis in previtellogenic and vitellogenic ovaries, thus affecting basal and acute steroidogenesis respectively. Our observations also demonstrated that cGMP was always present in blowfly ovary, reaching its maximal levels at the end of

vitellogenesis, in close correlation with the physiological decrease in ovarian steroidogenesis. Experiments using an inhibitor of protein kinase G clearly indicated that the effects of cGMP were mediated by this enzyme. On the contrary, these effects did not seem to involve cGMP-regulated PDEs or ion channels. Our results also indicated that ovarian cGMP concentrations were not controlled by brain factors, suggesting a probable involvement of paracrine/autocrine factors. Nitric oxide (NO) appeared to be a good candidate for such a control, because an NO donor was able to stimulate ovarian cGMP concentrations and to drastically decrease ovarian ecdysteroid biosynthesis in blowflies. These data thus demonstrate, for the first time in invertebrates, a potent role of cGMP in the negative control of ovarian steroidogenesis and suggest a possible co-regulation with NO.

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Introduction

Steroidogenesis is often activated by peptidic hormones, which bind to specific membrane receptors on endocrine gland cells and exert their stimulatory action via the involvement of second messengers, in particular cAMP and calcium (for review see Cooke 1999). However, other signaling molecules, either stimulatory or inhibitory, also intervene for a fine tuning of this vital biosynthesis. Among them, cGMP, an ubiquitous second messenger involved in numerous physiological responses (for review see Drewett & Garbers 1994, Lucas *et al.* 2000) may also have a modulatory role on steroidogenesis. Furthermore, cGMP is frequently linked with another important messenger, nitric oxide (NO), which is known to be a potent stimulator of soluble guanylate cyclases (for review see Bredt & Snyder 1994) and thus a possible regulator of steroid biosynthesis.

Regarding more particularly the control of steroidogenesis in the gonads, the effects of cGMP have been studied in several vertebrate models, but not yet in invertebrates. In the former, however, the situation appears very confused: indeed, stimulatory effects of cGMP analogs have been sometimes observed (Mukhopadhyay *et al.* 1986, Khurana & Pandey 1993) and interpreted as resulting from an interference with the protein kinase A pathway (Schumacher *et al.* 1992); conversely, inhibitory effects of cGMP have also been described (LaPolt & Hong 1995) and related to a potential stimulation of soluble guanylate cyclases by NO (Welch *et al.* 1995, Grasselli *et al.* 2001, Ishimaru *et al.* 2001). However, in numerous other cases, it has also been observed that cGMP has no significant effect on steroidogenesis in gonads and that NO is able to play an inhibiting role alone, directly at the level of P-450 enzymes (Van Voorhis *et al.* 1994, Del Punta *et al.* 1996, Masuda

et al. 1997). In this complex context, the study of invertebrate models may be of great interest for a comparative point of view.

In arthropods, steroid hormones, known as ecdysteroids or molting hormones, play a pivotal role not only in the control of embryonic and postembryonic development, but also in the regulation of their reproductive physiology. In particular, ecdysteroids are known to stimulate vitellogenin biosynthesis in the fat body of higher Diptera (for review see Hagedorn 1985). Biosynthesis of these hormones occurs in specialized glands known as molting glands, but also in several other sources (for review see Delbecque *et al.* 1990) and particularly in gonads, as demonstrated in adult insects (Hagedorn *et al.* 1975, Loeb *et al.* 1982). In insect molting glands (also known as prothoracic glands), steroidogenesis is mainly regulated by stimulatory brain peptides acting through calcium and cAMP (for review see Gilbert *et al.* 1996), whereas in crustacean molting glands (also known as Y-organs), it is negatively regulated by molt-inhibiting hormones (MIHs), which seem to act through either cGMP or cAMP (Sedlmeier & Fenrich 1993, Spaziani *et al.* 1999).

In our previous studies on the control of steroidogenesis and vitellogenesis in our model, the blowfly *Phormia regina*, we have demonstrated that ovarian biosynthesis of ecdysteroids is stimulated by several (unidentified) brain factors, triggering either cAMP-dependent or cAMP-independent signaling cascades (Manière *et al.* 2000) and is also subjected to an unusual inhibitory control by calcium (Manière *et al.* 2002). During these investigations, we also observed that 3-isobutyl-1-methylxanthine (IBMX), a broad spectrum inhibitor of phosphodiesterases (PDEs), failed to mimic the steroidogenic effects of cAMP analogs or of forskolin, whereas some specific PDE inhibitors were able to do so (Manière *et al.* 2002). Attempts to elucidate this paradox have led us to the hypothesis that IBMX could have simultaneously two opposite pharmacological effects on steroid biosynthesis in blowfly ovaries, a stimulatory effect via the cAMP-signaling pathway and an inhibitory effect, possibly via cGMP involvement. The following study was thus undertaken to test this hypothesis and to investigate the potential role of cGMP in the control of ovarian steroidogenesis in blowflies.

