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Modulation of FSH Receptor Phosphorylation Correlates with Hormone-Induced Coupling to the Adenylate Cyclase System

Natarajagounder Selvaraj and Abraham Amsterdam

Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

The authors have recently demonstrated that an inhibitor of protein phosphorylation, staurosporine (SSP), can dramatically enhance follicle-stimulating hormone (FSH) stimulated cyclic adenosine monophosphate (cAMP) accumulation in rat granulosa cell line (GFSHR-17) overexpressing about 20-fold FSH receptor than primary granulosa cells. Moreover, incubation with SSP can partially release the cells from FSH-induced desensitization. In this work, it was examined whether coupling of FSH receptor to the adenylate cyclase is correlated with the degree of receptor phosphorylation. Immunoprecipitation of FSH receptor after metabolic labeling of the cells with ^{32}P-orthophosphate revealed that preincubation of the cells with SSP resulted in pronounced reduction in FSH receptor phosphorylation compared to control cells, concomitantly with a dramatic increase in FSH-stimulated cAMP accumulation. In contrast, incubation of the cells with saturating dose of FSH, which leads to uncoupling between the receptor and the adenylate cyclase, resulted in enhanced receptor phosphorylation. Moreover, cells preincubated with FSH could be released from desensitization by further incubation with SSP and a significant reduction in FSH receptor phosphorylation. Immunostaining of the cells with FSH receptor antibody reveal a homogenous distribution of the receptor on the surface of SSP-treated cells. Some aggregation of the receptor was evident in control cells that were not treated with SSP. In contrast, massive clustering and capping of the receptor molecules were observed on the surface of FSH-stimulated cells. The current data suggest that phosphorylation–dephosphorylation of the receptor molecules play an important role in the degree of coupling between the receptor and the adenylate cyclase system. Moreover, desensitization to FSH stimulation that is implicated with high degree of receptor phosphorylation may lead to aggregation of the receptor molecules on the cell surface.

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Author to whom all correspondence and reprint requests should be addressed: Dr. A. Amsterdam, Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel. E-mail: lclesley@weizmann.weizmann.ac.il

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Introduction

Gonadotrophin receptors are members of the serpentine G-protein coupled receptors *(1,2)*. These receptors are characterized functionally by their association with adenylate cyclase through G-stimulatory (Gs) protein *(3,4)*. Continuous exposure of cells to gonadotrophin results in desensitization to the agonist, characterized by reduction in cyclic adenosine monophosphate (cAMP) accumulation *(5–8)*. This phenomenon occurs as a consequence of regulatory processes at the gonadotrophin receptor level that could be either caused by uncoupling of the receptor from the effector system *(9,10)*, or the aggregation and subsequent internalization of the hormone receptor complexes from the cell surface *(7,11)*. However, since gonadotrophin-induced desensitization could be demonstrated in isolated follicular membrane preparations, internalization may not serve as a crucial step in this process *(12,13)*. It was also demonstrated, in primary rat granulosa cells, that desensitization to the hormone precede internalization of the hormone-receptor complex *(6)*.

The gonadotrophin receptors possess multiple potential phosphorylation sites for protein kinase A (PKA) as well as protein kinase C (PKC) in the cytoplasmic loop and the C-terminal tail of the receptor *(1,14)*. It was observed that serine and threonine residues are phosphorylated in purified testicular and ovarian luteinizing hormone/chorionic gonadotrophin (LH/CG) receptor by the catalytic subunit of PKA *(15)*. However, it was recently demonstrated in human embryonic kidney cells transfected with LH/CG receptor, that hCG and phorbol ester-stimulated phosphorylation of the receptor maps to only serines 635, 639, 649, and 652 in the C-terminal tail of the receptor in intact cells *(16)*. Further, recent evidence suggests a positive correlation between receptor phosphorylation and desensitization to hCG *(17)*. In human embryonic kidney cells transfected with follicle-stimulating hormone (FSH) receptor, hFSH and phorbol ester can both phosphorylate and uncouple FSH receptor from the adenylate cyclase *(18,19)*. Phosphorylation of the FSH receptor was observed at both serine

and threonine residues of the receptor *(19)*. Removal of all but one of the potential phosphorylation sites in the C-terminal tail, by truncation of the FSH receptor at residue 635, had no effect on agonist-induced phosphorylation or uncoupling of the FSH receptor *(19)*.

