Calcium inhibits ovarian steroidogenesis in the blowfly *Phormia regina*

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Abstract

Calcium is frequently involved in the stimulation of steroidogenesis in gonads and endocrine glands, generally in association with cAMP. However, our present observations show that it has the opposite effect in the ovary of the blowfly *Phormia regina*. Our *in vitro* experiments first showed that extracellular calcium does not play a role during the stimulation of steroidogenesis in fly ovaries; indeed steroidogenesis was activated *in vitro* as efficiently in a medium with or without calcium, either by pharmacological compounds mimicking cAMP signaling or by active brain extracts. When calcium was experimentally introduced into biosynthetic cells by ionophores or liberated from internal stores by thapsigargin, it did not activate, but clearly inhibited both basal and acute steroidogenesis respectively in previtellogenic and in vitellogenic ovaries. Our experiments also demonstrated that calcium decreases cAMP concentrations in the ovaries of *Phormia*, by stimulating its degradation, without modifying its biosynthesis. Moreover, inhibitors of calcium–calmodulin phosphodiesterases (PDEs) increased steroid biosynthesis *in vitro*, whereas inhibitors of calcium-insensitive PDEs did not. These data thus demonstrate that, in blowfly ovaries, calcium ions inhibit cAMP-stimulated steroidogenesis by activating a calmodulin-sensitive (type I) PDE.

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Introduction

Steroid hormones are a very ancestral class of regulatory molecules, mainly involved in the control of development, homeostasis and/or reproduction in living organisms. Thus, a precise regulation of steroid biosynthesis is of crucial importance for the perpetuation of life. In animals, steroidogenesis is generally under the control of various regulators, principally peptidic hormones, which generally activate (or eventually inhibit) biosynthetic cells after binding to specific membrane receptors and triggering their corresponding transduction pathways. The two main second messengers involved in the control of steroidogenesis are cAMP and calcium ions, which frequently cooperate in the stimulation of biosynthetic cells, aside from several other independent mechanisms (for a recent review, see Cooke 1999). In many biosynthetic systems, external calcium is required for maximal stimulation of steroidogenesis by peptide hormones, and its entry into the cells via specialized membrane channels is necessary either before or after cAMP biosynthesis (Cooke 1999). Although calcium is frequently considered to have a permissive role in cAMP signaling, it has been also suggested to have its own effects, including genomic effects, as for example the ability to induce transcription of the steroidogenic acute regulatory protein (Cherradi *et al.* 1998).

Until now, our knowledge of insects was consistent with this overview. In these animals, as well as in many other invertebrates, the steroid hormones, also known as molting hormones or ecdysteroids, play a great role in the control of development (reviewed in Gilbert *et al.* 1996). In larvae, a brain neurohormone named PTTH (prothoracicotropic hormone) triggers the molting gland (frequently named the prothoracic gland) to synthesize and to secrete ecdysone and/or very close compounds such as 3-dehydroecdysone (Kiriishi *et al.* 1990) or 2-deoxyecdysone (Aribi *et al.* 1997). These ecdysteroids are the precursors of the main molting hormone, 20-hydroxyecdysone. It has been more particularly established in the larvae of the lepidopteran *Manduca sexta* that purified extracts of PTTH first lead to an influx of extracellular Ca^{2+} ions into molting gland cells, which activates a calcium-dependent adenylate cyclase; the

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resulting increase of cAMP then induces a cascade of protein phosphorylations, in particular that of the S6 ribosomal protein, which finally leads to the activation of steroidogenesis (reviewed in Gilbert *et al.* 1996). In such a model, the application of calcium ionophores to molting glands *in vitro* is sufficient to stimulate maximal steroidogenesis. The effect of purified brain extracts on external Ca^{2+} influx, also observed in *Galleria mellonella* (Birkenbeil 1996), has been recently confirmed in *Bombyx mori* (Gu *et al.* 1998) and *Manduca sexta* (Gilbert *et al.* 2000) using recombinant PTTHs.

In most insects, molting glands generally disappear in adults and animals do not molt any more. However, ecdysteroids remain present, with the same chemical structures as larval molting hormones, although generally in lower quantities; they are then essentially produced by the gonads and they exert a control on reproductive physiology (reviewed in Hagedorn 1985). Several peptidic hormones, reported to have a steroidogenic function in adult insects, have been identified and sequenced (Loeb *et al.* 1994, Girardie & Girardie 1996, Brown *et al.* 1998), but, particularly in females, their respective roles and their mode of action are still poorly understood.

Our previous investigations on ovarian ecdysteroidogenesis in the adult female blowfly *Phormia regina* (Manière *et al.* 2000), as well as studies from other researchers (Yin *et al.* 1990), have shown that this insect model is among the most suitable for the study of ovarian steroidogenesis. Indeed, it is very easy in this species to obtain previtellogenic or vitellogenic females, by changing their diet; previtellogenic flies, which only have a low (basal) biosynthesis of ovarian ecdysteroids, are maintained on sugar and water, whereas vitellogenic flies, characterized by an acute synthesis of ecdysteroids, can be induced by a single protein meal. Although the steroidogenic neurohormones have not yet been characterized in adult flies, crude brain extracts of *Phormia regina* have been demonstrated to stimulate acute ecdysteroid biosynthesis and to trigger both cAMP-dependent and -independent signaling mechanisms (Manière *et al.* 2000). The present study was originally started in order to verify the links between cAMP and calcium during the stimulation of steroidogenesis. However, our experiments progressively showed that, contrary to the generally accepted hypotheses, calcium was not involved in a positive regulation of ecdysteroid biosynthesis in blowflies, but in its inhibition.

Materials and Methods

Animals

Phormia regina maggots were regularly purchased from the firm La Verminière de l'Ouest (Tremblay, France). They were obtained as wandering (3rd instar) larvae, which pupated within 2–3 days in controlled conditions (25 °C, 16 h light:8 h darkness cycle), without needing any food supply, and which transformed into adults after 5–6 further days. Adult blowflies were then maintained under the same conditions and the two sexes were not separated; they were only supplied with sugar and water after adult eclosion, which prevented females from undertaking vitellogenesis and their oocytes from growing. Our experiments were performed on such sugar-fed females, 5–7 days after adult eclosion (named thereafter previtellogenic females), or on females which, after a similar sugar diet for 5–7 days, received a protein meal (liver) for 1 h or more (thus becoming vitellogenic females).

