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Mechanisms of desensitization of follicle-stimulating hormone (FSH) action in a murine granulosa cell line stably transfected with the human FSH receptor complementary deoxyribonucleic acid

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Abstract

The desensitization of follicle-stimulating hormone (FSH)-evoked cAMP synthesis occurs upon continuous or repeated hormonal stimulation, and it involves the hormone-receptor interaction and post-receptor events. These mechanisms were studied in a murine granulosa cell line (KK-1) stably transfected with the human FSH receptor (hFSHR) complementary deoxyribonucleic acid (cDNA) under a powerful viral promoter. Hence, the FSHR transcriptional regulation was eliminated from the experimental model. Stimulation of the cells with recombinant human FSH (rhFSH) or a phorbol ester, 12-*O*-tetradecanoylphorbol-13 acetate (TPA), resulted in clear desensitization, i.e. subsequent rhFSH-stimulated cAMP formation was $73.4 \pm 2.2\%$, ($P < 0.001$) and $66.3 \pm 3.4\%$, ($P < 0.0001$), respectively, of that of cells preincubated in medium. TPA prestimulation evoked also clear inhibition (65–74% of control) of rhFSH or forskolin (a non-specific activator of adenylate cyclase) induced progesterone production. The suppression by TPA preincubation of the rhFSH-induced cAMP synthesis was completely abolished by the protein kinase C (PKC) inhibitor staurosporine (STR). Preincubation with STR exhibited a significant ($P < 0.0001$) increasing effect on the rhFSH-stimulated cAMP accumulation. The specific involvement of PKC was further evidenced by other inhibitors, all of them exerted significant elevation of cAMP synthesis following rhFSH restimulation. Furthermore, only the PKC β isoform appeared to be constitutively expressed in these cells during desensitization. Prestimulation of the G-protein activity by sodium fluoride (NaF) or cholera toxin (CT), followed by rhFSH challenge, accounted for a decrease in the cAMP-mediated responsiveness, down to 69.4 ± 2.8 or $74.2 \pm 1.9\%$ of control ($P < 0.001$), respectively, indicating that the post-receptor events are critical for desensitization. [^{125}I]iodo-rhFSH binding to the cells did not change significantly during desensitization and the different stimulations. In contrast, $\approx 50\%$ increase ($P < 0.001$) occurred in the steady-state levels of FSHR mRNA in the cells stimulated with FSH. This was apparently due to prolonged half-time of mRNA, and not to altered transcription, since the FSHR cDNA was driven by a powerful viral promoter. In accordance, the cells transfected with Simian Virus (SV40) promoter-driven luciferase gene did not display alterations in luciferase activity following stimulatory treatments. The effects of the post-receptor stimulations (NaF or CT) on [^{125}I]iodo-rhFSH binding were minor (8–12% reduction). Taken together, these data provide evidence that the agonist-responsive hFSHR desensitization appears through a PKC-β isoform-mediated modulation of cAMP production. The desensitization of FSH action involves modifications of functional properties of the existing components of the FSH signal transduction complex, and does not require concomitant suppression of transcription or translation of the FSHR gene.

Keywords: hFSHR expressing cell line; Desensitization; PKC; Uncoupling

1. Introduction

The response of the target cell to a hormone wanes upon repeated or prolonged stimulation due to regulatory modifications in the signal transduction cascade.

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This phenomenon is termed desensitization, and it may occur at several sites, including the receptor itself, and the various proximal and distal components of the signal transduction cascade. The process of desensitization seems to involve changes in functional properties of the persistent cell surface receptors (un-coupling) and it can be differentiated from the ligand-induced reduction in receptor density, i.e. down-regulation.

The relationship of the ligand-induced FSHR desensitization in Sertoli or granulosa cells to the second messenger responsiveness has been extensively studied, along with that of the structurally related receptor for luteinizing hormone/choriogonadotropin (LH/CG) and thyroid-stimulating hormone (TSH) in their respective target cells (Freeman and Ascoli, 1981; Huhtaniemi et al., 1982; Le Gac et al., 1985; Rebois and Fishman, 1986; Chazenbalk et al., 1990; Tezelman et al., 1994; Cadoret and Guillou, 1995). These receptors belong to the family of G-protein coupled seven transmembrane domain receptors and employ the adenylate cyclase (AC) signaling pathway (Dohlman et al., 1991; Segaloff and Ascoli, 1993; Baldwin, 1994). The activation of the transmembrane signaling is initiated by hormone binding to its receptors, after which Gs-protein activation at the cell membrane leads to the activation of AC. These events also involve Ca^{2+} mobilization, activation of the inositol phosphate cycle, and functional changes in cell membrane potassium and chloride channels (Mattioli et al., 1991; Flores et al., 1992; Gudermann et al., 1992). However, the specific roles of the different signaling systems in the diverse cellular responses to a specific hormonal stimulus are still poorly understood.

There is increasing evidence that the gonadotropin receptor desensitization occurs through alterations in the level of phosphorylation (Hipkin et al., 1993; Sánchez-Yagüe et al., 1993; Quintana et al., 1994; Keren-Tal et al., 1996). Receptor phosphorylation is mostly assumed to be involved in the uncoupling of signal transduction rather than receptor down-regulation (Benovic et al., 1985; Hipkin et al., 1995). The latter process involves receptor internalization and subsequent degradation upon prolonged stimulation (Freeman and Ascoli, 1981; Rodriguez et al., 1992; Rozell et al., 1995; Wang et al., 1996). In accordance with the β-adrenergic receptor (Bouvier et al., 1988; Hausdorff et al., 1990; Lohse et al., 1990; Lefkowitz, 1993), removal of the C-terminal tail of the LH/CGR, containing a phosphorylation site, delays the onset of agonist-induced desensitization (Sánchez-Yagüe et al., 1992). In contrast, in the case of FSHR, the respective phosphorylation site is not involved in the agonist or phorbol ester induced uncoupling (Hipkin et al., 1995).

Much evidence has been provided recently that the PKC signaling pathway plays an important role in desensitization of the agonist-induced AC activity (Rebois and Patel, 1985; Monaco and Conti, 1987; Inoune and Rebois, 1989; Themmen et al., 1991; Eskola et al., 1993; Würthner et al., 1995). PKC is a family of phospholipid-dependent serine–threonine kinases with isoforms that exhibit specific responses of cellular expression, localization and cofactor dependence, and they play a unique role in signal transduction (Nishizuka, 1992; Dekker and Parker, 1994; Nishizuka, 1995). Davis (1994) demonstrated that in gonadal cells LH/hCG appears to be capable of stimulating phospholipase C, indicating further that it is able to modulate the PKC activity. The increase in PKC activity by TPA leads to an inhibitory effect of LH/hCG stimulated cAMP formation and steroidogenesis in rat and mouse Leydig tumor cells (Mukhopadhyay and Schumacher, 1985; Themmen et al., 1986). Recent data on a human embryonic kidney cell line stably transfected with rat FSHR or LH/CGR demonstrate that both gonadotropins and phorbol ester are able to phosphorylate and uncouple FSHR and LH/CGR from the AC signaling pathway (Sánchez-Yagüe et al., 1993; Quintana et al., 1994; Hipkin et al., 1995; Selvaraj and Amsterdam, 1997). However, the precise identity of the PKC isoform involved in FSHR-mediated AC activity is yet to be determined.

