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Rapid communication

Insulin stimulates ecdysteroid production through a conserved signaling cascade in the mosquito *Aedes aegypti*

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Abstract

Selective activators and inhibitors of insulin signaling cascades in mammalian cells were tested for their effects on insulin stimulated steroidogenesis by ovaries of *Aedes aegypti*. Bovine insulin in the concentration range of 1.7 μM to 85 μM stimulated ecdysteroidogenesis in vitro. Pervanadate, an inhibitor of tyrosine kinase phosphatase, stimulated ecdysteroid production at concentrations of 250 μM to 1 mM. Okadaic acid, a serine/threonine phosphatase inhibitor, stimulated steroidogenesis with an ED_{50} of 77.39 nM. A selective inhibitor of tyrosine kinase activity, HNMPA-(AM₃), inhibited ecdysteroid production with an IC_{50} of 14.2 μM . Two selective inhibitors of phosphatidylinositol 3-kinase, wortmannin and LY294002, inhibited ecdysteroid production at low concentrations (IC_{50} = 1.6 nM and 30 nM, respectively). These concentrations are similar to those inhibiting insulin action in mammalian cells. A selective inhibitor of mitogen-activated protein kinase, PD098059, had no effect on ecdysteroid production even up to 100 μM . Thus, insulin stimulation of ecdysteroid production by ovaries in vitro appears to be controlled by the tyrosine kinase activity of the mosquito insulin receptor and the signaling cascade involving phosphatidylinositol 3-kinase and protein kinase B. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Insect; Diptera; Phosphatidylinositol 3-kinase; Protein kinase B; Insulin receptor; Tyrosine kinase

1. Introduction

Homologues of vertebrate insulin receptors have been characterized from the fruit fly, *Drosophila melanogaster* (Fernandez et al., 1995), the silkworm, *Bombyx mori* (Fullbright et al., 1997), and the mosquito, *Aedes aegypti* (Graf et al., 1997). The mosquito insulin receptor cDNA was cloned, expressed, and characterized as a proreceptor (200 kDa) consisting of an α subunit with a putative insulin binding domain, a β subunit with a tyrosine kinase domain, and a putative processing site (Graf et al., 1997). Similar domains exist in the insulin proreceptor of vertebrates (200 kDa), which is proteolytically cleaved into α and β subunits (135 kDa and 95 kDa, respectively). Two α subunits and two β subunits are covalently bound through disulfide bridges to form the mature receptor. The mosquito insulin receptor has been localized in follicle cells surrounding the ovaries and in

the nurse cells by immunocytochemistry and in situ hybridization (Helbling and Graf, 1998).

The proreceptor of the *Drosophila* insulin receptor is proteolytically cleaved into an α subunit (120 kDa) and a β subunit (170 kDa), with a 300 amino acid extension on the carboxy terminus. This extension may play a role in signal transduction similar to that of insulin receptor substrate 1 (IRS1) in vertebrates (Fernandez et al., 1995). Insulin receptors of vertebrates and mosquitoes do not have comparable extensions on the carboxy terminus, but other members of the receptor tyrosine kinase family, which bind epidermal growth factor and platelet derived growth factor, possess similar extensions. Although the *Drosophila* insulin receptor has been shown to bind mammalian insulin specifically (Fernandez-Almonacid and Rosen, 1987), endogenous ligands for the insulin receptors in either *Drosophila* or *Aedes aegypti* have not been identified to date.

An insulin-like peptide, bombyxin, was originally isolated from the silkworm, *Bombyx mori*, based on its “molting hormone”-like activity, and it stimulates ecdysone production by the prothoracic glands of another

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silkworm, *Samia cynthia ricini* (Yoshida et al., 1998). Bombyxin also has been shown to affect the metabolism of carbohydrate stores in silkworms (Satake et al., 1997). At least 32 bombyxin genes, classified into seven families (A–G), have been identified from *Bombyx mori* (Yoshida et al., 1998). Binding of bombyxin to ovarian receptors has been demonstrated for three different species of Lepidoptera (Fullbright et al., 1997). The bombyxin receptor has a mass of approximately 300 kDa which, when reduced, forms two distinct bands of 90 kDa and 116 kDa (Fullbright et al., 1997). In the tobacco hornworm, *Manduca sexta*, a 178 kDa protein immunoreactive to anti-phosphotyrosine and human insulin receptor antisera has been identified in the prothoracic glands, fat body, and muscle of larvae (Smith et al., 1997).

