Induction of Promiscuous G Protein Coupling of the Follicle-Stimulating Hormone (FSH) Receptor: A Novel Mechanism for Transducing Pleiotropic Actions of FSH Isoforms

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Under physiological conditions, FSH is secreted into the circulation as a complex mixture of several isoforms that vary in the degree of glycosylation. Although it is well established that the glycosylation of FSH is important for the serum half-life of the hormone and coupling of the receptor to adenylate cyclase, little is known concerning how physiologically occurring glycosylation patterns of this hormone affect receptor signaling. In this study, we have examined the biological activity of deglycosylated human FSH (DeGly-phFSH), recombinant mammalian-expressed hFSH (CHO-hFSH), and insect cell-expressed hFSH (BV-hFSH, alternatively glycosylated) as compared with that of purified human pituitary FSH (phFSH) using a Chinese hamster ovarian cell line stably expressing the hFSH receptor (3D2 cells). Differentially glycosylated forms of FSH did not bind to the FSH receptor in the same manner as phFSH. Although all hormones showed similar potency in competing for [^{125}I]phFSH binding to the hFSH receptor, competition curves for deglycosylated and insect cell-produced FSH were steeper. Similarly, glycosylation of FSH had a profound effect on bioactivity of the hormone. Purified hFSH produced a sigmoidal dose-dependent stimulation in cAMP production, whereas DeGly-phFSH and BV-hFSH induced biphasic (bell-shaped) dose-response curves. BV-hFSH also elicited biphasic effects on steroidogenesis in primary cultures of rat granulosa cells. The cellular response to BV-hFSH was dependent on the degree of receptor-transducer activation. BV-hFSH bioactivity was strictly inhibitory when combined with the ED_{80} of phFSH. Lower concentrations of phFSH resulted in a gradual shift from inhibition to a biphasic activity in the presence of the ED_{20} of phFSH. Biphasic responses to BV-hFSH were attributed to activation of different G protein subtypes, since treatment of 3D2 cells with cholera toxin or pertussis toxin differentially blocked the two phases of BV-hFSH bioactivity. These data suggest that alternative glycosylation of FSH leads to a functionally altered form of the hormone. Functionally different hormones appear to convey distinct signals that are transduced by the receptor-transduction system as either stimulatory or inhibitory intracellular events *via* promiscuous, glycosylation-dependent G protein coupling. Promiscuity in signaling of the FSH receptor, in turn, may represent a potentially novel mechanism for FSH action, whereby the gonad may respond in diverse ways to complex hormonal signals such as those presented by circulating FSH isoforms. (Molecular Endocrinology 11: 517–526, 1997)

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INTRODUCTION

FSH, LH, human CG (hCG), and thyroid-stimulating hormone make up the heterodimeric glycoprotein hormone family (1). These proteins contain a common α-subunit that is linked in a noncovalent manner with a hormone-specific β-subunit. Despite their similar structures and sequence homologies, there is only marginal cross-reactivity among the receptors (Ref. 2 and B. J. Arey, D. C. Deecher, E. S. Shen, and F. J. López, unpublished observations). The presence of multiple glycosylation sites on both subunits is one of the common structural features of these hormones. FSH, for example, contains four such glycosylation sites, two each on the α- and β-subunits (3). The action of FSH in the gonad involves an initial binding event with a specific FSH receptor. The human (h) FSH receptor (hFSH-R) is a large, integral membrane protein comprised of multiple hydrophobic regions con-

sistent with the seven transmembrane-spanning domains identified in G protein-coupled receptors (4). Indeed, the FSH receptor has been shown to be positively coupled to G_s, resulting in activation of adenylate cyclase and cAMP production (5, 6).

In vivo, FSH is secreted into and maintained in serum as a series of isoforms of differing isoelectric points (pI) (for review see Refs. 7 and 8). These charge differences have been attributed to the presence of differently glycosylated forms of the hormone (9, 10). It is clear that the relative abundance of FSH isoforms in the circulation is dependent on the physiological status of the subject (11–14). For example, in the rat, a shift in the pituitary content of FSH isoforms is observed on proestrus from those of lower pI to those of higher pI (12, 13). Because forms of FSH possessing greater pI values have a decreased sialic acid content, this represents a shift in circulating FSH from those species having a more complex glycosylated structure (more acidic) and lesser bioactivity to those of higher pI and greater bioactivity (15, 16). These observations suggest that feedback from the ovary signals an increase in bioactive FSH before ovulation. Therefore, the effect of a given stimulus on the gonad depends not only upon the amount of circulating gonadotropins but perhaps, more importantly, on the qualitative aspects of the stimulus such as the relative distribution of diverse hormone isoforms under various physiological conditions.

Several studies have provided evidence to indicate that FSH isoforms exhibit different properties, which, in turn, modify their biological activity. The data available suggest that hormone glycosylation is important for both serum half-life (17–19) and signal transduction (7, 20–23). Indeed, it has been shown that chemically deglycosylated FSH acts as an antagonist at the FSH-R (24, 25). Because FSH binding to its receptor involves multiple interactions between the proteins, one possible explanation for these observations is that deglycosylation of the hormone decreases its intrinsic activity, producing an isoform that retains affinity for the receptor. In this respect, isoforms of FSH are known to contain differing degrees of glycosylation and also a range of receptor-binding characteristics (for review see Ref. 8). Therefore, the deglycosylated hormone would act as a competitive blocker of the native hormone. Under this hypothesis then, the trophic signal to the gonad consists of a mix of agonists and antagonists with varying affinities and intrinsic activities that would determine gonadal response. Alternatively, it is plausible that certain FSH isoforms may bind to the FSH-R in a unique manner that induces ligand-specific conformational changes to the ligand-receptor complex. Distinct ligand-receptor complex conformations, in turn, could provide the molecular foundation for either activation or deactivation of alternative signaling pathways. These, therefore, may serve as a means to provide pleiotropic responses to complex signals consisting of multiple or varying glycosylated forms of FSH. In this study, we have evaluated whether different glycosylated forms of hFSH are capable of activating multiple different signal transduction pathways. For this purpose, dose-effect relationships for differently glycosylated forms of FSH were evaluated, not only in recombinant cell lines expressing the hFSH-R, but also in some physiologically relevant *in vitro* models of hFSH action.

RESULTS

The role of alternatively glycosylated forms of FSH in FSH-R signaling was evaluated by studying the biological activity of various preparations of FSH. For this purpose, Chinese hamster ovarian (CHO) cells stably expressing the hFSH-R (3D2 cells) were incubated in the presence of varying concentrations of either purified human FSH (phFSH), chemically deglycosylated (hydrogen fluoride-treated) phFSH (DeGly-phFSH), recombinant hFSH produced using a baculovirus expression system (BV-hFSH), or recombinant hFSH expressed in CHO cells (CHO-hFSH). Purified hFSH, DeGly-phFSH, BV-hFSH, and CHO-hFSH were assayed by hFSH immunoradiometric assay (IRMA) simultaneously, and the concentrations of stock solutions normalized to the detected levels of immunoreactive α/β hFSH. Purified hFSH induced a sigmoidal dose-dependent increase in cAMP accumulation that reached maximal levels more than 300-fold greater than control (Fig. 1, *upper left* panel), consistent with the coupling of the FSH-R to G_s (5, 6). The estimated ED_{50} for phFSH in 3D2 cells was 9.55 ng/ml. Similarly, DeGly-phFSH induced a dose-dependent elevation in cAMP accumulation apparent at doses ranging from 1–10,000 ng/ml (ED_{50} = 9.61 ng/ml; Fig. 1, *upper right* panel). However, unlike the native hormone, DeGly-phFSH produced a biphasic (bell-shaped) dose-response curve. Concentrations of DeGly-phFSH greater than 100 ng/ml were less efficacious in bioactivity when compared with that elicited by this dose (Fig. 1, *upper right* panel). Maximal stimulation of cAMP accumulation was approximately 20-fold greater than control. Thus, DeGly-phFSH is approximately 10-fold less efficacious than the native hormone. The IC_{50} for DeGly-phFSH, as estimated using a seven-parameter logistic model, was 7.62 μg/ml.

