

Effect of Adenylate Cyclase Inhibitor and Protein Kinase C Inhibitor on GnRH-induced LH Release and LH β Subunit Biosynthesis in Rat Anterior Pituitary Cells

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According to our previous studies together with others, GnRH, a hypothalamic decapeptide, has been known to be a major regulator for LH release and its subunit biosynthesis in anterior pituitary gonadotropes. But the precise mechanisms by which GnRH exerts stimulatory effects on LH release and its subunit biosynthesis have not been clearly understood. In the present study we examined the effect of GnRH on protein kinase C (PKC) activity and intracellular cAMP content in cultured anterior pituitary cells of rat to clarify whether PKC or cAMP are involved in GnRH action. Moreover, we examined the effects of staurosporine (ST), a PKC inhibitor and 2',3'-dideoxyadenosine (2',3'-DDA), an adenylate cyclase inhibitor, on LH release and steady state LH β subunit mRNA levels in cultured anterior pituitary cells of rat. PKC activity was rapidly increased within 30 min after GnRH treatment whereas intracellular cAMP level was elevated 18 h after GnRH treatment. ST significantly inhibited GnRH-induced LH release and LH β subunit mRNA levels in a dose-dependent manner, showing a half maximal response at 50 nM ST. 2',3'-DDA inhibited GnRH-induced LH release and LH β subunit mRNA levels in a dose-dependent manner in pituitary cells. From these results, it is suggested that GnRH stimulates LH β subunit mRNA level as well as LH release in anterior pituitary cells and this GnRH action might be mediated by PKC activation and cAMP stimulation.

Key Words: GnRH, LH, staurosporine, 2', 3'-dideoxyadenosine, protein kinase C, cAMP, pituitary cells

Luteinizing hormone (LH) is a pituitary gonadotropin that plays an important role in gonadal function, mainly through its influence on steroidogenesis and gametogenesis. LH re-

lease and its subunit biosynthesis have been known to be modulated directly by GnRH (gonadotropin-releasing hormone), a hypothalamic decapeptide (Gharib *et al.* 1990; Ryu *et al.* 1990; Kim *et al.* 1994) and indirectly by ovarian steroids (Ryu *et al.* 1989; Gharib *et al.* 1990; Kim *et al.* 1993). Although a number of studies have investigated the intracellular mechanism for the regulation of LH release and its subunit biosynthesis, the nature of GnRH action mechanism has not been clearly understood. Therefore, the intracellular signaling pathways mediating GnRH-induced LH release and its subunit biosynthesis have been the subjects of intensive investigations. It has been demon-

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strated that the interaction of GnRH with the membrane receptor initiates cAMP accumulation (Borgeat *et al.* 1972), inositol phospholipid hydrolysis (Catt and Stojilkovic, 1989), activation of PKC (Andrews *et al.* 1988; Dan-Cohen and Naor, 1990), arachidonic acid release (Naor and Childs, 1986) and Ca⁺⁺ mobilization (Naor *et al.* 1988) in pituitary cells. The production of multiple second messenger molecules seemed to be responsible for LH release and synthesis. However, conflict reports are also available. It was reported that GnRH stimulated LH release in PKC-depleted anterior pituitary cells (McArdle *et al.* 1987) and staurosporine, a PKC inhibitor enhanced GnRH-stimulated LH exocytosis in intact sheep gonadotropes (Merve *et al.* 1990). These results suggest that PKC does not play a major role in mediating GnRH-stimulated LH release. In addition, Conn *et al.* (1979) showed that there was no detectable increase in cAMP content after GnRH treatment in anterior pituitary cells. Furthermore, cholera toxin, an adenylate cyclase activator, failed to stimulate LH release from pituitary cells in culture (Conn *et al.* 1979). These results raise questions about the nature of intracellular signaling pathways operated in the course of GnRH-induced LH release and its subunit biosynthesis within anterior pituitary gonadotropes.

Therefore, the present study was attempted to determine the effect of GnRH on PKC activity and intracellular cAMP content in cultured anterior pituitary cells of rat. Moreover, we examined the effects of staurosporine, a PKC inhibitor and 2',3'-dideoxyadenosine (2',3'-DDA), an adenylate cyclase inhibitor, on LH release and steady state LH β subunit mRNA levels in cultured anterior pituitary cells of rat.