Materials and Methods

Animals

Phormia maggots were regularly purchased from La Verminière de l'Ouest (Tremblay, France) and reared to adulthood as previously described (Manière *et al.* 2000). Adult blowflies (both sexes together) were maintained in controlled conditions (25 °C, 16 h light:8 h darkness cycle) and supplied with only sugar and water, this diet being known to prevent females from undertaking vitellogenesis and their oocytes from growing. Our experiments were

performed on such sugar-fed females (named hereafter previtellogenic females), 5 to 7 days after adult eclosion, or on females which, after a similar sugar diet for 5–7 days, received a single protein meal (bovine or porcine liver) for 1 h or more; such a diet was necessary and sufficient to induce steroidogenesis and vitellogenesis. Liver-fed females were then named vitellogenic females and, unless otherwise stated, they were generally used 24 h after the meal. For more details, see Manière *et al.* (2000). Before any operation, flies were first anesthetized with CO₂ and then maintained on crushed ice.

In vitro experiments

Ovaries from the same pair were extirpated under sterile conditions, rinsed four times and incubated separately in 50 µl culture medium, using multiwell plates. Incubations were made at 26 ± 1 °C in Grace's insect culture medium (Gibco, Life Technologies-France, Cergy-Pontoise, France). Some experiments were also carried out using a defined saline solution without calcium (128 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM Na₂HPO₄, 0.34 mM KH₂PO₄, 26.8 mM glucose, see Manière *et al.* 2002). Our biotests were made as previously described (Manière *et al.* 2000): in general, one ovary from an individual pair was tested for its response to a precise pharmacological treatment, whereas the contralateral ovary was used as its corresponding untreated control. These ovaries were generally named, respectively, treated and control ovaries. In some cases, two ovaries from the same pair were incubated separately in pure medium and were designated as blanks. After an appropriate incubation time (1 h for cyclic nucleotides, 3 h for ecdysteroids in order to take account of their slower anabolism), ovaries were removed for the individual determination of cAMP and cGMP concentrations, whereas ecdysteroids were measured in incubation media.

Preparation of crude brain extracts was made as previously described (Manière *et al.* 2000): adult 'brains' (defined here as the whole cephalic nervous mass, including subesophageal ganglion) were extirpated under sterile conditions, either from previtellogenic or vitellogenic females. Pools of approximately 50 brains were homogenized in Grace's insect culture medium (at a final concentration of three brain equivalents in 50 µl medium, i.e. per culture) and were centrifuged; then the supernatants were filtered on 0.2 µm sterile cartridges (Millipore, St Quentin en Yvelines, France).

Chemicals

All chemicals used in this study were purchased from Sigma (distributed by Sigma-Aldrich-France, St Quentin Fallavier, France), except 4-{(3',4'-(methylenedioxy)benzyl)amino}-6-methoxy-quinazoline (MDBAMQ) from Calbiochem (France Biochem,

Meudon, France) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) from RBI (distributed by Sigma-Aldrich-France). When necessary, chemicals were previously dissolved as concentrated stock solutions in dimethyl sulfoxide or 50% ethanol, then appropriately diluted into the culture medium: in these cases, a similar amount of solvent was added in the corresponding control incubations.

Immunoassays

Ecdysteroids, cAMP and cGMP were measured using immunoassays. Ecdysteroids were measured as previously described (Manière *et al.* 2000), from the incubation medium of a single ovary, by enzyme immunoassay using L2 antibody. This polyclonal antibody, raised against an ecdysone conjugate, is particularly convenient for ecdysone measurements (Pascual *et al.* 1995); it was chosen as being the most suitable for our experiments among various other antibodies with different specificities (authors' unpublished observations). Results were obtained by comparison with calibrated solutions of ecdysone and thus expressed as fmol ecdysone equivalents per culture. Taking account of sample dilution (which was made in order to measure them several times if necessary), the inferior limit of sensitivity of our assay was approximately 2 fmol/ovary.

cAMP was also measured as previously described (Manière *et al.* 2000), after extraction of a single incubated ovary and acetylation of the extract, using the immunoassay of Kingan (1989). Results were obtained as fmol/ovary. The inferior limit of sensitivity of this method was approximately 1–2 fmol/ovary.

cGMP was measured using RPN-226 enzyme immunoassay kits from Amersham-Pharmacia (Orsay, France). The extraction and acetylation of extracts were similar to those used with cAMP (Kingan 1989). The rest of the protocol followed the recommendations given with the commercial kits. Results were also obtained as fmol/ovary. The inferior limit of sensitivity of this method was also 1–2 fmol/ovary.

