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ANTIGONADOTROPIC ACTIONS OF GnRH AGONIST ON OVARIAN CELLS *IN VIVO* AND *IN VITRO*

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Summary—GnRH and its agonists have recently been shown to inhibit a variety of reproductive functions, in addition to its well-known gonadotropin releasing action in the pituitary. In order to determine the inhibitory mechanism and the site of action of these peptides in ovary, the direct actions of an agonistic analogue [D-Leu_6, des-Gly-NH_2] GnRH ethylamide on hypophysectomized immature rat ovaries *in vivo* and on rat luteal cells as well as porcine granulosa cells incubated *in vitro* were investigated.

^{125}I-Labeled GnRH agonist, when injected to immature female rats, bound specifically not only to pituitary but also to ovaries. GnRH agonist inhibited hCG stimulation of progesterone production and ovarian weight augmentation in hypophysectomized immature female rats *in vivo*. FSH-stimulated induction of ovarian LH/hCG receptors and ovarian weight gain in diethylstilbestrol (DES)-treated hypophysectomized immature female rats were also suppressed by GnRH agonist.

Moreover, treatment with GnRH agonist inhibited hCG-stimulated progesterone production by rat luteal cells incubated *in vitro*. In short time incubation of porcine granulosa cells obtained from medium follicles, the capacity and affinity of LH/hCG receptors were not affected by GnRH agonist. However, in long term culture of porcine granulosa cells obtained from small follicles, concomitant treatment with GnRH agonist markedly inhibited the induction of LH/hCG receptors stimulated by FSH and insulin. It may be possible that GnRH agonist prevents the differentiation of granulosa cells and subsequent acquisition of LH/hCG receptors, but has no effect on the LH/hCG receptors already induced. On the other hand, GnRH agonist delayed hCG-stimulated accumulation of cyclic AMP in porcine granulosa cells obtained from medium follicles. This delay of cyclic AMP accumulation may be responsible for the inhibition of progesterone production by ovarian cells. These findings suggest that GnRH agonist acts directly on ovarian cells and inhibits the action of FSH and LH/hCG, independently.

INTRODUCTION

Since pituitary gonadotropins are indispensable to gonadal functions and gonadotropin-releasing hormone (GnRH) stimulates pituitary release of gonadotropin, treatment with GnRH and its agonist was initially expected to be a potential means for the enhancement of gonadal functions. However, clinical applications of GnRH and its agonist to the induction of ovulation in anovulatory women has met with only limited success. Paradoxically, GnRH and its agonists have recently been shown to inhibit a variety of reproductive functions.

These peptides suppress the gonadotropin receptor level and steroidogenesis in rat gonads [1–7], prevent implantation and/or cause resorption of fetus in female rats [2, 8, 9] and shorten the luteal phase in normal cycling woman [10, 11]. Nevertheless, the mechanism by which GnRH and its agonist exert these multiple antigonadal actions remain uncertain.

Thus, we have examined the multiple antigonadal effects of a superactive agonistic analogue, [D-Leu_6, des-Gly-NH_2^{10}] GnRH ethylamide upon ovaries of hypophysectomized immature female rats *in vivo*, cultured porcine granulosa cells and rat luteal cells *in vitro*. In this paper, evidence will be presented to demonstrate the direct inhibitory actions of a GnRH agonist on *in vivo* ovarian functions in hypophysectomized rats and on *in vitro* ovarian cell functions in porcine granulosa cells and rat luteal cells.

IN VIVO SPECIFIC UPTAKE OF [^{125}I] GnRH AGONIST BY RAT OVARY

First, *in vivo* specific uptake of radiolabelled GnRH agonist in the ovary was examined by the administration of [^{125}I]GnRH agonist to immature female rat. The GnRH agonist was iodinated by the lactoperoxidase method. The labelled GnRH agonist was purified by elution through a CMC column. 10 μCi of [^{125}I]GnRH agonist was given intravenously into immature female rats with or without an excessive amount (100 μg) of unlabelled GnRH agonist. Thirty minutes after the administration, the rats were sacrificed and the uptake of radioactivity of [^{125}I]GnRH agonist by the various organs of rats was determined.

