Signaling and Phosphorylation-impaired Mutants of the Rat Follitropin Receptor Reveal an Activation- and Phosphorylation-independent but Arrestin-dependent Pathway for Internalization*

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We have previously shown that the rat follitropin receptor (rFSHR) expressed in transfected cells becomes phosphorylated upon stimulation of the cells with agonist or a phorbol ester. Peptide mapping and mutagenesis studies have also shown that the agonist- or phorbol ester-induced phosphorylation of the rFSHR maps to Ser/Thr residues present in the first and third intracellular loops.

The experiments presented herein were initially designed to test for the presence of additional phosphorylation sites on the second intracellular loop of the rFSHR. Analysis of two new mutants in which the two threonines in the second intracellular loop (rFSHR-2L) or the two threonines in the second intracellular loop and the seven Ser/Thr residues in the third intracellular loop (rFSHR-2L + 3L) were mutated showed that one or more of the two threonines in the second intracellular loop are phosphorylated in response to phorbol ester, but not in response to agonist stimulation.

Since rFSHR-2L and rFSHR-2L + 3L displayed a reduction in agonist-induced signaling, two additional mutants (rFSHR-D389N and rFSHR-Y530F) were constructed in an attempt to better understand the relationship between the agonist-induced activation, phosphorylation, and internalization of the rFSHR. These point mutations impaired agonist-stimulated signal transduction and abolished agonist-induced phosphorylation. Co-transfection studies revealed that the phosphorylation of these mutants can be rescued by overexpression of G protein-coupled receptor kinase 2, but this increased phosphorylation only rescues the internalization of rFSHR-D389N. The internalization of both mutants could be rescued by overexpression of arrestin-3, however. Taken together, these results argue that the agonist-induced activation and phosphorylation of the rFSHR are not essential for internalization, while the interaction of the rFSHR with a nonvisual arrestin is essential for internalization.

Agonist binding to G protein-coupled receptors (GPCRs) ultimately results in the phosphorylation of the receptor on Ser/Thr residues. Agonist-induced GPCR phosphorylation can be catalyzed by second messenger-dependent kinases as well as by a family of G protein-coupled receptor kinases (GRKs) (reviewed in Refs. 1 and 2). The phosphorylation of GPCRs catalyzed by GRKs facilitates the interaction of the receptors with a family of inhibitory proteins called arrestins. The complex formed by the phosphorylated receptor and arrestin promotes the uncoupling of the receptors from their cognate G proteins and targets the activated receptor to clathrin-coated pits for subsequent internalization (1–4). Thus, the complex formed by the phosphorylated GPCR and arrestin serves as a common molecular intermediate for the uncoupling of the receptor from its cognate G protein and for receptor internalization.

Previous results from this laboratory have shown that the rat FSH receptor (rFSHR) expressed in transfected cells becomes phosphorylated upon agonist activation or upon pharmacological activation of protein kinase C with PMA (5–7). When engaged by hFSH, the rFSHR expressed in transfected cells activates the cAMP and the inositol phosphate/diacylglycerol signaling pathways (5, 6). Although the cAMP signaling pathway is thought to be the only pathway activated in cells that express the endogenous FSHR (8) and it is the most sensitive to hFSH stimulation in transfected cells (5, 6), it does not appear to be involved in the phosphorylation of the rFSHR. Thus, the hFSH-induced phosphorylation of the rFSHR proceeds normally in a transfected cell line that does not respond with an increase in cAMP accumulation (because of overexpression of cAMP phosphodiesterase), and the pharmacological activation of A kinase with cAMP analogs does not result in the phosphorylation of the rFSHR (5). In contrast, the pharmacological activation of protein kinase C with PMA enhances the phosphorylation of the rFSHR (see above), and the “down-regulation” of protein kinase C by chronic treatment of transfected cells with PMA impairs (but does not abolish) the hFSH-induced phosphorylation (5). Thus, it appears that the agonist-induced phosphorylation of the rFSHR is mediated by protein kinase C and one or more other kinases. These other kinases include members of the GRK family.

The rFSHR belongs to a small subfamily of GPCRs that are phosphorylated in the intracellular loops instead of the C-terminal tail. Like the α2A-adrenergic (9) and the m2 muscarinic (10, 11) receptors, the agonist-induced phosphorylation of
the rFSHR occurs in the third intracellular loop (6, 7). The rFSHR is unusual, however, in that agonist activation also promotes the phosphorylation of sites present in the first intracellular loop (7). Functional analysis of several phosphorylation-deficient mutants of the rFSHR have shown that, like other GPCRs, the phosphorylated rFSHR serves as a common molecular intermediate in FSH-induced uncoupling and FSH-induced internalization (6, 7). The phosphorylation sites that mediate these two responses are distinct, however. Thus, the phosphorylation of the third intracellular loop facilitates FSH-induced uncoupling but is not necessary for FSH-induced internalization. The phosphorylation sites that mediate FSH-induced internalization are not as well defined, but they are located either in the first intracellular loop only or in the first and third intracellular loops.

