Truncation of the C-terminal Tail of the Follitropin Receptor Does Not Impair the Agonist- or Phorbol Ester-induced Receptor Phosphorylation and Uncoupling*

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R. William Hipkin, Xuebo Liu, and Mario Ascoli

From the Department of Pharmacology, The University of Iowa College of Medicine, Iowa City, Iowa 52242-1109

We have recently shown that addition of follitropin (FSH) or a phorbol ester (phorbol 12-myristate 13-acetate (PMA)) to cells expressing the recombinant follitropin receptor (FSHR) results in both phosphorylation and uncoupling of the FSHR from adenyl cyclase. In the light of findings reported with other G protein-coupled receptors we have proposed that phosphorylation of the FSHR mediates the uncoupling from adenyl cyclase. The experiments described herein represent the first attempt to determine the location of the amino acid residues that become phosphorylated in FSHR and to test the hypothesis that phosphorylation is responsible for uncoupling of FSHR from adenyl cyclase.

As a first step in identifying which residues may be phosphorylated in response to hFSH and PMA, we constructed a mutant of the FSHR cDNA in which the C-terminal cytoplasmic tail was truncated at residue 635 (FSHR-t635), thus removing all but one of the potential phosphorylation sites present in the C-terminal tail. Cells expressing FSHR-t635 bind hFSH with the appropriate affinity and respond with increases in cAMP and inositol phosphate accumulation. The maximal cAMP and inositol phosphate responses of cells expressing FSHR-t635 are higher than those of cells expressing the wild type FSHR, but the concentration of hFSH required to elicit these responses is similar in both cell lines.

Immunoprecipitation of FSHR-t635 shows that the truncated receptor is still effectively phosphorylated in response to hFSH or PMA. Phosphoamino acid analysis revealed that, like the wild-type FSHR, FSHR-t635 phosphorylation occurs on serine and threonine residues. Peptide mapping suggests that the phosphorylated residues in the FSHR and FSHR-t635 are located within the same areas of the intracellular regions of the receptors. In addition to stimulating phosphorylation of FSHR-t635, hFSH and PMA also effectively uncouple the truncated receptor from adenyl cyclase. Taken together, these data show that hFSH and PMA can both phosphorylate and uncouple a FSH receptor species with a cytoplasmic tail truncated at residue 635.

When target cells are exposed to a hormone their responsive-ness wanes with time, despite the continuous presence of hormone. This phenomenon, referred to as desensitization, is due to regulatory events that occur at the level of the hormone receptor as well as at postreceptor steps. While postreceptor events may be specific for the metabolic pathways that are activated in a given target cell, receptor events are more general in nature and conserved for a given family of receptors. There are at least two categories of regulatory events that occur at the level of the receptor and contribute to the process of desensitization. One of them, henceforth referred to as uncoupling, is defined as a change in the functional properties of a constant number of receptors resulting in a reduction in the ability of the receptor to activate its effector system. The other, henceforth referred to as down-regulation, is defined as a reduction in the density of cell surface receptors. Uncoupling is generally faster than down-regulation and is believed to be due to post-translational modifications of the receptor. Down-regulation, on the other hand, is slower and could be due to an increase in the rate of receptor sequestration/internalization or receptor degradation and/or a decrease in the rate of receptor externalization, processing, or synthesis.

It is now generally accepted that the phosphorylation of the β2-adrenergic receptor that results as a consequence of agonist binding is an important event in the agonist-induced uncoupling of this receptor (1–3). Although the phosphorylation of other G protein-coupled receptors (GPCRs) has not been studied in as much detail, it is rather clear that phosphorylation plays a central role in the regulation of the functions of many other GPCRs. Thus, in all other members of this family that have been studied so far, the removal or mutation of phosphorylation sites leads to a decrease in agonist-induced phosphorylation and an impairment in the process of agonist-induced uncoupling (see Refs. 4–11 for a few examples).

