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HORMONE-INDUCED DESENSITIZATION OF CULTURED RAT GRANULOSA CELLS TO FSH

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SUMMARY

Monolayers of granulosa cells (GC) derived from immature hypophysectomized diethylstilbestrol-treated rats became refractory in terms of FSH-stimulable cyclic AMP production following exposure to the homologous hormone. In the presence of ovine FSH (5 µg/ml), maximal refractoriness was attained after 4 h of incubation. Upon removal of the FSH from the medium, the cells regained their full responsiveness within 24 h. The extent of desensitization was dependent upon the dose of FSH, and could not be overcome by increasing the dose of the hormone during the challenge period. Exposure of GC monolayers for 2-4 h to the protein synthesis inhibitors actinomycin D (8 µg/ml) and cycloheximide (5 µg/ml) on their own enhanced FSH-stimulable cyclic AMP production. When added together with FSH, the inhibitors did not prevent the process of desensitization to the hormone. The results suggest that the initial phases of FSH-induced desensitization do not require *de novo* protein synthesis.

Several tissues have been shown to undergo a decrease in hormonal responsiveness following continued exposure to the homologous hormone (1,2). Such desensitization has proven to be a widespread biological phenomenon. Refractoriness to hormones which act via the adenylate cyclase system is associated with a marked decrease in hormone-stimulated cyclic AMP production (2-9).

Prolonged exposure of intact ovaries or isolated follicles to LH or PGE *in vitro* (5-9) induced refractoriness to a subsequent challenge with fresh hormone, and this desensitization was shown to be homospecific and dose- and time-dependent (5-7,9-12). FSH was shown to exhibit a similar effect (5,8,9), but information concerning the mode of action of this hormone in the process of refractoriness is more scanty.

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Since most ovarian preparations previously selected for studies of hormone-induced desensitization were multicomponential, consisting of several cell types, the results obtained are not completely unequivocal. Isolation and culture of granulosa cells (GC) from ovarian follicles provide a more homogenous model system. In fact, we have shown (13) that refractoriness to hCG can be induced by the homologous hormone in monolayers of GC from preovulatory follicles. Nevertheless, studies with cultures of GC from such follicles are complicated by (i) the existence of both LH and FSH receptors, which may be located in different sub-populations of the cells; and (ii) spontaneous luteinization of the cells during culture. GC from follicles of immature-hypophysectomized estrogen-treated rats (Hx-DES) are endowed with FSH receptors, contain few or no LH binding sites and do not undergo spontaneous luteinization in culture (14-16).

In the present study we have used monolayers of GC from Hx-DES rats to explore the mechanism underlying FSH-induced refractoriness with respect to hormone stimulable cyclic AMP production.

MATERIALS AND METHODS

Ovine FSH (NIH-FSH-S-12) was kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md. Actinomycin D was obtained from Merck, Sharpe and Dohme, Rahway, N.J. and cycloheximide was obtained from Sigma Chemical Co., St. Louis, Missouri. 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Aldwich Chemical Co., Milwaukee, Wisconsin.

Granulosa cells were collected from immature (28-30 day-old) hypophysectomized rats treated with diethylstilbestrol (0.5 mg/rat/day) for 5 days (Hx-DES cells) and cultured as described by Nimrod (16). Cells ($3\text{-}6 \times 10^5$ cells/ml) were cultured in replicate for 48 h in 3 cm tissue culture dishes (Falcon), containing 1 ml medium. The medium was then removed and the monolayers (n=6 for each experimental group) were incubated for the times specified in 1 ml of tissue culture medium with or without FSH. In some experiments, actinomycin D (8 µg/ml) or cycloheximide (5 µg/ml) were included. The cells were then washed 3 times with Krebs-Ringer bicarbonate buffer, containing 1 mg/ml glucose, and taken for assay of FSH-stimulable cyclic AMP accumulation as described by Nimrod *et al.* (17). In this assay, 3 replicates of an experimental group served as the unstimulated controls, while the remaining 3 dishes were exposed to FSH challenge.