The phosphorylation of the rFSHR induced by the pharmacological activation of protein kinase C also maps to Ser/Thr residues located in the first and third intracellular loops (7) and, like the hFSH-induced phosphorylation, the PMA-induced phosphorylation of the rFSHR uncouples the receptor from Gs (stimulatory G protein) (5, 6). There are several important differences in the functional effects of the PMA- and agonist-induced uncoupling, however. Agonist-induced uncoupling is detected mostly as a reduction in the efficacy of hFSH without a change in potency, while the PMA-induced uncoupling is detected mostly as a reduction in potency of hFSH rather than efficacy (5, 6). Moreover, the agonist-induced uncoupling is impaired in rFSHR-1L or rFSHR-3L (mutants where the Ser/Thr residues present in the first or third intracellular loops were substituted with nonphosphorylatable residues), while the PMA-induced uncoupling is not (7).

The experiments presented herein were initially designed to test for the potential presence of additional phosphorylation sites on the second intracellular loop of the rFSHR that may be phosphorylated in response to agonist or PMA stimulation. In the process of doing these experiments, we found that mutation of the two threonine residues present in the second intracellular loop of the rFSHR impaired hFSH-mediated signal transduction. Additional studies were thus conducted to test for the involvement of the activation of the FSH receptor on FSH-induced receptor phosphorylation and FSH-induced internalization.

MATERIALS AND METHODS

Plasmids and Cells—The cloning of the rFSHR cDNA containing the full-length coding region plus portions of the 5′- and 3′-untranslated regions of the wild-type rFSHR cDNA have been previously described (12). This cDNA was subcloned into the eukaryotic expression vector pcDNA/Neo (Invitrogen) and used as a template for mutagenesis and for transfactions. The rFSHR mutants used here were constructed by PCR with overlap extension (13). Rat FSHR-2L was constructed by mutating the nucleotide sequence within the second intracellular loop of the rFSHR from 1405 ACC ATACA 1413 to 1405 GCC ATCG TA 1413, thus changing the amino acid sequence within the second loop from T451 I T453 to T451 A T453. Rat FSHR-2L + 3L was constructed by introducing this same change in the context of rFSHR(3L), a previously described rFSHR mutant in which all Ser/Thr residues in the third intracellular loop were simultaneously mutated to Ala (7). Rat FSHR-D389N and Y530F were constructed by mutating codons 389 and 530 from GAT to CAA and from TAT to TTT, respectively. The sequence of the entire region of each mutant cDNA generated by PCR was verified by automated DNA sequencing.

A full-length bovine GRK2 (14) was subcloned into pcDNA1.1/Amp for expression studies. The expression vectors (all in pcDNA3.1) encoding for arrestin-3 and β-arrestin-(319–41B), a dominant-negative mutant of β-arrestin, have been described (4). Transient co-transfections of human embryonic kidney (293) cells were done using calcium phosphate as described by Chen and Okayama (15). Cells plated in 100-mm dishes were transfected when 70–80% confluent using 10–20 µg of each plasmid (the total amount of plasmid transfected was kept constant by including the appropriate amounts of empty expression vector). After an overnight incubation, the cells were washed and incubated in growth medium for 1–2 h at 37°C. The cells were then maintained in the same dishes for an additional 24 h or trypsinized, distributed into 35 mm wells (5–10 × 10^4 cells/well), and used 24 h later. Stably transfected cell lines were obtained following G418 selection and cloning as described elsewhere (5–7). Hormone Binding and cAMP Accumulation—Binding parameters for hFSH were measured during a 1-h incubation of intact cells (plated in 35-mm wells) with increasing concentrations of 125I-hFSH at 37°C. All binding assays were corrected for nonspecific binding, which was measured in the presence of 100 nM pregnant mare serum gonadotropin. The binding of hFSH to intact cells at 37°C is clearly not reversible and the bound hormone is internalized. Since the irreversible nature of the binding reaction precludes the measurement of equilibrium binding parameters (i.e. binding affinity and maximal binding capacity), we simply fitted the binding data to a sigmoidal equation (16) using the DeltaGraph software (Delta Point, Monterey, CA) and used this equation to calculate the maximal amount of cell-associated hormone and the concentration of hFSH required to attain half of this value (EC50). Measurements of 125I-FSH binding in transiently transfected cells were done during a 1-h incubation of intact cells (plated in 35-mm wells) with a single concentration (100 ng/ml) of 125I-hFSH at room temperature.

Concentration-response curves for the hFSH-induced increases in cAMP accumulation were obtained by measuring total cAMP levels in cells that had been incubated with five different concentrations of hFSH for 15 min at 37°C in the presence of a phosphodiesterase inhibitor. The different parameters that describe the concentration response curves were calculated as described elsewhere (6).

Intact Cell Phosphorylation Assays—Clonal lines expressing the rFSHR (or mutants thereof) or transiently transfected cells (all in 100-mm dishes) were metabolically labeled with 200–300 µCi/ml of ^32P for 3 h at 37°C. The methods used to lyse the cells, to immunoprecipitate the rFSHR, and to resolve the immunoprecipitates on SDS gels have been described (5, 6, 17). Receptor phosphorylation was ascertained after incubating the ^32P-prelabeled cells at 37°C with buffer only for 15–60 min, 100 ng/ml hFSH for 60 min, or 200 nM PMA for 15 min (6). When using clonal cell lines the amount of wild-type and mutant receptor used for the immunoprecipitation experiments was equalized based on the binding data shown on Table I. When using transiently transfected cells, the amount of wild-type and mutant receptor used for the immunoprecipitation experiments was equalized based on one-point binding assays as described above.