Similar to DeGly-phFSH, a differently glycosylated [under-glycosylated (26)] form of hFSH expressed in Hi5 insect cells (BV-hFSH) also produced a biphasic stimulation of cAMP accumulation in 3D2 cells (Fig. 1, *lower left* panel). Maximal stimulation of cAMP accumulation by BV-hFSH was approximately 200-fold greater than control at a dose of 100 ng/ml. Higher doses of BV-hFSH were less bioactive, leading to a bell-shaped dose-response curve. The ED_{50} for the ascending phase of the BV-hFSH bioactivity was 1.37 ng/ml, whereas the ID_{50} for the descending phase of the BV-hFSH curve was 532.59 ng/ml. Moreover, in a

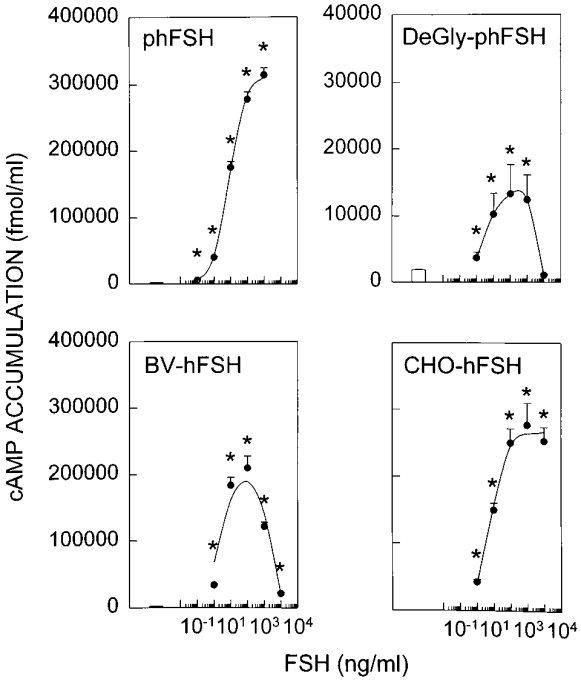

Fig. 1. Different Glycosylation Patterns of hFSH Induce cAMP Accumulation in a Biphasic Manner

Purified human FSH (phFSH, *top left*, ED_{50}= 9.55 ng/ml). Chemically deglycosylated phFSH (DeGly-phFSH, *top right*, ED_{50} = 9.61 ng/ml and ID_{50}= 7.62 μg/ml). Insect cell expressed hFSH (BV-hFSH, *bottom left*, ED_{50} = 1.37 ng/ml and ID_{50} = 0.53 μg/ml). Mammalian cell expressed hFSH (CHO-hFSH, *bottom right*, ED_{50} = 7.29 ng/ml). *Open bars* denote cAMP accumulation in the control group. *, $P < 0.05$ *vs*. control by ANOVA followed by the Dunnet's test on log-transformed data .

cell-free adenylate cyclase assay using isolated 3D2 cell membranes, phFSH induced a sigmoidal dose-dependent stimulation in adenylate cyclase activity (Table 1). In contrast, increasing concentrations of BV-hFSH elicited a biphasic effect on adenylate cyclase activity with distinct ascending and descending phases to the dose-response relationship (Table 1). Supernatants of Hi5 cells that were infected with baculovirus without the coding region of hFSH did not increase cAMP accumulation as compared with control in the whole-cell assay (data not shown). Furthermore, purification of BV-hFSH from Hi5 cell supernatants by ion exchange and subsequent size exclusion chromatography revealed that a single peak was responsible for the biphasic dose-responses induced by crude Hi5 cell supernatants (data not shown). In contrast, FSH expressed in a mammalian cell line (CHO cells) induced a sigmoidal dose-dependent increase in cAMP accumulation that was nearly indistinguishable from that observed for phFSH (ED_{50} = 7.29 ng/ml; Fig. 1, *lower right* panel). Maximal induction of cAMP production by CHO-hFSH was approximately 300-fold greater than that observed in control wells.

To evaluate whether altered affinities of the various FSH preparations could account for the diverse biological activities, the ability of the ligands to bind to the hFSH receptor was studied by radioligand-binding assay using 3D2 cell membranes. Purified hFSH, DeGly-phFSH, and BV-hFSH dose-dependently competed for [^{125}I]hFSH binding to 3D2 cell membrane FSH-R (Fig. 2). Purified hFSH competed for [^{125}I]hFSH binding with an ID_{50} = 869 $\pm$ 7 pM. Deglycosylated and BV-hFSH were slightly more potent with an estimated ID_{50} of 395 $\pm$ 5 pM and 207 $\pm$ 7.8 pM, respectively. The slopes of competition curves for DeGly-phFSH and BV-hFSH, however, were not parallel to that of phFSH.

Table 1. Insect Cell-Expressed Human FSH (BV-hFSH) Activates Adenylate Cyclase in a Biphasic Manner, Whereas Purified Human FSH (phFSH) Induces a Sigmoidal Dose-Dependent Elevation of Enzyme Activity in 3D2 Cell Membrane Preparations

Treatment	phFSH-Induced Adenylate Cyclase Activity[a]	BV-hFSH-Induced Adenylate Cyclase Activity[a]
Control	17.97 $\pm$ 0.27	15.80 $\pm$ 1.37
0.001 μg/ml	18.75 $\pm$ 1.41	16.23 $\pm$ 0.03
0.10 μg/ml	35.68 $\pm$ 1.78[b]	37.87 $\pm$ 0.70[b]
10.0 μg/ml	91.85 $\pm$ 5.97[b]	13.29 $\pm$ 1.54

[a] Adenylate cyclase activity was determined following a 10-min incubation of 3D2 cell membranes with varying concentrations of the indicated hormones and expressed as picomoles of cAMP produced per mg protein per min. The determinations were performed in four replicate wells for each concentration. Data presented are representative of three independent experiments.

[b] Activity is statistically different from the respective control group (ANOVA followed by the Dunnet's test on log-transformed data).

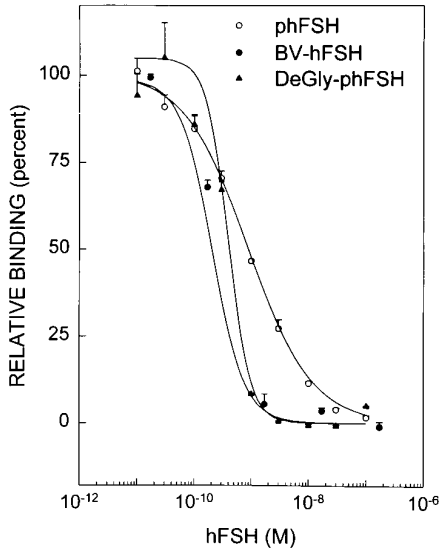

Fig. 2. Binding Properties of Diverse FSH Preparations to the FSH-R Are Different

Binding of [^{125}I]phFSH in the presence of phFSH (*open circles*), BV-hFSH *(solid circles*), or DeGly-phFSH *(solid triangles*).

