Control of ovarian steroidogenesis by insulin-like peptides in the blowfly (*Phormia regina*)

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Abstract

This study investigated the ability of insulin and of insect insulin-like peptides (ILPs) to stimulate ovarian steroidogenesis in the blowfly *Phormia regina*. Bovine insulin was active on ovaries isolated *in vitro*, which showed an age-dependent sensitivity; this peptide progressively stimulated steroidogenesis in ovaries isolated from the third day after adult molt, but not in younger ones, and had maximal activity after the fifth day. This stimulatory effect was observed equally from females reared in the presence or in the absence of males, excluding a regulatory effect of mating. The mode of action of insulin in blowflies did not involve cAMP, but triggered a specific and well-conserved transduction cascade. In particular, a peroxovanadium compound, known to activate specifically the insulin receptor in mammals, also stimulated blowfly ovarian steroidogenesis *in vitro*. Conversely, chemicals known to inhibit the mammalian insulin receptor or downstream elements of its signaling pathway, such as LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K), were able to prevent the steroidogenic action of bovine insulin on fly ovaries. Extracts from the median neurosecretory cells (MNCs) of blowfly brains, which are known to contain endogenous ILPs, stimulated ovarian steroidogenesis very efficiently and were also sensitive to inhibition by LY294002. These experiments indicated the involvement of PI3K in the mode of action of MNC extracts and substantiated that their endogenous ILPs are involved in the regulation of ovarian steroidogenesis. This conclusion was corroborated by the effects of synthetic bombyxin II, an ILP originating from silkworm MNCs, which also stimulated steroidogenesis in isolated blowfly ovaries. Altogether, these data suggest that insulin-like neurohormones from MNCs play a crucial role as steroidogenic gonadotropins in female flies.

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Introduction

Peptide gonadotropins that regulate vitellogenesis and oogenesis in insects are generally considered to originate from the median neurosecretory cells (MNCs) of the brain, also known as pars intercerebralis, and to be liberated from the corpora cardiaca neurohemal complex (for review see Girardie 1983, De Loof *et al.* 2001). The major roles of these neurohormones include the stimulation of ovarian steroidogenesis, leading to the release of ecdysteroids into hemolymph. Indeed, beside their crucial function as molting hormones during immature stages, ecdysteroids also control several important aspects of reproduction in adult insects (for review see Hagedorn 1989). In particular, they are directly involved in the regulation of vitellogenin biosynthesis in the fat body of higher Diptera. Although in other insects this important role is played by a sesquiterpenoid hormone (juvenile hormone), there is some evidence, at least in two species of locusts (Girardie & Girardie 1996, Girardie *et al.* 1998), that ecdysteroids still intervene and may be necessary for the onset of vitellogenesis.

Until now, only two unrelated steroidogenic gonadotropins have been identified in two different insect species, ovarian maturating parsin (OMP) in *Locusta migratoria* (Girardie & Girardie 1996) and ovarian ecdysteroidogenic hormone (OEH) in *Aedes aegypti* (Brown *et al.* 1998). However, such neurohormones have not yet been isolated

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in flies. In particular, no putative homolog has been identified in *Drosophila*, despite the availability of the complete sequence of its genome (for review see Nässel 2002).

Nevertheless, our previous investigations in the blowfly *Phormia regina* have shown that at least two different brain factors are involved in the stimulation of ovarian steroidogenesis: the main one indeed originates from MNCs, whereas the other is elaborated in another, as yet unknown, part of the brain. Thus far, these factors have only been distinguished by their mode of action on ovarian steroidogenic cells, more particularly by their relationships with cAMP, the former acting via a cAMP-independent pathway, the latter via a cAMP-dependent one (Manière *et al.* 2000).

The aim of the present study was to obtain more information on the nature of the MNC factor in blowflies and on its mode of action in ovaries. More particularly, our experiments were designed to examine the hypothesis that this factor could be an insulin-like peptide (ILP). In insects indeed, ILPs are among the major products synthesized by MNCs and are thus considered as true neurohormones (for review see Smit *et al.* 1998, Claeys *et al.* 2002, Nässel 2002). In particular, they have been detected in the MNCs of a blowfly species (Duve & Thorpe 1979, Duve *et al.* 1979) and of *Drosophila* (Brogiolo *et al.* 2001). Variations in the hemolymph concentrations of ILPs have been correlated with the ovarian biosynthesis of ecdysteroids in locusts (Sevala & Loughton 1992). Bombyxin, an ILP isolated from silkworm brain was found to stimulate steroidogenesis in the molting glands of lepidopteran larvae (Nagasawa *et al.* 1986) and to have also some unknown role during reproduction, as indicated by the detection of specific receptors in ovaries (Fullbright *et al.* 1997). Several studies have also demonstrated that ILP receptors and their transduction mechanisms are involved in the control of reproduction in several insect species, in particular the fruitfly (for review see Claeys *et al.* 2002, Garofalo 2002). The ILP receptor gene is mainly expressed in adult ovaries, particularly in the follicle cells (Fullbright *et al.* 1997, Graf *et al.* 1997, Helbling & Graf 1998, Riehle & Brown 2002) which are considered as the source of ovarian ecdysteroids (Goltzené *et al.* 1978, Chavez *et al.* 2000). More particularly, mutations on the ILP receptor gene in *Drosophila* provoke deficiencies in ovarian ecdysteroidogenesis (Tu *et al.* 2002). Furthermore, it has been demonstrated that bovine or porcine insulins, as well as pharmacological compounds mimicking the insulin signaling pathway in mammals, are able to stimulate ecdysteroid biosynthesis in the ovaries of female mosquitoes (Graf *et al.* 1997, Riehle & Brown 1999).