MATERIALS AND METHODS

Cell culture

Sprague Dawley female rats (150~200 g; Yuhan Research Center, Korea) were used in these experiments and anterior pituitary glands were obtained by decapitation. Anterior

pituitary cells were prepared by enzymatic dispersion, as a modification of previously described methods (Wilfinger *et al.* 1984). Following decapitation, pituitary glands were pooled and washed in Spinner's Minimal Essential Medium (S-MEM; Gibco) containing 0.5 % BSA (fraction V, Sigma) and 10 mM HEPES (Sigma). Anterior lobes of the pituitary were cut to several pieces in S-MEM/BSA and then digested with 20 ml S-MEM/BSA containing 0.25% trypsin (1:250, Difco) and DNase (10 μ g/gland, Sigma) for 1 h at 4°C and further 30 min at 37°C. Cell dispersion was facilitated by repeated aspiration and expulsion of tissue fragments with a fire-polished pasteur pipette. Dispersed cells were then centrifuged at 400 \times g for 10 min. The pellet was resuspended in 20 ml S-MEM/BSA containing trypsin inhibitor (100 μ g, Sigma) and filtered through a lense paper to remove residual tissue fragments. The cell suspension was briefly centrifuged and the pellet was resuspended in alpha Minimal Essential Medium (α -MEM; Gibco) containing 2.5% fetal calf serum (Gibco), 10% horse serum (Gibco) and antibiotics (100 units penicillin and 100 μ g streptomycin/ml, Sigma). All sera were dextran-charcoal stripped for the removal of residual steroids as mentioned previously (Audy *et al.* 1990). The cells were more than 95% viable, as measured by trypan blue exclusion. Aliquotes of cell suspension (1 \times 10⁶ cells/ml) were placed in multiwell culture plates (Falcon) and incubated in 5% CO₂/air at 37°C. Following the 48 h culture period, the cells were washed twice with 2 ml/well of Dulbecco's phosphate buffered saline (D-PBS; Sigma) to remove serum and nonadherent cells and then subjected to experiments. GnRH (LHRH; Sigma), staurosporine (ST; Sigma) and 2',3'-dideoxyadenosine (2',3'-DDA; Sigma) were dissolved in absolute ethanol as a stock solution and diluted with culture medium. The final concentration of ethanol was adjusted to less than 0.1% to avoid toxicity to cultured cells.

Radioimmunoassay (RIA) for LH and cAMP determination

LH was measured by a double antibody RIA using reagents kindly provided by the NIDDK

(USA). As a tracer, rat LH (NIDDK-rLH-I-9) was iodinated with ^{125}I by chloramine T. The antiserum was NIDDK-rLH-S-10 and the reference preparation was NIDDK-rLH-RP-2. Assay sensitivity was 0.1 ng/ml.

cAMP content was measured by a double antibody RIA using antiserum (CV-27 Pool) kindly provided by NIDDK (USA). 2,0'-mono succinyl-adenosine-3',5'-monophosphate tyrosine methyl ester (Sigma) was iodinated with ^{125}I to serve as a tracer. Reference preparation was adenosine 3',5'-cyclic monophosphate (Sigma). Assay sensitivity was 0.1 pmole/tube.

LH β mRNA analysis

Total RNA from anterior pituitary cells was isolated as previously described (Sambrook *et al.* 1989). Briefly, the cells were scraped with rubber policeman and collected by centrifugation at $12,000\times g$ for 1 min. The cell pellet was resuspended in 200 μl digestion buffer (0.14 M NaCl, 1.5 mM MgCl_2 , 10 mM Tris.Cl, pH 8.6, 1 mM DTT) supplemented by nonidet P-40 (0.5 %, Sigma) for 5 min on ice. After centrifugation at $12,000\times g$ (4°C, 5 min), equal volume of proteinase digestion buffer (0.01% proteinase K, 0.2 M Tris.Cl, pH 8.0, 25 mM EDTA, 0.3 M NaCl, 2% SDS) was added to the supernatant fraction and incubated for 30 min at 37°C. The proteins were removed by extracting once with equal volume of phenol:chloroform and cytoplasmic RNA was precipitated with 2 volumes of ice-cold ethanol and 0.1 volume of 3 M sodium acetate. RNA pellet was collected by centrifugation at $12,000\times g$ for 5 min and washed once with 70% ice-cold ethanol. After brief drying the RNA pellet was dissolved in TE buffer (pH 7.6) and quantitated. RNA samples were transferred to nylon filters (Hybond, Amersham) using a multiplace manifold apparatus (Hoefer) for dot blot analysis of LH β mRNA. The filters containing the immobilized RNA were baked at 80°C for 2 h and hybridized with LH β cDNA. The LH β cDNA was radiolabelled with α - ^{32}P -dCTP (3,000 Ci/mmol, Amersham) to a specific activity $0.5\text{--}1.0\times 10^9$ cpm/ μg cDNA by a multiprime labelling system (Amersham). Prehybridization was performed for 3 h at 42°C in prehybridization buffer (50% forma-