A temporal relationship between LH and FSH receptor desensitization and receptor phosphorylation by PKC was demonstrated in human embryonic kidney cells transfected with LH or FSH receptor *(18,20)*; however, other experiments fail to show such a correlation between these processes *(16,21,22)*. Moreover, careful kinetic studies on gonadotrophin-induced desensitization in LHR 11/6 cells expressing murine LH receptor, show that this phenomenon occurs independently of PKA or PKC activity *(23)*.

It was recently demonstrated in rat granulosa cells overexpressing FSH receptor (GFSHR-17), that a potent kinase inhibitor, SSP, was able to enhance the hormone sensitive cAMP accumulation *(24)*. However, specific inhibitors of PKA and PKC had marginal or no effect on FSH-induced cAMP formation. In addition, SSP was able to markedly reduce the rate of FSH-induced desensitization suggesting a role for a specific SSP sensitive kinase in the FSH receptor desensitization.

In the present study, it is shown that enhancement of cAMP response to FSH in SSP-treated GFSHR-17 cells is correlated with a significant reduction in receptor phosphorylation, whereas stimulation of the cells with FSH, for a period which is sufficient to induce desensitization, resulted in pronounced enhancement of receptor phosphorylation. Thus, cycles of phosphorylation–dephosphorylation of the FSH receptor may play an important role in coupling of the receptor to the adenylate cyclase system.

Results

Effect of SSP on cAMP Accumulation and Phosphorylation of the FSH Receptor in GFSHR-17 Cells

In response to 30 min stimulation of FSH, there was an elevation in both secreted (Fig. 1A) and the intracellular levels (Fig. 1B) of cAMP. Preincubating the cells with SSP had no effect on the basal level of cAMP production. However, SSP preincubation increased the subsequent FSH-induced cAMP formation by about 180% compared to the FSH-stimulated cells, which were not preincubated with SSP (Fig. 1). This increase in cAMP was observed both in the cell culture medium and within the cells (Fig. 1A,B). In parallel, the degree of phosphorylation of FSH receptors was examined in the same batch of cells treated as above. Phosphorylation of the FSH receptor was observed even in cells that were not stimulated by FSH (Fig. 2A, lane 1). However, preincubation of the cells with SSP reduced the levels of receptor phosphorylation to 35% of control levels (Fig. 2A, lane 3 and Fig. 2B). A fourfold increase in receptor phosphorylation was evident subsequent to 30 min stimulation of the cells by FSH (Fig. 2A, lane 5 and Fig. 2B), whereas preincubation of the cells by SSP prior to FSH stimulation reduced the levels of phosphorylation by 31% (Fig. 2A, lane 7 and Fig. 2B). Lysates of cell membranes that were treated with normal rabbit serum instead of the FSH antiserum show no labeling in the appropriate lanes (Fig. 2A, lanes 2, 4, 6, and 8) at the expected position of the migration of the receptor molecule.

