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DIACYLGLYCEROL RATHER THAN Ca^{2+} MEDIATES GnRH INHIBITION OF FSH INDUCED STEROIDOGENESIS IN OVARIAN GRANULOSA CELLS

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SUMMARY: Treatment of cultured granulosa cells with PLC or GnRH stimulated the rapid generation of DAG and phosphoinositide turnover. The PKC activators PLC (3 mU/ml) and TPA (10^{-7}M) or the decapeptide GnRH (10^{-6}M) elicited similar inhibitory responses on FSH or cAMP stimulated granulosa cell steroidogenesis. Mobilization of intracellular Ca^{2+} with A23187 (10^{-8}M) was followed by a slight increase in the steroidogenic activity of cultured granulosa cells, whereas elevation of extracellular K^+ (50 mM) largely augmented the steroid biosynthetic activity of the granulosa cells. These results suggest that the inhibitory effect of GnRH on granulosa cell steroidogenesis is mediated by generation of DAG, rather than by increases in intracellular Ca^{2+} concentrations.

Interaction of FSH with its plasma membrane receptor on granulosa cell generates an increase in intracellular cAMP which elicits the activation of steroidogenic enzymes and the expression of peptide hormone receptors (reviewed in Refs. 1,2). These biochemical events elicited by the gonadotropin are accompanied by morphological changes of the immature cell and its transformation into its fully mature counterpart. After binding to specific receptors on granulosa cell membranes (3,4) the decapeptide GnRH can exert stimulatory (5,6) or inhibitory (7-9) control of the differentiation process, depending on the duration of GnRH treatment or the presence of other endocrine, paracrine or autocrine regulatory ligands (reviewed in Refs. 10,11). In the presence of FSH, GnRH prevents gonadotropin-stimulated granulosa cell steroidogenesis, and receptor expression processes (6,7) but the intracellular mechanism(s) whereby the decapeptide inhibits granulosa cell function are not completely

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Abbreviations: GnRH, gonadotropin-releasing hormone; FSH, Follicle stimulating hormone; DAG, diacylglycerol; PLC, phospholipase C; TPA, 12-O-tetradecanoylphorbol-13- acetate; PKC, protein kinase C; 3ß-HSD, 3ß-hydroxystroid dehydrogenase /Δ^{5-4} isomerase.

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understood (1,2,10,11). Nevertheless, since GnRH-receptor activation stimulates phosphatidylinositol hydrolysis and the subsequent generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) production (12-14) followed by increases in intracellular Ca^{2+} (14-16) an involvement of the calcium-phospholipid dependent PKC pathway as intracellular mediator of GnRH action has been proposed (15-18). Although this hypothesis is widely accepted, steroidogenesis is a calcium-dependent event (15,19,20) and therefore it is doubtfull that IP_3 mobilization of calcium from intracellular stores may be involved in the inhibitory effects of GnRH on granulosa cell function. The present study was undertaken to provide additional information about the relative roles of DAG and calcium on the inhibitory effect of GnRH on granulosa cell steroidogenesis.

MATERIAL AND METHODS

Materials

Radiolabeled steroids, [1-^{14}C]stearate (50 mCi/mmol) and [2-^{3}H]myoinositol (14.6 Ci/mmol) were from Du Pont-New England Nuclear (Bad Homburg, Germany). Cholera toxin (CTX), A23187, dibutyril-cyclic AMP (But_2-cAMP), Forskolin (FK), PLC (*c. perfringens*) and TPA, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). FSH (NIADDK-oFSH-S16) was donated by the National Hormone and Pituitary Agency (Baltimore, MD, U.S.A.).

Cell culture procedures

Granulosa cells were obtained by follicle puncture from the ovaries of inmature (22-24 days) rats, implanted for 5 days with diethylestylbestrol filled Silastic capsules (17,18) and cultured in Mc Coy's 5a medium (modified, without serum) for the time periods indicated for each experiment (21). At the end of the incubation period the media were collected and stored frozen (-20°C) until assayed for steroid content (see below).