Presentation of results and statistics

The majority of the results obtained after immunoassay measurements are presented as means ($\pm$ S.E.) calculated from the individual ratios obtained by dividing the concentration obtained from one ovary with that obtained from the corresponding contralateral one. Such concentration ratios (or treated/control ratios), independent of the size of flies (or of ovaries) and of incubation times, appeared more convenient than absolute amounts for comparisons between various experiments (see Manière *et al.* 2000, for more details). Statistical analysis was

generally made using Student's *t*-tests by comparing means of concentration ratios to the theoretical value of 1 (equivalent to a paired test).

Results

Effects of IBMX and of cGMP-mimicking compounds

In order to confirm our hypothesis according to which IBMX could activate simultaneously both cyclic nucleotide-signaling cascades in our model, measurements of cAMP and cGMP, together with ecdysteroids, were performed from blowfly previtellogenic ovaries explanted *in vitro*, in the absence or the presence of IBMX. At first, blanks in the absence of IBMX verified an important property of this model: when explanted separately *in vitro*, previtellogenic ovaries from the same pair (Fig. 1A) maintained very similar concentrations of cGMP, as well as of cAMP, and secreted the same amounts of ecdysteroids in the medium (means of individual concentration ratios between ovaries of the same pair were not significantly different from 1, Student's *t*-test, $P>0.05$). Then, when similar ovaries (one per pair) were individually treated with IBMX (10^{-3} M) and compared with their respective contralateral untreated ovaries (Fig. 1B), they presented a significant increase of both cyclic nucleotides (ratios significantly higher than 1, Student's *t*-test, $P<0.05$), whereas ecdysteroid secretion remained unchanged (ratio not significantly different from 1, $P>0.05$).

In order to investigate the potential role of cGMP, a cGMP analog, chloro-phenyl-thio-cGMP (CPT-cGMP), was tested on previtellogenic ovaries (explanted from sugar-fed females), as well as on vitellogenic ones (explanted from liver-fed females 24 h after the beginning of the protein meal). Results, given in Fig. 2A, show that this cGMP analog had a significant inhibitory effect on steroidogenesis (at 10^{-5} and 10^{-4} M) in both previtellogenic and vitellogenic ovaries. Blank experiments (results given at concentrations 0) also verified that both ovaries from similar flies, either in previtellogenesis or in vitellogenesis, secreted the same quantities of ecdysteroids (ratios not significantly different from 1, $P>0.05$). Then, experiments with a selective inhibitor of cGMP-specific (type V) phosphodiesterases, MDBAMQ (Takase *et al.* 1994), were performed on previtellogenic as well as on vitellogenic ovaries. Figure 2B shows that this specific inhibitor, unlike IBMX, reduced ecdysteroid secretion (at 10^{-5} and 10^{-4} M) very efficiently in vitellogenic and, to a lesser extent, in previtellogenic ovaries.

CTP-cGMP and, more efficiently, MDBAMQ (Fig. 3) were also able to reduce the stimulation of ovarian ecdysteroid biosynthesis triggered by a cAMP-mimicking compound such as forskolin (used here at the optimal concentration of 10^{-5} M, see dose-response curve in Manière *et al.* 2000). Interestingly, a capacity to inhibit steroidogenesis was also revealed for IBMX in the

Figure 1 Effects of the absence (A) or the presence (B) of IBMX on ecdysteroid, cAMP and cGMP biosyntheses by previtellogenic ovaries *in vitro*. Ovaries were incubated in Grace's medium for 1 h for cAMP and cGMP measurements or 3 h for ecdysteroid measurements. Each bar represents a mean $\pm$ S.E.M. obtained from six to eight pairs of ovaries. The set without IBMX (A) was made to verify whether ecdysteroids, cAMP and cGMP were synthesized in equivalent amounts in both ovaries of the same pair. For IBMX treatment (B), 10^{-3} M IBMX was added to one ovary of each pair and the contralateral one was used as its corresponding untreated control. Results (ordinate) are expressed by dividing ecdysteroid quantities secreted by the two ovaries of the same pair for series A, in order to obtain treated/control ratios for series B. The ordinate represents the means of these ratios and asterisks above error bars (standard errors) indicate results significantly different from the theoretical value of 1 (Student's *t*-test, $P < 0.05$). When expressed in absolute amounts, ecdysteroids secreted into the medium after a 3-h incubation of untreated ovaries (controls) represent 11.3 ± 2.5 fmol ecdysone equivalents per ovary ($n=8$), whereas internal concentrations of cyclic nucleotides (after 1 h of incubation) represent 12.8 ± 3.7 fmol cAMP per ovary ($n=8$) and 13.5 ± 1.8 fmol cGMP per ovary ($n=8$).

presence of forskolin: IBMX (at 10^{-3} M) even appeared to be as efficient as 10^{-4} M CTP-cGMP in reducing forskolin stimulation. Altogether, these observations suggested that an inhibitory effect of IBMX on ovarian steroidogenesis, obviously due to a rise in cGMP concentrations, can be observed in particular conditions such as an excess of cAMP, but that this effect is not visible when cAMP and cGMP concentrations rise with a similar intensity.