Autoradiograms of the dried gels were obtained using Kodak BioMax MR. Autoradiograms were scanned and quantitated using a Bio-Rad Molecular Imaging System. All images were captured in a digital format for presentation.

Internalization Assays—Stable or transiently transfected cells, plated in 35-mm wells, were preincubated in 1 ml of Waymouth’s MB752/1 containing 1 mg/ml bovine serum albumin and 20 mM Heps, pH 7.4, for 60 min at 37°C. Each well then received 40 ng/ml of ^125I-hFSH, and incubation was continued at 37°C. Groups of cells were placed on ice at 3-min intervals and washed twice with 2-ml aliquots of cold Hanks’ balanced salt solution containing 1 mg/ml bovine serum albumin. The surface-bound hormone was then released by incubating the cells in 1 ml of cold 50 mM glycine, 100 mM NaCl, pH 3, for 2–4 min (18, 19). The acidic buffer was removed, and the cells were washed once more with another aliquot of the same buffer. The acid buffer washes were combined and counted, and the cells were solubilized with 100 µl of 0.5 N NaOH, collected with a cotton swab, and counted to determine the amount of internalized hormone. Six different data points collected at 3-min intervals were used in each experiment, and the rate constant for internalization (k0) was calculated from the slope of the line obtained by plotting the internalized radioactivity against the integral of the surface-bound radioactivity (20). The half-life of internalization (t1/2) is defined as 0.693/k0.

Single point internalization assays were done using an incubation time of 9 min. For these assays, the data are expressed as percentage of internalization (i.e. the internalized radioactivity divided by the sum of the internalized plus surface-bound radioactivity).

Immunochemicals—Expression of the transfected arrestins was ascertained by using immunochemicals (17) using the Enhanced Chemiluminescence (ECL) system of detection. Arrestin 3 was detected with a mouse monoclonal antibody (F4C1) directed against an epitope common to all known arrestins (21). Rat FSHR-D389N and -Y530F were constructed by mutating codons 389 and 530 from GAT to CAA and from TAT to TTT, respectively. The sequence of the entire region of each mutant cDNA generated by PCR was verified by automated DNA sequencing.
Thr453 were mutated to Ala and Val, respectively. This mutant that was comparable with or higher than that detected in cells ble I). These two mutant receptors bound hFSH with an affinity clonal line expressing an equivalent density of rFSHR-wt (Ta-}

Mutation of Two Threonines in the Second Intracellular Loop of the rFSHR Impairs Signal Transduction as Well as Agonist- and PMA-stimulated Receptor Phosphorylation—Peptide mapping and mutagenesis experiments have previously shown that the FSH- and PMA-induced phosphorylation of the rFSHR occurs on serine and threonine residues present in the first and third intracellular loop (5–7). Since the second intracellular loop contains two threonine residues (at positions 451 and 453) that have not been formally excluded as potential phosphorylation sites, we prepared and analyzed two new rFSHR mutants. In one mutant (designated rFSHR-2L) Thr451 was mutated to Ala and Thr453 to Val. In the other mutant (designated rFSHR-2L + 3L), all Ser/Thr residues present in the third loop were mutated to Ala, and Thr451 and Thr453 were mutated to Ala and Val, respectively. This mutant was constructed and analyzed because we have previously shown that mutation of the Ser/Thr residues present in the third loop reduces FSH- and PMA-induced phosphorylation by 40–60% (7).

Clonal lines of transfected 293 cells stably expressing rF-}

Results

| Cell line              | hFSH Binding | cAMP responsiveness |
|------------------------|--------------|---------------------|
|                        | EC50 pmol/10^6 cells | Bmax molecules/cell |
| 293F(wt-103)           | 293 ± 100    | 44,000 ± 5000       |
| 293F(2L)               | 520 ± 450    | 60,000 ± 6300       |
| 293F(2L + 3L)          | 590 ± 200    | 32,000 ± 2600       |
| 293F(D389N)            | 1150 ± 170   | 53,000 ± 2000       |
| 293F(Y530F)            | 1360 ± 270   | 58,000 ± 5000       |

has been described (17). Purified hFSH (AFP-5720D) was kindly pro-vided by the National Hormone and Pituitary Agency of the NIDDK (National Institutes of Health). Labeled FSH was prepared as described previously (23). Pregnant mare serum gonadotropin was obtained from the National Hormone and Pituitary Agency of the NIDDK (National Institutes of Health) or purchased from Sigma. [35S]Orthophosphate was obtained from NEN Life Science Products. Phosphate-free Dulbecco’s modified Eagle’s medium was purchased from ICN Biomedicals (Irvine, CA). Nonidet P-40, protease inhibitors, N,N,N-triacetylcysteinate, protein A-agarose, and bovine serum albu-min were from Sigma. Okadaic acid and cypermethrin were purchased from Alexis Biochemicals (Woburn, MA). Wheat germ agglutinin-agarose was from Vector Laboratories. Cell culture supplies and reagents were obtained from Corning (Corning, NY) and Life Technologies, Inc., respectively. The enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech, and the horseradish peroxidase-labeled secondary antibodies were from Bio-Rad. Human embryonic kidney cells (293) were obtained from the American Type Culture Collection (CRL-1573). All other materials were obtained from commonly used suppliers.

