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Rapid Paper

Follitropin receptor down-regulation involves a cAMP-dependent post-transcriptional decrease of receptor mRNA expression

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Summary

The regulation by FSH (follitropin; follicle-stimulating hormone) of FSH receptor mRNA and protein (FSH binding) was studied using cultured Sertoli cells isolated from 21-day-old rats. FSH induced a dose-dependent and almost complete down-regulation of receptor mRNA at 4 h after addition of the hormone. At subsequent time points (16 h and later) the FSH receptor mRNA levels had returned close to control values. The effect of FSH was mimicked by dibutyryl cyclic AMP (dbcAMP) and forskolin, and the phosphodiesterase inhibitor methyl-isobutylxanthine (MIX) prolonged the FSH action. These findings indicate that the effect of FSH on its receptor mRNA was mediated by cAMP. A down-regulatory effect of FSH and dbcAMP on FSH receptor mRNA was also observed in the presence of the protein synthesis inhibitor cycloheximide, suggesting a direct effect of FSH/dbcAMP on the expression of the FSH receptor gene. Transcriptional run-on experiments revealed that FSH did not inhibit initiation of the FSH receptor gene; hence a post-transcriptional mechanism is involved. Binding of ^{125}I-FSH to the cultured Sertoli cells was rapidly (4 h) decreased when the cells were incubated with FSH or FSH in combination with MIX. This effect can be explained by ligand-induced receptor sequestration. In contrast, incubation of Sertoli cells with dbcAMP had no effect on binding of ^{125}I-FSH after 4 h, but resulted in a 60% loss of FSH binding sites after 24 h, probably caused by decreased mRNA expression.

In conclusion, FSH receptor down-regulation in Sertoli cells is effected not only by the well-documented ligand-induced loss of receptors from the plasma membrane, but also involves a cAMP-mediated decrease of FSH receptor mRNA through a post-transcriptional mechanism.

Introduction

Follicle-stimulating hormone (follitropin; FSH), a glycoprotein hormone produced by the pituitary gland, is essential for normal reproductive function. FSH acts on testicular Sertoli cells and ovarian granulosa cells, the only cells in the body that express a detectable amount of receptors for FSH. It is generally assumed that FSH acts through the stimulating GTP-binding protein G_s on adenylyl cyclase, resulting in increased levels of cAMP, although more than one second messenger pathway may be involved in the transduction of the FSH signal (Grasso et al., 1991).

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Several mechanisms exist to control the level of stimulation of Sertoli cells by FSH. In the hypothalamus-pituitary-testis axis, testosterone acts as a major negative feedback regulator of FSH secretion (Drouin and Labrie, 1981), and the Sertoli cell product inhibin also may play a role in this respect (de Jong, 1988). In addition, FSH sensitivity of Sertoli cells is regulated at the cellular level. Upon binding of FSH to its receptor and stimulation of the adenylyl cyclase enzyme, the hormone-receptor complex is thought to be internalized and transported to an intracellular compartment, where the bound FSH is degraded (Fletcher and Reichert, 1984; Shimizu and Kawashima, 1989); there are indications that the unoccupied receptor is recycled to the plasma membrane (Saez and Jaillard, 1986). Yet another mechanism of cellular control of sensitivity is the increase of phosphodiesterase activity observed in Sertoli cells after stimulation with FSH (Conti et al., 1981, 1983).

In the present study, we investigated the involvement of regulation of FSH receptor mRNA expression in the cellular regulation of FSH sensitivity, using cultured Sertoli cells. It was found that the loss of FSH receptors at the plasma membrane is the initial site of regulation, while a subsequent contribution results from a decrease of FSH receptor mRNA expression.