RESULTS

Time-course of desensitization and recovery from the refractory state induced by FSH in GC cultures. Cultures of GC from follicles of Hx-DES rats

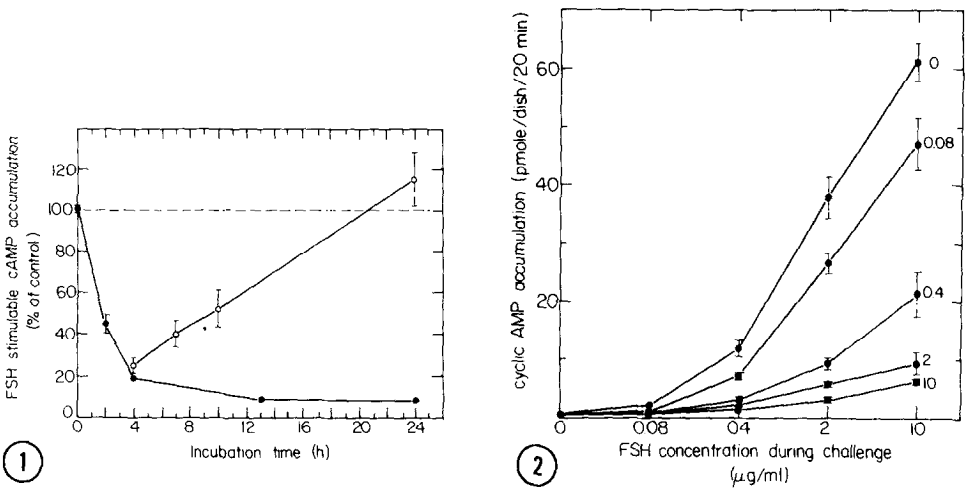

Figure 1: Time course of desensitization to FSH of GC monolayers from Hx-DES rats, and of the recovery of FSH-stimulable cAMP accumulation. Cell monolayers ($4x10^5$ cells/ml dish) were either incubated for 2-24 h in the presence of 5 μg/ml of oFSH (full circles), or incubated first for 4 h with the hormone, then washed thoroughly and incubated in hormone-free medium (open circles). Control cells were incubated for the times specified without FSH addition. Results are expressed as the percent accumulation of cyclic AMP in the cells during a FSH challenge (20 min) compared to control cultures. Values represent the mean ± S.E. of triplicate cultures, each assayed in duplicate.

Figure 2: Dose dependence of desensitization of GC monolayers to FSH. Cells were first desensitized by a 4 h exposure to oFSH (0-10 μg/ml, shown by the numbers in the right part of the graph, top to bottom), then washed and challenged for 20 min with varying doses of oFSH (0-10 μg/ml). Values represent the mean ± S.E. (n=3) of cyclic AMP levels found in the cells after the 20 min FSH-challenge.

responded to exposure to FSH (5 μg/ml) for 20 min in the presence of phosphodiesterase inhibitor (IBMX) with an approximately 16-fold rise in cyclic AMP accumulation (1.0 ± 0.1 versus 15.8 ± 0.4 pmol cAMP/20 min/dish, respectively; mean ± S.E.M.). Refractoriness to FSH challenge developed gradually in the cells during culture in the presence of FSH (5 μg/ml): a 50% decrease in FSH-stimulable cAMP production was attained after 2 h of exposure and maximal desensitization (92% decrease; cf. Fig. 1) was reached within 12 h. The prolonged exposure of the cells to FSH did not affect basal cAMP accumulation during a 20 min incubation without FSH addition (data not shown). When cells refractory to FSH were washed and further cultured in hormone-free medium, they progressively regained responsiveness, attaining control values by 24 h (Fig. 1).

Dose dependency of the desensitization of GC monolayers by FSH. Monolayers of GC from Hx-DES rats were cultured for 24 h in the presence of varying concentrations of FSH (0.08-10 μg/ml). Each concentration group was then washed and challenged for 20 min with a similar concentration range of fresh FSH. The results (Fig. 2) indicate that partial desensitization was attained following a continuous exposure to FSH at a concentration as low as 0.08 μg/ml. The extent of refractoriness was further augmented at increasing concentrations of FSH during the 24 h culture period. In each concentration group, the percent decrease of FSH-stimulable cAMP accumulation was unaffected by varying the doses of the hormone during the 20 min challenge period. Thus, cells that have been exposed for 24 h to 0.4 μg/ml FSH exhibited a similar decrease in cAMP accumulation (65-75%; cf. Fig. 2) during a 20 min challenge with 0.4, 2 or 10 μg/ml of FSH.

