In this report, we review our present effort in the field of molecular reproductive endocrinology: to identify a small molecular weight follicle stimulating hormone (FSH) agonistic molecule. To achieve this goal we require a number of molecular tools. We have cloned and expressed the human gonadotrophin, FSH and the human FSH receptor and developed a reliable high throughput assay. We have also proposed a model to explain FSH receptor activation and from that model, begun to create small molecules predicted to induce FSH signal transduction without binding to the extracellular domain of the membrane protein. In this report, we summarize our efforts to date and discuss our future research efforts in this area.

Key words: assay/FSH/FSH receptor/luciferase

Introduction

Purified follicle stimulating hormone (FSH) is administered to women to stimulate the growth and maturation of ovarian follicles and thus increase the frequency of conception in couples with fertility problems. In the past, human FSH was only available through purification from urine obtained from post-menopausal women. With the advent of molecular biology methods, it has become possible to clone the genes for the common α and the hormone-specific β subunit of human (h)FSH. When the appropriate expression vectors that contain either the cDNA or the genomic DNA that encodes these subunits are inserted into mammalian cells, it is possible to produce biologically active human FSH (Chappel, 1988; Keene et al., 1989; Galway et al., 1990; Howles, 1996).

We have cloned the genes for the common α subunit as well as the hormone-specific β subunits and expressed each of the human gonadotrophins. Indeed, recombinant human FSH is now available commercially and is being used in assisted reproduction (Loumaye et al., 1996). Human chorionic gonadotrophin (HCG) and luteinizing hormone (LH) registrations are soon to follow.

As with other therapeutic proteins, it is necessary to administer gonadotrophins either s.c. or i.m. From several perspectives, it would be advantageous to activate
FSH and its receptor

the receptor with a small molecule that could be administered through the oral or transdermal route. The current challenge in biotechnology is to reduce the size of these proteins by developing small functional mimetic synthetic molecules. Success in identifying a small peptidomimetic of erythropoietin has been reported (Livnah et al., 1996). In this case, it has been shown that a 20 amino acid cyclic peptide can be created which binds to and dimerizes the receptor for erythropoietin to elicit signal transduction. The biological response is identical to that obtained with the hormone erythropoietin; however, the binding site of the peptide mimic is unrelated to the natural ligand. We believe it may be possible to activate the FSH receptor with a mimic that does not bind to the classical extracellular domain binding domain of the receptor. To achieve this objective for FSH, we must develop a working model to explain how the gonadotrophin activates its receptor, develop a high throughput assay specific for FSH and create a large number of samples to test. Our hope is to identify molecules with some activity which will then be optimized.

To develop this model, we have reviewed published reports available on the structure-function of FSH and have proposed a mechanism by which FSH may activate its receptor. We have also used information obtained from the study of the activation of other G protein-coupled receptors such as catecholamines (Strader et al., 1994). Using this information, we have predicted that a specific portion of the FSH molecule interacts with and activates its receptor. Using that portion of the molecule as a template, we have synthesized a family of related peptides and synthetic organic molecules that may exhibit agonistic activity.

To test these chemicals for agonistic activity, we need a well-controlled and reliable assay. Several years ago, we cloned and expressed the human FSH receptor (Kelton et al., 1992). When inserted into an adrenal cell line (Y1), activation of the FSH receptor results in the increased biosynthesis and secretion of the steroid hormone, progesterone, which can be measured by radioimmuno-assay. A dose–response relationship exists between the amount of FSH added to Y1 cells that express the FSH receptor and progesterone release. This cell line has been used to better understand the manner by which FSH binds to and activates the ovarian FSH receptor. We will now use it to search for molecules with FSH agonistic activity.

More recently, we have expressed the human FSH receptor in a Chinese hamster ovarian (CHO) cell line. Within the cell, we have inserted a cAMP response element operatively linked to the gene that encodes firefly luciferase (Brasier et al., 1989; Benzakour et al., 1995). Binding of FSH to the receptor expressed on this engineered cell line results in the production of intracellular cAMP and expression of the enzyme luciferase. Receptor activation induced by increasing doses of FSH, results in an increase in the intensity of luminescence within the cell. This cell based assay can be configured to function as a high throughput screen to test thousands of compounds for FSH receptor stimulatory activity.