mid, $5\times$ SSC, 0.1% BSA, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate (pH 6.5), 1 mM EDTA, 0.1% SDS, 200 $\mu\text{g}/\text{ml}$ denatured salmon testis DNA). Radiolabelled cDNA probes were added to fresh prehybridization buffer for the preparation of hybridization solution. Filters were hybridized overnight at 42°C, and thereafter washed twice (30 min each) in $2\times$ SSC with 0.1% SDS at 25°C and twice (15 min each) in $0.1\times$ SSC with 0.1% SDS at 52°C. The filters were then dried and exposed to X-ray film (Kodak X-Omat) at -70°C . Slot intensity in autoradiogram were assessed by scanning densitometer analysis. The integrated peak area of each slot was expressed as a arbitrary densitometric unit (ADU) over control group.

PKC assay

Determination of PKC activity was performed as previously described (Andrews *et al.* 1990). Adherent cells were removed from the culture dishes and washed with ice-cold phosphate-buffered saline. After a brief centrifugation, cell pellet was homogenized in 200 μl buffer (25 mM Tris-Cl, pH 7.5, 0.25 M sucrose, 2.5 mM MgCl_2 , 2.5 mM EDTA, 50 mM β -mercaptoethanol, 1 mM phenylmethyl sulfonyl-fluoride) with hand-hold glass homogenizer and recentrifuged ($100,000\times g$, 1 hr). 200 μl of 1% triton X-100 solution in homogenizing buffer was added to the supernatant and sonicated for 1 min at 4°C. Membrane fraction collected from the supernatant after centrifugation ($6,000\times g$, 20 min) of the suspension was assayed for membrane-associated PKC activity. PKC activity was determined by measuring phosphorylated histone after incubation of membrane fraction with γ - ^{32}P -ATP. The assay mixture (250 μl) was contained 5 μmol Tris-HCl, pH 7.5, 1.25 μmol MgCl_2 , 50 μg histone(III-S), 2.5 nmol γ - ^{32}P -ATP (3,000 Ci/mmol, Amersham), 10 μg phosphatidylserine, 1 μg diolein, 1 mM CaCl_2 and 150 μl sample (membrane fraction). The assay was carried out for 5 min at 30°C. The reaction was stopped by the addition of 2 ml trichloroacetic acid (25% TCA) and radioactivity on nitrocellulose membrane filters (0.45 μm , Millipore) was counted in β -counter. Specific protein kinase C activity

was determined by the removal of basal activity in Ca^{++} -depleted condition. All reagents were purchased from Sigma except for specified.

Data analysis

All values are given as means \pm SE. Statistical comparisons between groups were performed by student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

As shown in Fig. 1A, all doses of GnRH (5×10^{-11} – 1×10^{-9} M) stimulated LH release from the pituitary cells. The LH response to GnRH was time- and dose-dependent, the release being sharply increased during the first 2 hrs after GnRH treatment. In the absence of

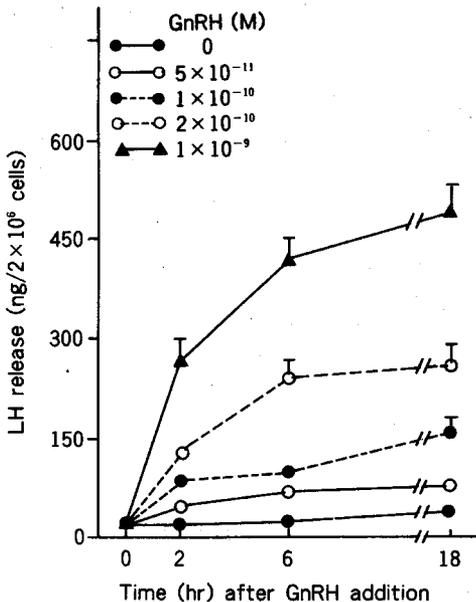


Fig. 1. Effect of GnRH on the LH release and contents in anterior pituitary cells in culture. Each point represents the mean \pm S.E. of repeated experiments ($n=3$ to 4).

