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Effects of Extracellular Ca^{++} on PKC or cAMP-stimulated Increases in LH Release and LHβ Subunit mRNA Levels in Rat Anterior Pituitary Cells

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ABSTRACT

We examined the effects of EGTA and verapamil on phorbol ester- and forskolin-stimulated LH releases and LHβ subunit mRNA levels in order to verify the role of extracellular Ca^{++} on PKC- or cAMP-induced increases in LH release and LHβ subunit mRNA levels in cultured anterior pituitary cells of rat. Forskolin-stimulated LHβ subunit mRNA levels as well as LH release were all suppressed by prevention of Ca^{++} mobilization from extracellular environment, after the treatment of EGTA as a Ca^{++} chelator or verapamil as a Ca^{++} channel blocker. PMA-stimulated LHβ subunit mRNA levels were also suppressed by the treatment of EGTA and verapamil, while PMA-induced LH release was not affected. From the present study, it is, therefore, suggested that PKC activation and cAMP elevation all stimulate LHβ subunit mRNA levels and these are extracellular Ca^{++}-dependent. However, LH releases by PKC activation and cAMP increase seem to be different each other. LH release by PKC activation is thought to be independent of extracellular Ca^{++}. On the other hand, cAMP stimulated-LH release is thought to be dependent on the entry of extracellular Ca^{++}.

Key Words: LHβ subunit mRNA, Ca^{++}, PKC, cAMP, Pituitary, Rat

INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide that regulates subunit biosynthesis and release of luteinizing hormone (LH) from the anterior pituitary. Although much has been known about the regulation of LH release, the precise mechanisms by which GnRH stimulates release of LH and biosynthesis of its subunits are not clearly understood (Conn *et al.*, 1981; Jutisz *et al.*, 1990; Naor, 1990). Phorbol ester such as phorbol myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC), has been known to increase LH release from pituitary cells (Naor and Catt, 1982), indicating that PKC is a mediator of GnRH action in anterior pituitary gonadotropes (Naor, 1990). But a few reports failed to confirm the involvement of PKC in GnRH-induced LH release since GnRH action was not affected by the depletion of PKC in cultured anterior pituitary cells (McArdle *et al.*, 1987). In addition, cAMP is thought to be an intracellular mediator in GnRH action in the rat pituitary gonadotropes (Borgeat *et al.*, 1972). It was shown that the activation of adenylate cyclase, or the treatment of cAMP analogues increased LH release and LHβ subunit biosynthesis in anterior pituitary cells (Starzec *et al.*, 1989; Naor, 1990). However, other investigators reported that cAMP did not play any substantial role in

GnRH-induced LH release (Wakabayashi *et al.*, 1973; Tang and Spies, 1974; Conn *et al.*, 1979). These results raise questions concerning the role of PKC and/or cAMP in GnRH action within anterior pituitary gonadotropes. Futhermore, a body of evidence suggests that Ca^{++} mobilization is an essential for the acute release of LH by GnRH (Conn *et al.*, 1981; Catt *et al.*, 1985; Conn, 1986). Ca^{++} chelators and Ca^{++} channel blockers have shown to diminish GnRH-induced LH release (Borgeat *et al.*, 1975; Kiesel *et al.*, 1984), whereas Ca^{++} ionophores (A23187 and inomycin) stimulated LH release (Kiesel *et al.*, 1984; Chang *et al.*, 1986). Our previous study found that phorbol ester and cAMP stimulated LH release as well as LHβ mRNA level in cultured pituitary cells (Park *et al.*, 1992).

In the present study, we examined the effects of Ca^{++} chelator and Ca^{++} channel blocker on phorbol ester- and forskolin-induced LH release and LHβ subunit mRNA in order to verify the role of extracellular Ca^{++} in PKC- or cAMP-induced LH release and LHβ subunit mRNA levels in cultured anterior pituitary cells of rats.

