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COMMENTARY

Functional Consequences of the Phosphorylation of the Gonadotropin Receptors

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ABSTRACT. When target cells are exposed to a hormone, their responsiveness wanes with time, in spite of the continuous presence of the hormone. This phenomenon, referred to as desensitization, is due to regulatory steps that occur at the level of the hormone receptor as well as at post-receptor steps. While post-receptor events may be specific for the metabolic pathways activated in a given target cell, receptor events are more general in nature and conserved within 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, referred to as receptor uncoupling, will be used here to denote 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 most proximal effector system. The other, referred to as receptor down-regulation, will be used here to denote 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 internalization, sequestration or degradation, and/or to a decrease in the rate of receptor externalization, processing or synthesis. In this paper, recent studies from my laboratory designed to directly address the potential involvement of gonadotropin receptor phosphorylation in the process of uncoupling have been reviewed. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1647–1655, 1996.

KEY WORDS. G protein-coupled receptors; phosphorylation; gonadotropins; desensitization; protein kinases; G proteins

It is now generally accepted that the phosphorylation of the β_2AR† that results as a consequence of agonist binding is an important event in the agonist-induced uncoupling of the β_2AR (reviewed in Refs. 1–3). The current model states that the phosphorylation of β_2AR is catalyzed by the agonist-induced activation of two different serine/threonine kinases: the PKA and the βARK. At low agonist concentrations, the β_2AR is phosphorylated by PKA at serine residues present in one or both of the PKA consensus sites present in its third cytoplasmic loop and in the proximal portion of the C-terminal cytoplasmic tail. The phosphorylation of these serine residues disrupts the coupling of the receptor to G_s. At high agonist concentrations, the dissociated βγ subunits of G_s recruit the βARK (βARK1 or βARK2, also known as GRK2 or GRK3, see Refs. 3 and 4) to the membrane. This second messenger-independent kinase further phosphorylates the agonist-occupied receptor on serine and/or threonine residues located in the extreme C-terminus. The phosphorylation of these residues also disrupts the coupling of the receptor to G_s and promotes the interaction of the receptor with β-arrestin, further uncoupling the receptor from G_s.

Although the phosphorylation of other 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 members of this family. Thus, in all other GPCRs that have been studied so far, the removal or mutation of potential phosphorylation sites leads to a decrease in agonist-induced phosphorylation, and an impairment in the process of agonist-induced desensitization (see Refs. 5–10 for a few recent examples). The involvement of phosphorylation in the uncoupling of GPCRs has also been conserved throughout evolution, since the phosphorylation of the α-factor receptor in *Saccharomyces cerevisiae* or the surface cAMP receptor in *Dictyostelium* is believed to be responsible for the desensitization or adaptation of the agonist-induced responses that are mediated by these GPCRs [11, 12].

As expected, these additional studies have revealed some interesting similarities and differences in the phosphorylation of different GPCRs that merit mention. For example, all of the GPCRs that have been examined thus far are substrates for GRKs, and this GRK-catalyzed phosphorylation is necessary for desensitization [3, 4]. Although GRK-catalyzed phosphorylation usually occurs in the C-terminal tail of these receptors [3, 4, 6], there are

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† *Abbreviations:* β_2AR, β_2-adrenergic receptor; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; βARK, β_2-adrenergic receptor kinase; GRK, G protein-coupled receptor kinase; GPCRs, G protein-coupled receptors; LHR, lutropin/choriogonadotropin receptor; rLHR, rat LHR; FSHR, follitropin receptor; rFSHR, rat FSHR; CG, choriogonadotropin; hCG, human CG; FSH, follitropin; hFSH, human FSH; PMA, phorbol 12-myristate-13-acetate; PKC, protein kinase C: PGE_2, prostaglandin E_2; and wt, wild type.

some GPCRs where GRK-catalyzed phosphorylation may occur in the third intracellular loop [13, 14]. In addition to GRKs and PKA, kinases such as PKC, a well as other unidentified kinases, are also involved in receptor phosphorylation [15–17]. These kinases may act independently [2] or sequentially [18] in the process of desensitization, and the phosphorylation events catalyzed by these kinases may or may not be redundant [2]. Lastly, other studies have also shown that unlike the desensitization of the β_2AR, which is partially mediated by PKA (see above), the desensitization of other receptors (such as the dopamine D_1 receptor) [19] does not require the participation of PKA. In summary, then, it is not yet known if the overall features of the model derived from the study of the β_2AR are readily applicable to other members of the GPCR family. This question is particularly relevant to members of the GPCR family that have or may have different mechanisms of activation. For example, the thrombin receptor is a member of the GPCR family that becomes activated by a tethered ligand that is generated by the thrombin-catalyzed proteolysis of the extracellular domain of this receptor [20]. Because of this different mechanism of activation, it was postulated that the processes involved in the desensitization of the thrombin receptor would be somewhat different than those involved in the desensitization of other GPCRs [20]. Recent studies, however, have shown that the thrombin receptor becomes phosphorylated in response to thrombin activation, and that receptor phosphorylation uncouples the thrombin receptor from its effector system [6].

