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GONADOTROPIN-RELEASING HORMONE AS A MODULATOR OF OVARIAN FUNCTION

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Summary—GnRH and its agonist analogs exert direct inhibitory and stimulatory effects on the ovaries of animals from several species. In the immature follicle, GnRH inhibits the actions of FSH on an integrated array of biochemical responses that lead to follicular development and corpus luteum formation. GnRH also suppresses gonadotropin action in mature follicles, and stimulates certain ovarian processes such as steroidogenesis and oocyte maturation. The inhibitory and stimulatory effects of GnRH are mediated through the binding of the peptide to high-affinity receptors in granulosa and thecal cells. Recent studies have shown that GnRH action in the ovary is dependent upon calcium mobilization and probably operates through stimulation of phospholipid turnover and activation of protein kinase C.

INTRODUCTION

Since the initial observation of Rippel and Johnson in 1976 [1] that exogenous GnRH prevents augmentation of ovarian and uterine weight gain by hCG in hypophysectomized rats, considerable effort has been accorded to studies of the direct involvement of GnRH in ovarian function [for reviews, see 2–4]. These investigations have indicated that GnRH and its agonist analogs can inhibit gonadotropin action in the developing follicle, and also stimulate specific ovarian functions under appropriate conditions. The major anti-reproductive actions of GnRH, as well as its stimulatory actions on ovarian function, are shown in Table 1. The presence of specific GnRH binding sites in ovarian tissue indicates that GnRH acts through receptor-mediated processes. However, the actual mechanism of GnRH action in the ovary remains to be elucidated. In this regard, the ability of GnRH to act as either an inhibitory or stimulatory ligand on the same ovarian processes, such as steroidogenesis and ovulation, complicates the development of a unifying hypothesis of GnRH action in the ovary. However, several recent studies have demonstrated that the nature of the response to GnRH is largely determined by the maturational status of the ovary. It is clear that the suppressive effects of GnRH predominate in immature follicles that are responsive to gonadotropic stimulation. As the follicle matures, stimulatory as well as inhibitory effects of GnRH begin to appear. In general, the stimulatory responses are largely the direct action of GnRH itself, whereas the inhibitory effects are due to the suppression of responses to both FSH and LH.

Table 1. Direct effects of GnRH on the ovary

1.	*Inhibition of reproductive function*
a.	Oocyte—inhibition of ovulation, ovum transport, implantation of the fertilized zygote;
b.	Follicular maturation—impaired cyclic nucleotide production, steroidogenesis, polypeptide hormone receptor formation;
c.	Corpora lutea function—inhibition of steroidogenesis; luteolysis; cycle length changes;
d.	Thecal/interstitial cells—suppression of steroidogenesis;
2.	*Stimulation of reproductive function*
a.	Oocyte—stimulation of oocyte maturation and ovulation, oocyte cleavage;
b.	Biochemical effects in the mature follicle—increased steroidogenesis, prostaglandin production, glycolysis and lactate production, phospholipid turnover.

SPECIES-SPECIFICITY OF GnRH ACTION

The original demonstration of a direct effect of GnRH in the rat ovary was followed by extensive studies on its gonadal actions in other species. Animals that exhibit responses to the direct gonadal effects of GnRH include the rat [2], pig [5, 6], hamster [7], rabbit [8], and chicken [9]. In these species, GnRH has effects on cyclic nucleotide production, steroidogenesis, gonadotropin receptor formation, follicular morphology, and the oocyte. Animals in which GnRH apparently does not act upon or bind to functional receptors in the ovary include the sheep [10], cow [10], mouse [11], and primates. The direct effect of GnRH on the primate ovary is still somewhat controversial. In several studies, no effects of GnRH upon gonadotropin-induced steroidogenesis were observed in human granulosa and luteal cells cultured from several hours for up to 12 days [12–15]. Consistent with these observations has been the lack of appreciable binding of labelled GnRH and its agonist analogs to human luteal tissue [13, 16]. However, other reports have suggested that a direct response of GnRH in the primate ovary may be observed under appropriate conditions. Thus, a GnRH agonist inhibited progesterone production by maturing human granulosa cells cultured for several days [17], and low-affinity binding sites for GnRH have been reported in human luteal tissue and