Before any operation, flies were first anesthetized with CO_2 and then maintained on crushed ice. A preparation of crude brain extracts was made as previously described (Manière *et al.* 2000).

In vitro experiments

Ovaries of the same pair were extirpated under sterile conditions, rinsed four times and then incubated separately in 50 µl culture medium, using multi-wells plates; incubations were made at 26 ± 1 °C, for 1–15 h, either in Grace's insect culture medium (Gibco) or in a defined saline solution (128 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM Na_2HPO_4, 0·34 mM KH_2PO_4, 1·83 mM $CaCl_2$, 25 mM glucose). The same saline without calcium ($CaCl_2$ replaced by glucose) was also used in some experiments. As a general rule, one ovary of an individual pair was tested for its response to a precise pharmacological treatment, whereas the contralateral ovary was used as the corresponding control. After incubation, the medium or the ovaries were removed for the determination of respectively ecdysteroid or cAMP concentrations.

Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich, except Ro-20-174 from Calbiochem (France Biochem, Meudon, France). When necessary, chemicals were previously dissolved as concentrated stock solutions in DMSO 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.

Ecdysteroid and cAMP measurements

Ecdysteroids and cAMP were measured using immunoassays, as previously described (Manière *et al.* 2000). Briefly, ecdysteroids were measured in the culture medium of a single ovary by enzyme immunoassay using the polyclonal L2 antibody, highly sensitive for ecdysone, the major ecdysteroid precursor secreted by blowfly ovaries. Results were expressed as fmol ecdysone-equivalents per culture.

Table 1 Effect of the presence or absence of extracellular calcium in the incubation medium on the *in vitro* ecdysteroid secretion of individual (previtellogenic) ovaries treated (or not) with CPT-cAMP (10^{-5} M) or forskolin (10^{-5} M). Ecdysteroids were measured in the culture media after 3 h of incubation at 26 °C. Each value is the mean ± S.E.M. obtained from eight separate ovaries

	Ecdysteroid secretion (fmol ecdysone equivalents/ovary)		
	Control ovaries	CPT-cAMP-treated ovaries	Forskolin-treated ovaries
With calcium	10·9 ± 1·3	18·2 ± 2·7	26·6 ± 2·4
Without calcium	10·7 ± 1·3	19·2 ± 2·4	27·4 ± 5·2

Values in the same column do not differ significantly ($P>0.05$, Student's *t*-test).

cAMP was measured, after extraction of a single incubated ovary and acetylation of the extract, using the immunoassay of Kingan (1989). Results were expressed as fmol per ovary.

Presentation of results and statistics

The majority of the results obtained after immunoassay measurements are presented as means ± S.E.M. calculated from the individual ratios obtained by dividing the result from a tested ovary with that of the corresponding contralateral control ovary (treated/control ratios). Such ratios appear more convenient than absolute amounts for comparisons between various experiments, because they are independent of several variable parameters, in particular the size of flies (or of ovaries) and the incubation time (for more details see Manière *et al.* 2000). Statistical analysis was generally made using Student's *t*-tests either by comparing two absolute mean values between them or by comparing means of treated/control ratios to the theoretical value of 1 (equivalent to a paired test).

Results

External Ca^{2+} ions are not necessary for the stimulation of ovarian steroidogenesis

Our first experiments were designed to test whether external calcium is necessary or not for the steroidogenic action elicited by cAMP signaling. For this purpose, the pharmacological agents CPT-cAMP (8-chlorophenylthio-cAMP, a cAMP analog) and forskolin (an activator of adenylate cyclase), known to stimulate ovarian steroidogenesis in previtellogenic (i.e. sugar-fed) flies (Manière *et al.* 2000), were tested in the presence or absence of calcium. Table 1 shows that these pharmacological compounds stimulated ecdysteroid biosynthesis very similarly in saline conditions with or without calcium (no significant difference using Student's *t*-test). As preliminary rinses and incubation in the medium without calcium probably eliminated extracellular ions that may have been trapped within the ovaries (evidence will be given below), our data suggest that the entry of calcium into biosynthetic cells is not necessary for steroidogenesis, at least downstream of adenylate cyclase.

In the absence of any known isolated fly neurohormone, complementary experiments were made with *Phormia* brain extracts, which have been previously shown to stimulate ovarian steroidogenesis *in vitro* by cAMP-dependent and -independent mechanisms (Manière *et al.* 2000). Previtellogenic ovaries (taken from sugar-fed female blowflies) were incubated with active brain extracts, in a saline solution with or without Ca^{2+}. Table 2 shows that the absence of calcium did not prevent these extracts from stimulating steroidogenesis very efficiently *in vitro*. Results are given either as means of absolute amounts per isolated ovary or as means of individual ratios, comparing the stimulated ovary with the unstimulated contralateral one (control); this second presentation allowed a better estimate of individual results and an easier comparison with other experiments (results given in this way are independent of the incubation time and of the ovary size). Although results suggested a slightly better stimulation without calcium, means were not significantly different using Student's *t*-test. These data thus indicate that entry of external calcium into ovarian biosynthetic cells is not required for steroidogenesis stimulation. Statistical analysis also established that basal ecdysteroid secretions (in controls without brain extracts) were not significantly different in the absence or the presence of calcium.

Calcium does not activate, but inhibits ovarian steroidogenesis

In order to better investigate the role of calcium in ovarian steroidogenesis in blowflies, pharmacological experiments were then performed on isolated ovaries, incubated either with ionophores (inducing calcium influx into

Table 2 Effect of the presence or absence of extracellular calcium in the incubation medium on the *in vitro* ecdysteroid secretion of individual (previtellogenic) ovaries treated (or not) with active brain extract. *n*=number of flies per experiment. From each fly, one ovary was incubated with brain extract (three brain-equivalents per culture), the other without (control), in the same sterile saline solution (either with or without calcium). Ecdysteroids were measured in the culture media after 15 h of incubation at 26 °C. Results are means ± S.E.M. of ecdysteroids secreted per ovary or means ± S.E.M. of individual experimental ratios (ratio of ecdysteroids secreted by the extract-treated ovary to that of the corresponding untreated control one)

		Ecdysteroid secretion (fmol ecdysone equivalents/ovary)		
	n	Control ovaries	Treated ovaries (incubated with brain extracts)	**Individual experimental ratios**
With calcium	7	34·2 ± 8·2	82·0 ± 15·6	2·9 ± 0·4
Without calcium	8	28·8 ± 5·0	101·2 ± 12·6	3·9 ± 0·5