Based on the available information, we found it important to examine the agonist-induced desensitization using a murine granulosa cell line [KK-1, (Kananen et al., 1995)] stably transfected with human FSHR cDNA, under a powerful viral promoter. The viral promoter maintains strong constitutive expression of the FSHR, which allows us to single out changes in the FSHR transcription from the desensitization mechanisms studied. The most intriguing observations presented herein are that hFSHR desensitization can be observed at the post-translational level in cells expressing this receptor in constitutive fashion. In addition, stimulation of the PKC-β isoform activity is the mechanism, at least in part, involved in the homologous desensitization of FSHR-mediated AC activity.

2. Materials and methods

2.1. Hormones and reagents

Recombinant human FSH (rhFSH, Org 32489, 10000 lU/mg) was kindly provided by Organon (Oss, The Netherlands). 12-*O*-tetradecanoylphorbol-13 acetate (TPA), staurosporine (STR), forskolin, 3-isobutyl-1-methyl xanthine (IBMX), sodium lauroylsarcosine, 2-mercaptoethanol, antifoam A, cholera toxin (CT), sodium fluoride (NaF) and geneticin (G418) were purchased from Sigma (MO). Guanidine thiocyanate was obtained from Fluka Chemie AG (Buchs, Switzerland). Na[^{125}l]-iodide and [$\propto$-^{32}P]-UTP (800 Ci/mmol) were purchased from Amersham (Amersham Bucks, UK) and [^{125}l]iodo-progesterone was obtained from Orion-

Farmos Diagnostica (Turku, Finland). Dulbecco's Modified Eagle's Medium (DMEM/F12) was obtained from Gibco (Scotland, UK), gentamycin from Biological Industries (Bet-Haemek, Israel), and the tissue culture plasticware from Greiner Labortechnik (Frickenhausen, Germany). All reagents used were of the best grade available.

2.2. Transfections and culture of stably hFSHR-transfected KK-1 cells

The KK-1 cells were derived from a murine granulosa cell tumor detected in a transgenic mouse expressing the SV40 virus T-antigen under the murine inhibin α-subunit promoter (Kananen et al., 1995). The cells were maintained in a growth medium [DMEM/F12, (1:1)] supplemented with 10% fetal calf serum (FCS; Biochlear, UK), gentamycin (0.1 g/l) and fungizone (2.5 mg/l), pH 7.4, at 37°C in a humidified atmosphere containing 5% CO_2. Transfection studies were initiated when the freshly plated cells (9 cm diameter) reached a confluency between 65–75% by using the lipofection method (Paukku et al., 1997). Six μg of hFSHR, inserted into expression plasmid, pSG5 (Stratagene, La Jolla, USA) were cotransfected with 0.6 μg of a neomycin resistance plasmid pPGKneobpA (Southern and Berg, 1982). Three μg of a β-galactosidase expression vector, pRSV-β-galactosidase (Promega, WI) were used to control the transfection efficiency. After 48–72 h of transfection, cells were studied for the transient hFSH receptor expression. For stable transfection, the cells were trypsinized and split into selection medium containing 600 mg/l of G418. The cells were maintained in the same medium for $\approx$2–3 weeks with changing the medium three times a week. Resistant colonies were isolated, expanded and screened for the hFSHR mRNA expression. The clonal line of choice (WT-11) was subsequently cultured for further studies in the medium containing 300 mg/l of G418.

To evaluate the involvement of the viral promoter in hFSHR transcription, the KK-1 cells were transiently transfected with pGL3 derived luciferase expressing reporter plasmid linked to SV40 promoter, under identical conditions, using lipofectamine (GibcoBRL, MD), and luciferase activity was determined. Two μg of FSHR expression plasmid pSG5-hFSHR, 0.4 μg of pPGKneobpa and 2 μg of reporter plasmid SV40-LUC were cotransfected together with 2 μg of pRSV-β-galactosidase, to correct for the possible variation in transfection efficiency. Forty-eight hours after transfection, the cells were examined for hFSHR expression, and followed by the stimulations with appropriate stimulants. The luciferase activity (chemiluminescence upon addition of luciferin substrate) was determined from the cell lysate, as described before, using 1251 luminometer (BioOrbit, Turku, Finland) (Koskimies et al., 1997). The corresponding β-galactosidase activity was measured from the same sample to correct for the variation in transfection efficiency.

2.3. Incubation and desensitization experiments

The stably transfected cells expressing hFSHR were detached from the plate at 85–90% confluency with 0.05% trypsin and their viability was examined by 0.4% trypan blue dye exclusion technique. The cells were cultured further in 24-well plates at a density of 6×10^4 cells/well and maintained in the regular medium $\approx$24 h before stimulations. Prior to stimulation, the cells were washed twice with 0.01 mol/l phosphate-buffered saline (PBS) and incubated in serum-free medium containing 0.1% BSA without or with increasing concentrations of rhFSH (0.1–1000 IU/l). A dose response curve for rhFSH stimulation was then determined in the presence of 0.5 mmol/l IBMX.

For the desensitization studies, prestimulated cells from the different groups, as indicated elsewhere, were washed twice with PBS and the medium was supplemented with the appropriate stimulants for another 20 min for cAMP, or 18 h for progesterone production. At the end of stimulation, the medium was collected, diluted (1:1) with 2 mmol/l theophylline for the determination of extracellular cAMP, and the cells were processed further for intracellular cAMP determination (see below). The media were collected, boiled and extracted for progesterone measurements.

2.4. Determination of the cAMP end progesterone levels

Extracellular cAMP was determined directly from the culture media and compared with the level of intracellular cAMP. For the measurement of intracellular cAMP, medium was removed and the cells were washed twice with cold assay medium containing 0.5 mmol/l IBMX. The washing medium was removed and intracellular cAMP was extracted by adding 0.5 ml of 10% TCA containing 2 mmol/l theophylline under ice. The cells were then scraped off under ice and centrifuged for 10 min at 4°C for $\times$3000 rpm. The supernatant was extracted 3 times with 5 volumes of diethyl ether and evaporated to dryness. After evaporation, the residues were dissolved in 0.5 ml of the cAMP assay buffer (PBS + 0.1% BSA). The insoluble residues were removed by centrifugation and 100 μl of the supernatant were directly employed in the assay. The levels of intracellular cAMP were determined using a standard radioimmunoassay method (Harper and Brooker, 1975).

For progesterone measurements, the media collected after 18 h were extracted with diethyl ether. The concentration of progesterone was determined by RIA as

described before (Vuorento et al., 1989). The reagents for progesterone RIA were obtained from Orion-Farmos Diagnostica (Turku, Finland).

2.5. [^{125}I]iodo-rhFSH binding experiments

Recombinant human FSH (rhFSH) was radio-iodinated with Na[^{125}I]-iodide (IMS 300, Amersham) using a solid phase lactoperoxidase method (Karonen et al., 1975). The specific activity of the labeled hormone was determined by a self displacement analysis in a radioreceptor assay using adult rat testis homogenate (Huhtaniemi et al., 1987) and was found to be 3782 cpm/ng, with maximum binding capacity of the [^{125}I]iodo-rhFSH to an excess of hFSHR was 20–30%. For the binding experiments, stably transfected cells were cultured in 9 cm plates in the regular medium and stimulated with or without the indicated substances. [^{125}I]iodo-rhFSH binding experiments were conducted according to an earlier report from this laboratory (Zhang et al., 1997) with minor modifications. Briefly, at the end of stimulation, the cells were washed, scraped off and resuspended in Dulbecco's PBS (DPBS) containing 0.1% BSA. The hFSHR expressing KK-1 cells, 0.25×10^6 (in triplicate), were incubated with [^{125}I]iodo-rhFSH (150000 cpm) either in the absence (total) or presence (non-specific) of 400-fold excess of the unlabeled rhFSH in a final volume of 250 μl. For determining the affinity and density of the receptors in response to rhFSH and PKC stimulation, Scatchard analysis was conducted by incubating similar aliquots of cell suspension with increasing amounts of [^{125}I]iodo-rhFSH (1.5–60×104 cpm/tube) along with fixed concentration of unlabeled rhFSH. The contents of the tubes were mixed by vortexing and incubated overnight at room temperature. The incubation was terminated by placing the tubes on ice, followed by the addition of 3 ml of ice-cold D-PBS containing 0.1% BSA. After centrifugation, the supernatant was discarded by careful aspiration, followed by washing of the pellet in the same medium. Finally, the radioactivity of the cell pellets was determined in a gamma spectrometer. Specific binding was corrected by subtracting the non-specific binding from total binding.