GnRH, LH release was not changed with time. In the presence of GnRH, LH contents in cultured anterior pituitary cell were negatively correlated with LH release (Fig. 1B). However, LH contents in the pituitary cells tended to be increased with time after GnRH addition except the highest dose of GnRH, which induced maximum LH release, suggesting a time-dependent accumulation of newly produced LH after rapid release.

To investigate whether GnRH increases LH β subunit mRNA levels in static culture of anterior pituitary cells, GnRH (5×10^{-11} – 1×10^{-9} M) was treated. GnRH increased LH β subunit mRNA levels in a dose-dependent manner, reaching the maximum with 2×10^{-10} M GnRH (Fig. 2).

To investigate the intracellular pathway by which GnRH exerts its effect on LH subunit biosynthesis as well as release from anterior pituitary cells, PKC activity and intracellular cAMP levels were examined. PKC activity in membrane fraction of anterior pituitary cells was significantly increased 30 min after GnRH treatment (2×10^{-10} M) and this increase in PKC activity was maintained up to 18 h (Fig. 3). Intracellular cAMP levels were also

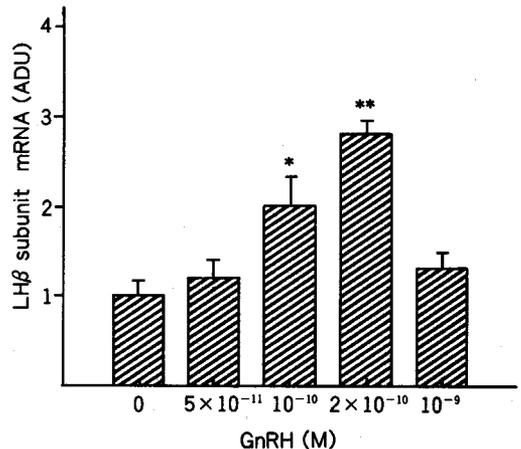


Fig. 2. Effect of GnRH on LH β subunit mRNA levels in anterior pituitary cells in culture. Each point represents the mean \pm S.E. of repeated experiments ($n=3$ to 4). * $P < 0.05$, ** $P < 0.01$; compared with control group.

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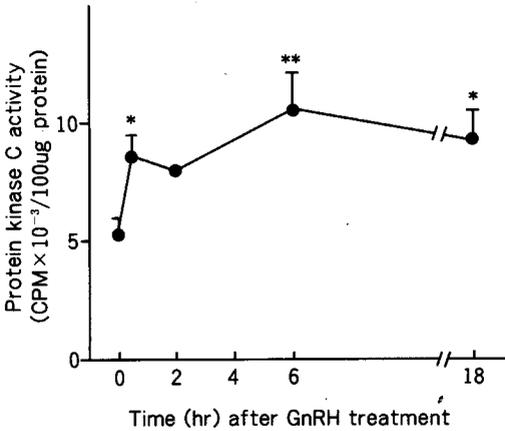


Fig. 3. Effect of GnRH on protein kinase C in membrane fraction of anterior pituitary cells. Anterior pituitary cells (5×10^6 cells) were incubated with GnRH (2×10^{-10} M) for time indicated. Each point represents the mean \pm S.E. of repeated experiments ($n=4$). * $P < 0.05$, ** $P < 0.01$; compared with control group.

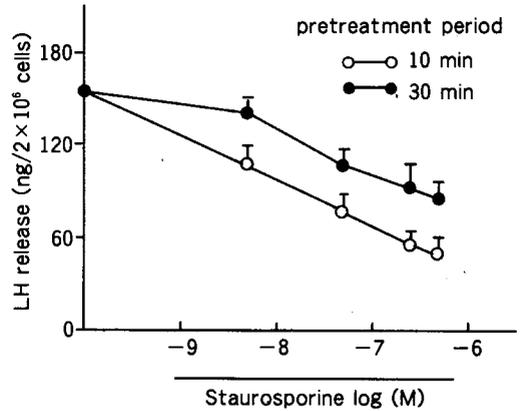


Fig. 5. Effect of staurosporine on GnRH-stimulated LH release from anterior pituitary cells in culture. Anterior pituitary cells pretreated for 10 min or 30 min with increasing dose of staurosporine were further incubated with 2×10^{-10} M GnRH. Each point represents the mean \pm S.E. of repeated experiments ($n=4$).