Values in the same column do not differ significantly ($P>0·05$, Student's *t*-test).

steroidogenic cells) or with thapsigargin (liberating calcium from intracellular storage sites). These experiments were first made on previtellogenic ovaries, in which only basal steroidogenesis occurs *in vitro*. Results obtained using various concentrations of the calcium ionophore A23187 did not show a stimulation but, on the contrary, a dose-dependent decrease of basal steroidogenesis in the presence of external calcium, either in Grace's insect culture medium (Fig. 1A) or in an appropriate saline (Fig. 1B). However, such an inhibition was not observable in the corresponding calcium-free saline (Fig. 1C), which additionally also shows that incubation in such a saline (including the appropriate preliminary rinses) was sufficient to eliminate most of the extracellular Ca^{2+} ions possibly trapped within the explanted ovaries. Altogether, the data of Fig. 1 thus clearly demonstrate that calcium influx induced by ionophores does not activate ovarian steroidogenesis in blowflies, but significantly inhibits basal steroidogenesis *in vitro*; the inhibitory effect, seen only in calcium-containing media, was obviously not a toxic side-effect of A23187. A similar inhibition was also observed using another ionophore, ionomycin (not shown).

Moreover, this inhibition of steroidogenesis was not due to a lethal effect of an excess of calcium ions. Indeed, ovaries remained sensitive to further stimulation by pharmacological factors, forskolin for example; after a preliminary incubation for 3 h with A23187 and a very short rinse (5 min) in normal culture medium, previtellogenic ovaries stimulated by 10^{-5} M forskolin for 3 h produced 1·72 ± 0·13-fold more ecdysteroids than their contralateral corresponding controls ($n=14$; significant value in Student's *t*-test, $P<0·05$).

Thapsigargin, mobilizing intracellular calcium, also did not increase ecdysteroid production in previtellogenic ovaries, but significantly reduced basal ovarian steroidogenesis at doses of 10^{-5} and 10^{-4} M (Fig. 2), confirming that calcium, whatever its origin and the pharmacological agent which put it into play, is an inhibitor of basal ecdysteroid biosynthesis in blowfly ovaries.

The effect of Ca^{2+} ions, induced by ionophores or thapsigargin, was also examined in vitellogenic ovaries engaged in acute steroidogenesis. In the following experiments, ovarian extirpation was made 1 h after *in vivo* stimulation (made by giving a protein meal to females). Figure 3A shows that calcium influx triggered by A23187 dramatically decreased *in vitro* ecdysteroid secretion in a dose-dependent manner. Thapsigargin, mobilizing intracellular calcium, also had a similar, although less pronounced inhibitory effect during acute steroidogenesis (Fig. 3B). The inhibitory effect of ionophores or of thapsigargin, illustrated here with explants taken 1 h after a protein meal stimulation (this time was chosen because it corresponds to a peak of ovarian cAMP, according to our previous data, see Manière *et al.* (2000)), was also observed later, namely 5 and 12 h after a protein meal, although in a slightly less pronounced manner (not shown). Calcium is thus a potent inhibitor of the different phases of ecdysteroid biosynthesis in blowfly ovaries and possibly interferes with cAMP signaling.

Then, the effects of calcium were also examined on previtellogenic ovaries stimulated either by forskolin or by CTP-cAMP. Results, given in Table 3, first showed that calcium ionophores significantly reduced ecdysteroid biosynthesis stimulated by forskolin. However, they only induced a slight (but significant) decrease of ecdysteroidogenesis with CTP-cAMP. Similar results were obtained with thapsigargin (not shown). These data thus suggest that calcium is an inhibitor of cAMP-stimulated steroidogenesis, probably acting on cAMP biosynthesis and/or on its degradation.

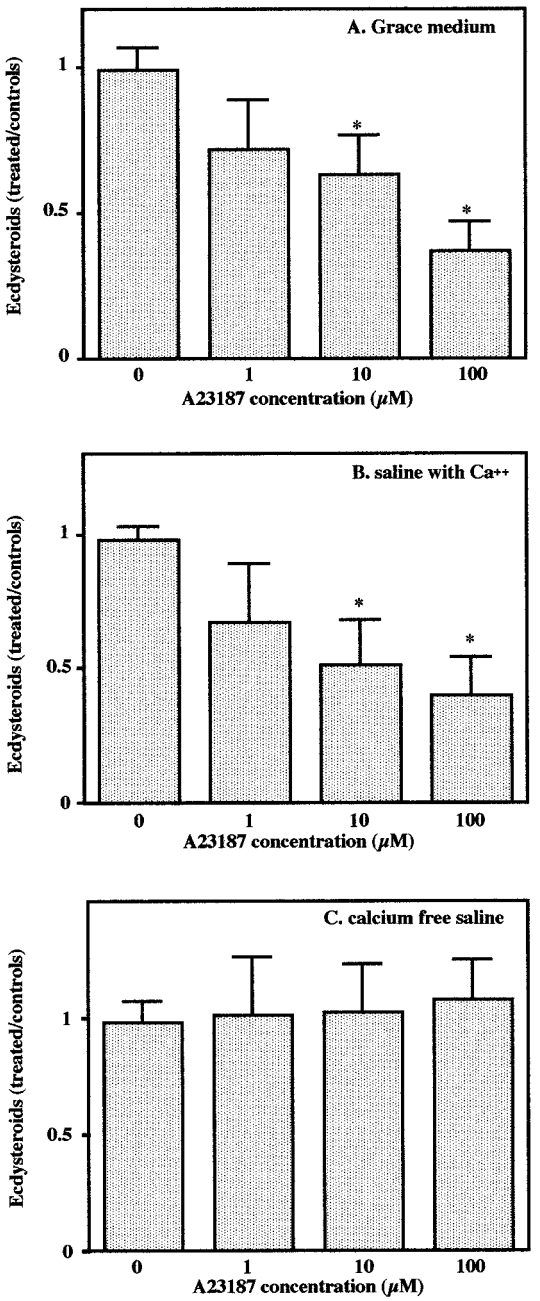

Figure 1 Effects of the ionophore A23187 at different concentrations on basal ecdysteroid secretion by previtellogenic ovaries *in vitro* (explanted from sugar-fed females). Ovaries were incubated for 3 h in (A) Grace's medium, (B) calcium-containing saline or (C) calcium-free saline. Means ± S.E.M. for six to eight ovary pairs; for each pair, one ovary had A23187 added and the contralateral one was used as its corresponding untreated control. Other controls without A23187 (concentration 0) were made on the two ovaries of the same pair to verify the similarity of ecdysteroid secretion in the series. Results are treated/control ratios, i.e. ecdysteroid secreted by a treated ovary divided by that of the untreated contralateral one. Asterisks indicate results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

