Using a cell line stably transfected with the rat follitropin (FSH) receptor cDNA we demonstrate that the FSH receptor becomes phosphorylated when cells are exposed to FSH. Since binding of FSH to its receptor results in an increase in cAMP and inositol phosphate accumulation, we examined the potential involvement of protein kinase A and C in mediating receptor phosphorylation. Stimulation of protein kinase A does not appear to be necessary because hFSH-induced receptor phosphorylation was minimally impaired in a cell line that overexpresses cAMP phosphodiesterase. Moreover, stimulation of the protein kinase A pathway with other agonists result in minimal phosphorylation of the FSH receptor. Stimulation of the protein kinase C with a phorbol ester did result in an increase in receptor phosphorylation, and down-regulation of the protein kinase C decreased, but did not abolish, the FSH-induced receptor phosphorylation.

The possible impact of phosphorylation on the functions of the receptor was examined by testing if conditions that lead to phosphorylation decrease the ability of FSH to stimulate cAMP synthesis. Our data show that as with the addition of FSH, addition of a phorbol ester also results in a decrease in the ability of FSH to stimulate cAMP synthesis.

It is now generally accepted that phosphorylation of catecholamine receptors is one of the events involved in the termination of catecholamine actions. A number of studies conducted using the β2-adrenergic receptor (reviewed in Refs. 1-4) gave rise to a model wherein receptor phosphorylation is catalyzed by two distinct classes of serine/threonine kinases which appear to be necessary because hFSH-induced receptor phosphorylation is minimally impaired in a cell line that overexpresses cAMP phosphodiesterase. Moreover, stimulation of the protein kinase A pathway with other agonists result in minimal phosphorylation of the FSH receptor. Stimulation of the protein kinase C with a phorbol ester did result in an increase in receptor phosphorylation, and down-regulation of the protein kinase C decreased, but did not abolish, the FSH-induced receptor phosphorylation.

The possible impact of phosphorylation on the functions of the receptor was examined by testing if conditions that lead to phosphorylation decrease the ability of FSH to stimulate cAMP synthesis. Our data show that as with the addition of FSH, addition of a phorbol ester also results in a decrease in the ability of FSH to stimulate cAMP synthesis.

It is now generally accepted that phosphorylation of catecholamine receptors is one of the events involved in the termination of catecholamine actions. A number of studies conducted using the β2-adrenergic receptor (reviewed in Refs. 1-4) gave rise to a model wherein receptor phosphorylation is catalyzed by two distinct classes of serine/threonine kinases which appear to be necessary because hFSH-induced receptor phosphorylation is minimally impaired in a cell line that overexpresses cAMP phosphodiesterase. Moreover, stimulation of the protein kinase A pathway with other agonists result in minimal phosphorylation of the FSH receptor. Stimulation of the protein kinase C with a phorbol ester did result in an increase in receptor phosphorylation, and down-regulation of the protein kinase C decreased, but did not abolish, the FSH-induced receptor phosphorylation.

The possible impact of phosphorylation on the functions of the receptor was examined by testing if conditions that lead to phosphorylation decrease the ability of FSH to stimulate cAMP synthesis. Our data show that as with the addition of FSH, addition of a phorbol ester also results in a decrease in the ability of FSH to stimulate cAMP synthesis.

It is now generally accepted that phosphorylation of catecholamine receptors is one of the events involved in the termination of catecholamine actions. A number of studies conducted using the β2-adrenergic receptor (reviewed in Refs. 1-4) gave rise to a model wherein receptor phosphorylation is catalyzed by two distinct classes of serine/threonine kinases which appear to be necessary because hFSH-induced receptor phosphorylation is minimally impaired in a cell line that overexpresses cAMP phosphodiesterase. Moreover, stimulation of the protein kinase A pathway with other agonists result in minimal phosphorylation of the FSH receptor. Stimulation of the protein kinase C with a phorbol ester did result in an increase in receptor phosphorylation, and down-regulation of the protein kinase C decreased, but did not abolish, the FSH-induced receptor phosphorylation.

The possible impact of phosphorylation on the functions of the receptor was examined by testing if conditions that lead to phosphorylation decrease the ability of FSH to stimulate cAMP synthesis. Our data show that as with the addition of FSH, addition of a phorbol ester also results in a decrease in the ability of FSH to stimulate cAMP synthesis.
EXPERIMENTAL PROCEDURES

Cells—The origin and handling of human embryonic kidney (293) cells permanently transfected with the rat FSH receptor cDNA (designated 293F(wt1)) are described elsewhere (29) and were a generous gift of Dr. Deborah Segaloff of this institution. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes, 10% newborn calf serum, 50 μg/ml gentamicin, and 700 μg/ml G418 in a humidified atmosphere containing 5% CO₂. An additional clonal line of 293F(wt1) cells which overexpress cAMP phosphodiesterase was obtained by re-transfection (24) of the 293F(wt1) cells with an expression vector for the rat CAMP phosphodiesterase 3 (designated 293F(wt1)+P) and is maintained in the medium described above further supplemented with 250 μg/ml hygromycin B.

