LIGAND-INDUCED STIMULATION OF GONADOTROPIN RECEPTORS

LIGAND-GEINDUCEERDE STIMULATIE VAN GONADOTROPINE RECEPTOREN

PROEFSCHRIFT

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HENDRIK JOHANNES VAN LOENEN
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PROMOTIE-COMMISSIE:

Promotor:	Prof. dr. J.A. Grootegoed
Co-promotor:	Dr. F.F.G. Rommerts
Overige Leden:	Prof. dr. B.A. Cooke (University of London, UK)
	Prof. dr. H.A. Drexhage
	Prof. dr. S.W.J. Lamberts
	Prof. dr. J.M.J. Lamers
	Dr. R. de Leeuw (Organon International bv)
	Dr. J.A. Maassen (Rijksuniversiteit Leiden)

The research described in this thesis was performed at the department of Endocrinology & Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, The Netherlands.

Aan mijn ouders
Aan Mirjam

CONTENTS

ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	adenosine cyclic-3':5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CG	chorion gonadotropin
DNA	deoxyribonucleic acid
DhCG	deglycosylated human chorion gonadotropin
FSH	follicle stimulating hormone
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
h (prefix)	human
IBMX	1-methyl-3-isobutyl-xanthine
Kd	equilibrium dissociation constant
KRB	krebs ringer buffer
LH	luteinizing hormone
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
TSH	thyroid stimulating hormone

CHAPTER ONE

GENERAL INTRODUCTION

Intercellular communication

In multicellular organisms, elaborate cell-to-cell communication networks coordinate the growth, differentiation, and metabolism of the multitude of cells in the diverse tissues and organs. The many cell types use a large variety of signalling molecules and mechanisms for intercellular communication. Besides direct cell-to-cell contacts, cellular communication is also conveyed by secreted chemical messengers. Signal molecules that are synthesized and secreted by signalling cells induce cellular responses only in those cells that express specific receptor molecules for them. Distinct types of intercellular communication are mediated through such a mechanism: (1) neural communication, in which neurotransmitters are released at synaptic junctions from nerve cells and act across a narrow synaptic cleft on a postsynaptic cell; (2) paracrine communication, in which secreted products of cells diffuse locally and affect neighboring cells of different type; (3) autocrine communication, when local chemical messengers act on the secreting cell itself or on neighboring cells of the same type, and (4) endocrine communication, in which specific cells of endocrine organs secrete signal molecules (hormones) that are carried by the blood to their specific target cells, which may be distributed widely throughout the body.

Glycoprotein hormones

In mammalian species, the anterior pituitary gland synthesizes and secretes three glycoprotein hormones under hypothalamic control. These are the gonadotropins lutropin (luteinizing hormone; LH) and follitropin (follicle-stimulating hormone; FSH), and thyrotropin (thyroid-stimulating hormone; TSH). LH and FSH play pivotal roles in mammalian reproduction (1,2), while TSH is the main regulator of thyroid gland activity (3). During gestation in primates and equidaes an additional gonadotropin, chorionic gonadotropin (CG), is secreted by the placenta and is essential for maintenance of pregnancy (4). In this thesis, the term glycoprotein hormones is used exclusively for the subfamily of gonadotropic and thyrotropic hormones, not including other hormones such as prolactin and erythropoietin which also exist in glycosylated forms.

The gonadotropins LH, FSH, and CG, along with TSH, constitute a family of structurally related glycoprotein hormones that are among the largest and most complex molecules possessing hormone activity. These hormones have molecular weights of 28,000-38,000 and are composed of two dissimilar noncovalently linked subunits, designated α and β (5). Both subunits consist of a single polypeptide chain that contains one or more oligosaccharide units, depending on the type of hormone (5-9).

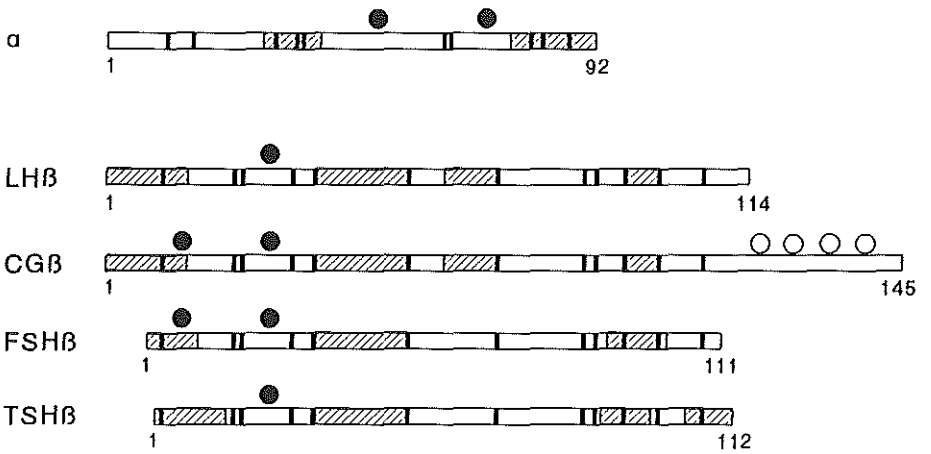

Figure 1. ***Glycoprotein hormone subunits.*** *Schematic representation of the primary structure of the human glycoprotein hormone subunits, showing domains that have been shown to be involved in receptor binding and/or activation (hatched boxes). The α subunit consists of 92 amino acid residues, and contains two asparagine-linked oligosaccharide units (●) at positions α52 and α78. The hormone specific ß subunits for LH, FSH, and TSH differ in length, and are aligned by their conserved cysteine residues (dark lines). The hCG ß subunit contains an additional C-terminal extension of 31 amino acid residues when compared to hLHß. The various ß subunits contain one (LH and TSH) or two (hCG and FSH) asparagine-linked oligosaccharide units at positions ß13 and ß30 of the LH/CG ß subunit sequence. The C-terminal extension of the hCG ß subunit contains for additional serine-linked oligosaccharide units (○).*

Within one species, the α subunit is identical in all glycoprotein hormones and is encoded by a single gene (10), whereas the various ß subunits arise from separate genes (11) and define hormone specificity (5-9). The α subunit consists of 92 amino acid residues in humans and of 96 amino acid residues in other species, and shows 80-85% sequence homology among different species (Figure 1). The hormone specific ß subunits vary in size. In humans the ß subunits of FSH, LH and TSH are composed of 111, 114, and 112 amino acid residues, respectively. The hCG ß subunit is composed of 145 amino acid residues, and contains an additional proline- and serine-rich C-terminal extension in comparison to the other glycoprotein hormones. hLH and hCG possess approximately 85% sequence homology through the first 114 amino acid residues of their ß subunits, and have similar receptor binding specificity. As in hCG, a C-terminal extension has also been found in equine LHß and CGß, which are encoded by a single gene (12) and possess identical

amino acid sequences (13,14). The gene encoding the hCG ß subunit probably originates from a single base deletion in the ancestral primate LHß gene, causing the loss of a stop codon and transcription of a region of the DNA that is not transcribed from the LHß gene (15,16). The additional amino acid residues of the C-terminal extension of the hCG ß subunit are therefore not very likely to be important for receptor interaction. Indeed, hLH and hCG possess similar biological properties as they bind and activate the same receptor. Moreover, removal of the C-terminal extension of the hCG ß subunit does not impair the interaction of the hormone with the LH/CG receptor (17-19).

As expected, the hormone specific ß subunits possess substantial heterogeneity in amino acid sequence (5-9), although some regions possess sequence homology. These homologous regions might be involved in the interaction of the different ß subunits with the common α subunit, but may also be involved in a general receptor-stimulating mechanism of the glycoprotein hormones. The α and ß subunits of the glycoprotein hormones contain 10 and 12 conserved cysteine residues (Figure 1), which form 5 and 6 intrachain disulfide bonds, respectively. A disulfide bond contributes to the stabilization of the three-dimensional structure of a protein, and the high conservation of the cysteine residues in the glycoprotein hormones suggests similar three-dimensional structures. The cysteine residues in the glycoprotein hormones are also highly conserved among different species. As the separated subunits possess no significant hormonal activity, subunit association appears to be a prerequisite for full expression of hormonal activity (5-9). Several domains on both the α and ß subunit have been shown to be important for receptor binding and activation (Figure 1) (5-9). A proper spatial orientation of these domains and of their relative position may only be obtained in the "active conformation" of the hormone which is achieved after subunit association (20).

Glycoprotein hormone specificity

While domains of both the α and ß subunits have been shown to be involved in the interaction of the glycoprotein hormones with their receptors, specificity exclusively depends on the ß subunit (5-9). A sequence within the disulfide bridge 93-100 of the hCG ß subunit, and its corresponding region in the other glycoprotein hormones, has been postulated to confer hormone specificity and was designated the *determinant loop* (21). The variable net charge of amino acid residues in this loop structure might act as a determinant of specificity: a positive net charge for LH/CG, and a distinguishable negative net charge for FSH and TSH. Recently, it has been shown by means of so-called cassette mutagenesis that substitution of the *determinant loop* in FSH by the corresponding

sequence of hCG results in a chimeric hormone that possesses hCG-like and no longer FSH-like activity (22-23). This observation is consistent with the "negative specificity" model which suggests that all glycoprotein hormones share the domains that are important for receptor binding and activation, while specificity of the interaction is defined by the *determinant loop*. The interaction of the *determinant loop* with its complementary receptor site would then determine whether the appropriate interactions of the homologous domains with the respective receptors can occur (Figure 2) (8,24). The α subunit may contribute the greater part of the homologous binding/activation domains of the various glycoprotein hormones.

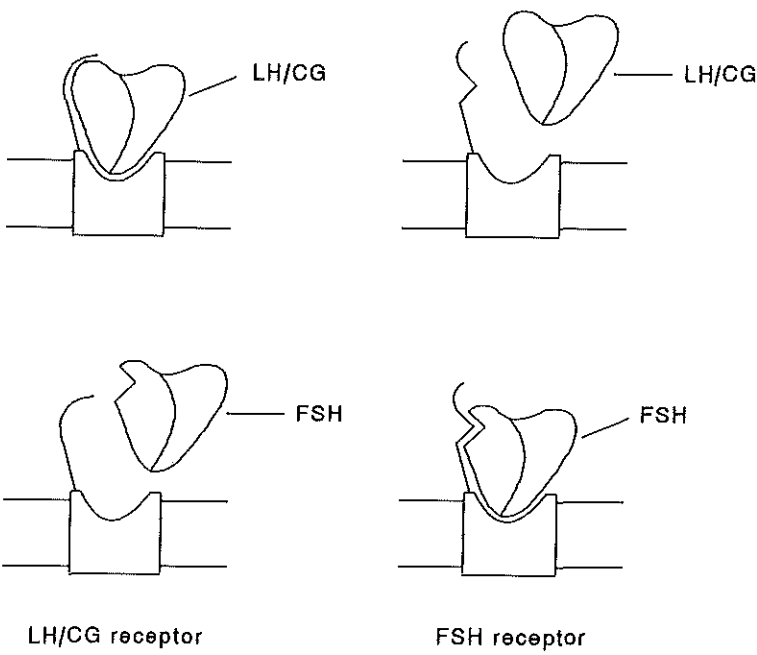

Figure 2. ***"Negative specificity model" for glycoprotein hormone-receptor interaction.*** *The model suggests that all glycoprotein hormones (and hence all glycoprotein hormone receptors) share an overall structural homology that is important for hormone-receptor binding and activation. Receptor stimulation only occurs when a domain on the hormone that defines specificity of the hormone towards its receptor interacts with the correct complementary interaction site on the receptor.*

Glycosylation of glycoprotein hormones

Both subunits of the glycoprotein hormones contain oligosaccharide units that are

covalently attached to the polypeptide chain (Figure 1). Two asparagine-linked (N-linked) oligosaccharide units are present on the human α subunit, and conserved in all other mammalian species. The hormone specific ß subunit contains either one (in LH and TSH) or two (in CG and FSH) N-linked oligosaccharide units depending on the type of hormone. The hCG ß subunit contains four additional serine-linked (O-linked) oligosaccharide units bound to its unique C-terminal extension (25). Similar O-linked glycosylation has also been found on equine LH/CG ß subunits, whereas the shorter FSH, LH, and TSH ß subunits do not contain O-linked glycosylation.

Substantial microheterogeneity in terms of the chemical composition of the oligosaccharide units results in a polymorphism of different isoforms of a specific glycoprotein hormone (25-29). Different degrees of sialylation or sulfation of the oligosaccharide side chains influence the molecular net charge and the *in vivo* biological activity of the hormones, whereas the receptor binding activity and the *in vitro* biological activity are not influenced (30-32).

Different levels of regulation are known for the control of the actual *in vivo* biological activity of glycoprotein hormones. The first level concerns regulation of the processes of synthesis and secretion of the hormones by the pituitary gland, as both processes are regulated by hypothalamic factors. The hypothalamic releasing hormones LH-releasing hormone and TSH-releasing hormone have been shown to regulate glycosylation of secreted LH (33) and TSH (34), respectively. In respect of the microheterogeneity in glycosylation, naturally occurring antagonistic isohormones of FSH (35) and TSH (36) have been described. The clearance rate from the circulation is a second level of control. The third level is the ability of the hormone to specifically recognize its respective receptor, while the fourth level is the intrinsic competence to stimulate the receptor. The *in vitro* biological activity of the glycoprotein hormones is defined by the latter hormone properties. As indicated above, glycosylation of glycoprotein hormones may affect hormonal activity at different levels. This aspect of glycoprotein hormone function has been most extensively studied for hCG.

Clearance of glycoprotein hormones from the circulation

Subsequent to the secretion of hCG by the trophoblastic cells of the placenta, the *in vivo* activity of the hormone is highly dependent on the half-life of the hormone in the circulation. Glycoproteins are removed from serum by desialylation and subsequent uptake through interaction with the hepatic asialo-glycoprotein receptor in the liver (37). Consistent with this clearance route, *in vitro* neuraminidase treatment of hCG, resulting in

removal of sialic acid, considerably diminishes the *in vivo* circulatory half-life and biological activity of hCG (30). However, neither the *in vitro* receptor-binding activity nor the *in vitro* receptor-stimulating activity of hCG are substantially affected by desialylation of the hormone (30). Pituitary glycoprotein hormones are less sialylated than hCG, but contain a high degree of sulfate groups at their oligosaccharide units. These sulfate groups are thought to play a similar role as sialic acid residues, as they decrease the rate of hormone clearance by masking the termini of their oligosaccharide units that are recognized by receptors of the hepatic clearance route (38). For the pituitary glycoprotein hormones, the liver is the main site of clearance from circulation, but hCG is predominantly excreted by the kidney. Urinary excretion of hCG occurs rapidly and might be a mechanism to control the high hCG concentrations during pregnancy. Glycoprotein excretion by the kidney might continuously occur at a low rate at physiological hormone concentrations, while at high hCG concentrations, as occur during pregnancy, it may become the main clearance route for hCG. Similar concentration dependent excretion of glycoprotein hormones by the kidneys may occur for FSH in menopausal women, as they possess significantly higher FSH serum levels than young women.

Compared to the other glycoprotein hormones, hCG exhibits a longer circulating half-life and *in vivo* biological activity. Recent studies using site-directed mutagenesis and gene transfer techniques demonstrated that the C-terminal extension of the hCG ß subunit, containing four O-linked oligosaccharide units is involved in the increased *in vivo* biopotency of hCG compared to that of LH, since deletion of this region decreases the circulation half-life and the *in vivo* activity (18). The O-linked oligosaccharide units and the C-terminal extension do not play a role in either receptor binding or the *in vitro* biological activity of the hormone (18). Consistent with the importance of the C-terminal extension of the hCG ß subunit for the *in vivo* half-life of hCG, and thus for the *in vivo* biological activity of hCG, recombinant chimeric FSH that contains the C-terminal extension of hCGß on its FSH ß subunit possesses a more prolonged half-life in the circulation and a higher *in vivo* biological activity in comparison to native FSH (39,40).

Intracellular signalling

G protein-coupled receptors (GPCRs) are functionally coupled to intracellular effector systems through guanyl nucleotide-binding proteins, termed G proteins. G proteins are heterotrimeric proteins composed of α, ß, and γ subunits. In the nonactivated state, the G protein oligomer is complexed with GDP, and the dissociation rate of GDP from the G protein is extremely low (41). An agonist-occupied receptor triggers interactions between

the receptor and the G protein, thereby facilitating the exchange of GTP for bound GDP at a site within the α subunit of the G protein (41). The binding of GTP to the α subunit promotes dissociation of this subunit from the ßγ complex (Figure 3). GTP-liganded α subunits, and in some cases also the ßγ complexes, are responsible for modulating the activity of distinct intracellular effector systems, including adenylyl cyclase, phospholipases, and ion channels. Changes in intracellular effector activity result in ionic and/or metabolic changes within the cell (42-44). Hydrolysis of bound GTP by the intrinsic GTP-ase activity of the α subunit acts as a timing mechanism for effector activation, as it leads to reassociation of the α subunit with the ßγ complex (45).

The glycoprotein hormones express their hormonal activity through interaction with specific G protein-coupled receptors in gonadal or thyroidal tissues. These receptors are coupled via G_S protein to adenylyl cyclase. The subsequent elevation of intracellular levels of cAMP results, through interaction with cAMP-dependent protein kinase and subsequent phosphorylation of endogenous proteins, in regulation of a diversity of cellular processes, of which steroidogenesis is the most investigated biological response for the gonadotropic hormones.

An activated G_S protein is able to sustain activation of adenylyl cyclase for up to a few seconds (41,45). Each cyclase molecule, in turn, catalyses the conversion of a large number of ATP molecules to cAMP molecules. Further downstream in this cascade, cAMP functions as an allosteric effector to activate cAMP-dependent protein kinases, which then can phosphorylate many proteins. A single extracellular signal molecule can in this way activate or inhibit the biological activity of many intracellular proteins, and the cascades of intracellular mediators provide several opportunities for amplification and regulation of the responses to extracellular signals (46). As such amplification cascades require tight regulation, it is not surprising that cells have efficient mechanisms for rapidly degrading cAMP by phosphodiesterases, as well as for inactivating phosphorylated proteins. FSH, for example, regulates cAMP degradation in Sertoli cells by induction of phosphodiesterase activity, thereby controlling its own intracellular signalling (47,48).

Glycoprotein hormone receptors have also been shown to be coupled via G_Q proteins to phospholipase C (49-52), an enzyme that catalyses the hydrolysis of polyphosphoinositides into inositol triphosphate and diacylglycerol, which are intracellular signalling molecules that induce release of calcium from intracellular stores and probably the entry of extracellular calcium into the cell, resulting in activation of calcium-dependent protein kinases. The ligand-occupied receptors might also be direct regulators of ion channels, thereby directly modulating transient ion currents across the cell membrane (43,53). The

type of intracellular signalling depends on which type of G protein is activated, since the various intracellular effector systems act through their specific G proteins (44). The ability of a ligand-occupied receptor to activate distinct intracellular signalling cascades is known as the dual signalling properties of the receptor.

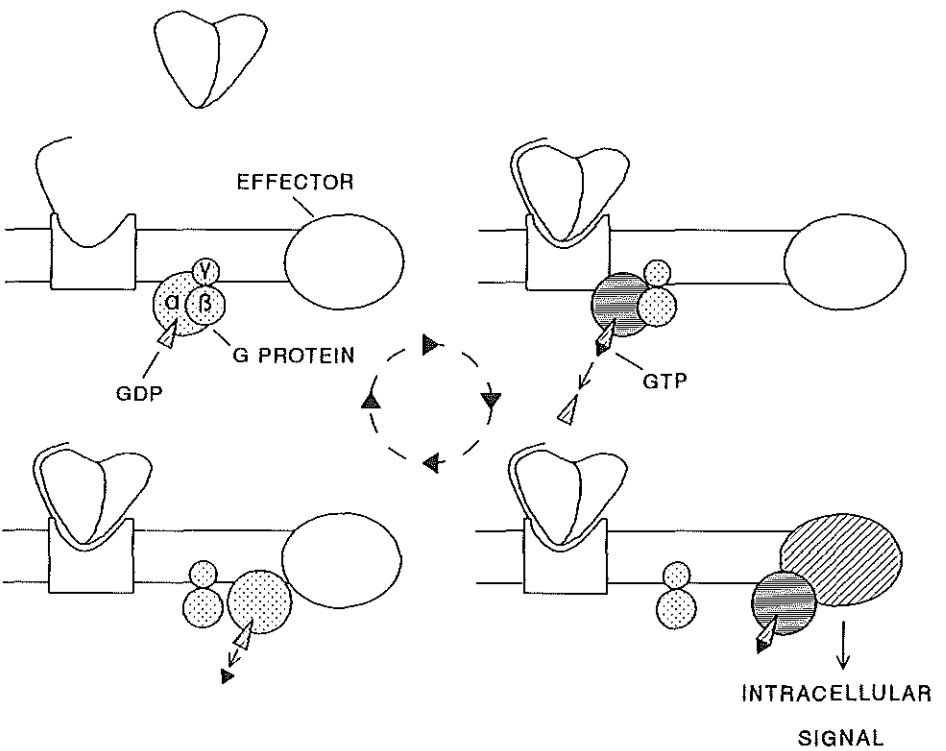

Figure 3. ***Activation cycle of G proteins.*** *G proteins are heterotrimeric proteins composed of an α, β, and γ subunit. A nonactivated G protein contains GDP at a GDP/GTP binding site on the α subunit. Ligand-induced stimulation of the receptor causes the G protein to exchange GDP for GTP, which activates the G protein. The activated G protein then dissociates and the GTP-bound α subunit activates an effector molecule (e.g. adenylyl cyclase). Intrinsic GTP-ase activity of the α subunit causes hydrolyses of bound GTP, and inactivation of the G protein. The α subunit then may reassociate with a βγ complex, and the G protein re-enters the activation cycle.*

At a given cell surface, different G protein-coupled receptors may be expressed, all of these with the intrinsic property to transduce an extracellular signal into an intracellular

signal. However, only a limited number of intracellular effectors have been characterized. Different receptors probably share similar effector systems for intracellular signalling. In this respect, intracellular signalling induced by a specific ligand may not only be determined by an overall change in the intracellular concentration of activated signalling molecules, but might also depend on the intracellular distribution of these molecules (54,55). Moreover, different temporal kinetics of the changes in intracellular concentrations of signalling molecules might offer further diversity in intracellular signalling. In conclusion, dual signalling properties of the receptor, compartmentalization of intracellular signalling, and temporal kinetics of intracellular signalling, offer several opportunities to convert a hormonal signal into a diversity of intracellular signals.

Importance of the oligosaccharide units in glycoprotein hormones for receptor binding

During the last decade, a major aim has been, and it still is, to define domains of the glycoprotein hormones that determine their intrinsic receptor stimulating activity. The question whether the glycosylation of hCG constitutes such domains has been studied by modulation of the oligosaccharides units in hCG. The effects of desialylation and/or deglycosylation have been extensively studied in terms of LH/CG receptor binding, adenylyl cyclase activation, and stimulation of steroid production. Asialo hCG obtained by neuraminidase treatment appeared fully active *in vitro*, and the sialic acid residues are therefore not important for hCG-induced receptor activation (30). However, asialo hCG has been reported to possess higher receptor binding affinity than native hCG (56,57).

More progressive deglycosylation of hCG has been obtained by removal of 70-80% of the oligosaccharide units of hCG by either enzymatic (58,59) or chemical (hydrogen-fluoride) treatment (60-62) of intact hCG. Deglycosylated hCG (DhCG) possesses 2 to 3-fold higher affinity for the LH/CG receptor than intact hCG, illustrating that the oligosaccharide units in hCG are not essential for receptor binding (30,63). It has been demonstrated that desialylated or deglycosylated hCG exhibit enhanced binding activities towards TSH (64,65) and FSH receptors (66). Deglycosylation of hCG resulted in an approximately 100-fold increase in the binding activity towards the TSH receptor, compared to intact hCG. However, the binding potency of DhCG to the TSH receptor is still approximately 3000-fold less than that of TSH. Thus, the oligosaccharide units in hCG play only a marginal role in hormone specificity towards the receptor, since even in their absence hCG possesses much higher affinity for LH/CG receptors than for TSH or FSH receptors.

Importance of the oligosaccharide units in glycoprotein hormones for receptor stimulation

Within the three-dimensional protein structure of hCG various domains have been shown to be important for LH/CG receptor binding and activation (67). A proper spatial orientation of these domains is likely a prerequisite for full hormonal activity, and the oligosaccharide units might be important for stabilization of the appropriate protein conformation. Physico-chemical studies and antibody binding studies have indicated conformational differences between hCG and DhCG (30,63). These conformational differences may underlay the discrepancy between the receptor binding and receptor activation properties of deglycosylated hormones. Subsequent to deglycosylation of hCG, the hormonal property to stimulate adenylyl cyclase is markedly reduced, while the receptor binding property is not impaired (30,63). Differences between hCG and DhCG in terms of receptor binding and activation might reflect differences in the functional interaction within the hormone/receptor complexes for hCG and DhCG. These ligands have been shown to bind to different domains of the porcine LH/CG receptor (68). Moreover, free hCG and DhCG share epitopes that are recognized by anti-hCG antibodies, and whereas all epitopes are no longer accessible in DhCG/receptor complexes, some of these epitopes remain exposed in hCG/receptor complexes (69). The association of DhCG with the LH/CG receptor occurs at a higher rate than observed for hCG (69-71). In addition, while the binding of hCG to the LH/CG receptor is partially reversible over a period of several hours (72,73), DhCG binding to the LH/CG receptor appears to be nonreversible (74). These data suggest different molecular interactions for DhCG and hCG with the LH/CG receptor. Glycopeptides and carbohydrate chains derived from hCG and other glycoproteins have been shown to bind to the LH/CG receptor, and to increase the rate of dissociation of receptor-bound hCG (75,76). Therefore, the oligosaccharide units of hCG might play a role in the dissociation of the ligand from the receptor.