This report summarizes the efforts that have been made in our laboratory to
S. Chappel et al.

develop all of the tools necessary to accomplish the task of identification of a small, synthetic molecule that specifically activates the FSH receptor.

**Cloning and expression of proteins necessary for identification of small FSH agonists**

To determine the manner by which FSH binds to and activates its receptor, it is essential to have cloned and expressed the FSH dimeric glycoprotein as well as its receptor. A review of the entire process used to produce recombinant FSH has been published (Howles, 1996).

*Construction of vectors for the expression of the glycoprotein α and FSHβ subunits in mammalian cells*

The human α subunit genomic fragment used for expression purposes was 11 kb pairs in length and was derived from a 17 kb genomic clone that was identical to that described previously (Fiddes and Goodman, 1981). The α gene promoter region was removed by cleavage at the unique *BamHI* site in exon I. The fragment which resulted included the remainder of exon I, all of the coding exons II, III, and IV, as well as the intervening sequences, and ~2 kb of 3' flanking sequence. The termini of the 11 kb fragment were converted to *SalI* sites prior to its insertion into the unique *XhoI* site of the CLH3AXSV2DHFR expression vector. In this construct, transcription of the α gene was directed by the mouse metallothionein-I (MMT-I) promoter and the endogenous α subunit gene polyadenylation signal was used for 3' processing of the mRNA. The expression vector also contained the mouse dihydrofolate reductase (*DHFR*) gene for a selectable and amplifiable marker. A restriction endonuclease map of the complete human α subunit expression vector plasmid is shown in Figure 1.

Alternatively, an expression vector containing the human α cDNA construct, such as CLH3AXSV2DHFR/hαIVSA/hαcDNA (Fiddes and Goodman, 1979; Cole et al., 1993), could be used for α subunit expression.

The human FSHβ coding region was obtained from a *DdeI-Sau3AI* subfragment of the genomic clone as described previously (Jameson et al., 1988). As the endogenous FSHβ polyadenylation signal was removed during engineering, the SV40 early polyadenylation signal supplied by the vector was used for 3' processing of the FSHβ subunit transcript. The murine ornithine decarboxylase (*ODC*) transcriptional unit was also part of the vector. The *ODC* gene, like the *DHFR* gene, is a selectable and amplifiable marker. A restriction map of the complete human FSHβ subunit expression vector is shown in Figure 2.

*Expression of FSH α and β genes in CHO cells*

A DHFR-deficient CHO cell line was transfected with the two expression vectors described above. Transfectants were selected with the addition of methotrexate
FSH and its receptor

Figure 1. A map of the human α subunit expression plasmid.

Figure 2. A map of the follicle stimulating hormone (FSH) β subunit expression plasmid.
S. Chappel et al.

(MTX). Colonies were selected and cultures were amplified with increasing amounts of MTX to obtain higher levels of FSH expression. Individual tranfectants were screened by assaying culture supernatants for the secreted hormone. The best expressor was expanded for use in bioproduction. The expressed FSH was purified by anion exchange and immunoaffinity chromatography, sequenced to show identity with the predicted amino acid sequence derived from DNA and analysed for purity using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining. A detailed review of the physicochemical analysis of recombinant FSH has been published (Siebold, 1996). The granulosa cell bioassay was used to assess in-vitro bioactivity (Keene et al., 1994). Activity of FSH was also assessed using the Y1 FSH receptor based bioassay (Kelton et al., 1992). In-vivo biological potency was determined using the rat ovarian weight gain assay of Steelman and Pohley (1953).