The LHR and the FSHR are also unusual members of the GPCR family, because they bind their respective ligands through their large extracellular domains [21] rather than the transmembrane pocket [22, 23], and their mechanism of activation is also proposed to be more complex [24, 25] than that of GPCRs which bind small ligands. Thus, it is important to determine if the mechanisms involved in the uncoupling of these receptors are similar to those that have been described for other GPCRs. The need for further studies in this area is also underscored by conflicting data on the biochemical properties of uncoupling. Thus, while some investigators have reported that the gonadotropin-induced uncoupling in cell-free systems requires ATP [26–28], a finding that is consistent with the notion that a phosphorylation event is involved, other investigators have reported that the gonadotropin-induced uncoupling in cell-free systems requires GTP rather than ATP [29–33], a finding that is not consistent with the notion that a phosphorylation event is involved.

TRANSFECTED CELLS AS A MODEL SYSTEM TO STUDY PHOSPHORYLATION AND UNCOUPLING OF THE GONADOTROPIN RECEPTORS

Up until recently, studies on the post-translational modifications of the gonadotropin receptors had been hampered by the low abundance of endogenous gonadotropin receptors expressed in freshly isolated or transformed gonadal cells [34–37] and by the lack of receptor antibodies. Both of these problems were solved recently by using the information and reagents gained from the cloning of the cDNAs for these receptors (reviewed in Ref. 21).

Useful polyclonal antibodies to the rLHR have been obtained using homogeneous preparations of rLHR purified from rat corpora lutea as antigen, [38] or by using 10–15 residue peptides synthesized based on the amino acid sequence of the receptor [39, 40]. For the rFSHR, a useful polyclonal antibody was generated against a 10-residue receptor peptide as well [41].

Human kidney 293 cells permanently transfected with the rLHR or the rFSHR cDNAs and expressing large amounts (100,000–500,000 surface receptors/cell) of these receptors can also be readily obtained. Cells expressing the transfected rLHR (designated 293L) bind hCG with the expected high affinity (K_d = 200–500 pM) and respond to hCG with increases in cAMP (EC_{50} ~ 50 pM) and inositol phosphate accumulation (EC_{50} ~ 5000 pM) [42]. Likewise, cells expressing the transfected rFSHR (designated 293F) bind hFSH with the expected high affinity (K_d ~ 10,000 pM) and respond to hFSH with increases in cAMP (EC_{50} ~ 100 pM) and inositol phosphate accumulation (EC_{50} ~ 5000 pM) [43]. The equilibrium parameters that describe the binding and cAMP responses detected in transfected cells are similar to those detected in normal or transformed gonadal cells and clearly show that the recombinant gonadotropin receptors expressed in non-gonadal cell lines function similarly to the endogenous receptors expressed in gonadal cells. The data on the gonadotropin stimulation of the inositol phosphate pathway in transfected cells are difficult to relate to endogenous gonadotropin receptors because it is not quite clear if this pathway is gonadotropin-sensitive in gonadal cells [44–48]. What is clear from the studies from transfected cells, however, is that the gonadotropin stimulation of inositol phosphate accumulation is observed only at high concentrations of hormone and in cells that express large numbers of receptors [42, 49].

Because we are interested in examining the hypothesis that the phosphorylation of the gonadotropin receptors is responsible for uncoupling, we routinely use measurements of one of the most proximal events in signal transduction (i.e. cAMP accumulation) as an index of desensitization, and measurements of hormone binding as an index of down-regulation. As shown in Fig. 1, when 293L or 293F cells are stimulated with the appropriate gonadotropin, there is a fast reduction in the gonadotropin-sensitive adenylyl cyclase (i.e. desensitization) that cannot be accounted for by a change in the number of receptors (i.e. down-regulation). Thus, we can readily conclude that under these experimental conditions the gonadotropin-induced desensitization is mostly (or entirely) due to uncoupling rather than down-regulation. The existence of the uncoupling phenomenon can also be readily demonstrated