2.6. RNA extraction and Northem hybridization analysis

Total RNA was isolated from the control and stimulated cells by homogenization in denaturing solution containing 4 mol/l guanidine thiocyanate, 25 mmol/l sodium citrate, pH 7.0, 0.5% sodium laurosyl sarcosin, 0.7% 2-mercaptoethanol and 0.1% antifoam A, and extracted by using the method of Chomczynski and Sacchi (1987). The extracted RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and total RNA was quantified at 260 nm, and its purity was determined by scanning with spectrophotometer at wave length 220–320 nm. Twenty micrograms of the total RNA obtained from the different groups of experiments were resolved on 1.2% formaldehyde denaturing agarose gel and transferred onto Hybond-N$^+$ nylon membrane (Amersham) by employing the capillary transfer method. An antisense cRNA probe corresponding to bases of 169–778, a *Hind* III fragment of the hFSH receptor cDNA, was produced by in vitro transcription (Promega, WI) with T7 RNA polymerase, dNTPs and [$\propto$-^{32}P]-UTP (Amersham). The labeled riboprobes were purified by using Sephadex G-50 nick columns (Pharmacia, Sweden). Prehybridization and hybridization were carried out under stringent conditions as previously described (Rannikko et al., 1995). In brief, prehybridization was performed at least for 4 h at 65°C in a solution containing 50% formamide, 3 X SSC (150 mmol/l sodium chloride, 50 mmol/l sodium citrate, pH 7.0), 5 X Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 0.1 g/l heat-denatured calf thymus DNA, and 100 mg/l yeast tRNA. Hybridization was performed at 66°C in the same solution after the addition of [^{32}P]-labeled cRNA probe and continued for $\approx$16 h. The membranes were washed twice at room temperature for 20 min with 2 X SSC containing 0.1% SDS, followed by 2 h at 66°C with 0.1 X SSC and 0.1% SDS until the removal of the background counts. Membranes were then exposed to the X-ray film (Kodak XAR-5, Eastman Kodak Rochester, NY) for 36–48 h at -80°C. The relative levels of hFSHR mRNA were determined by densitometric scanning of the autoradiograms (TINA 2.0 package, Straubenhardt, Germany).

To determine the specific involvement of PKC, isoforms α, β, γ, δ, and ε were examined for analysis. Different EcoRI-EcoRI fragments of the rat PKC cDNAs (α, 1.3 kb; β, 1.7 kb; γ, 1.4 kb; δ, 25 kb; ε, 2.4 kb) were labeled with [$\propto$-^{32}P]-CTP (3000 Ci/mmol) by the random priming method using Klenow fragment (Promega). After 6 h of prehybridization of the membranes at 42°C, the labeled probes were added and hybridized overnight in a buffer containing the same ingredients used for prehybridization. After hybridization, the membranes were washed with a buffer containing $1 \times$ SSC and 0.1% SDS at room temperature for 15 min followed by the washing with $0.2 \times$ SSC and 0.1% SDS for 20 min twice at 42°C. Membranes were then exposed to the X-ray films and relative levels of PKC isoform messages were determined by densitometric scanning as mentioned earlier. To normalize the variation in the levels of hFSHR and PKC-β mRNA expression, the membranes were subjected to rehybridization to the cDNA probe for human GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

2.7. Presentation of the results

The results are expressed as means ± SEM from representative cultures carried out in triplicate or quadruplicate. All the experiments were repeated four to six times. Statistical analysis was performed by using one-way ANOVA and the post hoc Fisher's PLSD (Statview-4.51) test for the comparison of the means. $P < 0.05$ was considered statistically significant.

3. Results

3.1. rhFSH-stimulated cAMP formation in KK-1/hFSHR cells

The responses of extra- and intracellular cAMP levels to increasing concentrations of rhFSH (0.1–1000 IU/l) are shown in Fig. 1. The stimulatory effect of rhFSH on cAMP accumulation was significant within 5 min, the maximum response was reached within 15–20 min, and after ≈90 min there was a significant decline of intracellular cAMP response (data not shown). The amount of intracellular cAMP was ≈1.7-fold higher than the extracellular cAMP, measured at the ED_{50} concentration of rhFSH of 32 IU/l. To elucidate the regulatory events involved in the process of desensitization, we chose the intracellular cAMP as the parameter to be monitored.

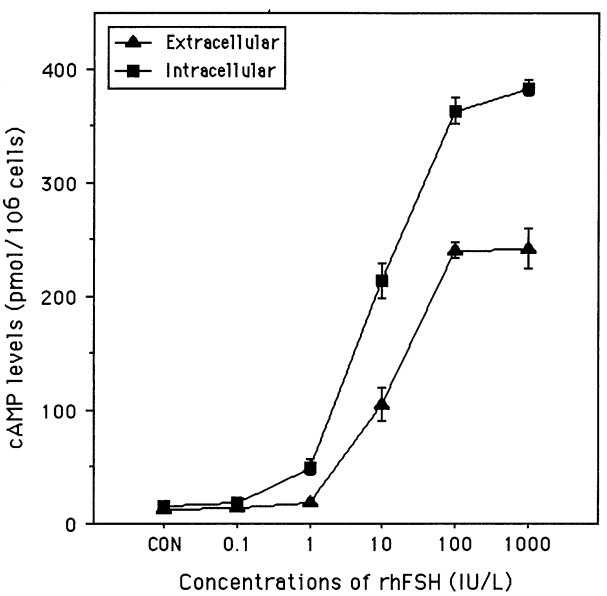

Fig. 1. The extracellular and intracellular cAMP levels of KK-1/hFSHR cells in response to rhFSH stimulation. The cells were cultured in 24-well plates at a density of 6×10^4 cells/well. After two washes with 0.01 mol/l PBS, the cells were stimulated for 20 min without (CON) or with increasing concentrations of rhFSH (0.1–1000 IU/l) in the presence of 0.5 mmol/l IBMX, and extracellular and intracellular cAMP levels were measured. The results represent the mean ± SEM of four independent experiments in quadruplicates.