Materials and methods

Isolation and culture of Sertoli cells. All glassware used for the Sertoli cell isolation procedure was sylanized (Oonk et al., 1985). Sertoli cells were isolated from testes of 21-day-old Wistar rats (substrain R-1 Amsterdam). Rats were sacrificed using cervical dislocation, and the testes were removed and decapsulated. 10–12 testes were shaken (120 cycles/min) at 37°C in 20 ml phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954), supplemented with 5 μg/ml DNase I (type DN25; Sigma, St. Louis, MO, U.S.A.) (PBS/DNase), 1 mg/ml collagenase (type CLS; Worthington, Freehold, NJ, U.S.A.), 1 mg/ml hyaluronidase (type I-S; Sigma) and 1 mg/ml trypsin (type TRL; Worthington) in a 100 ml Erlenmeyer flask for 20 min. The tubule fragments obtained by this enzyme treatment were washed 4 times with 40 ml PBS/DNase by sedimentation for 2 min at unit gravity. The volume was readjusted to 20 ml PBS/DNase and made 1 mg/ml collagenase and 1 mg/ml hyaluronidase, and incubated for 20 min as described above. The tubule fragments then were washed 4 times (see above), and fragmented further using a dounce homogenizer (five strokes) as described by Oonk et al. (1985). The small fragments were washed 4 times with PBS/DNase, and once with Eagle's minimal essential medium (MEM; Gibco, Grand Island, NY, U.S.A.) by sedimentation at $100 \times g$ for 2 min in a calibrated glass tube to measure the cell pellet volume. MEM was supplemented with non-essential amino acids, glutamine, fungizone, streptomycin and penicillin (Oonk et al., 1985). The cell pellet was resuspended in 9 volumes of MEM with 1% fetal calf serum (FCS; Gibco), and plated at a density of 1 ml cell suspension per 75 cm^2 culture flask area. The cells in the culture flasks were incubated in MEM with 1% FCS at 37°C, under 5% CO_2 in air for 48 h. To eliminate contaminating germinal cells, the Sertoli cell cultures were exposed to an osmotic shock, using 10-fold diluted MEM in water for 2 min (Galdieri et al., 1981; as modified by Toebosch et al., 1989). Cells were then incubated for a further 24 h period in MEM with 0.1% (w/v) BSA (bovine serum albumin; fraction V; Sigma), followed by the incubations and the experiments described in the Results and Discussion section. The experiments were repeated 2–3 times, using different cell preparations.

The Sertoli cell isolation method described above yielded a highly purified preparation of Sertoli cells, as evidenced by staining of the cultures for alkaline phosphatase activity as described by Blok et al. (1990). The number of alkaline phosphatase-positive cells, representing peritubular myoid cells (Palombi and Di Carlo, 1988), was less than 0.1% up to the end of the culture period.

RNA analysis. All general molecular biology techniques were carried out as described in Sambrook et al. (1989) and Davis et al. (1986). Recently, the cDNA encoding the rat FSH receptor was cloned (Sprengel et al., 1990). In a study using a PCR approach to obtain the cDNA of the TSH receptor, a human partial cDNA clone was isolated that was expressed only in ovary and

testis (Parmentier et al., 1989). This sequence is homologous with the rat FSH receptor sequence. We have used this human probe (pHGMP09) to study the regulation of expression of FSH receptor mRNA in cultured rat Sertoli cells.

Total RNA was isolated from flash-frozen cells (−80°C) using the LiCl/urea method (Aufray and Rougeon, 1980). RNA was separated on 1% agarose/formaldehyde gels, and blotted using Hybond N^+ nylon membrane filters (Amersham, 's-Hertogenbosch, The Netherlands) or Biotrans nylon membrane filters (ICN, Irvine, CA, U.S.A.). The blots were hybridized with the ^{32}P-labelled pHGMP09 probe for 48 h. After hybridization, the blots were washed to a final stringency of 1 × SSC/0.5% (w/v) SDS (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 42°C, and autoradiographed using Hyperfilm-MP film (Amersham). The hybridization signals of the 3.5 kb FSH receptor mRNA band were quantified using a BioRad-1D gel scanner (BioRad, Utrecht, The Netherlands). Hybridization of the blots with an actin probe showed that in all lanes similar amounts of RNA were applied.

^{125}I-FSH binding. ^{125}I-FSH binding was measured using a whole cell binding assay, essentially as described for porcine Sertoli cells by Saez and Jaillard (1986). In brief, the attached cells were washed twice with 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1% (w/v) BSA (TB 7.4) at 37°C, and once with 10 mM Tris-HCl pH 3.9, 5 mM $MgCl_2$, 0.1% (w/v) BSA (TB 3.9) at 4°C to remove bound FSH from the cell surface, and subsequently twice with TB 7.4 at 37 °C. These washed cells were incubated for 1 h in 1 ml of TB 7.4 containing 50,000 cpm ^{125}I-FSH (specific activity: 135 μCi/μg; NEN-Du Pont de Nemours, 's-Hertogenbosch, The Netherlands) per well (Costar 12-well plate), at 37°C under 5% CO_2/95% air. To remove unbound ^{125}I-FSH, the wells were rinsed twice with TB 7.4 at 4°C. Bound ^{125}I-FSH was recovered by dissociation from the membrane in TB 3.9 for 10 min at 4°C. The supernatant was collected and the radioactivity was determined. Routinely, the remaining cells were dissolved in 1 N NaOH to determine the remaining amount of cell-associated ^{125}I-FSH. Under all incubation conditions less than 1% of the added ^{125}I-FSH was found in the NaOH lysate. Binding assays were performed in triplicate, with non-specific binding controls in triplicate containing 500-fold excess of unlabelled ovine FSH (NIH S16; NIH, Bethesda, MD, U.S.A.). Maximal specific binding was 3–5% of total added ^{125}I-FSH.