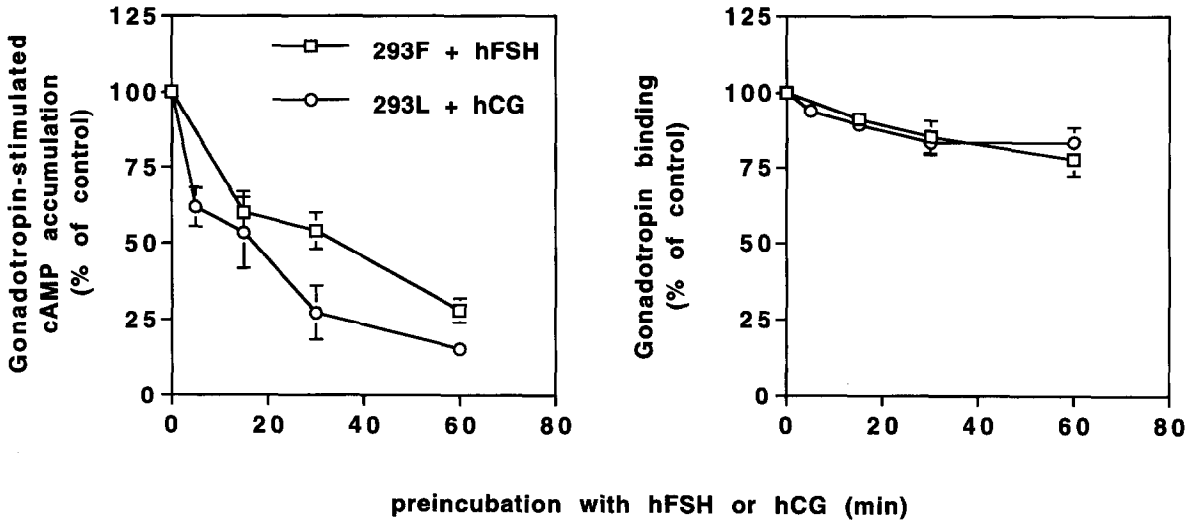

FIG. 1. Gonadotropin-induced desensitization of gonadotropin responses and down-regulation of gonadotropin receptors in human kidney 293 cells transfected with the rat gonadotropin receptor cDNAs. In the left panel, 293F or 293L cells were preincubated with 100 ng/mL hFSH or 100 ng/mL hCG, respectively, for the times indicated. The free hormone was removed by washing, and the receptor-bound hormone was removed during a brief incubation with an isotonic acidic buffer [37, 50, 51]. A second incubation was then performed in which each group of cells was divided into two groups and incubated with or without 100 ng/mL hFSH (293F cells) or 100 ng/mL hCG (293L cells) for 15 min prior to measuring intracellular cAMP. The amount of cAMP present in the cells incubated without hormone was subtracted from that present in the cells incubated with hormone, and this corrected response was expressed as a percentage of the response of cells that were treated similarly, but did not receive hormone during the preincubation. In the right panel, 293F or 293L cells were preincubated with 100 ng/mL hFSH or 100 ng/mL hCG, respectively, for the times indicated. The free hormone was removed by washing, and the receptor-bound hormone was removed during a brief incubation with an isotonic acidic buffer [37, 50, 51]. The density of cell surface gonadotropin receptors was then determined during an overnight incubation with 100 ng/mL of radiolabeled hFSH (293F cells) or radiolabeled hCG (293L cells). Results are expressed as the percent of the binding detected in cells that had been preincubated without hormone. The data presented were redrawn with permission from Ref. 43 [Hipkin RW, Liu X and Ascoli M, Truncation of the C-terminal tail of the follitropin (FSH) receptor does not impair the agonist- or phorbol ester-induced receptor phosphorylation and uncoupling. *J Biol Chem* 270: 26683–26689, 1995; © The American Society for Biochemistry & Molecular Biology] and from Ref. 52 [Wang Z, Hipkin RW and Ascoli M, Progressive cytoplasmic tail truncations of the lutropin-choriogonadotropin receptor prevent agonist or phorbol ester-induced phosphorylation, impair agonist- or phorbol ester-induced desensitization and enhance agonist-induced receptor down-regulation. *Mol Endocrinol* 10: 748–759, 1996; © The Endocrine Society].

by the finding that gonadotropins desensitize the homologous gonadotropin-responsive adenylyl cyclase in isolated membranes where receptor down-regulation does not occur [27]. Lastly, it is clear that the gonadotropin-induced uncoupling is mostly (or entirely) due to functional changes that occur at the level of the gonadotropin receptors rather than G_s or adenylyl cyclase. Thus, following incubation of cells or membranes with FSH or hCG, there is no change in the cAMP response induced by NaF, cholera toxin, or forskolin [53, 54]. Moreover, the functional activity of G_s from 293F membranes treated with FSH, as well as from 293L or Leydig tumor cells treated with hCG, has been measured directly by reconstitution of the isoproterenol-sensitive adenylyl cyclase in cyc^- membranes, and found to be normal [27, 54].