Figure 1 shows the uptakes of [^{125}I]GnRH agonist by rat pituitary, ovary, uterus and liver which are expressed as the ratio of tissue cpm/mg to blood cpm/μl (tissue–blood ratio). The radioactive tissue–blood ratio of pituitary was suppressed by concomitant injection of unlabelled GnRH agonist ($P < 0.05$). A similar result was obtained in the radioactive tissue–blood ratio of ovary ($P < 0.05$), whereas no suppression by unlabelled GnRH agonist

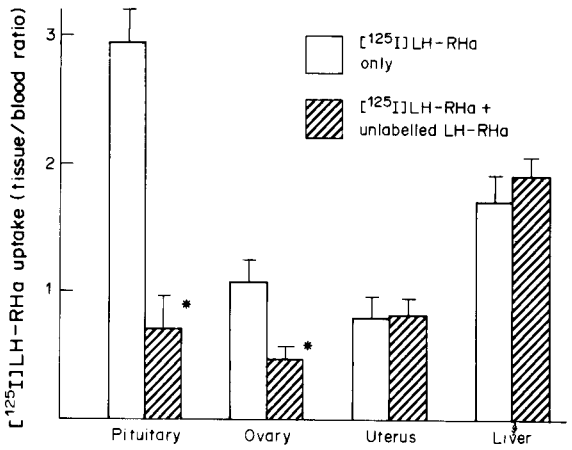

Fig. 1. Uptake of [^{125}I]GnRH agonist by pituitary, ovary, uterus and liver of immature female rats 30 min after i.v. administration of [^{125}I]GnRH agonist. Shaded column represents the uptake of [^{125}I]GnRH agonist in rats received [^{125}I]GnRH agonist together with excess of unlabelled GnRH agonist. Bars represent the mean ± SE. *; $P < 0.05$ as compared to the results obtained in rats treated with [^{125}I]GnRH agonist alone. From [14].

was observed in the uptake of radioactivity by liver and uterus. These results suggest that specific binding sites of GnRH agonist do exist not only in the pituitary but also in the ovary.

EFFECTS OF GnRH AGONIST ON hCG-STIMULATED *IN VIVO* OVARIAN RESPONSES IN HYPOPHYSECTOMIZED RATS

Immature female rats were hypophysectomized on day 21 of life and hormone treatment was initiated

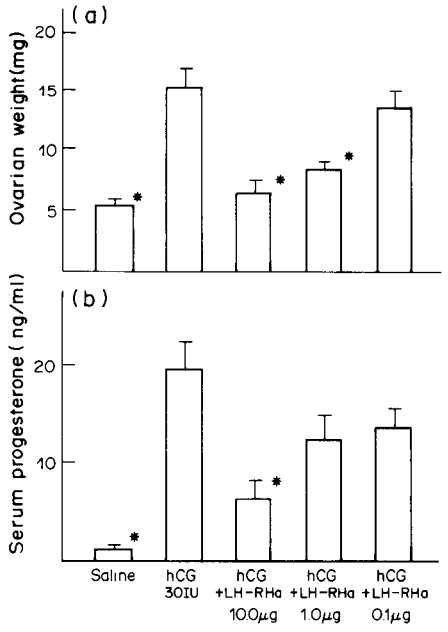

Fig. 2. Ovarian weight and serum progesterone concentrations of hypophysectomized immature female rats treated for three days with saline, hCG alone or both hCG and GnRH agonist. Bars represent the mean ± SE. *; $P < 0.01$ as compared to the results obtained in rats treated with hCG alone. From [14].

4–5 days after hypophysectomy. Rats received twice daily s.c. injection of 30 IU of hCG with or without varying amounts of GnRH agonist for three days. Twenty-four hours after the last injection, the rats were sacrificed, sera were collected and ovaries were removed and weighed.

In hypophysectomized rats, daily injections of 30 IU of hCG for three days increased ovarian weight from 5.1 ± 0.6 mg of saline treated control to 15.1 ± 1.5 mg ($P < 0.01$), whereas concomitant treatment with GnRH agonist inhibited the hCG-stimulated ovarian weight augumentation in a dose dependent manner (Fig. 2a).