Until now, however, no direct evidence has been obtained that an insect ILP could really play such a gonadotropic role in an adult insect. The following study was thus undertaken, initially to verify if such a steroidogenic response to mammalian insulin also exists in blowflies and, further, to examine if MNCs could be the source of an insulin-like stimulation of ecdysteroid biosynthesis in ovaries. In this context, we also investigated the effects of a synthetic bombyxin on blowfly steroidogenesis.

Materials and Methods

Animals

Last-instar larvae of *P. regina* were purchased from La Verminière de l'Ouest (Tremblay, France) and reared to adulthood in controlled conditions (25 °C, 16 h light:8 h darkness cycle). Flies were supplied with sugar and water, a diet preventing oocyte growth and vitellogenesis in females. The flies used in this study were thus only previtellogenic females, generally maintained in the presence of males (except when otherwise stated). (For more details, see Manière *et al.* 2000).

After anesthesia with CO_2, experimental flies were placed on crushed ice and were decapitated immediately. Dissections of brains, MNCs and/or ovaries were performed rapidly under a dissecting microscope, in dry conditions.

Chemicals

The chemicals used in this study were purchased from Sigma, except mpV(pic) (monoperoxo(picolinato) oxovanadate (V)) and HNMPA-$(AM)_3$ (hydroxy-2-napthalenylmethylphosphonic acid-tris acetoxymethyl-ester) which were obtained from Calbiochem (France Biochem, Meudon). Bombyxin II was synthesized as previously described (Büllesbach 1999). LY294002: 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one.

In vitro *experiments*

Ovaries of the same pair, extirpated under sterile conditions, were rinsed four times with Grace's insect culture medium (Gibco), placed separately in 50 µl culture medium into 96-well plates and incubated at 26 ± 1 °C, over 1 h for cAMP experiments and 3 h or overnight for ecdysteroids.

Our bioassays were performed as previously described (Manière *et al.* 2000): in general, one ovary from an individual pair was treated and tested for its response, whereas the contralateral ovary was used as its corresponding untreated control. For every new batch of flies, blank experiments were also carried out to verify regularly that the two ovaries from the same pair, incubated separately in medium, secreted the same amounts of ecdysteroids.

MNC extracts were prepared as previously described (Manière *et al.* 2000): pools of approximately 50 pars

intercerebralis were homogenized in Grace's insect culture medium and centrifuged (at 6000 ***g*** for 10 min at 4 °C); then the supernatants were filtered through 0·2 μm sterile cartridges (Millipore).

Immunoassays

Ecdysteroids were measured as previously described (Manière *et al.* 2000), with an enzyme immunoassay based on the polyclonal L2 anti-ecdysone antibody (Pascual *et al.* 1995). Each measurement was made on the conditioned medium of a single ovary, preliminary experiments having shown that isolated previtellogenic ovaries did not contain significant amounts of ecdysteroids before culture in our conditions and did not store them during incubation. The amount of hormones was determined by comparison with dose–response curves using ecdysone as standard, this compound being the main ecdysteroid secreted by blowfly ovaries (unpublished observations). Consequently, the data were calculated as picograms ecdysone-equivalents per culture. The lower detection limit of our assay was about 1 pg per ovary.

cAMP was determined as previously described (Manière *et al.* 2000), from the acid extract of a single ovary, with the immunoassay described by Kingan (1989). Data are reported as fmol cAMP per ovary.

Statistics

For each individual experiment, the ratio of the concentration of ecdysone, or of cAMP, in the experiment over control was calculated and the data presented as relative means (± S.E.M.). Such treated/control ratios are independent of the size of flies (or of ovaries) and from incubation times (see Manière *et al.* 2000 for more details). Statistical analysis was generally made using either Student's *t*-test, by comparing means of concentration ratios to the theoretical value of 1 (which is equivalent to a paired *t*-test), or, for multiple comparisons, one-way ANOVA.

Results

Steroidogenic effects of bovine insulin in blowflies

The ability of bovine insulin to stimulate ecdysteroid biosynthesis was first verified on isolated previtellogenic ovaries from 5-day-old blowflies. As outlined in Table 1, the secretion of ecdysteroids into the culture medium increased in the presence of 10^{-5} M insulin by about 2·5-fold over their corresponding contralateral controls. By comparison, BSA at the same concentration was ineffective. Insulin at 10^{-6} M or at lower concentrations had no significant effect. Due to the limited solubility of insulin under physiological pH conditions, concentrations higher than 10^{-5} M insulin could not be tested.