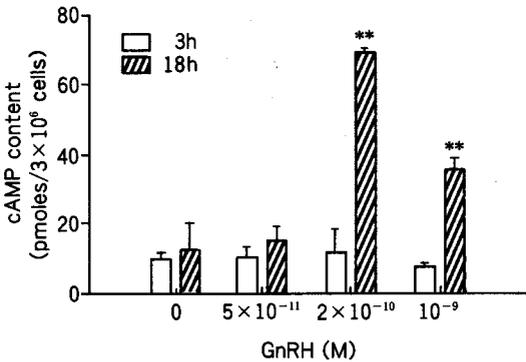


Fig. 4. Effect of GnRH on cAMP content in anterior pituitary cells in culture. Anterior pituitary cells were treated with increasing dose of GnRH for 3 hrs or 18 hrs. * $P < 0.05$, ** $P < 0.01$; compared with control (0) group

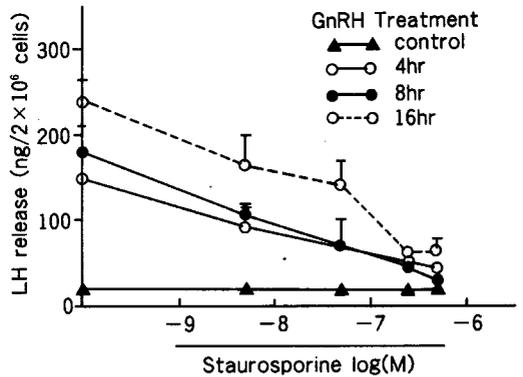


Fig. 6. Inhibition of GnRH-stimulated LH release by staurosporine from anterior pituitary cells in culture. Anterior pituitary cells pretreated for 10 min with staurosporine were further incubated with 2×10^{-10} M GnRH for various time. Each point represents the mean \pm S.E. of repeated experiments ($n=4$).

determined at 3 h and 18 h after GnRH treatment (Fig. 4). When pituitary cells were treated with different doses of GnRH (5×10^{-11} – 10^{-9} M), a significant increase in intracellular cAMP content was observed after 18 h incu-

bation (about 5.5 fold increase at 2×10^{-10} M; 2.8 fold at 10^{-9} M), while after 3 h incubation, no detectable increase in intracellular cAMP level was shown.

Then, to confirm that PKC activation and/

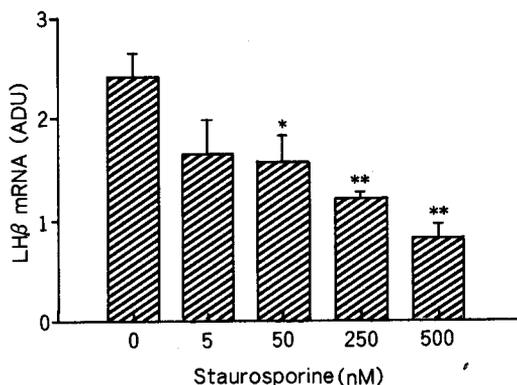


Fig. 7. Inhibition of GnRH-stimulated LHβ subunit mRNA by staurosporine in anterior pituitary cells. Anterior pituitary cells pretreated for 10 min with staurosporine were further incubated with 2×10^{-10} M GnRH for 16 hrs. Each point represents the mean \pm S.E. of repeated experiments ($n=4$). * $P < 0.05$, ** $P < 0.01$; compared with control group.

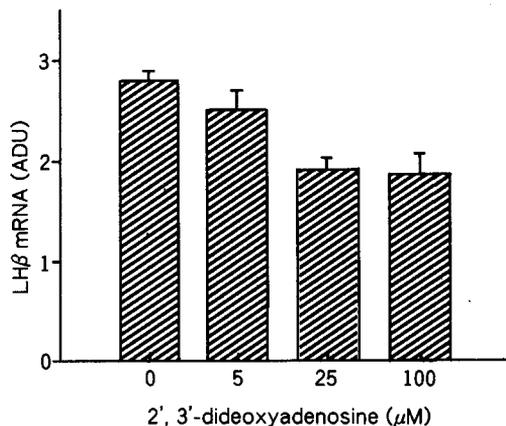


Fig. 9. Effect of 2',3'-dideoxyadenosine on GnRH-induced LHβ subunit mRNA in anterior pituitary cells. Each bar represents the mean \pm S.E. of repeated experiments ($n=4$).