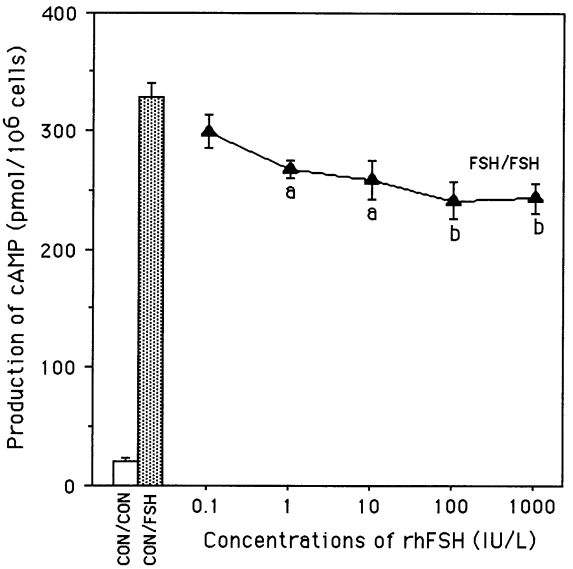

Fig. 2. Effect of preincubation of KK-1/hFSHR cells in the presence of rhFSH on their cAMP response to increasing concentrations of rhFSH in a second incubation. The cells were prestimulated with medium (CON) or rhFSH (100 IU/l) for 90 min. After washing the cells twice, one group was again stimulated for 20 min with the medium (CON/CON), the other control group for 20 min with 100 IU/l rhFSH (CON/FSH). The third group of cells which were prestimulated with rhFSH, were further stimulated for 20 min in the second incubation with increasing concentrations rhFSH (0.1–1000 IU/l) (FSH/FSH). Intracellular cAMP of the samples was determined. The data are expressed as the mean ± SEM of six independent experiments in triplicates. a, $P < 0.01$; b, $P < 0.001$ indicate significant differences from the CON/FSH level.

3.2. Homologous desensitization of rhFSH-stimulated cAMP production

Fig. 2 shows that when the KK-1/hFSHR cells were preincubated in the absence or presence of rhFSH (100 IU/l) for 90 min, followed by a second 20 min incubation with increasing concentrations of rhFSH (0.1–1000 IU/l), a clear desensitization was induced by rhFSH. The concentration of rhFSH in the first incubation (100 IU/l) was chosen based on its maximal ability to elicit cAMP synthesis. To evaluate the dose dependent effect of rhFSH in desensitization, varying concentrations of rhFSH were used in the second incubation. The cAMP response in desensitized condition, at the saturating dose (100 IU/l) of rhFSH after a 20 min of second incubation was 73.4 ± 2.2% ($P < 0.001$) of that measured in medium preincubated cells (CON/FSH). The high cAMP production in the second incubation with the low rhFSH concentrations (0.1 and 1 IU/l), as compared to the dose-response of Fig. 1, is explained by remaining receptor-bound FSH from the first incubation. The dose dependent decline of rhFSH-stimulated cAMP production in the second incubation thus indicates desensitization of these cells (Fig. 2). On the other hand, in cells pretreated with the lower concen-

trations of rhFSH (0.1–1.0 IU/l), desensitization could not be observed even after the subsequent second incubation with 100 IU/l of FSH (data not shown). The result implies that preincubation of the cells with hFSHR results in a dose-dependent loss of cAMP responsiveness to subsequent rhFSH stimulation.

3.3. rhFSH-induced cAMP synthesis and its relation to PKC

The relationship of the agonist-induced FSHR desensitization to PKC function was assessed next. The concentration of the PKC activator TPA (100 nmol/l), used in the present experiments was based on earlier observations from our laboratory (Nikula et al., 1990; Eskola et al., 1993). The concentration of STR, a PKC inhibitor (50 nmol/l), was based on its maximal effect on PKC activity (Rüegg and Burgess, 1989). After a prestimulation with TPA or STR, a second incubation for 20 min was carried out in the presence or absence of saturating concentration of rhFSH (100 IU/l), and the cAMP response was then determined. The function of the post-receptor part of the signal transduction cascade was examined by 50 μmol/l forskolin stimulation.

TPA pretreatment (30 or 60 min) significantly suppressed the subsequent rhFSHstimulated cAMP synthesis, but not that evoked by forskolin, which, in contrast, was enhanced (Fig. 3A). To eliminate the possibility of cAMP degradation, a phosphodiesterase inhibitor (0.5 mmol/l, IBMX) was included in the incubations. The inhibitory effect of TPA on rhFSH stimulation was similar after 30 or 60 min preincubations, $\approx$63–67%, as compared to the cells preincubated for 60 min with medium ($P < 0.0001$). In contrast, forskolin-stimulated cAMP production was significantly enhanced (127–131%) by pretreatment with TPA ($P < 0.0001$) (Fig. 3A). On the other hand, TPA prestimulation (30 or 60 min) significantly (65–74% of control) inhibited both rhFSH- and forskolin-stimulated progesterone accumulation in the media (Fig. 3B).

The possible effects of staurosporine were examined for comparison, as TPA exhibited an inhibitory effect on rhFSH-stimulated cAMP synthesis. STR significantly (143%; $P < 0.0001$) increased the rhFSH-induced cAMP production. Interestingly, the inhibitory effect of TPA on rhFSH-induced cAMP formation was completely abolished in combination with STR (Fig. 3C). Alternatively, the forskolin-stimulated cAMP production moderately increased by preincubation with TPA, STR and their combination ($P < 0.01$). Since STR increased the rhFSH-stimulated cAMP production, apparently by inhibiting PKC activity, we next examined the precise action of STR using highly specific inhibitors of PKC. It could be clearly seen that a 90 min prestimulation with PKC inhibitors i.e. STR (50 nmol/l), N-(2-aminoethyl)-5-isoquinoline-sulfonamide [(H-9), 1 μmol/l] (Hidaka et al. 1984), clelerythrine (5 μmol/l) (Herbert et al. 1990) and calphostin C (50 nmol/l) (Kobayashi et al. 1989), followed by an additional 20 min restimulation with rhFSH (100 IU/l) significantly elevated, by 126–143%, the cAMP levels, as compared to rhFSH stimulated controls (Fig. 4). In addition, inhibition of PKC activity evoked CT-stimulated cAMP accumulation in a dose-dependent manner, indicating its involvement directly at the Gs-protein level (data not shown).

3.4. Effects of rhFSH and the modulators of PKC activity on SV40 promoter activity

The possible changes in the activity of the viral promoter during the hFSHR expression was evaluated in transient transfection of the cells with an SV40 promoter-driven luciferase reporter construct as described in Section 2. The cells were preincubated for 90 min with medium (CON), rhFSH (100 IU/l), TPA (100 nmol/l) and STR (50 nmol/l), followed by reincubation with medium or the same stimulating factors in different combinations for an additional 20 min. The results of Table 1 clearly demonstrate that the relative levels of luciferase activity did not differ significantly between the different treatment groups, suggesting constitutive expression of the viral promoter-driven hFSHR cDNA during the progress of desensitization.

3.5. Determination of PKC isoforms expression

The expression of the PKC isoforms (α, β, γ, δ, and ε) was evaluated with Northern hybridization analysis. The KK-1/hFSHR cells were prestimulated for 90 min in medium (CON), rhFSH and TPA followed by a 20 min reincubation without or with rhFSH, at concentrations used in the previous experiments. Northern analysis demonstrated that among the isoforms tested, only the PKC-β mRNA was detected. The Eco Rl-Eco Rl fragment of rat PKC cDNA-β (1.7 kb) probe hybridized with a major RNA transcript of $\approx$2.6 kb size, and two minor transcripts of $\approx$2.1 and 1.8 kb sizes were detected with longer exposure (72 h) of the membranes (Fig. 5). TPA or rhFSH prestimulations increased the expression of all the trancripts by $\approx$ 40%, whereas an additional rhFSH incubation for 20 min simultaneously diminished the expression of the transcripts over TPA stimulation by 23–30%. On the other hand inhibition of PKC activity by STR diminished by $\approx$60% the expression of all the PKC-β transcripts (Fig. 5).