Table 1 Effects of bovine insulin on ecdysteroid biosynthesis in isolated previtellogenic ovaries from 5-day-old female blowflies, after overnight incubation *in vitro*. Absolute amounts of ecdysteroids secreted by one ovary in control cultures after overnight incubation was 40·9 ± 6·5 pg ecdysone-equivalents (n=20)

	n	**Individual treated/control ratios** (means ± S.E.M.)
Experimental treatments		
Null (blanks)	8	0·99 ± 0·08
BSA (10^{-5} M)	10	1·08 ± 0·15
Insulin (10^{-6} M)	10	1·19 ± 0·09
Insulin (10^{-5} M)	10	2·44 ± 0·34*

*Result significantly different from the ideal value of 1 (Student's *t*-test, P<0·05).

The effects of 10^{-5} M insulin were then examined at different days following adult ecdysis. As shown in Fig. 1, the steroidogenic properties of insulin were dependent on female age. In fact, insulin was unable to stimulate steroidogenesis in ovaries explanted during the 2 days following ecdysis, but ovaries progressively acquired the capacity to respond to insulin stimulation during the following days, reaching a plateau 5–6 days after ecdysis.

The capacity of ovaries to respond to insulin was not conditioned by a previous contact of females with males. Indeed, 10^{-5} M insulin produced similar stimulatory effects on ovaries isolated from 5-day-old females previously maintained either in the presence or in the absence of males; treated/control ratios of ecdysteroid production by fly ovaries of a same batch were 2·75 ± 0·45, n=6, in the presence of males and 2·80 ± 0·45, n=7, in their

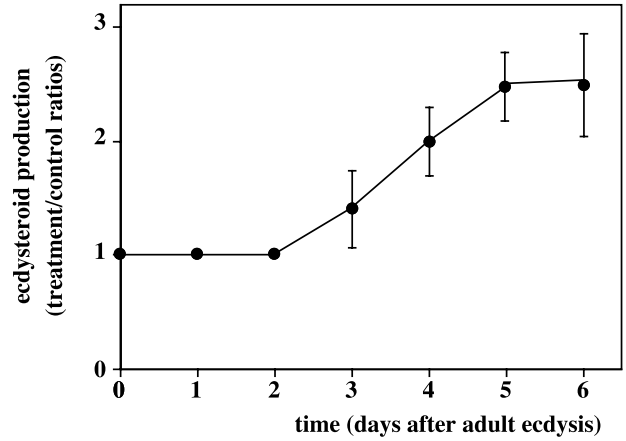

Figure 1 Progressive acquisition of the steroidogenic response to insulin (10^{-5} M) by previtellogenic ovaries isolated from *Phormia* at different days after adult ecdysis. Same experimental conditions as in Table 1.

absence; these data were not significantly different when evaluated by Student's *t*-test ($P > 0{\cdot}05$).

Insulin effects were not mediated by cAMP

Surprisingly, the insulin-stimulated physiological properties reported above were very similar to those previously observed with cAMP-dependent steroidogenesis in our model (Manière *et al.* 2000). Thus it was important to evaluate if insulin treatment would cross-talk with the cAMP pathway. Concentrations of cAMP in isolated ovaries were determined after 1 h incubation with or without 10^{-5} M insulin and no significant difference was observed ($18{\cdot}0 \pm 3{\cdot}6$ and $16{\cdot}6 \pm 3{\cdot}1$ fmol cAMP per ovary respectively; $n=9$, $P > 0{\cdot}05$). Similar measurements were also made after the addition of 3-isobutyl-1-methylxanthine (IBMX) at 10^{-3} M, a concentration known to inhibit phosphodiesterases and to enhance cAMP concentrations in our model (Manière *et al.* 2000, 2003). Results showed that insulin did not increase ovarian cAMP concentrations in the presence of IBMX ($28{\cdot}4 \pm 5{\cdot}0$ fmol cAMP in insulin-treated ovaries vs $27{\cdot}4 \pm 6{\cdot}1$ fmol in controls; $n=13$, $P > 0{\cdot}05$).

Moreover, experiments with the cAMP antagonist adenosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS), used at doses known to be effective on cAMP-stimulated biosynthesis (Manière *et al.* 2000, 2002), did not decrease insulin-stimulated steroidogenesis. Amounts of ecdysteroids secreted by ovaries simultaneously treated with 10^{-5} M insulin and with either 10^{-4} M or 10^{-3} M Rp-cAMPS ($64{\cdot}2 \pm 10{\cdot}4$ pg ($n=4$) and $72{\cdot}0 \pm 5{\cdot}1$ pg ($n=6$) respectively) were not significantly different from those secreted by ovaries treated with 10^{-5} M insulin only ($63{\cdot}2 \pm 8{\cdot}2$ pg; $n=6$, $P > 0{\cdot}05$), but were different from those of untreated controls ($28{\cdot}1 \pm 3{\cdot}1$ pg; $n=14$, $P < 0{\cdot}05$). Altogether, these experiments clearly demonstrated that the steroidogenic action of insulin was not mediated via the cAMP pathway.