The 293L and 293F cells are useful models to study the uncoupling of the gonadotropin receptors because, like normal gonadal cells [46, 54–58], they uncouple in response to gonadotropins and during pharmacological activation of PKC with phorbol esters. The general properties of gonadotropin- or PMA-induced uncoupling are similar to those observed in gonadal cells, as illustrated in Fig. 2 for 293F and rat granulosa cells.

Having established that the 293L and 293F cells faithfully reproduce the process of uncoupling detected in normal gonadal cells, we set out to determine if the gonadotropin receptors become phosphorylated under conditions that result in receptor uncoupling. Using the receptor antibodies described above and 293L or 293F cells metabolically labeled with $^{32}P_i$, we were able to readily demonstrate that the rLHR and the rFSHR become phosphorylated when cells are exposed to the appropriate gonadotropin (i.e. hCG or hFSH, respectively) [42, 43, 52, 59, 60]. Increased phosphorylation of the rLHR or rFSHR can be detected within 2 min of exposure of 293L cells to hCG or of 293F cells to FSH, and the stimulated level of phosphorylation is sustained for at least 30 min [59, 60]. While we had reported initially that phosphorylation could be detected only at high concentrations of hormone, improvements in the methodology have now allowed us to detect receptor phosphorylation at lower hormone concentrations. The lowest concentration of gonadotropins at which

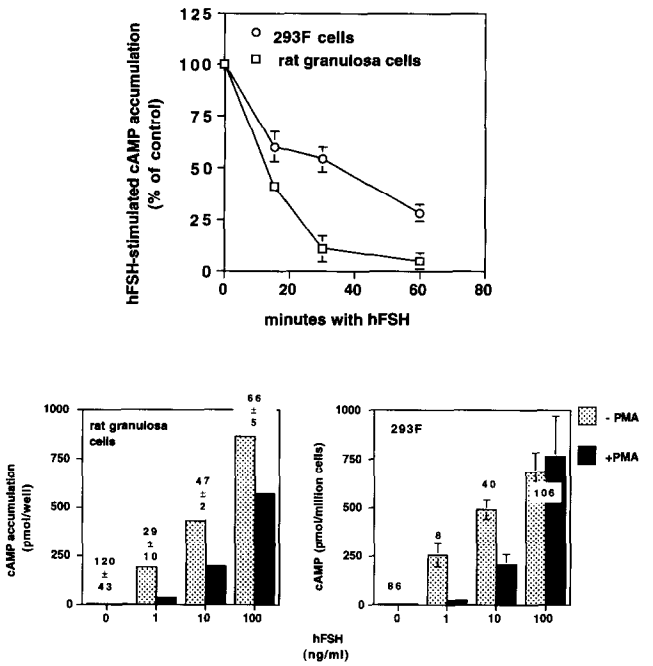

FIG. 2. Human FSH- and PMA-induced desensitization of hFSH responses in primary cultures of rat granulosa cells and human kidney 293 cells transfected with the rFSHR cDNA (293F). In the top panel, cells were preincubated with 100 ng/mL hFSH for the times indicated. The free hormone was removed by washing and the receptor-bound hormone was removed during a brief incubation with an isotonic acidic buffer [37, 50, 51]. A second incubation was then performed in which each group of cells was divided into two groups and incubated with or without 100 ng/mL hFSH for 15 min prior to measuring intracellular cAMP. The amount of cAMP present in the cells incubated without hFSH was subtracted from that present in the cells incubated with hFSH, and this corrected response was expressed as the percentage of the response of cells that were treated similarly, but did not receive hFSH during the preincubation. In the bottom panels, cells were preincubated with 200 nM PMA for 30 min in medium containing a phosphodiesterase inhibitor. The indicated concentrations of hFSH were then added, and the incubation was continued for 15 min prior to measuring total cAMP. For 293F cells, each bar shows the average ± SEM of three experiments, and the numbers shown above the bars represent the average percent desensitization calculated by comparing the cAMP response of cells preincubated with PMA with that of cells preincubated without PMA. For rat granulosa cells, the results of a representative experiment are shown, but the numbers shown above the bars represent the average (± range) percent desensitization obtained in two experiments. The data shown for 293F cells was redrawn with permission from Ref. 43 [Hipkin RW, Liu X and Ascoli M, Truncation of the C-terminal tail of the follitropin (FSH) receptor does not impair the agonist- or phorbol ester-induced receptor phosphorylation and uncoupling. *J Biol Chem* 270: 26683–26689, 1995; © The American Society for Biochemistry & Molecular Biology].