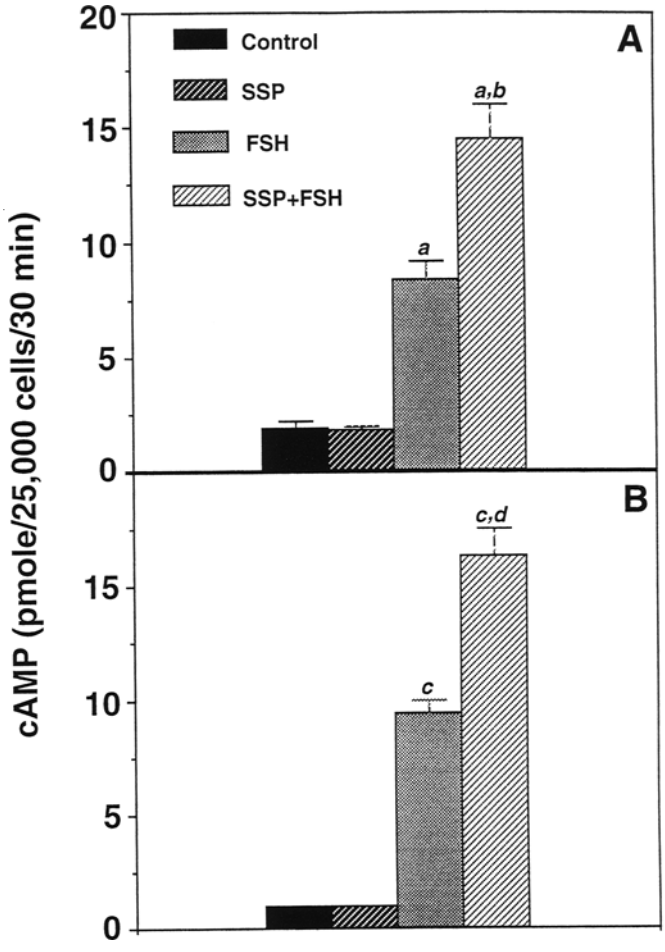

Fig. 1. Effect of SSP on cAMP accumulation in GFSHR-17 cells. Cells (25×10^3 cells/well) were preincubated in the presence or in the absence of SSP (50 n*M*) for 1 h and then the medium was replaced with or without hFSH (2.4 n*M*) for 30 min cAMP in the culture medium **(A)** and within the cells **(B)** were determined as described in the materials and methods. Data are mean ± SEM of triplicate cultures (SEMs in control and SSP groups are within the histogram bar). $^{a}p < 0.05$ compared to control; $^{b,d}p < 0.05$ compared to FSH alone; $^{c}p < 0.01$ compared to control.

Immunolocalization of the Receptor

In order to examine whether the degree of receptor activation and phosphorylation affect the distribution of the receptor molecules on the surface of GFSHR-17 cells, the cells were incubated with antibodies to the receptor and visualized the receptor molecule by immunofluorescence subsequent to incubation of the cells with FITC-coupled second antibody. As shown in Fig. 3, FSH receptor was localized uniformly on the cell membrane of most of the