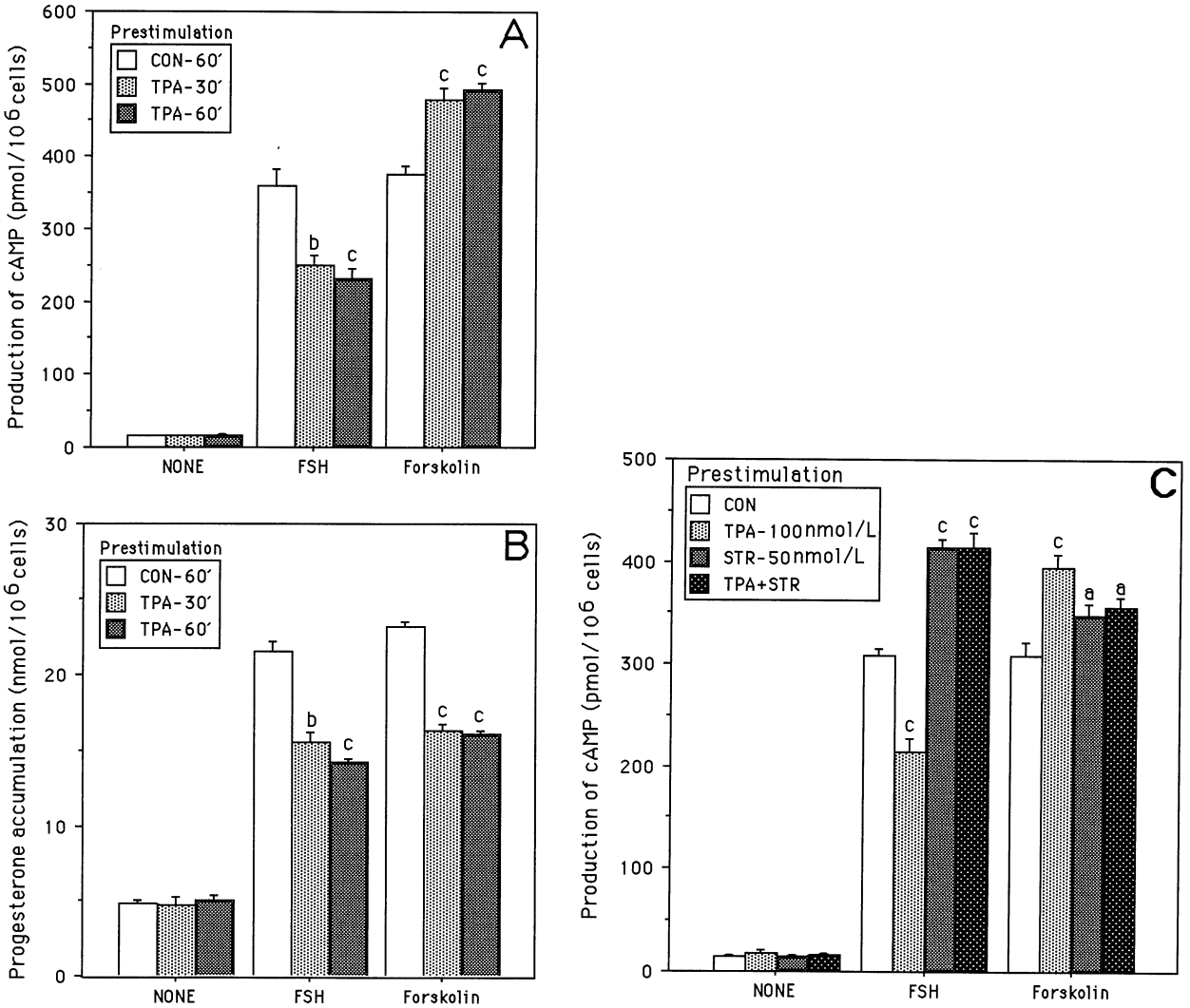

Fig. 3. Effect of preincubation of KK-1/hFSHR cells with TPA and STR on rhFSH- and forskolin-stimulated cAMP and progesterone production in a second incubation. The prestimulation was carried out with medium for 60 min (CON-60′), and with 100 nmol/l TPA for 30 or 60 min. At the end of prestimulation, the cells were washed and medium was replaced with fresh medium (none), 100 IU/l rhFSH or 50 μmol/l forskolin, all in the presence of 0.5 mmol/l IBMX, and cAMP was measured after a 20 min incubation (A). Progesterone concentration from the same culture media was determined after 18 h of the second stimulation (B). The data presented are the mean $\pm$SEM of six independent experiments in quadruplicates. b, $P < 0.001$; c, $P < 0.0001$ indicate the level of significance of differences from the control group. In the second set of incubations (panel C), the influence of preincubation with TPA, STR or their combination was studied on rhFSH- and forskolin-induced cAMP production in the second incubation. The cells were preincubated in the control medium, 100 nmol/l TPA, 50 nmol/l STR or their combination. The cells were washed after preincubation and the medium was replaced by control medium (none), 100 IU/l rhFSH or 50 μmol/l forskolin. Intracellular cAMP was determined after a 20 min second stimulation. The data expressed are the mean $\pm$SEM of four independent experiments in triplicates. a, $P < 0.01$; c, $P < 0.0001$ indicate the level of significance of differences from the control group.

3.6. Functional correlation between cAMP-mediated desensitization and receptor binding

In this experiment we examined the level of FSH binding and cAMP responsiveness during the process of desensitization (Table 2). The cells were prestimulated for 90 min with medium, rhFSH, TPA or STR, and divided thereafter into two groups. One was reincubated for 20 min in the absence or presence of rhFSH, and the cAMP response was determined. The other group was assessed for [^{125}I]iodo-rhFSH binding. The data demonstrate that under the conditions where cAMP response was clearly desensitized by rhFSH or TPA treatments, the number of available FSHR on the cell surface did not show clear changes. No significant changes were observed in the Kd values of the different experimental groups, either. The suppression of [^{125}I]iodo-rhFSH binding during the process of desensitization was minimal compared to the loss of cAMP responsiveness, which was reduced to 73.4% by rhFSH

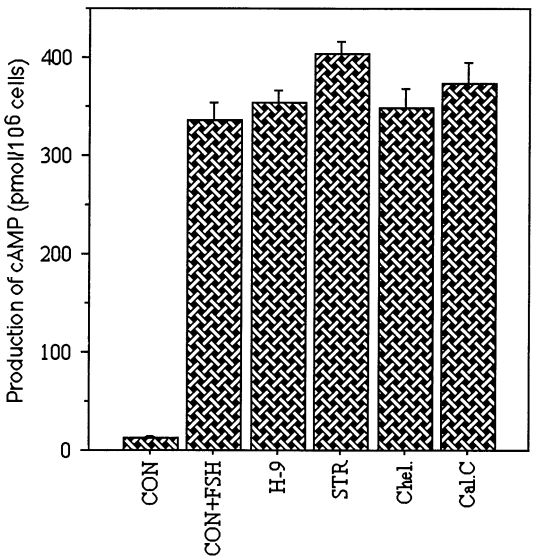

Fig. 4. Involvement of PKC inhibitors in the synthesis of cAMP in the KK-1/hFSHR cells. The cells were preincubated for 90 min in medium (CON), staurosporin (STR, 50 nmol/l), H-9 (1 μmol/l), chelerythrine (Chel., 5 μmol/l) and calphostin C (Cal.C, 50 nmol/l). The cells were washed and reincubated in the absence or presence of 100 IU/l rhFSH for another 20 min, and intracellular cAMP was determined. The experiments are the mean $\pm$SEM of four independent determinations in triplicates.

and to 66.3% by TPA in comparison to the medium preincubated controls. In contrast, STR increased the cAMP production with subsequent rhFSH stimulation significantly, by $143 \pm 2.8\%$ ($P < 0.0001$), whereas only a small concomitant increase (10% of control) was observed in FSH binding.