Effects of pharmacological compounds interfering with insulin signaling

Pharmacological compounds mimicking insulin action in mammals were found to stimulate ovarian steroidogenesis in blowflies. In particular, mpV(pic), a potent inhibitor of protein phosphotyrosine phosphatase and activator of insulin receptor kinase (Posner *et al.* 1994), was found to stimulate ecdysteroid biosynthesis very efficiently in isolated ovaries; as shown in Fig. 2, a significant increase of steroidogenesis was observed with 10^{-4} M and 10^{-3} M mpV(pic). Moreover, okadaic acid, a known inhibitor of serine/threonine phosphatases, including those regulating the activity of protein kinase B (Haystead *et al.* 1990, Tanti *et al.* 1997), was also found to stimulate ovarian ecdysteroid biosynthesis significantly at concentrations higher or equal to 10^{-7} M (treated/control ratio of $2{\cdot}24 \pm 0{\cdot}49$ at 10^{-7} M; $n=8$, $P < 0{\cdot}05$).

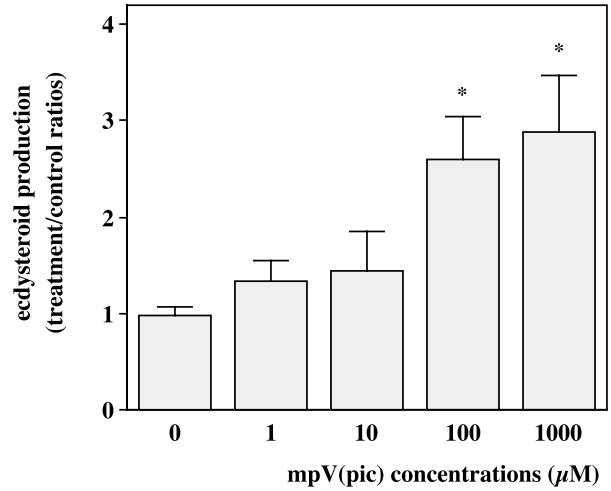

Figure 2 Effects of various concentrations of mpV(pic), a potent activator of mammalian insulin receptor, on steroidogenesis in blowfly previtellogenic ovaries. Each histogram bar, obtained from immunoassay measurements, represents the mean (± S.E.M.) of 8–12 individual ratios; these ratios were calculated by dividing the ecdysteroid amounts secreted by one ovary, submitted to mpV(pic) (except for blanks at concentration 0), with those of the other ovary of the same pair, being the corresponding untreated control. *Results significantly different from the ideal value of 1 (Student's *t*-test, $P < 0{\cdot}05$).

Conversely, pharmacological compounds having inhibitory effects on insulin signaling in mammals reduced insulin-stimulated ecdysteroid secretion in blowfly ovaries. This was observed with HNMPA-$(AM)_3$, a potent and specific inhibitor of insulin receptor tyrosine kinase (Saperstein *et al.* 1989). When used at 10^{-3} M, this compound almost completely inhibited the stimulation of steroidogenesis by insulin, but had no effect on basal steroidogenesis in the absence of insulin, and thus, no apparent toxicity (Table 2). The IC_{50} (concentration

Table 2 Effects of HNMPA-$(AM)_3$, an inhibitor of insulin receptor tyrosine kinase, either used alone or in combination with 10^{-5} M bovine insulin, on ecdysteroid production by isolated previtellogenic ovaries from 5-day-old female blowflies, after overnight incubation *in vitro*

	n	**Treated/control ratios** (means ± S.E.M.)
Experimental treatments		
Null (blanks)	8	$1{\cdot}00 \pm 0{\cdot}08$
HNMPA-$(AM)_3$ (10^{-4} M)	8	$1{\cdot}02 \pm 0{\cdot}11$
HNMPA-$(AM)_3$ (10^{-3} M)	6	$0{\cdot}95 \pm 0{\cdot}10$
Insulin alone	8	$2{\cdot}57 \pm 0{\cdot}45$*
Insulin + HNMPA-$(AM)_3$ (10^{-4} M)	7	$2{\cdot}19 \pm 0{\cdot}37$*
Insulin + HNMPA-$(AM)_3$ (10^{-3} M)	8	$1{\cdot}11 \pm 0{\cdot}13$

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

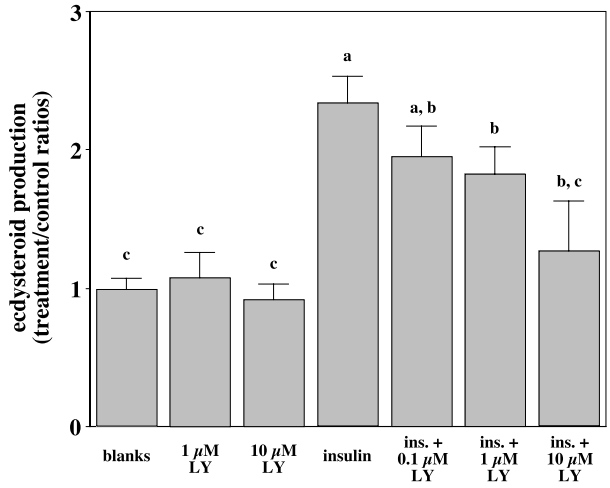

Figure 3 Effects of LY294002 (LY), a potent inhibitor of PI3K and of the insulin signaling pathway, on insulin-stimulated ovarian steroidogenesis. Production of ecdysteroids by untreated individual ovaries (blanks), by ovaries incubated only with 1 or 10 μM LY, by ovaries incubated with 10^{-5} M insulin, or by ovaries incubated simultaneously with 10^{-5} M insulin (ins.) and with 0·1, 1 or 10 μM LY, was compared with corresponding controls and expressed as treated/control ratios. Each histogram (mean ratios ± S.E.M.) was calculated from five to seven measurements. Same letters over error bars indicate values which were not significantly different (ANOVA, $P<0·05$).