Effect of cycloheximide and of actinomycin D on the development of refractoriness to FSH. Culture of cells in hormone-free medium for 2 and 4 h

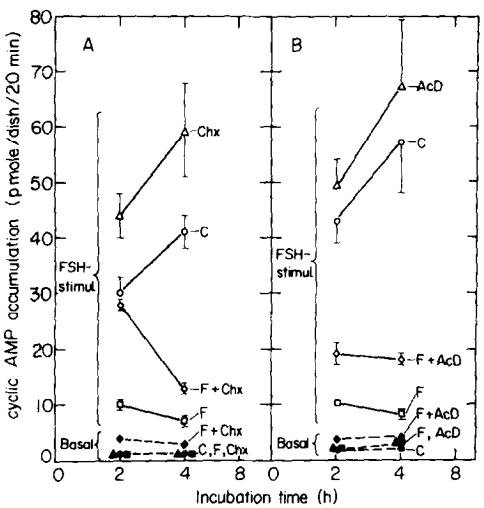

Figure 3: The effect of protein synthesis inhibitors on the desensitization of GC by FSH. Cells ($3x10^5$ cells/ml dish) were cultured for 2 or 4 h with: (i) unsupplemented medium (○,●); (ii) 5 μg/ml oFSH (□,■); (iii) an inhibitor (△,▲); or (iv) both oFSH and inhibitor (◇, ◆). In experiment A, cycloheximide (Chx; 5 μg/ml) was used while in B actinomycin D (AcD; 8 μg/ml) was the inhibitor. Following incubation, the monolayers were thoroughly washed and re-incubated for 20 min in the presence of IBMX with (full lines) or without (broken lines) 5 μg/ml oFSH. Values represent the mean ± S.E. (n=3) of cAMP levels found in the cells after the 20 min incubation period.

with cycloheximide (8 µg/ml) or actinomycin (5 µg/ml) did not affect cAMP accumulation during a subsequent 20 min incubation without FSH-challenge (Fig. 3). By contrast, an augmentation by the protein synthesis inhibitors was observed in these cells when FSH-stimulable cAMP accumulation was measured (45% and 16% for cycloheximide and actinomycin D, respectively; cf. Fig. 3).

When FSH was included in the 2 and 4 h culture periods, the cells became markedly desensitized to FSH stimulation. Addition of the inhibitors with FSH during the desensitization period resulted in FSH-stimulable cAMP accumulation that was higher than that observed after exposure to FSH alone. These values, however, were markedly lower than those obtained from cells treated with the inhibitors on their own. Thus, cycloheximide and actinomycin D enhanced the stimulatory action of FSH on cAMP production, but did not prevent the desensitization process.

DISCUSSION

It is well recognized that follicular development and granulosa cell proliferation are dependent on the sequential action of FSH and LH (18). Of the two gonadotropic hormones, the action of FSH appears to be more critical for the initiation of follicular growth (15,18). Furthermore, rat ovarian cells become exposed during the reproductive cycle to FSH levels that are more sustained than those of LH (19,20). An appreciable amount of data has been accumulated on the desensitization process induced in ovarian tissue by LH, while the information concerning FSH is more meagre. In the isolated GC system described here, FSH was shown to rapidly induce desensitization of the FSH-stimulable adenylate cyclase system and this effect was reversed upon removal of the desensitizing agent (cf. Fig. 1). The transient nature of this process is in contrast with the course of desensitization to LH induced *in vitro* by the homologous hormone in isolated rat Graafian follicles (6) and by hCG in GC monolayers derived from rat preovulatory follicles (13): in the latter systems, no recovery from the refractory state was observed after removal of the hormone. The transient response of the GC derived from Hx-DES

rats in terms of refractoriness to FSH may reflect the need of a functional FSH-responsive system for the continued growth of the immature follicle. Similar results were observed by Jonassen and Richards (21) for the time course of desensitization *in vivo* in Hx-DES-type rats by FSH and for the recovery from the refractory state, thus indicating that the response of the Hx-DES cells was not affected by the culture conditions.

Actinomycin D and cycloheximide, at concentrations known to block protein synthesis in ovarian tissue (22), were ineffective in preventing the FSH-induced desensitization in cultured GC from Hx-DES rats (Fig. 3). This is in contrast to previous observations (6,7,9) that in whole ovaries and cultured Graafian follicles FSH- and LH-induced desensitization could be abolished by protein synthesis inhibitors. The reason for this discrepancy is not clear. However, it may indicate that the mechanism of desensitization operating in Graafian follicles differs from the FSH-induced process in GC from preantral follicles. Alternatively, since it has been postulated that multiple mechanisms of refractoriness exist in hormone-responsive tissues (1-3), it may well be that one of them - not dependant on protein synthesis - became predominant over others in the GC as an adaptation to the culture conditions. A discrepancy between the effect of protein synthesis inhibitors on agonist-induced refractoriness in whole tissue and isolated cells has also been described for TSH in the thyroid gland (23,24).

The stimulatory action of the inhibitors on cyclic AMP accumulation is not surprising, since such an effect has also been observed in other hormone-responsive systems (24-26).

In conclusion, the studies reported here indicate that FSH-induced refractoriness in GC of preantral follicles is a rapid and reversible phenomenon, and suggest that the initial phases of this process do not require *de novo* protein synthesis.

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