Diacylglycerol determination and phosphoinositide turnover

Cells were cultured in medium alone for 48 h and labeled for the last 3 h with [^{14}C]stearate (5 μCi/ml). GnRH (10^{-6}M) or PLC (3 mU/ml) were added at decreasing times from appropriate stock solutions and the experiments terminated by adding 1 ml cold methanol supplemented with [^{3}H]DAG (~ 1,000 dpm) to correct for procedural losses (22). After standing for 30 min at 4°C, granulosa cells were scraped from the dishes, transferred to clean glass tubes and the lipids extracted by two succesive washes with chloroform/methanol (2:1, by vol.). The organic phases were combined, dried under nitrogen and DAG purified by TLC as described previously (23). Phosphoinositides were extracted from cultured granulosa cells labeled with [^{3}H]myoinositol for 48 h. To terminate experiments, media were aspirated and 1 ml ice-cold 5% TCA added to each culture. Phospholipids were extracted twice with 3 ml chloroform/methanol (1:2, by vol.) containing 0.03 N HCl and PI, PIP and PIP_2 separated by sequential TLC in the solvent systems described (24).

Assay of 3ß-hydroxystroid dehydrogenase /Δ^{5-4} isomerase

In selected experiments, cells were scraped from the dishes and assayed for 3ß-hydroxystroid dehydrogenase activity as the rate of conversion of [^{3}H]pregnenolone to [^{3}H]progesterone (25). Briefly, triplicate aliquots of cell homogenates (80-100 μg protein) from the different experimental groups were assayed for 30 min at 37°C in a final volume of 100 μl, containing 0.05M potassium phosphate (pH 7.4), 1 mM EDTA, 100μM NAD^+

and 50 μM pregnenolone ($\sim$2 x 10^4 cpm/nmol) dissolved in dimethyl sulfoxide (final concentration 3%, by vol). The reaction was stopped by placing the tubes in an ice bath, and [^{14}C]progesterone ($\sim$ 500 cpm) added to determine recovery. Reaction samples were extracted with diethyl ether (20 vol.) and radiometabolites separated by two succesive developments in the system chloroform/ether (5:1, by vol.) as described previously (21,25). Results (mean $\pm$ S.E.M.) are expressed as nmol progesterone formed/30 min/mg protein. Protein content as determined by the Bio-Rad (Richmond, Ca, U.S.A.) dye binding assay and was unaffected by the different treatments.

Steroid quantification

Steroid levels in the culture media were determined by RIA, using specific antisera for progesterone (antiprogesterone-11-BSA #337), a gift from Dr. G.C. Niswender (Colorado State University) and estradiol (anti-17ß-estradiol lot #50605-59), from Arnel Products Co.(New York, NY, U.S.A.). When estradiol production was measured, the cells were incubated for 48 h with the different treatments, washed twice with prewarmed medium, and cultured for an additional 8 h period in the presence of Δ^4-androstendione (10^{-5}M) as substrate for aromatases (1,2). Intra and inter assay coefficients of variation were 4% and 6.3% for progesterone and 5% and 5.8% for estradiol assay respectively.

RESULTS AND DISCUSSION

In the present investigation we show that treatment of granulosa cells with GnRH or PLC induced a significant rise in DAG generation 5 min later which was sustained for the following 30 min period (Figure 1). The effect of GnRH was preceded by the rapid (30 sec) hydrolysis of PIP_2 (circa 50%) which returned to baseline levels 2 min later. As shown in Figures 2 and 3, the full replication of FSH-induced stimulation on 3ß-HSD enzyme activity and steroid production by the membrane permeable cAMP analog (But_2-cAMP) as well as by agents which increase intracellular cAMP by nonreceptor-mediated activation of adenylate cyclase (CTX and FK) was prevented by simultaneous treatment of granulosa cells with GnRH (10^{-6}M) , PLC (3 mU/ml) and TPA (10^{-7}M). Nevertheless the inhibitory effect of these agents on gonadotropin stimulated steroidogenesis contrasted with a consistent stimulation of steroidogenesis observed when granulosa cells were cultured in the absence of FSH (Figure 3). These results confirm that activation of PKC by phorbol esters (18,26), GnRH (9,17,18) or PLC stimulated DAG generation (Figures 2 and 3) exerts both stimulatory or inhibitory control on granulosa cell function depending on the presence or absence of FSH-induced maturational stage of these cells (3-9,12-17). Since phosphodiesterasic cleavege of phosphoinositides appears to be an early step in the action of GnRH in ovarian granulosa cells, the generation of DAG and IP_3-stimulated burst in the level of intracellular free calcium have been implicated as mediators of GnRH activation of the calcium-phospholipid dependent PKC inhibitory pathway which prevents FSH-cAMP induced maturation and steroidogenic function of the rat granulosa cell (1,2,10,11). Steroidogenesis is a calcium-dependent pathway however (15,19,20), and in the absence of