in monkey ovaries [3, 18]. It is not clear whether the lack of consistent responses in the primate ovary is largely due to methodological differences or to physiological variations in receptor abundance. For example, the suppressive effects of GnRH in the rat ovary are extremely marked in cells from immature follicles, suggesting that follicular rather than luteal tissue may be a more appropriate source to investigate GnRH effects in the primate ovary. The relative ease of obtaining synchronized, immature granulosa cells from preantral follicles of hypophysectomized, estrogen-treated rats has demonstrated the importance of a well defined model for following hormonal responses during follicular development. Alternatively, the evolutionary pattern of putative endogenous GnRH or GnRH-like peptides in the primate ovary may have resulted in the development of distinct differences in the responsiveness of this organ to the hypothalamic peptide.

BIOLOGICAL ACTIONS OF GnRH ARE MEDIATED BY GnRH RECEPTORS

GnRH receptors of high affinity and specificity are distributed in all ovarian compartments. Autoradiographic studies have localized GnRH binding sites in the rat ovary to the theca interna and externa, and to the granulosa and luteal cells [19, 20]. Analysis of the binding of a labeled GnRH agonist to ovarian slices from immature, diethylstilbestrol-primed rats and to ovaries from animals treated with FSH or PMSG–hCG is shown in Fig. 1. Photoaffinity labeling of rat granulosa cells has shown that the GnRH receptor consists of two components with apparent mol. wts of 53,000 and 42,000 [21]. The molecular weights of the two components of the ovarian GnRH receptor were similar to those observed for GnRH receptors solubilized from the anterior pituitary. When examined by fluorescence microscopy, labeled GnRH receptors were initially distributed uniformly over the cell surface, with subsequent patch formation and apparent internalization into endocytic vesicles [22]. The similarity of ovarian and pituitary GnRH receptor sites is also indicated by the correlation between the responsiveness of ovarian granulosa cells and pituitary gonadotrophs to various GnRH agonists and antagonists [23, 24]. A recent study has suggested that the GnRH receptor of both the pituitary and ovary may contain a cAMP binding site [25]. Cyclic AMP and its derivatives in millimolar amounts can inhibit the binding of a GnRH agonist to membrane fractions and to solubilized GnRH receptors. Since cAMP is the primary regulator of granulosa cell maturation [26], an interaction of cAMP with the GnRH receptor may be of physiological importance.

The presence of GnRH receptors in ovarian tissues correlates with the effects of the hypothalamic peptide on reproductive function. In granulosa and luteal cells of the rat, increasing concentrations of GnRH

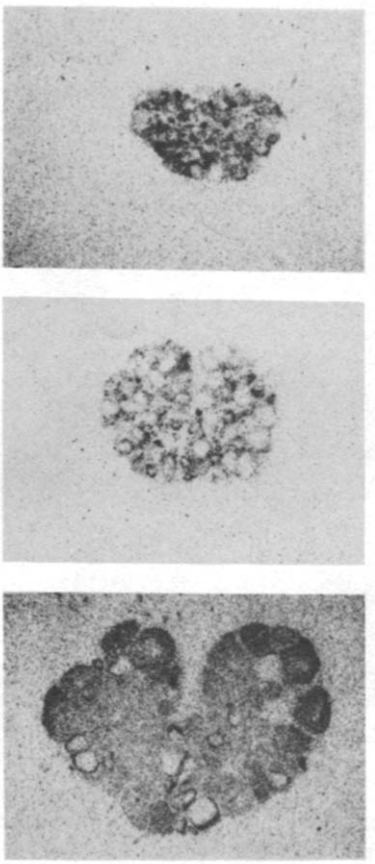

Fig. 1. Binding of ^{125}I-[D-Ala^6]des-Gly^{10}-GnRH *N*-ethylamide (GnRHa) to ovarian slices from diethylstilbestrol-implanted (top), FSH-treated (middle), and PMSG-hCG-treated (bottom) rats. Ovaries were frozen and sliced in 20 μm sections. Slices were incubated with [^{125}IGnRHa (50,000 cpm) at 0°C for 2 h, washed twice to remove unbound hormone, and exposed for 3 days to X-ray film. Non-specific binding was determined in the presence of 10^{-7} M unlabeled GnRHa and showed no detectable staining.

and its agonist analogs inhibit gonadotropin-induced steroidogenesis [2], while in the interstitial cells of the hamster [7] and rat [27] ovary, a GnRH agonist significantly reduced steroid production. These direct effects of GnRH can be completely prevented by specific GnRH antagonists [2].