Development of a high throughput assay system: CHO–FSH receptor–luciferase

To rapidly and accurately assess the potential ability of proteins, chemicals, peptides or peptoids to activate the FSH receptor, a high throughput cell based assay is required. The FSH receptor is a G protein-coupled receptor that stimulates the conversion of intracellular ATP to cAMP and in turn increases the transcription of all cAMP-dependent genes. A CHO cell-based reporter system was developed that employed a cAMP response element that controlled the expression of the firefly luciferase gene (Brasier et al., 1989; Benzakour et al., 1995). Luciferase was used as the reporter gene of choice in this assay because of the ease and speed of luminescence measurements and the sensitivity of detection (0.01 attomoles of luciferase). Luciferase gene expression in the CHO–FSH receptor positive cells was placed under control of a single c-fos cAMP response element. In addition, control cells which contained the reporter gene but do not express the FSH receptor were developed to serve as a control.

CHO-DUKX cells were transfected with the human FSH receptor (Kelton et al., 1992). Transfectants were expanded in minimal essential medium (MEM) α medium (without ribonucleosides or deoxyribonucleosides) supplemented with 10% dialysed fetal bovine serum (FBS), 1% L-glutamine and 0.02 μM MTX.

Routine cell culturing

CHO hFSHR Luc1-1 cells were grown in MEM α medium without Phenol Red supplemented with 10% dialysed FBS, 2 mM L-glutamine, 0.02 μM MTX and 600 μg/ml of G418 (growth medium). Adherent cells were recovered by removing serum-containing medium from the flasks and rinsing the cell monolayer with calcium and magnesium-free phosphate-buffered saline (PBS). Cells were dislodged by adding ~ 2 ml trypsin/EDTA solution to the flasks (just enough to cover the monolayer) and incubating at 37°C for 2 min. Trypsin was neutralized.
by adding back 5 volumes of serum-containing growth media and resuspending cells by pipetting. Cells were routinely maintained by passaging twice a week by splitting them down to 2–3x10⁴ cells/ml.

CHO–FSHR Luc 1-1 cells were cultured and plated overnight. As demonstrated in Figure 3, when cultures of these cells were incubated with FSH, an increase above matrix control was seen in luminescence signal, as measured by the Wallac microbeta plate reader. The same degree of induction was also seen in the presence of forskolin (1 μM) which directly activates adenylate cyclase resulting in cAMP production.

**Development of a high throughput assay to assess FSH stimulation**

For analysis of the response of the clones to FSH receptor activation, cells were seeded at a density of 4x10⁴ cells/well in 96-well tissue culture plates in 200 μl of appropriate selection medium at 37°C for 48 h.

The CHO–Luc assay meets most of the objectives set forth as ideal features of a high throughput screening assay: ease of performance, rapid read out within 4–5 h, high sensitivity to FSH stimulation (pM range) very reproducible (approximately 30 independent assays have been performed with the isolate) and adaptable to automation with robotics. Further improvements of the assay are ongoing along with screening of compound libraries for FSH agonists using this assay.

The same approach was used to develop a high throughput assay using a CHO cell line that had not been transfected with the human FSH receptor expression
vector but contained the cAMP response element operatively linked to the luciferase gene. As example, forskolin was able to active luciferase expression in this cell line. This cell line served as a negative control for this high throughput assay.

**Construction of a model for FSH receptor binding and activation**

*Background*

With the positive control (recombinant human FSH protein) and CHO-FSH receptor-luciferase cell line now in place, a review of literature in the gonadotrophin and G protein-coupled receptor field allowed us to develop a model to test.

The family of pituitary/placental glycoprotein hormones which include FSH, LH, thyroid stimulating hormone (TSH) are heterodimers composed of an α and β subunit that are both glycosylated. The α subunits have an identical primary sequence (92 amino acids) while the β subunits share regions of similarity as well as a conservation of cysteine position indicating that they fold similarly (Lustbader et al., 1993).

Upon dissociation of the α/β dimer, each subunit loses high affinity binding (Pierce and Parsons, 1981). Photoaffinity labelling and immunological studies have shown that both subunits make contact with the receptor. Further, synthetic peptide studies (as detailed below) have shown that there are two or more hormone-receptor contact points (Yoo et al., 1993).

Although the primary structure of the gonadotrophins had been known for many years (Pierce and Parsons, 1981), their tertiary structure remained unknown until recently. The crystal structure of HCG is now known (Lapthorn et al., 1994). As a result, we can infer a great deal about the structure of the other members of this family, such as FSH.