In contrast to the marginal effects of deglycosylation of hCG on the receptor binding properties of the ligand, DhCG showed a substantially reduced receptor stimulating activity when measured in terms of ligand-induced adenylyl cyclase activation, in comparison to the activation obtained with intact hCG (30,63). In addition, especially the oligosaccharide units of the α subunit appeared to be important for efficient stimulation of adenylyl cyclase (59,77). Based on these observations, DhCG is considered as a hormone devoid of receptor-stimulating activity, or as a hormone with substantially impaired receptor-stimulating activity. Because of the discordant properties of receptor binding and receptor

activation, DhCG acts as a competitive inhibitor of hCG-induced activation of adenylyl cyclase. Thus, in terms of adenylyl cyclase activation DhCG possesses antagonistic activity towards intact hCG. Similar results as for DhCG have been reported for deglycosylated LH (77-79), FSH (80-82), and TSH (64,83). Although consensus has been obtained for the less efficient adenylyl cyclase stimulation by DhCG, variable steroidogenic activities have been reported for DhCG. Maximal steroidogenic activity obtained with DhCG varied from 0-5% of that of intact hCG when measured in mouse Leydig tumor cells (MA-10 cells) (84,85), to 30-50% when estimated in isolated rat Leydig cells (86-88). Differences in fitting between hCG and DhCG and the LH/CG receptor in different species might underlay the different receptor-stimulating activities of DhCG when compared to that of native hCG in various species. In addition, species dependent differences in the coupling efficiency between adenylyl cyclase activation and the steroidogenic pathway might explain the variable steroidogenic activities of DhCG. In isolated rat Leydig cells maximal hCG-induced stimulation of steroidogenesis coincides with nonmeasurable elevations of the intracellular concentration of cAMP (89). On the other hand, in MA-10 cells approximately 70% of the maximal stimulation of adenylyl cyclase is required for full stimulation of steroid production (90). Although cAMP is the primary second messenger for LH/CG induced steroidogenesis we can not exclude other second messenger to be involved in LH/CG-induced steroidogenesis (91). However, thus far no evidence has been obtained for the involvement of other second messenger pathways than cAMP in DhCG-induced steroidogenesis.

It is not clear to what extent DhCG possesses biological activity *in vivo*. Some antagonistic activities have been reported, since DhCG has been shown to inhibit hCG-induced steroidogenesis *in vivo* (92), and to inhibit implantation and terminate pregnancy in rats (93). However, DhCG failed to block endogenous steroidogenesis in male monkeys (94) and cycling women (95).

More recently, the individual roles of the asparagine-linked oligosaccharide units in hCG and FSH in receptor binding and hormone-induced receptor activation have been studied by site-directed mutagenesis of the asparagine residues that are essential for glycosylation (84,96-98). Mutant hormone derivatives were obtained from cells that had been transfected with complementary DNA (cDNA) constructs encoding the α and ß subunit genes containing substitution mutations at the individual glycosylation sites, preventing glycosylation at those sites. For both hCG and FSH, it appeared that the absence of any of the various oligosaccharide units did not impair the binding of the hormone to the receptor. Of all four oligosaccharide units, the one attached to asparagine

52 of the α subunit appears to be the most important for ligand-induced receptor activation. Loss of this oligosaccharide unit markedly impairs the receptor-stimulating activity of the mutant hormones (84,96-98).

How the oligosaccharide units in hCG influence hormone-induced LH/CG receptor activation, is still unknown. They may interact directly with lectin-like domains on the receptor (99) and/or on other plasma membrane components, thereby inducing aggregation of hormone-receptor complexes and efficient transmembrane signalling (76,77). Alternatively, the oligosaccharide units may stabilize the three-dimensional protein conformation of the hormone that is properly recognized by the LH/CG receptor. Consistent with the latter hypothesis, immunological and physico-chemical evidence suggests different conformations of hCG and DhCG (80,62,100-104). Polyclonal antibodies directed against hCG (105) and more specific those against the hCG ß subunit (106,107) were able to enhance the receptor-stimulating activity of DhCG. Antibody induced receptor aggregation leading to activation of the receptor (108) is not involved in the effect of anti-hCG antibodies on the receptor-stimulating activity of DhCG, since Fab fragments that were derived from these antibodies showed similar activity as the bivalent intact antibodies (105). Thus, antibody binding to DhCG apparently enhances the receptor-stimulating activity of the hormone, suggesting that conformational changes in the protein backbone of DhCG, induced by deglycosylation, might be reversed. However, thus far the use of polyclonal antibodies hampered characterization of the epitopes on the hormone that are involved in this process. The use of monoclonal antibodies could help to define these epitopes.

Glycoprotein hormone receptors

Receptors for the glycoprotein hormones have been demonstrated on ovarian granulosa (FSH and LH) and theca cells (LH), on testicular Sertoli (FSH) and Leydig (LH) cells, and on thyroidal cells (TSH). It has recently been found that various nongonadal and nonthyroidal tissues also express glycoprotein hormone receptors at a low level, but the biological significance of this expression remains to be established (109-111).

During the past four years, the cDNAs for the LH/CG (99,112,113), FSH (114-117), and TSH (118-122) receptors in various species have been cloned and sequenced. From these data it appeared that glycoprotein hormone receptors are composed of a single polypeptide chain of 650-750 amino acid residues. Hydropathy analysis of the primary structure of the LH/CG, FSH, and TSH receptors predicts the existence of seven regions of

20-25 amino acid residues each, which probably traverse the membrane and form membrane-spanning α helices, with the intervening hydrophilic loops exposed alternately intracellularly and extracellularly. The three exoplasmic and three cytoplasmic loops might be involved in the spatial restriction of the transmembrane domains that form a barrel-shaped pocket embedded in the phospholipid bilayer (Figure 4). Within the α helices, the hydrophobic amino acid residues on one side of the helix might interact with the membrane, while the more hydrophilic amino acid residues are exposed to the inside of the pocket, and are able to interact with hydrophilic surfaces on the ligands. The predicted structure of the transmembrane domain, as based on hydropathy analysis, has been independently confirmed for bacteriorhodopsin, rhodopsin, and $ß_2$-adrenergic receptors (122-125).

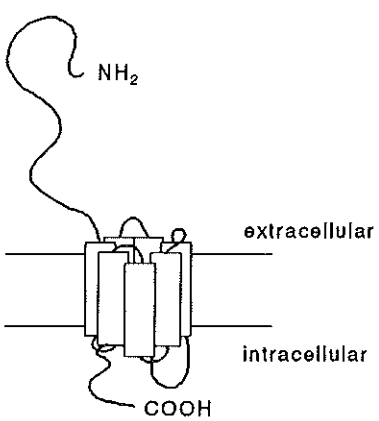

Figure 4. ***Glycoprotein hormone receptor.*** *Schematic representation of the postulated structure of a glycoprotein hormone receptor, containing a large extracellular N-terminal domain, a transmembrane domain that is likely to traverse the membrane seven times with three intervening loops alternatively located intracellularly and extracellularly, and an intracellular C-terminal domain.*

The overall structure of the glycoprotein hormone receptors is similar to that off all G protein-coupled receptors. However, the LH/CG, FSH, and TSH receptors share two characteristics that distinguish them from other GPCRs: 1) their precursors contain a signal peptide (20-30 amino acids in length), and 2) they possess a large extracellular N-terminal domain of approximately 340 (LH/CG and FSH receptors) and 390 (TSH receptor) amino acid residues. Characteristic for the N-terminal domain is the presence of 14 (LH/CG and

FSH receptors) and 16 (TSH receptor) copies of an imperfectly repeated sequence of about 25 amino acid residues, that is similar to a so-called "leucine-rich repeat" motif that exists in various leucine-rich proteins (126). The functional importance of the leucine-rich repeat motif is unclear, however, by forming amphipathic α helices or ß sheets (127) this structure might be functional in the interaction with both hydrophobic and hydrophilic surfaces, and might contribute to protein-protein and/or protein-membrane interactions. The C-terminal part of the extracellular domain of the glycoprotein hormone receptors may then function as a hinge region, connecting the hormone binding site to the membrane-spanning domains. The predicted extracellular location of the N-terminal domain and intracellular location of the C-terminal domain of the receptor have been confirmed for the rat LH/CG receptor, by using polyclonal antibodies directed against peptides corresponding to regions of the specific domains (128).

The intracellular loops and C-terminal domain of the receptors are most likely involved in G protein coupling. Especially, the third cytoplasmic loop has been demonstrated to be a major contact site between GPCRs and G proteins (129,130). Within the intracellular domains of the receptors different regions might be involved in stimulation of distinct intracellular signalling pathways. In the TSH receptor an intracellular region adjacent to transmembrane helix VII appeared to be essential for hormone-induced stimulation of the phosphoinositide pathway but not for stimulation of adenylyl cyclase (131).

Recently, mutations in TSH and LH receptors have been found in patients, that result in constitutively activated receptors even in the absence of ligands (131,132). Mutations in GPCRs that cause constitutive activation of the receptors have also been shown in *in vitro* mutagenesis studies of these receptors (134,135). Domains within the receptors, among which are sites in the third cytoplasmic loop, are likely to be essential for maintaining the receptor in a constrained conformation (135). Ligand occupation of the receptor might then induce conformational changes in these domains, causing receptor activation and subsequent coupling to G proteis. The cytoplasmic loops and the C-terminal intracellular domain of the glycoprotein hormone receptors also contain several potential phosphorylation sites. Phosphorylation of these sites might be involved in desensitization and downregulation of the receptor (136). Recent studies have demonstrated that LH/CG and FSH receptors are phosphorylated upon hormonal stimulation, however, the implications for receptor function remain to be established (137,138).

The genes encoding the glycoprotein hormone receptors consist of 10 (FSH and TSH receptors) and 11 (LH/CG receptor) exons (139-142). Perhaps the most remarkable feature of these genes is that in all glycoprotein hormone receptors the extracellular domain with

its leucine-rich sequences is encoded by 9 (FSH and TSH receptors) or 10 (LH/CG receptor) exons, while the remaining part of the receptor, the transmembrane and intracellular C-terminal domain, is encoded by a single exon. Many members of the superfamily of GPCRs which bind small ligands, contain short extracellular domains, and are encoded by a single exon of intronless genes. The extracellular N-terminal domain of the LH/CG receptor contains most, if not all, of the binding site(s) for hCG (143-146). The structural organization of glycoprotein hormone receptor genes suggests that they evolutionary arose by coupling a gene encoding a soluble, leucine-rich ligand-binding protein (9 or 10 exons) to an intronless gene (one exon) of the ancient GPCR family.

The glycoprotein hormone receptors are highly homologous in their transmembrane domains, with an overall amino acid sequence identity of 60-70%, while the N- and C-terminal domains possess only 20-40% sequence identity. Conserved locations of cysteine residues in the receptors might indicate structural homology between different receptors. However, the total number of cysteine residues varies among the different receptors, and there is only partial conservation of the positions of these residues. Although the low number of conserved cysteine residues between the different glycoprotein hormone receptors suggest otherwise, there probably is substantial structural homology between the FSH, LH/CG, and TSH receptors, as would be consistent with the structural homology of their ligands.

The extracellular N-terminal domains of the LH/CG, FSH, and TSH receptors contain several consensus sequences for N-linked glycosylation. Only one of these potential glycosylation sites has been fully conserved in all known glycoprotein hormone receptors. Although it is clear that the receptor is a glycoprotein (147-150), it is not known which and how many of the potential glycosylation sites contain an oligosaccharide unit. The oligosaccharide moiety is likely the reason for the discrepancy between the molecular mass as predicted from the cDNA (70-80 kD) and the apparent mass as deduced from sodium dodecylsulfate gel electrophoresis of the purified glycoprotein hormone receptors (90-100 kD). By either N-glycosidase F treatment or site-directed mutagenesis, it has been shown that none of the oligosaccharide units is important for the affinity or specificity of the receptor towards its ligand (151-154). Deglycosylated LH/CG receptors can be stimulated by hCG, indicating that the oligosaccharide units of the receptor are not important for ligand-induced receptor activation (151-154).

Glycoprotein hormone-receptor interaction

For more than 25 years, scientists have investigated the interaction between the

glycoprotein hormones and their receptors without having information on the spatial interactions between hormone and receptor. The glycoprotein hormone receptors bind large ligands (28-38 kD) and contain a large extracellular N-terminal domain, constituting approximately half of the receptor protein, that distinguishes them from all other GPCRs. Most GPCRs only contain a small extracellular N-terminal domain (30-50 amino acids in length), and bind a relatively small ligand (200-300 dalton) through interactions with amino acid residues that are located inside the transmembrane pocket. It was hypothesized that the large extracellular N-terminus of the glycoprotein hormone receptors would be responsible for specific recognition and binding of the respective hormones. This hypothesis is supported for hCG binding to the LH/CG receptor, by the observation that truncated LH/CG receptor proteins which represent only the extracellular N-terminus of the receptor, bind hCG with similar affinity as the full-length receptor (143-146). Moreover, the specificity of the glycoprotein hormone receptors for their respective ligands appears to be defined by the extracellular N-terminal domain, since substitution of a large part of the N-terminal domain of the FSH receptor for the corresponding region of the LH/CG receptor, and *vice versa*, yielded chimeric receptors of which the specificity was defined by the origin of the N-terminal part of the extracellular domain (155). Similar results have been established for chimeric LH/CG-TSH receptors (156,157).

Subsequent to hormone binding to the extracellular N-terminal domain of the receptor, the hormone might be properly positioned towards an activation domain of the receptor that is defined by the spatial orientation of amino acid residues within the transmembrane pocket (Figure 5) (155). Photoaffinity labeling studies (158), and studies with peptides that represent domains of the receptor (159) have revealed that both the extracellular N-terminal domain and the transmembrane pocket of the LH/CG receptor contain contact sites for hCG. Moreover, a truncated LH/CG receptor that lacks the extracellular N-terminal domain, binds hCG with low affinity and can be activated by it (160,161). These results suggest that hCG interacts with its receptor through at least two distinguishable sites, located on the extracellular N-terminal and the transmembrane domain. The extracellular N-terminal domain might function as an antenna that binds the hormone, while receptor activation and transmembrane signalling might depend on structural elements in the transmembrane-spanning domains of the receptor. This latter site of interaction within the transmembrane pocket could be relatively small for small ligands such as neurotransmitters, and more complex for the large glycoprotein hormones. The transmembrane pocket might also be a rather flexible structure that adjusts to the size of the ligand. A two site binding model for the glycoprotein hormone receptors, in which the

ligand binds to the extracellular N-terminal domain but also interacts with the transmembrane domain, is appealing. It conserves the ligand binding properties of the transmembrane domain of the GPCR superfamily, and provides an evolutionary bridge for ligand-induced receptor stimulation between those family members that bind small ligands and those that bind large glycoproteins.

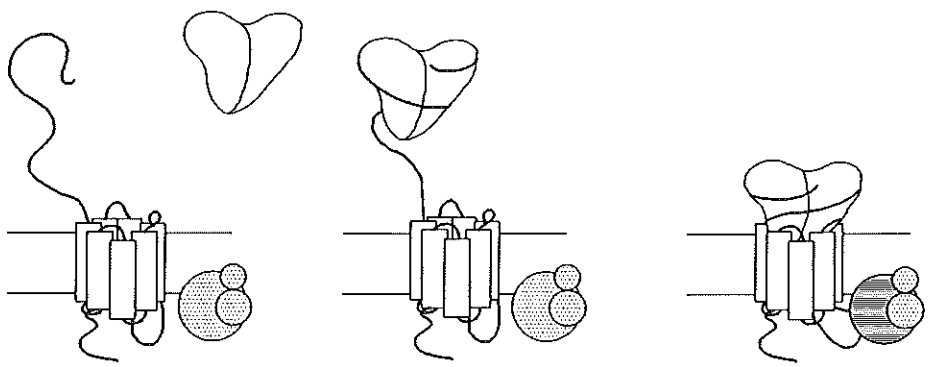

Figure 5. ***Model for gonadotropin receptor stimulation.*** *Sequential steps in the hormone-receptor interaction lead to hormone-induced receptor stimulation: the hormone 1) binds to the extracellular N terminal domain of the receptor, 2) becomes properly positioned towards the transmembrane domain, and 3) is docked into the transmembrane pocket, thereby inducing receptor activation and subsequent coupling between the receptor and a G protein.*

NaCl modulation of glycoprotein hormone-receptor interaction

The binding of a hormone to its receptor is essential for hormone-induced receptor stimulation, and has been extensively studied. Under low salt conditions, using either membrane preparations, solubilized receptors, or intact cells, a high affinity binding complex of glycoprotein hormones and their respective receptors is detectable (162,163). These binding characteristics are generally considered to represent the functional receptor that is involved in signal transduction. However, it should be noted that formation of high affinity binding complexes is favoured by nonphysiological low salt conditions. In this respect, a common feature of glycoprotein hormone receptors, and in general of GPCRs, is that the ligand binding properties are influenced by local concentrations of monovalent and divalent cations (164-168). In this thesis, we have focused on the effects of NaCl, since NaCl contributes most to the physiologic ionic milieu.

For the glycoprotein hormones, it is known that NaCl markedly affects the binding of the hormone to its receptor. NaCl has been shown to decrease the affinity constant for LH binding to the LH/CG receptor (169-172), and the actual number of TSH binding sites that is able to bind ligand (173,174). Thus, the high affinity binding properties of the glycoprotein hormone receptors, as measured in binding assays under low ionic strength conditions apparently do not reflect properties of the functional receptors. The exact binding properties of the receptors that mediates signal transduction under physiological conditions remain to be established.

In the literature, discrepancies have been described between the binding properties of a receptor towards its ligand and the receptor-stimulating potency of that ligand. LH and hCG activate the LH/CG receptor with equimolar concentrations (172). However, this is in contradiction with the observation that in the presence of NaCl the receptor possesses approximately 10-fold higher affinity for hCG than for LH. Also for the human TSH receptor, it has been reported that receptor binding and activation are dependent of the ligand (175). Under low ionic strength conditions bovine TSH binds with high affinity to the human TSH receptor, whereas the binding of human TSH to the human TSH receptor was undetectable. Yet, both ligands possess the potency to stimulate the human TSH receptor.

An electrostatical basis for glycoprotein hormone-receptor interaction

Several hormones and neurotransmitters that bind to GPCRs contain positively charged groups that are pivotal for hormonal activity (176-179). Electrostatic interactions between positively charged residues in the ligand and negative amino acid residues in the receptor might be involved in hormone/receptor interaction. For the glycoprotein hormones, several charged residues have been shown to be important for hormone binding to the receptor and hormone-induced receptor stimulation (67). These residues might come into close proximity in the three dimensional conformation of the "active center" of the hormone. The "active center" might then be positioned into the transmembrane pocket of the receptor, and act in a similar fashion as small charged ligands that activate GPCRs. A large part of the protein backbone, and probably also the oligosaccharide units of the glycoprotein hormones, would then be important for the maintenance of the proper spatial conformation of the "active center" and the correct positioning thereof towards the transmembrane pocket. Substitution mutagenesis of lysine 91 in the α subunit of hCG by either a neutral methionine residue or a negatively charged glutamic acid residue, revealed that a positive charge at this position is essential for hormone-induced activation of the LH/CG receptor,

whereas it appeared not to be important for high affinity receptor binding (176). Substitution mutagenesis of lysine α91 into a positive arginine residue was without any significant effect on receptor stimulation and binding.

Several negatively charged amino acid residues are located in the transmembrane domains of the receptor. Site-directed mutagenesis and covalent chemical labeling studies on adrenergic and muscarinic receptors indicate that an aspartic acid residue located in the third transmembrane domain is most probably directly involved in ligand binding (180-183). Moreover, since this aspartic acid or glutamic acid residue is highly conserved among GPCRs, a negatively charged amino acid residue at this position might be essential for the interaction of GPCRs with ligands. In contrast to negatively charged residues that are directly involved in ligand binding, a highly conserved aspartic acid residue located in the interior of the transmembrane pocket in the second helix is involved in allosteric modulation of ligand binding (172,181). In unoccupied GPCRs the negatively charged carboxyl group of this aspartic acid residue might form an ionic bond with Na^+ ions, assuming that Na^+ ions can enter the transmembrane pocket. As a consequence of this shielding of the negative charge, the ligand-binding affinity of many GPCRs for agonists is lower in the presence than in the absence of NaCl. For the dopamine receptors and the α_2- and $ß_2$-adrenergic receptors, substitution of this negative aspartic acid residue by the corresponding neutral amide abolished the effect of NaCl on the affinity for agonists (180,181,184-186). Similar results have been obtained for the corresponding aspartic acid residue in the LH/CG receptor (172). All these data support the possible importance of electrostatic interactions between hCG and the LH/CG receptor. Consistent with this hypothesis, a hCG molecule with lysine α91 substituted by either a negatively charged aspartic acid or glutamic acid residue can bind to and activate a LH/CG receptor containing a lysine or arginine residue instead of an aspartic acid residue in the second transmembrane domain, with similar affinity and potency as native hCG binds to and activates the wild type LH/CG receptor (187).

Different receptor binding for hCG as compared to other glycoprotein hormones

For the binding of hCG to the LH/CG receptor it has been well established that a high affinity binding site is located in the extracellular N-terminal domain of the receptor (143-146). However, a negatively charged aspartic acid residue in the first extracellular loop of the LH/CG receptor has been shown to be indispensable for hCG-induced adenylyl cyclase stimulation (161). In addition, a truncated LH/CG receptor that lacks its extracellular N-terminal domain is able to bind hCG with low affinity and is stimulated by hCG, albeit at

much higher hormone concentrations than the wild type receptor (162). On this truncated receptor, the positioning and the docking of the "active center" of the hormone into the transmembrane domain might be less efficient than for the wild type receptor.

For the other glycoprotein hormones it remains to be established whether a high affinity binding site is located in the extracellular N-terminal domain of their respective receptors. It has been shown that while hCG binds with high affinity to a truncated LH/CG receptor that represents only the extracellular N-terminal domain, the binding affinity of the truncated receptor towards LH was substantially lower than that of the full-length LH/CG receptor (172). This may indicate that the binding sites on the LH/CG receptor for LH and hCG are different. Whereas for hCG the binding site is primarily located in the extracellular N-terminal domain of the receptor, the binding site for LH is very likely composed of regions contributed by both the extracellular N-terminal domain and the transmembrane domain. This could explain why NaCl modulates only LH binding and not hCG binding to the same receptor, since the modifying role of Na^+ ions has been demonstrated to be mediated by an aspartic acid residue in the second transmembrane spanning helix (172). The ligand binding affinity of truncated FSH and TSH receptors has not yet been tested. However, the ligand binding site of these receptors is very likely also composed of regions on both the N-terminal and transmembrane domains, as it is for LH binding to the LH/CG receptor, since NaCl influences ligand binding to the TSH and FSH receptors.

A binding site defined by both the extracellular N-terminus and the transmembrane domain of the glycoprotein hormone receptors would be in contradiction to the idea that the extracellular N-terminal domain functions as an extended antenna for the hormone. As indicated before, the extracellular N-terminal domain contains a "leucine-rich repeat" motif. Recently, the molecular structure of porcine ribonuclease inhibitor, a member of the "leucine rich repeat" protein family has been elucidated (188). Based on this finding it might be deduced that the extracellular N-terminal domain of the glycoprotein hormone receptor is much more a collar-like structure on top of the transmembrane domain, rather than an extended antenna-like structure. Considering a collar-like structure of the extracellular N-terminal domain, the interaction site of the receptor might be a spatial orientation of amino acid residues located on the extracellular domain as well as on the transmembrane domain (Figure 6). The extracellular N-terminus could confer hormone specificity, whereas the interaction site can mediate both hormone binding to the receptor and hormone-induced stimulation of the receptor.

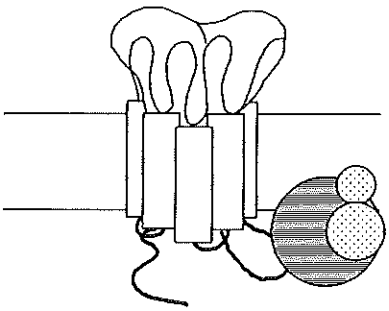

Figure 6. ***Alternative model for hormone-receptor interaction.*** *Based on its "leucine-rich repeat" motif, the extracellular domain of the glycoprotein hormone receptors might be an ordered structure that forms a collar-like structure on top of the transmembrane domain. Such a conformation would allow for the presence of one interaction site for the hormone on the receptor that is involved in both ligand binding and ligand-induced receptor stimulation. The interaction site might be composed of domains in both the extracellular N-terminal domain and the transmembrane domain.*

Scope of this thesis

The studies described in this thesis have been carried out in order to increase our understanding of the interaction between glycoprotein hormones and their respective receptors. This interaction has been studied in terms of ligand binding to the receptor and ligand-induced receptor stimulation. Ligand binding to the glycoprotein hormone receptors is pivotal for ligand-induced receptor stimulation, however, discrepancies between the receptor binding properties of a ligand and its receptor-stimulating potency have been reported. Subsequent to deglycosylation of hCG, the binding of the hormone to the LH/CG receptor is of similar or even higher affinity when compared to that of native hCG. Yet, the interaction of DhCG with the LH/CG receptor leads to a substantial lower stimulation of adenylyl cyclase in comparison to that induced by hCG. Moreover, LH and hCG stimulate the LH/CG receptor with equimolar potencies in the presence of 0.15 M NaCl, whereas under the same conditions the binding affinity of the LH/CG receptor for LH is approximately 10-fold lower than that for hCG. This discrepancy in binding to the LH/CG receptor between LH and hCG is abolished in the absence of NaCl. Similar as for LH, 0.15 M NaCl has been shown to impair the binding properties of FSH and TSH to their respective receptors.