Variations in cGMP concentrations during the vitellogenic cycle

Time correlations between variations in ovarian concentrations of cGMP and changes in ecdysteroid biosynthesis were examined after a protein meal. In particular, comparisons were made between the cGMP content of indi-

Figure 2 Effect of (A) CTP-cGMP, a cGMP analog, and of (B) MDBAMQ, a selective PDE V inhibitor, on ecdysteroid biosynthesis (treated/control ratios) by previtellogenic (broken lines) and vitellogenic (solid lines) ovaries, after 3 h of incubation. Each point represents the mean $\pm$ S.E.M. obtained from seven to fifteen pairs of ovaries: from each female fly, one ovary of the pair was incubated with a known (or null for blanks) concentration (in μ M) of respective pharmacological compounds, whereas the contralateral one was used as its corresponding untreated control. Asterisks above error bars indicate results significantly different from the theoretical value of 1 (Student's *t*-test, $P < 0.05$).

vidual ovaries (one per pair) at the time of extirpation (made at different times after a liver meal) and the steroidogenic capacity of corresponding contralateral ovaries, incubated for 3 h *in vitro*: results (Fig. 4) showed that a peak in ovarian cGMP concentrations at the end of the vitellogenic cycle was associated with a dramatic decrease in steroidogenic capacity *in vitro*.

Correlations between cGMP and cAMP concentrations were also examined at the beginning of the vitellogenic cycle, our previous studies having established that the stimulation of steroidogenesis is associated with a peak in cAMP concentrations during the first 3 h after a protein meal, then followed by a return to basal levels during the rest of the cycle (Manière *et al.* 2000). Results (Fig. 5) demonstrated that no drop of cGMP was associated with

Figure 3 Effects of forskolin (10^{-5} M), either alone or in combination with CTP-cGMP (10^{-4} M), MDBAMQ (10^{-4} M) or IBMX (10^{-3} M), on ecdysteroid secretions by previtellogenic ovaries incubated for 3 h in Grace's insect culture medium. Each bar represents a mean $\pm$ S.E.M. obtained from eight pairs of ovaries: from each female fly, pharmaceutical compound(s) was added to one ovary of the pair, whereas the contralateral one was used as its corresponding untreated control. Ecdysteroids were measured by immunoassay for each ovary. Results are expressed on a relative scale, as treated/control ratios (ordinate). A series of blanks was also incubated without any drug addition (blanks) to verify the regularity of ecdysteroid secretions between untreated ovaries extirpated from similar flies. Asterisks above error bars indicate results significantly different from the theoretical value of 1 (Student's *t*-test, $P < 0.05$). When expressed in absolute amounts, ecdysteroids secreted after a 3-h incubation of untreated ovaries (blanks) represent 11.4 ± 1.1 fmol ecdysone equivalents per ovary ($n = 16$).

cAMP and ecdysteroid increases during the 3 h following a protein meal: unlike cAMP and ecdysteroids, cGMP values measured from samples taken 1 or 3 h after the protein meal did not significantly differ from those taken at time 0 (Student's *t*-test, $P > 0.05$).

cGMP targets

It is generally admitted (for reviews see Vaandrager & de Jonge 1996, Lucas *et al.* 2000) that major targets for cGMP include cGMP-regulated PDE isoforms, cyclic nucleotide-gated ion channels and/or protein kinase G (PKG). We have thus briefly examined these three possibilities. First, we have studied the possibility of a direct activation of a type II PDE (i.e. a cGMP-stimulated, cAMP-specific PDE), in order to investigate whether cGMP could exert its inhibitory effect by antagonizing cAMP signaling: for this purpose, experiments were performed with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), a potent inhibitor of type II PDEs (Mery *et al.* 1995, Podzuweit *et al.* 1995). Results showed that EHNA (tested from 10^{-6} to 10^{-4} M) was not able to increase cAMP and ecdysteroid biosyntheses in previtellogenic ovaries (for example, a dose

Figure 4 Temporal correlation between ovarian contents of cGMP, measured immediately after extirpation (expressed in fmol/ovary, right ordinate) and ecdysteroids secreted after an *in vitro* incubation of 3 h following extirpation (expressed in ecdysone equivalents per ovary, left ordinate). The abscissa represents the time of extirpation, expressed in hours after the beginning of a protein meal. Each point represents the mean $\pm$ S.E.M. from four to eight individual measurements.