The FSH-induced desensitization of the FSH-responsive adenyl cyclase has been studied in some detail in target cells (Sertoli or granulosa cells) or membranes prepared therefrom (12–17). Moreover, since the agonist-induced desensitization of the isoproterenol-responsive adenyl cyclase of rat Sertoli cells was also studied (15, 18), it is relatively straightforward to compare the properties of the homologous desensitization induced by these two ligands in the same cell and to compare these data with what we know now about the mechanisms of desensitization of the β2-adrenergic receptor. These studies showed that the time course, magnitude, and other biochemical properties of the agonist-induced desensitization of the FSH receptor (FSHR) and β2-adrenergic receptor are very similar. Although in both cases one can demonstrate that uncoupling

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‡To whom correspondence should be addressed: Dr. Mario Ascoli, Dept. of Pharmacology, University of Iowa, 2–512 BSB, Iowa City, IA 52242-1109. Tel.: 319-335-9907; Fax: 319-335-9925; E-mail: mario-ascoli@uiowa.edu.

1 The abbreviations used are: GPCR, G protein-coupled receptors; FSH, follitropin; hFSH, human FSH; FSHR, FSH receptor; PMA, phorbol 12-myristate 13-acetate; LH, luteinizing hormone; LHR, LH receptor.
and down-regulation are involved in desensitization, uncoupling seems to be quantitatively more important than down-regulation (13, 19). Last, in agreement with what was reported for the agonist-induced desensitization of β2-adrenergic receptor in turkey erythrocyte membranes (20), the FSH-induced desensitization of FSHR in Sertoli cell membranes was reported to require Mg$^{2+}$ and ATP (15).

Based on these findings, it is reasonable to propose that phosphorylation of the FSHR is responsible for the agonist-induced uncoupling of the FSHR. In fact, this hypothesis was initially proposed by Hansson and co-workers in a paper published 11 years ago (15), but it has proven difficult to test until now. It is also reasonable to propose that the phosphorylation of the FSHR is involved in the heterologous regulation of the actions of FSHR. A particularly relevant example of this heterologous type of regulation of the actions of FSHR is that of agonists that activate the inositol phosphate/diacylglycerol pathway (such as gonadotropin-releasing hormone in granulosa cells or purinergic agonists in Sertoli cells) or pharmacological agents (such as phorbol 12-myristate 13-acetate (PMA)) that activate protein kinase C (21–23). In Sertoli or granulosa cells, the activation of this pathway results in an attenuation or desensitization of FSH actions, which seems to be due to a decrease in the functional properties (i.e., uncoupling) of the FSHR rather than to the down-regulation of the FSHR (21–23).

Recent studies from this laboratory (24–26) have established a transfected cell system that (i) faithfully reproduces the process of FSH- or PMA-induced desensitization that has been documented in gonadal cells bearing the FSHR, (ii) allows for biochemical analyses of the phosphorylation of the FSHR, and (iii) can be used in conjunction with site-directed mutagenesis of the FSHR cDNA, to conclusively determine if FSHR phosphorylation is involved in the desensitization of FSHR. The experiments described herein represent the first attempt to determine the location of the amino acid residues that become phosphorylated in FSHR and to test the hypothesis that phosphorylation is responsible for uncoupling of FSHR from adenylyl cyclase.

**EXPERIMENTAL PROCEDURES**

A wild-type rat FSH receptor cDNA (27) was subcloned into the pcDNA1/Neo expression vector (Invitrogen, San Diego, CA). A cDNA encoding for the FSH receptor truncated at residue 635 (designated FSHR-635) was constructed using the polymerase chain reaction (28) by introducing a stop codon after the codon for amino acid residue 635. The identity of this construct was verified by sequencing the entire open reading frame (29). The origin and handling of the parental human embryonic kidney cells (293 cells) and the methods used for transfection and isolation of clonal cell lines stably transfected with the wild type or mutant FSH receptor cDNAs have been described in detail elsewhere (11). All transfected cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10 mM HEPES, 10% newborn calf serum, 50 μg/ml gentamicin, 700 μg/ml G418, pH 7.4, at 37 °C in a humidified atmosphere containing 5% CO$_2$. Experimental cultures were plated on day 0 and used on day 3 or 4.