In vertebrates, the pleiotropic effects of insulin are controlled, in part, by the phosphatidylinositol (PI) 3-kinase and Ras/mitogen activated protein (MAP) kinase cascades (Avruch, 1998). The tyrosine kinase activity of the insulin receptor and the subsequent phosphorylation of IRS-1 stimulate these two pathways. A variety of agonists and antagonists have known effects on each of these pathways. Vanadium compounds and okadaic acid have been shown to stimulate the insulin receptor and protein kinase B, respectively, and mimic many of the effects of insulin (Molero et al., 1998; Tanti et al., 1997). The molecule, (hydroxy-2-naphthalenyl-methyl) phosphonic acid trisacetoxymethyl ester (HNMPA-(AM)₃), inhibits the tyrosine kinase activity of the insulin receptor (Saperstein et al., 1989). The PI 3-kinase pathway is inhibited by the fungal metabolite, wortmannin (Okada et al., 1994), and the compound 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) (Vlahos et al., 1995). An inhibitor of MAP kinase kinase (MEK), PD098059, is capable of inhibiting the Ras/MAP kinase cascade (Avruch, 1998).

A direct stimulatory effect of bovine insulin on ecdysteroid production and protein synthesis of ovaries has been demonstrated in vitro for *Aedes aegypti* (Graf et al., 1997). Using activators and inhibitors of critical steps in the signaling pathways of the model insulin receptor for vertebrates, we investigated the signaling pathways stimulated by bovine insulin to initiate ecdysteroid production by the ovaries of *Aedes aegypti*.

2. Materials and methods

Mosquitoes were maintained at 27°C in a 16L/8D photoperiod, and larvae were fed ground rat chow/lactalbumin/brewers yeast (1:1:1). Adults fed at will on a 10% sucrose solution via wick for 2 days, and were then given only distilled water. For the following assays, three to five day old female mosquitoes were immobilized on ice, and ovaries were dissected in a

saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM CaCl₂).

The bioassay for ecdysteroid production by ovaries in vitro (Brown et al., 1998) was modified as follows. Four pairs of ovaries were incubated in 60 µl of medium (139 mM NaCl, 4.05 mM KCl, 1.85 mM CaCl₂, 12.5 mM HEPES, 2.5 mM trehalose, 0.3 mM MgCl₂, and 0.9 mM NaHCO₃; pH 6.5, adjusted with NaOH) for 6 hours in the caps of 1.5 ml polypropylene microcentrifuge tubes. The caps were placed in moistened, 24 well microtitre plates and gently shaken in a water bath at 30°C. Fifty µl of the medium were analyzed for ecdysteroid content using a radioimmunoassay (RIA) with an ecdysteroid antiserum at a 1:9000 final concentration.

Bovine insulin (Sigma) was prepared as a 1 mM stock solution in medium, and serially diluted concentrations (170 µM–0.17 µM) were tested, as described above. Stock solutions of inhibitors were prepared in ethanol: PD098059 (20 mM, Calbiochem), wortmannin (2 mM, Sigma), LY294002 (2 mM, Sigma), and HNMPA-(AM)₃ (50 nM, Calbiochem). Inhibitors were stored at –80°C and diluted serially in medium immediately before use. Ovaries were tested for ecdysteroid production in the presence of bovine insulin (17 µM) and various concentrations of the four inhibitors. Each dose of the test substance was performed in triplicate and replicated three to seven times. For negative controls, ovaries were incubated in medium alone or in 1.6% EtOH both with and without bovine insulin. Stock solutions of 20 µM okadaic acid (Sigma) and 50 mM sodium ortho-vanadate (Sigma) were prepared in medium. Pervanadate was synthesized by incubating sodium ortho-vanadate with equimolar amounts of H₂O₂ at room temperature for 15 minutes. A serial dilution of pervanadate (2 mM to 125 µM) and okadaic acid (250 nM to 19nM) in medium only were tested for ecdysteroid production.