In order to better understand ligand-induced stimulation of glycoprotein hormone

receptors, the binding of a glycoprotein hormone to its receptor should be studied under similar conditions as in which ligand-induced receptor stimulation occurs.

The molecular details of the mechanisms underlying hormone-induced receptor activation will probably remain unknown until the three-dimensional structures of the hormones, the receptors, and the hormone/receptor complexes are elucidated. Recently, the crystal structure of hCG in its deglycosylated form has been elucidated, although that of the natural ligand, hCG, remains unknown. We have attempted to gain further insight in hormonal stimulation of the gonadotropin receptor by two different indirect approaches.

First, we have studied ligand-receptor interaction by manipulation of the ligand (deglycosylation of hCG) and measurement of the biological effects of this modified ligand (DhCG). As indicated in this Introduction, the receptor binding and activation properties of DhCG might be dependent on the matching between hCG and DhCG with LH/CG receptors of different species origin. To study this, the biological effects of different preparations of deglycosylated hCG have been measured in rat Leydig cells and mouse Leydig tumor cells. Conformational changes in the protein backbone of DhCG, induced by deglycosylation of hCG may cause an improper fitting of DhCG to the LH/CG receptor, resulting in less efficient stimulation of adenylyl cyclase. Antibody binding to a protein may affect the three-dimensional structure of that protein, and the effects of monoclonal anti-hCG antibodies on DhCG-induced receptor stimulation have been studied.

As a second approach, we have studied the interactions between FSH and its receptor, in terms of binding and activation, by modulation of the ionic conditions. The effect of lowering the physiologic concentrations of NaCl on FSH-induced receptor stimulation and on the binding of the hormone to its receptor have been studied. Moreover, the kinetics of FSH receptor stimulation and binding have been compared in the absence and presence of physiologic concentrations of NaCl.

Based on our experimental data on the effects of modulation of the ligand and the ionic conditions on hormone-receptor interaction, compared with data reported in the literature, we propose a model involving sequential but distinguishable hormone/receptor complexes for glycoprotein hormone receptor binding and receptor activation.

CHAPTER TWO

THE RELATIVE IMPORTANCE OF THE OLIGOSACCHARIDE UNITS IN hCG FOR LH/CG RECEPTOR ACTIVATION IN RAT LEYDIG CELLS AND MOUSE LEYDIG TUMOR CELLS

H.J. van Loenen[1], S. van Gelderen-Boele[1], J.F. Flinterman[1], W.E. Merz[2] & F.F.G. Rommerts[1].

[1] Department of Endocrinology & Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, The Netherlands.
[2] Department of Biochemistry II, University of Heidelberg, Germany

Submitted

SUMMARY

The biological properties of deglycosylated human chorionic gonadotropin (DhCG), obtained by hydrogen-fluoride treatment (HF-DhCG) of intact hCG or by oligonucleotide-directed mutagenesis (CHO-DhCG), and that of their fully glycosylated counterparts, were tested in rat Leydig cells and in mouse Leydig tumor cells (MA-10 cells), in terms of cAMP and steroid production. In both cell types, HF-DhCG and CHO-DhCG showed comparable biological activities. The maximum for DhCG-induced cAMP production was approximately 12% of that of intact hCG when tested in rat Leydig cells, and only 2% when tested in MA-10 cells. DhCG possessed significant steroidogenic activity, with a maximal response being 30-50% of that of intact hCG in MA-10 cells, whereas in rat Leydig cells DhCG and hCG showed similar steroidogenic maxima. Based on its ED_{50}, DhCG possessed 10-17% of the steroidogenic potency of intact hCG in rat Leydig cells, while in MA-10 cells DhCG was only 2-fold less potent than hCG. When accurate binding data are absent, the intrinsic receptor-stimulating activity of a ligand can still be estimated at full receptor occupancy, provided that over the whole dose range the biological response is proportional to receptor stimulation. The present data show that in transfected MA-10(P^+29) cells which over-express rat phosphodiesterase, the cAMP and steroidogenic responses are directly coupled to receptor activation up to maximal occupation of the LH/CG receptor. The intrinsic receptor-stimulating activity of DhCG, measured in these cells in terms of DhCG-induced steroidogenesis, appeared to be 7 to 10-fold lower than that of intact hCG. It is known from literature that DhCG possesses 2 to 3-fold higher affinity to the LH/CG receptor than intact hCG. In MA-10 cells, this 2 to 3-fold increased binding affinity of DhCG may partly compensate for the 7 to 10-fold reduction in the intrinsic receptor-stimulating activity, resulting in a only 2-fold reduction in steroidogenic potency of DhCG. In terms of adenylyl cyclase stimulation, DhCG had a much lower (50-fold) receptor-stimulating activity. The present data illustrate that the oligosaccharide units in hCG are not essential for LH/CG receptor activation, and that the relative receptor-stimulating activity of DhCG to that of hCG is highly dependent on whether cAMP or steroid production is measured as the biological response, and whether rat Leydig cells or MA-10 cells are used as bioassay system.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a highly glycosylated protein hormone that

stimulates target cells via the G_S protein-coupled LH/CG receptor. Following hCG occupation of the receptor and coupling of the receptor to G_S protein, adenylyl cyclase becomes activated, which results in an increase in intracellular cAMP concentration. Through activation of cAMP-dependent protein kinases and subsequent phosphorylation of endogenous proteins, a diversity of cellular processes is regulated, among which steroidogenesis is by far the most intensively investigated. Although cAMP is the main second messenger for hCG-induced steroidogenesis, other intracellular signalling cascades, such as the phosphatidylinositol pathway, may also be involved (50,51,91).

hCG consists of two non-covalently linked subunits, α and ß, which both contain two asparagine-linked oligosaccharide units, while the carboxyl-terminus of the ß subunit also contains four additional serine-linked oligosaccharide units (6,8,63). The carboxyl-terminus of hCGß with its serine-linked glycosylation is not important for LH/CG receptor activation, but appears to play a crucial role in the circulatory half-life of the hormone (18).

The current interest with regard to hCG action, is to define the domains on the hormone that are involved in hormone-induced receptor-stimulation. Many studies using oligosaccharide deficient hormone preparations have been carried out in order to resolve the question whether the oligosaccharide units in hCG constitute one of these domains. For this purpose, deglycosylated hCG (DhCG) has been obtained from hCG by enzymatic procedures (58,59) or by treatment with hydrogenfluoride (60-62,189) or trifluormethan sulfonic acid (190). Recently, *De novo* synthesis of recombinant DhCG was obtained after stable transfection of chinese hamster ovary cells with cDNA constructs encoding the hCG α and ß subunit genes that contained oligonucleotide mutations resulting in the absence of the asparagine residues that are essential for asparagine-linked glycosylation (84,96).

Many studies have shown that DhCG possesses 2 to 3-fold higher affinity towards the LH/CG receptor than intact hCG (6,8,63). However, the increased receptor-binding affinity of the ligand appears not to result in an increased potency to stimulate the LH/CG receptor. In contrast, deglycosylation of hCG results in a substantial reduction in receptor-stimulating activity of the ligand. DhCG possesses a relatively low *in vitro* biological activity when compared to that of hCG, and the property to inhibit hCG-induced receptor stimulation (63,84). In particular the antagonistic properties of DhCG have been emphasized in literature, and the oligosaccharide units in hCG are therefore considered to be very important or even essential for LH/CG receptor activation (59-62,85,94). However, although consensus has been obtained for the markedly impaired adenylyl cyclase-stimulating activity of DhCG, discordant steroidogenic properties for DhCG have been found. The reported steroidogenic potencies of DhCG varied tremendously, being 0-5% in

terms of the maximal hCG-induced steroidogenesis in mouse Leydig tumor cells (MA-10 cells) (85,96,107), whereas in rat Leydig cell preparations the maximal steroidogenic activity of DhCG was 20-50% of that of intact hCG (62,71,87). For rat Leydig cells, it has been shown that the LH/CG receptor binding and receptor activation properties are highly dependent on the species specificity of the stimulating hormone (170,191). Hence, the large variation in the steroidogenic properties of DhCG when compared to that of intact hCG might to a substantial extent depend on the use of Leydig cells of different species origin.

The present report describes, to our knowledge for the first time, a comparative study on the receptor-stimulating properties of intact hCG and two different types of DhCG (HF-DhCG, obtained by hydrogen-fluoride treatment of intact hCG, and recombinant CHO-DhCG), in terms of stimulation of cAMP and steroid production by MA-10 cells and rat Leydig cells.

MATERIALS AND METHODS

Hormones:

Highly purified urinary hCG (95% pure) was kindly provided by Organon International b.v. (Oss, The Netherlands). Chemically deglycosylated hCG (HF-DhCG) was obtained by hydrogen fluoride treatment of intact hCG and purified by gel filtration as described before (189). DhCG lacking the asparagine residues that are essential for oligosaccharide attachment on the α and ß subunits was produced via oligonucleotide-directed mutagenesis and cell transfection (84). Stable transfected CHO- cells that produce either wild type hCG or hCG lacking all four asparagine-linked oligosaccharides were obtained from Dr. I. Boime (Washington University, St. Louis, MO, USA), and were cultured essentially as described by Matzuk et al. (84). In brief, cells were maintained in Ham's F-12 medium supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 5% (v/v) fetal calf serum and G-418 (0.25 mg/ml) at 37°C. For hormone collection, confluent cell layers were incubated in α-minimal essential medium supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), in the absence of serum. After 48 hours of incubation, the medium was collected, centrifuged to remove cell debris, and concentrated approximately 10-fold with an Amicon centriprep with a 10 kD cut-off (Amicon, Lexington, MA, USA). The total protein contents of the concentrated CHO-hCG and CHO-DhCG preparations were 1.3 ± 0.1 and 1.5 ± 0.1 mg/ml (mean ± SD, of three independent protein determinations), respectively. The hCG concentrations in the different hormone preparations were determined in a double antibody fluorometric enzyme immunoassay for dimeric hCG (Baxter Diagnostics Inc., Deerfield, USA) according to the instructions of the manufacturer. The assay has been standardized to the World Health Organization 1ST international reference preparation (IRP#75/537), and cross-reactivity of free ß subunits was approximately 1.4%. Immunological activities were converted to molar concentrations based on a molecular weight of 38.000 for hCG and 30.000 for DhCG (5).

Isolation and culture of cells:

Rat Leydig cells were isolated from testes of 21-23 days old Wistar rats, as described by Rommerts et al. (192). Cells were plated in RPMI-1640 medium, supplemented with streptomycin

(100 µg/ml), penicillin (100 IU/ml), L-glutamine (2 mM), and bovine serum albumin (0.1% w/v). After 1 hour, nonattached cells were removed by washing twice with RPMI-1640 medium containing 0.1% BSA, and the incubations were started by the addition of the hormone-containing media.

Mouse Leydig tumor cells, MA-10 cells (193), were grown in RPMI-1640 medium supplemented with streptomycin (100 µg/ml), penicillin (100 IU/ml), L-glutamine (2 mM), and horse serum (10% v/v). The cells were subcultured after trypsin treatment (0.05% w/v trypsin), and experimental cultures were plated and cultured for 2 days. After washing twice with RPMI-1640 containing 0.1% BSA, the cells were used for the experiments.

Stable transfectants of MA-10 cells that over-express rat cAMP-phosphodiesterase [MA-10(P^+29) cells] were obtained from Dr. M. Conti (University of Iowa, Iowa City, USA), and grown essentially as described by Swinnen et al. (194). In brief, the cells were grown in RPMI-1640 medium supplemented with streptomycin (100 µg/ml), penicillin (100 IU/ml), L-glutamine (2 mM), and horse serum (10% v/v) in the continuing presence of 200 µg/ml G-418 and 5 µM Ro20-1724. The cells were subcultured after trypsin treatment (0.05% w/v trypsin), and experimental cultures were plated and cultured for 2 days in the absence of G418 but the presence of 5 µM Ro20-1724. Prior to the experiments the cells were washed twice with RPMI-1640 containing 0.1% BSA.

cAMP production:

Rat Leydig cells and MA-10 cells were incubated for 1 hour at 37°C with or without hormones, in the presence of 0.25 mM of the phosphodiesterase inhibitor 1-methyl-3-isobutyl-xanthine (IBMX). For determination of the total amount of cAMP, concentrated perchloric acid was added to the cells and medium at a final concentration of 5% (v/v). The cell lysate was treated with 1.1 M Tris-containing KOH sufficient to neutralize the perchloric acid, and cAMP was subsequently measured by radioimmunoassay essentially as described by Stoof et al. (195).

Steroid production:

Rat Leydig cells and MA-10 cells were incubated with or without hormone for 3 hours at 37°C, in the presence of the inhibitors of pregnenolone metabolism epostane and SU-10603 at concentrations of (5 µM and 5 µM) and (5 µM and 20 µM), respectively (196). These compounds prevent metabolism of pregnenolone by 3ß-hydroxy-steroid dehydrogenase and 17α-hydroxylase, respectively. Pregnenolone was measured in the medium by radioimmunoassay (197).

RESULTS

Ligand-induced cAMP and steroid production:

The biological properties of hCG, CHO-hCG, HF-DhCG, and CHO-DhCG were tested in terms of stimulation of cAMP and pregnenolone production in MA-10 cells and rat Leydig cells. In both the rat Leydig cell bioassay and the MA-10 cell bioassay the glycosylated hormones hCG and CHO-hCG were equipotent, with similar ED_{50} values (the hormone concentration that is required for half-maximal stimulation) and E_{MAX} (maximal response), when either cAMP or pregnenolone production was measured (Table 1). The E_{MAX} for the DhCG-induced cAMP production was much less than for intact hCG, being only 2 and 12% of the maximal response to hCG in MA-10 cells and rat Leydig cells,

respectively. Accurate comparison of the ED_{50} values of the adenylyl cyclase-stimulating activities of DhCG and hCG was not possible, due to the severe nonparallelism of their dose/response curves.

Table 1. ***Half-maximal dose (ED_{50}) and maximal response (E_{MAX}) in the rat Leydig cell and MA-10 cell bioassay.***

		Pregnenolone production		cAMP production	
		ED_{50} [pM]	E_{MAX} [pmoles]	ED_{50} [pM]	E_{MAX} [pmoles]
Rat	hCG	0.3 ± 0.02	208.9 ± 9.5	16.3 ± 3.0	1006.4 ± 24.9
	CHO-hCG	0.3 ± 0.01	216.5 ± 4.5	15.7 ± 2.5	1000.7 ± 16.8
	HF-DhCG	4.3 ± 0.7	215.8 ± 45.5	ND	124.0 ± 2.4
	CHO-DhCG	1.8 ± 0.6	231.1 ± 11.1	ND	115.5 ± 14.4
MA-10	hCG	18.4 ± 1.8	4040.5 ± 207.7	97.5 ± 8.3	936.1 ± 48.1
	CHO-hCG	21.8 ± 3.0	4483.9 ± 741.0	123.6 ± 23.2	961.6 ± 123.0
	HF-DhCG	40.0 ± 1.6	1376.5 ± 103.4	ND	16.6 ± 0.6
	CHO-DhCG	38.0 ± 4.9	2683.1 ± 186.7	ND	21.0 ± 1.2

ND. Due to the low E_{MAX} for the DhCG-induced cAMP production we considered it inaccurate to estimate ED_{50} values.

Ligand-induced pregnenolone and cAMP production are measured as described in Materials and Methods. It should be noted that the pregnenolone production is measured in the absence of IBMX, whereas the cAMP production is mesured in the presence of IBMX. All E_{MAX} values are presented as the amount of pregnenolone or cAMP production produced by 10^6 cells, and represent the mean ± SD of two independent experiments. Basal progesterone and cAMP production were 4.4 ± 1.0 and 3.5 ± 0.8 pmoles/10^6cells in rat Leydig cells, and 108.0 ± 15.5 and 22.5 ± 2.0 pmoles/10^6 cells in MA-10 cells.

The steroidogenic E_{MAX} was much less affected by deglycosylation of hCG, being still 30-50% of that of fully glycosylated hCG in MA-10 cells, while in the rat Leydig cell bioassay DhCG and hCG possessed similar steroidogenic maxima. For the ligand-induced steroidogenic response the dose/response curves of the deglycosylated ligands are shifted to

higher dose range, and the extent of this shift was dependent on the cell type. In the rat Leydig cell steroid bioassay, DhCG possessed a 6 to 10-fold higher ED_{50} value than hCG, with CHO-DhCG being slightly more active than HF-DhCG. Although the effect of deglycosylation of hCG on the E_{MAX} of the ligand-induced steroidogenesis was larger in MA-10 cells than in the rat Leydig cell bioassay, the effect on the steroidogenic ED_{50} value was the smallest in MA-10 cells, as the ED_{50} value for both types of DhCG was only 2-fold higher than their fully glycosylated counterparts.

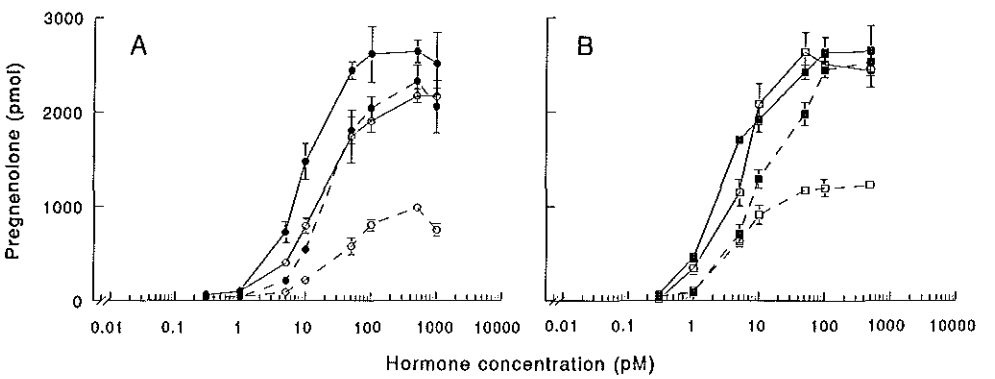

Figure 1. ***Effects of a phosphodiesterase inhibitor on hormonal stimulation of steroidogenesis in MA-10 cells.*** *MA-10 cells were incubated with increasing concentrations of hCG (●) and HF-DhCG (○) (Panel A), or CHO-hCG (■) and CHO-DhCG (□) (Panel B), in the presence (closed lines) or absence (broken lines) of 0.25 mM IBMX. The pregnenolone production was measured in the presence of the pregnenolone metabolism inhibitors epostane (5μM) and SU-10603 (20μM). Values are presented as the amount of pregnenolone produced by 10^6 cells in 3h, and represent the mean ± SD of triplicate estimations in a representative experiment. Basal pregnenolone production was 39.1 ± 2.9 and 62.7 ± 6.5 pmol (10^6 cells/3h) in the absence or presence of IBMX, respectively.*

Discrimination between potencies and intrinsic activities of hormones:

The results as described above indicate that the dose-response curves for the different hCG preparations are highly dependent on the cell type and biological response. The E_{MAX} of the ligand-induced biological response can give information about the intrinsic receptor-stimulating activity of the ligand, provided that the responses is limited only by receptor occupancy and not by other response limiting factors.

In order to compare the different hCG preparations in terms of their intrinsic receptor-stimulating activity, we have manipulated the conditions for the MA-10 cell bioassay. At first, inhibition of phosphodiesterase activity by addition of IBMX has been used to abolish the marginal stimulation of cAMP production by DhCG as a limiting factor for its steroidogenic maxima. Alternatively, over-expression of rat phosphodiesterase in MA-10 cells (194) results in submaximal levels for hCG induced steroid and cAMP production, suggesting that both responses are only limited by receptor occupancy.

Addition of IBMX to MA-10 cells enhanced the steroidogenic E_{MAX} for DhCG, while it did not affect that of hCG. In some experiments both types of DhCG showed similar steroidogenic maxima as their fully glycosylated counterparts in the presence of IBMX (Figure 1). Under these conditions, the dose/response curves for the ligand-induced steroidogenesis were shifted to a lower dose range, however, the relative steroidogenic potency of DhCG over that of hCG was unaffected. Since IBMX may stimulate cAMP production not only via inhibition of phosphodiesterase but also via stimulation of the adenosine receptor, we have also tested Ro20-1724 which is a more specific phophodieste-rase inhibitor. Ro20-1724 caused similar changes as IBMX in the DhCG dependent dose/response curves (data not shown).

Since the maximum steroidogenic responses in MA-10 cells to 8-Br-cAMP, forskolin, or cholera toxin are similar to the steroidogenic maximum that is obtained with hCG (90), maximal hCG induced steroidogenesis in MA-10 cells might be limited by other factors than receptor occupancy, for instance the availability of cholesterol as substrate. We have therefore compared the maximal steroidogenic responses to hCG and DhCG under conditions of a limited cAMP response and thus under conditions of a submaximal steroidogenic response in the MA-10 cell bioassay. For this purpose MA-10(P^+29) cells with high phosphodiesterase activity, have been used to determine the steroidogenic maxima of the different types of hCG. The approximately 2-fold upward shift of the maximally hCG-stimulated steroid production after addition of 100 μM Ro20-1724 (a maximally effective concentration) to MA-10(P^+29) cells, shows that, in the presence of high phosphodiesterase activity, the maximum steroidogenic response to hCG is probably limited by the cellular level of cAMP (Figure 2). Hence, the maxima of the steroidogenic responses to hCG and DhCG, when tested in the absence of Ro20-1724 in MA-10(P^+29) cells, are direct reflections of the receptor-stimulating activities of different types of hCG.

As shown in Figure 2, under these conditions HF-DhCG and CHO-DhCG possess 10 and 15%, respectively, of the intrinsic steroidogenic activity of intact hCG. Due to the high phosphodiesterase activity in the MA-10(P^+29) cells it was impossible to measure the

effects of DhCG on cAMP production in the absence of Ro20-1724. However, in the presence of 10 μM Ro20-1724 the cAMP production to DhCG was stimulated over basal levels, whereas the hCG induced cAMP production was still submaximally. As the maximum levels of cAMP production in MA-10 cells, induced by HF-DhCG or CHO-DhCG were similar, we only tested HF-DhCG. In the presence of 10 or 100 μM Ro20-1724, HF-DhCG possessed 2.2 ± 0.4 or 2.1 ± 0.3 % of the activity of intact hCG, respectively. These are similar values for the adenylyl cyclase stimulating properties of DhCG as observed in normal MA-10 cells, when tested in the presence of 0.25 mM IBMX (Table 1).

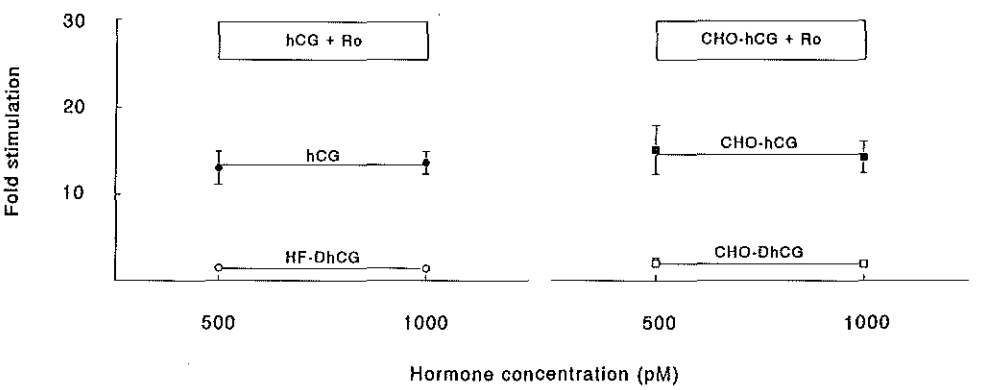

Figure 2. ***Comparative steroidogenic activities in MA-10(P$^+$29) cells.*** *MA-10(P$^+$29) cells were incubated with maximally stimulating concentrations (500 and 1000 pM) of hCG (●), HF-DhCG (○), CHO-hCG (■), or CHO-DhCG (□), for 3h at 37°C. The degree of stimulation of steroidogenesis was tested in the absence or presence of 100 μM Ro20-1724, and the steroidogenic activity is represented as the fold stimulation over basal production. In the presence of Ro20-1724, hCG and CHO-hCG showed a 27 ± 3.3 and 26.6 ± 3.3 fold stimulation, respectively. The boxed areas indicate this range of stimulation values. When hCG and HF-DhCG were tested in the absence of Ro20-1724, steroidogenesis was stimulated 13.7 ± 1.8 and 1.4 ± 0.2 fold, respectively. For CHO-hCG and CHO-DhCG these values were 14.8 ± 2.2 and 2.0 ± 0.5 fold, respectively. All values represent the mean ± SD of duplicate determinations in three independent experiments.*

DISCUSSION

One of the main interests concerning hCG action is to define the domains on hCG that

are involved in transmembrane signalling. This study shows that the relative receptor-stimulating activities of DhCG are highly dependent on the biological response and the species origin of the Leydig cells. In terms of its potency to stimulate cAMP production, DhCG possessed 10% of the activity of intact hCG in rat Leydig cells, and only 2% in MA-10 cells. In addition, DhCG possessed significant steroidogenic activity in both cell types, with an E_{MAX} being 30-50% of that of intact hCG in MA-10 cells, while in rat Leydig cells DhCG and hCG induced similar maximum steroidogenic responses. The oligosaccharide units in hCG appear not to be essential for expression of hormone action, but the glycosylation can modulate the receptor-stimulating activity of the ligand differently in rat Leydig cells and MA-10 cells.