receptor phosphorylation can be detected is 1–3 ng/mL; the EC_{50} for receptor phosphorylation is 30–50 ng/mL and maximal phosphorylation is attained using gonadotropin concentrations of 100 ng/mL (Ref. 43 and unpublished observations). As already mentioned above, concentration–response curves for the hFSH- or hCG-induced increases in cAMP accumulation are characterized by EC_{50} values of 1–3 ng/mL.

Exposure of 293L or 293F cells to PMA also results in a robust and rapid phosphorylation of the rLHR or rFSHR, respectively. The time–course of PMA-induced phosphorylation is similar to that of the gonadotropin-induced phosphorylation [59, 60]. Exposure of 293F cells to different concentrations of 8Br-cAMP, A23187, or PGE_2 (an agonist that increases endogenous cAMP levels in 293 cells) does not stimulate the phosphorylation of the rFSHR [60]. Likewise, 293L cells fail to respond to A23187 with an increase in the phosphorylation of rLHR [59]. Data on the effects of 8Br-cAMP and PGE_2 on the phosphorylation of rLHR are somewhat variable, but maximally effective concentrations of these two compounds appear to elicit a small increase in rLHR phosphorylation [59]. A summary of the different stimuli that increase the phosphorylation of the rLHR and rFSHR in transfected cells is shown in Table 1.

The finding that pharmacological activation of PKC, but not PKA, leads to the phosphorylation of the rLHR and rFSHR should not be used to argue for the involvement of PKC and the lack of involvement of PKA as mediators of the gonadotropin-induced phosphorylation of their cognate receptors. This issue is better addressed by determining if gonadotropin-induced receptor phosphorylation is decreased when either one of these two kinases is inhibited. To address the involvement of PKA, we examined gonadotropin-induced receptor phosphorylation in 293L or 293F cells that overexpress cAMP phosphodiesterase, a manipulation that prevents the expected increase in gonadotropin-induced cAMP accumulation [59–61]. 293F cells that overexpress cAMP phosphodiesterase respond normally to hFSH with an increase in rFSHR phosphorylation [60]. 293L cells that overexpress cAMP phosphodiesterase showed a small reduction in hCG-stimulated rLHR phosphorylation [59]. The meaning of this reduction is difficult to interpret, however, because "basal" phosphorylation was also affected. Thus, if the extent of hCG-stimulated rLHR phosphorylation is measured as a fold-stimulation over basal, rather than as an absolute level of stimulation, it can

TABLE 1. Effects of different stimuli on the phosphorylation of the rat gonadotropin receptors expressed in human kidney 293 cells

	Fold-increase in receptor phosphorylation	
Stimulus	**rFSHR**	**rLHR**
hCG	NT*	3–4
hFSH	6–8	NT
8Br-cAMP	1	1–2
PGE_2	1	1–2
A23187	1	1–2
PMA	4–6	6–12

Note that 1 means no increase in receptor phosphorylation.
* NT = not tested.

be concluded that the overexpression of cAMP phosphodiesterase has little or no effect on the hCG-induced phosphorylation of the rLHR. To address the involvement of PKC we examined gonadotropin-induced receptor phosphorylation in 293L or 293F cells that been pretreated with a high concentration of PMA, a condition that "down-regulates" PKC. Although this treatment clearly blocked the effect of a subsequent PMA addition on the phosphorylation of rLHR or rFSHR, it had no effect on the hCG-induced phosphorylation of rLHR and a small effect on the hFSH-induced phosphorylation of the rFSHR [42, 60]. The lack of involvement of PKC on the hCG-induced phosphorylation of the rLHR is also supported by the finding that hCG-induced receptor phosphorylation can be readily detected in transfected cells expressing a low density of rLHR (where hCG-induced increase in inositol phosphates cannot be detected) or when the rLHR has been mutated to render it inactive in eliciting increases in inositol phosphates [42, 52].