These hormones bind G protein-coupled receptors which contain seven transmembrane domains with homology to the β adrenergic receptor and activate primarily the adenylate cyclase second messenger system. The extracellular domain of the pituitary/placental glycoprotein hormone receptors (>350 amino acids) is much longer than those of most other G protein-coupled receptor and contains multiple cysteines and glycosylation sites offering potential for disulphide bond formation and carbohydrate attachment (Segaloff and Ascoli, 1993). Despite the similarity of these hormones and their receptors, each must bind specifically to its own receptor to maintain independent function. We have drawn upon data obtained from the study of all of the human dimeric glycoproteins and their receptors, as well as other G protein-coupled receptors to construct our hypothesis for hormone-induced receptor activation.

**The β subunit**

Specific regions of the β subunit are important to receptor binding and subunit contact. The FSH β subunit contains 111 amino acids and the large inter-cysteine
loop formed by the disulphide bonds between Cys$^{32}$ and Cys$^{51}$ of this subunit is a region of heterogeneity amongst the glycoprotein β subunits. This region contains amino acids that are accessible on the surface of the FSH heterodimer. This region is antigenic and antibodies that map to it inhibit binding of hFSH to its receptor (Vakharia et al., 1990). Thus, it is proposed that this region is involved in the specific binding to the receptor. Other sites of receptor binding have been mapped at β amino acids 38–57 (Santa-Coloma et al., 1990) and 81–99 (Lindau-Shepard et al., 1994). No reports could be identified by us in which FSH β sequences were shown to be involved with FSH receptor activation with the exception of one. Boniface and Reichert (1990) found that a four amino acid region of FSH β (Cys$^{84}$-Gly$^{85}$-Lys$^{86}$-Cys$^{87}$) has structural and functional homology to the active centre of thioredoxin and has implicated this region in the process of signal transduction.

**The α subunit**

Several laboratories have shown that a region of the α subunit from amino acids 37–40 is involved in assembly with the β subunit (Bielinska and Boime, 1992; Xia et al., 1994).

The α subunit has been mapped for receptor binding by site-directed mutagenesis and epitope mapping studies. To date, the regions that appear to be involved in receptor binding are α 26–46 (Leinung et al., 1991; Kikuchi et al., 1994; Xia et al., 1994; Cattini-Schultz et al., 1995; Liu and Dias, 1996) and 81–92 (Morris et al., 1988; Chen et al., 1992). Other studies have concluded that residues of the α subunit Phe$^{33}$, Arg$^{35}$, Arg$^{42}$, Ser$^{43}$ and Lys$^{44}$ are not required for binding of FSH to its receptor (Liu et al., 1993).

The sequences described above, α 26–46 and 81–92 are linked by two disulphide bonds (Cys$^{28}$-Cys$^{82}$ and Cys$^{32}$-Cys$^{84}$) and form part of β sheet structures. In particular, α Tyr$^{88}$, Tyr$^{89}$, His$^{90}$ and Lys$^{91}$ of FSH have been shown to play an essential role in cAMP induction (Chen et al., 1992; Yoo et al., 1993; Zeng et al., 1995). This cystein knot motif is characteristic of the family of proteins that includes the glycoprotein hormones as well as TGFβ, PDGF, NGF, activin and inhibin. This suggests that due to their structural similarities, their recognition by specific receptors is the result of subtle differences in the conformational orientation of the two subunits that arises as a result of their heterodimeric association.

Substitutions of a basic amino acid (lysine) in a specific region of the α subunit (residues 13, 16 and 20 or 13, 14, 16 and 20) and then combined with the β subunit resulted in an increase in cAMP accumulation within the target cells compared with wild type α subunit (Szkudlinski et al., 1996). These regions are in the β1 strand and the L1 loop of the α subunit.