DhCG shows a 2 to 3-fold higher affinity than hCG towards the LH/CG receptor does not lead to an increased biological activity of the ligand. On the contrary, DhCG possesses less biological activity than intact hCG (6,8). Although there is general consensus about the less efficient stimulation of adenylyl cyclase by DhCG (198), discordant results have been obtained for the steroidogenic potencies of DhCG (62,63,85-87). As we show here, the use of different types of Leydig cells and the use of different DhCG preparations might have contributed to the large variation in the steroidogenic properties of DhCG.

Damages of the protein in the purified HF-DhCG preparation are unlikely but cannot be excluded. Therefore, a direct comparison of its biological properties to that of CHO-DhCG is of interest. In CHO-DhCG the asparagine-linked glycosylation of the hormone was prevented by site-directed mutagenesis. However in this case, the protein composition of the CHO-DhCG preparation is less defined since concentrated media of transfected CHO cells were used, which also contain other secreted proteins (84,107). For estimation of the biological potencies of the different types of hCG, we measured the hormone concentrations using a double antibody fluorometric enzyme immunoassay specific for the holo form of hCG. Deglycosylation does not change the pattern of hCG immunological epitopes (103), but small differences in antibody binding have been reported and might influence the results of the immunoassay (101,103,107). However, validation of the immunoassay with the highly purified intact hCG and HF-DhCG preparations showed that the immunological activities were directly related to the protein masses. Moreover, inaccuracies in the estimation of the protein mass dos not affect the comparative aspects on the receptor-stimulating activity of the ligand in terms of cAMP and steroid production, nor or the comparison between the ligand activity in rat Leydig cells and MA-10 cells.

For a comparison between hCG and DhCG it is of particular interest that DhCG possesses a higher affinity towards the LH/CG receptor than intact hCG, while its potency

to stimulate the receptor is decreased. Since the binding properties of the different types of hCG are unknown, it is not possible to obtain directly information on the relative receptor-stimulating potencies from the estimated ED_{50} values. However, a comparison of the receptor-stimulating activities of hormones, with unknown affinities for a receptor, can still be made, provided that all receptors are fully occupied and functionally coupled to the biological response of interest. In rat Leydig cells, maximal stimulation of steroidogenesis occurs at approximately 1% of the maximal stimulation of cAMP production (89). On the contrary Pereira et al. (90) have shown that in MA-10 cells approximately 70% of the maximal stimulation of cAMP production is required to sustain maximal steroidogenesis, indicating that for stimulation of steroidogenesis in MA-10 cells more cAMP is required than in rat Leydig cells. It can thus be understood that a marginal stimulation of cAMP production, as induced by DhCG, might limit the steroidogenic maxima in MA-10 cells but not in rat Leydig cells. The enhancement of the steroidogenic maximum for DhCG in MA-10 cells under the influence of a phosphodiesterase inhibitor, and the reduction of the steroidogenic maxima in MA-10(P^+29) cells which over-express phosphodiesterase, further illustrate the importance of an efficient coupling between the cAMP and steroid production in MA-10 cells. It was surprising that in the presence of a phosphodiesterase inhibitor the steroidogenic maximum for DhCG in MA-10 cells was close to that of intact hCG while in terms of cAMP production DhCG possesses only 2% of the activity of hCG. These observations are in contradiction to the tight correlation between cAMP and steroid production in MA-10 cells as shown by Peirera et al (90). The presence of the phosphodiesterase inhibitor may have increased the efficiency in coupling between cAMP and steroidogenesis.

In MA-10(P^+29) cells the maximal stimulation of steroidogenesis by intact hCG is 2 to 3-fold lower than in wildtype MA-10 cells (present results; 194). In MA-10(P^+29) cells, the steroidogenic activity is only restricted by receptor activation. A comparison of the steroidogenic maxima induced by DhCG and hCG in these cells, showed that the steroidogenic activity of HF-DhCG and CHO-DhCG was 10-15% of that of intact hCG. The biological potency of DhCG measured in MA-10 cells in the presence of a phosphodiesterase inhibitor was 2-fold lower than that of intact hCG (Figure 1). This marginal effect of deglycosylation on the biological potency, while the intrinsic steroidogenic activity is 7 to 10-fold reduced, might be the result of a compensating effect of a 2 to 3-fold increased affinity of DhCG towards the receptor (63,85).

Our data clearly show that although DhCG has marginal biological activity in terms of adenylyl cyclase stimulation, it has still partial agonistic activity concerning steroid

production. The relative E_{MAX} of DhCG for steroidogenesis to that of intact hCG depends on the celltype. Support for the substantial steroidogenic activity of DhCG comes from the observations that DhCG stimulates and not inhibits steroidogenesis in monkeys (94) and cycling women (95).

In many studies in the literature on the steroidogenic properties of DhCG MA-10 cells have been used. However, almost all investigators have measured progesterone as an index for steroid production, whereas we have measured pregnenolone production in the presence of pregnenolone metabolism inhibitors. In an attempt to clarify the discrepancies between the steroidogenic properties of DhCG as determined by us and others, we have compared the production of pregnenolone and progesterone in MA-10 cells. It was found that especially the basal levels of progesterone production was much lower than those for pregnenolone production. Preliminary results indicate that metabolism of progesterone occurs in MA-10 cells (199). It might be possible that progesterone is metabolized at different rates, depending on the progesterone concentration (85). This might lead to an under estimation of the basal activity, but also of the steroidogenic activity of DhCG, when progesterone is measured as an index for steroid production. We are currently investigating the optimal conditions for measurement of hCG induced steroidogenesis in MA-10 cells.

It is not clear why the intrinsic receptor-stimulating activity of DhCG in terms of adenylyl cyclase stimulation is only 2% of that of intact hCG while the steroidogenic activity (10-15%) is much higher. This discorrelation could represent a new argument in favour of alternative second messengers systems in control of steroidogenesis in addition of cAMP (91). However, the relationship between cAMP and steroid production is beyond the scope of this study. In this regard, it should be stressed that in our experiments, total cAMP production has been measured as an index of adenylyl cyclase activity, while, in order to study the role of cAMP in the control of steroidogenesis, one should focus on the intracellular levels of cAMP.

It remains unknown how deglycosylation of hCG alters LH/CG receptor activation. Calvo and Ryan (75) suggested that a lectin-like binding site for oligosaccharides on the membrane of gonadotropin responsive target cells would be required for receptor mediated adenylyl cyclase activation. As a result of the reports of Thotakura et al. (76) and Seth and Bahl (198), this lectin-like binding site might be a part of the receptor itself (99), but it has not yet been proven that the oligosaccharides of the hormone interact with this domain.

Since the interaction between hCG with the LH/CG receptor involves several different domains (6,8), changes in the spatial structure of hCG, induced by deglycosylation, may influence the subtle protein-protein interactions between domains on the hormone and

receptor. This could result in a decreased stimulation of G_S, as has been described for deglycosylated hCG in rat ovary plasma membranes (198). Indirect support for conformational changes in the protein back bone of the hormone is the observation that the bioactivity of DhCG preparations can be enhanced with either intact anti-hCG polyclonal antibodies or with Fab fragments produced from them (70,105-107). Studies with specific anti-hCG monoclonal antibodies and LH-receptors from different species may help to define the domains and mechanisms that are involved in LH/CG receptor activation.

Acknowledgments:

This study would not have been possible without cooperation of Dr. I Boime (Washington University, St. Louis, MO, USA) who kindly provided stable transfected CHO cells producing either wild type or fully deglycosylated hCG. We also thank Dr. M. Ascoli (Vanderbilt Medical School, Nashville, Tennessee, USA) for kindly providing us the MA-10 cells and Dr. M. Conti (University of Iowa College of Medicine, Iowa City, Iowa, USA) for kindly providing us the MA-10(P^+29) cells.

CHAPTER THREE

MONOCLONAL ANTIBODIES DIRECTED AGAINST THE ß SUBUNIT OF hCG ENHANCE THE RECEPTOR-STIMULATING ACTIVITY OF DEGLYCOSYLATED hCG.

H.J. van Loenen[1], J.F. Flinterman[1], J.M. Bidart[2] & F.F.G. Rommerts[1]

[1] Department of Endocrinology & Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, The Netherlands.
[2] Service d'Immunologie Moléculaire, Institute Gustave-Roussy, Rue Camille Desmoulins, 94805 Villejuif, France.

Submitted

SUMMARY

Deglycosylated hCG preparations, obtained either by hydrogen-fluoride treatment of intact hCG (HF-DhCG) or by oligonucleotide-directed mutagenesis of the asparagine residues that are essential for asparagine-linked glycosylation of hCG (CHO-DhCG), exhibited reduced receptor-stimulating activity in mouse Leydig tumor cells (MA-10 cells). Both types of DhCG were approximately 20 to 30-fold or 2 to 3-fold less active than intact hCG, depending on whether stimulation of cAMP production or steroidogenesis was measured. In the present study, the effect of monoclonal antibodies (mAbs) directed against hCG on the receptor-stimulating activity of DhCG was investigated. Two mAbs, that recognize epitopes on the hCG ß subunit, designated FBT10 and D1E8, enhanced the stimulation of cAMP production over that by DhCG alone, in a concentration dependent way, when added to MA-10 cells that had been preincubated with DhCG. Fab fragments of these antibodies were as effective as intact antibodies in enhancing the receptor-stimulating activity of DhCG. Both mAbs did not fully enhance the cAMP production up to the level as obtained with intact hCG, but the mAb-enhanced steroidogenic activity of DhCG was similar to the activity of intact hCG. Antibodies recognizing either the α-subunit or the αß dimer were ineffective. As we used mAbs instead of polyclonal antisera, it was also possible to study the effects of preformed mAb/hormone complexes. When using either intact antibodies or Fab fragments of FBT10 and D1E8, preformed mAb/hormone complexes enhanced the receptor-stimulating activity of DhCG. Antibodies recognizing either the α subunit or the αß dimer inhibited both DhCG and hCG action, when tested as mAb/hormone complexes. Thus, antibodies directed against specific epitopes on the ß subunit of hCG enhanced the biological activity of DhCG, either when added to cells that had been preincubated with DhCG or when added to cells as mAb/DhCG complexes. A region of the hCG ß subunit, comprizing amino acid residues at positions 22, 74-77, and 95, is defined as the hypothetical epitope that is expressed on DhCG/receptor complexes, and that is involved in antibody-induced enhancement of the receptor-stimulating activity of DhCG. It is postulated that the antibodies can influence the conformation of hCG that has changed after deglycosylation, thereby restoring the correct three-dimensional structure of the protein backbone of the hormone that is properly recognized by the LH/CG receptor.

INTRODUCTION

Removal of the oligosaccharide units in lutropin (luteinizing hormone, LH) or its

analogue human chorionic gonadotropin (hCG) increases the affinity of the hormone towards the LH/CG receptor, but reduces its receptor-stimulating activity (6,8,63). Deglycosylated hCG (DhCG), obtained either by hydrogen-fluoride treatment of intact hCG (HF-DhCG) or by oligonucleotide-directed mutagenesis of the asparagine residues that are essential for asparagine-linked glycosylation of hCG (CHO-DhCG), possess reduced receptor-stimulating activity, but the magnitude of the change in activity is dependent on the biological response that is measured. DhCG is a weak agonist in terms of cAMP production when tested on mouse Leydig tumor cells (MA-10 cells), exhibiting approximately 5% of the maximum response of intact hCG (84,85,Chapter II). On the other hand, DhCG exhibits much stronger agonistic properties in terms of its steroidogenic activity, which is approximately 30-50% as compared to the activity of intact hCG (Chapter II).

It is unknown how the oligosaccharide units in hCG influence LH/CG receptor activation. It has been postulated that they interact directly with the receptor, or with other plasma membrane components that influence receptor stimulation (75,76). Alternatively, the oligosaccharide units may stabilize a three-dimensional conformation of the protein backbone of the hormone that is properly recognized by the LH/CG receptor. Consistent with the latter hypothesis, different conformations for hCG and DhCG have been proposed from differences in UV absorbance spectra (60,189), circular dichroism spectra (100,200), subunit-subunit association and dissociation (200), peptide bond cleavage susceptibility (189), or from differences in antibody affinity (100-103). Moreover, removal of the oligosaccharide units may cause the hormone to become more rigid (9). Hattori et al. (70) showed that conformational changes are mainly located in the ß subunit of the hormone. Additional evidence for conformational effects of oligosaccharide units on the three-dimensional structure of hCG came from the observation that different hormone/receptor complexes are formed after interaction of hCG and DhCG with the LH/CG receptor (68,74).

Polyclonal antibodies directed against hCG (105), and in particular those directed against the hCG ß subunit (70,106), were found to be able to enhance the receptor-stimulating activity of DhCG. These observations support the hypothesis that a conformational disturbance in the protein backbone of DhCG is involved in the reduced receptor-stimulating activity of the hormone. However, the precise epitopes on DhCG that are involved in the antibody-induced increase of its receptor-stimulating activity remain to be elucidated. Although deglycosylation of hCG appeared not to affect the number and topography of antigenic epitopes (103), this does not exclude the possibility that receptor stimulation is more dependent on subtle changes in the three-dimensional structure than

interactions with antibodies. Thus, monoclonal antibodies (mAbs) directed against hCG and cross-reacting with DhCG might be useful in identifying the epitope(s) that are involved in the antibody-induced enhancement of the receptor-stimulating activity of DhCG. In this study we have tested four mAbs, all recognizing intact hCG, for their effect on the hormone-induced LH/CG receptor activation in MA-10 cells.

MATERIALS AND METHODS

Hormones:

Highly purified urinary hCG (95% pure) was obtained from Organon International b.v. (Oss, The Netherlands). Chemically deglycosylated hCG (HF-DhCG) was obtained by hydrogen fluoride treatment of intact hCG (189), and was kindly provided to us by Prof. Dr. W.E. Merz (University of Heidelberg, Heidelberg, Germany). Stable transfected CHO cell lines that produce either wild-type hCG (CHO-hCG) or hCG that lacks all asparagine-linked oligosaccharides (CHO-DhCG) were kindly provided by Dr. I. Boime (Washington University, St. Louis, MO, USA). The cells were cultured essentially as described by Matzuk et al. (84). In brief, cells were maintained in Ham's F-12 medium supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 5% (v/v) fetal calf serum and G-418 (0.25 mg/ml) at 37°C. For hormone collection, confluent cell layers were incubated in α-minimal essential medium supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) in the absence of serum. After 48 hours of incubation, the medium was collected, centrifuged to remove cell debris, and concentrated approximately 10-fold with an Amicon centriprep with a 10 kD cut-off (Amicon, Lexington, MA, USA). The total protein contents of the concentrated CHO-hCG and CHO-DhCG preparations were 1.3 ± 0.1 (mean ± SD, of three independent protein determinations) and 1.5 ± 0.1 mg/ml, respectively. The hCG concentrations in the different hormone preparations were determined in a double antibody fluorometric enzyme immunoassay for dimeric hCG (Baxter Diagnostics Inc., Deerfield, USA) according to the instructions of the manufacturer. The assay has been standardized to the World Health Organization 1ST international reference preparation (IRP#75/537) and cross-reactivity of free ß subunits was approximately 1.4%. Immunological activities were converted to molar concentrations based on a molecular weight of 38.000 for hCG and 30.000 for DhCG (5).

Monoclonal antibodies:

Monoclonal antibodies were obtained by immunizations of mice using hCG (HT13), hCG ß-subunit (FBT10, D1E8), or LH (LH05), as described before (201-203). The characteristics of these antibodies are presented in Table 1. Highly purified mAbs were prepared from mAb-containing ascitic fluids as described previously (202). Fab fragments from mAbs were generated by using the Pierce Immunopure Fab Preparation Kit according to the instructions of the manufacturer.

Effects of mAbs on hormone preincubated MA-10 cells:

Mouse Leydig tumor cells; MA-10 cells (193), were grown in RPMI-1640 medium supplemented with streptomycin (100 µg/ml), penicillin (100 IU/ml), L-glutamin (2 mM), and horse serum (10% v/v). The cells were subcultered by trypsin treatment (0.05% w/v trypsin) and experimental cultures were plated and cultured for two days. After washing twice with RPMI-1640 containing 0.1% BSA, the cells were used for experiments. The cells were preincubated with a maximally stimulating concentration (200 pM) of hCG, CHO-hCG, HF-DhCG or CHO-DhCG for 30 min at 37°C in RPMI-Hepes (RPMI-1640 medium buffered with 25 mM Hepes and

supplemented with 0.1% w/v bovine serum albumin). The cells were subsequently washed twice with RPMI-Hepes to remove free hormone, before they were incubated with the mAbs for 3 hours at 4°C. The incubations for estimation of the LH/CG receptor activation in terms of cAMP and steroid production were started by incubating the cells at 37°C.

Stimulation of MA-10 cells by preformed mAb/hormone complexes:

Hormones (200 pM) were incubated with mAbs for 3 hours at 4°C in RPMI-Hepes, and after warming up to 37°C the solutions were subsequently added to MA-10 cells.

Steroid production:

MA-10 cells were incubated for 2 hours in RPMI-Hepes at 37°C, in the presence of the inhibitors of pregnenolone metabolism epostane (5 μM) and SU-10603 (20 μM) (196). Pregnenolone was measured in the incubation medium by radioimmunoassay (197).

cAMP production:

MA-10 cells were incubated for 2 hours at 37°C in the presence of 0.25 mM of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX). For determination of the total amount of cAMP, concentrated perchloric acid was added to the cells and the medium at a final concentration of 5% (v/v). The cell lysate was treated with 1.1 M Tris-containing KOH sufficient to neutralize the perchloric acid, and cAMP was measured by radioimmunoassay as described by Stoof et al. (195).

Table 1. ***Characteristics of mAbs directed against hCG.***

mAb	Specificity	Hypothetical epitope	Reference
LH05	αß	α(64-76)ß(49-50,59-60,86-87)	203
FBT10	ß,αß	ß(22,74-75,95)	206
D1E8	ß,αß	overlapping or similar to FBT10	202
HT13	α,αß	α(16-17,64-66,73-76)	206

RESULTS

Effects of monoclonal antibodies on hormone stimulated cAMP production:

When cells were preincubated with hormone and subsequently tested for hormone-induced stimulation of cAMP production, the maximal stimulation of cAMP production by DhCG was approximately 5% of that obtained with intact hCG (Figure 1). Addition of mAb FBT10 or D1E8 to MA-10 cells that had been preincubated with a maximally stimulating concentration of HF-DhCG or CHO-DhCG, further stimulated the cAMP production over that of DhCG alone. Addition of increasing concentrations of antibody to the hormone-preincubated cells enhanced the cAMP production up to 40-60% of that obtained with intact hCG (Figure 1). Fab fagments of FBT10 an D1E8 showed comparable

activities as intact antibodies (Figure 1). FBT10 and D1E8 had no stimulatory activity on the cAMP production when added to MA-10 cells that had not been pre-incubated with hormone. Other antibodies, directed against either the α-subunit (HT13) or the αß dimer (LH05) were ineffective in enhancing the biological activity of DhCG (data not shown).

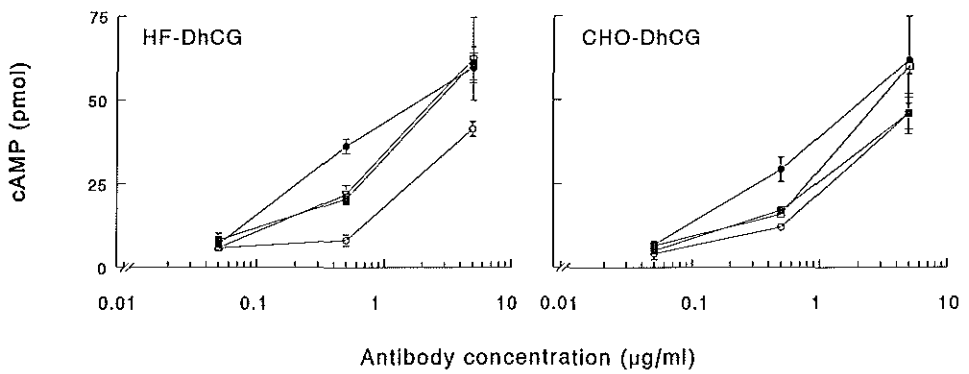

Figure 1. ***Effects of monoclonal antibodies on DhCG-stimulated cAMP production.*** *MA-10 cells were incubated with a maximally stimulating concentration of DhCG (200 pM) for 30 min at 37°C. The cells were then washed twice with medium to remove free hormone, and subsequently incubated with or without increasing concentrations of FBT10 (●), D1E8 (■), FBT10-Fab (○), or D1E8-Fab (□), for 3 h at 4°C. The cells were then incubated for 2 h at 37°C. The total amount of cAMP was measured as described in Materials and Methods. Values are expressed as the percentage stimulation of the cAMP production obtained with intact hCG under similar conditions, and represent the mean ± SEM of duplicate determinations in two independent experiments. Basal, hCG (200 pM), and CHO-hCG (200 pM) stimulated cAMP production was 22 ± 6, 1340 ± 56, and 1356 ± 49 pmol/10^5 cells (mean ± SEM), respectively.*

Effects of preformed mAb/hormone complexes on cAMP production:

Maximal stimulation of cAMP production by DhCG was approximately 5% of that of intact hCG. When maximally stimulating concentrations of DhCG were preincubated with increasing concentrations of FBT10 or D1E8 antibodies, prior to addition to MA-10 cells, both mAbs stimulated the cAMP production over that of DhCG alone. FBT10/DhCG and D1E8/DhCG complexes stimulated cAMP production in a concentration-dependent fashion, up to approximately 30% of that induced by intact hCG (Figure 2). The effects of FBT10 and D1E8 were similar when either HF-DhCG or CHO-DhCG were used, and Fab

fragments obtained from FBT10 and D1E8 antibodies showed comparable activities as obtained with intact antibodies (Figure 2).

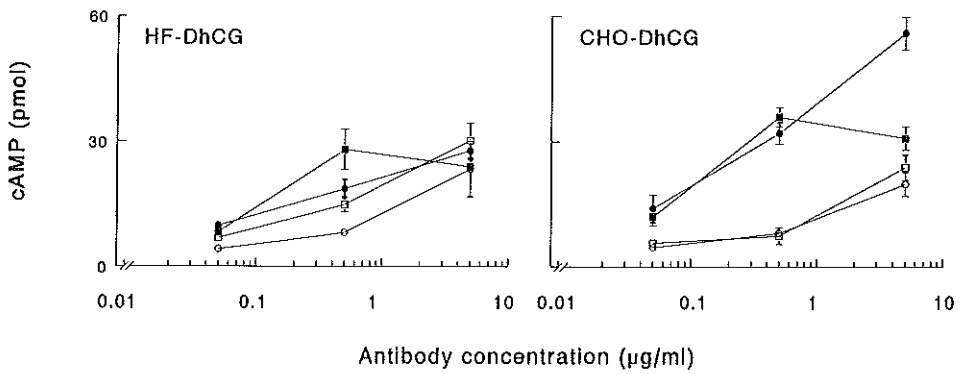

Figure 2. ***Effect of preformed mAb/hormone complexes on cAMP production.*** *A maximally stimulating concentration of DhCG (200 pM) was incubated with increasing concentrations of FBT10 (●), D1E8 (■), FBT10-Fab (○), or D1E8-Fab (□), for 3 h at 4°C. The total mixture was then added to MA-10 cells at 37°C, and cells were subsequently incubated for 2 h, and the total amount of cAMP was measured as described in Materials and Methods. Values are expressed as rge percentage stimulation when compared to the cAMP production observed with intact hCG, and represent the mean ± SEM of duplicate determinations in two indepenent experiments. Basal, hCG (200 pM), and CHO-hCG (200 pM) stimulated cAMP production was 41 ± 8, 2400 ± 93, and 2550 ± 107 pmol/10^6 cells (mean ± SEM), respectively.*

Effect of preformed mAb/hormone complexes on steroid production:

After incubating the hormone with mAbs, no attempt has been made to isolate the preformed hormone/mAb complexes from free hormone and mAbs. However, some mAbs inhibited hormonal stimulation of MA-10 cells after preincubating the hormone with mAbs, illustrating that most, if not all, hormone was complexed with antibodies.

The steroidogenic maxima for HF-DhCG and CHO-DhCG were approximately 30% and 50%, respectively, of the maximal stimulation observed with their fully glycosylated counterparts (Figure 3). When HT13 and LH05 antibodies were tested as mAb/hormone complexes, complete inhibition of the steroidogenic activity of both hCG and DhCG was found (Figure 3). In agreement with their ability to enhance the DhCG-induced cAMP production, FBT10 and D1E8 markedly enhanced the steroidogenic activity of both types

of DhCG. Both antibodies did not affect the maximal steroidogenic activity of intact hCG when added as mAb/hCG complexes (Figure 3). Consistent with their effects on hormone-induced cAMP production, Fab fragments were as active as the intact antibodies.