Transcriptional run-on. Transcriptional run-on experiments were performed essentially as described by Marzluff and Huang (1984) and Ausubel et al. (1989). Typically, 25×10^6 nuclei were recovered from 100×10^6 Sertoli cells. Elongation of the in vivo initiated mRNAs was performed in the presence of [^{32}P]α-GTP, and labelled pre-mRNAs were separated from unincorporated nucleotides. Hybrid selection was performed as follows. Plasmid DNA (5 μg of pRK-FSHR-NH2 and 5 μg of pRK-FSHR-COOH, rat FSH receptor cDNA (Sprengel et al., 1990) for determination of FSH receptor pre-mRNAs and 10 μg pTZ as a control for background hybridization) was bound to nylon membrane filter using a dot blot apparatus (Schleicher and Schuell, 's-Hertogenbosch, The Netherlands). The filters were hybridized to $3–10^6$ cpm of labeled RNA for 65 h at 55 °C, washed at 55 °C using 1 × SSC for 15 min, and autoradiographed.

Results and discussion

A time course study using the cultured rat Sertoli cells from 21-day-old rats showed a rapid, but transient, down-regulatory effect of FSH on FSH receptor mRNA expression (Fig. 1). This effect was maximal after 4 h incubation with FSH, while the amount of FSH receptor mRNA had returned to 80% of control at the 16 h time point. The ED_{50} of FSH in down-regulating FSH receptor mRNA expression was between 5 and 50 ng/ml FSH (Fig. 2). This is close to the ED_{50} for other effects of FSH, such as stimulation of cAMP, c-*fos* expression, aromatase activity, glycolysis and α-inhibin expression (Oonk et al., 1985; Hall et al., 1988; Toebosch et al., 1989).

FSH is thought to act mainly through the cAMP pathway (Means et al., 1976). Concurrent with this, the down-regulatory effect of FSH was mimicked by dbcAMP (Fig. 3*A*) and forskolin (not shown). This dbcAMP/forskolin induced down-regulation was effective at 4 h and persisted up to at least 24 h. The effect of dbcAMP

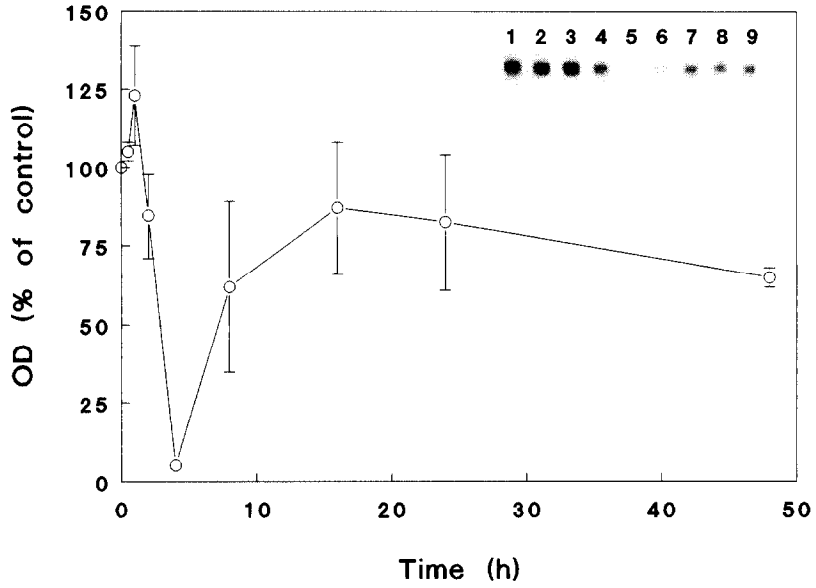

Fig. 1. Time course of the effect of FSH on FSH receptor mRNA expression in Sertoli cells. Cells were incubated for different time periods in the presence of 500 ng/ml FSH. Subsequently, the cells were harvested and total RNA was isolated. The FSH receptor mRNA levels were assessed by Northern analysis, using 20 μg of total RNA per lane. The FSH receptor mRNA band was scanned and the resulting optical density (OD) measurements were expressed relative to the control value (100%). Inset: Autoradiogram of one representative experiment. To lanes 1–9 RNA from cells incubated for 0, 0.5, 1, 2, 4, 8, 16, 24 and 48 h was applied. In the graph the combined results of three experiments are represented. Values given are means ± SD for three experiments.