The GnRH receptor content of the ovary is regulated by gonadotropins and by GnRH itself [28, 29]. Thus, FSH and low concentrations of a GnRH agonist (less than 10^{-8} M) increased GnRH binding activity in cultured granulosa cells [29]. In contrast, high concentration of GnRH decreased it homologous receptors in cultured granulosa cells [29], while hyperstimulation of pituitary function using daily injections of GnRH for three weeks had little effect on ovarian binding sites for GnRH [20]. Although the ovarian GnRH receptors do not change during the rat estrous cycle [19, 28], marked changes in GnRH receptors are observed during fetal life and puberty [28, 30]. GnRH receptor content is low in the

neonatal rat ovary (days 5–15), probably due to the binding of a GnRH-like substance derived from milk [30]. Dissociation of endogenously bound milk GnRH by magnesium chloride, or prevention of suckling, resulted in a rise in ovarian GnRH binding capacity, similar to that observed in 25-day old rats. The milk GnRH substance was biologically active since it released FSH and LH from pituitaries *in vitro*, inhibited FSH-stimulated steroidogenesis in granulosa cells, and displaced the binding of a labeled GnRH agonist to ovarian membranes. These results demonstrate the importance of ectopic GnRH peptides in the direct regulation of normal ovarian function and development.

INHIBITORY AND STIMULATORY EFFECTS OF GnRH IN THE OVARY

Inhibitory effects of GnRH

GnRH markedly inhibits the maturation of ovarian follicles both *in vivo* and *in vitro* [2, 3]. These effects of GnRH are more prominent upon the immature follicle and its responses to FSH, and include the disruption of cyclic nucleotide production, steroidogenesis, receptor formation for LH, FSH, and prolactin, morphological maturation, and ovulation. The inhibitory action of GnRH on granulosa cell differentiation is essentially irreversible and can occur at any point within the maturation process mediated by FSH [31]. In cultured granulosa cells from estrogen-treated hypophysectomized rats, FSH induces a marked clustering of individual cells into multilayered aggregates (Fig. 2). After 6 h of culture (a), granulosa cells are round and in small clumps, while at 24 h (c), FSH-treated cells are highly aggregated with an epithelial appearance. GnRH has minimal effects on this restructuring process for the first 24 h of culture (b,d). However, with continued times of culture GnRH disrupts cellular aggregates (f) and the granulosa cells assume a fibroblastic appearance. Since newly formed LH receptors induced by FSH are largely localized on the microvilli of aggregated cells [32], the inhibitory effects of GnRH on follicular morphology and cell-to-cell com-