Experiments were done using cells that had been plated 3–4 days earlier. Phosphorylation experiments were done using cells plated in 100-mm dishes. Experiments involving the measurements of inositol phosphates or cAMP were done using cells plated in 35-mm wells.

Labeling of Cells with ³²P, and Immunoprecipitation of the FSH Receptor—Cells plated in 100-mm dishes were labeled in 4 ml of phosphate-free Dulbecco's modified Eagle's medium containing 1% newborn calf serum and 100 μCi/ml [³²P]orthophosphate for 3 h at 37 °C under 5% CO₂. The medium was then replaced with nonradioactive medium containing the indicated stimuli, and the cells were further incubated at 37 °C. The plates were placed on ice, the medium was aspirated, and the cells were scraped into ice-cold buffer A (0.15 M NaCl, 20 mM Hepes, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, 1 mM leupeptin, 5 mM EDTA, 3 mM EGTA, 50 mM β-glycerophosphate, 10 mM sodium fluoride, 0.1 mM orthovanadate, and 100 μM okadaic acid. The cells were collected by centrifugation and lysed with buffer A supplemented with 0.5% Nonidet P-40. The lysate was then centrifuged at 100,000 × g for 30 min, and the protein content of the supernatants was assayed by the method of Bradford (27). Aliquots of the lysates containing equal amounts of protein (1–2 mg) were then adsorbed to and eluted from a wheat germ agglutinin-agarose column, and immunoprecipitated with an antibody to the FSH receptor (Anti-F) as described elsewhere (15, 28). Immunoprecipitates were resolved on sodium dodecyl sulfate-polyacrylamide gels in the presence of thiol-reducing agents. Autoradiograms of the dried gels were obtained using intensifying screens.

Other Methods—The methods used to measure cAMP (30), inositol phosphates (31), and adenyl cyclase activity have been described (11, 12). The different parameters that describe the concentration-response curves were determined using the curve-fitting routine of the computer program Deltagraph and the equations initially used by De Lean and co-workers (32). Phosphoamino acid analysis was performed using standard methodology as described elsewhere (33).

Hormones and Supplies—Purified hFSH (AFP-5720D) was kindly provided by the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases. The antibody to the FSH receptor has been recently described (28). The expression vector for the rat cAMP phosphodiesterase 3 (designated pCMV-rat-PDE3, see Ref. 25) was a gift of Dr. Marco Conti of Stanford University. The expression vector that confers hygromycin B resistance was generously donated by Dr. Bill Sugden of the University of Wisconsin. 8-Br-cAMP, phorbol 12-myristate 13-acetate (PMA), prostaglandin E₂, A23187, and protein A-agarose were obtained from Sigma. Okadaic acid and hygromycin B were from Calbiochem. Phosphate-free Dulbecco's modified Eagle's medium was from ICN. [³²P]Orthophosphate and myo-[³H]inositol were from DuPont NEN. Tissue culture plasticware and supplies were obtained from Corning and Life Technologies Inc., respectively.

RESULTS

Agonist-induced Phosphorylation of the FSH Receptor—In a recent publication, we described a polyclonal antibody (Anti-F) that can be used to immunoprecipitate the FSH receptor from metabolically labeled cells (28). Using this experimental approach we showed that the mature FSH receptor expressed by a cell line (designated 293F(wt1)) permanently transfected with the rat FSH receptor cDNA is a 74-kDa glycoprotein (28).

Fig. 1 shows that the FSH receptor is phosphorylated in 293F(wt1) cells incubated without hFSH, and that receptor phosphorylation is stimulated by hFSH. An increase in phosphorylation can be detected within 2 min of hormone addition, a maximal increase (about 4-fold over basal) is attained by 15 min and this increase is sustained for at least up to 30 min after hFSH addition. Receptor phosphorylation was not examined using incubations longer than 30 min. These short incubations were chosen to avoid or minimize the depletion of the surface receptors that occurs as a consequence of the receptor-mediated endocytosis of the hormone. Fig. 1 also shows that preimmune serum does not immunoprecipitate the phosphorylated FSH receptor from cells incubated without hFSH or from cells incubated with hFSH for 30 min. Last, although the 74-kDa band previously identified as the FSH receptor (28) is the most prominent band present in the Anti-F immunoprecipitates of ³²P-labeled cells, a 171-kDa band is also present. This band was detected in some, but not all, of the experiments presented here.
Phosphorylation of Gonadotropin Receptors

and it is not detectable in immunoprecipitates of [35S]cysteine-labeled cells or on Western blots (28). The identity of this protein(s) has not been investigated.