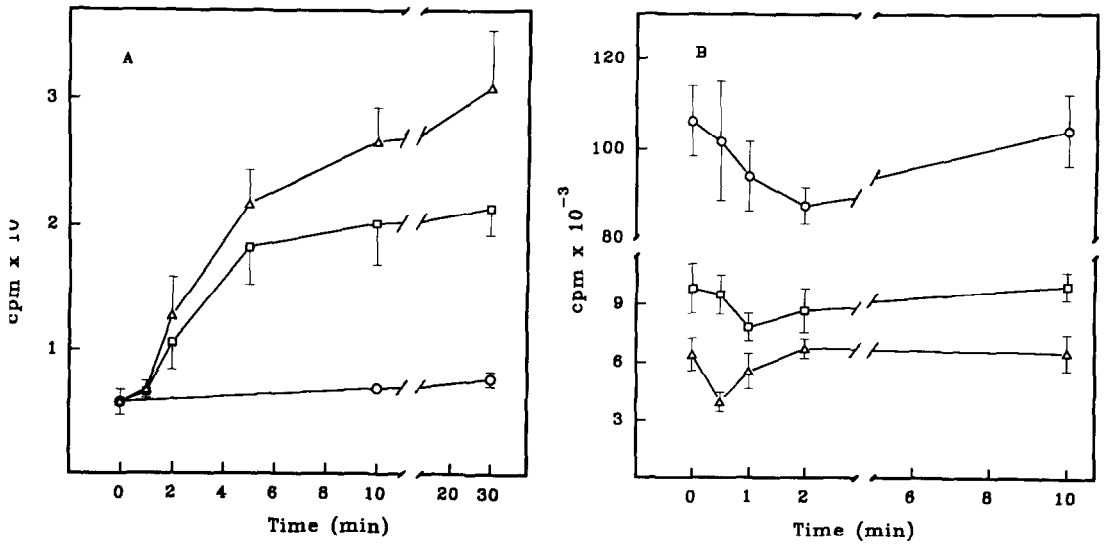

Figure 1. Effects of GnRH and PLC on DAG generation and phosphoinositide turnover.

A: Granulosa cells ($\sim 10^6$ viable cells/dish) were labeled for 3 hours with [^{14}C]stearate (5μCi/ml) and treated for the times indicated with Mc Coy's 5a medium (O-O), 10^{-6}M GnRH (□-□) or 3mU/ml PLC (Δ-Δ). The lipids were extracted from individual cultures and DAG separated as described in methods. Similar results (mean ± S.E.M.) were obtained in three additional experiments.

B: Cultured granulosa cells were labeled for 48 h with [^{3}H]myoinositol (10 μCi/ml) and GnRH added at decreasing times to triplicate or quadruplicate cultures in a retrograde fashion. Phospholipids from individual cultures were extracted and the phosphoinositide PI (○-○) PIP (□-□) and PIP_2 (Δ-Δ) separated and quantitated by liquid scintillation counting.

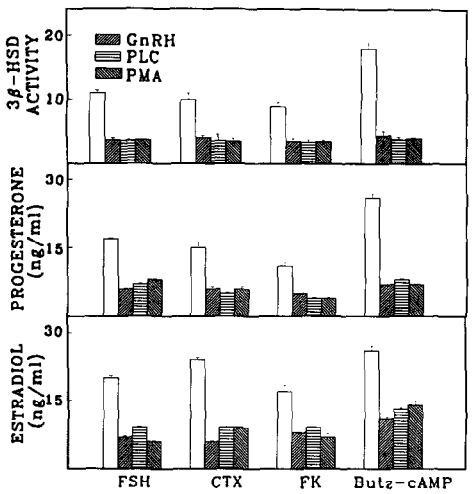

Figure 2. Effect of GnRH, PLC or TPA on FSH, CTX or But_2cAMP stimulated steroidogenesis of cultured granulosa cells.