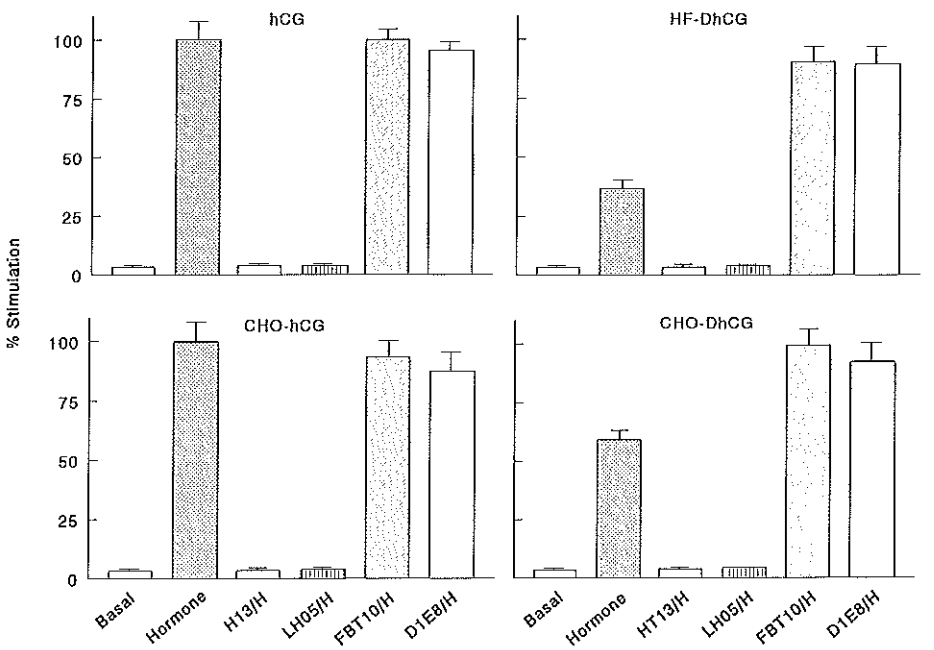

Figure 3. ***Effects of preformed mAb/hormone complexes on steroid production.*** *A maximally stimulating concentration of hormone (200 pM) was incubated with or without 5 µg/ml mAb for 3h at 4°C. The cells were subsequently incubated with the preformed mAb/hormone complex for 2 h at 37°C in the presence of pregnenolone metabolism inhibitors. The amount of pregnenolone produced was measured in the incubation medium. Values are expressed as the percentage stimulation of the steroid production obtained with intact hCG, and represent the mean ± SEM of duplicate determinations in two independent experiments. Basal, hCG, and CHO-hCG stimulated pregnenolone production were 67 ± 11, 2786 ± 191, and 2834 ± 173 pmol/10^6 cells (mean ± SEM), respectively.*

Fab fragments prepared from FBT10 and D1E8 antibodies, when tested as HF-DhCG/Fab complexes, enhanced the steroidogenic activity of HF-DhCG up to 96.7 ± 2.3 and 91.1 ± 4.0 percent (mean ± SD of duplicate determinations of two independent experiments) of

that obtained with intact hCG. The steroidogenic activity of CHO-DhCG was enhanced up to 97.2 ± 1.7 and 97.5 ± 2.8 percent by the FBT10 and D1E8 Fab fragments, respectively.

Table 2. ***Effects of FBT10 and D1E8 mAbs on hormonal stimulation of steroidogenesis.***

Inc.1	Inc.2	Pregnenolone (pmoles/10^6 cells)
-	-	97 ± 5
hCG	-	2624 ± 105
HF-DhCG	-	761 ± 46
HF-DhCG	FBT10	2511 ± 96
HF-DhCG	D1E8	2320 ± 63
HF-DhCG	FBT10 (Fab)	2307 ± 84
HF-DhCG	D1E8 (Fab)	2299 ± 91
CHO-hCG	-	2743 ± 112
CHO-DhCG	-	1512 ± 61
CHO-DhCG	FBT10	2356 ± 93
CHO-DhCG	D1E8	2375 ± 82
CHO-DhCG	FBT10 (Fab)	2348 ± 82
CHO-DhCG	D1E8 (Fab)	2238 ± 107

After preincubating MA-10 cells with or without hormone for 30 min at 37°C (Incubation 1) the cells were washed to remove free hormone, and either medium (-) or mAb was added, and the cells were incubated for 3 h at 4°C (Incubation 2). The cells were subsequently incubated for 2 h at 37°C, and the total amount of pregnenolone production was measured as described in Materials and Methods. Values represent the mean ± SEM of triplicate determinations in two independent experiments.

Effects of FBT10 and D1E8 antibodies on hormonal stimulation of steroid production:

When either intact antibodies or Fab fragments of FBT10 and D1E8 mAbs were added to MA-10 cells that had been preincubated with HF-DhCG or CHO-DhCG, the pregnenolone production was increased up to the level that was obtained with intact hCG (Table 2). Both mAbs possessed no steroidogenic activity when added to MA-10 cells that were not

preincubated with hormone.

DISCUSSION

Removal of the oligosaccharide units from hCG results in a ligand that possesses increased affinity towards the LH/CG receptor, whereas its receptor-stimulating activity is reduced. The decrease in receptor-stimulating activity appears to be highly dependent on whether cAMP or steroid production is measured as the biological response, and the cell type that is used in the bioassay (Chapter II). DhCG possesses increased affinity towards the LH/CG receptor and the association rate for DhCG to the LH/CG receptor is higher than that for hCG (70). In addition, while the binding of hCG to the LH/CG receptor is reversed slowly over a period of several hours, receptor binding of DhCG is nonreversible (74). Conformational differences in the three-dimensional structure of DhCG/receptor and hCG/receptor complexes might be involved in the different association and dissociation kinetics for DhCG and hCG (68,69).

The exact role of the oligosaccharide units in hCG in hormone-receptor interaction is still poorly understood. The oligosaccharide units may either stabilize the "active protein conformation", or they may directly interact with the receptor itself and/or with other plasma membrane components (75,76). It might also be possible that more tight interactions within the DhCG/receptor complex limit the conformational changes that may be required for efficient signal transduction.

Antibodies are valuable tools for studying the conformation and structure-activity relationship of proteins. In this regard, Rebois and Fishman (105) and Sairam (63) have demonstrated that polyclonal antibodies raised against intact hCG, and in particular antisera directed against the hCG ß subunit (70,106) were able to enhance the receptor-stimulating activity of receptor-bound DhCG. However, as polyclonal antibodies are directed against many antigenic determinants or epitopes on hCG, including sites that are involved in the interaction with the LH/CG receptor, the effects of antibody/hormone complexes on receptor activation could not be studied. Indeed, Rebois and Fishman (105) showed that preincubation of DhCG with stimulating polyclonal anti-hCG antibodies completely abolished the receptor-stimulating activity of DhCG. The availability of a wide range of monoclonal antibodies against hCG and its subunits has led several groups to study hormone-receptor interactions in more detail. Identification of the epitopes on the hCG/receptor complex showed that of all epitopes that are exposed on hCG only two remain exposed when hCG was bound to the LH/CG receptor (69,204,205). These epitopes

were located in the ß subunit of hCG. In DhCG/receptor complexes all epitopes were masked (69), illustrating conformational differences between hormone/receptor complexes for hCG and DhCG.

We have investigated the effects of monoclonal antibodies directed against hCG on the receptor-stimulating activity of deglycosylated and intact hCG. To this aim, four mAbs were selected: one recognizing an epitope present on hCGα and intact hCG (HT13), an antibody specifically directed against the αß dimer and recognizing both hLH and hCG (LH05), and two mAbs which bind to free hCGß and intact hCG (FBT10 and D1E8). Among these antibodies, only FBT10 and D1E8 substantially increased DhCG-induced stimulation of cAMP production, while the steroidogenic activity of the hormone was almost fully restored. These effects were observed when the antibodies were added to cells that had been preincubated with hormone, but also when cells were incubated with preformed antibody/hormone complexes. Furthermore, Fab fragments, prepared from these antibodies, were as effective as the intact antibodies, confirming the observations from Rebois and Fishman (105). This indicates that the bivalent structure of the intact antibody is not involved in cross-linking and/or aggregation of DhCG/receptor complexes (108). It is noteworthy that FBT10 and D1E8 were capable to restore the receptor-stimulating activity of both HF-DhCG and CHO-DhCG. Thus, although HF-DhCG has been obtained by hydrogen fluoride treatment of intact hCG whereas CHO-DhCG has been obtained by site-directed mutagenesis of all four asparagine residues that are essential for asparagine-linked glycosylation of the hormone, the disturbance in their conformations, reflected by a less efficient receptor activation, might be similar. In contrast, Sairam and Jiang (107) reported that polyclonal anti-DhCG antibodies which cross-reacted with hCG, were without effect on the steroidogenic activity of CHO-DhCG while that of HF-DhCG was fully restored. The reason for the discrepancy between the results presented here and those of Sairam and Jiang (107) are unclear.

The present results clearly indicate that a portion of the ß subunit remains accessible when either hCG or DhCG is bound to the LH/CG receptor, and that antibody binding to DhCG is capable of enhancing its receptor-stimulating activity. Antibody binding studies have revealed that most immunogenic regions of hCGß reside on the ß core fragment of hCG (ß6-40 linked to ß55-92) (206). Moreover, antigenic regions that project away from the hormone/receptor interface will be most accessible to antibody binding. Although Schwarz et al. (69) concluded from antibody binding studies that all epitopes present in free DhCG are masked in the DhCG/receptor complex, our data indicate that at least one epitope remains exposed when DhCG is bound to the LH/CG receptor, since FBT10 and

D1E8 antibodies recognize the same epitope or overlapping epitopes on the hCG ß subunit (202). Although we do not have a clear explanation for these discrepancies, the epitopes as defined by Schwarz et al. (69) might be slightly different from those recognized by FBT10 and D1E8. Moreover, Schwarz et al. (69) studied the interaction of mAbs with DhCG bound to the rat LH/CG receptor, while in the present study DhCG was bound to the mouse LH/CG receptor.

Several observations indicate that a region of hCGß comprising amino acid residues around positions 22 and 74-77 might be exposed at the surface of hCG and DhCG in their receptor-bound form. Mutagenesis studies indicated that $ßAsn_{77}$ is probably involved in the epitope that is exposed on the hormone/receptor complex (9). Interestingly, $ßGly_{22}$, $ßArg_{74}$ and $ßArg_{95}$ are potential residues for interaction with FBT10, as revealed by the cross-reactivity binding pattern of this antibody (207). It should be noted that $ßCys_{23}$ and $ßCys_{72}$ form a disulfide bond, and an asparagine-linked carbohydrate structure is present at $ßAsn_{30}$. Antibodies directed against a conformational epitope comprising $ßGly_{22}$, $ßArg_{74}$ and $ßArg_{95}$ might thus be able to modify the improper conformation of the DhCG/receptor complex. Recently, the crystal structure of deglycosylated hCG has been elucidated and the amino acid residues comprising the postulated epitopes come into close proximity in the spatial conformation (240). Indeed, it is likely that the epitopes recognized by FBT10 and D1E8 comprised amino acid residues around positions $ßCys_{22}$ and $ßCys_{72}$, as these regions are constituted from two disulfide-linked loop structures and are accessible at one side of the molecule. Moreover, the oligosaccharide unit at positions $ßAsn_{13}$ and $ßAsn_{30}$ are closely spatially related.

Other regions of hCG appear to be in close contact with the LH/CG receptor, since anti-α antibodies are more effective inhibitors of hCG binding to the LH/CG receptor than antibodies directed against the ß subunit (204,208-210). We showed that HT13 (anti-α) and LH05 (anti-αß) mAbs were effective inhibitors of the hormonal activity of either intact or deglycosylated hCG, indicating that both these antibodies hinder the interaction of hormone and receptor. Interestingly, HT13 mAb recognizes hCG bound to a truncated LH/CG receptor representing only the extracellular domain of the receptor (207), while it fails to recognize hCG when bound to the entire receptor (205). Thus, HT13 may bind to a part of hCGα that is buried at the interface between the hormone and the transmembrane domain of the LH/CG-receptor.

Finally, since antibodies directed against hCG were able to enhance the receptor-stimulating activity of DhCG, it would be tempting to speculate that the conformation of DhCG, which is likely different from that of hCG due to the deglycosylation, has been

restored by antibody binding. It is not clear how the antibodies can exert such an effect on the DhCG conformation. It might be that since anti-hCG antibodies are directed against epitopes on the "active conformation" of the intact hormones, they force the disturbed conformation of DhCG into the native conformation of the hormone. However, this hypothesis is not in line with the observation that polyclonal antisera directed against deglycosylated hCG and cross-reacting with intact hCG, also enhanced the receptor-stimulating activity of DhCG, since in this case the "disturbed conformation" is stabilized (70,106,107). Therefore, the question of how mAbs can affect the conformation of DhCG requires more knowledge about the conformational structure of the hormone.

CHAPTER FOUR

FOLLICLE-STIMULATING HORMONE STIMULATES RAT SERTOLI CELLS VIA RELATIVELY LOW AFFINITY BINDING SITES.

H.J. van Loenen, J.F. Flinterman & F.F.G. Rommerts.

Department of Endocrinology & Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, The Netherlands.

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SUMMARY

FSH receptor binding and hormone-induced activation of the FSH receptor have been studied in the absence and presence of NaCl. Physiological concentrations of NaCl inhibited FSH receptor binding to either intact rat Sertoli cells or Sertoli cell membrane preparations. Analysis of FSH receptor binding, after a 3 hours incubation period at 37°C to both FSH receptor sources revealed a single class of high affinity binding sites ($K_d \approx 30$ pM) in the absence of NaCl. A gradual increase in NaCl concentration resulted in a progressive decrease in the ligand binding affinity of the FSH receptor and a concomitant 3 to 4-fold reduction in the actual number of FSH binding sites. Estimation of the specific FSH receptor binding became less accurate at physiological NaCl concentrations, since total binding declined while non-specific binding was not affected. Therefore, accurate estimations of the ligand binding affinity and the number of FSH binding sites were not possible at physiological NaCl concentrations.

NaCl also affected functional properties of the FSH receptor. The half-maximal FSH concentration for hormone-induced receptor endocytosis was increased from 31 ± 4 pM, when tested in the absence of NaCl, up to 660 ± 27 pM at physiological NaCl concentrations. Similarly, the half-maximal concentration for FSH-induced stimulation of cAMP production was increased from 14 ± 2 pM up to 259 ± 21 pM when 0.15M NaCl was added. Forskolin-stimulated cAMP production was independent of the NaCl concentration. It thus appears that the functional FSH receptor is of relatively low affinity, as illustrated by the 15 to 20-fold higher ED_{50} values for hormone-induced stimulation of adenylyl cyclase activity and receptor endocytosis in the presence of 0.15 M NaCl. Moreover, in the presence of physiological concentrations of NaCl only approximately 30% of the FSH binding sites that are detectable under low ionic strength conditions are bound by the ligand. The interaction between FSH and its receptor, as occurring under low and physiological ionic strength conditions are so different that it is not warranted to apply the high ligand binding properties of the receptor at low ionic strength conditions to evaluate physiological receptor function.

INTRODUCTION

Follicle-stimulating hormone (FSH, follitropin) is a pituitary glycoprotein hormone that is pivotal for gonadal function. FSH acts on testicular Sertoli cells, and ovarian granulosa cells, the only cell types in mammalian species that express detectable amounts of the FSH

receptor. The FSH receptor belongs to a family of three glycoprotein hormone receptors (follitropin, lutropin/chorionic gonadotropin, and thyrotropin) (99,114,118), which in turn belongs to the large super family of G protein-coupled receptors (130,211,212). As for the other glycoprotein hormone receptors, ligand occupation of the FSH receptor results in stimulation of a membrane-associated G_s protein and adenylyl cyclase activity, and ensuing cAMP production (213,214). Although cAMP is considered to be the main second messenger for FSH action, it has been shown that FSH also elicits an increase in intracellular calcium (215-217). The different second messengers in turn regulate a variety of cellular processes, including aromatase activity. Binding of FSH to its receptor is considered to be a prerequisite for hormonal action, and FSH receptor binding has been extensively studied (218-220). It is known that the binding of FSH to its receptor can be modulated by various monovalent and divalent cations, and that low ionic strength buffers favour formation of a high affinity hormone/receptor complex (165,168,221). Probably as a result of these findings, FSH binding to cell membranes or solubilized receptors has mainly been studied under low ionic strength conditions (162,222). Since the binding properties of the FSH receptor under these low salt conditions may not reflect the properties of the functional receptor under physiological conditions, we have compared the ligand binding and functional properties of the FSH receptor in intact rat Sertoli cells and Sertoli cell membrane preparations under conditions of different ionic strength.

MATERIALS AND METHODS

Isolation and culture of Sertoli cells:

Sertoli cells were isolated from testes of immature (21-23 day-old) Wistar rats as described by Themmen et al. (223). Cells were plated in 24-well plates (Costar Corp., Cambridge, USA) in Eagle's minimal essential medium (MEM; Gibco, Grand Island, NY, USA) supplemented with nonessential amino acids, glutamine, fungizone, streptomycin, penicillin, and 1% fetal calf serum (224), and cultured for 3 days at 37°C under 5% CO_2 in air in a humidified incubator. To eliminate contaminating germ cells, cell cultures were exposed to an osmotic shock using 10-fold diluted MEM in water for 2 min (225,226). Cells were further incubated in MEM containing 0.1% (w/v) bovine serum albumin (BSA; fraction V; Sigma) for another 1-4 days.

FSH receptor binding:

Binding of ^{125}I-hFSH, (Du Pont Canada Inc., Markham, Ontario, Canada) with a specific activity of 130-140 μCi/μg (Mw≈30,000), to rat Sertoli cells was studied in isotonic Hepes-buffered Krebs Ringer buffer (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 25 mM Hepes) containing 0.1% (w/v) BSA, and indicated as KRB. Other buffers were KRB-N (exclusion of 140 mM NaCl from KRB) and KRB-N+S (addition of 200 mM sucrose in order to compensate for the low osmotic value of KRB-N). Cells were washed twice with the specific buffer before ^{125}I-hFSH was added. Since cells were incubated in buffers of

different ionic strength, cell viability at the end of the 3 hours incubation period is unknown. Consistent with the low ionic strength of KRB-N, severe swelling of the cells was observed after incubation in this buffer, however, most of the cells remained attached to the plastic of the culture plates. Swelling of cells was not observed after a 3 hours incubation period in KRB-N+S. After incubation in both buffers the cell membranes were found to be intact, as indicated by the accumulation of an intracellularly formed fluoresceine fluorescent dye after hydrolysis of fluoresceine-diacetate (FDA) (227). On the other hand, the morphological appearance of cells that had been incubated in KRB-N was not restored after a subsequent incubation period of 24 hours in MEM containing 0.1% (w/v) BSA. In addition, when the FSH-induced stimulation of aromatase activity was used as an index of cell viability, only the cells that had been incubated in KRB responded well to FSH. Cells that had been incubated for 3 hours in KRB-N or KRB-N+S did not show an FSH dependent oestradiol production. It thus appears that the biochemical integrity gradually diminishes when cells were kept in KRB-N+S, but that in KRB-N this process is accelerated.

Table 1. *Binding and internalization of ^{125}I-hFSH.*

	Acid-releasable			Acid-resistant		
Control	260	19	(42*)	319	81	(58)
sodium azide (5mM)	304	48	(79)	81	18	(21)
air exposure (15min)	669	11	(89)	85	16	(11)

* Values in between brackets represent the percentage of total specific cell-associated radioactivity.

Intact rat Sertoli cells in the absence or presence of 5 mM sodium azide, and air-dried cells were incubated with 35 pM ^{125}I-hFSH (approximately 25000 cpm/250 µl) in KRB-N+S for 3 hours at 37°C. At the end of the incubation period, specific acid-releasable and acid-resistant radioactivity (cpm) were measured as described in Materials and Methods. Values represent the mean ± SD of triplicate determinations in each of two independent experiments.

All binding studies were carried out for 3 hours at 37°C, to ensure equilibration of FSH receptor binding. At the end of the incubation period the buffer was aspirated, and the cells were washed twice with ice-cold buffer. Cells were subsequently dissolved in 1N NaOH, and total cell-associated radioactivity (the sum of surface-bound radioactivity and internalized radioactivity) was measured. Nonspecific association of ^{125}I-hFSH was determined in the presence of an approximately 300-fold molar excess of unlabeled hFSH (NIDDK-hFSH-I-SIAFP-1). Values for the apparent affinity constant and the number of binding sites were calculated by the method of Scatchard (228), and least squares analysis using the LIGAND computer program (229). Where indicated, surface-bound radioactivity and internalized radioactivity were estimated separately. Surface-bound radioactivity was determined as acid-releasable radioactivity after incubating the cells for 10 min with ice-cold 0.05 M glycine-HCl, 0.1 M NaCl, pH 3.0 (230). Acid-resistant radioactivity was considered as internalized ^{125}I-hFSH, and was measured by dissolving acid washed cells in 1N NaOH.

Stimulation of cAMP production:

Rat Sertoli cells were incubated for 1 hour at 37°C with or without rec-hFSH (Organon 32489, Organon International B.V., Oss, The Netherlands) in the presence of 0.25 mM of the

phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX). For determination of the total amount of cAMP, concentrated perchloric acid was added to the cells and medium at a final concentration of 5% (v/v). The cell lysate was treated with 1.1 M Tris-containing KOH sufficient to neutralize the perchloric acid, and cAMP was subsequently measured by radio immunoassay essentially as described by Stoof et al. (195)

RESULTS

Association of ^{125}I-hFSH to rat Sertoli cells:

Saturation of specific cell-associated radioactivity was found after incubation of rat Sertoli cells for 3 hours at 37°C with increasing concentrations of ^{125}I-hFSH in hypotonic KRB-N (Figure 1A). Nonspecific association accounted for less than 10% of total cell-associated radioactivity. Scatchard analysis of the specific cell-associated radioactivity revealed one class of high affinity binding sites with an apparent affinity constant of 32 ± 9 pM. The estimated number of binding sites was 19 ± 4 fmol/mg protein. The specific association of ^{125}I-hFSH to rat Sertoli cells was 3-4 fold lower when 140 mM NaCl was added to the incubation buffer (KRB; Figure 1A). The total amount of nonspecifically associated ^{125}I-hFSH was similar in KRB and KRB-N (data not shown). Thus, at physiological salt concentrations the contribution of the nonspecific association of ^{125}I-hFSH to the total cell-associated radioactivity was increased from 10% up to approximately 30%. An accurate estimation of the affinity constant and number of binding sites for the cellular associated radioactivity in KRB was not possible from the Scatchard plot. However, the data clearly indicate that the ligand binding affinity of the FSH receptor and the apparent number of binding sites were decreased under the influence of NaCl. The saturation curve of the specific cell-associated radioactivity indicates, that the number of binding sites was approximately 4-fold lower than in KRB-N, but it was not possible to accurately measure the ligand binding affinity.

When using intact Sertoli cells, internalization of ^{125}I-hFSH is likely to occur, and cell-associated radioactivity will thus be the sum of surface bound and internalized ^{125}I-hFSH. For accurate estimation of the binding properties of the FSH receptor, corrections must be made for the internalization of labeled hormone, or internalization must be inhibited. Incubation at low pH was used to separate surface-bound (acid releasable) from internalized (acid resistant) radioactivity. In KRB-N+S (KRB without NaCl but with 200 mM sucrose in order to compensate for the low osmolarity of KRB-N), 58% of the cell-associated radioactivity is likely to be internalized (acid resistant).