**Immunoprecipitation of the FSH Receptor from 32P-Labeled Cells—**

Metabolic labeling of cells and subsequent immunoprecipitation of the FSH receptor was achieved as described previously (24, 25). Cells plated in 100-mm plates were labeled using 3.5 ml of phosphate-free Dulbecco’s modified Eagle’s medium containing 100–400 μCi/ml [32P]orthophosphate for 3 h. All incubations were done at 37 °C under 5% CO$_2$. Unless otherwise stated in the figure legends, the various hormones and pharmacological agents were then added directly to the medium. Cells were further incubated for the indicated times. At the end of the incubation, the cells were scraped into ice-cold buffer A (0.15 M NaCl, 20 mM HEPES, pH 7.4) containing various protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptin, 5 mM EDTA, 3 mM EGTA, 50 mM β-glycerophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 100 μM okadaic acid, and 1 mM cypermethrin), pelletted by centrifugation, and lysed by vortexing in Buffer A containing 0.5% Nonidet P-40 and the aforementioned inhibitors. The detergent lysates were clarified by centrifugation at 100,000 × g for 30 min, and the protein content of the supernatants was assessed by the method of Bradford (30). Equal amounts of lysate protein were then immunoprecipitated with a polyclonal antibody to the rat FSH receptor as described before (24, 25). Immunoprecipitates were resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gels in the presence of thiol-reducing agents as described previously (31). Autoradiograms of the gels were obtained by placing the wells on ice, the medium was aspirated, and the gel pieces were then rehydrated with water for 60 min, and the paper backing was removed. The receptor polyepitope within the gel slice was then chemically cleaved at tryptophan residues with N-chlorosuccinimide using a modification of the method described by Lischwe and Ochs (32) as follows. The receptor protein was first denatured by incubating the gel slices in 4 M guanidine hydrochloride in 80% acetic acid (33) for 20 min, with one change of denaturing solution. The gel slices were then incubated in the presence of 50 mM N-chlorosuccinimide in the same denaturing solution for 1–2 h, washed twice with 20 ml of water for 10 min each time, and equilibrated in 1.5 ml of Tricine gel sample buffer (12% glycerol [w/v], 4% sodium dodecyl sulfate [w/v], 2% mercaptoethanol [w/v], and 0.02% Bromphenol Blue [w/v]) in 50 mM Tris-HCl, pH 6.8) for 15 min. This sample buffer was changed three times during the 90-min incubation.

The peptides within the gel slices were then separated using the discontinuous Tricine-urea SDS-polyacrylamide gel electrophoresis system described by Schägger and von Jagow (34) using a 16.5% acrylamide, 6% urea resolving gel. Ultrafloc molecular mass standards (Ref. 34; 700–2500 daltons) (Sigma) were included in each experiment to determine the molecular weights of the generated peptides. Following electrophoresis, the Tricine gels were fixed by incubating them in 50% methanol, 10% acetic acid for 60 min followed by staining with 0.025% Bromphenol Blue in 10% acetic acid and destaining with 10% acetic acid. Autoradiograms of the wet gels were then obtained using intensifying screens.

**FSH-induced Desensitization of the FSHR—**

The responsive Adenylyl Cyclase—Cells were plated in 35-mm wells that had been coated with a fibronectin-like engineered protein polymer (5 μg/ml) according to the manufacturer’s instructions. Two to three days after plating, the cells were washed twice with 2-ml portions of warm assay medium (Waymouth MB 752/1 containing 1.12% gelatin, 50 μg/ml gentamicin, 24 mM HEPES, and 1 mM bovine serum albumin, pH 7.4) and incubated at 37 °C in 1 ml of assay medium in the absence or presence of hFSH for various time periods. The free hormone was removed by placing the wells on ice and washing the cells three times with 2-ml portions of ice-cold Hank’s balanced salt solution containing 1 mg/ml bovine serum albumin. The receptor-bound hormone was then removed by washing twice with 2 ml of cold Tricine acetate, pH 3.0, followed by a 2-ml wash with the same cold acid buffer (35). Finally, the cells were washed twice with 2-ml portions of warm assay medium, and 1 ml of warm assay buffer was added to each well. The cells were then incubated in the presence or absence of various concentrations of hFSH for 15 min at 37 °C. At the end of this incubation the wells were placed on ice, the medium was aspirated, and the cells were washed once with a 2-ml aliquot of cold assay medium containing 0.5 mM isobutylmethylxanthine. The washing solution was removed, and the intracellular cAMP was extracted by adding 1 ml of 1 N perchloric acid containing 180 μg/ml theophylline. The samples were then processed, and the cAMP was assays as described previously (35). TSF-induced Desensitization of the FSHR—Cells plated on 35-mm plates were placed in coated dishes and treated as described above. Following a preincubation with or without hFSH for increasing periods of time at 37 °C, the free and receptor-bound hormones were removed as described above. The binding of $^{125}$I-FSH was then measured during an overnight incubation at 4 °C as described previously (24, 25).