Whether this portion of the hormone projects into the space between the transmembrane domains is not known. A recent study with HCG suggests that several regions of the α subunit (loop 1 and loop 3) are exposed following binding of the ligand to the extracellular domain of the receptor. According to a
published model (Jiang et al., 1995), these portions of the α subunit are available for interaction with the transmembrane domains of the receptor. In fact, these regions contain some of the residues shown in the studies described above to enhance cAMP accumulation when they are mutated to basic amino acids (Szkudlinski et al., 1996). Further, mutations in this area α 28–32 resulted in a loss of the gonadotrophin’s ability to activate its cognate receptor (Kikuchi et al., 1994). These regions are masked to antibody binding when HCG binds to the membrane bound receptor however, they are exposed when HCG is bound only to the extracellular domain of the truncated receptor (Pantel et al., 1993).

From the information summarized above, and that to be discussed below, we conclude that portions of the α and β subunits are required for high affinity binding to the extracellular domain. However, a specific portion of the α subunit, not involved in subunit assembly or binding to the extracellular domain, transduces binding into intracellular activation through a direct interaction with portions of the receptor other than the extracellular domain (Valove et al., 1994; Ji and Ji, 1995).

Role of carbohydrates

Since carbohydrates appear to play a critical role in receptor activation, that area of research was also considered. FSH is a heterodimer that contains a common α subunit and a hormone specific β subunit. The human α subunit is a 92 amino acid polypeptide with glycosylation sites at Asn-52 and Asn-78, while the β subunit contains two asparagine-linked carbohydrates at positions 7 and 24 (Pierce and Parsons, 1981; Green and Baenziger, 1988).

These carbohydrates have been shown to play a different role in receptor activation and binding (Galway et al., 1990; Bishop et al., 1994). Deglycosylation of FSH by acid treatment (Sairam, 1980; Sairam and Bhargavi, 1985) or site-directed mutagenesis (Keene et al., 1994) changes FSH from an agonist into an antagonist. A mutant FSH lacking the N-linked asparagine residue as Asn-52 on the α subunit exhibits 10-fold less bioactivity than the fully glycosylated wild-type. Further, an FSH devoid of all four N-linked carbohydrates on the α and β subunit is a potent antagonist of FSH action in vitro (Galway et al., 1990; Bishop et al., 1994; Keene et al., 1994).

Interestingly, forms of FSH that have oligosaccharides removed by either acid treatment or site-directed mutagenesis have usually been shown to exhibit an increased receptor binding affinity but a decreased ability to activate the FSH receptor. These observations are consistent with the hypothesis that binding and activation of the FSH receptor by the hormone are two separate and distinct events (Valove et al., 1994; Ji and Ji, 1995).

Deglycosylation of only the α subunit by hydrogen fluoride or removal of Asn-52 on the α subunit by site-directed mutagenesis results in an inhibition of activity but not binding. It has been suggested that these carbohydrate moieties on α project away from subunit binding site (Petaja-Repo et al., 1991; Lustbader et al., 1993).
FSH and its receptor

The loss of critical oligosaccharides, such as that on Asn-52 may alter the conformation of the gonadotrophin necessary for receptor activation while not affecting regions that are necessary for receptor binding. This conclusion is supported by the observation that deglycosylated HCG can be converted from an antagonist into an agonist by treatment with a specific monoclonal antibody. It is thought that antibody binding to the HCG antagonist somehow corrects the conformation to allow for both receptor binding and activation (Rebois and Fishman, 1984; Rebois and Liss, 1987).

Data generated in this field support the contention that binding and signal transduction are separate events and that the α subunit’s interaction with the receptor is critical for the latter. Removal of single residues on the α subunit may compromise the ability of the intact molecule to interact with the receptor.

**Binding of FSH to a G protein-coupled receptor**

The family of G protein-coupled receptors is composed of members involved in such diverse physiological functions as neurotransmission, vision and chemotaxis as well as endocrine signalling. Mutations of either the receptor or the ligand can lead to pathological conditions (Strader et al., 1994). The family of gonadotrophic hormones all interact selectively with specific receptors which comprise a subclass in the superfamily of G protein-coupled receptors. They all display seven transmembrane spanning segments (McFarland et al., 1989; Kelton et al., 1992; Segaloff and Ascoli, 1993).