at 24 h was more pronounced than that of FSH itself, suggesting that FSH down-regulates the activity of the FSH receptor signal transduction system, while dbcAMP does not have such an effect.

Experiments in the presence of the phosphodiesterase inhibitor methyl-isobutylxanthine (MIX) yielded results comparable to those observed using dbcAMP (Fig. 3*A*). This indicates that the transient FSH effect on receptor mRNA expression is related to a transient FSH effect on cAMP production, which is explained by increased levels of phosphodiesterase activity (Conti et al., 1981, 1983).

The intracellular and secreted amounts of cAMP were determined at different time points after addition of FSH or FSH in combination with MIX (Fig. 4). Treatment with FSH increased cAMP rapidly; the maximal intracellular concentration was reached within 15 min, while cAMP had returned to control values at 2 h. Secreted cAMP levels raised slower, reaching a plateau value at 2 h after addition of FSH. Phosphodiesterase activity in Sertoli cells is able to rapidly decrease intracellular cAMP concentrations, as is evidenced by the effects of MIX (Fig. 4). These results confirm earlier observations, that cultured Sertoli cells are rapidly desensitized to FSH with

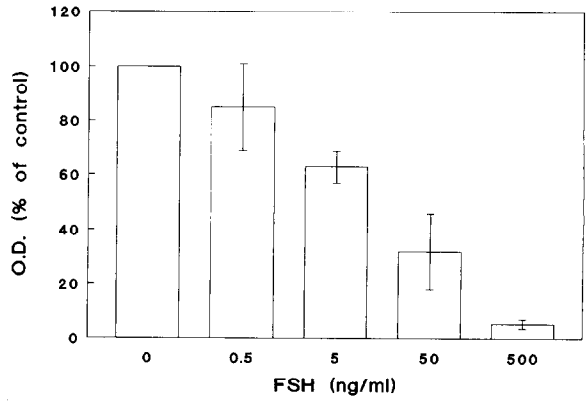

Fig. 2. Effect of FSH dose on the FSH receptor mRNA level in Sertoli cells. Cells were incubated for 4 h in the presence of different concentrations of FSH. Subsequently, the cells were harvested and the FSH receptor mRNA was measured as described in the legend to Fig. 1. Values given are means ± SD for three experiments.