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A binding parameters were determined during a 1-h incubation (37 °C) of intact cells with increasing concentrations of [125I]hFSH as described under "Materials and Methods." The maximal hFSH-induced cAMP response of cells expressing rFSHR-wt, rFSHR-2L, or rFSHR-2L + 3L was calculated by dividing the maximal hFSH response by the maximal cAMP response. Each number represents the average ± S.E. of 3–11 independent experiments.

The results presented in Figs. 1 and 2 show that cells expressing rFSHR-wt respond to hFSH with an average 8-fold increase in the [32P] content of the rFSHR. This response is reduced to 60% in cells expressing rFSHR-2L. Since we have previously shown that the agonist-induced phosphorylation of rFSHR-3L is reduced to ~60% (7), we expected the response of rFSHR-2L + 3L to be reduced by 80–90%. As shown in Figs. 1 and 2, however, the agonist-induced phosphorylation of this mutant was reduced only to 45%. This lack of additivity implies that Thr451 and Thr453 (the two residues mutated in rFSHR-2L) are not phosphorylated in response to agonist stimulation and that there are other reasons for the reduction in agonist-induced phosphorylation observed in rFSHR-2L. The inactivating nature of the 2L mutation is one potential reason for this decrease that is explored below.

In agreement with previous results from this laboratory (5–7), the data presented in Figs. 1 and 2 document that the pharmacological activation of protein kinase C results in the phosphorylation of rFSHR-wt. These data also show that the PMA-induced phosphorylation of rFSHR-2L and rFSHR-2L + 3L is reduced to ~60% of rFSHR-wt, respectively. Since the 3L mutation alone has been previously shown to reduce PMA-induced phosphorylation to ~60% (7), the additivity of the results obtained with the 2L + 3L mutation suggests that both the second and third intracellular loops become phosphorylated in response to PMA stimulation. Likewise, mutation of the phosphorylation sites present in the first intracellular loop also reduces PMA-induced phosphorylation to ~60% of rFSHR-wt (7). Thus, in contrast to the data presented above for hFSH-induced phosphorylation, the results presented here and elsewhere (7) for the PMA-induced phosphorylation are consistent with the hypothesis that all three
intracellular loops of the rFSHR contain Ser/Thr residues that are phosphorylated upon pharmacological activation of protein kinase C.

Two Mutations of the rFSHR That Impair Signal Transduction but Preserve the Phosphorylation Sites Block Agonist-induced Receptor Phosphorylation—Since cells expressing rFSHR-2L show an impairment in agonist-induced receptor activation (Table I) and since there is a positive correlation between agonist-induced receptor activation and agonist-induced receptor phosphorylation in many other GPCRs (1, 2, 28–30), it is possible that the impairment in agonist-induced phosphorylation detected in rFSHR-2L is caused by the inactivating nature of this mutation rather than by the mutation of putative phosphorylation sites.

In order to gain a better understanding of the relationship between agonist-induced activation and phosphorylation of the rFSHR, we prepared and analyzed two single point mutations of the rFSHR that impair signal transduction but retain all potential phosphorylation sites. Two residues that are highly conserved in the rhodospin-like subfamily of GPCRs (an Asp in the second transmembrane domain and a Tyr in the boundary of the fifth transmembrane domain and the third intracellular loop; see Refs. 31 and 32) were chosen for mutagenesis. Rat FSHR-D389N was prepared because mutation of the highly conserved D in the equivalent position of many other GPCRs, including the highly related lHR, impairs signal transduction without affecting agonist binding affinity (see examples cited in Refs. 33–35). Rat FSHR-Y530F was prepared because mutation of the highly conserved Y in the equivalent position of the angiotensin AT1A receptor (36) and the highly related rLHR3 have also been shown to impair signal transduction without affecting agonist binding affinity.

Clonal cell lines expressing rFSHR-D389N or rFSHR-Y530F were prepared and matched with a clonal cell line expressing an equivalent density of rFSHR-wt (Table I). As predicted from the foregoing analysis, cells expressing these mutant receptors bound hFSH with an affinity comparable with that of rFSHR-wt but had reduced basal levels of cAMP and responded poorly to hFSH with an increase in cAMP accumulation. The EC50 for the hFSH-induced cAMP accumulation was increased 3–4-fold, and the cAMP response attained in response to maximally effective concentrations of hFSH was reduced by 50–70% (i.e. the hFSH/cholera toxin response ratio fell from 2.65 in the cells expressing rFSHR-wt to 1.23 and 0.89 in cells expressing rFSHR-D389N or rFSHR-Y530F, respectively). Thus, we can readily conclude that, like cells expressing rFSHR-2L and rFSHR-2L + 3L, cells expressing rFSHR-D389N or rFSHR-Y530F display a reduction in hFSH responsiveness. It is worth noting that although all three mutations reduce the maximal cAMP response to hFSH, only the latter two increase the EC50 for this response. Unlike rFSHR-2L and rFSHR-2L + 3L, which are inactivating mutations with substitutions in potential phosphorylation sites, the mutations introduced into rFSHR-D389N and rFSHR-Y530F are inactivating but retain all potential phosphorylation sites. Although we did not examine the mechanisms underlying the inactivating nature of these mutations, detailed studies conducted with the α2-adrenergic and AT1A angiotensin receptors show that mutation of these highly conserved residues impairs the ability of these receptors to couple to their cognate G proteins (33, 36). Regardless of the reasons why these mutations impair signal transduction, however, the characterization of rFSHR-D389N and rFSHR-Y530F allowed us to examine the potential importance of receptor activation on hFSH-induced phosphorylation as detailed below.