Deglycosylated-phFSH and BV-hFSH competed with [^{125}I]hFSH for the hFSH-R with slope factors of 2.25 and 1.56, respectively. Purified hFSH competed for binding to the receptor with a slope factor of 0.77. Thus, these data suggest that differently glycosylated forms of FSH may bind to the receptor in a hormone-specific fashion and, perhaps, elicit diverse conformations to the ligand-receptor complex.

The possibility that the biphasic bioactivities of under-glycosylated FSH may be restricted to the genetically engineered cell line (3D2) used for these studies was ruled out by evaluating the biological activity of phFSH and BV-hFSH in an aromatase bioassay. In this more physiologically relevant system, incubation of rat granulosa cells with phFSH resulted in a dose-dependent increase in estradiol secretion with a 3-fold elevation as compared with cells cultured in the presence of medium alone (ED_{50} = 0.30 ng/ml, Fig. 3, *top* panel). In contrast, treatment of granulosa cells for 72 h with BV-hFSH resulted in a bell-shaped dose-response relationship (Fig. 3, *bottom* panel) similar to that observed in 3D2 cells. BV-hFSH stimulated estradiol secretion above control at all but the lowest dose tested (Fig. 3, *bottom* panel). Maximal stimulation of estradiol secretion was apparent at a dose of 10 ng/ml with an ED_{50} for the ascending portion of the BV-hFSH dose-response curve of 0.97 ng/ml. Concentrations of BV-hFSH greater than 10 ng/ml were less efficacious such that the highest dose of BV-hFSH induced only a 1.4-fold increase in estradiol secretion over control levels. The estimated ID_{50} for the descending phase of the BV-hFSH dose-response curve was 295.44 ng/ml. A similar dichotomy between phFSH and BV-hFSH in terms of the qualitative dose-response profile on progesterone secretion was evident in an adrenal cell line (Y1) stably expressing the hFSH-R (data not shown).

To study whether the ascending and descending bioactivities of BV-hFSH could be separated by altering the degree of receptor-transducer system activation, 3D2 cells were incubated with the ED_{20}, ED_{50}, or ED_{80} of phFSH (representing low, medium, or high receptor-transducer activation, respectively). In the presence of the ED_{20} of phFSH, BV-hFSH induced a bell-shaped dose-response curve (Fig. 4, *top* panel). However, when the ED_{50} of phFSH was incubated in the presence of the same doses of BV-hFSH, an intriguing difference in biological activity was observed (Fig. 4, *middle* panel). The recombinant hormone had

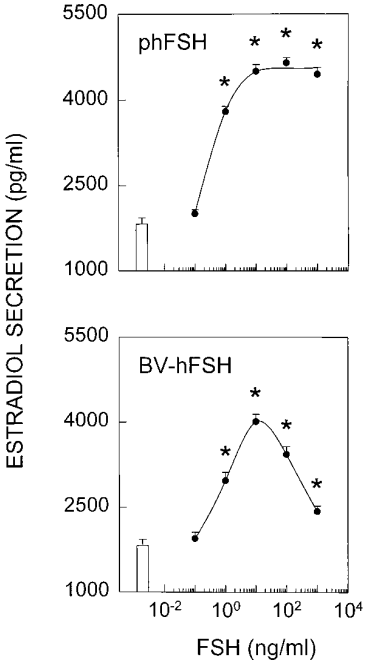

Fig. 3. BV-hFSH in Primary Cultures of Rat Ovarian Granulosa Cells Induces Estradiol Secretion in a Biphasic Manner

Top, Granulosa cells incubated in the presence of phFSH for 72 h. *Bottom,* Granulosa cells incubated in the presence of BV-hFSH for 72 h. *Open bars* denote estradiol release by the respective control group. *, $P < 0.05$ *vs*. respective control group by ANOVA followed by the Dunnet's test.

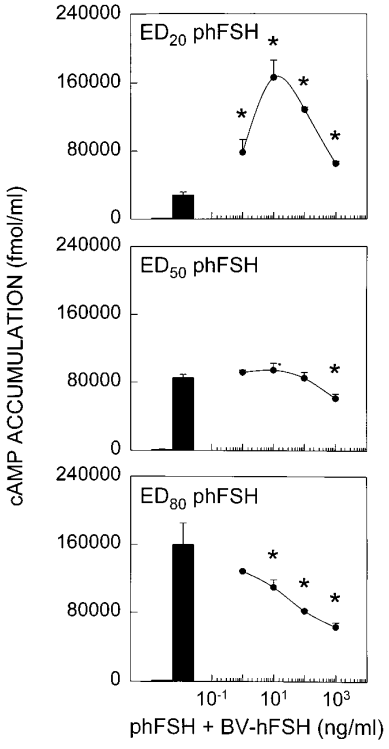

Fig. 4. The Biphasic Activity of BV-hFSH Is Dependent on the Degree of Receptor-Transducer Activation

Top, 3D2 cells treated with the ED_{20} of phFSH (representing low receptor-transducer activation) in the presence of varying concentrations of BV-hFSH (1–1000 ng/ml). *Middle*, 3D2 cells treated with the ED_{50} of phFSH (representing medium receptor-transducer activation) in the presence of varying concentrations of BV-hFSH. *Bottom*, 3D2 cells treated with the ED_{80} of phFSH (representing high receptor-transducer activation) in the presence of varying concentrations of BV-hFSH. *Open bars* denote cAMP accumulation of cells treated with medium, whereas *solid bars* indicate cells treated with the respective concentration of phFSH alone. *, $P < 0.05$ *vs*. phFSH alone by ANOVA followed by the Dunnet's test on log-transformed data.

no effect on cAMP accumulation except at the highest dose tested, where it inhibited cAMP accumulation induced by the ED_{50} of phFSH by 40%. In contrast, when the ED_{80} of phFSH (representing high receptor-transducer activation) was incubated in the presence of varying concentrations of BV-hFSH, the recombinant hormone was strictly inhibitory, with a maximal inhibition of phFSH-induced cAMP accumulation of 60% at a dose of 1.0 μg/ml (Fig. 4, *bottom* panel). Thus, when the receptor is only partially activated (low concentrations of phFSH) BV-hFSH displays agonistic activity, and accumulation of cAMP is observed, which is consistent with the coupling of the FSH-R to G_s. However, when the receptor is more fully activated (higher concentrations of phFSH), BV-hFSH becomes antagonistic, suggesting that at high receptor-transducer activation this hormone is either capable of uncoupling the receptor from the stimulatory signaling pathway and/or coupling it to an inhibitory cascade.