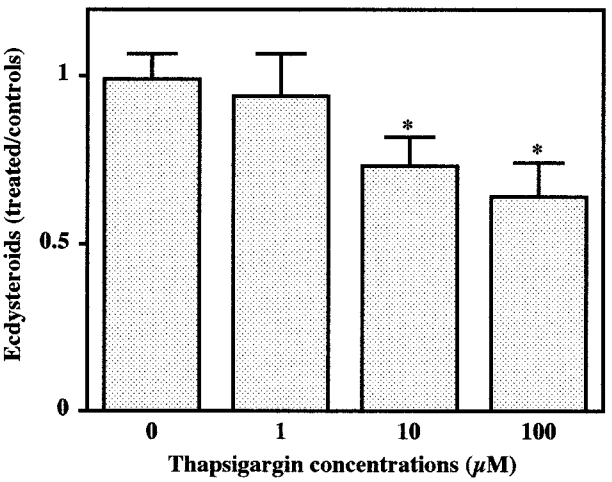

Figure 2 Effects of thapsigargin, an inhibitor of endoplasmic reticulum calcium-ATPase, at different concentrations, on basal ecdysteroid secretion by previtellogenic ovaries *in vitro* (explanted from sugar-fed females). Ovaries were incubated for 3 h in Grace's medium; for each pair, one ovary had thapsigargin added and the contralateral one was used as its corresponding untreated control. Other controls without thapsigargin (concentration 0) were made on the two ovaries of the same pair to verify the similarity of ecdysteroid secretion in the series. Results are treated/control ratios, i.e. ecdysteroid secreted by a treated ovary divided by that of the untreated contralateral one. Means ± S.E.M for six to eight ovary pairs. Asterisks indicate results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

Ca^{2+} interferes with cAMP signaling by increasing cAMP degradation

In order to better investigate whether calcium is able to influence cAMP concentrations, measurements of this cyclic nucleotide were performed in previtellogenic ovaries (explanted from sugar-fed blowflies) or in vitellogenic ones (explanted from 1 h liver-fed females) after incubation in Grace's culture medium with or without the ionophore A23187 (at two concentrations) and with the broad spectrum phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX). Results, given in Fig. 4, show that in previtellogenic, as well as in vitellogenic ovaries, the ionophore decreased cAMP concentrations in the absence of IBMX, but not in its presence. It can be inferred from these data that calcium acted on cAMP concentrations, not at the biosynthetic level (which appears unaffected in the presence of IBMX), but at the catabolic level (as observed in the absence of IBMX).

As the previous experiment suggested the involvement of specific enzymes, namely calcium–calmodulin-dependent PDEs (known as type I PDEs or PDE I), the effect of inhibitors of such PDEs, in the form of chlorpromazine (a calmodulin inhibitor) or 8-methoxymethyl-IBMX (a more specific PDE I inhibitor), was compared with that of some other PDE inhibitors. Results, given in Fig. 5, clearly indicated that chlorpromazine and

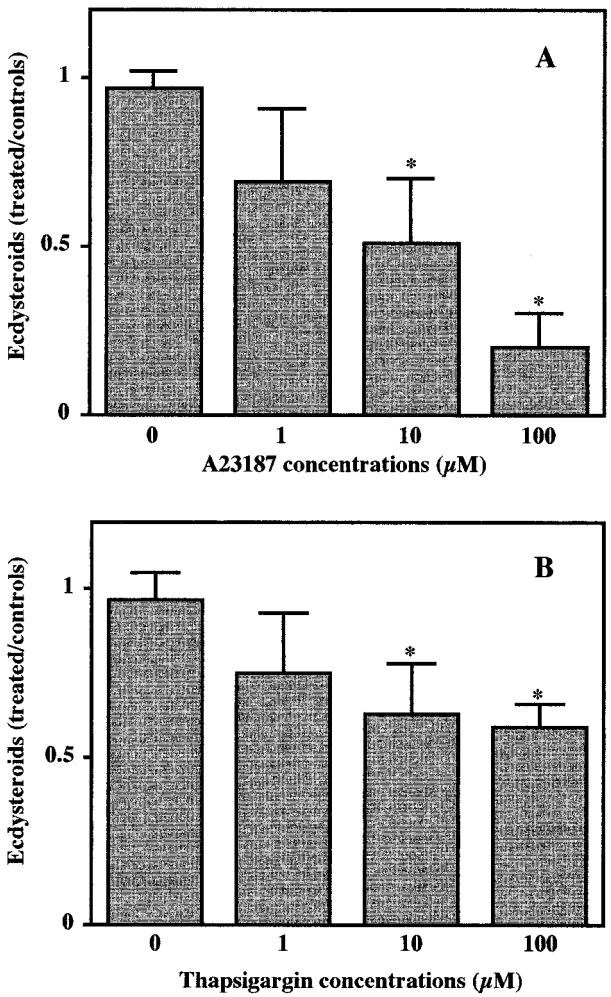

Figure 3 Effects of (A) A23187 ionophore and (B) thapsigargin, at different concentrations, on acute ecdysteroid secretion by vitellogenic ovaries *in vitro* (explanted from 1 h liver-fed females). Same protocols as Figs 1 and 2 respectively. Results are treated/control ratios, i.e. ecdysteroid secreted by a treated ovary divided by that of the untreated contralateral one. Means ± S.E.M. for six to eight ovary pairs. Asterisks indicate results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

8-methoxymethyl-IBMX were capable of increasing ecdysteroid secretion in previtellogenic ovaries *in vitro*. On the contrary, other PDE inhibitors, either active specifically on cAMP-specific, calcium–calmodulin-independent, PDEs (type IV), i.e. Ro-20-1724, or even active non-specifically on a wide range of PDEs, i.e. IBMX, were completely unable to increase ecdysteroid secretion. Other experiments (not shown), using various chemicals known to inhibit PDE II or III, also failed to modify ovarian steroidogenesis in the same conditions. Thus, among the different inhibitors tested, only those acting on calcium–calmodulin-dependent PDEs were found to increase ecdysteroid secretion, which strongly