MATERIALS AND METHODS

Preparation of pituitary cell cultures

Pituitary glands from Sprague Dawley female rats (150~200 g; provided by Yuhan Research Center, Korea) at random stages of the estrous cycle were used for the preparation of cell cultures. Following decapitation, anterior pituitary glands were pooled and washed in Spinner's Minimal Essential Medium (S-MEM; Gibco) containing 0.5% BSA (Sigma) and 10 mM HEPES (Sigma). Anterior lobes were minced in medium S-MEM/BSA and then digested enzymatically with 20 ml S-MEM/BSA containing 0.25% trypsin (1:250, Difco) and DNase (10 mg/gland, Sigma) for 1 h at 4 ℃ and further 30 min at 37℃. Dispersion was facilitated by repeated gentle aspiration and expulsion of the tissue fragments with a fire-polished pasteur pipette. Dispersed cells were then centrifuged at 400×g for 10 min. The pellet was resuspended in 20 ml S-MEM/BSA containing trypsin inhibitor (100 mg, Sigma) and filtered through lens paper to remove residual tissue fragments. The cell suspension was briefly centrifuged and cell 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 mg streptomycin/ml, Sigma). Endogenous steroids in sera were removed by charcoal absorption as previously described(Audy *et al.*, 1990). The cells were more than 95% viable, as measured by trypan blue exclusion. Aliquotes of this cells (1×10^6 cells/ml) were incubated in multiwell culture plates (Falcon) in 5% CO_2/air at 37℃. Following the 48 h preincubation, the cells were washed twice with 2 ml/well with Dulbecco's phosphate buffered saline (D-PBS; Sigma) to remove serum and nonadherent cells, thereafter further incubated in serum-free medium (α-MEM) for experiments.

Radioimmunoassay of LH

LH was measured by a double antibody RIA using reagents kindly provided by the National Pituitary Agency/NIDDK. Tracer (rLH-I-9) was iodinated by chloramine T. Radiolabelled tracer (^{125}I-rLH) was separated by a gel permeation chromatography with Sephadex G-50. The antisera (rLH-S-10) and the reference preparations (rLH-RP-2) were dissolved in 0.01 M phosphate buffer containing 0.1% (w/v) BSA. The antisera were diluted from the stock solutions (1:18.75), so that the final tube dilution was 1:135,000. For the measurement of LH concentration, radiolabelled tracer (10,000~15,000 cpm/100 μl), antisera (100 μl/1:45,000) and unknown samples (100 μl) were incubated for 16~20 h at room temperature. The antigen-antibody complex was precipitated by adding 0.1 ml EDTA, 0.1 ml normal rabbit serum (2%), 0.1 ml anti-rabbit IgG (Sigma) and 0.6 ml polyethyleneglycol (PEG; M. W. 8,000). After centrifugation at 3,000×g for 20 min, the supernatant was discarded and the radioactivity present in the precipitate was counted in γ-counter (Packard, USA). Assay sensitivity was 0.1 ng/ml. The intra-and interassay coefficient was 6.8% and 9.5%, respectively.

mRNA analysis

Cytoplasmic RNA in anterior pituitary cells was isolated as followed. Briefly, cells were scraped with rubber policeman in ice-cold PBS and collected by centrifugation at 12,000×g for 1 min. The cell pellet was resuspended in 200 μl NP-40 lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris.Cl, pH 8.6, 0.5% nonidet P-40, 1 mM DTT) for 5 min on ice, and cytoplasmic fraction was separated by centrifugation at 12,000× g for 5 min. Proteinase digestion buffer (200 μl; 0.2 M Tris.Cl, pH 8.0, 25 mM EDTA, 0.3 M NaCl, 2% SDS) was added to cytoplasmic fraction and incubated for 30 min at 37℃. Proteins were removed by extracting once with equal volume of phenol: chloroform and aqueous phase was removed and precipitated with 2 volumes of ice-cold ethanol and 0.1 volume of 3 M sodium acetate (pH 4.2). RNA pellet was collected by centrifugation at 12,000×g for 5 min and washed once with 70% ice-cold ethanol. RNA pellet was dissolved in Tris-EDTA (pH 7.6) and quantitated. RNA samples were transferred to nylon filters (Hybond, Amersham) by the capillary transfer method after electrophoresis (1.2% agarose) of RNA samples for Northern blot hybridization. RNA was then immobilized by the incubation of filters at 80℃ for 2 h and hybridized with a cloned rat LHβ cDNA (kindly provided by Dr. J. Roberts, Mt. Sinai, New York, USA). The LHβ cDNA was radiolabelled to a specific activity of 0.5~1.0× 10^9 cpm/mg cDNA by random primer labeling system (Megaprime kit, Amersham). Prehybridization was performed for 3 h at 42℃ in prehybridization solution (50% formamide, 5x SSC, 0.1% BSA, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate, pH 6.5, 1 mM EDTA, 0.1% SDS, 200 μg/ml denatured salmon testis DNA). Radiolabelled cDNA probes were added to fresh prehybridization solution for the preparation of hybridization solution. Filters were hybridized overnight at 42℃ and thereafter washed twice (30 min each) in 2x SSC with 0.1% SDS at 25℃ and twice (15 min each) in 0.1x SSC with 0.1% SDS at 52℃. Relative LHβ mRNA levels were determined by measuring the intensity of each band from the autoradiograph and expressed as an arbitrary densitometric unit (ADU).