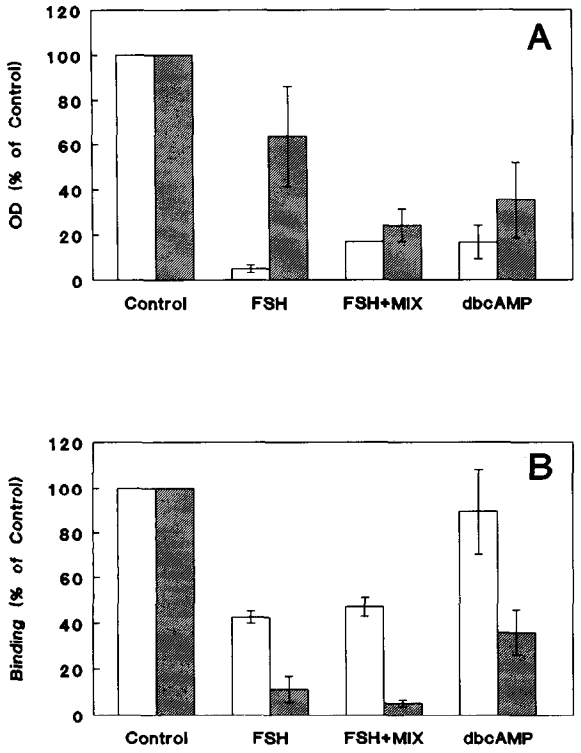

Fig. 3. Involvement of the cAMP pathway in the regulation of FSH receptor mRNA expression and FSH binding in Sertoli cells. *A*: FSH receptor mRNA. Cells were incubated for 4 h (open bars) or 24 h (hatched bars) in the presence of 500 ng/ml FSH, 500 ng/ml FSH in combination with 0.2 mM MIX, or 0.5 mM dbcAMP. Subsequently, the cells were harvested and the FSH receptor mRNA was measured as described in the legend to Fig. 1. Values given are means ± SD for three experiments. *B*: FSH binding. Cells were incubated for 4 h (open bars) or 24 h (hatched bars) in the presence of 500 ng/ml FSH, 500 ng/ml FSH in combination with 0.2 mM MIX, or 0.5 mM dbcAMP. Subsequently, ^{125}I-FSH binding was measured as described in Materials and Methods. Values given are means ± SD for three experiments.

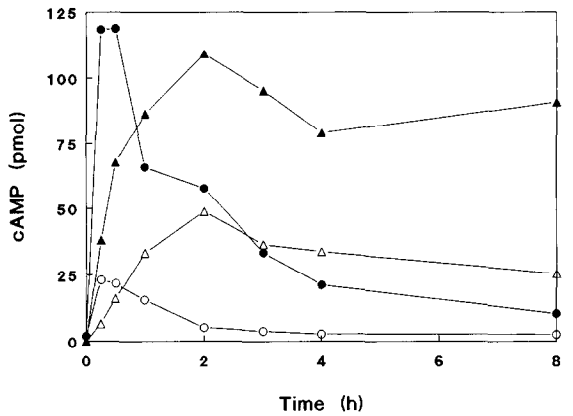

Fig. 4. Effect of FSH on cAMP production by Sertoli cells. Cells were incubated in the presence of 500 ng/ml FSH (open symbols) or 500 ng/ml FSH in combination with 0.2 mM MIX (closed symbols). At different time points, cAMP was determined in the cells (cellular; circles) and in the medium (secreted; triangles) as described previously (Themmen et al., 1986). Values given are means of duplicate determinations of total production of cAMP per well. Differences between duplicates were not larger than 5% of the mean.

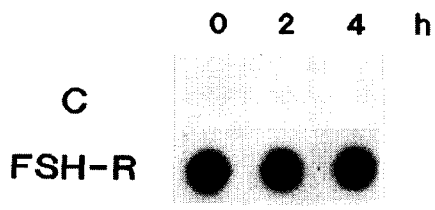

Fig. 5. Initiation of FSH receptor gene transcription. Sertoli cells were incubated for 0, 2 or 4 h with 500 ng/ml FSH. Nuclei were isolated and transcriptional run-on experiments were performed as described in Materials and Methods. C: control plasmid without cDNA insert; FSH-R: two plasmid DNAs containing the complete FSH receptor cDNA.

respect to cAMP formation (Verhoeven et al., 1980, 1981; Oonk et al., 1985).

In cells treated with the protein synthesis inhibitor cycloheximide, FSH receptor mRNA still was down-regulated by FSH or dbcAMP, although CX treatment resulted in an overall increase of the receptor mRNA levels (Table 1). The latter effect of CX, that has been observed in many other cell types as well (Almendral et al., 1988), is probably caused by a general effect on transcription and/or mRNA stability (Wilson and Treisman, 1988). The present results indicate that

TABLE 1

EFFECT OF CYCLOHEXIMIDE ON DOWN-REGULATION OF FSH RECEPTOR mRNA

	Addition	
	−CX	+CX
Control	100	328 ± 45
FSH	5 ± 2	169 ± 2
dbcAMP	17 ± 7	215 ± 2

Cells were incubated for 4 h with 50 μg/ml cycloheximide in the presence of 500 ng/ml FSH or 0.5 mM dbcAMP. Subsequently, the cells were harvested and the FSH receptor mRNA was measured as described in the legend to Fig. 1. Values given are means ± SD for three experiments.

the homologous down-regulation of FSH receptor mRNA operates through a direct mechanism which does not require de novo protein synthesis.

The attenuation of FSH receptor mRNA expression in cultured Sertoli cells after incubation with FSH or dbcAMP might be the result of a rapid decrease in transcription. Transcriptional run-on experiments were performed to investigate this mechanism. No decrease in transcription initiation of the FSH receptor gene could be observed, using nuclei isolated from Sertoli cells that had been incubated for 2 or 4 h in the presence of FSH. In the same series of experiments, initiation of α-inhibin gene transcription was consistently induced by FSH (not shown). These results indicate that decrease of initiation of transcription is not the dominant mechanism of FSH receptor mRNA down-regulation.