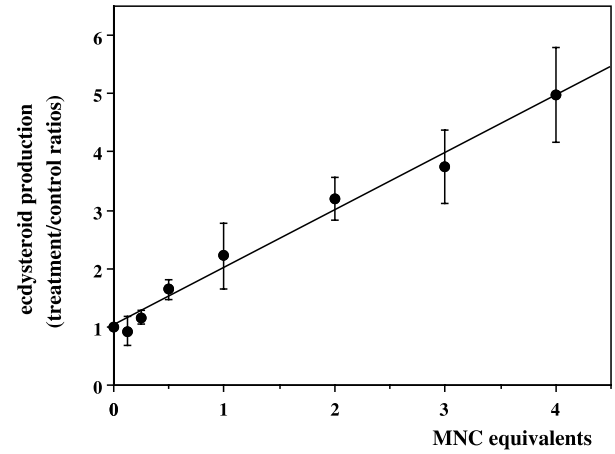

Figure 4 Stimulation of ecdysteroid biosynthesis in isolated previtellogenic ovaries by increasing quantities of crude extracts from blowfly MNCs, expressed in brain-equivalents per ovary. Each histogram was calculated from five to eight ovary pairs. Results obtained from 0·5 MNC-equivalents and more were significantly different from the ideal value of 1 (Student's *t*-test, $P<0·05$).

giving 50% of maximal inhibition) of this inhibitor ranged between 0·1 and 1 mM in our model, which is higher than reported for mosquitoes (14·2 μM, Riehle & Brown 1999), but seemed to be consistent with the concentration reported for vertebrates (200 μM, Saperstein *et al.* 1989).

Inhibition of insulin-stimulated steroidogenesis was also observed with a specific inhibitor of phosphatidylinositol 3-kinase (PI3K), LY294002 (Vlahos *et al.* 1994). When administered alone, this compound had no effect, showing no apparent toxicity. However, in the presence of insulin, it was able to inhibit the hormonal stimulation of ovarian steroidogenesis in a dose-dependent manner (Fig. 3). Its IC_{50} was estimated to be in the range 1–3 μM in our experiments, which is higher than the value reported in mosquitoes (30 nM, Riehle & Brown 1999), but in better agreement with vertebrate studies (1·4 μM, Vlahos *et al.* 1994). A comparable inhibition of insulin-stimulated steroidogenesis was also observed with wortmannin, another inhibitor of PI3K (not shown). Altogether, these results corroborated the involvement of several components of the insulin transduction pathway during the stimulation of ovarian steroidogenesis.

Insulin-like peptides from brain MNCs regulate ovarian steroidogenesis

Previous studies have demonstrated that blowfly MNCs elaborate a neurohormonal factor able to stimulate ovarian steroidogenesis through a cAMP-independent pathway (Manière *et al.* 2000). Complementary experiments showed that MNC extracts were able to trigger ovarian steroidogenesis with a regular, almost linear, dose–response curve within the range tested, allowing higher activation ratios than insulin (Fig. 4). As with insulin experiments, these results were obtained with ovaries extirpated 5 days after ecdysis, whereas those extirpated during the first two days remained insensitive to MNC extracts.

In order to verify that MNC gonadotropic activity could be due, at least in part, to an insulin-like neurohormone, ovaries were incubated with MNC extracts, in the presence or absence of the PI3K inhibitor LY294002. Results, given in Fig. 5, showed that LY294002 at 10^{-5} M significantly attenuated MNC-stimulated steroidogenesis. These data indicate that PI3K is involved in the stimulation of ovarian steroidogenesis by MNC extracts. As this kinase is a key component of the insulin-like signaling pathway, our data corroborated the hypothesis that MNC activity was due to endogenous ILPs.

Since blowfly ILPs have not yet been isolated, bombyxin II, an ILP previously identified from silkworm MNCs and prepared by chemical synthesis, was studied in our *in vitro* assay. As outlined in Fig. 6, a micromolar concentration of bombyxin was able to stimulate steroidogenesis in blowfly ovaries significantly. Bombyxin was obviously more active than bovine insulin and allowed higher activation ratios, comparable with those obtained with MNC extracts. These results showed for the first time that an insect ILP is able to stimulate the ovarian biosynthesis of ecdysteroids.