Table 3 Effects of A23187 ionophore, used at two concentrations, on *in vitro* ecdysteroid secretion of previtellogenic ovaries stimulated by forskolin (10^{-5} M) or by CTP-cAMP (10^{-5} M). From each fly, one ovary was incubated in Grace's medium simultaneously with either forskolin or CTP-cAMP and a known (or null) concentration of A23187 ionophore, whereas the contralateral control ovary was only incubated with forskolin or CTP-cAMP. Ecdysteroids were measured in the culture media after 3 h of incubation at 26 °C. Results are means ± S.E.M. of individual experimental ratios (ratio of ecdysteroids secreted by each A23187-treated ovary to that of its corresponding contralateral one)

	Ecdysteroid (treated/control ratio)	
	With forskolin	With CTP-cAMP
A23187 concentration		
0 (controls)	1·00 ± 0·12 (n=8)	1·01 ± 0·18 (n=8)
10^{-5} M	0·78 ± 0·09 (n=8)*	1·03 ± 0·21 (n=11)
10^{-4} M	0·41 ± 0·07 (n=8)*	0·77 ± 0·09 (n=19)*

Asterisks indicate results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

supports the involvement of PDE I in the negative regulation of blowfly ovarian steroidogenesis.

Moreover, the effect of chlorpromazine and 8-methoxymethyl-IBMX was also investigated on previtellogenic ovaries stimulated by forskolin and on vitellogenic ones (stimulated after a protein meal). Table 4 shows the data obtained with 8-methoxymethyl-IBMX; those obtained with chlorpromazine (not shown) were quite similar. These compounds were found to enhance acute ovarian ecdysteroid secretion, whereas inhibitors of other PDE types, such as Ro-20-1724, had no effect in the same conditions (not shown).

PDE I inhibitors were also able to stimulate ecdysteroid biosynthesis in the presence of the calcium ionophore A23187 (Fig. 6); this experiment demonstrates that the inhibitory effects of calcium can be (at least partially) reversed by a PDE I block, confirming the involvement of this enzyme in the regulation of steroidogenesis, and, additionally, that calcium effects were not due to a peculiar toxicity to biosynthetic cells.

Finally, our last experiment also verified that PDE I inhibitors acted on ovarian ecdysteroidogenesis via cAMP signaling modifications (Fig. 7); indeed, their steroidogenic effect was significantly (although partly) reduced by Rp-cAMPS, a protein kinase A (PKA) inhibitor, which had no effect alone on previtellogenic ovaries (controls). These results thus confirm that PDE I participated in the regulation of the cAMP pathway, controlling acute as well as basal steroidogenesis in blowfly ovaries.

Discussion

It is now well established that the mechanisms which control steroidogenesis are complex and involve the

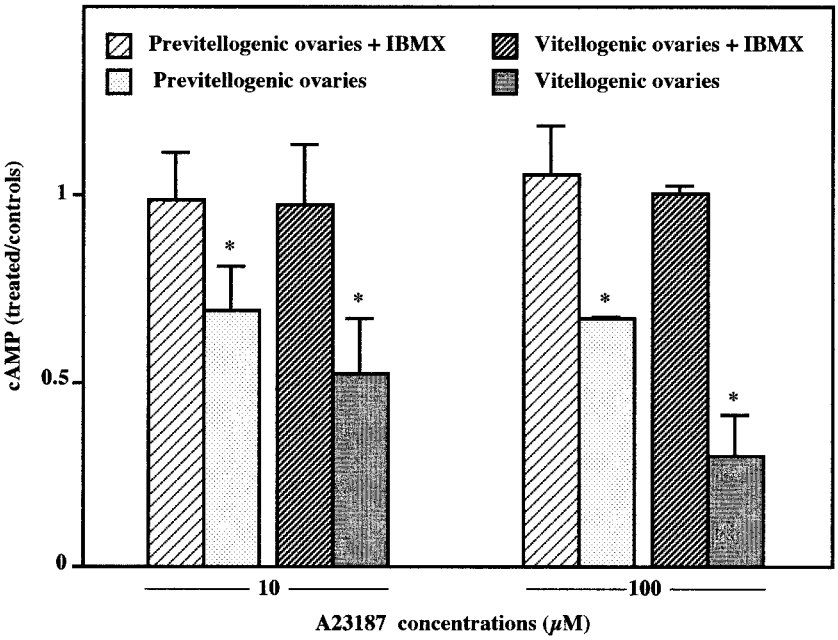

Figure 4 Effects of A23187 ionophore at two concentrations on cAMP content either in previtellogenic ovaries (sugar-fed females) or in vitellogenic ovaries (1 h liver-fed females), in the presence or absence of IBMX, a phosphodiesterase (PDE) inhibitor. Ovaries were incubated for 1 h in Grace's medium; for each pair, one ovary had A23187 added and the contralateral one was used as its corresponding untreated control. In the IBMX series, both ovaries of the same pair received the same amount of this PDE inhibitor. Results are treated/control ratios, i.e. cAMP secreted by an A23187-treated ovary divided by that of the untreated contralateral one. Means ± S.E.M. from eight ovary pairs. *Results significantly different from the theoretical values of 1 (Student's *t*-test, $P<0{\cdot}05$).

participation of several pathways, either stimulatory or inhibitory, which interact for a fine tuning of this vital process. Calcium is generally considered to participate in this regulation and to have a stimulatory role (reviewed in Cooke 1999). However, the present study in blowfly ovaries demonstrates that, contrary to this very frequent observation, calcium may also be a signal involved in the inhibition of steroid biosynthesis.