Taken together, these data suggest that glycosylated isoforms of FSH confer particular conformations to the receptor that may involve subsequent postreceptor mechanisms to produce the observed biphasic responses to De-Gly- and BV-hFSH. Therefore, the role of activation of hFSH-R/G protein-signaling pathways in transducing the signal of alternatively glycosylated forms of FSH was studied. 3D2 cells were challenged with FSH in the presence or absence of either cholera toxin (CTX) or pertussis toxin (PTX), which selectively abolish either G_s- or G_i/G_o-mediated signal transduction mechanisms, respectively. Pretreatment of 3D2 cells with CTX completely blocked the ascending phase of the BV-hFSH dose-response curve when performed in the presence of the ED_{20} of phFSH (Fig. 5, *top* panel, *solid symbols*). Low concentrations of BV-hFSH (1–10 ng/ml) had no effect on cAMP accumulation as compared with cells treated with the ED_{20} alone. However, higher concentrations of BV-hFSH led to a decrease in basal cAMP accumulation (ID_{50} = 2.02 μg/ml). Cells not pretreated with CTX responded to BV-hFSH in a biphasic manner in the presence of the ED_{20} of phFSH (Fig. 5, *top* panel, *open symbols*). Thus, CTX pretreatment had no effect on the descending phase of the dose-response curve, suggesting that this portion of the bioactivity of BV-hFSH is independent of G_s-mediated signaling.

Pretreatment of 3D2 cells with PTX completely blocked the inhibitory effect of BV-hFSH in the presence of the ED_{80} of phFSH (Fig. 5, *bottom* panel, *solid symbols*). In cells not treated with PTX, BV-hFSH was inhibitory to cAMP accumulation induced by the ED_{80} of phFSH (Fig. 5, *bottom* panel, *open symbols*). As a confirmation of these observations, we also tested the effect of PTX on BV-hFSH and phFSH bioactivity in the absence of additional phFSH. As observed in Fig. 6, in the absence of PTX (*solid symbols*), phFSH (*top left* panel) produced a sigmoidal dose-dependent increase in cAMP accumulation, while BV-hFSH (*top right* panel) induced its typical biphasic dose-response relationship. However, PTX pretreatment completely

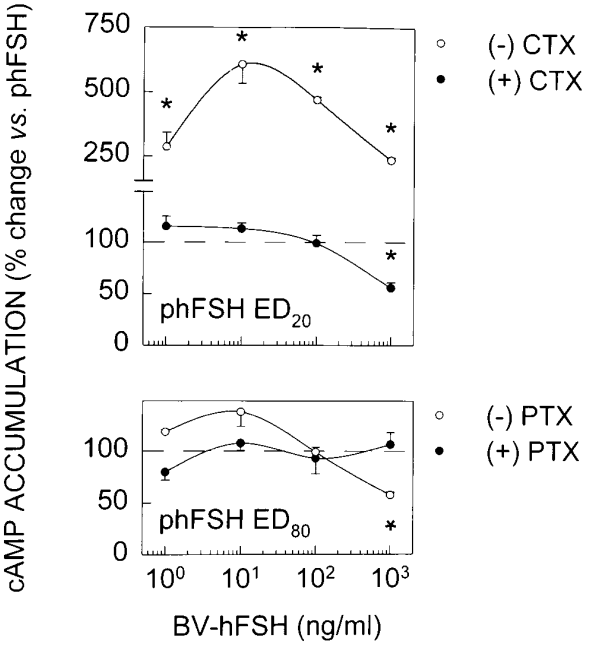

Fig. 5. CTX and PTX Differentially Abolish the Ascending or Descending Components of BV-hFSH Bioactivity

Top, 3D2 cells pretreated 24 h with (*solid symbols*) or without (*open symbols*) 2 μg/ml CTX before challenge with the ED_{20} of phFSH in the presence of varying concentrations of BV-hFSH (1–1,000 ng/ml). *Bottom*, 3D2 cells pretreated 24 h with (*solid symbols*) or without (*open symbols*) 5 μg/ml PTX before challenge with the ED_{80} of phFSH in the presence of varying concentrations of BV-hFSH. The *dashed line* denotes the mean of cells treated with the ED_{20} and ED_{80} of phFSH alone with or without toxins for the *top* and *bottom* panels, respectively. *, $P < 0.05$ *vs*. phFSH alone by ANOVA followed by the Dunnet's test on log-transformed data.

abolished the descending phase of BV-hFSH bioactivity. In the presence of PTX (Fig. 6, *open symbols*), BV-hFSH (*bottom right* panel) induced a sigmoidal dose-dependent increase in cAMP accumulation that was nearly identical to that of phFSH (*bottom left* panel). Interestingly, PTX treatment decreased the efficacy of both phFSH and BV-hFSH, as well as forskolin (data not shown), to increase cAMP levels approximately 6-fold when compared with control cells. Taken together, these data suggest that the biphasic activity of BV-hFSH is mediated through recruitment of differing G proteins.

DISCUSSION

During recent years it has become apparent that the actions of FSH on the gonad represent a multifaceted phenomenon. Rather than a classic ligand-receptor interaction in which a single ligand docks onto a specific receptor, the FSH-R is exposed to a multitude of FSH isoforms with varying characteristics that are present in different proportions depending on the

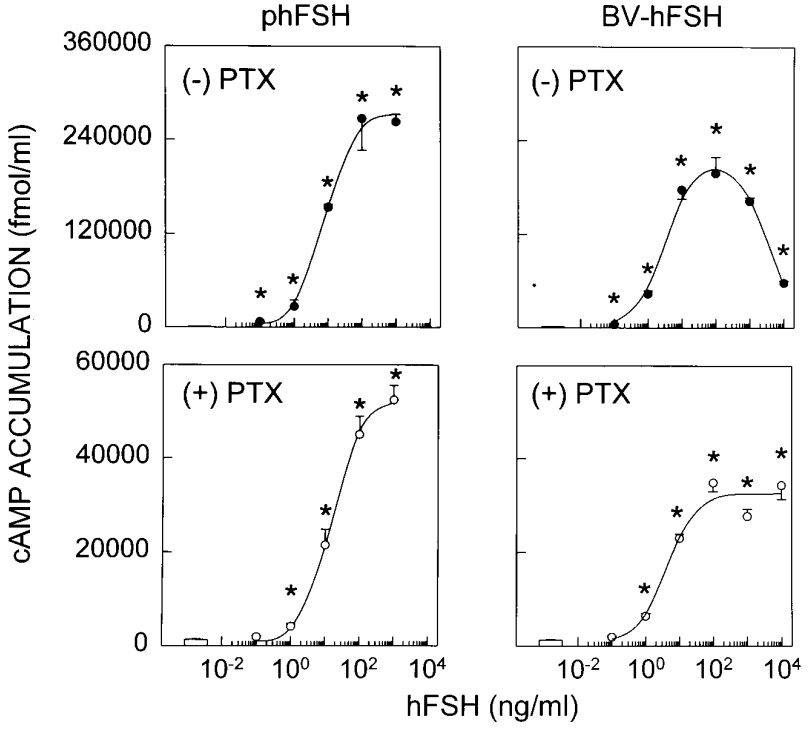

Fig. 6. PTX Abolishes the Descending Phase of BV-hFSH Bioactivity

Top left, 3D2 cells pretreated in the absence of PTX before challenge with varying concentrations of phFSH (0.1–1,000 ng/ml). *Top right*, 3D2 cells pretreated in the absence of PTX before challenge with varying concentrations of BV-hFSH (0.1–10,000 ng/ml). *Bottom left*, 3D2 cells pretreated 24 h with 5 μg/ml PTX before challenge with varying concentrations of phFSH. *Bottom right*, 3D2 cells pretreated 24 h with 5 μg/ml PTX before challenge with varying concentrations of BV-hFSH. *, $P < 0.05$ *vs*. the control group by ANOVA followed by the Dunnet's test on log-transformed data.

physiological status (8). Therefore, integration of complex signals represents a challenge to the target organ in general and to the receptor in particular. Such a richness of signals complicates our understanding of how these inputs are transduced. Many studies, in partial explanation of the relative roles of different FSH isoforms, have implicated differences in binding characteristics and serum half-life as the major properties of diverse FSH isoforms (8). However, studies aimed at evaluating whether the signal transduction system has sufficient versatility to respond to diverse FSH signals are scarce (20, 21). In our current studies, we have evaluated the role of glycosylation in activation of downstream signaling components by the FSH-R. Our data demonstrate a glycosylation-dependent coupling of the ligand-receptor complex with multiple signaling pathways, which provides evidence for the existence of a versatile coupling of the FSH-R in conveying FSH signals to the gonad.