Statistics

All values are given as means±SE. Statistical comparisions between groups were performed with student's t-test. P value<0.05 was considered significant.

RESULTS

At first, it was examined whether the mobili-

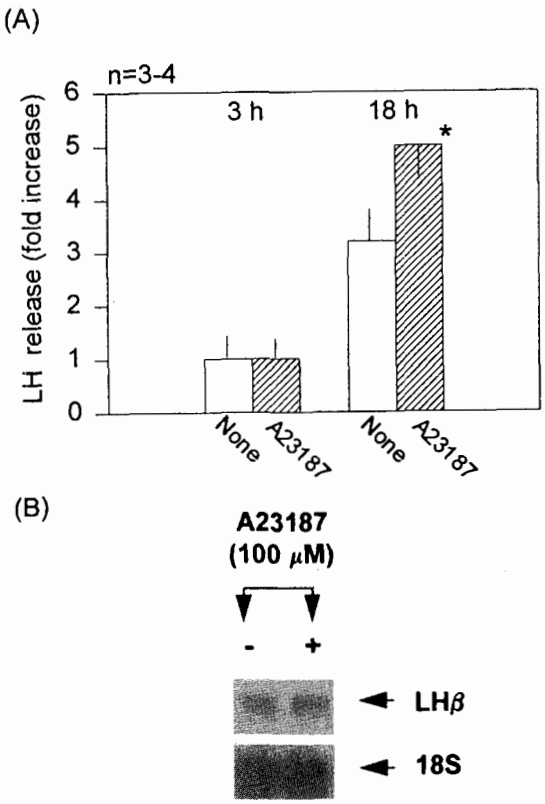

Fig. 1. Effect of A23187 on the basal release of LH (A) and LHβ mRNA levels (B). Each response of LH release to A23187 is expressed as a fold increase of the basal release of LH. Each bar represents the mean±S.E. of repeated experiments(n=3). *P<0.05 compared with the control without A23187. A23187 (100 mM).

zation of extracellular Ca^{++} into the cytoplasm could stimulate the release of LH, or the steady state levels of LHβ subunit mRNA. When pituitary cells were treated with A23187 (100 mM) , Ca^{++} ionophore, for 18 h, LH release was significantly increased ($P<0.05$) but this increase in LH release was not shown at 3 h treatment (Fig. 1A). However, such an increase in LH release by A23187 is not dramatic compared with the stimulation of LH release by a