Statistical analysis, using the SAS statistical software package (SAS institute), was performed on the treatments using the general linear model with analysis of variance (ANOVA). Treatment means were compared using least square means, Bonferroni, and Tukey's studentized range test. Means were considered significantly different if $p < 0.05$. The ID₅₀ values and dose-response curves of okadaic acid, HNMPA-(AM)₃, wortmannin, and LY294002 were determined by nonlinear regression using a sigmoidal dose-response equation. These values were calculated using the Prism program (GraphPad).

3. Results and discussion

Bovine insulin stimulates ecdysteroid production by the ovaries of *Aedes aegypti* in a dose dependant manner in vitro. A dose-response curve comparing insulin concentration to ecdysteroid production in ovaries demonstrated that a concentration of bovine insulin as low as

1.7 μM could significantly increase ecdysteroid production compared to control ovaries in medium only (Fig. 1). Maximum ecdysteroid production was achieved in the presence of 17 μM insulin, the concentration used for subsequent experiments with inhibitors. The highest concentration of insulin tested (170 μM) showed no stimulation of ecdysteroid production. Studies with mammalian cells typically use insulin concentrations between 10 nM and 100 nM for optimal activity. Considering bovine insulin is a distantly heterologous ligand, it is surprising that the concentration required to stimulate ecdysteroid production in *Aedes aegypti* is less than twenty fold higher than those used to stimulate physiological responses in mammalian cells.

In mammalian cells, the binding of insulin to the vertebrate insulin receptor triggers a cascade of events beginning with the autophosphorylation of the β subunit and the subsequent activation of the tyrosine kinase domain. Pervanadate, a tyrosine phosphatase inhibitor, stimulates the insulin signaling cascade in vertebrates by preventing the constitutive dephosphorylation of the insulin receptor. Pervanadate stimulated steroidogenesis in *A. aegypti* ovaries at concentrations as low as 250 μM with the greatest stimulation occurring at 500 μM (Fig. 2A). However, ecdysteroid production was approximately 50% less than the insulin control. HNMPA-(AM)₃ is a potent inhibitor of tyrosine kinase activity that completely blocks the action of insulin. HNMPA-(AM)₃ inhibited ecdysteroid production in the presence of 17 μM insulin at an IC_{50} of 14.2 μM (Fig. 2B). Complete inhibition was achieved at 840 μM . Thus, HNMPA-(AM)₃ is a more potent inhibitor of the tyrosine kinase activity of the mosquito insulin receptor (IC_{50} = 14.2 μM) than that of the insulin receptor in mammalian cells (IC_{50} = 200 μM) (Saperstein et al., 1989). These results strongly suggest that a tyrosine kinase

receptor, most likely the mosquito insulin receptor, binds bovine insulin, and that phosphorylation of the receptor is a critical step leading to the stimulation of ecdysteroidogenesis by mosquito ovaries.

Our studies demonstrate the involvement of a PI 3-kinase and protein kinase B (PKB) homologue in stimulating ecdysteroid production. In mammalian cells, the PI 3-kinase/PKB pathway regulates the metabolic effects of insulin, including glucose transport and glycogen synthesis, as demonstrated by the use of cell permeable, relatively selective agonists and antagonists of PI 3-kinase and PKB. PKB is activated when phosphorylated on several serine and threonine residues. Okadaic acid, a serine/threonine phosphatase inhibitor, has been shown to activate PKB and GLUT 4 translocation independent of insulin and PI 3-kinase (Tanti et al., 1997). Okadaic acid stimulated ovarian steroidogenesis in vitro at an ED_{50} of 77.39 nM, and at 125 nM was capable of stimulation comparable to bovine insulin (Fig. 3A). Concentrations of okadaic acid greater than 125 nM were less stimulatory.