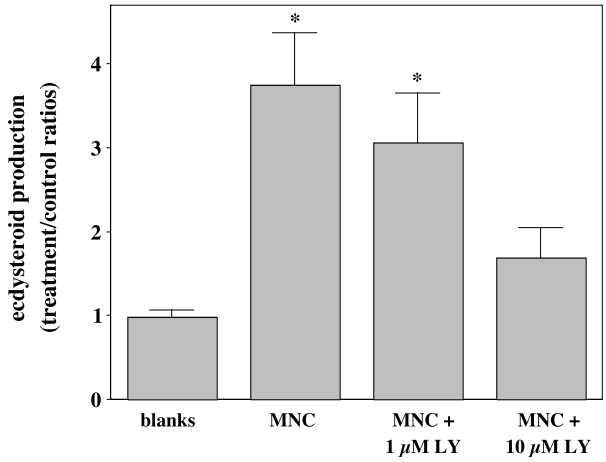

Figure 5 Inhibition of MNC-stimulated steroidogenesis (MNC, at a single concentration of 3 brain-equivalents) by the PI3K inhibitor LY294002 (LY) at two concentrations. See Fig. 3 for the effects of LY used alone at these concentrations. Each histogram was calculated from five to eight ovary pairs. *Results which are significantly different from the ideal value of 1 (Student's *t*-test, $P<0{\cdot}05$).

Discussion

The physiological significance of the ovarian response to insulin

It is now well established that ILPs are important signaling molecules present in insects and in numerous other invertebrates (for review see Ebberink *et al.* 1989, Claeys *et al.* 2002, Nässel 2002). As in vertebrates, insect ILPs seem to control several interrelated, well-conserved functions (such as the regulation of growth, body size and metabolism or aging) and also play a role during reproduction (for review see Smit *et al.* 1998, Claeys *et al.* 2002, Garofalo 2002). Because of its discovery in silkworms (Nagasawa *et al.* 1986), the first ILP identified in insects was named bombyxin, but those found later have been more frequently designated according to their resemblance with the mammalian hormone. However, there is no argument indicating that bombyxins might not be homologous to other insect ILPs and the following discussion will thus consider that insect ILPs include bombyxins.

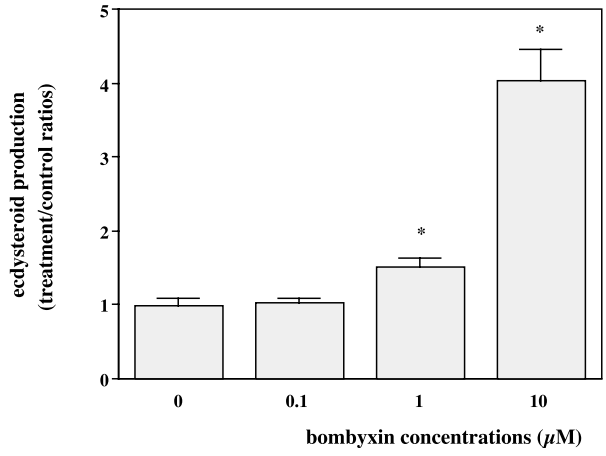

Figure 6 Stimulation of ovarian steroidogenesis by bombyxin II. Each histogram was calculated from seven or eight ovary pairs. *Results which are significantly different from the ideal value of 1 (Student's *t*-test, $P<0{\cdot}05$).

Several studies have also shown that insulin and/or insulin-like growth factors from mammals are able to elicit various physiological responses in insects (e.g. Bhakthan & Gilbert 1968, Vanhems *et al.* 1990, Hatt *et al.* 1997), emphasizing some of the pleiotropic functions played by ILPs in animals. More particularly, investigations on the mosquito *A. aegypti* (Graf *et al.* 1997, Riehle & Brown 1999) revealed ovarian steroidogenesis as a remarkable target of insulin in insects. Our present observations in blowflies are in perfect agreement with these reports and suggest that the steroidogenic property of insulin – and, probably, of endogenous ILPs – may be widespread in Diptera, if not in other insects.

However, the high concentration at which bovine insulin was active on blowfly ovaries could call into question the physiological significance of this response. Indeed, this concentration is an order of magnitude higher than the concentration active in mosquitoes (Graf *et al.* 1997) and both are higher than the physiological concentrations in mammals. However, these absolute concentrations of insulin are probably not very significant, since they probably result from a weaker affinity of blowfly receptors for the bovine peptide than for their natural ligands. This is substantiated by the results obtained with bombyxin and with MNC extracts, provided that the same receptors are involved in these different treatments (this will be discussed below). Indeed, bombyxin is an insect ILP and, in spite of the fact that it originates from a very distant species, it is more active than mammalian insulin. Moreover, extracts from blowfly MNCs, which most probably do not store large quantities of endogenous ILPs (the site of storage corresponding to corpora cardiaca being outside the brain), obviously acted at much lower and therefore physiologically more relevant concentrations.

Other results confirm that the response elicited by insulin has true physiological significance. In particular, the progressive appearance of insulin responsiveness in blowfly ovaries suggests that this property is precisely controlled over time. These data are in agreement with numerous reports that showed that the first 2 or 3 days following blowfly eclosion correspond to a progressive maturation of the whole reproductive system, under the control of corpora allata and/or of brain MNCs (Thomsen 1942, 1948, 1952, Thomsen & Lea 1969, Pappas & Fraenkel 1978, Yin *et al.* 1990). They are also in agreement with recent findings in mosquitoes, showing a

progressive expression of ILP receptor in ovarian follicular cells during previtellogenesis (Riehle & Brown 2002).