During a 15-min incubation an increase in receptor phosphorylation could be first detected with about 100 ng/ml hFSH, and the maximal response (a 4-fold increase in phosphorylation) appear to occur with 1000–2000 ng/ml hFSH (Fig. 2). The concentrations of hFSH required to elicit a detectable increase in receptor phosphorylation are rather high compared to those required to elicit an increase in cAMP synthesis, but are comparable to those required to increase inositol phosphates (see below).

Phosphorylation of the FSH Receptor by Stimulation of Second Messenger-activated Protein Kinases—In the next series of experiments, we examined the possibility that activation of the second messenger pathways utilized by FSH could result in phosphorylation of the FSH receptor. Since it is clear that the LH/CG (15, 18, 19) and the thyrotropin receptors (20, 21) mediate an increase in cAMP and inositol phosphate accumulation in transfected cells, we first examined the possibility that hFSH can also elicit these effects in 293F(wt1) cells.

The effects of hFSH on cAMP accumulation shown in Fig. 3 are consistent with previous data on the functional expression of the FSH receptor cDNA (34, 35) while the results presented with inositol phosphates demonstrate that hFSH stimulates this pathway in cells transfected with the FSH receptor cDNA. In agreement with results obtained with the thyrotropin and LH/CG receptors (18–21), the data presented in Fig. 3 show that the concentrations of hFSH needed to elicit an increase in inositol phosphates are higher than those needed to elicit an increase in cAMP accumulation.

A potential role of the cAMP signaling system in the agonist-induced phosphorylation of the FSH receptor was first examined using a novel cell line (designated 293F(wt1)+P), co-transfected with the rat FSH receptor cDNA and with a cDNA encoding for the rat cAMP phosphodiesterase 3 (25). The phenotype of 293F(wt1)+P cells is very similar to that of MA-10 cells transfected with the rat CAMP phosphodiesterase 3 (26), or to that of 293 cells co-transfected with the LH/CG receptor and the rat cAMP phosphodiesterase 3 (15). Thus, the cAMP response of 293F(wt1)+P cells to a saturating concentration of hFSH is only about 10% of the response of 293F(wt1) cells, but this response can be completely restored by addition of a phosphodiesterase inhibitor (data not shown). The data presented in Fig. 4 show that in spite of a 90% reduction in the cAMP...
Phosphorylation of Gonadotropin Receptors

**FIG. 4.** Effects of several stimuli on phosphorylation of the FSH receptor in 293F(wtl) and 293F(wtl)+P cells. Cells were metabolically labeled with [32P]orthophosphate for 3 h. Human FSH (1000 ng/ml), A23187 (10 μM), PMA (200 nM), 8-Br-cAMP (1 mM), or prostaglandin E2 (PGE2) (5 μM) were then added and the incubation was continued for an additional 15 min. Lysates were prepared and the FSH receptor was immunoprecipitated with Anti-F as described under “Experimental Procedures.” The results of a representative experiment showing only the relevant portion of the gel are presented.

response to hFSH, the degree of phosphorylation of the FSH receptor induced by hFSH in 32P-labeled 293F(wtl)+P cells is similar to that induced in the parental 32P-labeled 293F(wtl) cells. The data presented in Fig. 4 also show that activation of the cAMP signaling system with prostaglandin E2, or by addition of 8-Br-cAMP results in little or no increase in the phosphorylation of the FSH receptor in 32P-labeled 293F(wtl) cells. Fig. 4 also shows that addition of a phorbol ester (PMA) leads to phosphorylation of the FSH receptor, while addition of a calcium ionophore (A23187) has little or no effect. An inactive phorbol ester (4α-phorbol 12,13-diacetate) was also ineffective (data not shown). In other experiments (not shown), we examined receptor phosphorylation as a function of time (2-30 min) after addition of 1 mM 8-Br-cAMP, or in cells incubated for 15 min with increasing concentrations of 8-Br-cAMP (0.05-2.0 mM). At best, we noted only a small (less than 2-fold) increase in receptor phosphorylation.