Following the administration of the same dose of hCG to hypophysectomized immature female rats, serum progesterone concentrations increased markedly ($P < 0.01$), (Saline treated, 0.9 ± 0.2 ng/ml; hCG treated, 19.6 ± 2.3 ng/ml). However, when GnRH agonist was injected concomitantly with hCG, serum progesterone concentrations decreased ($P < 0.01$) in relation to the dose of GnRH agonist given (Fig. 2b). These results indicate that GnRH agonist prevents hCG-stimulated ovarian responses *in vivo*.

EFFECTS OF GnRH AGONIST ON FSH-STIMULATED *IN VIVO* OVARIAN RESPONSES IN HYPOPHYSECTOMIZED RATS

In this experiment, sialastic capsules containing diethylstilbestrol (DES) were implanted into the rats on the day of hypophysectomy. Starting from the fourth postoperative day, the rats received twice daily s.c. injections of 100 μg of ovine FSH (NIH-FSH-S11) with or without varying amounts of GnRH agonist for three days. Twenty-four hours after the last injection, ovarian weight and [^{125}I]-binding to crude membrane preparation of the ovaries were measured.

Twice daily s.c. injections of 100 μg of ovine FSH increased ovarian weight from 11.6 ± 0.5 mg in saline treated control to 22.0 ± 2.5 mg ($P < 0.01$), whereas concomitant treatment with GnRH agonist suppressed ($P < 0.01$) the ovarian weight augmentation (Fig. 3a).

On the other hand, Fig. 3b shows the effect of GnRH agonist on FSH induction of ovarian [^{125}I]hCG binding. An aliquot of the crude membrane preparation representing 10 mg ovarian tissue was incubated with 3×10^5 cpm of [^{125}I]hCG for 3 h at 37°C. FSH administration to the DES-treated hypophysectomized immature female rats increased ($P < 0.01$) the [^{125}I]hCG binding capacity of crude membrane preparation of the ovaries. Ovarian crude membrane preparation obtained from saline treated control rats and FSH treated rats bound 5.0 ± 1.5 pg hCG/mg ovarian tissue and 23.5 ± 3.7 pg hCG/mg ovarian tissue, respectively. Concomitant treatment with GnRH agonist, however, inhibited ($P < 0.01$) the FSH stimulation of [^{125}I]hCG binding to ovarian

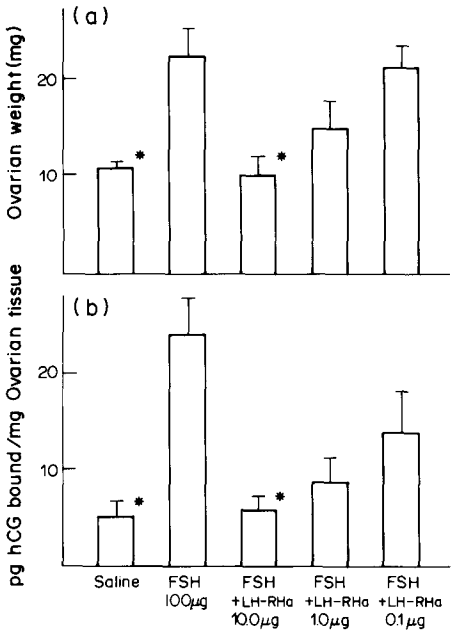

Fig. 3. Ovarian weight and [^{125}I]hCG binding to ovarian homogenate of DES-treated hypophysectomized immature female rats treated for 3 days with saline, FSH alone, or both FSH and GnRH agonist. Bars represent the mean ± SE. *; $P < 0.01$ as compared to the result obtained in rats treated with FSH alone. From [14].

crude membrane preparation in a dose dependent manner.

These results obtained in *in vivo* experiments with hypophysectomized immature female rats indicate that GnRH agonist inhibits not only hCG-dependent responses but also FSH-dependent responses in ovaries *in vivo*. Thus, GnRH agonist seems to block two independent actions of both gonadotropins of LH/hCG and FSH.