Based on these findings we conclude that the hFSH-induced phosphorylation of the rFSHR is not mediated by PKA. PKC appears to (directly or indirectly) mediate the actions of hFSH, but the contribution of this kinase is minimal. The converse appears to be true for the hCG-induced phosphorylation of the rLHR, which does not involve PKC, but probably needs a minimal contribution by PKA. In either case it is clear that another kinase(s) is the principal mediator of the phosphorylation of the gonadotropin receptors by their respective hormones (Table 2). Although the identity of this kinase(s) is currently unknown, it is likely that it will be a member of the GRK family (see above).

IDENTIFICATION AND MUTATION OF THE PHOSPHORYLATION SITES

The findings summarized above, together with our ability to express wild-type as well as mutant receptors, fulfill all the requirements needed to use a classical structure–function approach to test the hypothesis that phosphorylation of the gonadotropin receptors is responsible for uncoupling. The results of our experiments utilizing this approach are summarized below.

TABLE 2. Involvement of different protein kinases on the gonadotropin-induced phosphorylation of the gonadotropin receptors

	Percent of gonadotropin-induced phosphorylation of:	
Protein kinase	**rFSHR**	**rLHR**
PKA	0	0–20
PKC	10–30	0
Other	70–90	80–100

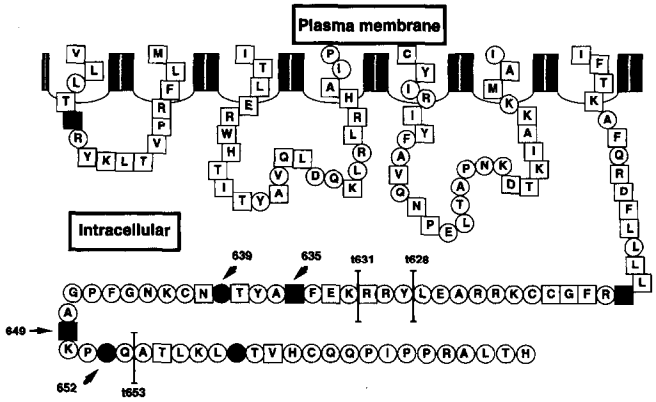

FIG. 3. Intracellular regions of the rLHR. The amino acid sequence of the intracellular regions (i.e. the three cytoplasmic loops and the C-terminal cytoplasmic tail) are shown. The 7 potential phosphorylation sites (i.e. serine residues) are shaded, and the 4 serine residues identified as phosphate acceptors in PMA- or hCG-stimulated cells (S^{635}, S^{639}, S^{649} and S^{652}) are marked with arrows. The location of truncation sites discussed in the text (t628, t631 and t653) are also shown. Amino acids enclosed in squares are identical in the rLHR and rFSHR. Those enclosed in circles are different. The amino acid sequence shown was taken from Ref. 62.

The rLHR

Phosphoaminoacid analysis of the rLHR phosphorylated in response to hCG or PMA stimulation revealed the presence of phosphoserine only [42]. Thus, one or more of the 7 serine residues in the intracellular regions of the rLHR have to be considered as potential phosphorylation sites (Fig. 3). In an initial attempt to localize the phosphorylation sites of the rLHR and to determine if phosphorylation is responsible for uncoupling, we constructed and analyzed three rLHR mutants with progressive deletions of the C-terminal cytoplasmic tails (Fig. 3 and Refs. 42 and 52]. A truncation at residue 653 (designated rLHR-t653) removes only 1 serine residue (S^{659}). The truncation at residues 631 or 628 (rLHR-t631 and rLHR-t628) remove 4 additional serine residues (S^{635}, S^{639}, S^{649} and S^{652}).

The truncated receptors were shown to bind hCG with the same affinity as the wild-type receptor, and clonal cell lines expressing a similar density of cell surface receptors were obtained and matched with clonal cells expressing an equivalent density of wt-rLHR prior to further analysis. The maximal cAMP response to hCG and the EC_{50} for this response in cell lines expressing rLHR-t653, rLHR-t631, or rLHR-t628 were found to be similar to those of cells expressing rLHR-wt [42, 52]. The inositol phosphate responses to hCG in cells expressing rLHR-t631 or rLHR-t628 were also comparable or higher than those of cells expressing rLHR-wt, but the inositol phosphate response to hCG of cells expressing rLHR-t653 was severely blunted compared with that of cells expressing rLHR-wt [42, 52].