The results shown in Figs. 3 and 4 show that the addition of hFSH to transiently transfected cells increased the phosphorylation of rFSHR-wt ~10-fold but failed to increase the phosphorylation of rFSHR-D389N or rFSHR-Y530F. Since the inability of agonists to promote the phosphorylation of their cognate GPCRs in transfected cells is a hallmark of a GRK-catalyzed phosphorylation event (1, 2, 28–30) we also examined the hFSH-promoted phosphorylation of rFSHR-D389N or rFSHR-Y530F upon co-expression of GRK2, one of the members of the GRK family that has been shown to phosphorylate the rFSHR-wt.3 Co-expression of GRK2 enhanced hFSH-stim-

3 K. S. Min and M. Ascoli, manuscript in preparation.
ulated phosphorylation of rFSHR-wt about 2-fold (Figs. 3 and 4) and decreased the mobility of the rFSHR (Fig. 3). This same co-transfection strategy greatly enhanced the hFSH-stimu-

lated phosphorylation of rFSHR-D389N or rFSHR-Y530F. Thus, when co-transfected with GRK2, hFSH induced a ~6-fold increase in the phosphorylation or rFSHR-D389N or rFSHR-Y530F, a value that approached ~60% of the hFSH-induced increase in the phosphorylation of rFSHR-wt detected in cells transfected with rFSHR-wt only or ~30% of the hFSH-induced increase in the phosphorylation of rFSHR-wt detected in cells co-transfected with rFSHR-wt and GRK2.

These results clearly show that the hFSH-induced activation of the rFSHR is important for subsequent receptor phosphorylation. As such, the partial reduction in the agonist-induced phosphorylation of rFSHR-2L reported above (cf. Figs. 1 and 2) could be readily explained by the inactivating nature of this mutation and/or by the mutation of putative phosphorylation sites. Initially, we had reasoned that we could use the GRK2 co-transfection strategy to discern the relative importance of agonist-induced activation versus mutation of phosphorylation sites on the reduction in agonist-induced phosphorylation detected in rFSHR-2L and rFSHR-2L + 3L. Thus, if the two threonines mutated in rFSHR-2L are not phosphorylation sites, we should fully restore phosphorylation of this mutant by co-transfection of GRK2. In contrast, GRK2 co-transfection should only partially restore the agonist-induced phosphorylation of rFSHR-2L + 3L, because the Ser/Thr residues mutated in the third intracellular loop are known to be phosphorylation sites (6, 7). This strategy was not possible, however, because the hFSH-induced phosphorylation of inactivating mutants that retain all phosphorylation sites (i.e. rFSHR-D389N and rFSHR-Y530F) could not be fully rescued by co-transfection with GRK2 (cf. Figs. 3 and 4).

We also assessed the ability of PMA to stimulate the phosphorylation of rFSHR-D389N and rFSHR-Y530F. As shown in Figs. 5 and 6, either of these two inactivating mutations had little or no effect on the PMA-induced phosphorylation. Since the two inactivating mutations that retain the full complement of potential phosphorylation sites can be readily phosphorylated upon PMA stimulation, we conclude that the decreased PMA-induced phosphorylation detected in rFSHR-2L and rFSHR-2L + 3L (cf. Figs. 1 and 2) cannot be explained by the inactivating nature of these mutations, and thus it must be due to the mutation of phosphorylation sites.

**Effect of Inactivating Mutations of the rFSHR on the rFSHR**

**FIG. 3.** Human FSH-induced phosphorylation of rFSHR-wt and mutants thereof in transiently transfected 293 cells. Cells were transiently co-transfected with rFSHR-wt with or without GRK2, rFSHR-D389N with or without GRK2, or rFSHR-Y530F with or without GRK2 as indicated. Two days after transfection, the cells were metabolically labeled with $^{32}$P for 3 h and further incubated with buffer only, or with 100 ng/ml hFSH for 60 min as indicated. Cell lysates were prepared, immunoprecipitated, resolved on SDS gels, and visualized by autoradiography as described under “Materials and Methods.” The results presented are densitometric scans of representative autoradiograms obtained by immunoprecipitation of equivalent amounts of receptor, which were calculated based on $^{125}$I-hFSH binding assays using intact cells as described under “Materials and Methods.”