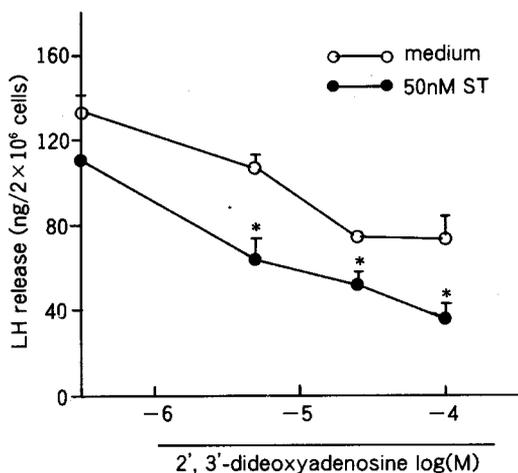


Fig. 8. Inhibition of GnRH-stimulated LH release by 2',3'-dideoxyadenosine in anterior pituitary cells. Anterior pituitary cells pretreated for 10 min with/without 50 nM staurosporine (ST) were further incubated for 6 hr with GnRH and 2',3'-dideoxyadenosine. Each point represents the mean \pm S.E. of repeated experiments ($n=4$). * $P < 0.05$; compared with medium-pretreated group.

or cAMP-dependent pathway may be mediated in GnRH action, PKC inhibitor and adenylylated cyclase inhibitor were treated with GnRH. Anterior pituitary cells were pretreated for 10 min or 30 min with staurosporine (ST), a relatively selective PKC inhibitor and then incubated for 6 hr with GnRH (2×10^{-10} M). ST significantly inhibited GnRH-induced LH release in a dose-dependent manner, showing half maximal response at 50 nM ST (Fig. 5). However, inhibition of GnRH-induced LH release was greater with 10 min pretreatment of ST than with 30 min pretreatment. When anterior pituitary cells were treated with 2×10^{-10} M GnRH for various time, GnRH-induced LH release was inhibited by ST in a time-dependent manner after GnRH treatment, while basal LH release was not altered (Fig. 6). Moreover, ST inhibited GnRH-induced LHβ subunit mRNA levels in a dose-dependent manner (Fig. 7).

On the other hand, anterior pituitary cells were incubated with GnRH and AC inhibitors, 2',3'-DDA. 2',3'-DDA significantly inhibited GnRH-induced LH release in a dose-dependent manner (Fig. 8), but 2',3'-DDA tended to inhibit GnRH-induced LHβ subunit mRNA levels (Fig. 9). However, even with high dose of 2',3'-DDA (25 μM), neither GnRH-induced LH

release nor LH β subunit mRNA levels were completely blocked. Furthermore, when anterior pituitary cells were pretreated for 10 min with ST and further incubated for 6 hrs with GnRH and 2',3'-DDA, the inhibition of GnRH-induced LH release was greater than 2',3'-DDA-only treated group (Fig. 8).

DISCUSSION

In the present study, we found that GnRH stimulated LH release as well as LH subunit biosynthesis in primary pituitary cell cultures. However, signaling pathway of GnRH action has not been clarified yet. It has been a subject of intensive investigations to determine whether PKC and/or cAMP mediate GnRH-induced LH release and its subunit biosynthesis in anterior pituitary gonadotropes.

It is well known that PKC activation mediates GnRH-induced LH release (Hawes and Conn, 1990) via modulating GnRH receptor up-regulation (McArdle *et al.* 1988). On the other hand, other studies reported that GnRH still induced LH release from PKC-depleted anterior pituitary cells (McArdle *et al.* 1987), suggesting that PKC activation was not necessarily involved in GnRH-induced LH release. The treatment of staurosporin, a relatively selective PKC inhibitor did not inhibit GnRH-induced LH release completely, suggesting a PKC-insensitive component in GnRH-induced gonadotropin release (Dan-Cohen and Naor, 1990).