Many investigators have shown the importance of secondary protein processing for bioactivity in several homeostatic systems (7, 24, 25). Glycosylation patterns found in FSH and the FSH-R are likely to play a significant role in ligand binding and possibly G protein coupling. Under physiological conditions, FSH is found in serum of many species as a myriad of different isoforms with differing pI values (7). The differences in pI have been shown to be primarily due to secretion of alternatively glycosylated forms of the hormone (10, 27). Therefore, the observed biological effects of FSH could be the result of a highly complex interaction between many different factors. For example, FSH bioactivity could depend upon the number of receptors present on the cell surface, the concentration of FSH in the serum, as well as the ratio of the isoforms of the hormone secreted. However, these factors would transduce signals solely as positive inputs to the target organ, since the FSH-R is thought to be coupled to a stimulatory G protein pathway (5, 6). An alternative mechanism to provide plasticity to the responsiveness of the target organ could conceivably involve coupling of the FSH-R with inhibitory transduction pathways (*i.e*. G proteins different than G_s). If such a mechanism operates within the FSH-R-transducer system, then the ability of the FSH-R to couple to different G proteins provides a means to respond in a pleiotropic manner to a complex ligand, a mix of multiple isoforms of FSH.

In our studies, we have taken advantage of chemically deglycosylated hFSH and the altered secondary processing of newly synthesized proteins in insect cells (26) to produce alternatively glycosylated forms of hFSH. Studies of the expression of other proteins have shown that insect cells have the ability to produce small truncated sugar side chains in place of the more complex oligosaccharide structures produced by

cells of higher organisms (26). Using this paradigm, we demonstrate that such alterations in glycosylation of FSH can have profound effects on its biological activity. Whereas phFSH and mammalian cell-expressed hFSH (CHO-hFSH) induced sigmoidal stimulation in cAMP accumulation in 3D2 and granulosa cells, DeGly-phFSH and BV-hFSH induced bell-shaped dose-response curves. Furthermore, the differences in bioactivity between phFSH and alternatively glycosylated FSH is evident at the membrane level, since adenylate cyclase activity in a cell-free paradigm revealed similar differences in bioactivity between phFSH and BV-hFSH. This may be due to an altered receptor-ligand conformation, since we have also shown that deglycosylation of the hormone had a notable effect on receptor binding. Both BV-hFSH and DeGly-phFSH competed for the hFSH-R with similar slope factors to each other, but different from that of phFSH, as has been described by others (28). It has been proposed that isoforms of hFSH or hLH could act in both a competitive or noncompetitive manner with native hormone for interaction with the receptor. In fact, chemically deglycosylated hFSH can antagonize the effects of native FSH (24, 25). Similarly, deglycosylated hCG acts as a noncompetitive antagonist (with equimolar affinity) to native LH, suggesting that these two ligands do not share the same binding site(s) (29). We have observed that, depending on the degree of receptor-transducer system activation, the underglycosylated BV-hFSH could behave either in a strictly inhibitory or stimulatory/inhibitory manner simultaneously. Thus, our data have extended earlier observations to clearly demonstrate that alternatively glycosylated hFSH is not a true antagonist of the hFSH-R, but actually an analog with partial agonistic activity capable of inducing the FSH-R to activate other signaling pathways than those already established for this receptor.

We hypothesized that the mechanism for the dual bioactivity of these hormones is related to the ability of the ligands to stabilize certain receptor conformations that permit interaction with multiple G proteins. This was evaluated by the use of two ADP-ribosylating toxins, PTX and CTX. These two toxins block either G_s- or G_i/G_o-activated signaling pathways, respectively. Treatment of 3D2 cells with CTX specifically blocked the ascending phase of BV-hFSH bioactivity, but did not abolish the descending phase. In contrast, PTX completely and selectively blocked the descending phase of BV-hFSH bioactivity. The two toxins, therefore, differentially affected BV-hFSH-induced responses in 3D2 cells. Interestingly, PTX treatment also altered the efficacy of both phFSH- and BV-hFSH-induced cAMP accumulation. These observations imply that a PTX-sensitive mechanism participates in maintenance of a fully responsive transduction system. The reason(s) for these findings is not readily apparent; however, two possible mechanisms could be invoked. First, it is possible that either multiple PTX-sensitive G proteins (*e.g.* G_o) or another PTX-sensitive pathway confers full responsiveness to the system. Alternatively, PTX-dependent chronic activation of adenylate cyclase (due to the removal of tonic G_i-dependent inhibitory inputs) could lead to desensitization of the enzyme. The latter hypothesis is supported by the observation that PTX treatment reduced forskolin responsiveness in terms of cAMP production (data not shown); however, this observation does not completely refute the first mechanism. Experiments are currently underway in our laboratory to address these possibilities in an attempt to discern whether other signaling pathways are involved in maintaining the gain of the FSH/FSH-R transduction system.

Our data provide strong evidence that the FSH-R is capable of activating alternate signaling cascades other than those activated through G_s. Moreover, the ability of the FSH-R to associate with alternative signaling molecules was dependent upon the degree of receptor-transducer system activation. At low levels of activation, BV-hFSH was capable of inducing both an ascending (stimulatory) or descending (reduced activity) bioactivity profile. At a midrange of receptor-transducer activation, addition of BV-hFSH did not increase cAMP accumulation over levels observed with phFSH alone. Furthermore, an inhibitory component was only identifiable at high concentrations of BV-hFSH. Similarly, at high receptor-transducer activation, BV-hFSH was inhibitory at all doses tested. Because in these studies cells were exposed to a mix of differently glycosylated forms of FSH, these conditions could resemble what occurs *in vivo*, *i.e.* circulating FSH isoforms with different glycosylation patterns. Taken together with the fact that biphasic responses were also evident in primary granulosa cells, these data suggest that this phenomenon may occur under physiological conditions. It is apparent from our data that high ratios of fully to incompletely glycosylated hormone result in positive intracellular signals, whereas lower ratios convey inhibition. Overall, it is tempting to speculate that, while the FSH receptor has an affinity for other G proteins, it has a higher affinity for G_s. Therefore, our data would define the FSH-R as a preferentially G_s-coupled receptor, but with capacity to associate with other (G_i/G_o) proteins as well. This so-called promiscuity in receptor-signal transducer coupling is well documented for catecholamine and adenosine receptors (30–32). However, unlike the catecholamine and adenosine receptors in which different ligands induce promiscuity, the ability of the FSH-R to couple to multiple G protein signaling pathways appears to be dependent on different physiological statuses (glycosylation) of the same ligand (*i.e.* the FSH molecule). Promiscuity of the FSH-R for coupling would be a physiologically relevant event by empowering the signal transduction system to respond in a positive or negative fashion, depending on the prevailing gonadotropic stimulus interacting with the system.