Granulosa cells ($\sim 10^6$ viable cells/dish) were cultured for 48 hours in the presence of FSH (30 ng/ml), CTX (50 μM) or But_2cAMP (0.1 mM) alone or in the presence of GnRH (10^{-6}M), PLC (3 mU/ml) or TPA (10^{-7}M). Media were assayed for progesterone and estradiol content, and cell homogenates assayed for 3ß-HSD activity as described under Materials and Methods. Results are the mean ± S.E.M. of quadruplicate cultures from an experiment representative of three others.

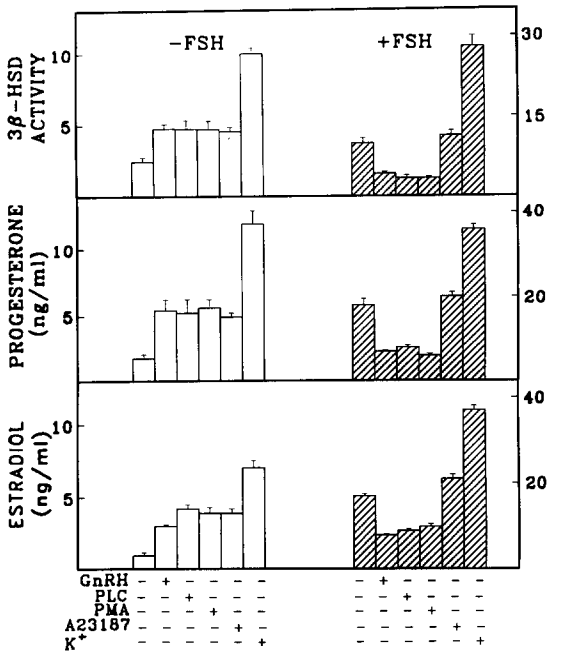

Figure 3. Effects of GnRH, PLC, PMA, A23187 and high extracellular K^+ on granulosa cell steroidogenesis.

Granulosa cells ($\sim 10^6$ viable cells/dish) were cultured in the absence or presence of FSH (30 ng/ml), alone or in combination with the different treatments. Media were assayed for steroid content, and cell homogenates assayed for 3ß-HSD enzyme activity as described under methods. Results are the mean $\pm$ S.E.M. of quadruplicate cultures from an experiment representative of three others.

FSH application of the non specific calcium mobilizing agent A23187 augmented granulosa cell steroidogenic activity, whereas elevation of external K^+ to depolarize granulosa cell membrane enhanced steroidogenesis in unstimulated cells and largely augmented steroid biosynthesis in FSH-activated cells (Figure 3). The stimulatory effect obtained after prolonged treatment of granulosa cells with A23187 or high extracellular K^+ concentration are not consistent with the rapid phosphoinositide breakdown observed herein (Figure 1) or the fast and transient rise in cytosolic calcium reported when IP_3 generation is stimulated by GnRH (13-16). These observations suggest that DAG generation in response to GnRH involves membrane phospholipids different from PIP_2 (27). In this regard, it is worthwhile notting that GnRH has been shown to activate PLC-mediated PIP_2 hydrolysis (12-16) as well as other G-protein coupled phospholipases (i.e. PLA_2 and PLD) in ovarian granulosa cells (28,29), and therefore it is possible that the initial effect of GnRH on PIP_2 hydrolysis triggers the subsequent activation of other phospholipases (27). Alternatively, agonists that stimulate the early activation of PKC either directly or indirectly via stimulation of phosphoinositide hydrolysis, can also stimulate phosphatidylcholine hydrolysis in intact cells (27,29,30). Since

the production of DAG from phosphatidylcholine provides additional opportunities for the regulation of PKC, it is tempting to speculate that the early GnRH-stimulated PIP_2 hydrolysis may represent a mechanism of activation of PKC isoforms involved in the inhibitory effect of the decapeptide in the absence of calcium mobilization (31). The identity of these GnRH-activated PKC isoforms in granulosa cells, and their role in controlling granulosa cell differentiation remains to be studied.

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