Calcium involvement in the control of ovarian steroidogenesis

Our experiments have first established that the presence of external calcium is not necessary for the acute stimulation of blowfly ovarian steroidogenesis. In a calcium-free medium, which was sufficient to wash away most of the extracellular Ca^{2+} ions (as seen in Fig. 1C), steroidogenesis was increased as efficiently as in calcium-containing media by pharmaceutical agents triggering the cAMP-signaling pathway or by active brain extracts (Tables 1 and 2). Since blowfly brain extracts stimulate ovarian steroidogenesis through both cAMP-dependent and -independent mechanisms, involving at least two different, but yet unknown, neurohormones (Manière *et al.* 2000), it can be concluded that these different pathways do not require calcium entry into steroidogenic cells. Concerning more particularly cAMP signaling, our data indicate that calcium influx is necessary neither before nor after the involvement of adenylate cyclase in blowfly ovaries. This is in opposition to numerous observations made in vertebrates as well as in insects; in vertebrates for example, testosterone production by Leydig cells is dramatically decreased in a calcium-free medium, after stimulation by luteinizing hormone or even after stimulation by cAMP analogs, which suggests that entry of calcium into steroidogenic cells is a post-cAMP event (Cooke 1999). In insects, as shown in Lepidoptera larvae, molting glands cannot be stimulated by the brain hormone PTTH to produce ecdysteroids in a calcium-free medium (Gilbert *et al.* 1996). Similarly, testes cannot be stimulated by active brain extracts containing testis ecdysiotropin without a low concentration of calcium in the culture medium (Loeb 1991). In molting glands, several studies have well established that PTTH stimulates steroidogenesis by triggering calcium influx into biosynthetic cells, which in turn stimulates a calcium–calmodulin-dependent adenylate cyclase (Gilbert *et al.* 1996); in such a case, calcium is thus a pre-cAMP event. The mode of action of PTTH on molting glands has not been studied in flies as extensively

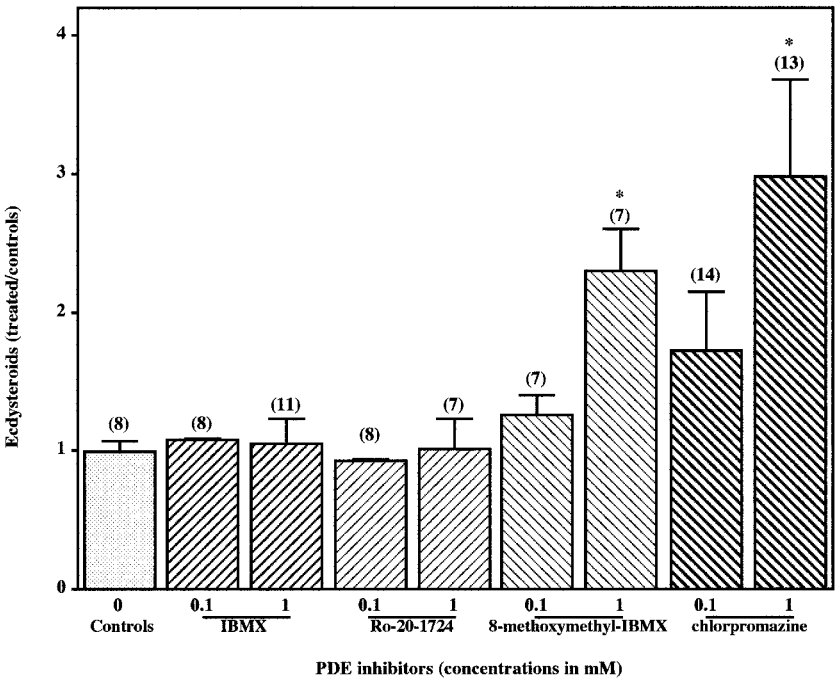

Figure 5 Effects of various PDE inhibitors (IBMX, Ro-20-1724, 8-methoxymethyl-IBMX and chlorpromazine), at two different concentrations, on ecdysteroid secretion by previtellogenic ovaries *in vitro* (explanted from sugar-fed females). Ovaries were incubated for 3 h in Grace's medium; for each pair, one ovary was treated with inhibitor, the contralateral remained untreated (control). Results are treated/control ratios. Means ± S.E.M. for (*n*) ovary pairs. *Results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

Table 4 Effect of 8-methoxymethyl-IBMX on acute ecdysteroid secretion in stimulated ovaries (either previtellogenic ovaries stimulated by forskolin *in vitro* or vitellogenic ovaries previously stimulated after a 1 h liver meal of the donor fly). *n*=number of flies per experiment. From each fly, one ovary was incubated in Grace's medium added with a known (or null) concentration of 8-methoxymethyl-IBMX, whereas the contralateral control ovary was incubated without this inhibitor. In the previtellogenic series, both ovaries from the same pair had 10^{-5} forskolin simultaneously added. Ecdysteroids were measured in the culture media after 3 h of incubation at 26 °C. Results are means ± S.E.M. of individual experimental ratios (ratio of ecdysteroids secreted by the 8-methoxymethyl-IBMX-treated ovary to that of the corresponding contralateral one)

	8-Methoxymethyl-IBMX	*n*	Ecdysteroid ratio
Ovary stage			
Previtellogenic	0	8	0·99 ± 0·03
(ovary stimulated by	10^{-4} M	14	1·41 ± 0·23
10^{-5} M forskolin *in vitro*)	10^{-3} M	14	2·88 ± 0·76*
Vitellogenic	0	8	0·97 ± 0·08
(female stimulated by	10^{-4} M	7	1·63 ± 0·64
liver meal)	10^{-3} M	8	2·19 ± 0·32*

*Results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

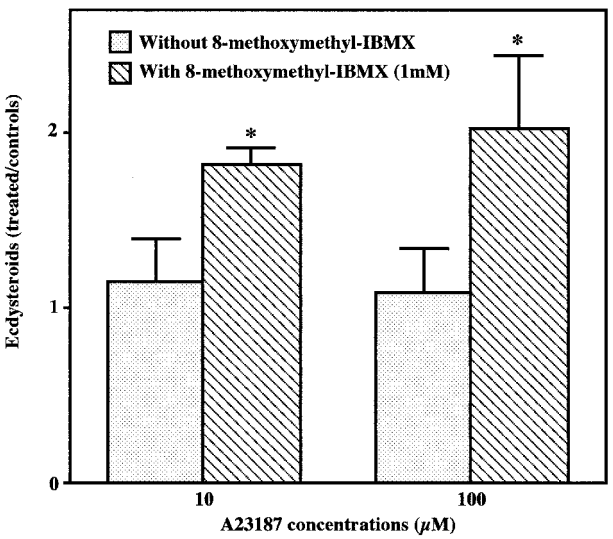

Figure 6 Combined effects of the PDE I inhibitor, 8-methoxymethyl-IBMX and of A23187 ionophore, at two concentrations, on ecdysteroid secretion *in vitro* (expressed as treated/control ratios). In this experiment, all ovaries received A23187 at a defined dose and only one ovary per pair received the PDE inhibitor. Incubation time 3 h. Means ± S.E.M. for 8 ovary pairs. *Results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