To study whether the observed down-regulation of FSH receptor mRNA finally resulted in down-regulation at the protein level, we have also measured FSH receptor binding sites on the cultured rat Sertoli cells (Fig. 3*B*). First, the binding characteristics of the FSH receptor was confirmed. A K_d of 45 pM and a B_{max} of 40 fmol per mg protein was generated from a straight Scatchard plot; these data are in agreement with values from the literature (Fletcher and Reichert, 1984). Incubation of the cells with either FSH or FSH in combination with MIX showed a rapid and marked down-regulation of FSH receptor number at 4 h, and a further decrease in binding sites at 24 h. dbcAMP had no effect at 4 h, but had markedly reduced FSH binding after a 24 h incubation period. Thus, the early effect of FSH on the receptor number is probably not mediated by cAMP, but rather reflects a direct effect at the plasma membrane, viz. receptor internalization. Down-regulation of the FSH receptor number by dbcAMP at 24 h can be explained by down-regulation of FSH receptor mRNA expression.

The results presented above indicate that FSH receptor expression is regulated through at least two independent mechanisms: a rapid down-regulation of FSH binding, which involves sequestration of membrane receptors, and a more slow regulation at the post-transcriptional level. The latter effect of FSH is mediated by cAMP.

Exposure of cells to hormones almost invariably leads to hormone-specific desensitization and down-regulation of the hormone receptors. The responsiveness of cells to hormones can be regulated through several mechanisms. Upon activation of a GTP-binding protein, the receptor may assume a low affinity state (Cerione et al., 1984; Zhang et al., 1991), and rapid phosphorylation also has been implicated in receptor desensitization (Benovic et al., 1987; Leeb-Lundberg et al., 1987). These rapid effects are followed by sequestration of the hormone-receptor complex (Lloyd and Ascoli, 1983).

In the case of the FSH receptor, several regulatory mechanisms have been studied. Pulse-chase studies with ^{131}I-FSH in cultured mouse Sertoli cells have shown that after hormone binding FSH receptors are internalized rapidly (within 50 min), and that all ^{131}I-FSH surface binding has disappeared at 100 min (Shimizu and Kawashima, 1989). Similar results have been obtained using ^{125}I-FSH and rat or pig Sertoli cells (Fletcher and Reichert, 1984; Saez and Jaillard, 1986). Furthermore, a decrease of the activity of the second messenger cAMP through activation of phosphodiesterase has been observed (Conti et al., 1981, 1983). In the present paper, we show yet another level of regulation of FSH receptor activity, viz. the expression of FSH receptor mRNA.

FSH acts on FSH receptor mRNA expression through a post-transcriptional mechanism, rather than through regulation of transcription. Both regulatory mechanisms have been shown to occur for other G-protein coupled receptors. The half-life of β_2-adrenergic receptor mRNA in DDT$_1$-MF-2 cells, for example, was decreased from 12 h to approximately 5 h after treatment of the cells with the β_2-adrenergic agonist isoproterenol (Hadcock et al., 1989). Interestingly, it has been found that β_2-adrenergic receptor gene transcription is stimulated acutely by cAMP in response to short-term agonist exposure (Collins et al., 1989). In the present experiments, a rapid induction of FSH receptor mRNA by FSH was not clearly observed, although FSH receptor mRNA expression seemed to be slightly increased (not statistically significantly different from controls) at 1 h after FSH addition (Fig. 1). This will be studied further using a more sensitive and quantitative solution hybridization method.

Similar to our results, it has been shown that LH/CG receptor mRNA in mouse MA-10 Leydig tumour cells also is under homologous down-regulatory control. In these cells, hCG and 8-bromo-cAMP transiently up-regulated LH/CG receptor mRNA, and caused a subsequent decline in mRNA level (Wang et al., 1990, 1991). In preovulatory follicles from hCG-treated immature rats, the situation is more complex: a low concentration of cAMP appeared to induce LH/CG receptor mRNA level, whereas a high dose of the second messenger inhibited the mRNA expression (Segaloff et al., 1990).

In conclusion, we have shown that, quantitatively, the primary cause of FSH receptor down-regulation in Sertoli cells is receptor internalization and/or degradation at the plasma membrane; a later contribution results from the loss of FSH receptor mRNA. This FSH-induced decrease of FSH receptor mRNA expression is mediated by cAMP through a post-transcriptional mechanism.

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