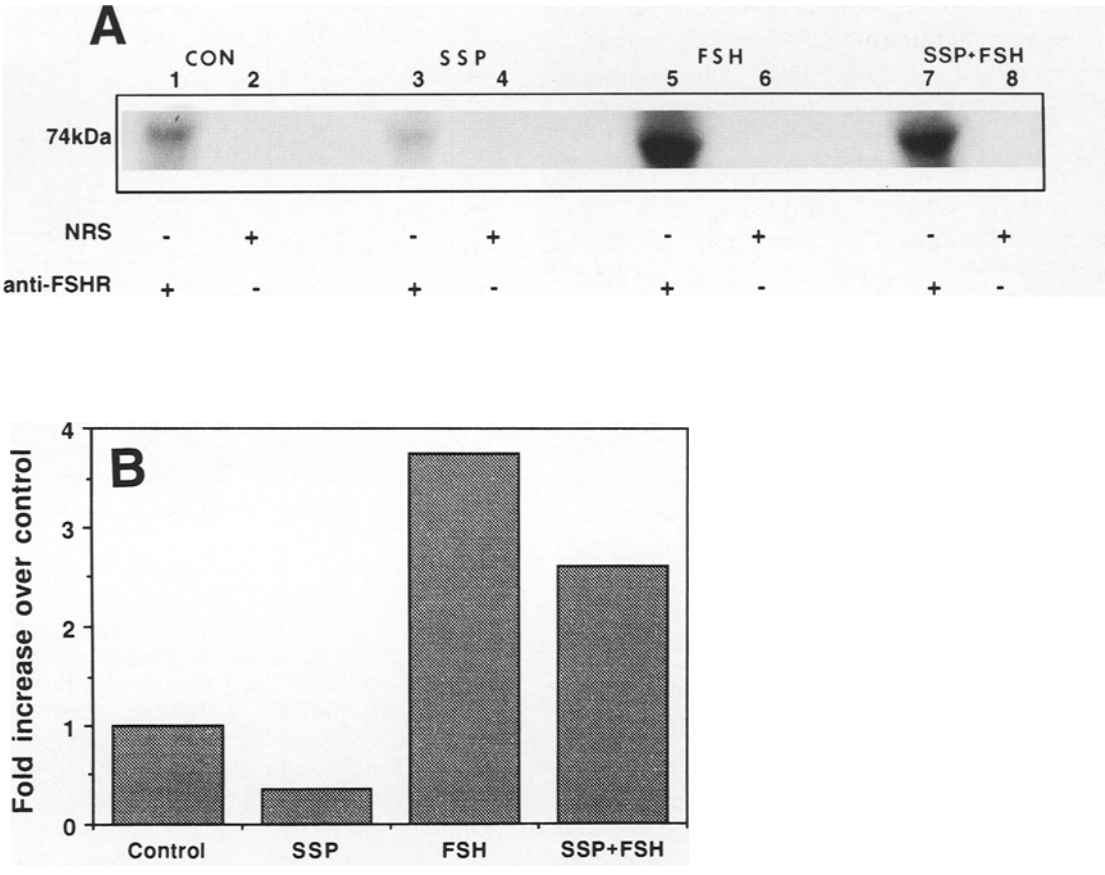

Fig. 2. Phosphorylation of the FSH receptor in GFSHR-17 cells. **(A)** Cells were metabolically labeled with ^{32}P-orthophosphate for 3 h and during the last 1 h they were preincubated with SSP when indicated (lanes 3, 4, 7, 8). Subsequently, the cells were stimulated with hFSH (2.4 n*M*) for 30 min. Cell lysates were prepared and ^{32}P-labeled proteins were immunoprecipitated with antibodies to FSH receptor (anti-FSHR) or normal rabbit serum (NRS) and resolved on SDS gel, as described in Materials and Methods. Labeled FSH receptor is seen as 74 kDa band. **(B)** Densitometric quantitation of the data represented as fold increase in FSH receptor phosphorylation over the control. The result of a representative experiment of two (which did not deviate by more than 15%) is presented.

cells. A significant portion of the cells, however, showed receptor clustering and capping at their circumference (Fig. 3A). Only traces of staining were observed when another cell line (GLHR-15) expressing the LH receptor, but not the FSH receptor, was examined indicating the specificity of the staining (Fig. 3B). No clustering of the receptor was evident in cells pretreated with SSP (Fig. 3C). Aggregation and capping of the receptor was evident following stimulation with FSH for 30 min (Fig. 3D). This phenomenon is reduced in cells incubated with SSP prior to FSH stimulation (Fig. 3E). As can be seen in Fig. 3F, the integrity of the cells following aldehyde fixation and the staining procedure was well preserved.

Statistical analysis carried on photographs of random fields of FSH-receptor immunostained cells showed that, whereas in nonstimulated cells, 34% of the cell population showed clustering and capping of the receptor, only 11% of receptor clustering was evident in cells pretreated with SSP. In contrast, 74% of cells treated with FSH showed clustering and capping of the FSH receptor. Treatment of FSH-stimulated cells with SSP reduced the incidence of capping of the FSH receptors to 59% (Fig. 4).