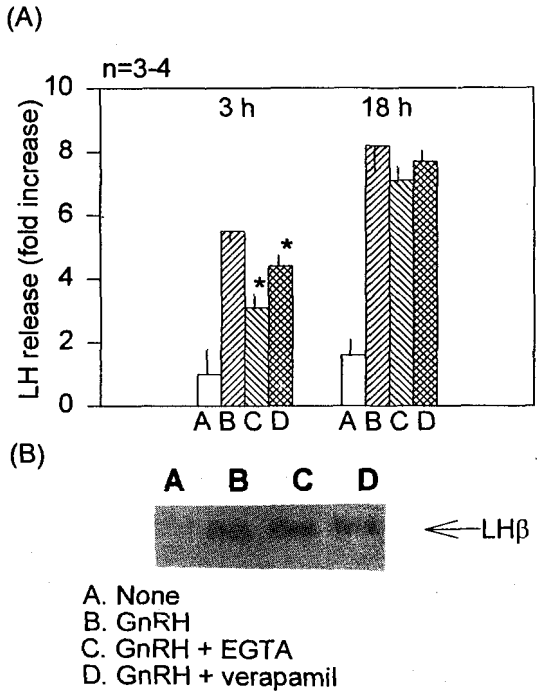

Fig. 2. Effect of EGTA or verapamil on GnRH-induced LH release (A) and LHβ mRNA levels (B) in anterior pituitary cells of rats. Pituitary cells were treated with GnRH, GnRH+ EGTA or GnRH+verapamil, respectively, for 18 h.

(A) The concentration of LH secreted into medium was measured at 3 h and 18 h after treatment. LH release is expressed as a fold increase of the basal release of LH (none). Each bar (A) represents the mean±S.E. of repeated experiments (n=3 ~4).

*$P<0.05$ compared with the control (GnRH alone).

GnRH (1 nM), EGTA (2 mM), verapamil (100 mM).

(B) LHβ mRNA levels at 18 h are expressed as the ADUs over the control (none).

GnRH (0.2 nM), EGTA (2 mM), verapamil (100 mM).

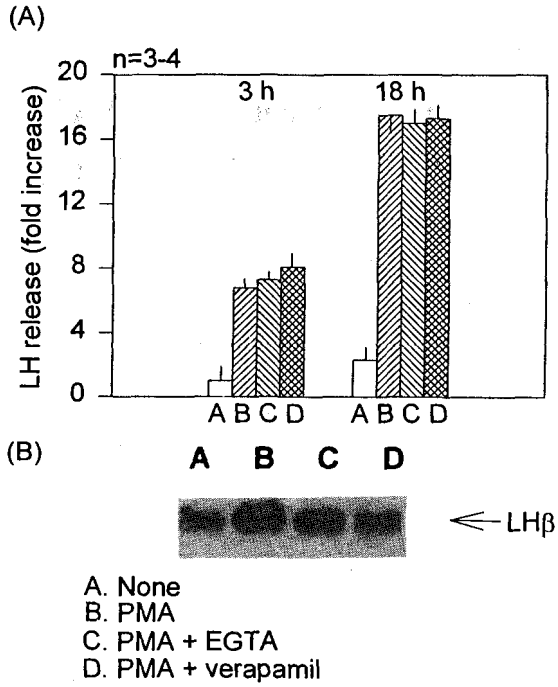

Fig. 3. Effect of EGTA or verapamil on PMA-induced LH release (A) and LHβ mRNA levels (B) in anterior pituitary cells of rats. Pituitary cells were treated with PMA, PMA+ EGTA or PMA+verapamil, respectively, for 18 h.

(A) The concentration of LH secreted into medium was measured at 3 h and 18 h after treatment. LH release is expressed as a fold increase of the basal release of LH (none). Each bar (A) represents the mean±S.E. of repeated experiments (n= 3-4).

*$P<0.05$ compared with the control (PMA alone) .

PMA (50 nM), EGTA (2 mM), verapamil (100 mM).

(B) LHβ mRNA levels at 18 h are expressed as the ADUs over the control (none).

PMA(5 nM), EGTA (2 mM), verapamil (100 mM).

number of secretagogues such as GnRH , PMA or forskolin in the following experiments. At the same experiment, LHβ subunit mRNA levels were not stimulated with the treatment of A23187 (Fig. 1B). These results suggest that the mobilization of extracellular Ca^{++} by itself does not seem to affect the steady state levels of LH β subunit mRNA as well as LH release.

Thus, Ca^{++} chelator (EGTA; 2 mM) or Ca^{++} channel blocker (verapamil; 100 mM) was treated simultaneously with GnRH for 18 h in order to examine the role of extracellular Ca^{++} in the course of GnRH-induced LH release and LHβ subunit mRNA levels in anterior pituitary cells (Fig. 2). LH release induced by GnRH (10^{-9}) was suppressed by EGTA ($P<0.05$) or verapamil (not statistically significant) at 3 h incubation, but not at 18 h. Steady state levels of LHβ subunit mRNA were also determined in the same experiments. Low dose of GnRH (2×10^{-10} M) treated for 18 h increased LHβ subunit mRNA levels, while higher concentrations of GnRH ($>10^{-9}$M) did not (data not shown). But this GnRH-stimulated increase in LHβ subunit mRNA levels was suppressed by EGTA or verapamil treatment.