Table 1
Determination of luciferase activity in KK-1 cells transiently transfected with the plasmid containing SV40 promoter linked to the luciferase reporter gene

Stimulations (90 min/20 min)	Relative luciferase response (luciferase/β-galactosidase)
CON/CON	1218 ± 275
CON/FSH	1412 ± 127
CON/TPA	1289 ± 228
CON/STR	1329 ± 198
FSH/FSH	1292 ± 215
TPA/FSH	1378 ± 198
STR/FSH	1224 ± 286

Forty eight hours after tranfection, the cells were prestimulated for 90 min, followed by 20 min restimulation in the presence of following stimulants: medium (CON), rhFSH (100 IU/l), TPA (100 nmol/l), STR (50 nmol/l) or their combinations as indicated.
Luciferase activity was measured and corrected with the corresponding β-galactosidase value, and expressed as relative luciferase response.
The data are the mean $\pm$SEM of three independent experiments in triplicates.

3.7. Agonist and PKC-dependent expression of FSHR mRNA

Besides receptor binding, the possibility was considered that the FSHR mRNA expression would change during desensitization (Fig. 6). Northem analysis revealed that the ≈ 2.4 kb hFSHR message increased ($P < 0.001$) ≈ 1.5-fold in the case of (CON/FSH), i.e. 90 min preincubation with medium, followed by 20 min incubation with saturating dose (100 IU/l) of rhFSH. The finding was similar when the first incubation was carried out in the presence of rhFSH followed by 20 min second incubation (FSH/FSH), indicating that a relatively short exposure of the cells to FSH increases the steady-state levels of FSHR mRNA. This increase in the FSHR mRNA was prevented by including TPA in the first incubation. In contrast, prestimulation with STR exhibited a significant ($P < 0.0001$) increase (1.9-fold) in FSHR mRNA expression after the second incubation (Fig. 6).

3.8. Studies of the post-receptor stimulation mechanisms involved in desensitization

The specific roles of the G-protein and the AC enzyme activities were analyzed using cell stimulations with NaF and CT. The cells were incubated, as above, in two steps with the medium (CON), NaF (10 mmol/), CT (50 μg/l) or their combinations with rhFSH (100 IU/l) (Fig. 7). Prestimulation of the cells with NaF caused $\approx 76\%$ reduction of cAMP formation ($P < 0.001$), followed by a 20 min second stimulation with rhFSH as compared to CON/NaF. Similarly, preincubation with CT diminished $\approx 69\%$ of the FSH-stimulated cAMP synthesis as compared to the preincubation in medium (CON/CT). Interestingly, prestimulation of cells with rhFSH (100 IU/l), following 20 min restimulation either by NaF or CT exhibited significant ($P < 0.001$) increase, rather than decrease in cAMP synthesis (Fig. 7).

In additional experiments, we determined the possible involvement of hFSHR binding in the process of NaF or CT-evoked desensitization. The results summarized in Fig. 8 show marginal decreases in the density of the FSHR (≈ 88–92% of control) following NaF and CT stimulations. The same experimental paradigm in FSHR mRNA expression did not exhibit significant changes (data not shown).

4. Discussion

Considerable progress has been made recently characterizing the mechanisms involved in agonist-induced desensitization of gonadotropin action (Huhtaniemi et al., 1982; Rebois and Fishman, 1986; Sánchez-Yagüe et

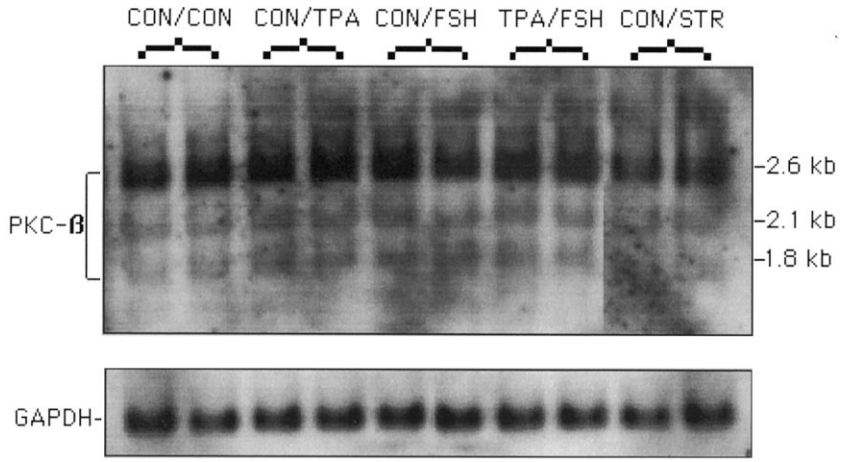

Fig. 5. Expression of PKC-β mRNA in KK-1/hFSHR cells. The cells were preincubated for 90 min in medium (CON), rhFSH (FSH, 100 IU/l) and TPA (100 nmol/l). After washing, the cells were restimulated without or with 100 IU/l of rhFSH for an additional 20 min. Total cellular RNA was extracted from each group separately, and Nothern blot analysis was conducted with [^{32}P]-labeled PKC-βcDNA probe. The GAPDH mRNA expression is indicated as loading control. The apparent molecular sizes of the different transcripts of PKC-β are indicated. The autradiogram was exposed for 48 h. The figure represents one of the three experiments with similar results.

Table 2
A comparison between rhFSH-stimulated cAMP synthesis and the level of FSH receptors in relation to the functional state of PKC

lst/2nd Stim.	cAMP (pmol/10^6 cells)	Relative response (%)	Kd (values)	No. of receptor (per cell)	Relative response (%)
CON/CON	7.4 ± 1.8	2.2	0.74×10^{-9} M	$26\,900 \pm 1870$	95
CON/FSH	338.2 ± 5.2	100	0.69×10^{-9} M	$28\,300 \pm 2700$	100
FSH/FSH	248.6 ± 4.6	73.4 [b]	0.62×10^{-9} M	$26\,600 \pm 3200$	94
TPA/FSH	224.2 ± 3.8	66.3 [c]	0.85×10^{-9} M	$24\,800 \pm 2860$	87.6
STR/FSH	485.5 ± 6.4	143.5 [c]	0.78×10^{-9}M	$31\,200 \pm 2320$	110

The prestimulation of KK-l/hFSHR cells for 90 min followed by 20 min second incubation with the indicated substances are considered 1st/2nd stimulations.
Intracellular cAMP was determined by RIA. The affinity (Kd) and the number of receptors were determined at the end of the second stimulation with rhFSH or in relation to PKC by Scatchard analysis.
The results are expressed as the relative response (%) of that of the control group (CON/FSH). CON/CON is shown for the comparison of the basal levels of cAMP and number of receptors per cell.
The data are considered for comparison represent the mean ($\pm$ SEM) of four independent experiments.
[b] $P<0.001$, [c] $P<0.0001$ represent the significant difference over CON/FSH value.

al., 1993; Hipkin et al., 1995). With regard to FSH, the changes occur both in the functional properties and in number of the cognate cell surface receptor (Rebois and Patel, 1985; Quintana et al., 1994; Hipkin et al., 1995). G-protein function, AC activity and PKC dependent signal transduction mechanisms are also involved in the desensitization of gonadotropin action (Rebois and Patel, 1985; Inoune and Rebois, 1989; Cadoret et al., 1994; Keren-Tal et al., 1996). To further understand the FSH-induced desensitization, we studied the hFSHR function and mRNA expression in relation to PKC and G-protein activation. The experiments were designed to elucidate the mechanisms of desensitization of the cAMP-mediated cellular responses. We used as the in vitro model a granulosa cell line, KK-1 (Kananen et al., 1995), not expressing the endogenous FSHR gene, which was stably transfected by a plasmid containing the coding sequence of the human FSHR cDNA driven by a strong viral promoter.