Interestingly, the activity of MNC extracts seemed to vary as a function of the age of ovaries, in a similar way to insulin. However, the sensitivity to cAMP-mimicking compounds also showed the same progressive responsiveness as insulin (Manière *et al.* 2000). These observations indicate that the responses to insulin and to endogenous ILPs have common physiological relationships with cAMP-dependent steroidogenesis. This could suggest that the receptors and/or the cellular components of the transduction pathways controlling ovarian steroidogenesis appear simultaneously and/or are regulated by the same factors. However, it may also indicate that common downstream effectors of these signaling pathways, in particular the biosynthetic enzymes, are developmentally controlled.

Our other results indicated that the sensitivity of blowfly ovaries to insulin is not directly controlled by factors depending on the presence of males. The insulin sensitivity of ovaries was indeed the same in virgin or in mated female blowflies. Here again, a similar observation has been made previously with the sensitivity to cAMP-mimicking substances (Manière *et al.* 2000), which again substantiates that the insulin response has a physiological significance and possible relationships with cAMP-dependent steroidogenesis.

Mode of action of insulin and ILPs

Several studies have emphasized that insects possess tyrosine kinase receptors displaying various similarities with mammalian insulin receptors (for review see Claeys *et al.* 2002, Garofalo 2002, Goberdhan & Wilson 2003), so much so that they can be activated by bovine insulin (Fernandez-Almonacid & Rosen 1987). They are frequently named 'insulin receptors', although the term 'ILP receptors' appears more appropriate in insects and will be preferred below. In lepidopterans, the bombyxin receptor has also shown similarities with the mammalian insulin receptor and can thus be considered as an ILP receptor (Fullbright *et al.* 1997). Interestingly, despite the presence of several different ILP genes, only one corresponding receptor gene has been identified so far in the genome of *Drosophila* and similar observations have been made in the other known invertebrate models, in contrast to what is described in vertebrates (for review see Nässel 2002, Goberdhan & Wilson 2003). If a single receptor is also present in *Phormia*, it appears very likely that bovine insulin and bombyxin acted on blowfly ovaries through this receptor, also sensitive to endogenous ILP ligands from MNCs.

In adult insects, the ILP receptor is expressed essentially in the ovaries, in close correlation with ecdysteroidogenesis (Fullbright *et al.* 1997, Graf *et al.* 1997, Helbling & Graf 1998, Riehle & Brown 2002). Moreover, it has been demonstrated that ILP receptor is involved in the steroidogenic response of ovaries to insulin (Riehle & Brown 1999, 2002, Tu *et al.* 2002). Our own experiments in blowflies are again in total agreement with these observations. Indeed, a potent and specific activator of insulin signaling in mammals, mpV(pic), a peroxovanadium compound inhibiting tyrosine kinase phosphatase and activating more specifically mammal insulin receptor (Posner *et al.* 1994), was found to stimulate ovarian steroidogenesis very efficiently in our model. This result confirms the observations made with another vanadate derivative in mosquitoes (Riehle & Brown 1999). Reciprocally, a potent inhibitor of the mammal insulin receptor, such as HNMPA-$(AM)_3$ (Saperstein *et al.* 1989), inhibited ecdysteroid production in our model, as also observed in the female mosquito (Riehle & Brown 1999). Altogether, these experiments clearly indicated that the steroidogenic effect of bovine insulin is mediated by an ILP receptor.

Downstream of the receptor, the cellular signaling mechanisms triggered by ILPs in insects also appear to be very well conserved (for review see Smit *et al.* 1998, Claeys *et al.* 2002, Garofalo 2002). In particular, observations on mosquitoes (Riehle & Brown 1999) have shown that ovarian steroidogenesis is controlled by a typical cascade of events involving a series of protein kinases, such as PI3K and protein kinase B (PKB). Using similar pharmacological experiments, our present observations are in absolute agreement with these findings. Indeed, the PI3K inhibitors LY294002 and wortmannin inhibited insulin-stimulated ovarian steroidogenesis in our model, indicating that PI3K plays an important role in the regulation of steroidogenesis by ILPs. Moreover, okadaic acid, a protein phosphatase inhibitor, was found to be an efficient activator of ecdysteroid biosynthesis in blowfly ovaries, which suggests the involvement of PKB. Indeed, okadaic acid is known to mimic insulin action when the inhibition of protein phosphatases favors the activation of PKB (Haystead *et al.* 1990, Tanti *et al.* 1997), in spite of possible inhibitory effects upstream from PKB (Mothe & Van Obberghen 1996).

Although the pharmacological compounds used during this study appeared less efficient in blowflies than reported in mosquitoes (Riehle & Brown 1999), the concentrations used in our model remained in agreement with those mentioned in vertebrate studies. More importantly, they were found to have no toxic effect, as judged by the fact that, when used alone, they did not decrease basal steroidogenesis, this parameter being easily measurable and extremely sensitive to any entry of calcium (Manière *et al.* 2002), which would be inevitable after cellular deteriorations. Thus, our observations corroborated that several components of the insulin-like signaling system, namely ILP receptor, PI3K and PKB, are involved in the control of ovarian steroidogenesis stimulated by bovine insulin in blowflies.