A more detailed characterization of the effects of PMA on the phosphorylation of the FSH receptor are shown in Fig. 5. These effects are easily measurable, and are similar in duration and magnitude to those attained with hFSH. A 2-4-fold increase in receptor phosphorylation can be attained within 15 min of addition of PMA and this response is sustained for up to 30 min. The concentrations of PMA that elicit receptor phosphorylation are similar to those that induced phosphorylation of the LH/CG receptor in cells transfected with the LH/CG receptor cDNA, and to those that uncouple the LH/CG receptor from adenylyl cyclase in Leydig tumor cells (15, 36).

A further test for the possible involvement of the protein kinase C in the FSH-induced receptor phosphorylation was performed by assessing receptor phosphorylation in 293F(wtl) cells that had been preincubated with a high concentration of PMA to "down-regulate" protein kinase C activity (37, 38). As shown in Fig. 6 this procedure enhanced basal phosphorylation while effectively down-regulating the protein kinase C such that freshly added PMA was unable to increase receptor phosphorylation. In contrast, the hFSH-induced receptor phosphorylation was reduced, but not abolished.

Taken together these data show that addition of hFSH and PMA to 32P-labeled 293F(wtl) cells leads to increased phosphorylation of the FSH receptor. Our data also suggest that: (i) protein kinase C, but not protein kinase A, is involved in the

---

2 At the concentration used, prostaglandin E2 stimulates cAMP accumulation to about the same extent as maximally effective concentrations of hFSH.
Phosphorylation of Gonadotropin Receptors

**Fig. 7.** Phosphoamino acid analysis of the FSH receptor. 293F(wt1) or 293F(wt1)+P cells were metabolically labeled with [32P]orthophosphate for 3 h. Human FSH (1000 ng/ml) or PMA (200 nm) were then added as indicated, and the incubation was continued for an additional 15 min. Lysates were prepared and the FSH receptor was immunoprecipitated with Anti-F. Samples were electrophoretically transferred to polyvinylidene difluoride membranes, hydrolyzed, and subjected to thin-layer electrophoresis as described under “Experimental Procedures.” Only the relevant portion of a representative autoradiogram is shown. The direction of migration is from bottom to top and the position of migration of authentic standards is shown.

buffer only, or stimulated with hFSH or PMA. Analysis was also performed on 293F(wt1)+P cells incubated with or without hFSH. These results are presented in Fig. 7 and show that phosphoserine and phosphothreonine, but not phosphotyrosine, can be detected in the FSH receptor phosphorylated in response to hFSH or PMA stimulation.

**Functional Correlates of FSH Receptor Phosphorylation**—

The possible effects of receptor phosphorylation on receptor functions were tested by measuring the ability of FSH to stimulate cAMP synthesis. This parameter was chosen because it is known that preincubation with FSH leads to a subsequent decrease in the ability of FSH to stimulate adenyl cyclase (a phenomenon that we call uncoupling, see Refs. 11 and 12) and because agonist-induced phosphorylation of the β2-adrenergic receptor is responsible for a similar phenomenon (reviewed in Refs. 1–4). A correlation between hFSH-induced receptor phosphorylation and changes in receptor function is difficult to test, because we do not yet have a paradigm wherein hormone binding occurs but the receptor is not phosphorylated. The current experiments indicate that like receptor phosphorylation (cf. Fig. 4), the hFSH-induced uncoupling of the FSH receptor from adenyl cyclase is not impaired in the 293F(wt1)+P cells (data not shown). A correlation between PMA-induced phosphorylation and changes in receptor function was more directly ascertained in the experiments summarized in Fig. 8. These experiments show that preincubation of 293F(wt1) cells with PMA results in a subsequent decrease in the ability of hFSH to increase cAMP synthesis. This effect is noticeable at all concentrations of hFSH tested, but its magnitude appears to decrease as the concentration of hFSH increases. We made two additional observations that suggest that the functional effects shown in Fig. 8 are largely due to changes in the ability of the FSH receptor to activate cAMP synthesis rather than to changes in the effector system or to changes in hormone binding. In order to discern possible changes in the effector system, we examined the effects of PMA on the ability of increasing concentrations of cholera toxin to increase cAMP accumulation. The results (not presented) showed that a preincubation with PMA resulted in a 1.5–2.0-fold enhancement of the effects of cholera toxin on cAMP accumulation. This effect, which is opposite to that shown in Fig. 8, was detected at all concentrations of cholera toxin tested. Using experimental conditions similar to those used in Fig. 8, we also examined the effects of PMA on the binding on increasing concentrations of 125I-hFSH to the surface (39) of 293F(wt1) cells. The results (not presented) showed that PMA reduces 125I-hFSH binding by about 40% regardless of the concentration of hFSH used. While this reduction in binding may contribute to the reduced ability of hFSH to increase cAMP accumulation, it is not of sufficient magnitude to fully explain the drastic reduction in function detected at low concentrations of hFSH.