Since hypophysectomized rat model was used, these inhibitory actions of GnRH agonist are not exerted via the pituitary. The inhibitory actions of GnRH agonist seem to be mediated through specific receptors in the ovaries, since specific uptake of [^{125}I]GnRH agonist was observed in the ovaries. However, one cannot completely rule out the possibility that GnRH agonist may act on another organ to cause secondary inhibition of ovarian function from the data obtained by these *in vivo* experiments.

EFFECTS OF GnRH AGONIST ON hCG-STIMULATED PROGESTERONE SECRETION BY RAT LUTEAL CELLS *IN VITRO*

The inhibition of ovarian functions by GnRH agonist was further investigated by *in vitro* incubation experiments with rat luteal cells. To prepare pseudopregnant rats, immature female rats received s.c. injection of 50 IU of PMSG, followed by 25 IU of hCG 65 h later. Seven days after the hCG injection, the pseudopregnant rat ovaries were removed and the suspension of rat luteal cells were prepared with collagenase treatment. An aliquot of the cell suspension in an appropriate concentration of 3×10^6 cells/ml was incubated with various amounts of hCG for three hours in the presence or absence of GnRH agonist (10 μg/ml).

Figure 4 shows the effect of GnRH agonist on hCG-stimulated secretion of progesterone by rat luteal cells *in vitro*. hCG stimulated progesterone secretion by dispersed rat luteal cells in a dose dependent manner. However, the addition of GnRH agonist to the incubation medium resulted in a significant decrease in progesterone secretion stimulated by hCG. This result indicates that GnRH agonist inhibits hCG-dependent progesterone production by rat luteal cells incubated *in vitro*, and demonstrates that GnRH agonist acts directly on ovarian cells to cause the inhibitory effects.

EFFECTS OF GnRH AGONIST ON INDUCTION OF LH/hCG RECEPTOR STIMULATED BY FSH AND INSULIN IN PORCINE GRANULOSA CELLS *IN VITRO*

Regarding the site of action of GnRH agonist in blocking LH/hCG dependent progesterone production, there may be three possibilities as follows: (1) GnRH agonist may change affinity or population of LH/hCG receptor in ovarian cells, (2) GnRH agonist may affect hormonal activation of cAMP accumulation in ovarian cells, (3) GnRH agonist may act on the site subsequent to cAMP accumulation. To determine which of these possibilities are actually responsible for the inhibitory action of GnRH agonist, *in vitro* experiments with porcine granulosa cells were carried out.

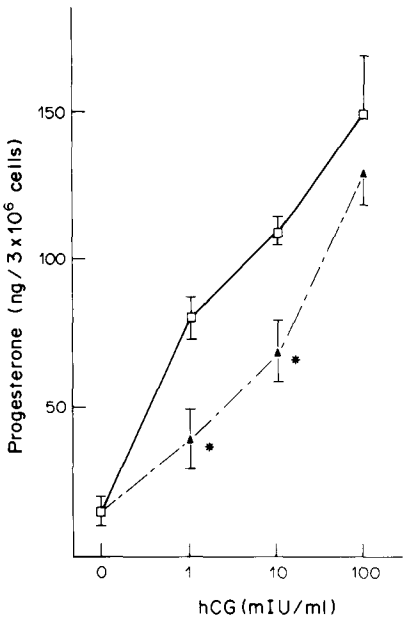

Fig. 4. Fig. 4. Progesterone production by rat luteal cells incubated *in vitro*. The cells were incubated in medium supplemented with hCG alone (□——□) or in medium containing hCG and GnRH agonist (▲---▲). Bars represent the mean ± SE. *; $P < 0.05$ as compared to the result obtained in incubation without GnRH agonist. From [14].