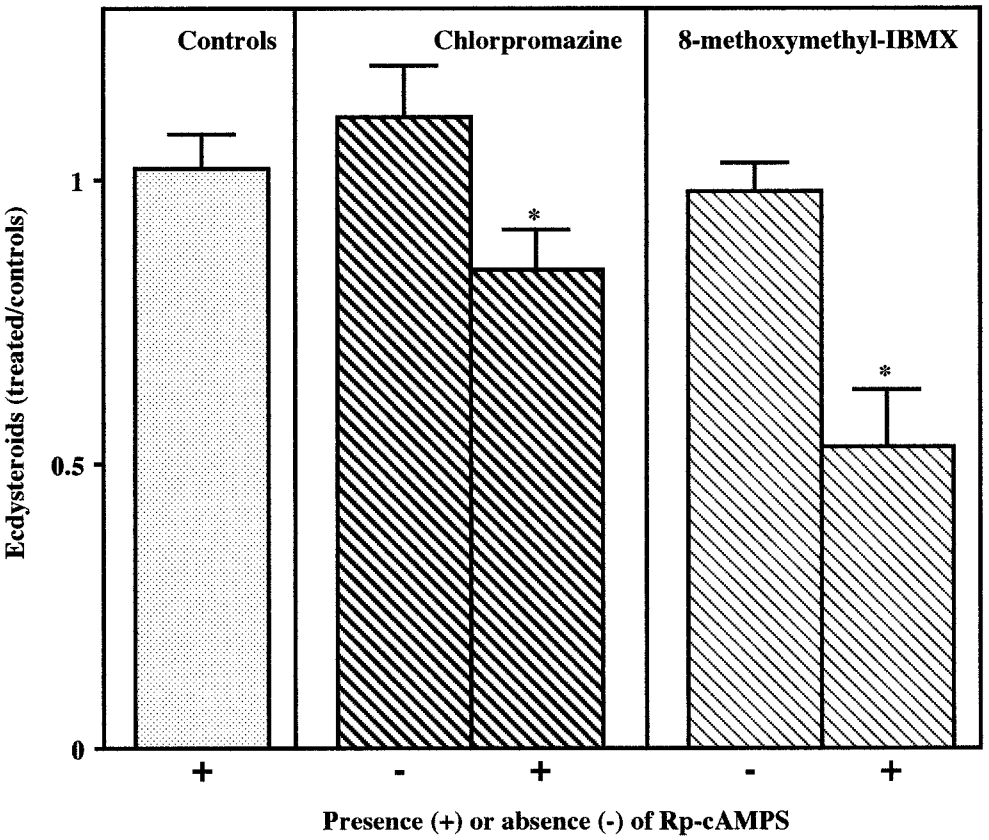

Figure 7 Effects of Rp-cAMPS, a protein kinase A antagonist, on the ecdysteroid secretion induced *in vitro* by PDE I inhibitors chlorpromazine (1 mM) and 8-methoxymethyl-IBMX (1 mM). PDE I inhibitors were applied to both ovaries of a same pair, the treated ovary being simultaneously incubated either in the absence (−) or in the presence (+) of Rp-cAMPS (1 mM), whereas the contralateral ovary remained always in the absence of Rp-cAMPS. In addition, controls (without PDE I inhibitor) were made to test the presence of Rp-cAMPS on one ovary against its absence in the contralateral one. Incubation time 3 h. Results are treated/control ratios, i.e. ecdysteroid secreted by a treated ovary divided by that of the untreated contralateral one. Means ± S.E.M. for seven or eight ovary pairs. *Results significantly different from the theoretical value of 1 (Student's *t*-test, $P<0{\cdot}05$).

as in Lepidoptera and may differ between different groups of insects; although the cAMP pathway appears to be inhibitory in *Calliphora* (Hua & Koolman 1995), it has also been observed that *Drosophila* molting glands require the presence of external calcium ions to respond to active brain extracts (Henrich 1995). These data thus suggest that, in Diptera, calcium could have a different role in the control of steroidogenesis in (larval) molting glands and in (adult) ovaries.

Our other observations also established that all experimental treatments mobilizing calcium in steroidogenic cells of blowfly ovaries, either by inducing its entry from the external medium or by liberating it from the intracellular stores, were strongly inhibitory for ecdysteroid biosynthesis, in opposition to what is generally observed in vertebrates (Cooke 1999) and also in insects, either in the molting glands, where ionophores have a strong steroidogenic property (Gilbert *et al.* 1996), or even in the testes, where they only have a slight stimulatory effect at low dose, but none at high dose (Loeb 1991).

One of our major cares during our study was to ascertain that the observed inhibitions in *Phormia* were not due to some artifactual toxicity either of the pharmacological agents used to mobilize calcium or of an excess of calcium itself. Indeed, several verifications were made which established, for example, that A23187 ionophore alone had no inhibitory effect on steroidogenesis in a calcium-deprived saline (Fig. 1C) and that ovaries remained sensitive to further stimulation by forskolin after incubation with ionophores. Other verifications (not shown), consisting of incubation of ovaries used in our various experiments with trypan blue (a dye which easily penetrates into dead cells, but not into live ones), did not show any increase of lethality in our various experiments.

Moreover, the fact that A23187 inhibited cAMP and steroid concentrations in the absence, but not in the presence, of PDE inhibitors (Figs 4 and 6) undoubtedly demonstrates that the ionophore and the mobilized calcium had no toxic effect by themselves in our conditions. It can thus be concluded from our experiments that calcium has a potential inhibitory function on blowfly ovarian steroidogenesis, but no role to play during its stimulation. Interestingly, our study also indicates that this strong inhibitory function can be efficacious both on basal and on acute ecdysteroid biosynthesis.

Calcium targets

Our experiments have also clearly established that calcium first triggers a decrease of blowfly ovarian cAMP content, in contrast to what has been observed in lepidopteran molting glands. As the existence of a calcium-inhibited adenylate cyclase has been recently documented in the central nervous system of *Drosophila* (Iourgenko & Levin 2000), the hypothesis of a calcium-inhibited cAMP biosynthesis should be taken into account in the blowfly ovary. However, our experiments using a broad spectrum PDE inhibitor (IBMX) have clearly shown that calcium (ionophore) does not modify cAMP biosynthesis in blowfly ovaries, but rather stimulates its degradation, and that the main, if not unique, target of calcium is located downstream of cAMP biosynthesis.