Figure 5 Evolution during the 3 h following a protein meal (time of ovarian extirpation on the abscissa) of the ovarian quantities (expressed in fmol/ovary, ordinate) of both cyclic nucleotides (measured immediately after extirpation) and of ecdysteroids secreted in the culture medium (measured after an *in vitro* incubation of 3 h following extirpation). Each point represents the mean $\pm$ S.E.M. from five to eight individual ovaries.

of 10^{-4} M EHNA tested on five to seven pairs of ovaries gave a cAMP treated/control ratio of 0.84 ± 0.09 ($n = 5$) and an ecdysteroid treated/control ratio of 0.84 ± 0.15 ($n = 7$), results not significantly different from 1, $P > 0.05$). EHNA also failed to increase ecdysteroid biosynthesis after activation of previtellogenic ovaries by 10^{-5} M forskolin, as well as failing to prevent the inhibition of steroidogenesis by MDBAMQ (data not shown). Altogether, these results did not corroborate the hypothesis of a cGMP effect on cAMP concentrations via the involvement of PDE II. The possibility of cGMP action on a type III PDE (i.e. a

cGMP-inhibited PDE), though unlikely considering our other results, was nevertheless investigated using milrinone, a specific inhibitor of PDE III: this compound also failed to alter ecdysteroid biosynthesis (data not shown), thus suggesting an absence of control of cGMP on PDEs in blowfly ovary.

The possibility of an action of cGMP on cyclic nucleotide-gated calcium channels was then examined. Considering that, in blowfly ovary, contrary to what happens generally in other models, an influx of external calcium is able to decrease cAMP concentrations into steroidogenic cells by activating a calmodulin-sensitive PDE (Manière *et al.* 2002), an indirect action of cGMP on cAMP concentrations via the regulation of calcium influx might have been suspected. However, CTP-cGMP and MDBAMQ were found to keep their inhibiting properties on steroidogenesis in spite of the incubation of blowfly ovaries in calcium-free saline (a treatment previously found to eliminate extracellular calcium trapped within the ovary; see Manière *et al.* 2002): for example, CTP-cGMP or MDBAMQ at 10^{-4} M reduced ecdysteroid treated/control ratios of previtellogenic ovaries to respectively 0.65 ± 0.08 ($n=7$) or 0.32 ± 0.09 ($n=8$) in calcium-free saline. Such values were significantly different from 1 (Student's *t*-test, $P<0.05$), but not significantly different from those observed in a calcium-containing medium (see Fig. 2 at 100 μ M). Thus, these results showed that cGMP does not act on steroidogenesis through the activation of a cyclic nucleotide-gated calcium channel.

Lastly, experiments were carried out to investigate the involvement of PKG. By adding a specific PKG inhibitor, KT 5823, a significant increase of ecdysteroid biosynthesis was triggered in previtellogenic and, more particularly, in vitellogenic ovaries (Table 1). Together, these results thus showed that PKG is the main, if not unique, target of cGMP for the regulation of ovarian steroidogenesis.

Potential cGMP activators

Experiments were then carried out to examine some possible factors which could activate the cGMP-signaling pathway. First, the ability of brain factors to stimulate cGMP biosynthesis was tested on ovaries incubated *in vitro*. For this purpose, crude brain extracts were prepared, either from previtellogenic or vitellogenic females, and added to *in vitro* cultures of ovaries also obtained from both types of females. Table 2 shows that, whatever the stage combination between ovaries and brain extracts, no significant effect was observed on ovarian cGMP biosynthesis (note that, in these experiments, the effects on ovarian ecdysteroid secretion were not examined, as it has been previously demonstrated that similar brain extracts strongly activate cAMP and ecdysteroid biosyntheses, see Manière *et al.* 2000). Our experiments thus showed that fly brain probably does not elaborate ecdysteroidostatic neuro-hormones acting on ovaries via cGMP, and consequently

Table 1 Effect of KT 5823, a PKG inhibitor, on ecdysteroid biosynthesis in previtellogenic (sugar-fed) and vitellogenic (liver-fed, 24 h after meal) flies. Each concentration of inhibitor was tested on 5 to 17 (*n*) flies. Ovaries of each pair were incubated separately in Grace's medium: one ovary was considered as a control, the other was given a known concentration of inhibitor (treated ovary). Using immunoassays, measurements of ecdysteroids were made after 3 h of incubation. Results are expressed as means $\pm$ S.E.M. of individual treated/control ratios

	KT 5823 concentrations	<i>n</i>	Ecdysteroids (treated/control ratios)
Ovary stage			
Previtellogenic	10^{-7} M	5	0.88 ± 0.31
	10^{-6} M	8	1.10 ± 0.29
	10^{-5} M	17	$1.61 \pm 0.29^*$
Vitellogenic	10^{-7} M	5	0.91 ± 0.31
	10^{-6} M	6	$1.66 \pm 0.25^*$
	10^{-5} M	10	$2.32 \pm 0.46^*$

*Results significantly different from the ideal value of 1 (Student's *t*-test, $P<0.05$).

suggest that this second messenger is rather activated by abdominal or intra-ovarian factor(s), probably involving a paracrine/autocrine mechanism.