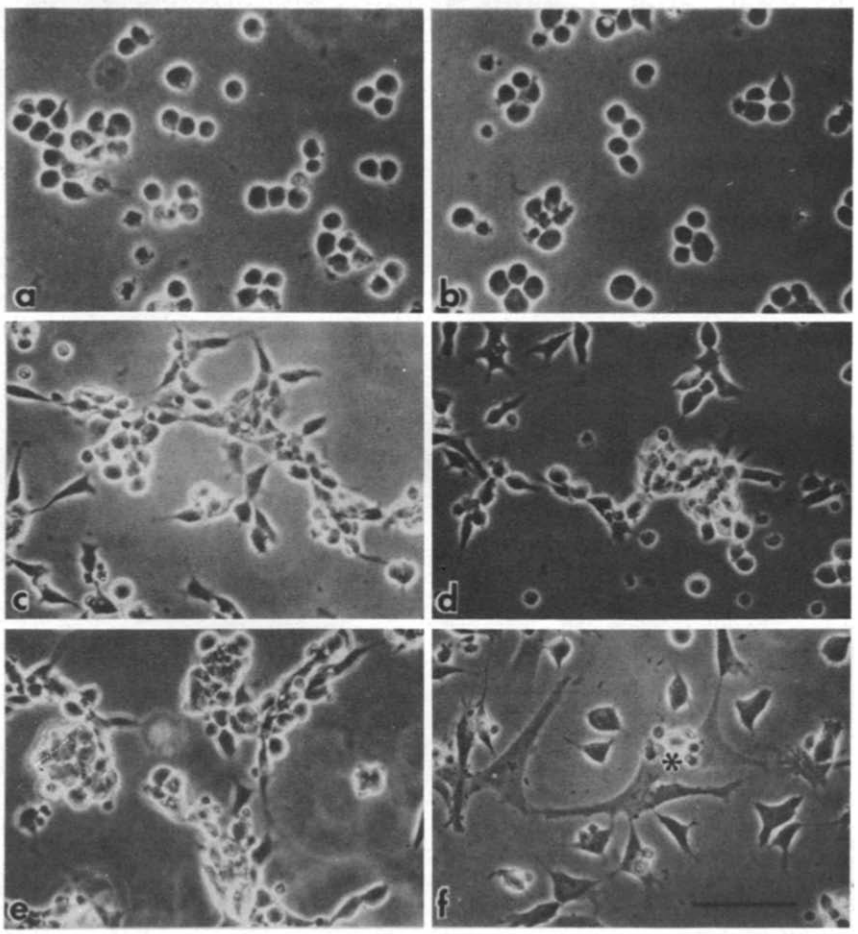

Fig. 2. Disruption of the morphological maturation of cultured granulosa cells by GnRHa. Granulosa cells were treated with FSH or FSH plus GnRHa for 6h (a,b), 24h (c,d), or 48h (e,f). Cells were then fixed with 3% glutaraldehyde in Millonig's phosphate buffer and visualized by phase contrast microscopy. *Indicates a disrupted cellular aggregate (f). Magnification = × 530, bar = μm. (Reprinted from *Endocrinology* **110,** 865–872, 1982).

munication are relevant to its suppressive role in the ovary.

Granulosa cell differentiation has been shown to be dependent upon cAMP-mediated processes [26], indicating that the effects of GnRH on the production and action of this cyclic nucleotide may directly alter ovarian function. GnRH suppresses cAMP formation in both granulosa and luteal cells of several species [3, 5, 33]. The decrease in cAMP is due to effects on both adenylate cyclase and phosphodiesterase, since GnRH inhibits gonadotropin-induced cAMP synthesis and reduces cAMP catabolism [33–35]. Although GnRH had no demonstrable effect on FSH-or LH-stimulated adenylate cyclase activities in granulosa or luteal cell membranes [33, 36], gonadotropin-dependent cAMP synthesis was inhibited in ovarian membranes from rats primed with FSH and a GnRH agonist [33]. The impairment of cAMP synthesis by GnRH was not mediated by alterations in the activity of the catalytic or guanine nucleotide regulatory subunits or adenylate cyclase, since GnRH did not affect cAMP synthesis induced by forskolin, GMP-P(NH)P, or sodium fluoride [33]. Although the reduction in both FSH and LH receptor content by GnRH [33] plays a major role in its inhibitory effects on cAMP synthesis, an as yet undefined effect of the peptide on the adenylate cyclase complex cannot be excluded. Thus, GnRH reduced adenylate cyclase activity in granulosa cells precultured with FSH for 48 h in the absence of an effect on FSH receptor content [34].

The actions of cAMP in ovarian cells are also impaired by GnRH, since steroidogenesis and LH receptor formation induced by cAMP derivatives or cAMP-inducing ligands were suppressed by GnRH agonist treatment [26]. Further, the FSH-stimulated increase in cAMP-dependent protein kinase activity was reduced by a GnRH agonist, with a delay in the activation of the enzyme during the first 60 min of granulosa cell culture [37]. GnRH also inhibited an FSH-induced rise in the regulatory subunit of type II cAMP-dependent protein kinase [37, 38]. These results suggest that GnRH may modify ovarian function by altering the phosphorylation of cellular proteins involved in maturation. In the luteal cell, the inhibitory actions of GnRH are predominantly due to impairment of cAMP production, rather than to suppression of cAMP action [36, 39]. Although GnRH has effects on multiple enzymes involved in steroidogenisis [40, 41], these changes are presumably secondary to the responsiveness of steroidogenic pathways to the cAMP induced by gonadotropins and to the alterations in cAMP production and action by GnRH.