These hormone receptors all stimulate adenyl cyclase to raise intracellular adenosine 3′,5′ cyclic monophosphate (cAMP) levels when activated which involves G proteins. Cloning of the genes for the receptors for LH (McFarland et al., 1989) and FSH (Sprengel et al., 1990; Kelton et al., 1992) show that each is a single polypeptide chain.

Like all other known families of G protein-coupled receptors, the glycoprotein hormone receptors have an extracellular domain and a cytoplasmic tail, in addition to the distinctive transmembrane domain. However, the glycoprotein hormone receptor family is distinguished from other G protein-coupled receptors such as rhodopsin, β-adrenergic receptors etc, by the large size (more than 300 amino acid residues) of its extracellular domain which also contains multiple cysteine residues and N-linked glycosylation sites (McFarland et al., 1989; Segaloff and Ascoli, 1993).

A comparison with the hLH and hTSH receptors reveal the conservation of nine cysteine residues in the extracellular domains and nine in the transmembrane/ cytoplasmic domain. This suggests a common pattern of protein folding with a C-terminus characteristic of G protein-coupled receptors, including seven transmembrane segments with sizeable intracellular portions and an N-terminal extracellular domain that belongs to the leucine-rich repeat superfamily (Braun et al., 1991; Kobe and Deisenhofer, 1994).

The overall sequence homology of the gonadotrophin receptors, as well as the G protein receptor superfamily, suggest a common mechanism of binding and
activation by the hormones. Binding of agonist has been considered essential for receptor activation and transmission of biological signal across plasma membranes. However, G protein-coupled activation of intracellular messengers occurring in the absence of an agonist has been reported (Dhenker et al., 1993; Lefkowitz, 1993; Parma et al., 1993; Tiberi and Caron, 1994; Yano et al., 1995; Gether et al., 1997). Indeed, experimental data demonstrates that these two events are separate and distinct (Valove et al., 1994; Ji and Ji, 1995).

Separation of ligand binding and receptor activation

It has been concluded, on the basis of deletion mutagenesis and chimeric constructions, that binding and activation processes are separable. There is strong evidence that the N-terminal portion of the FSH and LH receptor is responsible for ligand specificity and high affinity binding (Braun et al., 1991; Ji and Ji, 1991; Segaloff and Ascoli, 1993).

The FSH receptor has a region of the extracellular domain that is not homologous to LH receptor (R265-S296). Antipeptide antibodies made against this region inhibit binding of labelled FSH suggesting that this region is near or at the hormone receptor binding site (Liu et al., 1994). Variants of the LH receptor molecule lacking the transmembrane domain and the C-terminal regions have been found to bind LH with high specificity and affinity (Pajot-Augy et al., 1995). Binding specificity has also been found to be conferred by the extracellular domain in chimeric glycoprotein hormone receptors created by interchanging N-terminal portions that include the extracellular domain. We have shown that expression of the extracellular domain of FSH receptor by itself is sufficient for high affinity interactions with human FSH (unpublished observation). In particular, high affinity binding of the ligand has been mapped to regions encoded by exons 1–8 of the LH and FSH receptor (Braun et al., 1991; Ji and Ji, 1991; Pajot-Augy et al., 1995). Thus, we feel it is well established that the N-terminal, leucine rich region of the FSH receptor binds the gonadotrophin and positions it to interact with the transmembrane domain region of the receptor to induce receptor activation. We believe these two events to be separate and distinct. Activation may occur through a ligand induced conformational change of the receptor or through direct interaction of the ligand with one or more of the seven membrane spanning receptors.

Site-directed mutagenesis of receptor that induces or prevents hormone binding and/or activation

Some experiments of Nature cause a reduction in gonadotrophin receptor signalling. A dramatic reduction in binding capacity of the human FSH receptor was observed when the gene for the FSH receptor was analysed from a person with hypergonadotrophic ovarian dysgenesis (Aittomaki et al., 1995). A missense mutation was found in the region that encoded the extracellular ligand binding domain of the receptor. Transfection experiments showed that the
FSH and its receptor

mutation led to a dramatic reduction in binding and cAMP production after FSH stimulation. This mutation was found in exon 7 of the FSH receptor gene.