As our previous observations had shown that calcium influx into ovarian cells (provoked by A23187 ionophore treatment) was a powerful inhibitor of steroidogenesis in our model (Manière *et al.* 2002), the following experiment was carried out to examine the effect of calcium on ovarian cGMP content. Vitellogenic ovaries were incubated with known concentrations of A23187 and their cGMP contents were compared with untreated corresponding controls. The results showed that A23187, at concentrations which drastically inhibited ecdysteroid secretion (from 10^{-5} M,

Table 2 Effect of crude brain extracts on cGMP biosynthesis by fly ovaries *in vitro*. Brain and ovaries were obtained from either previtellogenic (sugar-fed) or vitellogenic (liver-fed, 24 h after meal) flies. Crude brain extracts were added at a dose of three brain equivalents per 50 μ l culture medium (as previously described in Manière *et al.* 2000). Each line of results was obtained from the ovaries of four individual flies. From each fly, one ovary was incubated in the presence of brain extract (treated), the other in its absence (control). Incubation time: 1 h. Individual measurements of cGMP are expressed as treated/control ratios (means $\pm$ S.E.M.)

Brain extract stage	Ovary stage	cGMP biosynthesis (treated/control ratios)
Previtellogenic	Previtellogenic	1.21 ± 0.34
Previtellogenic	Vitellogenic	0.90 ± 0.23
Vitellogenic	Previtellogenic	1.14 ± 0.34
Vitellogenic	Vitellogenic	0.98 ± 0.10

Values did not significantly differ from the ideal value of 1 (Student's *t*-test, $P>0.05$).

Figure 6 *In vitro* effects of sodium nitroprusside (concentrations in μM on the abscissa) on (A) cGMP ovarian levels and (B) secretion of ecdysteroids, obtained from incubations of previtellogenic (broken lines) and vitellogenic (solid lines) ovaries. Incubation times: 1 h for cGMP, 3 h for ecdysteroids. Each point represents the mean ± S.E.M. of treated/control ratios obtained from five to thirteen pairs of ovaries. Asterisks indicate values significantly different from the theoretical value of 1 (Student's *t*-test, *P* < 0.05).

see Manière *et al.* 2002), did not significantly increase the ovarian levels of cGMP: for example, treatment with 10^{-5} M A23187 corresponded to a cGMP concentration ratio of 1.27 ± 0.30 ($n=8$) and treatment with 10^{-4} M to 1.44 ± 0.44 ($n=7$), ratios which were not significantly different from the theoretical value of 1 (Student's *t*-test, *P* > 0.05). These data thus suggest that cGMP is not involved in the regulation of steroidogenesis by calcium.

Lastly, the possibility of control of cGMP concentrations by NO was examined with experiments using sodium nitroprusside, a potent source of NO. When added to the incubation medium of previtellogenic or of vitellogenic ovaries (Fig. 6), this compound was found to significantly enhance cGMP biosynthesis (from 10^{-4} M) and also to inhibit ecdysteroidogenesis (from 10^{-5} M). Thus, a possible steroidostatic effect of NO via the involvement of cGMP can be hypothesized in blowfly ovary.

Discussion

The IBMX paradox and the regulation of steroidogenesis by cyclic nucleotides

The presence of cGMP in blowfly ovary and its capacity to inhibit ovarian steroidogenesis was revealed by the careful examination of the effects of IBMX. As this PDE inhibitor was able to increase the ovarian concentrations of both cyclic nucleotides in our model, the absence of its effect on steroidogenesis may not be explained by the presence of IBMX-insensitive PDEs, but suggests that the steroidogenic effect of cAMP is prevented by cGMP. Indeed, our present experiments, showing that a cGMP analog is able to inhibit ovarian steroidogenesis and that a specific PDE V inhibitor has a powerful steroidostatic effect, allow an explanation of the IBMX paradox by the opposite effects of both cyclic nucleotides. This interpretation is confirmed by the observation of an inhibitory capacity of IBMX (as well as of other cGMP-mimicking compounds) when associated with a cAMP-mimicking substance such as forskolin.

Thus, cGMP- and cAMP-signaling pathways constitute, in blowfly ovarian follicles, two opposite mechanisms of the control of steroidogenesis. The existence of such a regulation by cyclic nucleotides is described here for the first time in an invertebrate model. Indeed, in insect molting (prothoracic) glands for example, the cAMP pathway is generally known to have a stimulatory role (for review see Gilbert *et al.* 1996), though sometimes possible inhibitory effects (Henrich 1995, Hua & Koolman 1995). The presence of cGMP has not been detected so far in prothoracic glands (Vedeckis *et al.* 1976). Moreover, cGMP analogs seem to have weak or null effects (Smith *et al.* 1984, Hua & Koolman 1995) and IBMX induces the same responses as cAMP analogs. Another series of examples is found in crustacean molting glands where both cyclic nucleotides seem to have inhibitory effects (Sedlmeier & Fenrich 1993, Spaziani *et al.* 1999): indeed, they probably co-operate in inhibiting steroidogenesis (Saïdi *et al.* 1994) and act through a single protein kinase (Baghdassarian *et al.* 1996), so that the pharmacological effects of IBMX on steroidogenesis in crustacean molting glands seem to be always inhibitory. From the above comparisons (as well as from others, detailed below), the regulation of steroidogenesis appears to be more complex in blowfly ovaries than in insect or crustacean molting glands.