PI 3-kinase phosphorylates phosphatidylinositol, creating a critical membrane binding site for the pleckstrin homology domain of PKB. The fungal metabolite, wortmannin, was found to completely inhibit the activity of PI 3-kinase in rat adipocytes (IC_{50} = 5 nM) (Okada et al., 1994). A more selective compound, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), was developed to inhibit PI 3-kinase (IC_{50} = 1.4 μM) (Vlahos et al., 1995). These inhibitors of PI 3-kinase completely inhibited ecdysteroidogenesis at concentrations comparable to those inhibiting the action of insulin in mammalian cells. Wortmannin inhibited ecdysteroid production with an IC_{50} of 1.6 nM (Fig. 3B), and complete inhibition was observed at 10 nM. LY294002 is approximately 20 fold less effective than wortmannin at inhibiting ecdysteroid production by ovaries (IC_{50} = 30 nM, Fig. 3C).

Our results suggest that a MAP kinase cascade does not control subsequent steps in the activation of ecdysteroid production. In mammalian cells, PD098059 is capable of inhibiting MEK activity at an IC_{50} of 2 μM (Avruch, 1998). Concentrations of PD098059 up to 100 μM displayed no inhibitory effect on ecdysteroid production (data not shown). The MAP kinase cascade stimulated by insulin in vertebrates is responsible for controlling mitogenesis and cell differentiation and does not appear to be involved in the metabolic functions of insulin (Avruch, 1998). In mammalian cells expressing a dominant negative Ras, both MAP and pp90^{S6} kinase activities were markedly reduced, but activation of glycogen synthase was unaffected. Furthermore, PD098059 had no effect on the activation of glycogen synthase (Avruch, 1998). Thus, it is not surprising that PD098059 had no effect on ecdysteroid production. In *Drosophila*, it has been demonstrated that the insulin receptor is

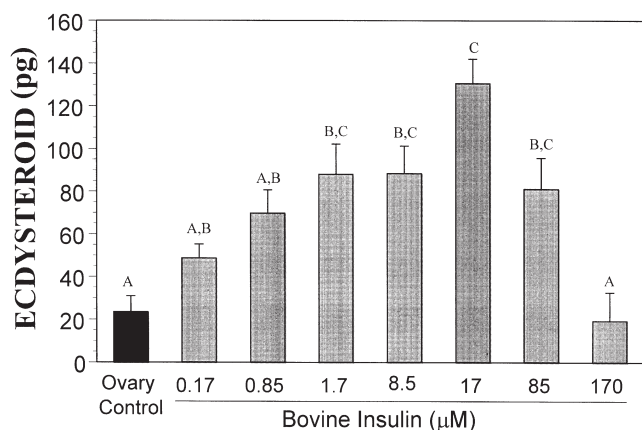


Fig. 1. Effects of different concentrations of bovine insulin on ecdysteroid production by ovaries in vitro. Error bars represent standard errors. Tukey's studentized range (HSD) test was performed between the treatment means. Treatments with different letters are significantly different. ($P < 0.05$; $n = 4$ sets of triplicates/dose).