Discussion

In this paper, it is demonstrated that the degree of FSH receptor phosphorylation is correlated with coupling to the adenylate cyclase. Earlier publications revealed a correlation between PKC mediated receptor phosphorylation and desensitization of the gonadotrophin receptors to hormonal stimulation in intact cells *(18–20)*. However, Birnbaumer and his colleagues demonstrated clearly that in cells, where kinase activity is completely abolished, LH-induced desensitization can take place *(23)*. Moreover, Hunzicker–Dunn and her collegues, working in a cell-free system, showed that receptor desensitization is independent of PKA or PKC activity *(21,22)*. It was recently demonstrated in GFSHR-17 cells that activation or inhibition of kinase C could affect only moderately the rate of cAMP accumulation in FSH stimulated cells, whereas cell pretreatment with SSP enhanced dramatically cAMP accumulation in FSH-stimulated cells and also decreased significantly the rate of desensitization *(24)*. In this work, it is shown for the first time that FSH receptor is substantially phosphorylated in nonstimulated GFSHR-17 cells. Moreover, SSP can dra-

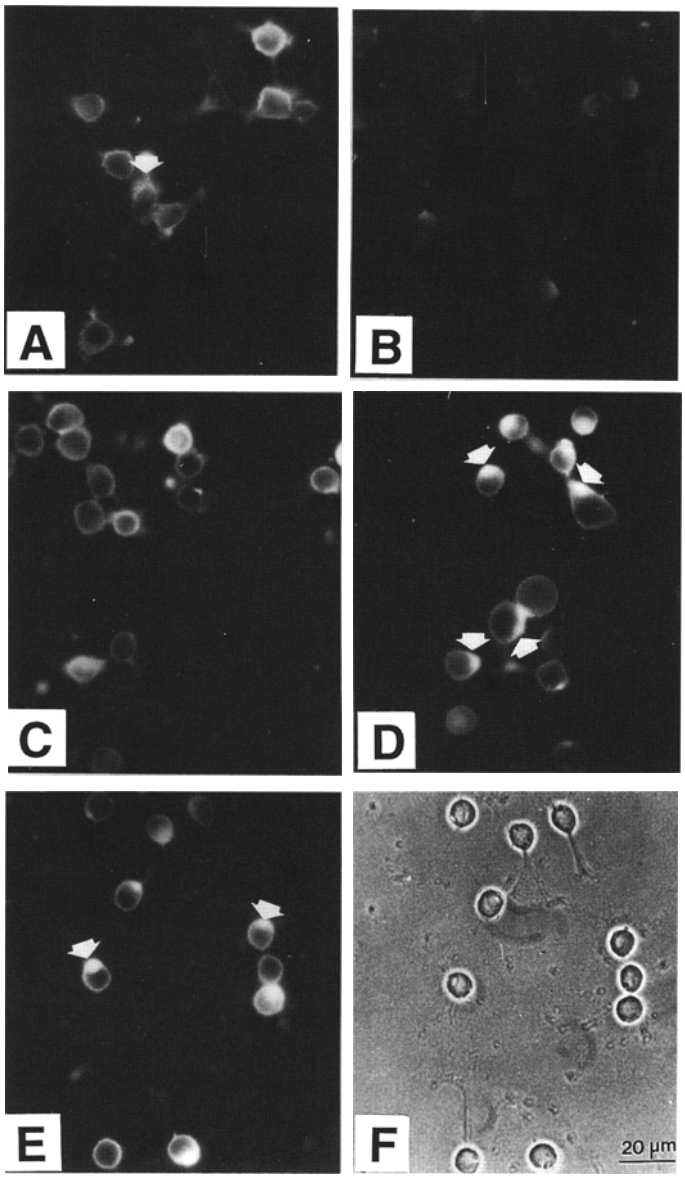

Fig. 3. Localization of FSH receptor in FSH responsive cells (GFSHR-17) by indirect immunofluorescent technique. **(A)** Nonstimulated GFSHR-17 cells show localization of the receptor at the circumference of the cells with moderate aggregation (arrow). **(B)** Control staining of GLHR-15 cells which express LH/CG receptor but not the FSH receptor. **(C)** GFSHR-17 cells treated with SSP. Note the homogenous distribution of the receptor at the circumference of the cells. **(D)** GFSHR-17 cells stimulated for 30 min with 2.4 n*M* of FSH. Note the capping of the receptor in one pole of the cell (arrows). **(E)** GFSHR-17 cells pretreated with SSP and FSH stimulated. Note that some cells show clustering of the receptor (arrows) and some show more homogenous distribution of the receptors on the cell surface. **(F)** the same field as in (E), but in phase contrast microscopy.

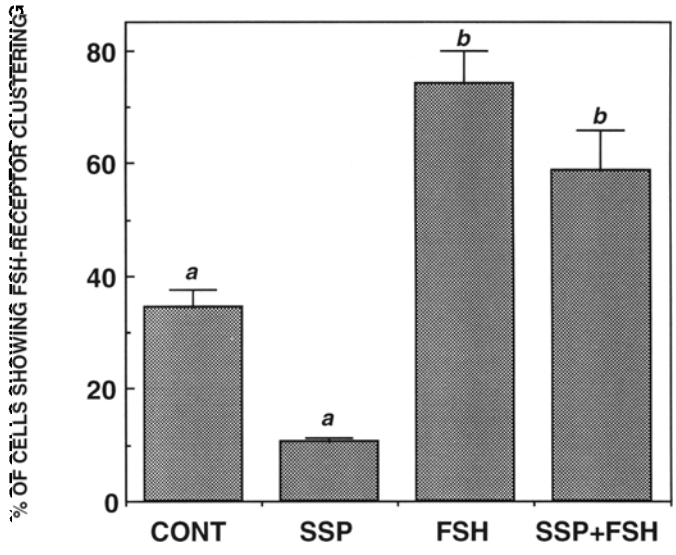