To further determine whether action of PKC in increasing LH release and LHβ subunit mRNA levels depends on the extracellular Ca^{++} mobilization, the effects of EGTA and verapamil on PMA-induced LH release and LHβ subunit mRNA levels were examined (Fig. 3).

PMA (100 nM)-stimulated LH release was not diminished by the treatment of EGTA (2 mM) or verapamil (100 mM) at 3 h as well as 18 h incubation. Low dose of PMA (10 nM) increased LHβ subunit mRNA level but this increase was suppressed by EGTA or verapamil.

When EGTA (2 mM) or verapamil (100 mM) was simultaneously treated with forskolin, an activator of adenylate cyclase, forskolin (50 mM) markedly stimulated LH release from pituitary cells at 18 h after the treatment, but this stimulation was not shown at 3 h after the treatment (Fig. 4). Forskolin-induced LH release was significantly suppressed by the treatment of EGTA or verapamil. LHβ subunit mRNA levels were increased by forskolin treatment and this increase was also suppressed by EGTA or verapamil (Fig. 4).

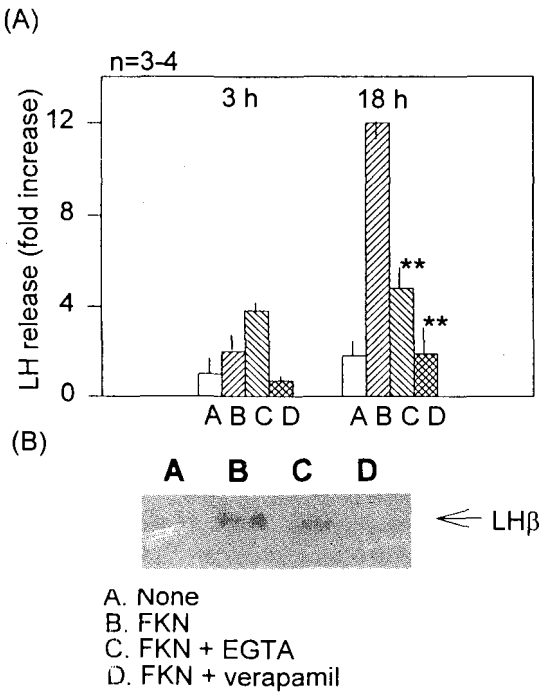

Fig. 4. Suppression of forskolin (FKN)-induced LH release (A) and LHβ mRNA levels (B) by EGTA or verapamil in anterior pituitary cells of rats. Pituitary cells were treated with FKN, FKN+EGTA or FKN+verapamil, respectively, for 18 h.

(A) The concentration of LH secreted into medium was measured at 3 h and 18 h after treatment. LH release is expressed as a fold increase of the basal release of LH (none). Each bar (A) represents the mean±S.E. of repeated experiments (n= 3-4).
**$P<0.01$ compared with the control (FKN alone).
FKN (10 mM), EGTA (2 mM), verapamil (100 mM).

(B) LHβ mRNA levels at 18 h are expressed as the ADUs over the control (none).
FKN (10 mM), EGTA (2 mM), verapamil (100 mM).