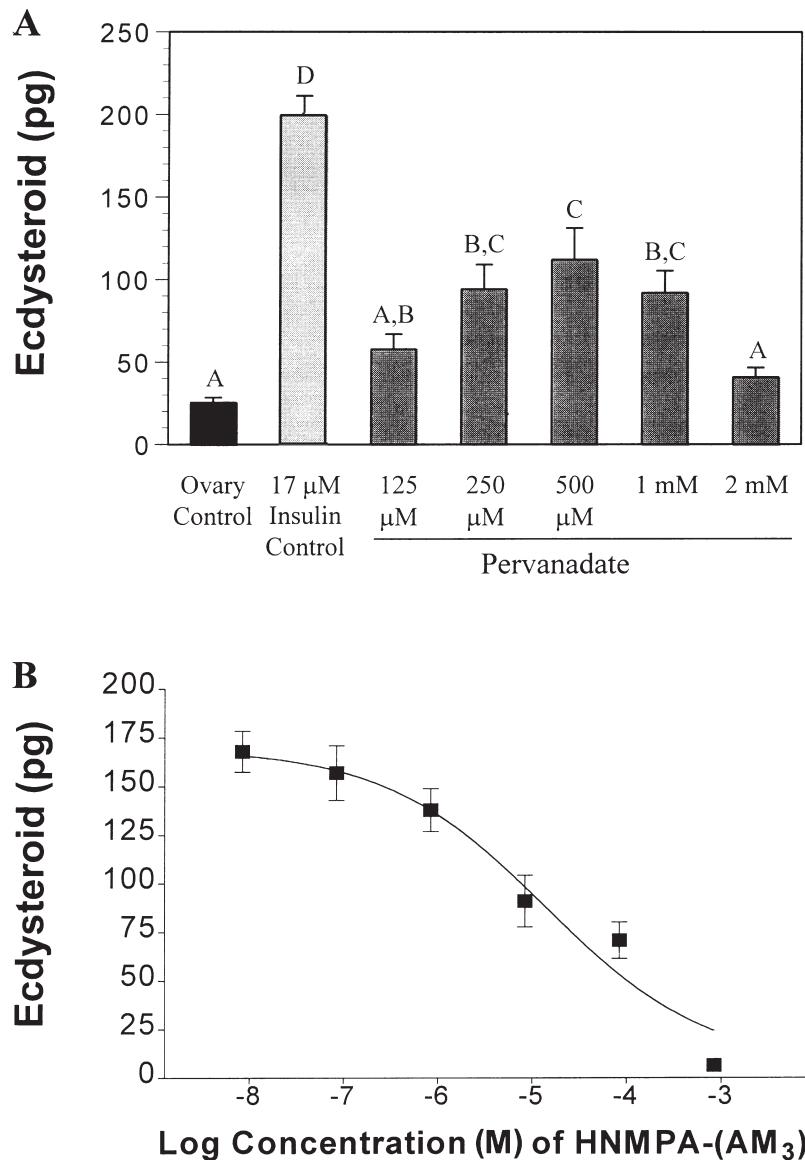


Fig. 2. Effects of a tyrosine kinase activator and inhibitor on the mosquito insulin receptor. A: Effects of pervanadate, a tyrosine kinase activator, on ecdysteroid production. Error bars represent standard errors. Treatments with different letters are significantly different. ($P < 0.05$; $n = 8$ sets of triplicates/dose). B: Dose-response curve for HNMPA-(AM₃), a tyrosine kinase inhibitor. ID₅₀ = 14.2 μ M. Ovaries were incubated in vitro with HNMPA-(AM₃) in the presence of 17 μ M bovine insulin ($n = 4$ experiments in triplicate/dose of each inhibitor).

critical for normal growth (Chen et al., 1996). Thus, the *Drosophila* insulin receptor, with a β subunit extension homologous to growth factor receptors in vertebrates, may utilize a MAP kinase cascade to regulate development.

Based on these results, we hypothesize that the insulin receptor in mosquito ovaries acts through a PI 3-kinase/PKB cascade that activates ecdysteroid biosynthesis. Three classes of PI 3-kinase have been described for vertebrate cells. In vertebrates, a class 1 PI 3-kinase has been shown to phosphorylate phosphatidylinositol, providing a binding site for PKB that, when constitutively active in 3T3-L1 adipocytes, stimulates the translocation of glucose transporters and glucose uptake (Kohn

et al., 1996). PI 3-kinases and PKB have been described from *Drosophila* (Franke et al., 1994; Leever et al., 1996). This information, coupled with the above studies, provides strong evidence for the involvement of a PI 3-kinase and PKB homologue in ecdysteroid production by mosquito ovaries.

This study has identified a likely signaling pathway for the putative insulin receptor in *Aedes aegypti* (Fig. 4). This pathway regulates ecdysteroidogenesis, an essential endocrine process in female mosquitoes. The mosquito insulin receptor is localized on follicle cells surrounding the oocytes (Helbling and Graf, 1998), and these cells in female locusts, and most likely other insects, are the primary source of ecdysteroids (Kappler et al., 1986).