When tested for phosphorylation, we found that the magnitude of the hCG- or PMA-induced phosphorylation in cells expressing rLHR-t653 was similar to that of cells

expressing rLHR-wt. Basal or stimulated phosphorylation was undetectable in cells expressing rLHR-t631 or rLHR-t628, however [42, 52]. Lastly, our experiments also showed that the hCG- and PMA-induced uncouplings are fully preserved in cells expressing rLHR-t653 (the phosphorylation-positive mutant), but are blunted in cells expressing rLHR-t631 and rLHR-t628 (the two phosphorylation-negative mutants). While the PMA-induced uncoupling is severely impaired or absent in the phosphorylation-negative mutants, only the rate of the hCG-induced uncoupling is affected in these mutants [52, 53]. For example, after a 5- and 60-min preincubation with hCG, the subsequent cAMP response to hCG is reduced to about 50 and 16% of control, respectively, in cells expressing rLHR-wt (cf. Fig. 1). In contrast, a 5- or 60-min preincubation with hCG reduces the subsequent cAMP response to hCG to about 90 and 36% of control, respectively, in cells expressing the phosphorylation-negative mutants [52]. This delay in the time–course of gonadotropin-induced uncoupling (as opposed to a change in magnitude) upon removal of phosphorylation sites is not peculiar to the rLHR nor unexpected from the data previously reported for the β_2AR [63]. In fact, removal or mutation of the C-terminal phosphorylation sites of the β_2AR was shown to delay the onset of agonist-uncoupling but had little or no effect on the magnitude of uncoupling provided upon prolonged incubation with agonist [63].

Our finding that phosphorylation affects mostly the time–course of hCG-induced uncoupling of the rLHR probably provides an explanation for the conflicting functional data reported by us when using rLHR-t631 and rLHR-t628 [52, 53] and Zhu *et al.* using mLHR-t628 [64]. Zhu *et al.* concluded that truncating the C-terminal tail of the mLHR at residue 628 did not prevent hCG-induced uncoupling, but their assays were performed at a single and late time point following exposure to a high hCG concentration. Our data argue that their conclusions would have been different had they examined a more complete time–course of uncoupling.

Taken together these results document that the PMA- and hCG-induced phosphorylation of the rLHR maps to the same area of the receptor, and implicate serines 635, 639, 649, and 652 as potential phosphorylation sites. This is an interesting finding in view of the fact that PKC does not appear to mediate the hCG-induced phosphorylation of the rLHR (see above). It is important to stress, however, that it is possible that the PMA- or hCG-induced phosphorylation maps to different serine residues within this locus. It is also clear that the phosphorylation of this locus is necessary for the PMA- or hCG-induced uncoupling of the rLHR. While the phosphorylation of this locus may also be sufficient for the PMA-induced uncoupling, the phosphorylation of this locus does not appear to be sufficient for the hCG-induced uncoupling.

All the data obtained with the C-terminal truncations of rLHR, however, have been confirmed recently using a full-length rLHR in which the 4 serine residues identified as potential phosphorylation sites were mutated simultaneously to alanine residues.[4]

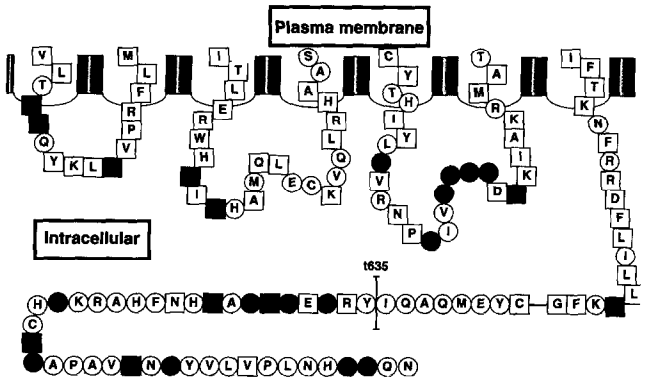

FIG. 4. Intracellular regions of the rFSHR. The amino acid sequence of the intracellular regions (i.e. the three cytoplasmic loops and the C-terminal cytoplasmic tail) are shown. The 25 potential phosphorylation sites (i.e. serine and threonine residues) are shaded. The location of the truncation site discussed in the text (t635) is also shown. Amino acids enclosed in squares are identical in the rLHR and rFSHR. Those enclosed in circles are different. A gap (designated by a dash) was introduced in the amino acid sequence for optimal alignment. The amino acid sequence shown was taken from Ref. 65.

The rFSHR

Phosphoaminoacid analysis of the rFSHR obtained from hFSH- or PMA-stimulated cells revealed the presence of both phosphoserine and phosphothreonine [60]. Thus, in contrast to the rLHR where only 7 serine residues needed to be considered as potential phosphorylation sites (cf. Fig. 3), we have to consider 14 serine and 11 threonine residues in the rFSH as potential phosphorylation sites (Fig. 4).