The inhibitory effects of GnRH in both immature and mature granulosa cells are highly dependent upon the extracellular calcium concentration [42]. In cells cultured for 48 h with FSH to induce LH receptors, a GnRH agonist progressively suppressed LH-stimulated cAMP production in the presence of 0.25–1 mM calcium. Omission of extracellular calcium completely prevented the inhibitory effect of the GnRH agonist upon cAMP production. The suppressive effects of GnRH on prostaglandin E_2 and isoproterenol-induced cAMP productions were also reduced when the extracellular calcium concentration was lowered, while a phosphodiesterase inhibitor reversed GnRH action. Thus, GnRH may activate a calcium-dependent phosphodiesterase in ovarian cells, thereby decreasing cAMP formation. In immature granulosa cells, FSH-induced formation is dependent upon the extracellular calcium concentration (Table 2). Lowering of the medium calcium concentration from 1.0 to 0.25 mM progressively inhibited the stimulatory effect of FSH on cAMP formation. In the presence of calcium, a GnRH agonist reduced FSH action by approx 80% during the 48 h culture period (Fig. 3). The inhibitory effects of GnRH were reduced by the calcium antagonist, verapamil, indicating the dependence of GnRH action upon calcium uptake. The importance of calcium in GnRH action was also demonstrated by the effects of the calcium ionophore, A23187, which increases cytosolic calcium levels (Fig. 4). The ionophore mimicked the inhibitory actions of GnRH on

Table 2. Dependence of FSH-stimulated cAMP accumulation in granulosa cells on the concentration of calcium

Calcium Level (mM)	cAMP (pmol)
0	Not detectable
0.25	6 ± 1
1.0	18 ± 1

2×10^5 granulosa cells were cultured for 48 h with FSH plus 0.1 mM EGTA in the presence of increasing concentrations of calcium in the extracellular medium. Data are the mean ± SE of 6 samples.

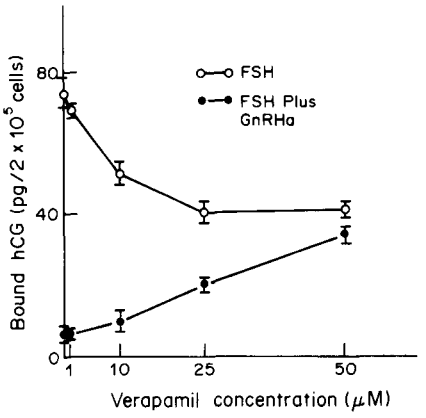

Fig. 3. Reversal of the direct inhibitory actions of GnRHa on LH receptor induction in granulosa cells by altering calcium flux. 2×10^5 granulosa cells were cultured with FSH (250 ng), FSH plus GnRHa (10^{-8} M), and increasing concentrations of verapamil for 4 h. Cells were then washed twice and cultured for a further 44 h with only FSH. The binding of [^{125}IhCG to LH receptors was then measured. Data are the mean ± SE from triplicate cultures.

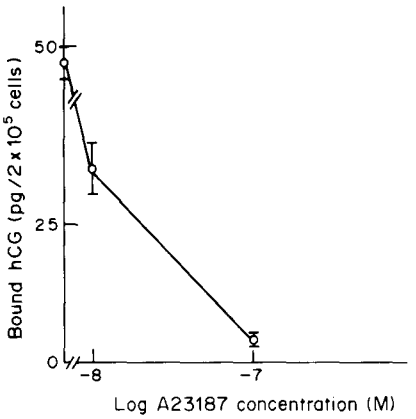

Fig. 4. Inhibitory effect of the calcium ionophore, A23187, on FSH-induced LH receptor formation. 2×10^5 granulosa cells were cultured with FSH (250 ng) plus increasing concentrations of A23187 for 4 h. Cells were then washed twice and cultured for a further 44 h with FSH alone. The binding of [^{125}I]hCG to LH receptors was then measured. Data are the mean $\pm$ SE from triplicate cultures.