Attenuation of the FSH-stimulated cAMP formation occurs upon continuous exposure of the target cells. The functional desensitization of the gonadotropin receptors has been reported to appear as soon as in 2 min, and 4 h, depending on the cell line used (Hipkin et al., 1993; Sánchez-Yagüe et al., 1993; Quintana et al., 1994; Wang et al., 1996). Our results demonstrate that hFSHR desensitization is dose and time dependent, i.e. from $\approx$90 down to 70% of maximal cAMP response was observed, as compared to the non-desensitized cell, in 20 min in the presence of 0.1–1000 IU/l rhFSH. The cAMP responsiveness was only partially blocked even after the highest concentrations of rhFSH. The results also show that rhFSH and a phorbol ester potentiate the desensitization of the FSHR in intact cells in a time

and dose dependent manner. Our results suggest that desensitization involves changes of functional properties of the receptors, and can take place without concomitant alteration in receptor number.

The action of FSH mainly involves the activation of the AC signaling pathway, although other signal transduction mechanisms, including PKC, are also involved (Rebois and Patel, 1985; Themmen et al., 1991; Eskola et al., 1993; Würthner et al., 1995; Keren-Tal et al., 1996). Reports from our laboratory demonstrate that the desensitization of the AC systems in rat Sertoli cells is dependent on the PKC activation pathway (Nikula et al., 1990), and activation of PKC also inhibits the FSH-stimulated cAMP production in the immature rat testis (Eskola et al., 1993). Contrary to that, Lamm and Hunzicker-Dunn (1994) reported that activation of PKC and PKA failed to exhibit desensitization of LH/CGR action in cell-free membrane preparations. Activation of the PKC dependent pathway by phorbol ester has also been reported in relation to phosphorylation of the rat FSHR in human embryonic kidney cell line expressing this receptor (Quintana et al., 1994), and with the desensitization of FSHR in a GFSHR-17 cell line, created by the co-transfection of primary granulosa cells with SV40 DNA (pSV Bam), Ha-ras oncogene (PEJ6.6) and FSHR expression plasmid (Keren-Tal et al., 1996; Selvaraj and Amsterdam, 1997).

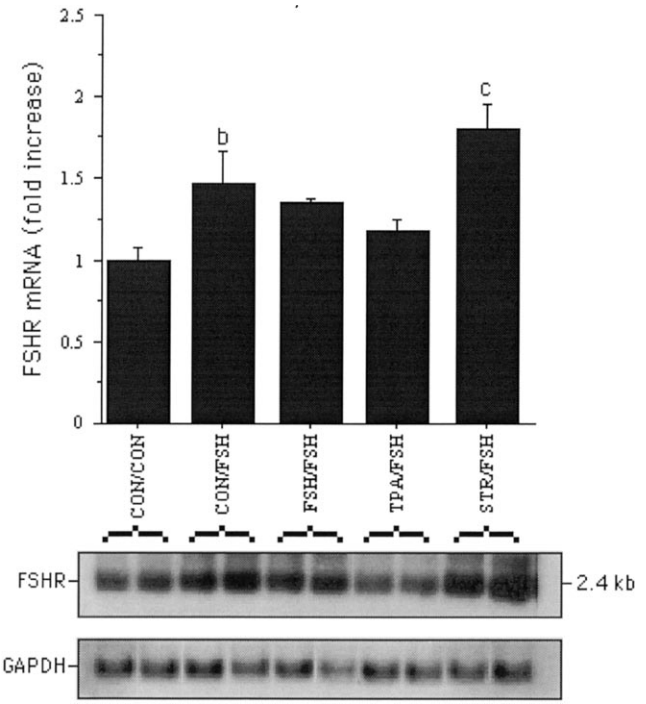

Fig. 6. Northern hybridization analysis of steady-state levels of hFSHR mRNA in KK1/hFSHR cells following preincubation for 90 min in medium (CON), rhFSH (100 IU/l), TPA (100 nmol/l) or STR (50 nmol/l). After washing, the second 20 min incubation was carried out in the presence of medium or rhFSH. Twenty micrograms of the total RNA isolated from the different groups were resolved in 1.2% agarose denaturing gel and transferred onto nylon membrane. Hybridization was carried out with [^{32}P]-labeled hFSHR cRNA probe. The data are presented as fold-increase (mean $\pm$SEM) in comparison to controls, calculated as arbitrary densitometric units. The intensity of the GAPDH mRNA expression is shown for equal loading of RNA. The migration of the specific hFSHR transcript of $\approx$2.4 kb and GAPDH expression are indicated. The exposure time was $\approx$48 h. b, $P < 0.001$; c, $P < 0.0001$ indicate significant differences from the CON/CON groups, and represent the mean $\pm$SEM of four experiments.

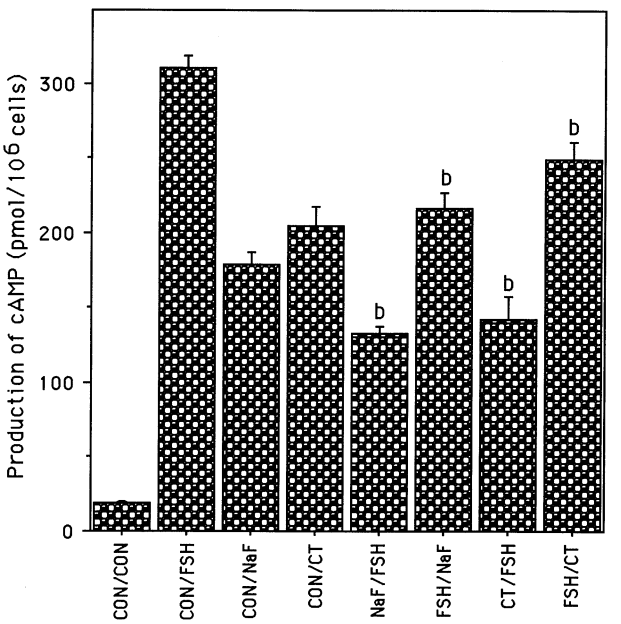

Fig. 7. Involvement of the post-receptor stimulation mechanisms in desensitization of the AC activity. The KK-1/hFSHR cells were preincubated for 90 min with medium alone (CON) or in the presence of 10 mmol/l NaF, 100 IU/l rhFSH or 50 μg/l CT. After washing, the cells twice with PBS, they were reincubated for 20 min with the above mentioned stimulants at different combinations in the presence of 0.5 mmol/l (IBMX), and measured for intracellular cAMP. Each bar represents the mean $\pm$SEM of five independent experiments in triplicates. b, $P < 0.001$ indicates significant differences in comparison to the respective controls.

In this study we have shown that PKC activation by TPA reduced the rhFSHstimulated cAMP formation, at the same time it inhibited rhFSH- or forskolin-stimulated progesterone production. In addition, among the isoforms of PKC examined, only PKC-β was detected in KK-1 cells expressing hFSHR, and it appeared to be constitutively involved in the process of desensitization. On the other hand, inhibition of PKC activity by specific inhibitors of PKC markedly increased the agonist-induced cAMP production, also suggesting the crucial involvement of PKC in this event. The role of the post-receptor components of the cyclase complex was also analyzed by using forskolin after PKC activation. Attenuation of the cAMP response to rhFSH stimulation was not accompanied by similar attenuation of the response to forskolin, an activator of AC. Clark et al. (1988) have reported that activation of the cAMP de-

pendent protein kinase is required for the heterologous desensitization of AC in S49 lymphoma cells. However, in rat testicular cells, the attenuation of the FSH-responsive cAMP production is clearly dependent on PKC activation (Eskola et al., 1993). Collectively, these results indicate that PKC is an important component responsible for the hFSHR desensitization.