In conclusion, these data provide evidence that the FSH-R is capable of coupling with more than one G protein subtype and that its association with other subtypes is dependent on the glycosylation pattern of the ligand bound and the degree of receptor-trans-

ducer activation. Perhaps, more importantly, our data provide some basis for a physiological role of alternatively glycosylated isoforms of circulating FSH. That is, depending on the prevailing physiological status of the subject, the ovary may be presented with and respond to differing pleiotropic signals from the pituitary that are sensed by the FSH-R and perceived as activation of alternative signaling pathways.

MATERIALS AND METHODS

Isolation and Expression of hFSH cDNA in Hi5 Insect Cells

The cDNAs for FSHα and β were isolated from human pituitary poly-A$^+$ RNA (Clontech, Palo Alto, CA) by RT-PCR using gene-specific primers: FSHα1, 5′CGCGGATCCGCCATGGATTACTACAGAAAATATGC3′; FSHα2, 5′CGCAAGCTTAGCAGTCATCAAGACAGCAC3′; FSHβ1, 5′CGCGGATCCCAGGATGAAGACACTCCAG3′; FSHβ2, 5′CGCAAGCT TCAGGACAAGGGTATGTGGC3′. The cDNA fragments were cloned into pCRII (Invitrogen, San Diego, CA) and sequenced to verify identity and fidelity of the cloned fragments. The fragments included restriction digestion sites (*Bam*HI-hFSH-*Hin*dIII) for use in subcloning into the corresponding sites of the baculovirus transfer vector, pBluBacIII (Invitrogen). The resulting transfer vectors containing hFSHα and hFSHβ were cotransfected into Sf9 insect cells with linearized AcNPV (Baculogold, Pharmingen, San Diego, CA) to generate recombinant virus. The latter was purified by successive rounds of plaque purification. A high titer viral stock was generated by two rounds of infection with the purified viral stock. For expression experiments, the high titer viral stock was used to infect Hi5 cells (Invitrogen), cultured in serum-free medium using a multiplicity of infection of 5 for each recombinant virus. The culture supernatant was collected at 96 h postinfection and analyzed for production of immunoreactive hFSHα/β dimer by a hFSH IRMA and by Western blot using antibodies specific for FSHα or FSHβ. The expression level of intact hFSHα/β dimer ranged from 1 to 5 mg/liter.

Deglycosylation of phFSH

Deglycosylated phFSH was obtained from Dr. P. M. Sluss (Massachusetts General Hospital, Charlestown, MA). Purified hFSH (2 mg, Cortex Biochem, San Leandro, CA) was deglycosylated by a 60-min exposure to hydrogen fluoride gas at room temperature. After exposure to hydrogen fluoride, the deglycosylated (all but the N-linked sugar) hormone was separated from all other carbohydrates by gel filtration chromatography. The mass of the purified material was determined by amino acid analysis and found to be consistent with acid hydrolysis of phFSH. The deglycosylated phFSH had an apparent molecular mass of 29 kDa, with no larger forms of the protein detectable by SDS-PAGE analysis. Dubois assay of phFSH revealed no detectable carbohydrate beyond the N-linked sugar of a 20-μg aliquot. The remaining material was lyophilized and reconstituted in 0.1 M acetic acid before use in bioactivity studies.

Primary Culture of Granulosa Cells and Aromatase Bioassay

All procedures using animals were approved by the Radnor Animal Care and Use Committee.Twenty one-day-old immature female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed under controlled light (12-h light, 12-h dark) and temperature (25 C) conditions. Food and water were available *ad libitum*. Animals were treated by single daily injections of 100 μg/kg diethylstilbestrol (DES) in olive oil for 3 days. On the fourth day, animals were euthanized by rapid CO_2 asphyxiation, and the ovaries were removed. Ovaries were washed three times in 50 ml of sterile HEPES-buffered saline (pH 7.4). Granulosa cells were harvested by incubating ovaries in a serum-free hypertonic medium consisting of McCoy's 5A medium (GIBCO Life Sciences, Grand Island, NY) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS, Sigma Chemical Co, St Louis, MO), 146 μg/ml L-glutamine, 100 nM testosterone, 100 nM DES, and 100 U/ml penicillin/10 mg/ml streptomycin/250 ng/ml amphotericin B (antibiotic/antimycotic, GIBCO) containing 0.5 M sucrose (Sigma) and 0.1 mM EGTA (Sigma). Ovaries were then incubated for 45 min at 37 C in a humidified incubator gassed with 95% air/5% CO_2. Subsequently, ovaries were washed three times with 10 ml isotonic medium (hypertonic medium without sucrose and EGTA) and incubated for an additional 45 min in isotonic medium at 37 C. Granulosa cells were harvested by puncture of swollen follicles using a 23 gauge needle. Isolated granulosa cells were placed in a 50-ml centrifuge tube and washed two times by the addition of 50 ml serum-free McCoy's 5A medium followed by centrifugation at 700 $\times$ *g* for 5 min. The final cell pellet was resuspended by gentle trituration in 25 ml serum-free isotonic medium. Cell number was determined using a hemocytometer, and viability was estimated by trypan blue exclusion. Cells were plated into 24-well Nunc (Naperville, IL) tissue culture plates at 100,000 viable cells per well.

The aromatase bioassay was performed according to the method of Hsueh *et al*. (33). Briefly, cells were challenged with test substances in isotonic McCoy's 5A medium supplemented with 0.1% BSA (Fraction V, Sigma), ITS, testosterone, DES, glutamine, and antibiotic/antimycotic mix in a total incubation volume of 500 μl. The cells were incubated for 72 h at 37 C with the test substances. At the end of the challenge period, the medium was assayed for estradiol concentration by RIA.

CHO Cell Line and cAMP Accumulation Assay

A CHO cell line expressing the hFSH-R was used to study effects of FSH on receptor activation (kindly provided by Dr. Kerry Koller, Affymax Inc., Palo Alto, CA). CHO cells were stably transfected with the cDNA for the hFSH-R, which was cloned by RT-PCR from human ovarian RNA. One clone (3D2) was found to express the hFSH-R and to respond to phFSH with a dose-dependent stimulation in cAMP production. 3D2 cells were maintained at 37 C in 1:1 DMEM/F12 medium supplemented with 10% FBS (GIBCO), 146 μg/ml L-glutamine, and 100 U/ml penicillin/10 μg/ml streptomycin. The cells were plated 1 day before each experiment into 24-well or 96-well Nunc tissue culture plates at 200,000 or 30,000 cells per well, respectively.

FSH activation of the FSH-R was studied by monitoring cAMP accumulation. Cells were washed twice with Optimem (GIBCO)/0.1% BSA. After the second wash, cells were preincubated in either 500 μl (24-well format) or 100 μl (96-well format) Optimem/0.1% BSA for 30 min at 37 C. The medium was removed from the wells, and the cells were challenged for 30 min at 37 C in Optimem/0.1% BSA containing test substances in a total incubation volume of 250 μl or 50 μl for the 24-well and 96-well formats, respectively. Experiments were terminated by the addition of an equal volume of 0.2 N HCl, and cAMP accumulation was measured by RIA.

