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CONTROL OF STEROIDOGENESIS IN LEYDIG CELLS

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Summary—Luteinizing hormone (LH) interacts with its plasma membrane receptor to stimulate steroidogenesis not only via cyclic AMP but also other pathways which include arachidonic acid and leukotrienes and regulation of chloride and calcium channels The same stimulatory pathways may lead to desensitization and down-regulation of the LH receptor and steroidogenesis The LH receptor exists in a dynamic state, being truncated, or internalized, degraded or recycled Desensitization is controlled by protein kinase C (PKC) in the rat and by cyclic AMP dependant protein kinase and PKC in the mouse Leydig cells Using an adapted anti-sense oligonucleotide strategy we have shown that the cytoplasmic C-terminal sequence of the LH receptor is essential for desensitization to occur In contrast, these sequences of the LH receptor are not required for the stimulation of cyclic AMP and steroid production We have also shown that the extracellular domain of the LH receptor is secreted from the Leydig cell and may act as a LH-binding protein

INTRODUCTION

It is well established that steroidogenesis in Leydig cells is regulated by luteinizing hormone (lutropin, LH), via the second messenger 3′,5′-cyclic AMP (cAMP) It has also been shown that other second messenger systems may be involved, including arachidonic acid and its metabolites [1], calcium [1] and efflux of chloride ions [2]. The release of arachidonic acid to the cytosol can occur by a hormone-mediated process, through the activation of phospholipase A_2 (PLA_2), phospholipase D (PLD) and/or phospholipase C (PLC) followed by hydrolysis of diacylglycerol by diacylglycerol lipase PLA_2 and PLC can be coupled to a common membrane receptor by distinct GTP-binding proteins and may be activated by the same membrane active hormone [3] Arachidonic acid can be further metabolized via cyclo oxygenase and lipoxygenase pathways to prostaglandins and leukotrienes, respectively Our previous results have shown that LH causes a rapid release of arachidonic acid from Leydig cells, probably via activation of PLA_2 [4] and that arachidonic acid lipoxygenase metabolites are involved in LH-induced steroidogenesis [5, 6]

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REGULATION OF STEROIDOGENESIS BY PROTEIN KINASE C (PKC)

Activation of PKC using the tumor-promoting phorbol ester, phorbol-12-miristate-13-acetate (PMA), has been reported to regulate, both positively and negatively, steroidogenesis in Leydig cells [7–9] In addition to diacylglycerol, arachidonic acid has been shown to be a physiological regulator of PKC in various tissues, via activation of the γ PKC subunit [10] In rat Leydig cells the γ subunit as well as the α and β subunit are present [11] and thus it is theoretically possible that arachidonic acid also regulates PKC in Leydig cells Recently [12] we have demonstrated that in rat Leydig cells arachidonic acid has a dose- and time-dependent biphasic effect on maximal and submaximal LH- and dibutyryl-cAMP-stimulated testosterone production The locus of the inhibition, which occurred during 3 h incubation, was prior to the side chain cleavage of cholesterol and after cAMP production The same inhibitory effect was found with the PKC activators, PMA and oleic acid, also with no change in LH-stimulated cAMP production Arachidonic acid, PMA and diolein, all stimulated PKC activity in a dose-dependent fashion in Leydig cell homogenates

The arachidonic acid potentiation of LH- and dibutyryl-cAMP-stimulated testosterone production, which occurred during 5 h, was also

mimicked by PMA PKC was down-regulated over 5 h (but not during 3 h) by pretreating Leydig cells with PMA or arachidonic acid in the presence of LH The stimulatory effect on steroidogenesis was specific for arachidonic acid, since oleic and elaidic acid did not have any effect on LH-stimulated testosterone production Lipoxygenase and cyclooxygenase inhibitors did not alter the stimulation effects of arachidonic acid We concluded that the short-term inhibitory effect of arachidonic acid is via activation of PKC, but when PKC is down-regulated, arachidonic acid potentiates steroidogenesis in response to LH