**Other Methods—**

The different parameters that describe the concentration...
Preparation and Functional Properties of FSHR(t635)—We have previously established that addition of hFSH or PMA to stably transfected 293 cells expressing the FSHR results in an increase in FSH receptor phosphorylation (25). However, in these initial experiments, pharmacological concentrations of the hFSH (EC$_{50}$ = 500 ng/ml) were required to increase receptor phosphorylation during a 15-min incubation. As it is highly unlikely that such high concentrations of FSH occur in vivo, we attempted to improve the sensitivity of our assay by increasing the length of the incubation with FSH to 1 h by using subconfluent cell cultures and by omitting the lectin purification step that was used prior to receptor immunoprecipitation (25). As shown in Fig. 1, these changes have now allowed us to detect an increase in FSHR phosphorylation with 3 ng/ml hFSH, a half-maximal increase with about 30 ng/ml hFSH, and a maximal increase with 100 ng/ml hFSH. While the maximal increase in receptor phosphorylation detected under these conditions is similar to that detected previously (25), the FSH concentration range that elicits receptor phosphorylation is now similar to the concentration range required to detect FSH-induced increases in cAMP, estradiol, or c-fos mRNA levels in Sertoli cells (41–43) as well as FSH-induced increases in cAMP accumulation in mammalian or insect cells transfected with the FSHR (25, 44). The reported EC$_{50}$ values for these responses vary between 2 and 30 ng/ml, and maximal responses are attained at 100–500 ng/ml hFSH.

Since truncations of the C-terminal tails of several G protein-coupled receptors have provided useful information about the location and functions of their phosphorylation sites (4, 5, 7, 11, 45), we constructed a mutant of the FSHR cDNA in which the C-terminal cytoplasmic tail was truncated at residue 635 (designated FSHR-t635). As shown in Fig. 2, this truncation removes all but one of the potential phosphorylation sites (serine and threonine residues; see Ref. 25) present in the C-terminal cytoplasmic tail of the FSHR. The possible phosphorylation of the remaining cytoplasmic tail serine cannot be examined by C-terminal truncation of the receptor because truncations done upstream of residue 635 generally result in mutant receptors that do not localize to the cell surface (data not shown).

Clonal, stably transfected cell lines expressing FSHR-t635 were prepared and matched with a clonal cell line stably transfected with the FSHR-wt expressing equivalent numbers of cell surface receptors. (Table I). Quantitation of the FSH receptors expressed in these cell lines by immunoprecipitation of the FSHR from $^{35}$S-labeled cells or by immunoblotting support the conclusion that 293F(wt-10) and 293F(t635–5) cells express equivalent numbers of the mature 74-kDa FSHR (data not shown).

The cAMP and inositol phosphate responses of 293F(wt-10) and 293F(t635–5) cells to FSH are shown in Fig. 3. The maximal responses of 293F(t635–5) cells to FSH are 2–3-fold higher than those of 293F(wt-10) cells, but there are no differences in the EC$_{50}$ values for these responses between the two cell lines. The EC$_{50}$ values for cAMP and inositol phosphate accumulation in both cell lines are 2–5 ng/ml and 150–250 ng/ml hFSH, respectively. Since the cAMP response to cholera toxin and the inositol phosphate response to UTP are similar for these two cell lines (Fig. 3), it can be safely concluded that the increased...
cellsthathadbeenprelabeledwith $[^3]H$inositolwereincubatedwiththe
effectiveto the 293F(t635–5) cells is not due to clonal
methylisobutylxanthine for 15 min and then incubated with the
phosphorylation of FSHR-wt and FSHR-t635—
hFSH and PMA each markedly
with what is known about other G protein-coupled receptors,
uncoupling of the FSHR from adenylyl cyclase.

Another interpretation of the finding that FSHR-t635 is
phosphorylated to about the same extent as FSHR-wt is that
to PMA, nor does it artificially introduce additional
phosphorylation sites.