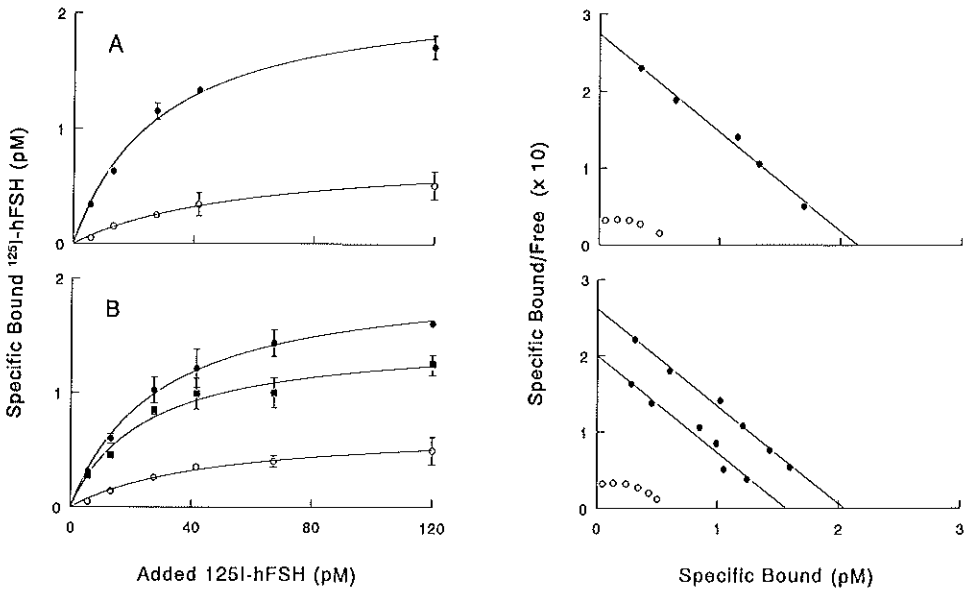

Figure 1. ***Binding of ^{125}I-hFSH to rat Sertoli cells and Sertoli cell membranes as a function of the concentration of ^{125}I-hFSH added.*** *Rat Sertoli cells (Graph A) were incubated with increasing concentrations of ^{125}I-hFSH in KRB without NaCl (KRB-N, ●), or in KRB (○), at 37°C for 3 h. Total and nonspecific binding were determined as described in Materials and Methods, and specific binding was calculated by subtracting nonspecific radioactivity from total radioactivity. Values in both Graph A and B represent the mean ± SD of triplicate determinations of a representative experiment. The apparent affinity constant and the maximal amount of cell-associated radioactivity KRB-N (Graph A, right panel) were 32 ± 9 pM and 19 ± 4 fmol/mg protein, respectively, as calculated from three independent experiments. These values could not be estimated from the Scatchard plot data when KRB was used. However, the data in the nontransformed binding curve (Graph A, left panel) indicate an approximately 4-fold lower value for the maximal cell-associated radioactivity than in KRB-N. In Graph B, ^{125}I-hFSH binding to Sertoli cell membranes was studied in KRB-N (●), KRB-N+S (■), and KRB (○). The apparent affinity constants for FSH receptor binding in KRB-N and KRB-N+S were 25 ± 4 and 28 ± 3 pM, receptively, as calculated from three independent experiments. The estimated numbers of binding sites as calculated from Scatchard plots were 19 ± 4 and 15 ± 4 fmol/mg protein in KRB-N and KRB-N+S, respectively.*

In order to inhibit internalization, which is an ATP dependent process, ATP synthesis was blocked by sodium azide. This procedure reduced internalization (Table 1). However, residual internalization, swelling, and potential loss of cells remained a problem. Therefore, FSH receptor binding was also studied in membrane preparations under similar conditions

as used for intact cells. Different procedures were tested for preparation of cell membranes. Fixation by either 3% paraformaldehyde or 10% formaline, frequently used in immunohistochemistry, completely destroyed the FSH binding properties. It appeared that after dehydration of rat Sertoli cells by brief exposure to air, cell membranes were strongly attached to the plastic of the culture plates and retained their binding properties. FSH receptor binding to Sertoli cell membranes was constant when the cells were exposed to air from 10 min up to 3 hours, but after a 12 hours exposure period to air, FSH receptor binding was lost (data not shown). Sertoli cells were routinely exposed to air for 15 min. It was a useful procedure for preparing a "plastic-attached FSH receptor preparation" with similar binding properties as receptors on intact cells (see below).

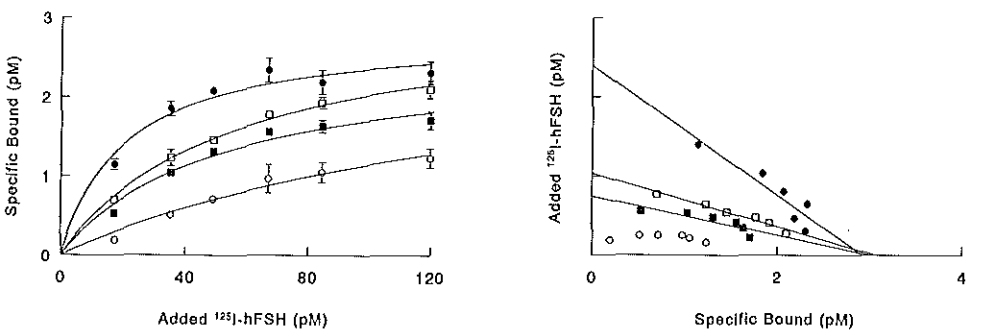

Figure 2. ***Concentration dependent effect of NaCl on ^{125}I-hFSH binding to Sertoli cell membranes.*** *Sertoli cell membranes were incubated for 3 h at 37°C with increasing concentrations of ^{125}I-hFSH in the presence of different concentrations of NaCl from zero up to 100 mM. Specific binding was measured as described in Materials and Methods, and analyzed as indicated in the Legend to Figure 1. Values represent the mean ± SD of triplicate determinations in a representative experiment, the affinity constants were approximately 25, 50 and 80 pM for 0 (●), 25 (□), and 50 (■) mM NaCl, respectively. It was not possible to give an accurate estimation of the affinity constant at 100 mM NaCl (○).*

^{125}I-hFSH binding to Sertoli cell membranes:

^{125}I-hFSH binding to Sertoli cell membranes was studied in KRB, KRB-N and KRB-N+S, at increasing concentrations of ^{125}I-hFSH. As in intact Sertoli cells, only the specific FSH receptor binding was influenced by NaCl while nonspecific binding was not affected. The apparent affinity constant for FSH receptor binding in KRB-N was 25 ± 4 pM (Figure 1B), which is similar to the affinity constant for cell-associated radioactivity in KRB-N

(Figure 1A). Saturation of the FSH receptors in KRB-N and KRB-N+S occurred at similar concentrations of ^{125}I-hFSH (Figure 1B), and the apparent affinity constant for FSH receptor binding in KRB-N+S was 28 ± 3 pM. In several experiments, the number of FSH binding sites was lower in KRB-N+S than in KRB-N, but the average values, being 15 ± 4 and 19 ± 4 fmol/mg protein, respectively, were not significantly different. The maximum number of FSH binding sites in KRB, as determined from the maxima of the saturation curves was approximately 4-fold lower than in KRB-N and KRB-N+S. However, as shown before for intact Sertoli cells, it was not possible using Scatchard analysis to make accurate estimations of the affinity constant and the number of FSH binding sites.

Rapid dissociation of hormone/receptor complexes might occur during the washing procedure when bound and free hormone are separated, and this might contribute to the low FSH receptor binding in KRB. We have therefore studied whether rapid dissociation occurs, particularly when KRB was used. Cell membranes were incubated for 3 hours with 35 pM ^{125}I-hFSH in KRB. At the end of the incubation period, the medium was aspirated without washing of the cell membranes. Values for total and nonspecific binding were measured as described in Materials and Methods, and were 976 ± 44 and 729 ± 25 cpm (mean ± SD of triplicate determinations in each of two independent experiments), respectively. After washing the cell membranes twice with KRB, these values were reduced to 428 ± 11 and 286 ± 25 cpm, respectively, indicating removal of similar amounts of radioactivity from the wells for total binding and nonspecific binding. Thus, specific binding appears not to be affected by the washing procedure, and so there is no evidence for rapid dissociation of a substantial number of hormone/receptor complexes during the washing procedure.

NaCl modulation of FSH receptor binding:

^{125}I-hFSH binding to Sertoli cell membranes was investigated at increasing concentrations of NaCl from zero up to 100 mM. Specific FSH receptor binding decreased at increasing concentrations of NaCl (Figure 2A). From the saturation curves for ^{125}I-hFSH binding it can be concluded that the maximal number of FSH binding sites decreased at increasing concentrations of NaCl. This is not apparent from the Scatchard plots, which give the impression that the number of binding sites is not affected by NaCl (Figure 2B). However, at higher NaCl concentrations, the accuracy of the specific binding data progressively declines, since the ratio specific/nonspecific binding decreases, and the Scatchard plots possess a progressive deviation from ideal linearity. Although it is not possible to give accurate values, it is clear that the affinity constant decreases at increasing

NaCl concentrations. Apparent nonlinearity of the Scatchard plots at high NaCl concentrations, may indicate that the interaction between FSH and its receptor differs from a simple reversible bimolecular interaction. We have not investigated this aspect. Instead we have focused on functional properties of the FSH receptor at different NaCl concentrations.

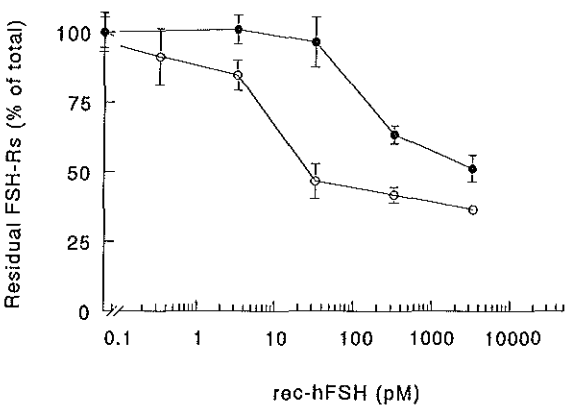

Figure 3. ***Effect of NaCl on FSH-induced receptor endocytosis.*** *Intact Sertoli cells were incubated with increasing concentrations of rec-hFSH in KRB (●) or in KRB-N+S (○), for 3 h at 37°C. Then the cells were washed, and incubated for 10 min with ice-cold KRB-N, at pH 3.0, to remove unlabelled hormone from the receptors. Residual FSH receptors on the cell surface were subsequently measured by incubating the cells with ^{125}I-hFSH in KRB-N+S in the presence of 5 mM sodium azide, for 3 h at 37°C. Specific binding was measured as described in Materials and Methods. Values represent the mean ± SEM of triplicate determinations in each of two independent experiments. The ED_{50} values for FSH-induced receptor endocytosis were 31 ± 4 pM and 661 ± 27 pM in KRB-N+S and KRB, respectively.*

Effect of NaCl on hormone-induced endocytosis of FSH receptors:

Internalization of FSH in rat Sertoli cells is very likely a receptor mediated process, and in this respect the amount of internalized FSH may reflect the integrated number of dynamic and reversible interactions between hormone and receptor over the incubation time. We have therefore measured FSH-induced endocytosis of the hormone/receptor complex under the influence of NaCl. Intact Sertoli cells were incubated with increasing concentrations of unlabeled FSH in either KRB or KRB-N+S, for 3 hours at 37°C. After this period of endocytosis, the cells were washed twice with the appropriate incubation buffer. Subsequently, the cells were incubated with the appropriate buffer at pH 3.0 for 10

min in order to displace the hormone from the receptor. The number of residual unoccupied surface receptors was then determined by incubating the hormone depleted cells with ^{125}I-hFSH in KRB-N+S, and in the presence of sodium azide to prevent further endocytosis. Under these conditions, cell-associated radioactivity reflects mainly receptor bound ^{125}I-hFSH (see Table 1). A hormone concentration dependent decrease in the number of surface receptors was observed in both KRB and KRB-N+S, as indicated by the decrease in cell-surface bound radioactivity after incubation with ^{125}I-hFSH (Figure 3). Moreover, in KRB, the ED_{50} value for hFSH-induced receptor endocytosis was 15-20 fold higher than in KRB-N+S. Cells incubated with 0.5 mM dbcAMP for 3 hours showed no decrease in the number of cell surface FSH receptors in either KRB or KRB-N+S.

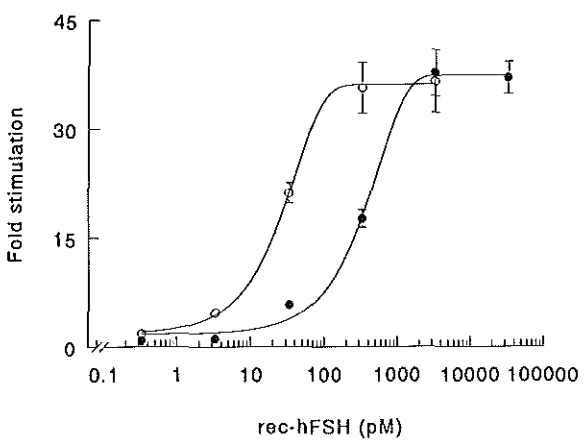

Figure 4. ***Effect of NaCl on FSH-induced cAMP production in isolated rat Sertoli cells.*** *Cells were incubated for 1 h at 37°C with increasing concentrations of rec-hFSH in KRB (●) or in KRB-N+S (○), and total cAMP production was measured as described in Materials and Methods. Basal and maximally FSH-stimulated cAMP productions were 55 ± 4 and 1725 ± 83 pmol/mg protein, respectively. Values represent the mean ± SEM of triplicate determinations in each of two independent experiments. ED_{50} values for FSH induced stimulation of cAMP production were 259 ± 21 pM and 14 ± 2 pM in KRB and KRB-N+S, respectively.*

Effect of NaCl on hormone-induced stimulation of cAMP production:

To investigate whether NaCl also affected the hormone-induced activation of the FSH receptor, hormonal stimulation of cAMP production in Sertoli cells was compared in KRB

and KRB-N+S. Total cAMP production in the presence of IBMX was measured as an index of FSH dependent receptor activation. The cAMP production was stimulated approximately 30-fold with hFSH in both KRB and in KRB-N+S. However, the dose-response curve for hFSH-stimulated cAMP production in KRB-N+S was shifted to the left when compared to stimulation in KRB (Figure 4). Thus, under low ionic strength conditions, receptor stimulation was observed at 15-20 fold lower FSH concentrations, than when tested in the presence of NaCl. The concentration-response curves for the forskolin-induced stimulation of the cAMP production were not affected by the incubation buffer (data not shown). This indicates that the 15-20 fold shift in sensitivity in KRB-N+S is specific for the interaction between FSH and the FSH receptor, and is not a consequence of ionic effects on adenylyl cyclase activation in general.

DISCUSSION

Under physiological conditions FSH interacts with its receptor in a milieu that contains 140 mM NaCl as the main ionic component. Therefore, the *in vitro* biological activity of FSH is normally studied in chemically defined media that mimic this physiological environment. In contrast, almost all FSH receptor binding studies have been carried out in low ionic strength buffers, which lack NaCl. Only under these low salt conditions, FSH binds with high affinity to its receptor, while in the presence of NaCl receptor binding is markedly reduced (165,168,221). However, it is not clear whether this is due to a reduction in the ligand binding affinity of the FSH receptor, or in the number of FSH binding sites, or both. We observed that the estimated affinity constant for FSH receptor binding to Sertoli cell membranes in the absence of NaCl was approximately 30 pM, which is well within the range of affinity constants reported in the literature, and reviewed by Reichert et al. (162,222). FSH receptor binding in the presence of 140 mM NaCl was substantially lower, and the specific binding data were less accurate since total binding declined while nonspecific binding was not affected by NaCl. Therefore, accurate estimation of the ligand binding affinity of the FSH receptor and of the number of FSH binding sites was not possible in KRB. In addition, a progressive decrease in both the ligand binding affinity and actual number of binding sites was observed when the NaCl concentration was gradually increased. At physiological NaCl concentrations, the number of FSH binding sites was approximately 4-fold lower than in the absence of NaCl.

The decrease in FSH receptor binding in the presence of NaCl is consistent with earlier studies (165,168,221). From this study it appears that both the affinity constant and

the number of FSH binding sites are reduced at physiological NaCl concentrations. Andersen and Reichert (165) reported that 100 mM NaCl reduced the affinity constant of the FSH receptor in bovine testicular membranes approximately 10-fold without affecting the actual number of binding sites. They reported a reduction in ligand binding affinity of the FSH receptor in the presence of various salts, however, the authors assumed the substantial variance in the maximal number of binding sites in the presence of different salts to be without any functional significance, thereby ignoring that the accuracy of the estimated values for the affinity constant and the number of binding sites depend on the same Scatchard plot. It is more likely that Andersen and Reichert (165) experienced similar problems when they analyzed Scatchard plots for FSH receptor binding at high salt concentrations, as we describe herein (see Results section).

Additional evidence for the existence of low affinity interactions between FSH and its receptor at physiological salt concentrations came from endocytosis studies. The rate of endocytosis might be a reflection of the dynamic interactions between hormone and receptor. It appears that in KRB-N+S approximately 20-fold lower FSH concentrations were required to induce a similar reduction in the number of surface receptors than in KRB. The existence of low affinity binding sites at physiological salt concentrations could also be deduced from the FSH-induced stimulation of cAMP production in KRB-N+S, which was observed at 15-20 fold lower hormone concentrations than in KRB.

The FSH receptor is a member of the super family of G protein-coupled receptors, and ligand binding to many other members of this receptor family has also been shown to be modulated by monovalent cations. For instance, sodium decreases the binding affinity of agonists to the α_2-adrenergic receptors and dopamine D_2 receptors by more than 10-fold (164,166). As for the FSH receptor, the NaCl-induced decrease in ligand-binding affinity of the receptor affinity parallels the decreased potency of ligands to stimulate α_2-adrenergic receptors (164). Modulation of ligand binding by NaCl has also been reported for LH/CG and TSH receptors. The apparent affinity of the LH/CG receptor for LH is markedly reduced in the presence of NaCl, while that for hCG is not affected (169-172). For both hormones the total number of binding sites is independent of the NaCl concentration. In contrast, NaCl decreased TSH binding to the FRTL5 rat thyroid cell line via a reduction of the number of binding sites while the ligand binding affinity of the receptors was not affected (173,174).

It is not at all understood how the binding affinity and the number of binding sites for the various glycoprotein hormone receptors can respond so differently to NaCl. Recently, it has been reported that an aspartic acid residue in the second transmembrane region of the

receptor is involved in Na^+ modulation of the ligand binding activity of several G protein-coupled receptors (172,181,185). Since this aspartic residue is conserved in FSH, LH/CG, and TSH receptors, it is likely that this aspartic acid residue mediates Na^+ modulation of the glycoprotein hormone receptor binding properties. Na^+ might interact with the carboxyl moiety of the aspartic acid residue, thereby allosterically hindering the binding of small ligands or particular domains of the large gonadotropin molecules to sites within the transmembrane domain of the receptor. The Na^+-induced reduction in interactions at the transmembrane level may contribute to the overall decrease in ligand binding affinity.

It is more difficult to envisage how the actual number of TSH (173,174) and FSH (this Chapter) binding sites can be regulated by Na^+ in a noncompetitive manner. In the present study, it was shown that in the presence of physiological concentrations of NaCl, only approximately 30% of the FSH binding sites that are detectable under low ionic strength are bound by FSH after a 3 hours incubation period at 37°C. It is well known that high affinity FSH receptor binding develops very slowly over a period of several hours (218-221). A relatively slow transformation process might be essential for formation of these high affinity complexes. The rate of the transformation process might then be influenced by Na^+. Experimental evidence for this hypothesis is the NaCl dependent rate of association of both hCG and LH with the rat LH/CG receptor, with LH binding being most dependent on low NaCl concentrations (170).

Since the properties of the FSH/receptor complex with respect to affinity and number of binding sites depend so much on the NaCl concentration, it is not warranted to apply properties of the FSH receptor as determined under low salt conditions to physiological conditions. Since 15-20 fold higher concentrations of FSH are required for stimulation of signal transduction in the presence of NaCl, it remains to be determined what the exact properties are of the relatively low affinity FSH/receptor complex that prevails under physiological conditions.

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CHAPTER FIVE

HIGH AFFINITY FSH RECEPTOR BINDING IS A SLOWLY NONREVERSIBLE PROCESS THAT APPEARS NOT TO BE IMPORTANT FOR RAPID RECEPTOR ACTIVATION.

H.J. van Loenen, J.F. Flinterman & F.F.G. Rommerts.

Department of Endocrinology & Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, The Netherlands.

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SUMMARY

The kinetics of FSH-induced stimulation of adenylyl cyclase and of FSH receptor binding have been studied, in order to test the hypothesis that FSH-induced receptor activation and high affinity FSH receptor binding are mediated by different hormone/receptor complexes. Within 2 minutes after addition of FSH to rat Sertoli cells, cAMP production was stimulated at a constant production rate for only 5 minutes. After 10 minutes adenylyl cyclase activity returned to basal levels. The steady state level of cAMP production was proportional to the FSH concentration. Since stimulation of adenylyl cyclase activity is correlated with receptor occupancy, different steady state levels of FSH/receptor complex must have been formed within 2 minutes after hormone addition. The rapid establishment of steady state receptor activation is in sharp contrast to the slow development of high affinity receptor binding, which requires several hours before an apparent steady state was reached. Steady state receptor activation occurs when FSH receptor binding is between 0 and 5% of maximal binding. Moreover, in the presence of steady state receptor activation, FSH receptor binding increases linearly in time. Since receptor binding of FSH was only partially (≈40%) reversible, the slow development of the high affinity binding complex suggests a transformation process of the initial FSH/receptor complex that is involved in transmembrane signalling, into a more stable binding complex. Consistent with the NaCl dependent affinity of the receptor for FSH (Chapter IV), 10-fold higher FSH concentrations were required to induce a similar rate of cAMP production when Krebs Ringer buffer was used instead of Krebs Ringer buffer without NaCl but with 200 mM sucrose. The kinetics of the cAMP response were not affected by NaCl.

On the basis of our experimental findings and those reported by other investigators, we propose a model with at least two distinct FSH/receptor complexes: **H + R ⇔ HR' (relatively low affinity and coupled with signal transduction and internalization) ⇒ HR" (higher affinity and nonreversible).** With this model the effects of NaCl on initial hormone/receptor complex formation and on the development of high affinity binding might be explained. The properties of the high affinity complex appear to be misleading for a proper understanding of the signal transduction properties of the receptor. The individual kinetic constants of the relatively low affinity complex, rather than the value of the apparent equilibrium constant of the high affinity complex, might be important.

INTRODUCTION

High affinity FSH receptor binding is only detectable under low ionic strength conditions, and requires several hours before an apparent equilibrium is reached (218-221). On the other hand, rapid functional interactions between FSH and its receptor might occur, since calcium transients are detectable within minutes after addition of FSH to target cells (215-217).

We postulate that FSH receptor binding and FSH induced receptor activation involve distinct hormone/receptor complexes, and that a relatively slow NaCl dependent process transforms the initial labile FSH/receptor complex that is involved in transmembrane signalling into a more stable binding complex. We have measured the kinetics of stimulation of adenylyl cyclase as a reflection of the functional FSH/receptor complex, and the kinetics of high affinity receptor binding as a reflection of the stable hormone/receptor complex. Since it is known that NaCl can alter the properties of the FSH receptor, these studies have been carried out at normal physiological concentrations of NaCl and in the absence of NaCl.

MATERIALS AND METHODS

FSH-induced stimulation of cAMP production:

Sertoli cells were isolated from testes of immature (21-23 day-old) Wistar rats and cultured as described previously (Chapter IV). Prior to the experiments, the cells were washed twice with isotonic Hepes-buffered Krebs Ringer buffer (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 25 mM Hepes) containing 0.1% (w/v) BSA, indicated as KRB, or with KRB-N+S (140 mM NaCl in KRB is replaced by 200 mM sucrose). Cells were incubated at 37°C for various periods with rec-hFSH (Organon 32489; Organon International B.V., Oss, The Netherlands) in the presence of 0.25 mM of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX). For determination of the total amount of cAMP, concentrated perchloric acid was added to cells and medium at a final concentration of 5% (v/v). The cell lysate was treated with 1.1 M Tris-containing KOH sufficient to neutralize the perchloric acid. cAMP was subsequently measured by radio-immunoassay as previously described (195).

FSH receptor binding:

Receptor binding experiments were carried out at 37°C with intact rat Sertoli cells in the presence of 5 mM sodium azide in order to block internalization (see Chapter IV). Cells were washed twice with KRB-N+S before ^{125}I-hFSH (Du Pont Canada Inc., Markham, Ontario, Canada) was added. At the end of the incubation period, the cells were washed twice with ice-cold KRB-N+S and subsequently dissolved in 1N NaOH. Nonspecific binding was determined in the presence of a 300-fold molar excess of unlabeled hFSH (NIDDK-hFSH-I-SIAFP-1). Specific binding was calculated by subtracting nonspecific binding from total binding.

For dissociation experiments, cells were first incubated for 3 h at 37°C with ^{125}I-hFSH. After aspiration of the buffer, fresh buffer with or without a 300-fold molar excess of unlabeled hFSH

was added. The amount of ^{125}I-hFSH bound to the receptor was measured at various time points. The residual amount of receptor bound ^{125}I-hFSH was calculated as a percentage of the amount at the start of the dissociation period, which is the addition of fresh buffer with or without unlabeled hFSH.

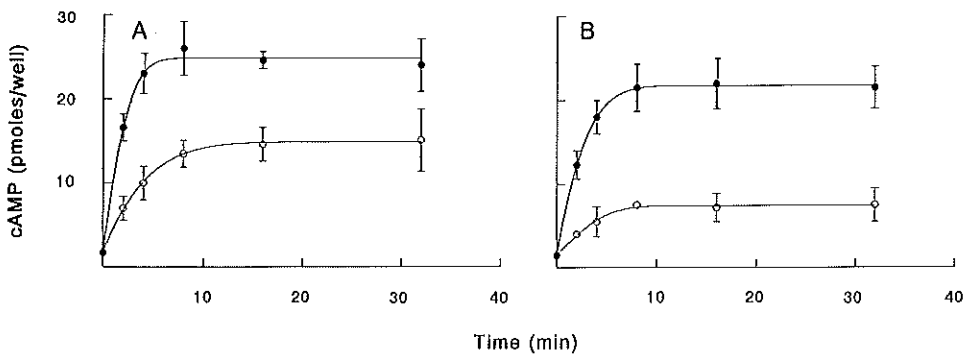


Figure 1. ***Time dependency of FSH induced stimulation of cAMP production.*** *Sertoli cells were incubated for 2, 4, 8 16, and 32 min with 100 (○) and 1000 (●) pM rec-hFSH in KRB (Panel A), or with 10 (○) and 100 (●) pM rec-hFSH in KRB-N+S (Panel B), in the presence of 0.25 mM IBMX. Total cAMP production was measured as indicated in Materials and Methods at the different time points. Values represent the mean ± SD of triplicate determinations in each of two independent experiments.*

RESULTS

Kinetics of the FSH-induced stimulation of cAMP production:

The FSH-stimulated cAMP production in rat Sertoli cells was studied as a function of time. A constant rate of cAMP production was observed up to approximately 5 minutes after addition of FSH, when cells were either incubated in KRB or KRB-N+S (Figure 1). Consistent with the previously observed lower ED_{50} value for FSH-induced cAMP production in KRB-N+S than in KRB (Chapter IV), approximately 10-fold higher FSH concentrations induced similar rates of cAMP production in KRB than in KRB-N+S. The hormone-induced cAMP production rates were hormone concentration dependent, since higher rates were found at higher hormone concentrations (Figure 1). The different steady states of cAMP production, as indicated by the linear increases in the amount of cAMP, were obtained almost instantaneously after addition of the FSH. The hormone induced cAMP production was terminated within less than 10 minutes after addition of FSH, and

the short period of steady state activation of adenylyl cyclase appeared to be independent of the buffer and the FSH concentration.