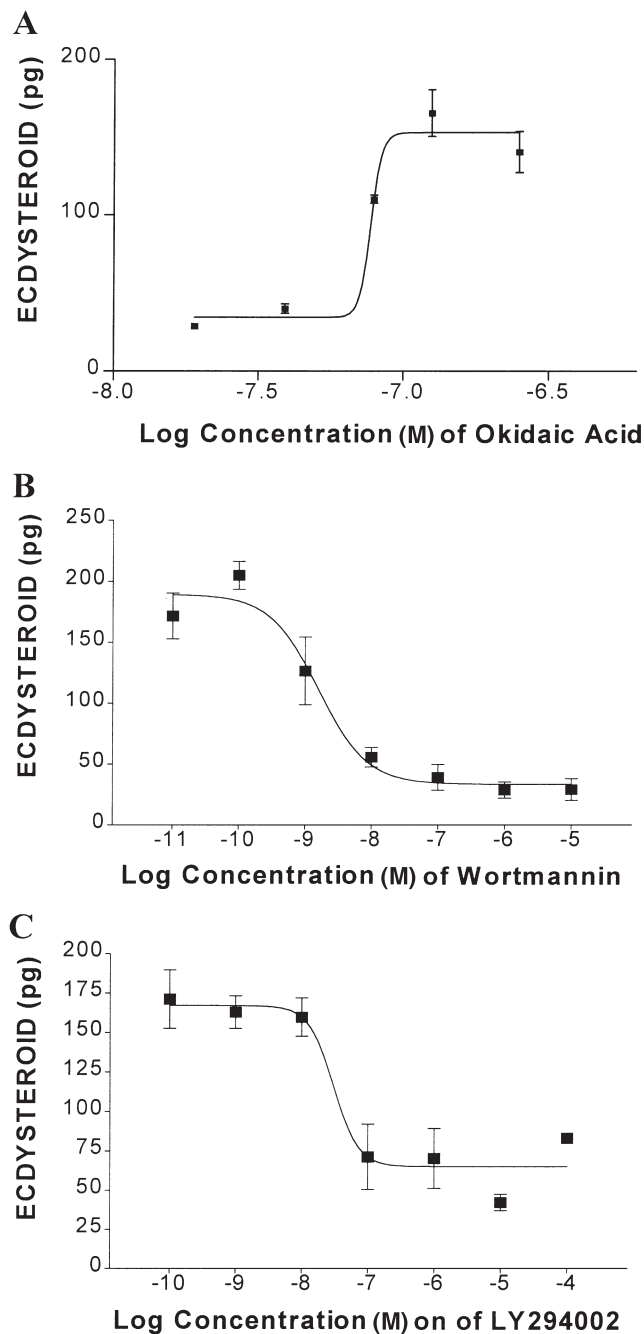


Fig. 3. Dose-response curves for the effects of a PKB activator and PI 3-kinase inhibitors on ecdysteroid production. Ovaries were incubated in vitro with PI 3-kinase inhibitors in the presence of 17 μ M bovine insulin ($n=4$ experiments in triplicate/dose of each inhibitor) and with okidaic acid ($n=3$ experiments in triplicate/dose). A: Dose-response curve for the PKB activator, okidaic acid. $ED_{50}=77.39$ nM. B: Dose-response curve for the PI 3-kinase inhibitor, wortmannin. $ID_{50}=1.59$ nM. C: Dose-response curve for the PI 3-kinase inhibitor, LY294002. $ID_{50}=30.25$ nM.

Bovine insulin was effective but is a distantly heterologous ligand for the receptor. Presumably, the corresponding insulin of mosquitoes may act through the proposed pathway to stimulate cholesterol uptake into the cell and to activate biosynthetic pathways, resulting in ecdystero-

idogenesis. Interestingly, ovary ecdysteroidogenic hormone I (OEH I), a neuropeptide with the same bioactivity as bovine insulin, is already known for *Aedes aegypti* (Brown et al., 1998). The amino acid sequence of OEH I exhibits no apparent similarity to invertebrate or vertebrate insulins, and the mode of action of OEH I is unknown. Characterization of a mosquito insulin and key proteins in the pathway identified by the present study are required for comprehensive understanding of the regulation of reproduction in female mosquitoes, including possible interactions between a putative insulin and OEH I.