However, the physiological characteristics of insulin-dependent steroidogenesis in blowfly ovaries also showed striking similarities with those previously observed for cAMP-dependent biosynthesis of ovarian ecdysteroids (Manière *et al.* 2000). It was then easily conceivable that the insulin signaling pathway could have an indirect influence on cAMP metabolism, causing the cAMP pathway to mediate insulin action ultimately. Examples of such an indirect action of insulin have been found in vertebrates as well as in invertebrates (Kuznetsova *et al.* 1999, Plesneva *et al.* 2001, Shpakov *et al.* 2002). In our model, however, bovine insulin did not increase the ovarian concentrations of cAMP and, furthermore, an efficient cAMP antagonist failed to prevent it stimulating ovarian steroidogenesis. Thus it seems definitely established that insulin-induced ovarian steroidogenesis in blowflies is a cAMP-independent process.

ILPs as potential steroidogenic gonadotropins in insects

Numerous reports have indicated that MNCs play an important role during the reproduction of insects (Thomsen 1948, Girardie 1983). The presence of insulin-like material in insect brain, and particularly in MNCs, has been detected in various species, including blowflies (e.g. Tager *et al.* 1976, Duve & Thorpe 1979, Duve *et al.* 1979). More recently, different ILPs encoded by different genes were described for *Bombyx* (Kondo *et al.* 1996) and *Drosophila* (Brogiolo *et al.* 2001). These genes have different time patterns of expression in MNCs and, therefore, different putative functions.

In spite of the wealth of arguments suggesting a possible involvement of ILPs from MNCs during insect reproduction (see Introduction), no direct evidence has been obtained before this study showing that ILPs could really regulate ovarian ecdysteroidogenesis. Consequently, they are generally not considered gonadotropins (see a recent review on gonadotropins in De Loof *et al.* 2001). On the contrary, the only neurohormones that have been identified as insect gonadotropins (i.e. OMP and OEH), although originating from MNCs, are not ILPs (Girardie & Girardie 1996, Brown *et al.* 1998). In mammals, insulin and/or insulin-like growth factors also modulate ovarian steroidogenesis and are not considered to be steroidogenic hormones or gonadotropins. In this case, however, they are generally supposed to act by potentiating the steroidogenic response of ovaries to pituitary gonadotropins, without having effects on their own (for review see Poretsky *et al.* 1999).

In contrast, our experiments provide two pieces of evidence that ILPs originating from MNCs are direct activators of steroidogenesis in blowfly ovaries and then, may be considered as potential gonadotropins. The first evidence is given by experiments showing that extracts from blowfly MNCs are able to stimulate ovarian steroidogenesis very efficiently and that this stimulation can be prevented by an inhibitor of PI3K, a key component of the mammalian insulin signaling pathway. These findings complete our previous study showing that the gonadotropic action of MNCs is cAMP independent (Manière *et al.* 2000), as also demonstrated in this study for insulin. Altogether, these results indicate that MNC extracts may stimulate ovarian steroidogenesis by acting mainly, if not only, through the ILP signaling pathway and, consequently, that the activity of these extracts is probably due to endogenous ILPs.

The second evidence is that synthetic bombyxin, an ILP originating from silkworm MNCs, is able to stimulate ovarian steroidogenesis in blowflies. Although the mode of action of bombyxin has not been investigated in our model, it has been clearly shown that it acts through an ILP receptor in lepidopterans (Fullbright *et al.* 1997). Since bombyxin has the same effect as bovine insulin and as MNC extracts in our model, it seems very probable that it is able to bind to the same receptor and to stimulate steroidogenesis through the same pathway.

Taking in account that bombyxin is originating from a distant species, the question is raised of the possible extent of the gonadotropic role of ILPs in insects. Other experiments indicate that ILPs extracted from *Locusta*, which are active in our blowfly model, are also able to stimulate ovarian steroidogenesis in locusts (Delbecque *et al.*, unpublished observations), corroborating the possibility of a steroidogenic role of ILPs in the ovaries of very different groups of insects. Moreover, as bombyxin is also a regulator of steroidogenesis in larval molting glands (Nagasawa *et al.* 1986), ILPs could participate in the control of ecdysteroid biosynthesis during both the development and the reproduction of insects, in synergy with prothoracicotropic hormone (PTTH) in larvae or with the cAMP-generating factor present in adults (see Manière *et al.* 2000).

Finally, it seems possible that many of the effects attributed to ILPs in insects may be mediated by ecdysteroids during their reproduction cycles but possibly also during their whole life. This interpretation may lead to a re-examination of the relationships between ILPs and ecdysteroids, for example in the control of metabolism, of body size or in life-span regulation (see for example Simon *et al.* 2003 and Tatar *et al.* 2003), particularly when ILPs and ecdysteroids are obviously involved in the control of the same functions.

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