Association kinetics of FSH receptor binding:

The time dependency of binding of 50 and 500 pM 125I-hFSH to rat Sertoli cells was studied in KRB-N+S at 37°C in the presence of 5 mM sodium azide, in order to prevent internalization of ^{125}I-hFSH (Chapter IV). In contrast to the rapid establishment of FSH induced steady state stimulation of adenylyl cyclase activity within minutes, high affinity receptor binding developed slowly over the first hour, and approximately 3 hours were required to obtain an apparent steady state of binding (Figure 2). The rate of association of FSH to the receptor was faster when cells were incubated with 500 pM instead of 50 pM ^{125}I-hFSH (Figure 2). Due to the lower affinity of the FSH receptor for FSH and the approximately 4-fold lower number of binding sites, when KRB instead of KRB-N+S was used (Chapter IV), it was not possible to establish accurate association kinetics for FSH receptor binding at physiological concentrations of NaCl.

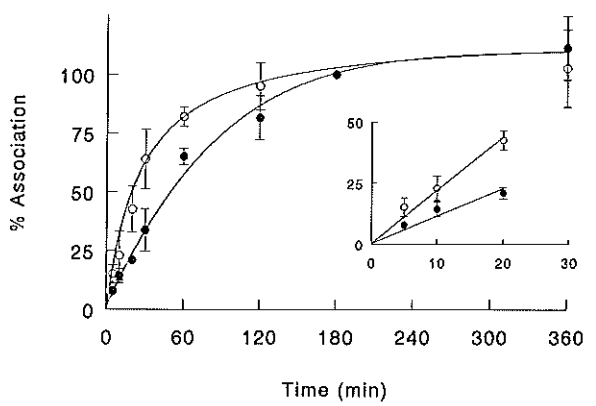


Figure 2. ***Binding of ^{125}I-hFSH to rat Sertoli cells.*** *Sertoli cells were incubated with 50 (●) or 500 (○) pM ^{125}I-hFSH for different time periods at 37°C, in KRB-N+S in the presence of 5 mM sodium azide in order to block internalization. Specific binding was measured as described in Materials and Methods. Specific binding at the various time points is indicated as the percentage of binding at 3 h (100%) for both ^{125}I-hFSH concentrations. Values represent the mean ± SD in each of triplicate determinations of three independent experiments.*

Dissociation kinetics of FSH receptor binding:

Sertoli cells were incubated with ^{125}I-hFSH for 3 hours at 37°C in KRB-N+S in the presence of 5 mM sodium azide, and dissociation of bound hormone was studied in the absence or presence of excess unlabeled FSH. The dissociation of preformed FSH/receptor complexes exhibited first-order kinetics, whereby part of the ligand is nonreversibly bound (Figure 3). The dissociation of pre-formed FSH/receptor complexes was independent of the amount of FSH that is present during the dissociation period (Figure 3). Within 1 hour approximately 40% of specifically receptor bound radioactivity was dissociated, and no further dissociation was observed during the next 3 hours. These FSH receptor binding properties in intact Sertoli cells confirm the observations made by others investigators using membrane preparations (see Discussion section).

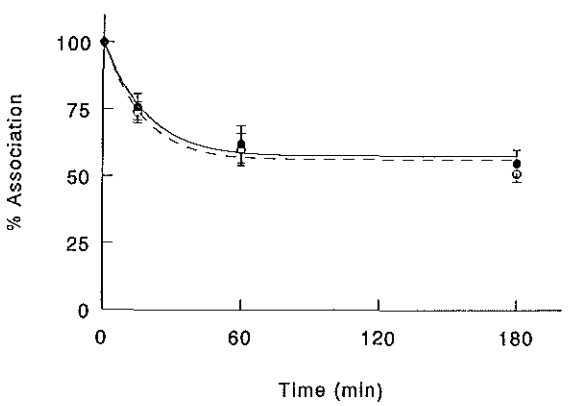


Figure 3. ***Dissociation kinetics of receptor-bound 125I-hFSH.*** *Sertoli cells were preincubated in KRB-N+S with 50 pM ^{125}I-hFSH for 3 h at 37°C in the presence of 5 mM sodium azide. After this incubation the cells were washed twice, fresh buffer with 15 nM hFSH (○; broken line) or without (●; closed line) was added, and the cells were subsequently incubated for 15, 60, or 180 min at 37°C. Specific binding was measured as described in Materials and Methods. Specific binding at individual time points is indicated as the percentage of the specific binding that was present at the start of the dissociation period. Values represent the mean ± SD of triplicate determinations in each of two independent experiments.*

DISCUSSION

Almost instantaneously after addition of FSH to rat Sertoli cells, a constant rate of

cAMP production was obtained. Since the cAMP production is an indirect reflection of receptor occupation, a constant production rate suggests a steady state in the number of FSH occupied receptors that mediate transmembrane signalling. Moreover, the rate of cAMP production is proportional to the FSH concentration, illustrating that the law of mass action applies for the initial FSH/receptor complex formation. Under conditions of relatively low receptor affinity (in normal KRB), 10-fold higher FSH concentrations are required for a similar degree of stimulation of cAMP production than in KRB-N+S, when the receptor possesses higher affinity for FSH (Chapter IV). It is striking that the steady state FSH receptor activation lasts for only approximately 5 minutes. After this brief period of constant stimulation, of which the time frame appeared to be independent of hormone concentration and buffer, the hormone-activated adenylyl cyclase is rapidly turned off. This phenomenon has been observed previously and is not related to loss of cell viability (223,224). In this respect, the response of Sertoli cells to FSH differs completely from the response that Leydig cells show after LH/hCG stimulation, since cAMP production in Leydig cells is stimulated for prolonged periods, up to several hours (231,232). We have not investigated the possible FSH-induced desensitization mechanisms, but it is important to note that when receptor activation is terminated, receptor binding is still linearly increasing and at that moment at 10-20% of the maximal binding. The rapid establishment of a steady state of receptor activation within less than 2 minutes is in sharp contrast to the slow development of the high affinity receptor binding that requires several hours before an apparent steady state was reached. Moreover, different levels of steady state receptor activation occur at a very low and almost undetectable level of FSH receptor binding. It should be noted, that receptor binding studies are carried out under optimal conditions for FSH receptor binding, which is in the absence of NaCl. In the presence of physiological amounts of NaCl, receptor binding will be much less (Chapter IV). These FSH receptor binding properties are consistent with the observations of others using membrane preparations (219-221).

Thus, the existence of at least two different complexes between FSH and its receptor can be postulated: one rapidly formed complex that is involved in signal transduction, and a slowly formed tight binding complex with unknown properties. If we assume that the slowly formed complex arose from the rapidly formed complex, then the following model can be postulated:

$$H + R \Leftrightarrow HR\alpha \Leftrightarrow HR\beta$$

Since the initial complex induces stimulation of adenylyl cyclase within less than 2 minutes without measurable hormone binding, we have assigned this as the labile relatively low affinity HRα complex. The slowly derived more stable binding complex, possessing higher affinity binding properties was assigned as HRß. Several observations for FSH receptor binding made by us and by other investigators support this model and/or can be explained by it.

The observation that higher FSH concentrations are required for stimulation of adenylyl cyclase in the presence than in the absence of NaCl (Chapter IV), might be explained by a reduction in affinity of the initial HRα complex under the influence of NaCl. Since the formation of HRß complexes depends on the amount of HRα complexes, it can also be understood that under physiological conditions more FSH is required to form binding complexes (218,220,Chapter IV). The model might even give an explanation for the puzzling observation that the apparent number of FSH binding sites is inversely proportional to the NaCl concentration (Chapter IV), if we assume that NaCl not only lowers the affinity of the initial HRα complex, but also inhibits transformation of HRα into HRß.

Interactions between hormones and receptors are often described by a reversible bimolecular interaction. Nevertheless, for several peptide hormones, including the gonadotropins, it has become clear that their interaction with specific receptors probably involves multiple states of hormone/receptor intermediates or complexes which differ in their degree of reversibility (72,73,219,233-236). We showed only partial reversibility of receptor bound FSH, which is consistent with the observations made by Andersen et al. (235) and Sanborn et al. (236). They showed that FSH receptor binding to bovine testis homogenates and solubilized bovine FSH receptor preparations was fully reversible when association took place at 4°C, but became progressively less reversible at higher temperatures, being essentially nonreversible at 37°C. Moreover, reversibility of FSH receptor binding progressively decreased at prolonged times of association (235,236). Reversibility of LH/CG (73,237) and TSH receptor binding (233,234) has also been shown to depend on time, temperature, and salt concentration. At present, the molecular basis for the slow formation of irreversible hormone/receptor complexes is not understood. It might be that the relatively low affinity hormone/receptor complexes can somehow aggregate and form more stable multimeric complexes. However, covalent binding between the hormone and receptors appears not to be involved, since bound hormone can be dissociated at low pH (pH3) (230).

Since the interaction of FSH, TSH, LH, and hCG with their receptors do not follow

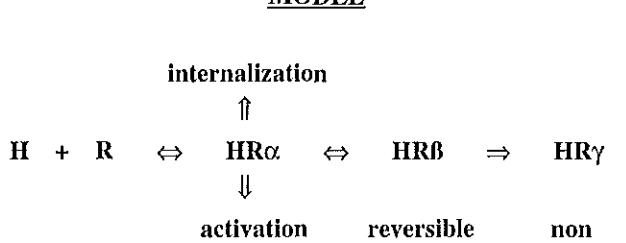
simple first order or second order association/dissociation kinetics, it is questionable whether such binding data can be properly analyzed by Scatchard analyses, which assumes a reversible bimolecular interaction. In this regard, deviation from the ideal Scatchard plot when analyzing FSH receptor binding at high NaCl concentrations (Chapter IV), might also be a reflection of the existence of different hormone/receptor complexes. In the present study, we have carried out only a limited number of binding experiments, since we did not consider it necessary to duplicate the many well conducted and detailed FSH receptor binding studies that have been carried out by others (235,236). It was of more importance for us, to make attempts to integrate many reported findings in a model. In the following paragraph we will briefly discuss some observations made with TSH, LH, and hCG, that support our model and extent it to other glycoprotein hormones.

It has been demonstrated for LH and hCG that both hormones activate the same receptor with virtually similar potency, whereas the receptor possesses approximately 10-fold higher binding affinity for hCG than for LH in the presence of NaCl (169-172). The disparity in affinity of the LH/CG receptor for hCG and LH is abolished when receptor binding is studied in the absence of NaCl. The formation of HRα complexes might be similar for hCG and LH, whereas NaCl mainly affects the transformation process of HRα into HRß for LH. Mock and Niswender (238) reported that internalization of oLH by ovine luteal cells is approximately 50-fold higher than that of hCG, while the affinity constant for hCG binding to these cells is 50-fold higher than for oLH binding. It might thus be that endocytosis is somehow mediated more by the low affinity HRα complex than by the higher affinity HRß complex. The findings of Genty et al. (171), that endocytosis of the LH/CG receptor is mediated by low affinity binding sites, supports this assumption. Another illustration of the ligand dependency of the transformation process comes from TSH receptor binding studies. It has been shown that bovine TSH and human TSH have similar potencies for activation of the human TSH receptor transfected in chinese hamster ovary cells (175), indicating that both hormones are able to form HRα complexes. However, the transformation of HRα complexes into higher affinity HRß complexes only occurs for bovine TSH, whereas human TSH fails to form a high affinity complex, even in the absence of NaCl (175,239). As a result of the observations made with LH, hCG, FSH, and TSH, we propose a more detailed model for different hormone/receptor complexes:

Within the transformation process, multimerization of the initial HRα complex might be involved in the formation of complexes that have increased affinity and stability in a time, temperature, NaCl, and ligand dependent fashion. Moreover, we postulate a non-

reversible HRγ complex, in order to explain the only partial reversibility of high affinity receptor binding.

MODEL



Although the properties of HRα are unknown, there is now abundant evidence that the high affinity binding complex is not a prerequisite for receptor activation. The relatively low affinity properties of the initial HRα indicates that the rate of association and dissociation are not much different, however, this ratio does not give information about the individual rate constants. Efficient activation of adenylyl cyclase, even under conditions of intermittent receptor activation might well be possible since activated G-proteins can have half lives in the order of seconds (239). The individual rate constants of the relatively low affinity complex, rather than the apparent equilibrium constant of the high affinity binding complex, might be important for a proper understanding of hormone induced receptor activation.

Acknowledgements:

The authors of the present paper would like to thank Organon International B.V. (Oss, The Netherlands) for kindly providing ^{125}I-hFSH and rec-hFSH.

CHAPTER SIX

GENERAL DISCUSSION

Glycoprotein hormone-receptor interaction

The gonadotropins (LH, FSH, and CG) and thyrotropin (TSH) constitute a subgroup of glycoprotein hormones that act on specific receptors on gonadal and thyroidal cells, and that are the main regulators of the gonads and the thyroid gland, respectively. As summarized in the Introduction to this thesis, many structural characteristics of hormones and receptors have been described during the last decades. Recently, the crystal structure of hCG in its deglycosylated form has been elucidated (240), although that of the native hormone remains unknown. It appeared that the α and ß subunits have similar structural conformations, with three disulfide bridges forming a so-called "Cystine knot". The heterodimer is stabilized by a segment of the ß subunit that wraps around the α subunit and covalently linked to another part of the ß subunit by disulfide linkage. Within the now known tree dimensional structure, domains at the protein backbone of the hormone which have been shown to be important for receptor binding and activation come into close proximity in the putative "active centre" of the hormone. The postulated position of the oligosaccharide unit attached to asparagine α52 is very close to this putative "active center", whereas the other oligosaccharide units are positioned at the opposite side of the molecule. Knowledge of the three-dimensional structure of both the ligand and the receptor will be essential for a proper understanding of their molecular interaction. Information about the mechanism of ligand-induced receptor stimulation can be derived from binding characteristics like specificity and affinity, and from evaluation of the functional properties of the receptor subsequent to ligand dependent stimulation.

Glycoprotein hormone-receptor binding and activation

Various domains within the three-dimensional protein structure of the glycoprotein hormones have been shown to be involved in receptor binding and activation (67). A proper spatial orientation of these domains is most likely pivotal for full hormonal activity. The oligosaccharide units, and especially that linked to asparagine α52, might be involved in the stabilization of an appropriate protein conformation. In this respect, deglycosylation of hCG most likely results in conformational changes, as indicated by physico-chemical and antibody binding studies (30,63). Subsequent to deglycosylation of hCG, the potency of the ligand to stimulate adenylyl cyclase is markedly reduced, whereas the receptor binding affinity is not impaired, but rather 2 to 3-fold increased when compared to the receptor binding properties of the native ligand (30,63). Conformational differences between hCG and DhCG may result in different fitting between DhCG and the LH/CG receptor than for hCG. Discrepancies in fitting might then result in structurally different

hormone/receptor complexes for hCG and DhCG. Ji and Ji (68) have demonstrated that DhCG binds to different protein parts of the porcine LH/CG receptor than hCG. Moreover, all immunological epitopes that are recognized by anti-hCG antibodies, and shared by hCG and DhCG, are masked in DhCG/receptor complexes, whereas some of these epitopes remain exposed in hCG/receptor complexes (69). Finally, hormone/receptor complexes for DhCG have been shown to be of nonreversible nature (74), while that for hCG possess partial reversibility within several hours (218).

Differences in fitting for hCG and DhCG and the LH/CG receptor might be dependent on the species origin of the LH/CG receptor, and this might influence the relative receptor-stimulating activity of DhCG in comparison to that of hCG, when tested in different species. We have studied the receptor-stimulating activity of hCG and DhCG in terms of cAMP and steroid production in Leydig cells obtained from various species (Chapter II). We observed a marginal adenylyl cyclase stimulating activity for DhCG when compared with hCG in mouse Leydig tumor cells and rat Leydig cells. Although the fitting of DhCG on the LH/CG receptor might be different for the mouse and rat LH/CG receptor, DhCG failed to induce efficient stimulation of adenylyl cyclase in either cell type. However, DhCG possessed variable but significant steroidogenic activity, depending on the species origin of the Leydig cells that were used. In rat Leydig cells the ED_{50} value for DhCG-stimulated steroidogenesis was approximately 6-fold higher than for native hCG, while the steroidogenic maximum was similar for both ligands. In mouse Leydig tumor cells, DhCG was only 2-fold less potent than native hCG based on its ED_{50} value, whereas the steroidogenic maximum for DhCG was approximately only 30% of that of hCG. The species dependent relative steroidogenic activity of DhCG when compared to hCG, might reflect different coupling efficiencies between cAMP production and stimulation of steroidogenesis in the various cell types.

Insight in the coupling efficiency between adenylyl cyclase activation and stimulation of steroidogenesis requires measurement of intracellular levels of cAMP, although even this may be insufficient since intracellular signalling may involve separate compartmentalized pools of cAMP (54,55). The intracellular level of cAMP should then be related to the steroidogenic activities of individual cells, rather than studying a population of cells. We have measured total cAMP production as an index for receptor activation in a population of cells, and our data can therefore not be used to resolve the coupling efficiency between cAMP production and steroidogenesis.

As discussed above, inappropriate fitting of DhCG on the LH/CG receptor might be

involved in the less efficient ligand-induced adenylyl cyclase activation. Support for this hypothesis has been obtained from experiments with anti-hCG polyclonal antisera. Antisera directed against hCGß possessed the ability to enhance the receptor-stimulating activity of DhCG. Since bivalent antibodies and Fab fragments of these antibodies showed similar effects, antibody-induced receptor aggregation appears not to play a major role in this mechanism of receptor stimulation (108). We have extended these studies by using monoclonal anti-hCG antibodies, and have defined an epitope on the ß subunit of DhCG that is involved in the antibody-induced increase of the receptor-stimulating activity of DhCG (Chapter III). Moreover, as we have used monoclonal antibodies, we were able to study the effect of preformed antibody/DhCG complexes. This would not have been possible using polyclonal antibodies, which also contain antibodies that are directed against epitopes that are important for receptor binding. The enhancing effects of specific antibodies on the receptor-stimulating activity of DhCG further support the hypothesis that the oligosaccharide units of the hormone are not directly involved in receptor activation, but that a particular spatial conformation of the protein is a prerequisite for receptor activation.

NaCl modulation of glycoprotein hormone-receptor interaction

The binding of a hormone to its receptor is the initial event in hormone-receptor interaction, and the high affinity binding complexes that are detectable in binding studies under low ionic strength conditions are assumed to mediate receptor activation. High affinity binding of FSH to its receptor, with an apparent affinity constant of approximately 30 pM, was detectable using a buffer without NaCl (Chapter IV). However, an accurate value of the affinity constant for the interaction between FSH and its receptor could not be estimated, when binding was studied in normal Krebs Ringer buffer (Chapter IV). Specific binding was substantially lower in the presence than in the absence of NaCl, due to a decrease in both the apparent ligand binding affinity of the FSH receptor and the maximal binding that could be measured. Approximately 70% of the FSH receptor binding signal that is measured in the absence of NaCl was not detectable in the presence of physiologic concentrations of NaCl (Chapter IV).

The FSH, LH/CG, and TSH receptors, are members of the large superfamily of G protein-coupled receptors. Modulation of ligand binding by monovalent and divalent cations has also been shown for other members of this superfamily. Na^+ ions have been found to decrease the binding affinity of agonists to the α_2-adrenergic receptor and

dopamine D_2 receptor by more than 10-fold (164,166). In addition, the reduction of the ligand-binding affinity of the α_2-adrenergic receptor correlated with a reduced receptor stimulating potency of the ligand (164). We have observed a similar parallelism of the FSH receptor binding affinity and the receptor stimulating activity of FSH. Consistent with the relatively low apparent ligand-binding affinity of the FSH receptor in the presence of 0.15 M NaCl, 15-20 fold higher FSH concentrations were required for stimulation of adenylyl cyclase in the presence of 0.15 M NaCl (Chapter IV). These data illustrate that the functional FSH receptor under physiologic conditions appears to be a receptor with a lower binding affinity than observed in FSH receptor binding studies carried out under low ionic strength conditions.

Glycoprotein hormone-receptor interaction involves sequential but distinguishable hormone/receptor complexes

The concentration of FSH that was required for stimulation of cAMP production in rat Sertoli cells was higher in the presence than in the absence of NaCl, whereas the maximum response appeared to be NaCl independent. If we assume that the degree of cAMP production is a direct reflection of functional receptor interaction, maximal cAMP production would be a reflection of a steady state in functional receptor occupation. The maximal response appeared independent of NaCl, indicating similar steady states for functional receptor occupation under both conditions. On the other hand, the maximum of detectable FSH receptor binding was substantially reduced in the presence of NaCl, indicating a reduced number of detectable binding sites. The fact that receptor stimulation is very similar in the presence and absence of NaCl, occuring at the same time and reaching the same maximum, whereas maximal FSH receptor binding is substantially impaired by NaCl, suggest the existence of different FSH/receptor complexes, involved in hormone-receptor binding and hormone-induced activation of the receptor. The observation that under physiologic conditions high affinity FSH receptor binding was not detectable, is probably not due to a fast dissociation of the binding complex under these conditions. Similar levels of FSH receptor binding were found with or without washing of the cells at the end of the binding period, indicating no fast dissociation during the washing procedure (Chapter IV).

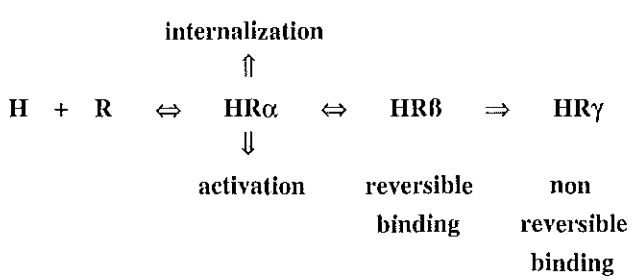
Additional evidence for the existence of at least two sequential but distinguishable hormone/receptor complexes came from kinetic studies on FSH-induced receptor activation and FSH receptor binding. Within 2 minutes after addition of FSH to rat Sertoli cells, either in the absence or presence of NaCl, cAMP production was stimulated at a constant

production rate (Chaper V). After approximately 10 minutes the stimulated adenylyl cyclase activity had returned to basal levels. It thus appeared that for the functional FSH/receptor complex that is involved in adenylyl cyclase stimulation a steady state was established within 2 minutes after hormone addition. Also for calcium transients induced by FSH in target cells, fast kinetics of stimulation within minutes after hormone addition have been reported (215-217). The steady state in cAMP production (from 2-10 min) was proportional to the concentration of FSH. In the presence of 0.15 M NaCl, when the affinity of the FSH receptor was found to be relatively low, approximately 10-fold higher hormone concentrations were required to obtain similar cAMP production rates than when tested in the absence of NaCl (Chapter 5). The distinguishable steady states at different concentrations of FSH, reflect different equilibria of subsequent functional interactions between FSH and its receptor at these concentrations. This would be consistent with the law of mass action for functional hormone-receptor interaction. At higher hormone concentrations a higher but constant frequency of functional interaction would occur at steady state, illustrated by a higher rate of cAMP production.

The rapid establishment of steady state receptor activation seems to be in contradiction to the slow development of high affinity receptor binding in the absence of NaCl, which requires several hours before an apparent steady state in binding is reached (Chapter V).

In order to explain the above findings we postulate the existence of at least three distinct hormone/receptor complexes:

MODEL



This model involves an initial HRα complex, that mediates the functional interaction between FSH and its receptor, that leads to activation of the receptor as reflected by cAMP production. Equilibrium of this initial interaction is obtained within minutes, whereas

formation of the binding complex HRß requires several hours before an apparent staedy state is obtained. The slow development of the binding complex would be consistent with a relatively slow transformation process of the initial complex into a binding complex with yet unknown properties. It is important to note that formation of HRα, that mediates adenylyl cyclase stimulation, and formation of HRß, both are affected by NaCl. In the presence of a physiologic concentration of NaCl, both the functional interaction between FSH and its receptor and the actual binding interaction apparently are of relatively low affinity, whereas in the absence of NaCl both are of higher affinity.