In additional experiments (not presented) we showed that the same experimental protocol used above to down-regulate the protein kinase C almost completely abolished the inhibitory effect of PMA on the FSH-induced cAMP accumulation shown in Fig. 8. These results correlate well with the inability of PMA to increase the phosphorylation of the FSH receptor following “down-regulation” of the protein kinase C (cf. Fig. 6). Since down-regulation of protein kinase C also reduced the ability of hFSH to increase phosphorylation of the FSH receptor (cf. Fig. 6), we attempted to use the same experimental paradigm to determine if a reduction in the FSH-induced phosphorylation of the FSH receptor resulted in a reduction in the magnitude of FSH-induced uncoupling. However, prior to performing these experiments, it was necessary to determine if the experimental approach used in Fig. 8 could be modified to study the FSH-induced uncoupling. To this end, 293F(wt1) cells were incubated with hFSH (first incubation) followed by removal of the free hormone by extensive washing with a isotonic buffer of neutral pH, and removal of the receptor-bound hormone by a brief exposure of the cells to an isotonic buffer at pH 3 (39). A second incubation of the cells with hFSH was then performed as shown in Fig. 8 and cAMP accumulation was measured. In spite of the fact that the acid treatment removes the majority of the receptor-bound hormone (as judged by measuring the release of 125I-hFSH) the use of the experimental approach used in Fig. 8 to study FSH-induced uncoupling was precluded because of the high “basal” levels of cAMP detected during the second incubation in the cells that had been preincubated with hFSH. This is probably due to the low levels of hFSH that are capable of increasing cAMP synthesis in intact cells (cf. Fig. 3). For example, if we use 100–1000 ng/ml hFSH in the preincubation used to induce uncoupling, a residual level of hFSH corresponding to only 1–0.1% (i.e. 1 ng/ml hFSH) is enough to significantly elevate the basal levels of cAMP observed during the second incubation (i.e. the incubation needed to measure uncoupling) because the EC₅₀ for the hFSH-induced increase in cAMP accumulation is only 2.9 ng/ml (cf. Fig. 3).

Another approach that can be used to study uncoupling is similar to that described above in which cells are preincubated with PMA or hFSH followed by extensive washing and removal of the receptor-bound hFSH. Unlike the approach described above, however, the second incubation is not done by measuring the effects of hFSH on cAMP accumulation in intact cells but rather by measuring the effects of hFSH on adenyl cyclase activity in cell membranes (11, 15). Under these conditions “residual hFSH” leftover from the first incubation is not much of a problem because the EC₅₀ for the hFSH stimulation of adenyl cyclase activity is about 50 ng/ml (12). As shown in Fig. 9 (upper panel) we can readily demonstrate FSH-induced uncoupling of the FSH-responsive adenyl cyclase using this approach. The results presented show that preincubation of 293F(wt1) cells with FSH has little or no effect on adenyl cyclase activity measured under basal conditions (i.e. in the presence of GTP) or when stimulated with NaF, but the magnitude of the FSH-stimulated adenyl cyclase activity is reduced by 40–60%. The same experimental approach does not allow for the measurement of the PMA-induced uncoupling, however (Fig. 9, lower panel). The basal, as well as the NaF- or FSH-stimulated adenyl cyclase activity of membranes prepared from PMA-treated 293F(wt1) cells is the same or higher than that of the control cells. We have published similar results.
for the LH/CG-sensitive adenyl cyclase (15). Thus, while the protocol described in Fig. 8 allows for the measurement of PMA-induced uncoupling, it cannot be used to measure FSH-induced uncoupling. Conversely, the protocol described in Fig. 9 allows for the measurement of FSH-induced uncoupling but it cannot be used to study PMA-induced uncoupling. Since we cannot measure the PMA-induced uncoupling using the protocol described in Fig. 9, we cannot use this approach to determine if down-regulation of the protein kinase C reduces the magnitude of the FSH-induced uncoupling because we cannot confirm that the PMA response is lost (or reduced) in the cells with down-regulated protein kinase C.

In summary, although we have been able to document that protein kinase C is partially responsible for the FSH-induced phosphorylation of the FSH receptor (cf. Fig. 6), we have not been able to determine to what extent this is responsible for the FSH-induced uncoupling of the FSH receptor from adenylyl cyclase. Since some of the biochemical properties of the PMA-induced uncoupling are different than those of the FSH-induced uncoupling (cf. Fig. 9) we can safely conclude that the FSH-induced uncoupling cannot be entirely explained by the activation of protein kinase C, however.