One possible explanation for the stimulatory effect of arachidonic acid is because of the down-regulation of PKC activity, the rat testis Leydig cell may normally be under tonic inhibitory control by this enzyme This may be by phosphorylation of regulatory proteins involved in the modulation of steroidogenesis When PKC is down-regulated this inhibition is removed and the cells become more active steroidogenically In accordance with this hypothesis we have shown that following down-regulation of PKC (with PMA for 5 h) followed by a 2 h incubation with dbcAMP (0 001 to 1 mM) there was an increase in basal-, submaximal- and maximal-stimulated steroidogenesis (unpublished observations)

In our previous studies [1] we found that phorbol esters desensitized LH-stimulated cAMP production In the above studies no effect of PMA or arachidonic acid on LH-stimulated cAMP was found This lack of effect on cAMP production was due to the high concentrations (1%) of albumin used In the presence of lower concentrations (0 1%), inhibition of LH-stimulated cAMP production by arachidonic acid (unpublished observations) and desensitization of LH-mediated cAMP production by PMA does occur

ACTIVATION AND DESENSITIZATION OF THE LH RECEPTOR (LHR)

Continued exposure of the Leydig cells to LH leads to desensitization [1, 13] Previously we demonstrated that desensitization of the rat Leydig cell involves an uncoupling of the LHR from its transducing system i e a G-protein coupled to adenylate cyclase [1] This process could be mimicked by stimulation of PKC but not cAMP-dependent protein kinase (PKA) The LH- or phorbol ester-induced desensitization led to a 50% decrease in cAMP production In mouse Leydig cells, stimulation of both PKA and PKC leads to complete desensitization with regard to cAMP and steroid production [14, 15] Thus, there are marked species differences Although the evidence suggests that the substrate for PKC/PKA-mediated phosphorylation may be the LHR itself [1, 16], this has not been directly demonstrated under physiological conditions The results could equally be explained by phosphorylation of a protein(s) that interacts with the LHR

DOWN-REGULATION OF THE LHR

It was previously shown that down-regulation i e loss of the LHR, only occurred after a considerable time period following exposure to LH/hCG i e 12–24 h [17, 18] We have recently shown that there is a rapid proteolytic cleavage of the LHR during culture of rat and mouse Leydig cells, which results in the release of the LHR extracellular domain [15] This process is continuous in the freshly isolated rat Leydig cells (up to 4 h), whereas in the mouse Leydig cell it is a cAMP-dependent process Activation of PKC is ineffective in causing truncation [15] Paradoxically, inhibition of LHR truncation with protease inhibitors, also inhibits cAMP production in the mouse (but not the rat) Leydig cell [19], indicating that proteolysis of the LHR is required for activation of adenylate cyclase to occur The depleted receptors in the mouse cells are replenished by a cAMP-dependent process It is possible therefore, that loss of receptor in the Leydig cell occurs at two levels acute, involving truncation, and chronic, involving internalization and degradation and also a decrease of LHR mRNAs [20, 21]

THE STRUCTURE OF THE LHR

The LHR has been cloned from human and rat ovaries and porcine Leydig cells Its sequence indicates that it is a member of the 7 transmembrane helix-G protein coupled receptor family [22, 23] The LH, (and FSH and TSH receptors) differ from other members of this receptor family in having a very large extracellular domain (50–60 kDa), which is the site of the hormone binding [22–27] There is also very little homology with the other receptors in the transmembrane domains and the C-terminal sequences which are the sites of binding of the other hormones and the G-proteins [28] There

is approx 85% homology between the cloned LHRs. A region spanning the 2nd and 3rd transmembrane domains is highly conserved among the LH, FSH and TSH receptors However, antibodies raised against the whole LHR show species and cell specificity [29] and the hLHR is unusual in that [^{125}I]hCG binding is not displaced by equine, rat or ovine LH [26]