**FIG. 4.** Quantitation of hFSH-induced phosphorylation of rFSHR-wt and mutants thereof in transiently transfected 293 cells. Cells were transiently co-transfected with rFSHR-wt with or without GRK2, rFSHR-D389N with or without GRK2, or rFSHR-Y530F with or without GRK2 as indicated. Two days after transfection, the cells were metabolically labeled with $^{32}$P for 3 h and further incubated with buffer only, or with 100 ng/ml hFSH for 60 min as indicated. The cells were lysed, and immunoprecipitates were obtained using equivalent amounts of receptor calculated based on $^{125}$I-hFSH binding assays performed on the transiently transfected cells as described under “Materials and Methods.” The immunoprecipitates were resolved on SDS gels and visualized by autoradiography. After scanning and quantitation by densitometry, the signal detected in the cells transiently transfected with rFSHR-wt alone and incubated with buffer only was arbitrarily set as 1, and all other signals were recalculated relative to this value. The bars represent the mean ± S.E. of five independent transfections for rFSHR-wt and three independent transfections for rFSHR-D389N or rFSHR-Y530F.
Cells were transiently transfected 293 cells. Two days after transfection, the cells were metabolically labeled with 32P for 3 h and further incubated with buffer only or with 200 nM PMA for 15 min as indicated. The cells were lysed, and immunoprecipitates were obtained using equivalent amounts of receptor calculated based on 125I-hFSH binding assays performed as described under "Materials and Methods." The immunoprecipitates were resolved on SDS gels and visualized by autoradiography. The results presented are densitometric scans of representative autoradiograms obtained using immunoprecipitates containing equivalent amounts of receptor calculated based on 125I-hFSH binding assays performed in the transiently transfected cells (see "Materials and Methods").

![Figure 5](image1.png)

**FIG. 5.** PMA-induced phosphorylation of rFSHR mutants in transiently transfected 293 cells. Cells were transiently transfected with rFSHR-wt, rFSHR-D389N, or rFSHR-Y530F as indicated. Two days after transfection, the cells were metabolically labeled with 32P for 3 h and further incubated with buffer only or with 200 nM PMA for 15 min as indicated. The cells were lysed, and immunoprecipitates were obtained using equivalent amounts of receptor calculated based on 125I-hFSH binding assays performed as described under "Materials and Methods." The immunoprecipitates were resolved on SDS gels and visualized by autoradiography. The results presented are densitometric scans of representative autoradiograms obtained using immunoprecipitates containing equivalent amounts of receptor calculated based on 125I-hFSH binding assays performed in the transiently transfected cells (see "Materials and Methods").

mediated Endocytosis of hFSH—Since the agonist-induced phosphorylation of several GPCRs and the association of the phosphorylated GPCRs with arrestin are important determinants of agonist-receptor internalization (3, 4, 37, 38) we also examined the internalization of hFSH mediated by each of the four mutants described above.

The data presented in Table II show that despite the observed reduction in agonist-induced activation and receptor phosphorylation detected in cells expressing rFSHR-2L or rFSHR-2L + 3L, there was no reduction in the rate of internalization of hFSH. In fact, cells expressing either of these two mutants internalized hFSH at a slightly faster rate than cells expressing rFSHR-wt. A similar increase in the rate of internalization of hFSH in cells expressing rFSHR-3L was previously reported (7). In contrast, the rate of internalization of hFSH is reduced about 2-fold in cells expressing rFSHR-1L, a mutant that displays ~90% reduction in hFSH-induced phosphorylation (7).

The internalization of the two inactivating mutations that retain a full complement of phosphorylation sites described above were measured in transiently transfected cells. Cells that are transiently transfected with the rFSHR-wt internalize 125I-hFSH with a rate very similar or identical to that measured in stably transfected cell lines (cf. Table II). Internalization can also be conveniently assessed by measuring the amount of 125I-hFSH that is internalized during a single 9-min incubation with a constant concentration of 125I-hFSH. The results presented in Fig. 7 show that under these conditions about 30% of the total 125I-hFSH associated with cells transiently transfected with rFSHR-wt has been internalized. This level of internalization is reduced to 11 and 7%, respectively, in cells transiently expressing rFSHR-D389N or rFSHR-Y530F. These results are consistent with the notion that activation and/or phosphorylation of many GPCRs is needed for internalization (25, 28–30, 36).

The results presented in Fig. 7 also show that the internalization of 125I-hFSH mediated by the rFSHR-wt is dependent on arrestin. Thus, co-transfection of rFSHR-wt with β-arrestin (319–418), a dominant-negative mutant of the nonvisual arrestins (4) reduces internalization from 30 to 13%. The internalization of rFSHR-D389N was reduced from 11 to 2% by co-transfection of β-arrestin (319–418), while the already low level of internalization detected in cells transfected with rFSHR-Y530F was only slightly reduced from 7 to 5% (Fig. 7). We also tested the effects of co-transfection with GRK2 and arrestin-3 on the internalization of rFSHR-wt and the two inactivating mutations. These results (Fig. 7) show that arrestin-3 overexpression increases the internalization mediated by rFSHR-wt from 30 to 46% and rescues the internalization mediated by rFSHR-D389N or rFSHR-Y530F to levels (25 and 27%, respectively) similar to those observed in cells transfected with rFSHR-wt only. In contrast, GRK2 overexpression, which partially rescues the phosphorylation of the two mutants to about the same extent (cf. Figs. 3 and 4) rescued the internalization of rFSHR-D389N to that observed in cells transfected with rFSHR-wt only but had little effect on the internalization of rFSHR-Y530F (Fig. 7). Last, the effect of co-expression of GRK2 and arrestin-3 was only slightly better than that of arrestin-3 alone (Fig. 7).