Finally, it is also possible that a preincubation with PMA or hFSH also affects the ability of hFSH to increase the levels of inositol phosphates. This possibility is difficult to test because PMA can also have direct effects on the different components of this effector system (i.e. the G protein(s) involved, phospholipase C, and/or the enzymes that degrade the inositol phosphates) (40) that cannot be independently ascertained. Thus, while the possible effects of PMA on the Gs/adenylyl cyclase effector system could be ascertained by using cholera toxin (see above) we do not know of another ligand (or pharmacologic agent) that could be used to test for these putative effects on the inositol phosphate cascade of 293 cells.

**DISCUSSION**

There are many biological effects that result from the binding of FSH to its cognate receptor. The results presented here add two novel effects to this list: an increase in inositol phosphate levels, and an increase in the phosphorylation of the FSH receptor.

The data showing that hFSH can increase inositol phosphate levels in cells transfected with the FSH receptor cDNA is consistent with recent observations made with the closely related LH/CG (18, 19) and the thyrotropin receptors (20, 21), and add this receptor to the growing list of G protein-coupled receptors that activate more than one signaling system (see Refs. 41–43 for additional examples).

From our perspective, the most interesting observation presented here is that the FSH receptor is phosphorylated in intact cells, and that its phosphorylation state can be modulated by stimulation with hFSH or with a phorbol ester. There are some important conclusions that can be made by comparing the data presented here with those previously published for the β-adrenergic receptor and the LH/CG receptor. First, there appears to be a clear difference between the phosphorylation of β-adrenergic and the FSH receptors in response to their respective agonists. In the case of the β-adrenergic receptor, protein kinase A is one of the two kinases involved in agonist-induced receptor phosphorylation (1–4) while the involvement

Fig. 8. Effect of PMA on hFSH-stimulated cAMP accumulation in 293F(wtl) cells. Cells were preincubated for 15 min at 37 °C with 0.5 mm 3-isobutyl-1-methylxanthine. PMA (200 nM) or vehicle were then added and the incubation was continued for another 30 min. At the end of this incubation (time = 0 in the figure) the cells received the indicated concentrations of hFSH and the incubation was continued for up to 15 min. Total cAMP (i.e. intracellular + extracellular) was measured at the times indicated as described under “Experimental Procedures.” Each point shows the average ± S.E. of six determinations (two experiments with triplicate wells in each). Note the different scales among the 3 panels.

Fig. 9. Effect of hFSH and PMA on adenylyl cyclase activity of 293F(wtl) cells. Cells were preincubated for 30 min at 37 °C with the indicated additions. In the top panel the concentration of hFSH used during the preincubation was 100 ng/ml. In the bottom panel, the concentration of PMA used during the preincubation was 200 nM. At the end of this incubation membranes were prepared and assayed for adenylyl cyclase activity (see “Experimental Procedures”) in the presence of 100 μM GTP, 100 μM GTP, and 1000 ng/ml hFSH, or 10 mM NaF as indicated. Each point shows the average ± S.E. of six determinations (two experiments with triplicate wells in each).
of this kinase in the phosphorylation of the FSH receptor appears minimal as demonstrated in the studies presented here. The same contrast can be made between the FSH receptor and the closely related LH/CG receptor, where experimental approaches similar to those utilized here lead us to conclude that protein kinase A is partially responsible for the hCG-induced phosphorylation of its receptor (15). Another potentially important difference between the phosphorylation of the two gonadotropin receptors is that the FSH receptor is phosphorylated in both serine and threonine residues (Fig. 7), while only phosphoserine can be detected in the phosphorylated LH/CG receptor. Second, while the β2-adrenergic receptor can be phosphorylated in vitro by protein kinase C, this kinase is not believed to be activated in cells stimulated with a β2 agonist or to be involved in the agonist-induced phosphorylation of the β2-adrenergic receptor in intact cells (44). In contrast, activation of the FSH receptor presumably results in the activation of protein kinase C because this receptor has now been shown to generate at least one of the two second messengers (inositol phosphates, see Fig. 8) associated with this signaling system. Moreover, as shown here, stimulation of intact cells with PMA results in phosphorylation of the FSH receptor while down-regulation of protein kinase C reduces the magnitude of the hFSH-induced receptor phosphorylation. Thus, it is likely that protein kinase C partly mediates the phosphorylation of the FSH receptor induced by hFSH. In this respect, the FSH receptor behaves in a similar fashion to the LH/CG receptor which is also phosphorylated in response to stimulation of cells with PMA (15). The putative roles of protein kinase A and C in the phosphorylation of the rat gonadotropin receptors are consistent with the existence of known consensus sequences for these kinases in the intracellular regions of these two receptors. Both gonadotropin receptors have only 2-3 weak consensus sequences for protein kinase A-catalyzed phosphorylation, but they have 10-12 weak and 1-2 strong consensus sequences of protein kinase C-catalyzed phosphorylation (see Refs. 15 and 16 for a partial list). Last, the results obtained in the cells with down-regulated protein kinase C suggest that, in addition to the protein kinase C, a second messenger-independent kinase(s), such as β-adrenergic receptor kinase, is also involved in the agonist-induced phosphorylation of the FSH receptor.