DISCUSSION

In our previous study, we demonstrated that

GnRH stimulated LHβ subunit mRNA levels as well as LH release in a dose-related manner *in vitro* (Kim *et al.*, 1994; Park *et al.*, 1996). Other investigators reported that GnRH is essential for the maintenance of LH release and subunit biosynthesis *in vivo* (Belchetz *et al.*, 1978; Wildt *et al.*, 1978; Clarke and Cummins, 1982). Additionally, it has been demonstrated that low levels of GnRH (<1 nM) stimulated LHβ subunit mRNA levels, whereas high concentrations of GnRH (>10 nM) decreased LHβ mRNA levels in cultured anterior pituitary cells of weaning rats (Andrews *et al.*, 1988). Furthermore, pulsatile GnRH treatment enhanced LHβ gene transcription (Shupnik, 1990) *in vitro* and the steady state levels of LHβ subunit mRNA *in vivo* (Heisenleder *et al.*, 1988). From these results, even though pulsatility of GnRH seems to be essential for the stimulation of LHβ subunit mRNA levels *in vivo* and *in vitro*, low concentrations of GnRH also seems to be able to stimulate LHβ subunit mRNA levels because pituitary gonadotropes might not be desensitized by the weak GnRH stimulus as shown in our and other studies (Andrews *et al.*, 1988; Kim *et al.*, 1994; Park *et al.*, 1996). However, the molecular mechanisms by which GnRH exerts its action within anterior pituitary gonadotropes are still poorly understood. A few studies reported that GnRH exerts effects on Ca^{++} mobilization, inositol phospholipid hydrolysis (Catt and Stojikovic, 1989), and cAMP accumulation (Borgeat *et al.*, 1972). In addition, the time course of cAMP accumulation following exposure to GnRH was similar to that of LHβ subunit biosynthesis (Starzec *et al.*, 1988), showing a lag period of 1~1.5 h. On the contrary, cAMP-dependent pathway does not seem to be involved in GnRH-induced LH release, since LH release was not stimulated by choleragen, an adenylate cyclase activator and GnRH failed to increase cAMP content in anterior pituitary cells (Conn *et al.*, 1979). Thus, the role of cAMP as an intracellular mediator in GnRH-induced LH release or LHβ subunit mRNA stimulation has not been clarified. Our present findings that forskolin, a potent adenylate cyclase activator, markedly stimulated LH release from cultured anterior pituitary cells after 18 h incubation provide an evidence for the involvement of cAMP-dependent pathway in LH release and LHβ subunit synthesis.

On the other hand, there are many reports that the activation of PKC mediates GnRH-induced LH release and/or stimulation of LHβ subunit biosynthesis. Hirota *et al.* (1986) reported that the activity of PKC was increased in membrane fraction of pituitary cells, while decreased in cytoplasmic fraction after GnRH treatment. It was also shown that phorbol ester, a potent PKC activator, stimulated LH release and LHβ subunit mRNA levels (Andrews *et al.*, 1988; Park *et al.*, 1992). However, the role of PKC activation in GnRH action is not fully understood. It has been demonstrated that the activation of PKC seems to affect up-regulation of GnRH receptors, but not down-regulation (McArdle *et al.*, 1988). However, it is uncertain how PKC mediates the GnRH action in LH release and LHβ subunit biosynthesis in anterior pituitary cells. GnRH has been known to initiate Ca^{++} mobilization (Stojikovic and Catt, 1992) and inositol phospholipid hydrolysis (Naor and Catt, 1982). An increase in intracellular Ca^{++} concentration is dependent on the entry from extracellular environments and/or its release from intracellular stores (Stojikovic and Catt, 1992). However, it has not been demonstrated how Ca^{++} mobilization is regulated and how LH release or LHβ gene transcription is regulated by Ca^{++}. Futhermore, little is known about the cross-talks among signaling pathways such as Ca^{++} mobilization, cAMP generation, and PKC activation in the course of LH release or LHβ subunit synthesis. In the present study, forskolin-induced LH release and LHβ subunit mRNA levels were dependent on the entry of Ca^{++} from extracellular environment. However, PMA-induced LH release was not suppressed by the prevention of Ca^{++} entry. These results indicate that although LH release or LHβ subunit biosynthesis may be mediated by cAMP and PKC in anterior pituitary cells, their interactions with extracellular Ca^{++} might be different each other. From our results, it is suggested that cAMP-dependent pathways might interact with extracellular Ca^{++} in order to stimulate LH release and/or LHβ subunit mRNA levels in anterior pituitary gonadotropes. However, supporting evidence is not