On the other hand, several reports show the presence of an activating mutation in the FSH receptor that allows for constitutive and ligand-independent signalling of the FSH receptor (Gromoll et al., 1996). In this and other studies, mutations within the transmembrane domain regions result in constitutive receptor signalling.

In reports that have studied the constitutive activation of TSH and LH receptor by mutation, it is clear that the third cytoplasmic loop between the fifth and sixth transmembrane domains can induce such an activity (Dhenker et al., 1993; Lefkowitz, 1993; Parma et al., 1993; Yano et al., 1995). This region appears normally to inhibit or prevent constitutive signalling as long as the wild-type sequence is maintained. Mutations in this area can prevent the inhibition of constitutive signalling, even in the absence of ligand. This area has been shown to be important for LH (Dhenker et al., 1993; Laue et al., 1995; Yano et al., 1995) and TSH receptors (Parma et al., 1993; Kopp et al., 1995) as well as α and β adrenergic receptors (O'Dowd et al., 1988; Kjelsberg et al., 1992).

Alterations in that sequence may induce a structural change in the receptor protein that elicits intracellular signalling. In other G protein-coupled receptors, the small ligands have been shown to interact directly with this portion of the receptor (Mirzadegan et al., 1992).

From the above studies, we have postulated that to activate the FSH receptor, we need to identify a small molecule that will interact with one or more of the transmembrane domain regions of the FSH receptor in a fashion analogous with that observed with the muscarinic, dopaminergic or rhodopsin receptors. We wish to induce a conformational change in one or more of the transmembrane loops specifically, to induce an activation signal.

FSH receptor activation

The mechanism of receptor activation by FSH is unknown. Some studies have suggested that by binding to the extracellular domain of the receptor, the hormone induces a conformational change in the structure of the receptor protein (Braun et al., 1991). Others believe that a portion of the hormone itself binds to a transmembrane domain region directly (Frasier et al., 1990), in a fashion similar to that seen with small ligands such as the rhodopsin or acetylcholine receptor (Strader et al., 1994). Several regions of the G protein-coupled receptor have been shown to control agonist-independent receptor activity but in some cases when mutated can actually confer oncogenic properties to the receptor (Lefkowitz, 1993).

How these conformational changes in the receptor protein induce signalling are unknown. It is assumed that that structural and molecular changes that occur somehow removes some of the stabilizing conformational constraints (Tiberi and Caron, 1994; Gether et al., 1997). We conclude from these observations that it should be possible to induce a conformational change in the receptor to induce signalling without the need for actual binding of FSH to the extracellular domain.
Figure 4. Schematic representation of the alternative models for the complex between a gonadotrophin and its receptor. (Left) side view of model a; (right) view of model a along the barrel axis of the receptor model. The α subunit of the gonadotrophin is shown in red, the β subunit is in blue, and the receptor is in yellow. Based on biochemical and mutational evidence, it is the lower portion in the side views, including loops L1α, L3α, L1β and the extended α chain C-terminus, that would contact the transmembrane portion of the receptor.

of the cognate receptor and are attempting to identify molecules that can interact directly with specific regions of the FSH transmembrane domain region, as has been observed with other G protein-coupled receptors (Mirzadegan et al., 1992). To this end, we have modelled the docking of the gonadotrophin to the extracellular domain of its receptor (see Figure 4). With this model (Jiang et al., 1995), we have been able to postulate that a portion of the α subunit directly interacts with one or more of the transmembrane domain regions of the receptor, to induce a physical change in the conformation of the receptor to elicit signal transduction (see below).

**Approaches to activate FSH receptor specifically and directly**

Based upon the above information, we set out to identify potential FSH agonistic molecules by three different approaches. Each approach will use the CHO–FSH receptor-luciferase described above to initially assess receptor activation. Activation will be compared with that obtained with the recombinant hFSH molecule. The specificity of that activation will be confirmed by the fact that the compound should fail to increase expression of the luciferase gene in CHO cells that have not been transfected with the FSH receptor gene.