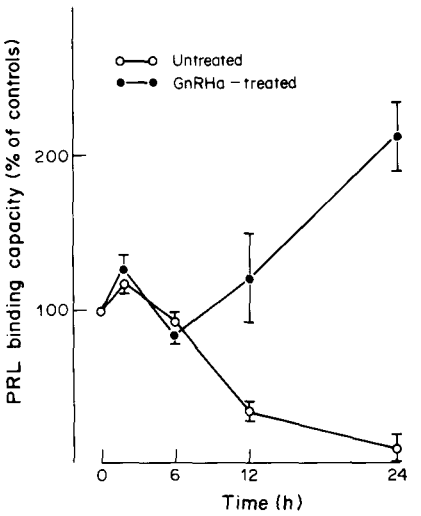

Fig. 5. Stimulation of prolactin receptor content in rat ovaries by GnRHa. Immature, diethylstilbestrol-implanted rats were treated with FSH for 48 h. Animals were then injected with 10 μg GnRHa or were untreated. At subsequent times, ovaries were removed and assessed for prolactin binding capacity. Within a 24 h period. GnRHa prevented the 90% fall in prolactin binding sites observed in untreated animals and instead stimulated a 2-fold rise in the levels of these receptors. (Reprinted from *Molecular and Cellular Endocrinology* **35**, 55–63, 1984).

FSH-induced LH receptor expression. In the luteal cell, inhibition of LH-stimulated cAMP production was dependent on an increase in intracellular calcium [43]. Thus, changes in cytosolic calcium concentration may be an early event in the mechanism of GnRH action.

Stimulatory effects of GnRH

The stimulatory effects of GnRH on ovarian function continue to be reported. Earlier observations of the minor enhancement by GnRH of progesterone and prostaglandin production [44, 45], cAMP formation [3, 46], and GnRH receptor content [29] were followed by reports on the effects of GnRH agonists on steroidogenic enzymes and steroid production [40], phosphodiesterase activity [35], phospholipid labeling and polyphosphoinositide turnover [47–50], follicular luteinization with elevated ovarian weight gain and prolactin receptor content [46, Fig. 5], glycolysis [51], and plasminogen activator production [52]. Stimulatory effects of GnRH on the oocyte include meiotic maturation [53], oocyte cleavage [46, Fig. 6], and ovulation [54]. Although the mechanisms of the stimulatory actions of GnRH in the ovary are not fully clear, the effects of GnRH on phospholipid and polyphosphoinositide

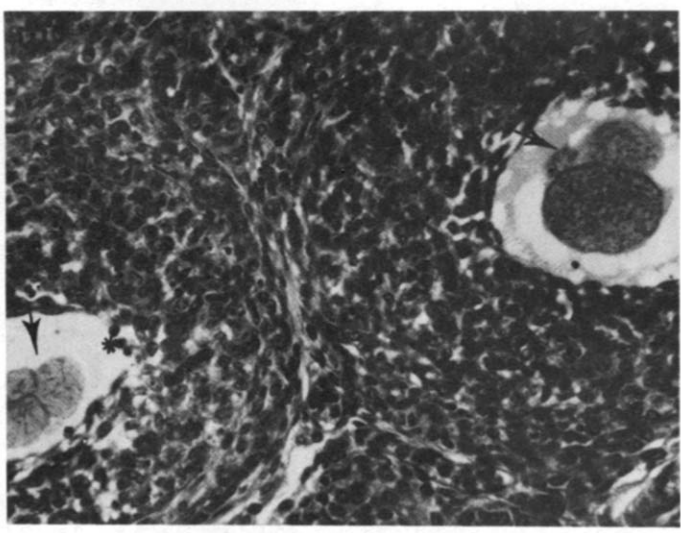

Fig. 6. GnRH stimulates oocyte cleavage. Immature, diethylstilbestrol-implanted rats were treated with GnRHa (10 μg, twice daily for 22 days). Ovaries were then removed, and fixed in Bouin's solution. The oocytes from 2 adjacent follicles are cleaved (arrows), and the granulosa cells are pyknotic (*). (reprinted from *Endocrinology* **112**, 956–964, 1983).