Porcine ovaries were collected within 30 min after slaughter and the follicular fluid was collected with the needle aspiration method of Channing and Kammerman[12]. Follicles were classified according to diameter as small (1–2 mm), medium (3–5 mm) and large (6–11 mm). Porcine granulosa cells obtained from small follicles were suspended in medium 199 containing 10% porcine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at a concentration of 1×10^6 cells/ml. Approximately 1×10^6 cells were plated on plastic multiwell plates, while approximately 3×10^6 cells were placed in plastic petri dishes. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Figure 5 shows the effects of GnRH agonist on [^{125}I]hCG binding to porcine granulosa cells obtained from small follicles in the course of culture for 6 days. [^{125}I]hCG binding to the cells decreased during the first two days of culture regardless of hormone treatment. [^{125}I]hCG binding to the cells cultured in medium supplemented only with FSH or insulin alone continued to decline through 6 days of culture. In contrast, when the cells were cultured in a medium supplemented with both FSH (1 μg/ml) and porcine insulin (25 mIU/ml), a substantial increase in [^{125}I]hCG binding to the cells was observed ($P < 0.01$). However, when the cells were cultured in a medium containing not only FSH and insulin but also GnRH agonist (10 μg/ml), no increase in [^{125}I]hCG binding to the cells occurred, demonstrating that concomitant treatment with GnRH agonist inhibits the increase in [^{125}I]hCG binding to the cells stimulated by the addition of FSH and insulin ($P < 0.01$). This result indicates that GnRH agonist prevents FSH induction of LH/hCG receptor in porcine granulosa cells.

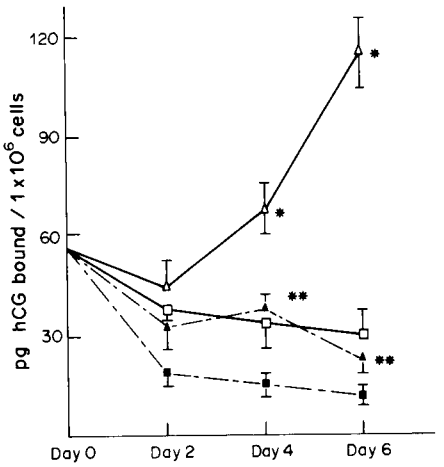

Fig. 5. [^{125}I]hCG binding to porcine granulosa cells obtained from small follicles in the course of culture for 6 days. The cells were cultured in medium without any supplement (■—·—■) or in medium containing insulin (□——□), insulin and FSH (△——△, insulin, FSH and GnRH agonist (▲—·—▲). Bars represent the mean ± SE. *; $P < 0.01$ as compared to control cultures. **; $P < 0.01$ as compared to cultures supplemented with insulin and FSH. From [14].

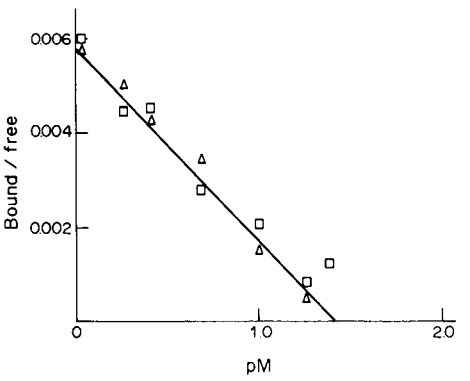

Fig. 6. Scatchard analysis of [^{125}I]hCG binding to porcine granulosa cells obtained from medium follicles. The cells were incubated for two hours with GnRH agonist (□) or without GnRH agonist (△). From [14].

ACUTE EFFECTS OF GnRH AGONIST ON LH/hCG RECEPTOR IN PORCINE GRANULOSA CELLS

In short time incubation experiments with porcine granulosa cells obtained from medium follicles, no acute effects of GnRH agonist on [^{125}I]hCG binding to the cells were observed. Figure 6 shows the Scatchard analysis of specific [^{125}I]hCG binding to porcine granulosa cells from medium follicles incubated for two hours with [^{125}I]hCG alone or with both [^{125}I]hCG and GnRH agonist. The dissociation constant obtained from the cells incubated with GnRH agonist and without GnRH agonist was 7.3×10^{-10} and 7.2×10^{-10}, respectively. No significant differences in the dissociation constant or in the number of hCG receptor sites between the cells incubated with GnRH agonist and without GnRH agonist were observed. This result suggests that GnRH agonist has no effect on LH/hCG receptor already induced.