Adenylate Cyclase Activity Assay

Adenylate cyclase assays were performed on isolated 3D2 cell membranes. 3D2 cells were grown to 90% confluency on 15-cm Nunc tissue culture dishes in growth medium. The medium was removed and cells were scraped from the plate into

30 ml FSH-binding buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA, and 0.025% sodium azide, pH 7.2), and the cells were homogenized. The homogenate was centrifuged at 15,000 × *g* for 10 min, and the pellet was resuspended in binding buffer and centrifuged again. The supernatant was discarded and the pellet resuspended to 100–150 μg/ml protein in binding buffer. At the start of the assay, 3D2 membranes were pelleted as above and resuspended in a volume of membrane buffer (50 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, and 2 mM EGTA) to give 2.5 mg membrane protein/ml. Assays were performed in 96-well plates (Nunc). The following additions were made to each well in order: 20 μl 0.25% BSA (Sigma), 20 μl of each concentration of hormone in 0.25% BSA, 20 μl of a solution containing 2,500 U/ml phosphocreatine kinase (Sigma), 50 mM creatine phosphate (Sigma) and 0.5% BSA, 40 μl of buffer containing 450 mM Tris-HCl, pH 7.4, 40 mM $MgCl_2$ and 5 mM isobutylmethylxanthine (Sigma), 10 μl 2 mM GTP (Sigma), and 50 μl 4 mM ATP (Sigma). Each hormone concentration was assayed in quadruplicate. The plates were incubated for 10 min at 37 C. After the incubation, the content of each well was rapidly transferred to a 1.5-ml centrifuge tube and spun at 12,000 × *g* for 5 min at room temperature. The supernatants were placed into fresh tubes and stored at −20 C until assayed for cAMP by RIA.

Radioligand-Binding Assay

Binding assays were performed using the same 3D2 cell membrane preparations used in the adenylate cyclase assays. To perform the binding assay, 100 μl/well (100 μg membrane protein) of the 3D2 membrane homogenate were added to a 96-well microtiter plate followed by the addition of 50 μl of either binding buffer (total binding), phFSH, DeGly-phFSH, or BV-hFSH at varying concentrations. Nonspecific binding was determined in the presence of 1 μM phFSH. Reactions were initiated by the addition of 50 μl [^{125}I]phFSH (50 pM; 55,000 cpm, 3500–4500 Ci/mmol; NEN, Boston, MA) in binding buffer, for a final reaction volume of 200 μl. Plates were incubated on an orbital shaker for 2 h at 25 C.

The binding assay was terminated by harvesting the cell membranes using a 96-well vacuum harvester (Skatron Instruments, Inc, Sterling, VA) onto presoaked (30 min in 50 mM Tris/1% BSA, pH 7.2) Skatron Blue mat 11740 glass fiber filters. Harvesting was completed by washing unbound radioactivity from the mats with five cycles of 3.5 ml of 50 mM Tris-HCl (4 C). Filters were individually punched out and the bound radioligand was determined by counting single disks for 1 min in a γ-counter (ICN Biomedical, Costa Mesa, CA).

RIAs

Estradiol levels in medium samples from the aromatase bioassay were measured using a commercially available Coat-a-Count kit with modifications (Diagnostic Products Corp., Los Angeles, CA). Medium samples were preincubated in the presence of assay buffer (100 μl total volume) in antibody-coated tubes for 1 h at 37 C and after the addition of [^{125}I]estradiol (1 ml), tubes were incubated for 2 h at room temperature. The assay was terminated by draining the tubes, and bound radioactivity was counted in an ICN γ-counter for 1 min. This assay has a sensitivity of 0.25 pg/tube. Intra- and interassay variability is 4.3% and 6.8%, respectively.

Cyclic AMP accumulation in the 3D2 cells was measured using a commercially available double-antibody RIA kit with some modifications (Amersham, Arlington Heights, IL). Medium samples were incubated in the presence of tracer and primary antibodies for 1 h at room temperature. Secondary antibodies were added, and the tubes were incubated for 10 min at room temperature and centrifuged at 1000 × *g* for 15 min. The supernatant was drained and the pellets counted in an ICN γ-counter for 1 min. This assay has a sensitivity of 2 fmol/tube. The intra- and interassay variation for this assay is approximately 6.7% and 10.8%, respectively.

For the adenylate cyclase activity assay, an acetylation step was performed on samples and standards before assay for cAMP as above (Amersham). Using this protocol, the assay did not detect ATP at the concentrations used in the adenylate cyclase activity reactions. This assay has a sensitivity of 0.25 fmol/tube. The intra- and interassay variation for the acetylated protocol is approximately 4.8 and 6.6%, respectively.

To normalize for FSH concentration of phFSH, CHO-hFSH, and DeGly-phFSH, as well as crude and purified Hi5 cell supernatants, hormones were assayed using a hFSH IRMA. This assay was performed using commercially available reagents (Diagnostic Products Corp.). Aliquots of the hormones were serially diluted in assay buffer and assayed as 5-μl aliquots in 95 μl assay buffer in tubes coated with primary hFSH antibodies. One milliliter of ^{125}I-labeled secondary hFSH antibodies was added to the tubes, and they were incubated on an orbital shaker at room temperature for 1 h. Thereafter, the tube content was discarded, and the tubes were washed twice by the addition of 500 μl assay wash buffer per wash and then counted in an ICN γ-counter for 1 min. This assay has a sensitivity of 0.019 mIU/tube. The intra- and interassay variation is approximately 2.4% and 4.0%, respectively.

Statistical Analysis

Statistical analyses were performed for bioassay data using the SigmaStat software package (Jandel Scientific, San Raphael, CA). Differences between treatment groups were analyzed by ANOVA. Differences *vs*. the control group were analyzed after a significant ANOVA by the Dunnet's test. In some instances, data were found to be skewed from normality or to have heterogeneous variance. In such cases, log transformation of the data was performed. Differences between treatment groups were considered significant if $P < 0.05$.

Sigmoidal dose-response curves were fitted and ED_{50}s determined mathematically using a four-parameter logistic equation and the SigmaPlot software (Jandel Scientific). Bell-shaped dose-response curves were fitted and ED_{50}s and ID_{50}s determined mathematically using a seven-parameter logistic equation as described by Rovati *et al.* (34) with some modifications: $\{((a - d_1)/(1 + (x/c_1)^{b1}) + d_1) - ((a - d_2)/(1 + (x/c_2)^{b2}) + d_2)\}$; where a = asymptotic maximum, b_1 = ascending slope factor, $c_1 = ED_{50}$ of ascending portion of the curve, d_1 = asymptotic minimum for the ascending portion of the curve, b_2 = slope factor of the descending portion of the curve, $c_2 = ID_{50}$ of descending portion of the curve, and d_2 = asymptotic minimum for the descending portion of the curve.

Data from radioligand binding studies were analyzed using the JMP software package (SAS Inc, Cary, NC). Square-root transformation of the data was performed in conjunction with a Huber weighting procedure. A four-parameter logistic equation was used to fit competition curves and calculate ID_{50}s from the transformed, weighted data.