It has also been observed that the LH/CGR desensitization is phosphorylation independent in porcine follicular membrane preparations (Lamm and Hunzicker-Dunn, 1994; Lamm et al., 1994). Our data document that the rhFSH-induced receptor desensitization mainly changes in the functional properties of the available receptors, which appeared to be a PKC dependent process, in support to the data of Hipkin et al. (1995). The receptor concentration during the desensitization of FSH and LH/CGR action does not show major changes (Rodriguez et al., 1992; Hipkin et al., 1995; Wang et al., 1996). In accordance, we showed that desensitization occurs in conditions where the FSHR gene is constitutively expressed under a powerful viral promoter. The results support the contention that the attenuation mechanism of rhFSH-sensitive AC activity is dependent on cAMP-mediated uncoupling of the hFSHR, not on changes in steady-state levels of the receptor protein. The elevation seen after rhFSH treatment in FSHR mRNA levels is apparently due to the prolonged half-time of the message, since it is unlikely that FSH would have specific stimulatory effects on function of the viral promoter controlling the FSHR expression. In fact, the cells transfected with luciferase reporter gene linked to SV40 promoter, also expressing hFSHR, did not exhibit alterations of luciferase levels with different stimulations. The data clearly reinforce the notion that viral promoter-driven FSHR transcription is constitutive in nature. A non-specific inhibitory effect on mRNA degradation can be ruled out because the FSHR message level was normalized to constant amout of β-galactosidase message.

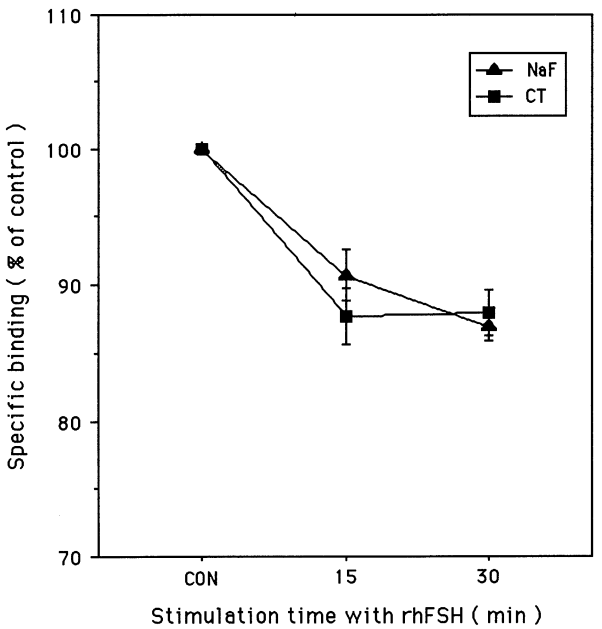

Fig. 8. [^{125}I]iodo-rhFSH binding to the intact cells during NaF and CT-induced desensitization. The KK-1/hFSHR cells were prestimulated with medium, 10 mmol/l NaF or 50 μg/l CT for 90 min separately. At the end, the cells were washed twice with 0.01 mol/l PBS and replaced with plain medium (CON) or one containing rhFSH (100 IU/l), and the incubation continued for 15 or 30 min. The unbound hormone was removed by washing and the binding of [^{125}I]-rhFSH was determined. The results are expressed as percent of specific binding over the control and represent the mean $\pm$ SEM of six independent experiments in triplicates.

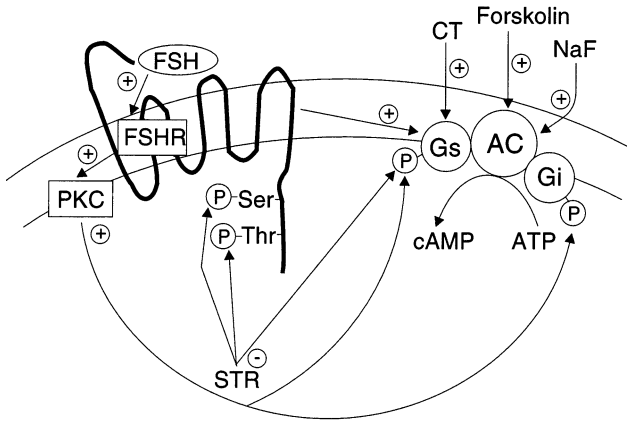

Fig. 9. A schematic presentation of the proposed mechanisms of hFSHR desensitization in the KK-1/hFSHR cells. Activation of PKC leads to the desensitization of FSHR, and the following pathways seem to be involved: Both the GTP-stimulatory (Gs) and inhibitory (Gi) GTP-binding proteins are inhibited through PKC-catalyzed phosphorylation, whereas no change occurs in AC activity. On the other hand, STR-sensitive receptor kinase(s) may phosphorylate Ser and Thr residues present in the FSHR. Inhibition of that kinase(s) by STR significantly increases the cAMP formation. CT activates directly the Gs protein, forskolin and NaF both activate AC and are also involved in desensitization.

The cAMP-dependent protein kinase regulates the rate of receptor phosphorylation following uncoupling of the receptor and the stimulatory G-proteins (Benovic et al., 1985). The desensitization in response to post-receptor stimulation of cAMP production is in agreement with previous findings (Monaco and Conti, 1987; Quintana et al., 1994; Tezelman et al., 1994). The extent of suppression of the cAMP response to rhFSH after pretreatment with NaF or CT is similar to that obtained with rhFSH pretreatment. This indicates that the process of desensitization of AC occurs, at least in part, through the involvement of FSHR, because of the similar nature of desensitization as obtained with rhFSH stimulation. The FSHR binding and mRNA measurements demonstrate that the attenuation of AC activity is not accompanied by significant changes in the synthesis or density of the FSHR.

A proposed model for the mechanisms of FSH action involved in desensitization is shown in Fig. 9. Activation of the PKC dependent pathway desensitizes the FSHstimulated cAMP formation. The PKC activation may phosphorylate both the stimulatory (Gs) and inhibitory (Gi) G-proteins. Multiple phosphorylation sites on serine and threonin residues are present in the FSHR (Quintana et al., 1994). Therefore, it may be possible that specific STR-sensitive receptor kinase(s) may phosphorylate these residues. Inhibition of the kinase(s) by STR elevates the FSH-induced cAMP formation in KK-1/hFSHR cells. There is apparently a tonic Gi-protein mediated inhibitory forms on AC activity. This is removed by PKC mediated inhibition of Gi-protein, which explains the increase of forskolin-stimulated cAMP production by TPA. In addition, PKC activation modulates negatively the steroidogenesis at a step(s) beyond cAMP formation (not illustrated in the figure).

In summary, the results provide evidence that the ligand-induced desensitization of hFSHR markedly involves the impairment of the signal transduction cascade distal to the hormone-receptor interaction, rather than changes in level of the receptor protein. Furthermore, the present results confirm and extend earlier findings that the changes in functional properties of the persisting receptors play a major role in desensitization during the activation of specific PKC-β isoform expression and post-receptor stimulations. Additional experiments will be required to establish in more detail the correlation between phosphorylation and uncoupling in the process of FSHR desensitization.

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