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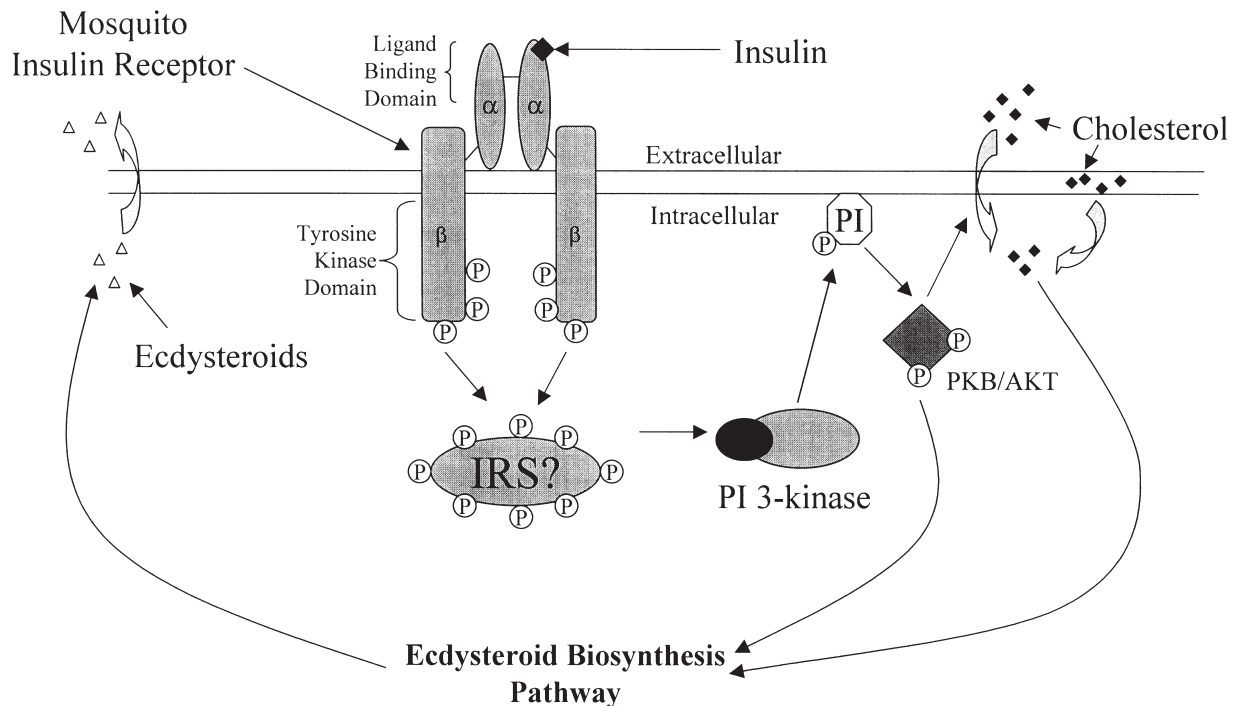


Fig. 4. Proposed signal transduction pathway for the mosquito insulin receptor based on a signaling cascade characterized for mammalian insulin receptors (Avruch, 1998). Insulin binds to the receptor on the surface of the follicle cells surrounding the oocytes, resulting in the autophosphorylation of the tyrosine kinase domain of the β -subunit as suggested by experiments with HNMPA-(AM₃). The activated tyrosine kinase domain, in turn, phosphorylates an IRS1 homologue. A PI 3-kinase homologue binds to phosphorylated tyrosine residues on IRS1, thus activating the catalytic subunit. The involvement of PI 3-kinase has been suggested by the use of the inhibitors, wortmannin and LY294002. The catalytic subunit of PI 3-kinase phosphorylates phosphatidylinositols (PI) in the inner cell membrane creating a binding site for PKB. When phosphorylated by unknown kinases, PKB may activate intracellular membrane movement, recruitment of cholesterol into the cell, and biosynthetic pathways for the conversion of cholesterol to ecdysteroids.

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