Since 13 of these 25 potential phosphorylation sites are present in the C-terminal cytoplasmic tail of the rFSHR, a mutant rFSHR cDNA with a truncated C-terminal cytoplasmic tail was constructed and analyzed [43]. This mutant cDNA encodes for a protein (designated rFSHR-t635) in which 12 of the 25 intracellular serine/threonine residues were removed (Fig. 4) and is analogous to two rLHR truncations (rLHR-t631 and rLHR-t628) already discussed above. Cells expressing rFSHR-t635 bind hFSH with the expected high affinity, and clonal cell lines expressing a high density of cell surface rFSHR-t635 were obtained and matched with a clonal cell line expressing an equivalent density of rFSHR-wt prior to further analysis. The EC_{50} values for cAMP and inositol phosphate accumulation in both cell lines were 2–5 ng/mL and 150–250 ng/mL hFSH, respectively, but the maximal cAMP and inositol phosphate responses were somewhat higher in cells expressing rFSHR-t635 than in cells expressing rFSHR-wt [43].

Immunoprecipitation experiments utilizing cells express-

[4] Wang Z, Liu X and Ascoli M, Manuscript submitted for publication.

ing rFSHR-t635 labeled with radioactive phosphate revealed that this truncated receptor is phosphorylated normally in cells stimulated with hFSH or PMA, suggesting that the 12 serine/threonine residues removed from this receptor are not phosphorylated [43]. A more complex interpretation of these results is that this C-terminal truncation of the rFSHR resulted in the exposure of phosphorylation sites that are not accessible in the full-length receptor. If this is true, then rFSHR-wt and rFSHR-t635 would be phosphorylated to the same extent but on different sites. We consider this possibility unlikely because we can detect both phosphoserine and phosphothreonine in rFSHR-t635 and rFSHR-wt and because both receptor species generate a similar phosphopeptide map upon cleavage with *N*-chlorosuccinimide [43]. Lastly, when tested for PMA- or hFSH-induced uncoupling, we found that rFSHR-t635 behaves very similar to rFSHR-wt.

Thus, in contrast to the rLHR, where some truncations of the C-terminal cytoplasmic tail prevent phosphorylation and retard or prevent the gonadotropin- or PMA-induced uncoupling, respectively, an equivalent truncation of the rFSHR does not affect phosphorylation or uncoupling.

SUMMARY

While it is now clear that both gonadotropin receptors become phosphorylated in transfected cells exposed to the cognate gonadotropin or to PMA, the identities and locations of the phosphorylated residues are different. In rLHR, hCG- or PMA-induced phosphorylation occurs on 1 or more of 4 serine residues: S^{635}, S^{639}, S^{649} and/or S^{652} (Fig. 3). In rFSHR, hFSH- or PMA-induced phosphorylation occurs on serine and threonine residues present in 1 or more of the 3 cytoplasmic loops and/or in S^{624} in the C-terminal cytoplasmic tail (Fig. 4). This finding is perhaps not surprising when one considers that the extent of amino acid sequence identity is only about 50% in the intracellular regions of these two receptors (Ref. 21 and Figs. 3 and 4).

Functional correlates of phosphorylation are rather clear for the rLHR, in that removal or mutation of phosphorylation sites affect the PMA- or hCG-induced uncoupling of the rLHR from adenylyl cyclase. The PMA-induced phosphorylation of S^{635}, S^{639}, S^{649}, and/or S^{652} appears to be necessary and sufficient for the PMA-induced uncoupling. On the other hand, while the hCG-induced phosphorylation of this locus is also necessary for the hCG-induced uncoupling, it is certainly not sufficient. Thus, other hCG-induced post-translational modifications of the rLHR need to be considered as contributors to the process of gonadotropin-induced uncoupling.

The possibility that phosphorylation of the rFSHR is involved in the hFSH- or PMA-induced uncoupling of the rFSHR from adenylyl cyclase cannot be tested formally until we identify the rFSHR residues that become phosphorylated. We are in the process of identifying these residues, however, and, as mentioned above, we have already eliminated 12 of the 25 serine/threonine residues present in the intracellular regions of this receptor as potential phosphorylation sites.

Lastly, although our initial efforts were concentrated on determining if the phosphorylation of the gonadotropin receptors is involved in uncoupling, we are now also considering the possibility that receptor phosphorylation affects other properties of these receptors (such as internalization).

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