Fig. 4. Clustering of FSH receptors in FSH responsive cells (GFSHR-17). GFSHR-17 cell cultures were stained with anti-FSH receptor antibodies and visualized by indirect immunofluorescence technique. CONT, nonstimulated cells. SSP, cells incubated with SSP. FSH, cells stimulated with FSH; SSP + FSH, cells pretreated with SSP and FSH stimulated. a, $p < 0.001$ when CONT and SSP treatments are compared; b, $p < 0.02$ when FSH and FSH + SSP treatments are compared. ($p < 0.001$ when control is compared to FSH or FSH + SSP treatment.)

matically reduce the phosphorylation of the FSH receptor concomitantly with increase in cellular response to FSH stimulation. Since significant phosphorylation is observed in nonstimulated cells, but not treated with SSP, it is suggested that the nonstimulated receptor is already partially desensitized. Stimulation of the cells for 30 min with FSH, when the rate of cAMP accumulation in these cells is dropped sharply because of desensitization *(24)*, leads to a pronounced enhancement of receptor phosphorylation. This process can be partially blocked by preincubation of the cells with SSP, implying that a SSP-sensitive specific kinase may play a role in the desensitization phenomenon in parallel to the β-adrenergic receptor system where a specific kinase activity can modulate the coupling between the receptor and the adenylate cyclase system *(25)*. It is also important to note, from recent evidences in the α_2-adrenergic receptor system, that the composition of the plasma membrane can play an important role in the coupling mechanism *(26)*. It is, therefore, suggested that the cell line used in the present study obtained by transfecting granulosa cells with the FSH receptor *(27)*, resembles more closely the physiological system compared to the expression of the receptor in nonrelated, nonsteroidogenic cells *(18,20,23)*. Moreover, since GFSHR-17 cells contain 20 times more receptors than primary granulosa cells, the detection of changes in receptor phosphorylation and aggregation should be much more pronounced and could be measured more accurately than in primary granulosa cells.