Taken together, these data suggest that the residues that
become phosphorylated in FSHR-wt or FSHR-t635 are located
within the same areas of the intracellular regions of the
receptor. Therefore, removal of the terminal 40 amino acids of
to PMA, both receptor species were found to be phosphorylated in
serine and threonine residues, however (data not shown). To
more stringently analyze potential differences in phosphoryla-
tion sites between these two receptor species, we generated
peptide maps of FSHR-wt and FSHR-t635 phosphorylated in
response to FSH or PMA. Peptide maps were obtained by
chemical cleavage of the immunoprecipitated receptor with
N-chlorosuccinimide, a reagent that cleaves polypeptides at
tryptophan residues (32, 33). Fig. 5 shows that the phosphopep-
tide maps generated upon cleavage of the full-length or trunc-
ated FSHR receptor isolated from FSH-stimulated cells are
indistinguishable from each other. In addition, the phos-
phopeptide maps of the full-length and truncated FSHR iso-
lated from PMA-stimulated cells were also the same, and were
very similar to those obtained from FSH-stimulated cells (data
not shown).

Deletion of the terminal 40 amino acids of the
to FSH or PMA, namely, the 10 C-terminal amino acids.
The results summarized in Fig. 6 (left panel) show that preincubation of 293F(wt-10) or 293F(t635–5) cells with hFSH results in a decrease in the cAMP response of these cells to a further stimulation with hFSH. Importantly, the data in Fig. 6 also show that the time course and magnitude of this phenomenon are very similar in both cell lines. Under these experimental conditions, however, the blunting of FSH-stimulated cAMP accumulation following preincubation with FSH could result from reduced numbers of cell surface receptors (receptor down-regulation) or from receptor uncoupling. To quantitate the contribution of these two pathways to the blunting of the cAMP response, we also measured the extent of FSH-induced FSHR down-regulation in cells incubated under identical conditions. As can be seen in the right panel of Fig. 6, the extent of FSHR down-regulation is similar in both cell lines, and the magnitude of down-regulation is rather small compared with the magnitude of the loss of cAMP responsiveness. For example, a 15-min preincubation with hFSH reduced 125I-FSH binding to only 91 ± 2% and 87 ± 3% of control levels in 293F(wt-10) and 293F(t635–5) cells, respectively. In contrast, under the same conditions, hFSH-stimulated cAMP accumulation was reduced to 60 ± 7% and 55 ± 2% of control levels in 293F(wt-10) and 293F(t635–5) cells, respectively. Therefore, the FSH-induced blunting of the FSH-sensitive adenyl cyclase appears to be due primarily to FSHR uncoupling rather than down-regulation.

In additional experiments, we showed that preincubation of 293F(wt-10) or 293F(t635–5) cells with hFSH induced a similar reduction in maximal FSH-stimulated cAMP accumulation between the two cell lines, (40 and 54%, respectively) but had no effect on the concentration of hFSH required to elicit a half-maximal response (Fig. 7).

Last, the data summarized in Fig. 8 show that preincubation of 293F(wt-10) or 293F(t635–5) cells with PMA effectively reduces the FSH-stimulated cAMP accumulation. In both cell lines, the blunting of FSH-stimulated cAMP accumulation by PMA was particularly evident with low stimulatory concentrations of FSH. In fact, a preincubation with PMA either had no effect or enhanced the stimulatory effects of high concentrations of FSH on cAMP accumulation (Fig. 8 and Ref. 25). This stimulatory effect of PMA, however, is not specific for the FSH receptor, as it can also be detected when cAMP synthesis is activated with any concentration of cholera toxin (25).

Taken together, these data show that hFSH or PMA can both phosphorylate and uncouple a FSH receptor species with a cytoplasmic tail truncated at residue 635.

FIG. 5. Phosphopeptide maps of FSHR-wt and FSHR-t635. The 32P-labeled FSHR-wt or FSHR-t635 was isolated from pre-labeled cells that had been stimulated with 1000 ng/ml hFSH for 15 min at 37 °C. Cell lysates were prepared, immunoprecipitated with AntiF, and resolved on SDS gels as described under "Experimental Procedures." The relevant portion of the gels was cut, digested with N-chlorosuccinimide, and subjected to electrophoresis on Tricine-urea-SDS gels as described under "Experimental Procedures." The results (densitometric scan of an autoradiogram) of a representative experiment are shown. The apparent molecular weights shown were calculated based on the migration of molecular weight standards.