Possible activators of cGMP

Several peptidic factors have been reported to inhibit ecdysteroid biosynthesis in the molting glands of arthropods. Beside MIH in crustaceans (for review see Keller 1992), several prothoracicostatic factors (generally named ecdysostatins) have also been characterized in

insects (Hua *et al.* 1994, 1999). However, no peptide has been isolated so far for its capacity to inhibit steroidogenesis in the gonads of arthropods, though partial purifications have suggested that such factors exist in flies and are probably elaborated in the abdomen (Adams & Li 1998). Our own experiments, which failed to show a regulation of ovarian cGMP by brain extracts, suggest that a cerebral control is unlikely and support the hypothesis of paracrine or autocrine regulations.

In mammals, NO has been described as an autocrine/paracrine regulator of ovarian steroidogenesis (Van Voorhis *et al.* 1994). NO signaling has been observed in several insect tissues (for review see Davies 2000), but, to the best of our knowledge, not yet in gonads. The above results have shown that an NO donor can be a powerful activator of cGMP biosynthesis in *Phormia* ovaries, indicating the presence of an NO-sensitive soluble guanylate cyclase. Moreover, an NO donor is also an efficient inhibitor of ecdysteroidogenesis, which is in agreement with a regulation of ovarian steroidogenesis by NO-cGMP signaling. However, these results are not sufficient to definitively conclude that NO could physiologically regulate ecdysteroid biosynthesis via cGMP in blowfly ovaries. Indeed, it has been frequently observed in vertebrates that NO (and NO donors) can have inhibitory effects on steroidogenesis by acting directly on P-450 biosynthetic enzymes and not necessarily via the cGMP pathway (Van Voorhis *et al.* 1994). Our preliminary experiments in blowfly ovaries with an inhibitor of NO-sensitive soluble guanylate cyclases did not prevent alterations of ecdysteroid biosynthesis by NO (G Manière, unpublished observations). Moreover, it remains to be demonstrated in our model that NO is really produced near or inside ovarian follicles. Other preliminary experiments using the NADPH-diaphorase staining method (G Manière, unpublished observations) have indicated the presence of NO-synthase in the blowfly ovary, mainly located in a few egg chambers which prematurely aborted vitellogenesis, thus suggesting a possible role in the regulation of egg chamber degeneration (which participates in the control of the number of mature oocytes and may influence steroidogenesis). However, a more precise investigation of the sites and the timing of NO production, as well as of the location of soluble guanylate cyclase, is needed to better analyse the exact role of NO during ovarian ecdysteroidogenesis in *Phormia*.

Calcium is also frequently known to be a regulator of NO-synthase, as well as of guanylate cyclase (for review see Stuehr 1999, Lucas *et al.* 2000). In a previous study (Manière *et al.* 2002), we have postulated the existence of an ecdysteroidostatic factor acting on ovarian steroidogenesis via calcium signaling. It could be hypothesized that the same factor may also be responsible for the involvement of cGMP. However, our present experiments demonstrated, on one hand, that cGMP signaling remains active in a calcium-free medium (which makes improbable a regu-

lation of steroidogenesis by cGMP via the control of calcium influx) and, on the other hand, they also indicated that calcium influx has no effect on cGMP levels (which makes a control of cGMP concentrations by calcium unlikely). Thus, our results are not in agreement with the existence of a single negative regulation involving both calcium and cGMP, but suggest the presence of two separate inhibitory pathways. This hypothesis is reinforced by the fact that calcium inhibition is known, in our model, to alter the cAMP pathway (because of the involvement of a calcium-sensitive type I PDE, Manière *et al.* 2002), whereas our present results indicate that cGMP failed to regulate cAMP concentrations. Nevertheless, other investigations also remain necessary to better understand the relationships between calcium and cGMP in blowfly ovary.

cGMP during the reproductive cycle

Our present experiments clearly indicated that the cGMP-signaling system (possibly linked with NO) is always present and is able to react at any time during the reproductive cycle, in previtellogenic as well as in vitellogenic ovaries. Similarly, our previous observations on calcium inhibition have also demonstrated its efficiency in previtellogenic and in vitellogenic flies (Manière *et al.* 2002). Interestingly, this dual inhibitory control is opposed to (at least) a dual stimulatory regulation, involving not only a cAMP-dependent pathway but also a cAMP-independent mechanism (Manière *et al.* 2000), which seems to involve insulin-like signaling (G Manière & J-P Delbecq, unpublished observations). So, a minimum of four different types of regulatory pathways, at least two inhibitory (maybe more if NO constitutes a separate pathway) and two stimulatory, control ovarian steroidogenesis during the blowfly reproductive cycle.