THE ANTI-SENSE INHIBITORY STRATEGY

This methodology involves incubating cells with anti-sense oligodeoxynucleotides (AS oligos) to the gene or mRNA coding for the N-terminal regions of the specific proteins The AS oligos are taken up by the cells and bind to the DNA/mRNA, preventing transcription/translation and thus completely blocking the synthesis of specific proteins This methodology has been used successfully in many investigations e g to study the effects of c-*myc* [30] and the proliferating cell nuclear antigen (cyclin) on cell proliferation and cell differentiation [31] It has also been successfully used to determine the role of type IIβ regulatory subunit mRNA of PK in the cAMP-induced differentiation of HL-60 leukaemia cells [32] We have shown that this strategy will also inhibit LHR synthesis [33] In MA10 cells depleted of their functional LHR by treatment with trypsin, complete resynthesis occurs during subsequent incubation for 48 h [34] When the incubation of the MA10 cells was carried out in the presence of an AS oligo directed against a LHR N-terminal sequence no resynthesis occurred and there was a complete loss of LH-induced cAMP production and steroidogenesis

We have adapted the strategy in an attempt to produce truncated LHRs with deleted C-terminal phosphorylation sites [33] We used AS oligos coding for 7 amino acids in the putative 3rd extracellular loop and to different sequences of the intracellular C-terminal loop of the rat ovarian LHR The presence of the AS oligos 2 or 3 was found to have no effect on the replenishment of the LH receptors, as measured by [^{125}]IhCG binding, or on the LH stimulation of cAMP or steroidogenesis However, they completely prevented the LH- and phorbol ester-induced desensitization of adenylate cyclase by these newly-synthesized (and presumably C-terminal truncated) receptors cAMP- and phorbol ester-induced desensitization of steroidogenesis was also prevented However, no effect was found on LH-induced desensitization of steroidogenesis, thus indicating that LH induces additional desensitization processes which are independent of PKA and PKC The losses of surface LHRs caused by LH and cAMP were also investigated It was found that pretreatment of the MA10 cells with all the C-terminal AS oligos including the sequence coding for the end C-terminal peptide, prevented the 50% loss of binding sites detected in the controls

The mechanisms of the AS oligos against the N-terminus sequence in preventing the synthesis of the LHR can be understood However, those involved in altering the function of the C-terminus are unknown It is possible that they act as a stop codon on the mRNA so that the LHR is synthesized up to the position where the AS oligo is bound and is then released Alternatively, mRNAs coding for the LHR lacking the cytoplasmic C-terminus may exist, addition of the AS oligos may completely prevent the synthesis of the full length LHR and thus allow the translation of the mRNAs coding for the truncated receptors Multiple mRNAs for LHR do exist

CONCLUSIONS

Because of the marked species differences in the mechanisms of desensitization and down-regulation found in the mouse and rat Leydig cells, it is apparent that these mechanisms cannot be studied in only one species It is well established however, that all Leydig cells do contain spare LHRs [18], full cAMP and steroid production can be maintained by low occupancy of the receptors In addition, the anti-sense experiments in our studies demonstrate that almost complete depletion of the LHR has to occur before cAMP or steroidogenesis is decreased The acute decreases in the ability of Leydig cells after exposure to LH and other ligands to form cAMP is therefore not due to a loss in the numbers of LHRs but to a loss in the ability of the LHR to couple to its transducing systems

In the rat Leydig cells, acute exposure *in vitro* to LH causes desensitization This desensitization can be induced by PKC activators but not by cAMP Prolonged exposure is required before down-regulation of the LHR However, continuous proteolytic cleavage of the LHR does occur during the first 4 h of incubation *in vitro* This results in the excretion of the extracellular binding domain of the LHR into the

incubation medium It is possible that this proteolysis is controlled *in vivo* via regulation with protease inhibitors, perhaps from adjacent cells e g seminiferous tubular myoid cells In the mouse Leydig cells, desensitization occurs after exposure to LH, cAMP and phorbol esters This results in very low cAMP and steroid production Proteolysis of the LHR occurs *in vitro* if internalization is inhibited This process is stimulated by cAMP but not phorbol esters Curiously, inhibition of proteolysis inhibits cAMP production, this does not necessarily imply that the proteolysis of the LHR extracellular domain is involved, other changes in the LHR structure or associated proteins may occur

The anti-sense strategy is obviously a powerful technique for investigating structure–activity relationships of regulator proteins in homologous expression systems The results of its application to the LHR imply that C-terminal residues are involved in the processes of desensitization and proteolytic cleavage of the extracellular domain Further work is required to identify the proposed truncated proteins and the mechanisms of inhibition of translation/transcription

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