FIG. 6. Time course of hFSH-induced desensitization and down-regulation. Left panel, cells were preincubated with 100 ng/ml hFSH at 37 °C for the times indicated. The free and receptor-bound hormones were then removed by washing at neutral and acid pH as described under "Experimental Procedures," and the cells were divided into two groups and incubated with or without 100 ng/ml hFSH for 15 min at 37 °C. At the end of this incubation the medium was removed, and the intracellular cAMP was measured. The amount of cAMP present in the cells incubated without hFSH was then subtracted from that present in the cells incubated with hFSH. Finally, this response was then expressed as the percentage of the response of cells treated in an identical fashion but preincubated for the same length of time without hFSH. Each point represents the mean ± S.E. of three independent experiments. Right panel, cells were preincubated with 100 ng/ml hFSH at 37 °C for the times indicated. The free and receptor-bound hormones were then removed by washing at neutral and acid pH, respectively, as described under "Experimental Procedures." Results are expressed as the percentage of the 125I-hFSH binding detected in cells preincubated for the same length of time, but without hFSH. Each point represents the mean ± S.E. of three independent experiments.

DISCUSSION

We have previously shown that the recombinant FSH receptor expressed in mammalian cells becomes phosphorylated on serine and threonine residues when cells are exposed to FSH or PMA (25). The experiments presented herein were designed as an initial attempt to identify the location of the phosphorylated residues and to test the hypothesis that phosphorylation is responsible for the uncoupling of FSHR from adenyl cyclase.

Since 13 of the 25 serine/threonine residues present in the intracellular regions of the FSHR are located in the C-terminal tail (Fig. 2), a mutant FSHR DNA construct with a truncated C-terminal cytoplasmic tail was constructed. This mutant DNA encodes for a protein (designated FSHR-t635) in which 12 of the 25 intracellular serine/threonine residues were removed. The analysis of these truncations was not possible due to the lack of expression of these truncated receptors at the cell surface. FSHR-t635 is fully functional, in that cells expressing this truncated receptor species bind hFSH with high affinity (Table 1) and respond to the bound hormone with the expected increases in cAMP and inositol phosphate accumulation (Fig. 3). In fact, while the potency of hFSH is similar in cells expressing FSHR-wt or FSHR-t635, the efficacy of hFSH is 2–3-fold higher in cells expressing FSHR-t635 (Fig. 3). These results are similar to those reported for several C-terminal truncations of the closely related LHR (11, 46).

FSHR-t635 appears to be phosphorylated normally in response to FSH or PMA stimulation (Fig. 4). The simplest interpretation of these results is that the 12 serine/threonine residues removed in this truncated receptor are not phosphate acceptors. Another more complex interpretation is that the truncation of the C-terminal cytoplasmic tail resulted in the exposure of phosphorylation sites that are not accessible in the full-length receptor. In this scenario, FSHR-wt and FSHR-t635 would be phosphorylated to the same extent but on different sites. The detection of phosphoserine and phosphothreonine...
residues in the full-length and truncated receptors as well as the similarity of the phosphopeptide maps generated when either receptor is cleaved with N-chlorosuccinimide (Fig. 5), however, strongly suggest that FSHR-wt and FSHR-t635 are phosphorylated in the same sites.

The finding that FSHR-t635 is phosphorylated normally in response to FSH stimulation is interesting because C-terminal truncations of many other GPCRs (4, 5, 7, 45), including the closely related LHR (11), have been previously shown to abolish (or at least drastically diminish) agonist-induced phosphorylation. In fact, to the best of our knowledge, there are only two other GPCRs that become phosphorylated upon agonist stimulation, in which the phosphorylated residues are not localized in the C-terminal tail. These are the human m2 muscarinic receptor (47), where the phosphorylation sites appear to be located in the third intracellular loop, and the human α2-adrenergic receptor, where the phosphorylation sites cannot be located in the C-terminal tail simply because this receptor does not have any serine/threonine residues in its C-terminal tail (48, 49). Based on the amino acid sequence of the rat FSHR (27), it can be predicted that N-chlorosuccinimide cleavage should generate three phosphopeptides: a 22-kDa peptide containing the third intracellular loop and the C-terminal cytoplasmic tail, a 7.4-kDa peptide containing the first intracellular loop, and a 2.96-kDa peptide containing the second intracellular loop. As shown in Fig. 5, however, there are at least four detectable peptides that are generated upon N-chlorosuccinimide cleavage, and their molecular weights do not correspond to the predicted molecular weights of the three theoretical peptides. These findings could be reconciled by assuming that the cleavage conditions used may not be optimal and by the knowledge that there is a fair amount of uncertainty in the molecular weight estimates derived from the separation system used (34). Thus, it is possible that the 18- and 8.8-kDa peptides shown in Fig. 7 correspond to the 22- and 7.4-kDa peptides predicted above, and the 14.4- and 12-kDa peptides may be incomplete degradation products. If this interpretation is correct, the data presented in Fig. 5 suggests that phosphorylation occurs in the first and third intracellular loops.