metabolism are the fastest cellular responses to GnRH described to date, making this effect a potential candidate for mediating GnRH action. A GnRH agonist rapidly stimulated the hydrolysis of triphosphinositide and diphosphoinositide, with effects as early as 30 s after GnRH addition to mature rat granulosa cells [50]. This response preceded GnRH-induced elevations in the labeling of phosphatidylinositiol, and a GnRH antagonist prevented the reduction in polyphosphoinositide by GnRH. The effects of GnRH on phosphatidylinositol and phosphatidic acid turnover occur within several minutes [47, 50]. In addition, GnRH caused enhanced labeling of several other phospholipids, including phosphatidylcholine and lysophosphatidylcholine [47]. These results can be correlated with other cellular systems in which receptor-mediated responses to hormones are associated with increases in phospholipid turnover [55]. Phospholipid turnover has been associated with calcium flux, and phosphatidic acid has been suggested to serve as a calcium ionophore [56]. The role of calcium in the actions of GnRH, as described above, suggests that calcium mobilization due to increased turnover of phospholipids may modify granulosa cell function. Such changes could lead to calcium-mediated alterations in adenylate cyclase and phosphodiesterase activities, and activation of phospholipase A_2 with elevations in arachidonic acid and prostaglandins. The presence of a calcium- and phospholiplid-dependent protein kinase C in ovarian cells is a further demonstration of the importance of phospholipids in follicular development [57]. Phosphorylation of specific ovarian substrates by protein kinase C in the presence of calcium and diacylglycerol has been observed [58]. In addition, an activator of protein kinase C, 12-*O*-tetradecanoylphorbol-13-aetate, impairs FSH-induced steroidogenesis and LH receptor expression, similar to the effects of GnRH and its agonist analogs [59].

Inhibitory and stimulatory effects of GnRH at specific stages of follicular development

Whether GnRH stimulates or inhibits ovarian function is largely determined by the stage of follicular development [46, 60]. The immature follicle is highly dependent upon gonadotropin action, and the major effects of GnRH in such tissue are inhibitory. However, the initiation of the cellular response to GnRH must involve the activation of certain functions, such as calcium and phospholipid fluxes. Maximal suppression of ovarian development was obtained when GnRH was administered to rats either concomitant with gonadotropin, or before a significant degree of follicular maturation had begun. In contrast, administration of GnRH to mature follicles resulted in ovulation and follicular luteinization. Similarly, the stimulatory effects of GnRH on steroidogenesis and prostaglandin production described above [44, 45] are predominantly seen in granulosa cells previously primed with gonadotropins. However, even in mature follicles, the major effect of GnRH on FSH and LH actions is inhibitory, as shown by the decreased activity of luteal cells to LH-induced cAMP and progesterone production in the presence of GnRH. Thus, the stimulatory effects of GnRH are largely a consequence of the direct actions of the peptide itself. Although these stimulatory responses are similar to those induced by gonadotropin, their mechanisms of action are probably distinct. Activation of steroidogenesis by LH is much greater and also more rapid than that by GnRH [61]. Also, LH-induced steroid production is mediated by cAMP, while GnRH has minimal stimulatory effects on the formation of this cyclic nucleotide and presumably acts through a calcium-dependent mechanism that converges on the steroidogenic pathway.

CONCLUSIONS

Although the direct control of ovarian function by locally formed GnRH would seem redundant in view of the multiple regulatory sites of reproductive function at the levels of the hypothalamus, pituitary, and gonad, there are several interesting possibilities for a role of GnRH-mediated actions within the ovary. Thus, endogenous GnRH-like materials may modulate follicular development to enhance the maturation of those follicles destined for ovulation by its effects on progesterone and prostaglandin production, and oocyte maturation. In contrast, the smaller follicles that are more sensitive to the inhibitory effects of GnRH may not respond to gonadotropins (in the presence of GnRH-like materials) and would undergo atresia, thereby ensuring the selection of only those mature follicles ready to progress towards ovulation. The validity of these potential direct functions awaits the isolation and characterization of an endogenous GnRH-like peptide that could mediate such actions. In addition, the possible role of other ectopic GnRH-like materials must be considered. The presence of a GnRH in milk, and the effect of such a ligand on the neonatal ovary, may be responsible for the delay in ovarian function until puberty commences [30]. Although the ovarian actions of GnRH are not manifested in all animals, the prominence of its receptor-mediated effects suggests that the peptide has a significant functional role in the control of follicular maturation in certain species.

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