While the concentrations of hFSH required to increase receptor phosphorylation (EC50 ≥ 500 ng/ml) are much higher than those that elicited cAMP accumulation (EC50 = 3 ng/ml), they are comparable to those that elicit increases in inositol phosphate accumulation (EC50 = 100 ng/ml). These data are consistent with our conclusions regarding the lack of involvement of protein kinase A, and the involvement of protein kinase C as well as second messenger-independent kinases in the hFSH-induced receptor phosphorylation (see above). An additional interpretation of these results is that the occupied receptor is a better substrate than the free receptor for the receptor kinases. Since the phosphorylation experiments were done using short incubations when hormone binding is not expected to be at equilibrium, only high hormone concentrations are likely to lead to a high fractional occupancy of the receptor population that is presumably needed to detect phosphorylation.

While it is clear that phosphorylation is responsible for the agonist-induced uncoupling of the catecholamine receptors from their respective effectors (1-4), a cause-effect relationship between agonist-induced phosphorylation and uncoupling of the LH/CG and FSH receptors has yet to be established (this paper and Refs. 11, 12, and 15). A requirement for ATP during uncoupling of the gonadotropin receptors (12), however, is consistent with the possibility that agonist-induced phosphorylation is involved in the agonist-induced uncoupling of these receptors. The phosphorylation of gonadotropin receptors induced by their respective ligands or PMA stimulation does correlate with a functional uncoupling of these receptors from at least one of their effector systems (Fig. 8 and Refs. 12 and 15).

Clearly, more work is needed to fully understand the involvement of protein kinase A and C, as well as second messenger-independent kinases (such as a β-adrenergic receptor kinase-type kinase) in the hFSH-induced phosphorylation of the FSH receptor. The location of the phosphorylated serine and threonine residues also needs to be determined. The work presented here, however, sets the foundation for the rational construction of gonadotropin receptor mutants that may not be phosphorylated. Structural and functional analysis of the phosphorylation of these mutant receptors in response to several stimuli will now be used to identify the specific residues that are phosphorylated and to conclusively determine if receptor phosphorylation has an impact on receptor function.

Acknowledgments—We thank Drs. Sue Sierke and John Koland for help with the phosphoamino acid analysis, Cheri Beth Whaler for technical assistance, and Dr. Deborah L. Segaloff for the gift of 293F( wt1) cells and for a critical reading of this manuscript. The services and facilities provided by the Diabetes and Endocrinology Research Center of the University of Iowa (supported by National Institutes of Health Grant DK-25295) are also gratefully acknowledged.