This finding was confirmed with more specific PDE inhibitors, which have also clearly indicated that the likely target of calcium in the cAMP signaling pathway is a calcium–calmodulin-dependent PDE (PDE I). Indeed, experiments with chlorpromazine, a calmodulin inhibitor, increased ecdysteroid biosynthesis in blowfly ovaries, demonstrating that calmodulin is involved in the inhibitory control of cAMP concentrations, during basal as well as acute steroidogenesis. Here again, the situation found in blowfly ovaries was opposite to what is generally accepted in other models, where calmodulin inhibitors usually decrease steroidogenesis (Hall *et al.* 1981, Cooke 1999). Our other experiments with a more specific inhibitor of PDE I, 8-methoxymethyl-IBMX, confirmed the involvement of this enzyme in the negative control of ovarian steroidogenesis. Moreover, among the various inhibitors of cAMP-selective PDEs that we tested, only those acting on PDE I appeared able to increase ecdysteroid concentrations. Even the broad-spectrum PDE inhibitor IBMX, although able to increase ovarian cAMP concentrations, was unable to increase ecdysteroid secretion; this surprising result, already observed in our previous study (Manière *et al.* 2000), can possibly be explained by the interaction of this non-selective inhibitor with both cAMP and cGMP signaling pathways, this latter being also involved in steroidogenesis inhibition, in probable synergy with calcium (Manière G & Delbecque JP, unpublished observations).

The involvement of PDE I was also confirmed by the results of Table 3, in which calcium ionophores show a greater inhibitory effect on acute steroidogenesis triggered by forskolin than by CTP-cAMP; indeed, this cAMP analog, which is much more resistant than cAMP to degradation by PDEs, is thus relatively protected from the effects of calcium. However, in spite of this resistance, a significant decrease of ecdysteroid biosynthesis persisted with CTP-cAMP, which probably suggests that calcium may have complementary targets. Other results have also shown that PDE I inhibitors were able to prevent, at least partly, the inhibitory effect of calcium ionophores and that their steroidogenic effect was itself, at least partly, mediated by PKA (Figs 6 and 7). These data thus confirm the involvement of PDE I in the control of blowfly ovarian steroidogenesis, but are again in agreement with the possibility that, together with its effects on cAMP degradation, calcium could also have complementary inhibitory roles, either downstream in the cAMP signaling cascade or in other pathways. As the control of ovarian steroidogenesis in blowflies involves at least two distinct signaling mechanisms (Manière *et al.* 2000), it remains possible that calcium could be also active on the cAMP-independent pathway or on other frequently involved targets, as for example protein kinase C (PKC). However, our preliminary experiments (Manière G & Delbecque JP, unpublished observations) with PKC activators did not argue for a major role of this signaling pathway in our model.

Control of calcium release

The main questions arising from the present study are due to our ignorance of the possible mechanism(s) by which calcium could be put into action in blowfly ovaries. In particular, nothing is known about the origin (extra- or intra-cellular?) of Ca^{2+} ions involved in this regulation and of the factors which lead to their mobilization; such factors could either be (neuro)hormones coming from (neuro)-endocrine centers, or various local paracrine factors regulating the interaction of the different cell categories, including oocytes, which constitute the ovary. In insects, several peptides, generally named ecdysiostatins, have already been described as having an inhibitory effect on steroid biosynthesis in the molting (or prothoracic) glands of larvae. Such peptides may come from the insect brain, as demonstrated in blowflies (Hua *et al.* 1997) and in *Bombyx* (Hua *et al.* 1999), but also from other organs, including the ovary itself, which is, for example, known to secrete TMOF (trypsin modulating oostatic factor), a powerful inhibitor of molting gland steroidogenesis (Hua *et al.* 1994). However, a possible inhibitory role of these ecdysiostatins has never been observed on adult sources of ecdysteroids (Bylemans *et al.* 1995) and our own experiments (Manière G, unpublished observations) with TMOF have also failed to show a significant effect on

Phormia ovaries. Yet, an ovarian ecdysteroidostatin has been partially purified from the abdomen of the adult house fly, *Musca domestica*, differing from TMOF, which efficiently blocks steroid biosynthesis in vitellogenic ovaries (Adams & Li 1998). The mode of action of this factor is still unknown, but it seems quite possible that calcium could be involved in its signaling pathway.

Mobilization of calcium by putative ecdysteroidostatic factors acting on ovaries might involve either calcium channels, triggering the influx of external calcium, or a complex transduction cascade in which 1,4,5-inositol trisphosphate (IP3) leads to the liberation of calcium from intracellular stores. Several studies have already demonstrated the involvement of calcium channels in the stimulation of steroidogenesis, either in insects (Gilbert *et al.* 1996) or in vertebrates (Cooke 1999), but to the best of our knowledge, never in its inhibition. Similarly, the involvement of IP3 in the stimulation of steroidogenesis has also been frequently demonstrated in several vertebrate models (Cooke 1999) or in crustaceans and insect molting glands (Mattson & Spaziani 1987, Venkatesh & Hasan 1997), as well as in lepidopteran testis (Loeb *et al.* 2001). Here again, however, this signaling pathway has never been put forward as a mechanism able to inhibit steroidogenesis. Because of the lack of information in adult insect females, further investigations are thus needed to identify the ecdysteroidostatic peptides active on ovaries, to determine their origin and to study their mode of action, particularly their possible interaction(s) with calcium.

The data obtained here in blowflies, as surprising as they looked at first, may, however, not represent a single exception; indeed, a few studies have also observed some inhibitory effects of calcium, more particularly related to female reproductive physiology, in the brook trout (Planas *et al.* 1997) and the domestic hen (Levorse *et al.* 1991), and in some mammalian models (Alila *et al.* 1990, De la Llosa-Hermier *et al.* 1991, Zosmer *et al.* 1997). In these examples, calcium was mainly supposed to exert its inhibitory action through PKC, but a possible effect on PDEs was not investigated at all. It would be worthy of interest to check whether calcium could also have such an inhibitory effect on steroidogenesis via PDE I in these (or other) models, in order to know whether our observations are only restricted to blowfly reproductive physiology or could be more widely applicable.

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