**DISCUSSION**

Previous mutagenesis and peptide mapping studies from this laboratory as well as the new data presented here have shown that, unlike most other G protein-coupled receptors, the Ser/Thr residues that become phosphorylated in the rFSHR are located in the intracellular loops rather than in the C-terminal tail (6, 7). The mutagenesis studies presented here and elsewhere are summarized in Table III and suggest a complex and overlapping pattern of phosphorylation sites for the PMA and hFSH stimuli.

Our data are consistent with a model where the hFSH-induced phosphorylation of the rFSHR occurs in the first and third intracellular loops. Thus, the removal or mutation of the 13 Ser/Thr residues present in the C-terminal tail does not affect hFSH-induced phosphorylation, while the mutation of

| TABLE II |
| --- |
| Rates of internalization of 125I-hFSH in stably transfected cell lines expressing rFSHR mutants |
| Cell line | Half-life of internalization (min) |
| 293F(wt-103) | 12 ± 1 |
| 293F(2L) | 7 ± 1 |
| 293F(2L + 3L) | 9 ± 3 |
Two days after transfection, the cells were incubated with 40 ng/ml $^{123}$I-hFSH for 9 min at 37 °C, and the amounts of surface-bound and internalized radioactivity were measured as described under "Materials and Methods." The internalized radioactivity was then expressed as a percentage of the internalized plus surface-bound radioactivity. The bars represent the average ± S.E. of results obtained in 3–11 independent transfections.

**Table III**

Summary of the signal-transducing properties and phosphorylation of several rFSHR mutants

| Receptor* | hFSH-stimulated cAMP accumulation | hFSH-promoted phosphorylation | PMA-promoted phosphorylation |
|-----------|----------------------------------|------------------------------|------------------------------|
| Wild type | Normal                           | 100                          | 100                          |
| 1L        | Normal                           | ~10                          | ~40                          |
| 2L        | Reduced                          | ~60                          | ~60                          |
| 3L        | Enhanced                         | ~60                          | ~60                          |
| 2L + 3L   | Reduced                          | ~50                          | ~20                          |
| CT        | Enhanced                         | ~100                         | ~100                         |

* Original data were published in Ref. 6.

The seven Ser/Thr residues present in the third intracellular loop reduce hFSH-induced phosphorylation to ~60%, and the mutation of three Ser/Thr residues present in the first intracellular loop reduces hFSH-induced phosphorylation to ~10% (Table III). Since peptide mapping experiments indicate the presence of phosphorylation sites in both the first and third intracellular loop (6, 7), the results obtained with the mutagenesis of the first loop suggest a complex hFSH-stimulated phosphorylation event where the first loop needs to be phosphorylated before the third loop becomes phosphorylated. Alternatively, the mutation of Ser/Thr residues present in the first loop may induce a generalized conformational change that prevents phosphorylation. The data presented here with the mutation of the two threonine residues present in the second intracellular loop show that the mutation of these residues reduces hFSH-induced phosphorylation to ~60%. The inability of hFSH to stimulate the phosphorylation of two inactivating mutations that retain all phosphorylation sites argues that the two threonine residues present in the second intracellular loop are not phosphorylated upon hFSH stimulation and that the reduction in hFSH-induced phosphorylation of rFSHR-2L is due to the inactivating nature of the 2L mutation. The lack of additivity in hFSH-induced phosphorylation observed when the 2L and the 3L mutation are introduced together also argues against the phosphorylation of the second intracellular loop upon agonist-induced activation. Last, it should be noted that the reduction in agonist-induced phosphorylation detected in rFSHR-1L, rFSHR-3L, or rFSHR-CT cannot be explained by changes in the agonist-induced activation of the receptor, because all of these other mutants display normal or enhanced activation (Table III).

In contrast to the model proposed above for the hFSH-induced phosphorylation of the rFSHR, the results obtained with PMA are consistent with a model where the PMA-induced phosphorylation of the rFSHR occurs in the first, second, and third intracellular loops. Thus, the removal or mutation of the 13 Ser/Thr residues present in the C-terminal tail does not affect PMA-induced phosphorylation, while the mutation of the potential phosphorylation sites present in the first, second, or third intracellular loops of the rFSHR each reduce PMA-induced phosphorylation to 40–60% (Table III), indicating that all three loops are phosphorylated in response to PMA. This conclusion is also supported by the finding that the effect of simultaneous mutation of Ser/Thr residues present in the second and third loops is roughly additive when compared with that of the individual loop mutations (Table III). Last, our data on the PMA-induced phosphorylation of the two inactivating mutations that retain all phosphorylation sites argues that the inactivating nature of the 2L or 2L + 3L mutations cannot explain the reduction in PMA-induced phosphorylation detected in these mutants.