In previtellogenic flies, these different regulations seem to maintain a complex equilibrium which keeps steroidogenesis at a basal but significant rate, from which it can be rapidly down- or up-regulated. Experiments with IBMX have shown that when two opposite mechanisms were put simultaneously into action with a similar intensity, they did not break this equilibrium. There is no doubt that, beyond the artefactual effects of IBMX, this capacity is probably very important in physiological conditions, as it may delay vitellogenesis under unfavorable conditions.

However, this equilibrium has to disappear for the stimulation of steroidogenesis and the consequential initiation of vitellogenesis. Indeed, at this step, a complete desynchronization was particularly observed for the two cyclic nucleotide pathways (nothing being presently known for the other mechanisms): our results indeed showed that cGMP concentrations remained constant during the stimulation of steroidogenesis, whereas cAMP levels increased significantly. This seems to be different from what is observed in crustacean molting glands, in which the stimulation of steroidogenesis needs the removal

of the inhibitory control by cyclic nucleotides (Spaziani *et al.* 1999), but appears to be in agreement with observations in the mammal reproductive cycle (Ling *et al.* 1980).

At the end of vitellogenesis, our experiments showed that cGMP reached its maximal levels, in correlation with the decline of steroidogenesis *in vitro*. A correlation was also observed *in vivo* between cGMP ovarian levels and hemolymph titers of ecdysteroids (authors' unpublished data). However, in this case, the decline in circulating ecdysteroids appeared to occur a few hours later than *in vitro*, approximately at the time of the cGMP peak. Such a delay is not surprising, because, as observed in the mosquito or in several other species (for review see Hagedorn 1989), including our model (authors' unpublished observations), stimulated ovaries generally secrete a precursor such as ecdysone into the hemolymph, then this precursor is transformed by other organs into the active hormone, 20-hydroxyecdysone, which is the main circulating and active hormone and which is then transformed into inactive (but immunodetected) metabolites. Thus, the delay between the peak of steroidogenesis *in vitro* and *in vivo* can be easily interpreted by the time necessary for these biochemical transformations and by the respective half-lives of these different ecdysteroids in the hemolymph.

The involvement of both cyclic nucleotides in the termination of normal vitellogenesis has been previously observed in the follicles of a lepidopteran species, *Hyalophora cecropia* and, more particularly, it was demonstrated that they regulate vitellogenin uptake (Wang & Telfer 1996). In *Phormia*, it seems unlikely that cAMP could play such a role due to its very low levels during this period (Manière *et al.* 2000), but our experiments strongly suggest that cGMP plays a major role in the termination of vitellogenesis by inhibiting ovarian steroidogenesis and, consequently, stopping the stimulation of vitellogenin biosynthesis by the fat body. Nevertheless, this interpretation does not exclude the possibility of a complementary role of cGMP on the control of vitellogenin uptake.

The very low levels of cAMP (Manière *et al.* 2000) at the time of the cGMP peak could also suggest that cGMP is able to down-regulate cAMP concentrations. However, such a possibility was not confirmed by our experiments, which failed to demonstrate that cGMP could stimulate cAMP degradation by specific PDEs. These arguments, however, must be considered with caution, because they are partly based upon the absence of effects of a pharmaceutical compound, EHNA, which is known to be active in mammals, but which has not been extensively studied in insects. Nevertheless, although cAMP and cGMP pathways appear to be antagonistic, no evidence has been obtained during this study that they really interact or alter each other's concentrations. However, as demonstrated previously for cAMP (Manière *et al.* 2000) and in this study for cGMP, both cyclic nucleotides seem to act essentially through their respective protein kinases: it is

thus probable that the antagonistic actions of the two signaling systems only intervene downstream from these kinases and not through a reciprocal regulation of their own levels.

Our data thus show that, contrary to the situation in previtellogenic flies, the two cyclic nucleotide-signaling pathways do not intervene simultaneously and symmetrically during vitellogenesis, but separately and alternatively: this difference in phase is undoubtedly essential for the regulation and completion of a normal vitellogenic cycle. Inversely, it can be hypothesized that a precocious activation of cGMP (or of NO or Ca^{2+} as well) before the completion of the reproductive cycle would probably result in an incomplete vitellogenesis. In this context, it would be interesting to verify the alterations of cAMP and cGMP pathways in blowflies with naturally or experimentally abortive vitellogenesis.

Finally, the involvement of cGMP in the regulation of steroidogenesis during the whole reproductive cycle appears unequivocal in blowfly ovary, in opposition to the complex situation observed in vertebrates (see Introduction). The involvement of NO in this regulation appears probable but remains to be fully demonstrated. Although it is premature to suggest that cGMP (and possibly NO) could have conserved their roles in the control of ovarian cycles during evolution, such a hypothesis deserves to be examined by further studies in other invertebrate models.

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