REFERENCES

1. Pakaczewski, K., and Benovic, J. L. (1991) Trends Biochem. Sci. 16, 387-391
2. Haussler, W. F., Caron, M. G., and Lefkowitz, R. J. (1990) PASER J. 4, 2881-2889
3. Lefkowitz, R. J., Haussler, W. F., and Caron, M. G. (1990) Trends Pharmacol. Sci. 11, 190-194
4. Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264-1269
5. Zhou, X., and Fishman, P. H. (1991) J. Biol. Chem. 266, 7462-7468
6. Richardson, R. M., and Hasey, M. M. (1991) J. Biol. Chem. 267, 22249-22255
7. Richardson, R. M., Prasenjeek, J., and Hasey, M. M. (1993) J. Biol. Chem. 268, 10127-10132
8. Richardson, R. M., Kim, C., Benovic, J. L., and Hasey, M. M. (1993) J. Biol. Chem. 268, 13650-13656
9. Kameyama, K., Haga, K., Haga, T., Konati, K., Kataeda, T., and Fukada, Y. (1993) J. Biol. Chem. 268, 7753-7758
10. Kwatra, M., Schiwein, D. A., Scheuwers, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 9161-9164
11. Sánchez-Yagüe, J., Rodriguez, M. C., Segaloff, D. L., and Ascoli, M. (1992) J. Biol. Chem. 267, 7214-7218
12. Sánchez-Yagüe, J., Hipkín, R. W., and Ascoli, M. (1993) Endocrinology 133, 1007-1015
13. Rassmussen, R. V., and Fishman, P. H. (1994) Endocrinology 118, 2340-2348
14. Ekstrom, R. C., and Hunsicker-Dunn, M. (1992) Endocrinology 113, 2470-2474
15. Hipkín, R. W., Sánchez-Yagüe, J., and Ascoli, M. (1993) Mol. Endocrinol. 7, 832-836
16. Segaloff, D. L., and Ascoli, M. (1993) Endocr. Rev. 14, 324-347
17. Nagayama, Y., and Rapport, B. (1993) Mol. Endocrinol. 6, 145-156
18. Gudermann, T., Birnbaumer, M., and Birnbaumer, L. (1992) J. Biol. Chem. 267, 4479-4484
19. Gudermann, T., Nebels, C., Levy, F. O., Birnbaumer, M., and Birnbaumer, L. (1993) Mol. Endocrinol. 6, 272-278
20. Kosugi, S., Okajima, F., Ban, T., Hodaka, A., Shenker, A., and Kohn, L. D. (1992) J. Biol. Chem. 267, 24159-24166
21. VanSandt, J., Rapee, P., Lejeune, C., Maenhaut, C., Vassart, G., and Dumont, J. E. (1990) Mol. Cell Endocrinol. 74, R1-R6
22. McFarland, K. C., Spongell, B., Phillips, H. S., Kobler, M., Rosembult, N., Nikolaes, K., Segaloff, D. L., and Sebbar, P. H. (1989) Science 245, 494-499
23. Shibata, E. F., Matsuda, J. J., Volk, K. A., Collison, K. A., and Segaloff, D. L. (1992) Endocrinology 134, 379-381
24. Chen, C., and Okajima, H. (1987) Mol. Cell. Biol. 7, 2745-2752
25. Swinnen, J. V., Joseph, D. R., and Cani, M. (1999) Proc. Natl. Acad. Sci. U.S.A. 86, 8197-8201
26. Swinnen, J. V., D'Souza, B., Cotti, M., and Ascoli, M. (1991) J. Biol. Chem. 266, 14385-14389
27. Bradford, M. M. (1976) Anal. Biochem. 51, 248-254
28. Quintana, J., Hipkín, R. W., and Ascoli, M. (1993) Endocrinology 133, 2098-2104
29. Hipkín, R. W., Sánchez-Yagüe, J., and Ascoli, M. (1992) Mol. Endocrinol. 6, 2210-2218
30. Wang, Z., Wang, H., and Ascoli, M. (1993) Mol. Endocrinol. 7, 85-93
31. Ascoli, M., Pignaturo, O. P., and Segaloff, D. L. (1989) J. Biol. Chem. 264, 6674-6681
32. De Lean, A., Munson, P. J., and Rodbard, D. (1978) Am. J. Physiol. 235, 823-832

R. W. Hipkín, Z. Wang, and M. Ascoli, unpublished observations.
Phosphorylation of Gonadotropin Receptors

33. Hoelscher, S. R., and Ascoli, M. (1993) Endocrinology 132, 2229-2238
34. Sprengel, R., Braun, T., Nikolics, K., Segaloff, D. L., and Seeburg, P. H. (1990) Mol. Endocrinol. 4, 525-530
35. Braun, T., Schofield, P. R., and Sprengel, R. (1991) EMBO J. 10, 1885-1890
36. Reuss, R. V., and Patel, J. (1986) J. Biol. Chem. 261, 3051-3054
37. Solanski, V., Slaga, T. J., Callahan, M., and Huberman, E. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1722-1725
38. Ballister, R., and Rosen, O. M. (1987) J. Biol. Chem. 260, 15194-15199
39. Ascoli, M. (1982) J. Biol. Chem. 257, 13306-13311
40. Majerus, P. W., Connolly, T. M., Bamsal, V. S., Ichhorn, R. C., Ross, T. S., and Lips, D. L. (1988) J. Biol. Chem. 263, 3051-3054
41. Ashkenazi, A., Winslow, J. W., Peralta, E. G., Peterson, G. L., Schimerlik, M. I., Capon, D. J., and Ramachandran, J. (1987) Science 238, 672-675.
42. Cotecechi, S., Kobilka, B. K., Daniel, K. W., Nolan, R. D., Laperina, E. Y., Caron, M. G., Lefkowitz, R. J., and Regan, J. W. (1990) J. Biol. Chem. 265, 63-69
43. Chabre, O., Conklin, B. R., Lin, H. Y., Ladish, H. F., Wilson, E., Ives, H. E., Catanzariti, L., Hemminga, B. A., and Bourne, H. R. (1992) Mol. Endocrinol. 6, 551-556
44. Bourier, M., Leeb-Lundberg, L. M., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1987) J. Biol. Chem. 262, 3106-3113