SSP, at the concentration used in our experiment, may block kinase A, C, and tyrosine kinases in addition to a putative specific receptor kinase; therefore, it will be of importance to determine in the future which phosphorylation site(s) on the FSH receptor is responsible for the modulation of desensitization and can be inhibited preferentially by this alkaloid. It should be pointed out that in our previous

work using specific inhibitors to PKA and to a variety of tyrosine kinases, we clearly indicated that the inhibition of these kinase activities did not affect the cAMP response to FSH stimulation in GFSHR-17 line *(24)*.

It has been observed that the distribution of FSH receptors on cell membrane is changed following 30 min of incubation with a saturating dose of FSH, namely enhanced aggregation and capping of the receptor compared to a more uniform distribution of the receptor in nonstimulated cells. These findings correlated with previous observation that enhanced clustering is associated with desensitization to LH receptor *(11)*. It has also been demonstrated that stimulation of pituitary cells by gonadotrophin-releasing hormone (GnRH) results in microaggregation of GnRH receptor and desensitization to further agonist stimulation *(28)*. Dias and his coworkers observed discreet patches of receptor aggregates when recombinant FSH receptor was expressed in Chinese hamster ovarian (CHO) cells *(29)*. The more homogenous distribution of the receptor in nonstimulated GFSHR-17 cells compared to clustered appearance of the receptor in CHO cells may arise because of possible difference in lipid composition of the cell membrane. On the other hand, from the resolution obtained in the immunofluorescence, the possibility cannot be absolutely ruled out that the receptor is organized in tiny clusters even in nonstimulated GFSHR-17 cells. In any case, there is no evidence for receptor internalization and disappearance from the cell surface during 30 min of incubation of the cells with FSH. Internalization of the FSH receptor may occur only after prolonged incubation with FSH, when the system is already desensitized, similar to the kinetics of internalization that was demonstrated in earlier work on the LH receptor *(6)*. In that work, it was clearly demonstrated that uncoupling between the LH/CG receptor and the adenylate cyclase system occurred, whereas most of the receptors still remain on the cell surface. These observations were confirmed recently, demonstrating that receptor phosphorylation and desensitization was evident when most of the receptors have not yet disappeared from the cell membrane *(17)*. In analogy to the gonadotrophin receptor system, it was demonstrated in the pituitary gonadotrope that loss of the GnRH receptor by internalization cannot account for GnRH mediated desensitization *(30)*.

It can be concluded from the current work that both in LH and FSH receptor systems desensitization precede the internalization of the hormone-receptor complexes and that phosphorylation of the receptors by specific kinase may be responsible for the desensitization phenomena that is also implicated in a massive aggregation of the receptor molecules. However, the site of phosphorylation and the putative enzyme(s) involved may not be identical for both LH and FSH receptors, as there are several nonidentical potential phosphorylation sites in the FSH receptor compared to the LH/CG receptor *(16,19)*.

Materials and Methods

Materials

Human FSH (hFSH) was a kind gift from TEVA Pharmaceuticals Industries Ltd. (Petach Tikva, Israel). SSP was purchased from Boehringer Mannheim (Germany). Protein A-sepharose, IBMX, PMSF, HEPES, NP 40, leupeptin, and orthovanvadate were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Biological Industries Co (Beit Haemek, Israel). Anti-FSH receptor antibody (W971) was kindly provided by Dr. J. A. Dias (State University of New York, NY). (^{32}P) Orthophosphate was from Amersham (Buckinghamshire, UK).

Cell Culture

GFSHR-17 cells were obtained by triple transfection of rat preovulatory granulosa cells with SV 40 DNA, Ha *ras* oncogene, and an FSH receptor expression plasmid as described earlier *(27)*. Cells were cultured on 10-cm Nunc Petrie dishes containing 8 mL DMEM/F12 medium (1:1) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 5% FCS in an incubator with 5%CO_2. For cAMP measurement, cells (25×10^3 cells/well) were cultured in medium containing 5% FCS on Nunc 24-well plates. After 24 h, the cells were washed with PBS and preincubated with SSP followed by stimulation with FSH for 30 min in serum free medium.