EFFECTS OF GnRH AGONIST ON MORPHOLOGICAL LUTEINIZATION OF PORCINE GRANULOSA CELLS CULTURED *IN VITRO*

Monolayer cultures of porcine granulosa cells obtained from small follicles were performed in plastic Petri dishes. Incubations were carried out in medium containing hCG (25 mIU/ml) and/or porcine insulin (25 mIU/ml) in the presence or absence of GnRH agonist (10 μg/ml).

The cells cultured in medium alone without hormone treatment showed a fibroblastic appearance by day 6 of culture (Fig. 7a). In contrast, when the cells were cultured in a medium supplemented with both hCG and insulin for 6 days, the cells became epitheloid (Fig. 7b). However, when the cells were cultured in a medium containing not only hCG and insulin but also GnRH agonist, the cells did not display the epitheloid change and maintained a fibroblastic appearance by day 6 of culture (Fig. 7c). This result suggests that GnRH agonist prevents the differentiation of porcine granulosa cells.

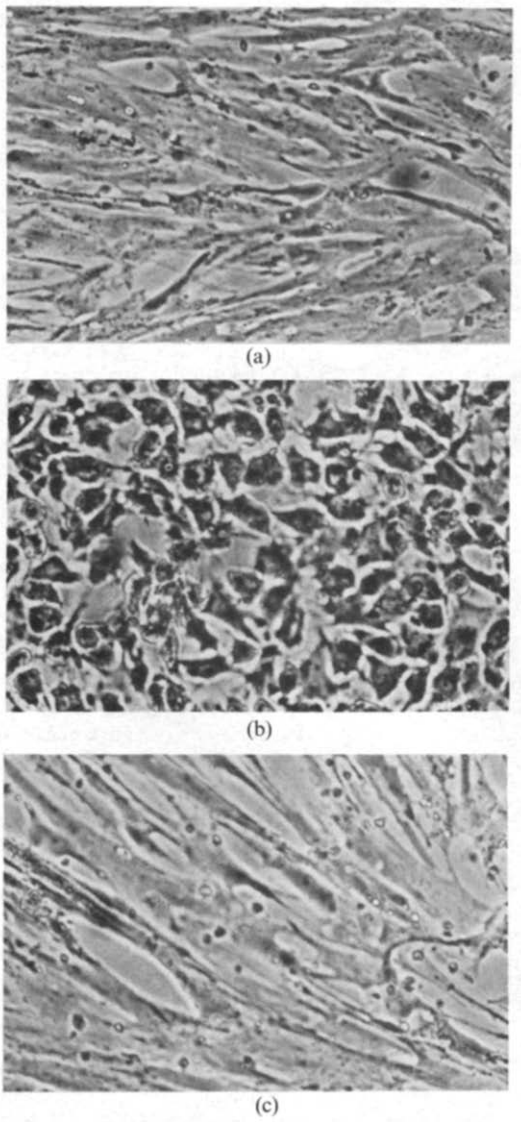

Fig. 7. Phase contrast photomicrographs of porcine granulosa cells cultured *in vitro* for 6 days. (a) culture in medium alone without hormone treatment, (b) culture in medium supplemented with hCG and insulin (c) culture in medium containing hCG, insulin and GnRH.

EFFECTS OF GnRH AGONIST ON hCG-STIMULATED cAMP ACCUMULATION IN PORCINE GRANULOSA CELLS *IN VITRO*

To examine the effects of GnRH agonist on hCG-stimulated cAMP accumulation in ovarian cells, porcine granulosa cells obtained from medium follicles were incubated in a Dubnoff metabolic shaker with hCG (30 mIU/ml) in the presence or absence of GnRH agonist (10 μg/ml). cAMP concentrations in the cells were assayed by the method of Honma *et al.*[13] after deproteinization with TCA. Radioimmunoassay of cAMP was carried out using antibody raised against succinyl cAMP.