available about the interaction of cAMP with extracellular Ca^{++} in rat anterior pituitary cells. It has been suggested that GnRH elicits two intracellular Ca^{++} phases, a fast "spike" phase and a delayed "plateau" phase in pituitary cells (Naor *et al.*, 1987; Limor *et al.*, 1987). The fast Ca^{++} "spike" within a few seconds after GnRH treatment is known to be elicited by Ca^{++} release from intracellular stores by inositol triphosphate (IP_3), a hydrolysis product of membrane phospholipid (phosphoino-sitide diphosphate; PIP_2) by phospholipase C (Shangold *et al.*, 1988). But it is not known how the delayed "plateau" phase is followed by the short "spike" phase. Even though the activation of membrane Ca^{++} channel may contribute to intracellular Ca^{++} elevation at the delayed " plateau" phase (Chang *et al.*, 1986; Naor *et al.*, 1987), any reasonable hypothesis has not been drawn. It was suggested that plasma membrane Ca^{++} channel is possibly potentiated by a PKC-dependent mechanism (Shangold *et al.*, 1988). If plasma membrane Ca^{++} channel is potentiated by PKC activation in anterior pituitary gonadotropes, LH release by PKC activation and Ca^{++} channel potentiation should be suppressed by the depletion of extracellular Ca^{++} or the inhibition of Ca^{++} channel function. However, our results that PMA-stimulated LH release was not suppressed by EGTA and verapamil at 3 h and 18 h incubation, suggest that LH release by PKC activation seems to be independent on extracellular Ca^{++} entry. But on the other hand, forskolin showed a delayed stimulatory effect on LH release at 18 h while any visible increase in LH release was not observed in the early phase (3 h) of forskolin treatment. This delayed stimulatory effect of forskolin on LH release was markedly suppressed by the treatment of EGTA or verapamil. These results indicate that cAMP-dependent pathway stimulates LH release by increasing the new synthesis of LHβ subunit rather than increasing the acute exocytosis of LH from the storage pools. Moreover, Ca^{++} entry through plasma membrane Ca^{++} channel seems to be necessary for the stimulation of LHβ subunit mRNA levels by PKC and cAMP, judged from the results that PMA and forskolin-induced increases in LHβ subunit mRNA were all suppressed by the treatment of EGTA or verapamil.

From the present study, it was, therefore, suggested that PKC activation and cAMP generation all stimulate steady state mRNA levels of LHβ subunit. These are dependent on extracellular Ca^{++}. However, LH release by PKC activation does not seem to depend on extracellular Ca^{++}. Whereas cAMP stimulated-LH release is thought to be dependent on the entry of extracellular Ca^{++}.

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=국문초록=

흰쥐 뇌하수체 전엽세포에서 PKC나 cAMP에 의한 LH 분비 및 LHβ Subunit mRNA 증가에 미치는 Ca^{++}의 영향

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흰쥐 뇌하수체 전엽배양세포에서 PKC나 cAMP에 의한 LH 분비와 LHβ subunit mRNA 증가과정에서 세포외 Ca^{++}의 역할을 검증하기 위하여 phorbol ester와 forskolin에 의해 촉진된 LH 분비와 LHβ subunit mRNA 수준에 미치는 EGTA와 verapamil의 영향을 조사하였다. Forskolin에 의해 촉진된 LH 분비와 LHβ subunit mRNA 수준은 Ca^{++} chelator인 EGTA나 Ca^{++} 채널차단제인 verapamil의 처리로 세포외부로부터 Ca^{++}의 이동을 억제시켰을때 모두 감소하였다. PMA에 의해 유도된 LH 분비는 EGTA와 verapamil 처리에 의해 영향을 받지 않았으나 PMA에 의해 촉진된 LHβ subunit mRNA는 억제되었다. 따라서 본 연구에 의하면 PKC 활성화나 세포내 cAMP 농도 증가는 LHβ subunit mRNA 수준을 증가시키며 이러한 과정은 세포외 Ca^{++} 의존적인 것으로 생각된다. 그러나 PKC 활성화나 cAMP 증가에 의한 세포외 Ca^{++}의 역할이 서로 다른 양상을 보인다. PKC 활성화에 의한 LH 분비는 세포외 Ca^{++}에 비의존적이나 cAMP에 의해 촉진된 LH 분비는 세포외 Ca^{++} 유입의 영향을 받는 것으로 사료된다.