The finding that truncation of the C-terminal tail of the FSHR does not affect the agonist- or PMA-induced uncoupling (Figs. 6–8) is in agreement with our hypothesis that phosphorylation is responsible for uncoupling. Admittedly, however, these results do not provide a rigorous test for our hypothesis, and additional experiments will be needed to conclusively determine if this hypothesis is correct. The main functional difference between FSHR-t635 and FSHR-wt is that the former is a better transducer of at least two FSH-induced signals: cAMP and inositol phosphate generation. Thus, while the concentrations of hFSH required to elicit half-maximal responses are similar in 293F(wt-10) and 293F(t635–5) cells, the maximal cAMP and inositol phosphate responses are higher in 293F(t635–5) cells (Fig. 3). At first glance these results may...
appear contradictory to the finding that the FSH-induced uncoupling of FSHR-t635 is normal because the increased responsiveness could be due to a loss of desensitization. Another interpretation that accommodates both sets of results, however, is that the C-terminal tail of the FSHR contains sequences that restrain the interaction of this receptor with G proteins. Thus, when these sequences are removed a more productive interaction takes place, and hormonal responsiveness is enhanced even if desensitization is normal. Enhanced hormonal responsiveness upon truncation of the C-terminal tail of GPCRs is, in fact, a rather common finding (4, 9, 11, 50–52), which does not always correlate with an impairment of desensitization. The best example of this lack of correlation is the closely related LHR, where truncations at residues 653 and 631 result in an enhanced human CG responsiveness but phosphorylation and desensitization are impaired only in LHR(t631) and not in LHR(t635) (Ref. 11).2

Last, it is interesting to note that the removal of the C-terminal tail of the FSHR did not affect either the agonist or the PMA-induced phosphorylation (Fig. 4) and that the phosphoamino acid analysis (not shown) and peptide maps of the FSHR phosphorylated in response to FSH or PMA (not shown) are very similar. Thus, it appears that regardless of the stimulus used (i.e. FSH or PMA), the FSHR becomes phosphorylated on serine/threonine residues present in one or more of the three cytoplasmic loops and/or in Ser626, the only potential phosphorylation site that remains in the C-terminal tail of FSHR-t635 (c.f. Fig. 2). These findings also suggest that at least some of the serine/threonine residues phosphorylated in response to either stimuli are the same. While this conclusion also remains to be confirmed, it is interesting to note that in the closely related LHR, the PMA- or agonist-induced phosphorylation maps to the same four serine residues in the C-terminal tail (11). In contrast to the similarities in the phosphoamino acid analysis and phosphopeptide maps of the FSHR isolated from cells stimulated with PMA or FSH, there is a rather obvious difference in the effects of FSH or PMA on the desensitization of the FSHR. A preincubation of cells with FSH leads to a reduction in the FSH-induced cAMP accumulation at all concentrations of FSH tested (Fig. 7), while a preincubation of cells with PMA leads to a reduction in the FSH-induced cAMP accumulation only at low concentrations of FSH (Fig. 8). This and other differences between the functional effects of PMA and FSH (25) suggest that the sites phosphorylated in response to PMA stimulation are not identical to those phosphorylated in response to FSH stimulation. This suggestion is also supported by the finding that protein kinase C is only partially responsible for mediating the FSH-induced phosphorylation of the FSHR (25).

In summary, then, the data presented herein have eliminated the 25 serine/threonine residues present in the FSHR as potential phosphorylation sites of agonist- or PMA-induced phosphorylation and as mediators of agonist- or PMA-induced uncoupling of FSHR from adenyl cyclase. Additional studies will be needed to determine the location of the phosphorylation sites, to determine if the same sites are phosphorylated in response to FSH- or PMA-stimulation, and to ascertain the functional impact of phosphorylation on the functions of FSHR.

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REFERENCES

1. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) FASEB J. 4, 2881–2889

2. Z. Wang, R. W. Hipkin, and M. Ascoli, unpublished observations.