The first approach will be to generate peptide libraries based upon the hormone receptor model described by Jiang et al. (1995). In this model, it is postulated that several of the loop structures in the α subunit of the FSH dimer are not involved in receptor binding and may play a role in the direct interaction with sequences found in the transmembrane domain regions of the FSH receptor, in areas thought to play a critical role in G protein-mediated increases in intracellular
FSH and its receptor

cAMP. Peptide libraries designed from this region will be synthesized and tested for activity with the CHO-FSHrec luciferase assay (Cwirla et al., 1990; Scott and Smith, 1990).

The second approach will be to test a large number of commercially available compounds for their ability to activate the FSH receptor. These compounds will be selected by their structural similarities to other small molecules that activate G protein-coupled receptors (Mirzadegan et al., 1997). This will be accomplished by adding each compound to an individual CHO cell culture that expresses the FSH receptor and contains the cAMP dependent luciferase expression cassette.

Another approach is to employ the technology that has been described for the thrombin receptor in which a tethered chimeric receptor is made based upon the model described by Chen et al. (1995) and Nanevicz et al. (1995). In this approach, the extracellular domain of the FSH receptor would be replaced by the thrombin receptor extracellular domain. However, the signal peptide portion of the extracellular domain of the thrombin receptor will be replaced with a random peptide library (Cwirla et al., 1990; Scott and Smith, 1990; Jayawickreme et al., 1994). A large number of transfectants will be generated, each with a random peptide sequence in it that may be able to activate the transmembrane domain of the FSH receptor in the same fashion as thrombin activates its transmembrane region. Positives can be identified by activation of luciferase and the peptide sequence can be obtained by recovery of the transfected receptor construct and polymerase chain reaction (PCR) sequencing of the activated receptor.

A final approach would be to begin with another G protein-coupled receptor with significant homology to the FSH receptor, such as vasopressin. One transmembrane region at a time is replaced with that of the FSH receptor. Using the vasopressin ligand as a template, a library of mutations is created looking for one that interacts with the vasopressin ΔTM1 receptor. Following that, the second TM region will be converted from vasopressin to FSH receptor and the mutant vasopressin with the highest activity in the TM1 receptor variant will be used as a template for the second. In this way, each of the TM regions is converted sequentially, and the ligand mutant that interacts best with the last is used as template for the next peptide library. Ultimately, the receptor will be converted to a complete FSH receptor transmembrane domain region and a mutant peptide originally derived from vasopressin, now with FSH receptor activating activity may be identified. This sort of approach has been employed in the past for other ligand receptor interactions (Balass et al., 1993; Hirschmann et al., 1996).

In each of these approaches, activation of the CHO cell will be detected through the expression of the luciferase gene. Specificity of response will be determined by the demonstration that a particular molecule activates luciferase in a CHO cell, only when the FSH receptor is present. After identification of a preliminary lead compound, structure-activity assessments will be initiated to increase the compound’s potency.

Any peptides that are found to exhibit agonistic activity will be converted to
S. Chappel et al.

peptoids. These synthetic monomers are substituted for amino acids and are thought to provide a more metabolically stable analogue than natural peptides (Simon et al., 1992).

We hope that through one or more of these techniques and the molecular tools that we have created to assess results, we will be well-positioned to identify lead candidates that can ultimately be converted into high potency therapeutic candidates to replace the dimeric glycoprotein hormones.

Conclusion

To achieve our goal of identifying a small molecular weight molecule that acts as a FSH agonist, we have built a number of tools. The first is the recombinant hFSH molecule from which to base biological properties. The second is the hFSH receptor expressed in a format that lends itself to a high throughput assay format. Finally, we need to develop a model of FSH receptor activation. We believe that due to the structural similarities between all G protein-coupled receptors and the evidence for gonadotrophin binding and receptor activation being distinct and separate events, it should be possible to identify a molecule that will interact with a catalytic region of the FSH receptor that controls G protein coupling and adenylate cyclase activation. This is thought to be a region of the transmembrane domain of the receptor. Efforts are now underway, with several of the approaches described above, to identify such a small molecule.

References


FSH and its receptor


S. Chappel et al.