Figure 8 shows the time course of cAMP levels in porcine granulosa cells obtained from medium follicles. hCG remarkably stimulated cAMP accumulation in the cells from the initial non-detectable level to 5.5 ± 0.6 pmol/10^6 cells at 5 min after the addition of hCG into the incubation medium. Concomitant incubation with GnRH agonist, however, delayed the accumulation of cAMP. The cAMP level at 5 min after the addition of hCG and GnRH agonist was 1.4 ± 0.4 pmol/10^6 cells, which was significantly lower than that obtained with the addition of hCG alone ($P < 0.01$).

This delay of cyclic AMP accumulation may represent one of the mechanisms involved in inhibiting progesterone production by ovarian cells. However, the cyclic AMP level in cells incubated with GnRH agonist rose to a level comparable to that in cells incubated without GnRH agonist at 30 min after the addition of hCG. Thus, whether or not such a short delay of cAMP accumulation is fully responsible for the GnRH agonist inhibition of progesterone production by ovarian cells is not clear. The possibility that there may be other sites of GnRH agonist action subsequent to cAMP accumulation cannot be excluded. Further study is needed to elucidate this point.

CONCLUSIONS

The findings obtained in the present studies are summarized as follows:

(1) There is specific uptake of radiolabelled GnRH agonist by rat ovaries, indicating the presence of specific receptor for GnRH agonist in the ovary.
(2) GnRH agonist inhibits gonadal functions in hypophysectomized immature female rats *in vivo*.
   (a) GnRH agonist inhibits LH/hCG-stimulated ovarian weight augmentation and progesterone production by rat ovaries *in vivo*.

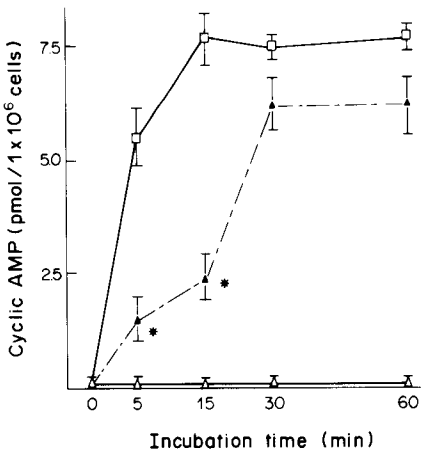

Fig. 8. Time course of cAMP accumulation in porcine granulosa cells obtained from medium follicles. The cells were incubated in medium supplemented with hCG alone (□——□) or in medium containing hCG and GnRH agonist. △——△; control incubation without hCG. Bars represent the mean ± SE. *; $P < 0.01$ as compared to the results obtained in cultures without GnRH agonist. From [14].

(b) GnRH agonist inhibits FSH-stimulated ovarian weight gain and *in vivo* induction of ovarian LH/hCG receptor by FSH.

(3) GnRH agonist inhibits ovarian cell functions *in vitro*

(a) GnRH agonist inhibits LH/hCG-dependent progesterone production by rat luteal cells *in vitro*.

(b) GnRH agonist inhibits *in vitro* induction of LH/hCG receptor stimulated by FSH and insulin in porcines granulosa cells obtained from small follicles.

(c) There are, however, no acute effects of GnRH agonist on the affinity or population of LH/hCG receptor in porcine granulosa cells obtained from medium follicles.

(d) GnRH agonist inhibits the morphological differentiation of porcine granulosa cells induced by hCG and insulin.

(4) GnRH agonist delays hCG-stimulated cAMP accumulation in porcine granulosa cells *in vitro*.

These finding suggest that GnRH agonist acts directly on ovarian cells through binding to its specific receptor sites in ovary and inhibits independently the gonadotropic actions of FSH and LH/hCG. It seems likely that GnRH agonist first inhibits cAMP accumulation in ovarian cells and subsequently inhibits LH/hCG-stimulated progesterone production by ovarian cells. Simultaneously, GnRH agonist prevents the differentiation of granulosa cells and FSH-stimulated LH/hCG receptor induction in poorly differentiated granulosa cells.

The physiological significance of the present observation is not yet known. However, these multiple antigonadotropic actions of GnRH agonist on ovarian cell function *in vivo* and *in vitro* will be considered in connection with its use as a regulator of fertility.

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