Labeling of Cells with ^{32}Pi and Immunoprecipitation of the FSH Receptor

Cells were cultured in 10 cm plates until it reached about 80% confluency and washed thrice with warm PBS followed by labeling at 37°C with ^{32}Pi for 3 h in 4 mL of phosphate free medium containing 1% FCS and 300 µCi/mL (^{32}P) orthophosphate. SSP (50 n*M*) was added to the culture 2 h after the change of the medium. At the end of 3 h labeling of the cells, the medium was replaced with serum free nonradioactive medium and the cells were stimulated with FSH (2.4 n*M*) for 30 min in presence of 0.1 m*M* IBMX. Following stimulation, the plates were transferred to ice, washed thrice with ice cold PBS, and the cells scrapped in 1 mL lysis buffer (0.15*M* NaCl, 50 m*M* HEPES, pH 7.2 containing 2.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* PMSF, 10 µg/mL leupeptin, 30 m*M* sodium pyrophosphate, 30 m*M* sodium fluoride, 1 m*M* orthovanadate, 10% glycerol, and 1% NP40). The cells were lysed in ice and snap frozen in liquid nitrogen. After all the samples were collected and frozen, they were thawed and centrifuged in a microfuge for 20 min at 4°C. The supernatant was incubated with 50 µL normal rabbit serum for 1 h at 4°C followed by additional 10 min incubation with formalin-inactivated (10%). Staphylococcus aureus and the suspension was centrifuged in microfuge at 4°C. The supernatant was transferred to a fresh Eppendorf tube. At this stage, each of the samples were equally divided into two aliquots, one was precipitated with 50 µL anti-FSH-R antibody

(W971) and the other with equal volume of normal rabbit serum (control) for overnight at 4°C in an end-to-end shaker. The immunoprecipitate was isolated by specific and complete adsorption to a large excess of protein A-sepharose matrix *(31)*. The adsorbed immunoprecipitate was released from the matrix by boiling for 5 min with sample buffer, centrifuged, and the supernatant was resolved on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Autoradiogram of the dried gel were obtained using an X-ray film.

Immunofluorescence

Immunofluorescence were carried out as described earlier *(27,32)*. In brief, cells were cultured and stimulated on square coverglasses (22 × 22 mm) in presence of 5% FCS, fixed in 3% paraformaldehyde, and reacted with anti-FSH receptor antibody followed by second antibody conjugated to FITC. The cells were visualized using a Zeiss photomicroscope equipped with a vertical illuminator.

FSH Receptor Aggregation and Capping

For each treatment, 4–5 random fields of immunostained cells were photographed in the Zeiss fluorescent microscope at ×400 magnification. Cells showing clustering and capping of the receptor at the cell circumference, and total cell number, were scored in each field. For each treatment, about 100 cells were scored. The significance of differences in the percentage of cells with aggregated receptors was evaluated by paired, Student's *t*-test. Differences between the treatment groups were considered statistically significant at $p < 0.05$.

cAMP Assay

cAMP in the medium and in the cells was measured by a protein binding method *(6,32,33)*. Cell were cultured in DMEM/F12 containing 0.1 m*M* IBMX. For measurement of intracellular cAMP, cells were washed twice with PBS, lysed with 0.5 mL of 50 m*M* sodium acetate (pH 4) containing 0.1 m*M* IBMX, harvested into Eppendorf tubes using a rubber policeman, and heated for 10 min at 90°C. The cell debris was precipitated by centrifugation for 5 min and cAMP in the supernatant was measured.

Statistical Analysis

Analysis of cAMP concentration was performed by paired Student's *t*-test. Difference between the treatment groups were considered statistically significant at $p < 0.05$.

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