It is now generally accepted that the agonist-induced activation of the β$_2$-adrenergic receptor facilitates the GRK-catalyzed phosphorylation of the β$_2$-adrenergic receptor, a modification that increases the affinity of the β$_2$-adrenergic receptor for β-arrestin. The formation of the phosphorylated β$_2$-adrenergic receptor-arrestin complex sterically hinders the β$_2$-adrenergic receptor-G protein association, thus leading to a functional “uncoupling” of the β$_2$-adrenergic receptor. Since β-arrestin can also bind clathrin with high affinity, the β$_2$-adrenergic receptor-arrestin complex is targeted for internalization through clathrin-coated pits (1–4, 39, 40). It should be stressed that not all GPCRs follow this pathway, however. Thus, while agonist stimulation of the AT$_1$A angiotensin (41) and the m2 muscarinic receptors (42) leads to receptor phosphorylation, the phosphorylated receptors can be internalized by a pathway that does not require the participation of arrestin or clathrin-coated
pits. However, overexpression of nonvisual arrestins forest the agonist-stimulated AT1A angiotensin and m2 muscarinic receptors to be internalized via clathrin-coated pits by a pathway that requires nonvisual arrestins (41, 42).

The data presented here provide the first evidence for the involvement of arrestin on the rFSHR-mediated internalization of hFSH and allows us to differentiate at least three steps in this process: (i) the agonist-induced activation of the rFSHR; (ii) the GRK-catalyzed phosphorylation of the rFSHR; and (iii) the association of the rFSHR with arrestin.

The involvement of arrestins on the rFSHR-mediated internalization of hFSH is documented by the finding that a dominant-negative mutant of the nonvisual arrestins reduces the internalization of hFSH mediated by rFSHR-wt, while the overexpression of wild-type arrestin-3 enhances internalization (Fig. 7). A role for the phosphorylation of the rFSHR is also indicated by the finding that overexpression of GRK2 enhances the agonist-induced phosphorylation or rFSHR-wt and the internalization of hFSH (Figs. 3, 4, and 7).

While it is clear that phosphorylation facilitates the internalization of the rFSHR and that internalization occurs through an arrestin-dependent pathway, the results presented here and elsewhere with rFSHR mutants suggest a rather complex role for the activation and the phosphorylation of the rFSHR on arrestin-mediated internalization. We have previously concluded that the agonist-induced phosphorylation of the Ser/Thr residues present in the third intracellular loop does not facilitate receptor internalization. Instead, the agonist-induced internalization of the rFSHR seems to require the phosphorylation of Ser/Thr residues present in the first intracellular loop or may require the combined phosphorylation of Ser/Thr residues present in the first and third intracellular loop (7).

The data presented here show that rFSHR-2L and rFSHR-Y530F, two mutants that display a reduction in agonist-induced activation and a partial loss of hFSH-induced phosphorylation are internalized at the same rate or faster than rFSHR-wt (Table II). This impairment in phosphorylation without a corresponding reduction in internalization is not surprising, because we have already concluded that not all phosphorylation sites are needed for internalization (see above). Likewise, the finding that rFSHR-2L and rFSHR-2L + 3L show an impairment in signal transduction but are internalized at a normal rate suggests that the agonist-induced activation of the rFSHR is not necessary for internalization. Additional experiments presented here with other inactivating mutations of the rFSHR argue against the overall importance of phosphorylation and activation to the process of internalization. Thus, rFSHR-D389N and rFSHR-Y530F, two inactivating mutations that retain all phosphorylation sites display a severe loss of hFSH-induced phosphorylation and internalization (Figs. 3, 4, and 7). Since GRK2 co-expression partially rescues the hFSH-induced phosphorylation of both mutants but rescues only the internalization of rFSHR-D389N (Figs. 3, 4, and 7), we can readily conclude that phosphorylation is not essential for internalization. Likewise, since the inactivating nature of these two mutants should remain unchanged upon GRK2 cotransfection, we can also conclude that receptor activation is not needed for internalization. Interestingly, arrestin overexpression rescues the internalization of rFSHR-D389N and rFSHR-Y530F to the levels detected with rFSHR-wt even under conditions (i.e. without GRK2 co-transfection) where these two mutants are not phosphorylated by agonist (Fig. 7).

Taken together, our results argue that of the three steps listed above (i.e. activation, phosphorylation, and arrestin binding), only the interaction of the rFSHR with arrestin is essential for internalization. The interaction of the rFSHR with arrestin seems to be facilitated by the agonist-induced activation and/or phosphorylation, but even if one or both of these steps are impaired or lost, internalization can be driven to normal levels by overexpression of arrestin. These states are best documented by the behavior of the rFSHR-Y530F mutant, which displays a substantial reduction in agonist-induced activation (Table I) and a complete loss of agonist-induced phosphorylation (Figs. 3 and 4). The internalization of hFSH mediated by this mutant is severely impaired but can be fully rescued by overexpression of arrestin-3, even in the absence of receptor phosphorylation (Fig. 7). Moreover, a substantial increase in agonist-induced phosphorylation induced by overexpression of GRK2 (Figs. 3 and 4) does not rescue the internalization mediated by rFSHR-Y530F unless arrestin-3 is overexpressed as well (Fig. 7).

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