A few studies also reported that LH β subunit mRNA level was stimulated by PKC activation in anterior pituitary gonadotropes. Andrews *et al.* (1988) reported that PMA markedly stimulated the steady state level of LH β subunit mRNA at low doses (2~20 nM) in cultured pituitary cells of weanling rats. In that study, LH β mRNA level was stimulated only by low doses of GnRH (<1 nM) which do not deplete PKC activity. It was also showed that TPA stimulated LH β mRNA level as well as ³⁵S-methionine incorporation into LH β subunit *in vitro* (Starzec *et al.* 1989). These findings strongly suggest that PKC

activation might be involved in GnRH-induced LH release and subunit synthesis. Then, a question is raised whether PKC activity in membrane fraction of pituitary cells is increased by the treatment of GnRH. The present study showed that PKC activity in membrane fraction of pituitary cells was started to increase significantly 30 min after GnRH treatment. The treatment of staurosporine inhibited GnRH-induced LH release and LH β subunit mRNA levels in dose and time dependent manners. The half-maximal inhibition was observed with 50 nM ST and 10 min preincubation time with ST was effective in inhibiting GnRH-induced LH release. This is in good agreement with other reports that have used ST to dissect PKC-mediated responses (50~200 nM; Dan-Cohen and Naor, 1990) and that prolonged incubation periods give rise to cytotoxic effect on pituitary cells (Tamaoki *et al.* 1986). Moreover, our laboratory found that LH β mRNA was also stimulated only with low doses (<20 nM) of PMA, and LH release was provoked by PMA in a dose-dependent manner (unpublished data). Thus, the present study together with others suggests that PKC activation is necessary for GnRH action on LH release and its subunit biosynthesis.

On the contrary studies on the role of cAMP in the process of GnRH action have been controversial for many years. Initially, the study showed that GnRH provoked an increase in cAMP levels in hemipituitaries paralleled to LH release (Borgeat *et al.* 1972). In addition, cAMP levels as well as gonadotropin release was observed to be decreased by the treatment of GnRH antagonists (Beaulier *et al.* 1975). The treatment of cAMP analogs was also observed to increase LH release (Starzec *et al.* 1981), although other studies failed to show an increase in LH release in other studies (Wakabayashi *et al.* 1973; Tang and Spies, 1974). The present study showed that cAMP level was not significantly increased by 3 h after GnRH treatment, but markedly increased by 18 h incubation. Furthermore the treatment of 2',3'-DDA, an AC inhibitor, inhibited GnRH-induced LH β subunit mRNA levels as well as LH release from anterior pi-

pituitary cells in a dose-dependent manner. It is assumed that intracellular cAMP content might be increased via activation of adenylate cyclase and/or inhibition of cytosolic phosphodiesterase (PDE). Although the former has been regarded as a major effector on the elevation of intracellular cAMP levels, the latter possibility should not be excluded in the case of the maintenance of elevated intracellular cAMP levels. However, the informations on the regulation of PDE are rarely available at this moment. The present findings support the previous suggestions that GnRH action might be mediated by PKC activation and cAMP stimulation.

One of interesting points of this study is that PKC activation and cAMP elevation were orderly induced by GnRH at different intervals. The present data showed that PKC activity in membrane fraction of anterior pituitary cells was increased in 30 min (Fig. 3) and intracellular cAMP content was elevated 18 hrs after GnRH treatment (Fig. 4). Although it is assumed that PKC activation and cAMP elevation might be involved in cascade reactions by GnRH (Counis and Starzec, 1991), the mechanism by which two intracellular pathways crosstalk in the processes of LH synthesis and release, remains to be elucidated. Summers and Cronin (1986) reported that phorbol esters enhanced basal and forskolin-stimulated adenylate cyclase activity in the rat 235-1 cells, a pituitary cell line. Recently, our laboratory found that PKC activation by PMA in cultured pituitary cells resulted in an elevation in cAMP levels. However, no evidences are available whether these cascade reactions are limited to GnRH-dependent gonadotropes of anterior pituitary glands, since there are heterogeneous cell populations including somatomammotropes, thyrotropes, and gonadotropes in anterior pituitary cells. Therefore, further studies which apply a gonadotrope-specific pituitary cell line would be required for the confirmation.

Conclusively, the present study suggests that GnRH may first induce PKC activation and then stimulate cAMP levels, thereby stimulating LH β subunit mRNA level as well as LH release in cultured anterior pituitary

cells.

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