Nonreversibility of glycoprotein hormone-receptor binding

Two gonadotropin receptor binding complexes with different reversibility are postulated, since receptor binding of FSH is only partially reversible after a 3 hours incubation in the absence of NaCl (Chapter V). Partial reversibility of receptor bound hormone has also been reported for the binding of TSH (233,234) and hCG (72,73,237), both in the absence of NaCl. Andersen et al. (235) carried out a detailed study on the thermodynamics of FSH receptor binding to bovine testis homogenates in the absence of NaCl. They showed that FSH binding was fully reversible when binding took place at 4°C, but became progressively less reversible at higher temperatures, being essentially non-reversible at 37°C. We and others only studied the nonreversibility of FSH/receptor complexes under low ionic strength conditions. It remains to be established whether nonreversible binding also occurs under physiologic conditions.

From the above, it appears that temperature stimulates the transformation of HRß into HRγ complexes. Due to the existence of more than one FSH/receptor complex, and the only partial reversibility of the FSH receptor binding at low salt conditions, Scatchard analysis of the binding data is not permitted. The significance of data from Scatchard analysis, when measuring glycoprotein hormone receptor binding, is a matter of debate.

A multistep model for glycoprotein hormone-receptor interaction

LH and hCG activate the LH/CG receptor with approximately equimolar potencies (172), indicating that the functional interactions between LH or hCG and the LH/CG receptor are of similar affinity. However, the ligand binding affinity of the LH/CG receptor, as measured in binding studies in the presence of NaCl, is approximately 10-fold higher for hCG than for LH. This discrepancy can now be understood if we assume that formation of the initial HRα complex in the presence of NaCl is similar for hCG and LH, while NaCl only affects the affinity constant of the HRß complex for LH, but not for hCG.

Ligand dependent transformation from HRα into HRß has also been observed for the human TSH receptor. Bovine TSH and human TSH both stimulated cAMP production in chinese hamster ovary cells that were transfected with cDNA encoding the human TSH receptor. However, only bovine TSH was able to bind with high affinity to the receptor, whereas for human TSH, even in the absence of NaCl, no high affinity towards its homologous receptor could be measured (175). It might thus be that, under physiologic conditions, formation of HRα is similar for bovine and human TSH, whereas binding is different. In the absence of NaCl, high affinity HRß complexes can be found for bovine TSH, while human TSH binding to its homologous receptor is not detectable.

Apparent discrepancies have been reported between receptor binding and hormone-induced receptor internalization for ovine LH and hCG. Mock and Niswender (238) reported that while hCG possessed approximately 50-fold higher affinity for the LH/CG receptor on ovine luteal cells than ovine LH, the hormone-induced receptor internalization was 50-fold higher for ovine LH than for hCG. This suggests that high affinity bound hormone is less available for internalization. In addition, Genty et al. (171) showed that endocytosis of the LH/CG receptor is mediated by low affinity binding. In the presence of NaCl, formation of functional HRα complexes is similar for LH and hCG, as illustrated by a similar potency of the ligands to stimulate the receptor. Under physiologic conditions, the affinity of detectable receptor binding is lower for LH than for hCG, and the lower affinity complexes for LH might thus be involved in the higher internalization of LH when compared to hCG.

It remains to be established whether HRß and/or HRγ complexes possess signalling properties. Anti-hCG antisera were able to enhance the receptor-stimulating activity of DhCG when added to preformed DhCG/receptor complexes (Chapter III). If we assume that these DhCG/receptor complexes were of the HRß and/or HRγ type, these complexes might have signalling properties under certain conditions. hCG has been shown to execute more prolonged receptor stimulation than LH, after a pulse exposure of superfused murine Leydig cells (238,241-243). The more stable binding of hCG to the LH/CG receptor than LH, and the prolonged residence on the plasma membrane of hCG/receptor complexes, might be involved in the more prolonged receptor stimulation by hCG (238,243). Moreover, it should be noted that, in the presence of NaCl, LH binding to the LH/CG receptor is fully reversible, whereas binding of hCG is only partially reversible (73). Based on the different reversibility of receptor binding for LH and hCG, it can thus be understood that removal of the hormone by washing of hormone-preincubated cells almost immediately results in abrogation of LH-induced but not hCG-induced receptor activation,

as the hormone/receptor complexes for LH will dissociate more quickly than for hCG. The more stable hCG/receptor complexes might continue to trigger receptor activation (238,241-243). Consistent with the above hypothesis, it can be understood that the persistent adenylyl cyclase activation in rabbit luteal membrane preparations by both LH and hCG after addition of hormone-neutralising antibodies (244) could be due to the fact that the adenylyl cyclase assay was carried out in the absence of NaCl, allowing both ligands to form HRß complexes of similar stability.

It is unknown which mechanisms are involved in the formation of nonreversible hormone/receptor complexes. Covalent interactions between the hormone and its receptor appear not to be involved, since receptor-bound hormone dissociates at low pH (pH 3) conditions (229). It might be possible that the hormone/receptor complexes aggregate in so-called coated pits prior to internalization. Alternativele, or in addition, aggregation might occur with other protein or nonprotein molecules present at the cell membrane. The major histocompatibility complex class I antigen is a possible candidate to become associated with the hormone/receptor complex. This heterodimeric protein, composed of a 45 kDa α chain noncovalently associated with a 12 kDa $ß_2$ microglobulin, functions in immunological self/nonself discrimination. It has been suggested that, following hormone binding, LH/CG receptors become associated with major histocompatibility complex class I molecules on Leydig cells (245). A similar association has been postulated for epidermal growth factor (246), glucagon (247), and insulin receptors (248-250). The major histocompatibility complex class I molecules may participate in multimeric receptor complex formation on the plasma membrane.

Functionality of low affinity binding sites

The multistep model for hormone-receptor interaction as postulated in this thesis, involves that a relatively low affinity interaction between the hormone and its receptor may mediate the signalling properties of glycoprotein hormone receptors. Such relatively low affinity binding sites might be involved in ligand-induced stimulation of many more G protein-coupled receptors, since functional low affinity binding has been described for muscarinic cholinergic receptors, cholecystokinin receptors, and atrial natriuretic factor receptors, which all are members of the G protein-coupled receptor family (251-253).

Relatively low affinity binding sites may be more efficient to sense physiologic variation in hormone concentrations than high affinity binding sites. At each hormone concentration, more high affinity binding sites than relatively low affinity binding sites will

binding sites. Therefore, the level of occupation of relatively low affinity binding sites can be affected over a larger range of hormone concentrations than that of high affinity binding sites (Figure 8). Relatively low binding sites allow physiological variation in hormone concentrations to cause substantial variation in receptor occupation. Different levels of receptor occupation might then be of functional importance.

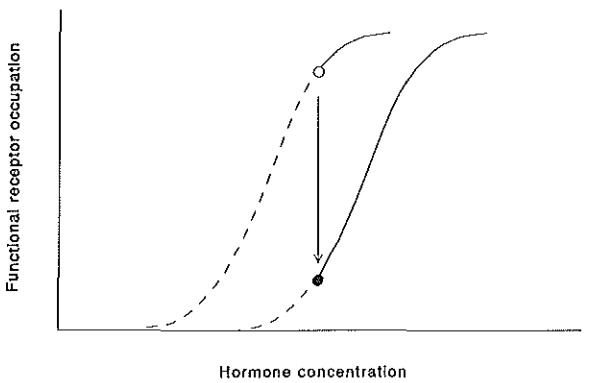


Figure 8. Receptor occupancy of relatively low affinity binding sites. *Two hypothetical curves represent hormone-receptor binding for a high affinity binding site (broken line), and a relatively low affinity binding site (closed line). When high affinity binding sites are almost fully occupied, only partial occupation of relatively low affinity binding sites occurs. Relatively low affinity binding sites allow better sensing of increases in hormone concentrations, since increases in hormone concentration would affect occupation of relatively low affinity binding sites more than that of high affinity binding sites. Increases in the level of receptor occupation might then be of functional importance.*

In a similar way as an enzyme reacts with a substrate via different enzyme/substrate intermediates with individual free energy states, the dynamic interaction between a hormone and its receptor might be characterized by sequential intermediates, each with a specific free energy state (Figure 9). Although highly speculative, it might be that different hormone/receptor complexes can activate specific intracellular signalling cascades. Within the transformation process, the free energy of the hormone/receptor complex might progressively decrease resulting in more stable hormone/receptor complexes. A high free energy of the transition state for the transformation process HR $\Leftrightarrow$ HRx, diminishes formation of HRx. NaCl might modulate the free energy of such a transition state, by

increasing this free energy, and thereby hampering formation of stable binding complexes.

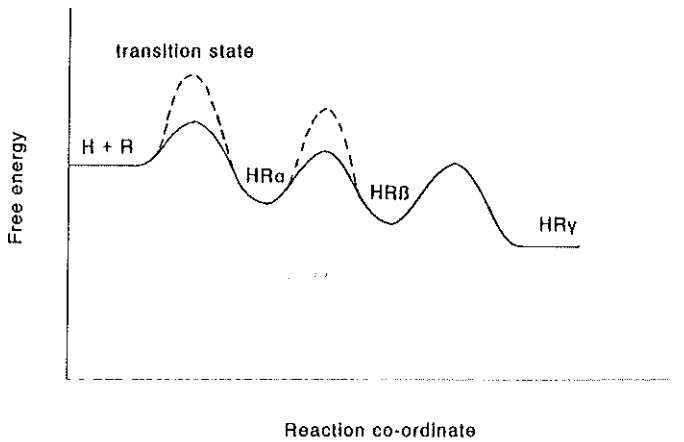


Figure 9. ***Postulated free energy diagram for different hormone-receptor complexes.*** *The interaction between a glycoprotein hormone and its receptor might involve different hormone/receptor complexes. For HRα, the state of free energy is relatively high. A progressive decrease in the free energy state would allow formation of more stable complexes. Part of the modulatory action of NaCl can be depicted as modulation of the free energy level of the transition state of the various complexes (broken line).*

If hormones have a relatively low affinity towards the receptor, efficient stimulation is still possible since the affinity constant, as a ratio, does not give any information about the individual rate constants of association and dissociation. In low affiny hormone/receptor complexes, the rates of association and dissociation might be both high or both low, but the rate constants are not substantialy different. Complexes with a low affinity might therefor still reflect a high frequency of hormone-receptor interactions. Efficient transmembrane signalling can be sustained even in cases of low frequency interactions between the hormone and the receptor, since activated G proteins can have a half-life up to seconds (45). Theoretically, full receptor stimulation is possible with only a few receptor-stimulating interactions per second. Not the dynamics of the hormone/receptor complex itself, but rather the activated α subunit of the G protein will be the regulating factor for hormone-induced transmembrane signalling.

The dynamics of ligand-receptor interaction, in addition to affinity, might be important

for ligand-induced receptor stimulation, and the induced biological effects. Results supporting the importance of the kinetics of hormone-receptor interaction have been reported by De Meyts et al. (254). They demonstrated that the dissociation kinetics of complexes between insulin analogues and the insulin receptor correlated with the biological activities of these ligands. Slow dissociation rates favoured enhanced mitotic over metabolic activity of the various ligand, and *vice versa.*

In future experiments, it might be important to put more emphasis on measurement of the kinetic properties of hormone-receptor interaction. In order to measure this real-time dynamics, new techniques for the detection of hormone-receptor interactions will be of critical importance. Using a light diffraction technique, it is possible to measure binding kinetics for binding of soluble antibodies to antigens coated on a sensor surface. Attempts to apply this technique for studying hormone-receptor interaction, have been published (255,256). Research on the dynamics of hormone-receptor interaction will prove to be an interesting field for future research.

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SUMMARY / SAMENVATTING

SUMMARY

The amino acid sequence of the gonadotropin hormones and their receptors have been determined, and very the crystal structure of hCG in its deglycosylated form was elucidated. However, the three-dimensional structures of fully glycosylated hormones and their receptors have not yet been resolved. Due to this shortage of structural information, many molecular details of hormone-receptor interaction are still unknown.

The binding of a ligand to its receptor is a prerequisite for receptor activation of that receptor, and a proper fitting between a hormone and its receptor probably is essential for efficient hormone-induced signal transduction. For the glycoprotein hormones, the oligosaccharide units appear to be important for stabilizing the overall conformation of the molecule, since structural differences between native and deglycosylated glycoprotein hormones have been reported. The different structure of DhCG in comparison of that of hCG does not impair the receptor binding potency of the ligand, on the contrary it slightly enhances its binding properties. However, differences in the three-dimensional conformations of hCG and DhCG may still result in an improper fitting of DhCG over that of hCG on the LH/CG receptor, as reflected by a markedly reduced potency to stimulate adenylyl cyclase for DhCG. We have shown that although DhCG possesses marginal adenylyl cyclase stimulating activity, it still possesses considerable steroidogenesis-stimulating activity, which is dependent on the cell type in which the ligand-induced steroidogenesis is measured (Chapter II). In rat Leydig cells the EC_{50} values of DhCG-induced steroidogenesis, are 6 to 10-fold higher than those of native hCG, while the steroidogenic maxima for hCG and DhCG were similar. In mouse Leydig tumour cells, the ED_{50} value of DhCG for steroidogenesis is only 2-fold higher than for hCG, while the maximal steroidogenic response to DhCG is only approximately 30% of that of hCG. Thus, DhCG possesses partial agonistic properties which depend on the type of Leydig cell.

Insight in the conformational changes of the hormone after deglycosylation will be possible when the crystal structures of both hCG and DhCG are available. Until then, indirect evidence supports the conformation stabilising function of the oligosaccharide units. We have studied whether monoclonal anti-hCG antibodies were able to affect the receptor-stimulating properties of DhCG, thereby suggesting that these antibodies alter the conformation of DhCG (Chapter III). Monoclonal anti-hCG antibodies and derived Fab fragments, enhanced the receptor-stimulating activity of DhCG in terms of adenylyl cyclase activity and steroidogenesis. This stimulating effect was shown either when antibodies were added to hormones prior to hormone addition to cells or when antibodies were added

to cells that had been preincubated with hormone. By using monoclonal antibodies we were able to define a region on DhCG that is still exposed in the DhCG/receptor complex. Antibody binding to this epitope enhances the receptor-stimulating activity of DhCG. When added as antibody/hormone complexes it is likely that antibody binding restored the active conformation" of the hormone. Moreover, it appears that the complex between DhCG and the LH/CG receptor is flexible as the active conformation of the hormone/receptor complex can be restored in a already existing DhCG/receptor complex.

Interaction between hormones and receptors can be characterized by measuring the affinity constant. High affinity binding of the glycoprotein hormones to their respective receptors has been demonstrated, when measured under low ionic strength conditions. However, when FSH binding to the FSH receptor is measured in the presence of physiologic concentrations of NaCl, no high affinity receptor binding is detectable (Chapter IV). Consistent with a relatively low affinity interaction between FSH and its receptor under physiologic conditions, the FSH concentrations that are required for activation of adenylyl cyclase in whole cells, are 10 to 15-fold higher in the presence of 0.15 M NaCl than in the absence of NaCl. Forskolin-induced stimulation of adenylyl cyclase is not affected by NaCl. These results illustrate that the high affinity FSH/receptor complex, as detectable under low ionic strength conditions, is not involved in physiological signal transduction. Under physiologic conditions, a relatively low affinity interaction between FSH and its receptor mediates stimulation of adenylyl cyclase. Kinetic experiments on FSH-induced adenylyl cyclase stimulation and FSH receptor binding indicate that the high affinity binding complex between FSH and its receptor evolves from a labile FSH/receptor complex that mediates transmembrane signalling (Chapter V). Based on our data and several observations in the literature concerning the interaction between LH, hCG, and TSH with their respective receptors, we postulate a new model for the interaction between the glycoprotein hormones and their receptors. In this model sequential but distinguishable hormone/receptor complexes are postulated to be involved in hormone-induced receptor activation and receptor binding. The initial relatively low affinity hormone/receptor complex may be processed into more stable binding complexes at low salt concentrations. It is unknown which molecular events are involved in the different steps of this transformation process.

Binding data have shown that hormone/receptor complexes are nonreveribly bound, indicating that scatchard analysis of these data is not allowed. Such tight binding is not required for glycoprotein hormone-induced receptor activation. The interaction between a glycoprotein hormone and its receptor, that causes signal transduction, appears to involve

much more dynamic interactions than suggested by the stable binding complexes. For future research on the mechanisms of glycoprotein hormone-induced receptor stimulation it will be important to investigate the dynamic aspects of the interaction between the glycoprotein hormones and their receptors, rather than the stable binding properties.

SAMENVATTING

In elk meercellig organisme, van relatief eenvoudige zoals een worm, tot complexe zoals de mens, is communicatie tussen cellen van groot belang voor de regulatie van processen in dat organisme. De verschillende celtypen in complexe organismen maken gebruik van een grote variëteit aan mechanismen voor communicatie. Naast directe contacten tussen de cellen vindt cellulaire communicatie ook in veel gevallen plaats via door de cellen uitgescheiden moleculen (o.a. neurotransmitters, hormonen, groeifactoren en cytokines). Deze moleculen worden geproduceerd door de ene cel en beïnvloeden andere cellen door te binden aan specifieke moleculen, receptoren. Onderzoek naar deze vorm van communicatie tussen cellen valt binnen het onderzoeksveld van het vakgebied Endocrinologie.

In zoogdieren worden in de hypofyse een aantal hormonen geproduceerd, waaronder de twee gonadotrope hormonen: follikel-stimulerend hormoon (FSH) en luteïniserend hormoon (LH). FSH en LH spelen een belangrijke rol bij de regulatie van het ovarium en de testis. In de testis komen FSH receptoren alleen voor op Sertoli cellen (in de zaadbuisjes), terwijl alleen de Leydig cellen (in het interstitium tussen de zaadbuisjes) receptoren voor LH bezitten. In de mens wordt, tijdens de zwangerschap, door de placenta nog een derde gonadotroop hormoon geproduceerd: het zwangerschapshormoon of humaan chorion gonadotropine (hCG), wat aan dezelfde receptor bindt als LH.

De drie genoemde hormonen zijn opgebouwd uit twee ketens van aminozuren (de bouwstenen van elk eiwit), waaraan op specifieke plaatsen suikergroepen zijn gekoppeld. Deze zogenoemde glycoproteine-hormonen binden aan specifieke receptoren in de plasmamembraan (aan het celoppervlak) van de doelcellen. De receptor moleculen steken door de plasmamembraan heen, met een deel buiten en een deel binnenin de cel. Hormoon-receptor binding vindt plaats aan de buitenkant van de cel. Door de binding van het hormoon aan de receptor veranderd deze van vorm, ook aan de binnenkant van de cel. Door deze vormverandering kan de receptor worden gestimuleerd, waardoor vervolgens andere moleculen binnenin de cel geactiveerd worden. Dit leidt tot een reactie van de cel op het signaal. In de testis zorgt LH voor stimulatie van de produktie van androgenen (de geslachtshormonen) door Leydig cellen, terwijl FSH door stimulatie van Sertoli cellen, van belang is voor de spermatogenese.

Inzicht in de interacties tussen glycoproteine-hormonen en hun receptoren is van belang om het proces van receptor stimulatie beter te kunnen begrijpen. De moleculaire mechanismen die betrokken zijn bij de stimulatie van gonadotropine receptoren zijn nog

grotendeels onbekend. Eerder onderzoek heeft laten zien dat bepaalde gebieden (domeinen) in de ruimtelijke eiwitstruktuur van het hormoon belangrijk zijn voor hormoon-receptor binding en activatie van de receptor. De juiste ruimtelijke structuur van deze domeinen en van hun positie ten opzichte van elkaar wordt bepaald door de drie-dimensionale structuur van het hormoon. Eén van de functies van de suikergroepen in de glycoproteine hormonen is waarschijnlijk het stabiliseren van een specifieke ruimtelijke structuur van deze hormonen. Een hCG molecuul zonder suikergroepen (gedeglycosyleerd hCG; DhCG) bindt beter dan intact hCG aan de receptor, maar stimuleert de receptor minder goed. Receptor stimulatie werd gemeten door de produktie van cyclisch AMP (een molecuul dat betrokken is bij de signaaloverdracht in de cel) en produktie van steroidhormoon in rat Leydig cellen en muize tumor Leydig cellen te bepalen (Hoofdstuk II). DhCG is ten opzichte van die van intact hCG een minder efficiënte stimulator van de LH/CG receptor, maar antilichamen gericht tegen hCG bleken in staat te zijn om de receptor-stimulerende activiteit van DhCG te verhogen (Hoofdstuk III). Hieruit is geconcludeerd dat door de binding van de antilichamen aan DhCG de ruimtelijke structuur van DhCG weer vergelijkbaar wordt met die van intact hCG, waardoor de receptor-stimulerende activiteit van DhCG toeneemt.

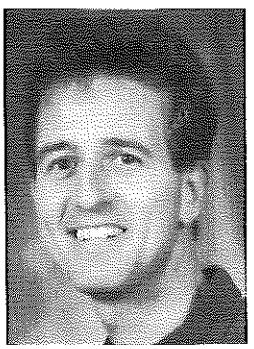
Interactie tussen hormoon en receptor is essentieel voor stimulatie van de receptor. In de literatuur zijn veel gegevens bekend over ondermeer de affiniteit en specificiteit. De gevonden affiniteit *in vitro* (buiten het lichaam) is hoog, wat betekent dat er een relatief sterke binding tussen de moleculen optreedt. Deze sterke binding werd echter vooral gevonden wanneer positief geladen ionen, zoals Na^+ ionen, grotendeels afwezig waren. In de vloeistof die de lichaamscellen omgeeft, is de concentratie Na^+ ionen echter hoog. De vraag kan dus gesteld worden met welke affiniteit de gonadotrope hormonen in het lichaam (*in vivo)* binden aan hun receptoren. De invloed van in het lichaam voorkomende NaCl concentraties op de interactie tussen FSH en de FSH receptor werd daarom onderzocht (Hoofdstuk IV). Dit is gebeurd door FSH receptor binding en de FSH-geïnduceerde receptor stimulatie te meten in aan- en afwezigheid van NaCl. Het bleek dat in aanwezigheid van NaCl, het hormoon FSH veel minder goed aan de FSH receptor kon binden dan in afwezigheid van NaCl. Ook het aantal FSH receptoren dat FSH kon binden bleek verlaagd in aanwezigheid van NaCl. In overeenstemming met een minder sterke binding van FSH aan de FSH-receptor, was voor het stimuleren van de receptor in aanwezigheid van NaCl een hogere concentratie hormoon nodig.

Ook werd de kinetiek van FSH-geïnduceerde receptor activatie en FSH-receptor binding bepaald in aan- en afwezigheid van NaCl (Hoofdstuk V). Op basis van het feit dat FSH-

geïnduceerde receptor stimulatie binnen enkele minuten resulteert in een constante produktie snelheid van cyclisch AMP, terwijl de vorming van een stabiel bindingscomplex pas maximaal is na enkele uren, wordt gepostuleerd dat het complex dat gevormd wordt tussen FSH en zijn receptor verschillende vormen kan aannemen. Een relatief instabiele (maar wel specifieke) hormoon-receptor interactie leidt tot stimulatie van de receptor, en dit complex kan vervolgens in de tijd een transformatie ondergaan tot een stabieler complex. Na^+ ionen verhinderen dit transformatie proces, waardoor het hormoon in aanwezigheid van NaCl niet stabiel aan de receptor kan binden. Het is nog onbekend welke moleculaire processen bij deze transformatie een rol spelen.

Wij postuleren daarom dat, in het lichaam, de interactie tussen een gonadotroop hormoon en zijn receptor, leidend tot receptor stimulatie, een instabiele dynamische interactie is, waarbij er geen stabiele complexvorming plaatsvindt. Ondanks dat het hormoon niet sterk aan zijn receptor bind, kunnen opeenvolgende kort durende interacties voldoende zijn voor stimulatie van de receptor. Van alle mogelijke interacties zullen alleen die interacties waarbij het hormoon goed op de receptor past de receptor activeren. Voor toekomstig onderzoek is het van belang meer aandacht te besteden aan de dynamiek van de interactie tussen hormonen en receptoren, om goed te kunnen begrijpen op welke wijze hormoon-receptor interactie kan leiden tot activatie van de receptor en een receptor-geïnduceerde cascade van cellulaire processen.

CURRICULUM VITAE



Hans van Loenen werd geboren op 16 mei 1965 te Leiden. In 1984 behaalde hij het VWO diploma aan het Rijnlands Lyceum te Oegstgeest. In datzelfde jaar begon hij met de studie Gezondheidswetenschappen (thans Biomedische wetenschappen) aan de Faculteit der Geneeskunde van de Rijksuniversiteit Leiden. Tijdens de studie zijn drie bijvakstages doorlopen, bij de afdelingen Anatomie en Fysiologie & Fysiologische Fysica van de Rijksuniversiteit Leiden en de afdeling Reumatologie van het Academisch Ziekenhuis Leiden. Bij deze laatste afdeling is tevens een 12-maanden hoofdvakstage afgerond en in mei 1989 werd het doctoraalexamen behaald. Vanaf augustus 1989 tot augustus 1993 heeft hij gewerkt als assistent in opleiding in dienst van de Erasmus Universiteit Rotterdam bij de afdeling Endocrinology & Voortplanting van de Faculteit der Geneeskunde en Gezondheids wetenschappen aan het onderzoek beschreven in dit proefschrift. Vanaf april tot augustus 1994 heeft hij onderzoek verricht bij het Istituto di Ricerca Cesare Serono te Rome, Italië. Vanaf november 1994 is hij gastmedewerker bij de vakgroepen Endocrinologie & Voortplanting en Immunologie van de Erasmus Universiteit Rotterdam en doet onderzoek naar de rol van cytokines in het ovarium.

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Hans

LIST OF PUBLICATIONS

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