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REFERENCES

1. Boothby M, Ruddon RW, Anderson C, McWilliams D, Boime I 1981 A single gonadotropin alpha-subunit gene in normal tissue and tumor-derived cell lines. J Biol Chem 256:5121–5127
2. Kammerman S, Canfield RE, Kolena J, Channing CP 1972 The binding of iodinated hCG to porcine granulosa cells. Endocrinology 91:65–74
3. Sairam MR 1981 Primary structure of the ovine pituitary follitropin alpha-subunit. Biochem J 197:535–539
4. Reichert Jr LE 1994 The functional relationship between FSH and its receptor as studied by synthetic peptide strategies. Mol Cell Endocrinol 100:21–27
5. Rao AJ, Ramachandran J 1975 Cyclic AMP production in isolated rat seminiferous tubule cell preparations: a potential *in vitro* assay for follicle stimulating hormone. Life Sci 17:411–416
6. Dufau ML, Catt KJ 1978 Gonadotropin receptors and regulation of steroidogenesis in the ovary. Vitam Horm 36:462–585
7. Dahl K, Stone MP 1992 FSH isoforms, radioimmunoassays, bioassays and their significance. J Androl 13:11–22
8. Ulloa-Aguirre A, Midgley AR, Beitins IZ, Padmanabhan V 1995 Follicle stimulating hormone isohormones-characterization and physiological relevance. Endocr Rev 16:765–787
9. Reichert Jr LE 1971 Electrophoretic properties of pituitary gonadotropins as studied by electrofocusing. Endocrinology 88:1029–1044
10. Chappell SC, Coutifaris C, Jacobs SJ 1982 Studies on the microheterogeneity of follicle-stimulating hormone present within the anterior pituitary gland of ovariectomized hamsters. Endocrinology 110:847–854
11. Chappel SC, Bethea CL, Spies HG 1984 Existence of multiple forms of follicle-stimulating hormone within anterior pituitaries of cyngomolus monkeys. J Med Primatol 14:177–194
12. Ulloa-Aguirre A, Espinoza R, Damian-Matsumura P, Larrea F, Flores A, Morales L, Dominguez R 1988 Studies on the microheterogeneity of anterior pituitary follicle-stimulating hormone in the female rat: isoelectricfocusing throughout the estrous cycle. Biol Reprod 38:70–78
13. Ulloa-Aguirre A, Damian-Matsumura P, Espinoza R, Dominguez R, Morales L, Flores A 1990 Effects of neonatal androgenization on the chromatofocusing pattern of anterior pituitary FSH in the female rat. J Endocrinol 126:323–332
14. Galle PC, Ulloa-Aguirre A, Chappell SC 1983 Effects of oestradiol, phenobarbitone and luteinizing hormone releasing hormone upon the isoelectric profile of pituitary follicle-stimulating hormone in ovariectomized hamsters. J Endocrinol 99:31–39
15. Chappel SC, Ulloa-Aguirre A, Ramaley J 1983 Sexual maturation in female rats: time-related changes in the isoelectric focusing pattern of anterior pituitary follicle-stimulating hormone. Biol Reprod 28:196–205
16. Ulloa-Aguirre A, Mejia JJ, Dominguez R, Guevara-Aguirre J, Diaz-Sanchez V, Larrea F 1986 Microheterogeneity of anterior pituitary FSH in the male rat: isoelectric focusing pattern throughout sexual maturation. J Endocrinol 110:539–549
17. Morell AG, Gregoriadis G, Scheinberg IH 1971 The role of sialic acid in determining the survival of glycoproteins in the circulation. J Biol Chem 246:1461–1467
18. Blum WFP, Gupta D 1985 Heterogeneity of rat FSH by chromatofocusing: studies on serum FSH, hormone release *in vitro* and metabolic clearance rates of its various forms. J Endocrinol 105:29–37
19. Wide L 1986 The regulation of metabolic clearance rate of human FSH in mice by variation of the molecular structure of the hormone. Acta Endocrinol (Copenh) 112:336–344
20. Sairam MR, Bhargavi GN 1985 A role of glycosylation of the α subunit in transduction of biological signal in glycoprotein hormones. Science 229:65–67
21. Padmanabhan V, Sairam MR, Hassing JM, Brown MB, Ridings JW, Beitins IZ 1991 Follicle-stimulating hormone signal transduction: role of carbohydrate in aromatase induction in immature rat sertoli cells. Mol Cell Endocrinol 79: 112–119
22. Stanton PG, Robertson DM, Burgon PG, Schmauk-White B, Hearn MTW 1992 Isolation and physicochemical characterization of human follicle-stimulating hormone isoforms. Endocrinology 130:2820–2832
23. Burgon P, Robertson D, Stanton PG, Hearn M 1993 Immunological activities of highly purified isoforms of human FSH correlate with *in vitro* bioactivities. J Endocrinol 139:511–518
24. Sairam MR, Manjunath P 1982 Studies on pituitary follitropin. XII. Enhanced thermal stability induced by chemical deglycosylation. Mol Cell Endocrinol 28:151–159
25. Sairam MR, Manjunath P 1982 Studies on pituitary follitropin. XI. Induction of hormonal antagonistic activity by chemical deglycosylation. Mol Cell Endocrinol 28:139–150
26. Kuroda K, Geyer H, Geyer R, Doefler W, Klenk H-D 1990 The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. Virology 174:418–429
27. Chappel SC, Ulloa-Aguirre A, Coutifaris C 1983 Biosynthesis and secretion of follicle-stimulating hormone. Endocr Rev 4:179–211
28. Manjunath P, Sairam MR, Sairam J 1982 Studies on pituitary follitropin. X. Biochemical, receptor binding and immunological properties of deglycosylated ovine hormone. Mol Cell Endocrinol 28:125–138
29. Dunkel L, Jia X-C, Nishimori K, Boime I, Hsueh AJW 1993 Deglycosylated human chorionic gonadotropin (hCG) antagonizes hCG stimulation of 3′,5′-cyclic adenosine monophosphate accumulation through a non-competitive interaction with recombinant human luteinizing hormone receptors. Endocrinology 132:763–769
30. Munshi R, Linden J 1989 Co-purification of A1 adenosine receptors and guanine-nucleotide binding proteins from bovine brain. J Biol Chem 264:14853–14859
31. Munshi R, Pang I-H, Sternweis PC, Linden J 1991 A_1 adenosine receptors of bovine brain couple to guanine nucleotide-binding proteins G_{i1}, G_{i2}, and G_o. J Biol Chem 266:22285–22289
32. Kimura K, White BH, Sidhu A 1995 Coupling of human D-1 dopamine receptors to different guanine nucleotide binding proteins: evidence that D-1 dopamine receptors can couple to both Gs and Go. J Biol Chem 270:14672–14678
33. Hsueh AJ, Bicsak TA, Jia X-C, Dahl KD, Fauser BCJM, Galway AB, Czwkala N, Pavlou SN, Pakoff H, Keene J, Boime I 1989 Granulosa cells as hormone targets: The role of biologically active follicle-stimulating hormone in reproduction. Recent Prog Horm Res 45:209–277
34. Rovati GE, Nicosia S 1994 Lower efficacy: interaction with an inhibitory receptor or partial agonism? Trends Pharmacol Sci 15:140–144