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Inhibition of gonadotropin-induced granulosa cell differentiation by activation of protein kinase C

(phorbol ester/diacylglycerol/cyclic AMP/luteinizing hormone receptor/progesterone)

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ABSTRACT **The induction of granulosa cell differentiation by follicle-stimulating hormone (FSH) is characterized by cellular aggregation, expression of luteinizing hormone (LH) receptors, and biosynthesis of steroidogenic enzymes. These actions of FSH are mediated by activation of adenylate cyclase and cAMP-dependent protein kinase and can be mimicked by choleragen, forskolin, and cAMP analogs. Gonadotropin releasing hormone (GnRH) agonists inhibit these maturation responses in a calcium-dependent manner and promote phosphoinositide turnover. The phorbol ester phorbol 12-myristate 13-acetate (PMA) also prevented FSH-induced cell aggregation and suppressed cAMP formation, LH receptor expression, and progesterone production, with an ID_{50} of 0.2 nM. In FSH-treated cells, PMA did not reduce the initial increase in cAMP formation during the first 24 hr of culture but prevented its secondary increase from 24 to 48 hr. PMA also inhibited LH receptor induction by cholera toxin, forskolin, and 8-bromo-cAMP, but it did not impair cAMP responses to the former two agents, indicating that the site of action of the phorbol ester is distal to adenylate cyclase. The early stimulation of cAMP-dependent protein kinase activity by FSH was also unaffected by PMA, consistent with its lack of effect on the initial cAMP response to FSH. However, PMA caused a marked decrease in cytosolic protein kinase C activity within 1 min of its addition to the cells. The permeant diacylglycerols, 1-oleoyl-2-acetoyl-*sn*-glycerol and *sn*-1,2-dioctanoyl glycerol, also inhibited LH receptor formation, while the nonpermeant diacylglycerol, diolein, was inactive. These results indicate that *in situ* activation of protein kinase C by PMA or permeant diacylglycerols inhibits cAMP-dependent granulosa cell differentiation, and suggest that the inhibitory actions of GnRH agonists on granulosa cell maturation are also mediated by protein kinase C.**

It is well established that follicle-stimulating hormone (FSH) induces granulosa cell differentiation and that this response is mediated by cAMP-dependent mechanisms (1, 2). The rat granulosa cell provides a valuable model to study the hormonal control of cellular differentiation, because maturation occurs within 48 hr and is manifested by morphological cytodifferentiation, expression of peptide hormone receptors [for luteinizing hormone (LH), prolactin, and catecholamines], and induction of steroidogenic enzymes leading to biosynthesis of progesterone and estrogen (1–3). Several ligands, including gonadotropin releasing hormone (GnRH) and growth factors, can inhibit or enhance the induction of granulosa cell differentiation by FSH, but their mechanisms of action are not completely understood (4–9). GnRH agonists are known to suppress FSH-induced cellular differentiation in a calcium-dependent fashion (10) and to stimulate phosphoinositide turnover in granulosa cells (11–13), suggesting that calcium- and phospholipid-dependent mechanisms are involved in the inhibition of granulosa cell differentiation. The abilities of tumor promoting phorbol esters and synthetic 1,2-diacylglycerols to stimulate calcium-activated phospholipid-dependent protein kinase C (14, 15) led us to examine the effects of these compounds on cellular maturation in the rat granulosa cell.

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MATERIALS AND METHODS

Granulosa cells were obtained from the ovaries of rats (Taconic Farms, Germantown, NY) implanted with diethylstilbestrol capsules (2 cm) at 21 days of age and sacrificed after 4–6 days (5). The cells were suspended in McCoy's 5A medium (without serum) supplemented with 10 mM Hepes, pH 7.4/4 mM L-glutamine/penicillin (100 units/ml)/streptomycin sulfate (100 μg/ml). The viability of the cell preparation, as indicated by Trypan blue exclusion, was ≈90%. Cultures were established in multiwell plates or polystyrene tubes (12 × 75 mm) at a concentration of 2×10^5 cells per ml and were incubated at 37°C in humidified 5% CO_2/95% air with or without hormones as indicated. At the indicated times, media were removed and centrifuged prior to analysis for cAMP and progesterone content by specific radioimmunoassay (5). LH receptor assays were performed by incubating the cells with 200,000 cpm of ^{125}I-labeled human choriogonadotropin (hCG) (50,000–70,000 cpm/ng) in a total vol of 0.3 ml of Dulbecco's phosphate-buffered saline (pH 7.3) containing 0.1% bovine serum albumin for 12–16 hr at room temperature (2). Nonspecific binding was determined in tubes containing 1 μg of hCG (10 international units of Pregnyl, Organon) and was <5% of the total radioactivity.

Cyclic AMP-dependent protein kinase A activity was measured in cytosol obtained by sonication of cell pellets (5×10^5 cells) as described (6, 16). Protein kinase C activity was assayed in 100 μl of buffer (20 mM Tris·HCl, pH 7.5/10 mM Mg acetate/50 μM [γ-^{32}P]ATP (2 μCi; 1 Ci = 37 GBq]/50 μg of histone IIIS [Sigma]). EGTA (1 mM) was added to control tubes, while 0.75 mM $CaCl_2$, phosphatidylserine (50 μg/ml) (Supelco, Bellefonte, PA), and 1,2-diolein (3 μg/ml) (Sigma) were added to stimulate protein kinase C activity. Phosphatidylserine and diolein were dissolved in chloroform and evaporated under a stream of nitrogen, and then they were sonicated at 30 W for 3 min in 20 mM Tris·HCl (pH 7.5). Samples were incubated for 3 min at 30°C. Controls included samples in which cytosol or histone was omitted from the assay.

Phorbol derivatives (P-L Biochemicals or Sigma) were dissolved either in ethanol or dimethyl sulfoxide (Me_2SO) at

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; GnRH, gonadotropin releasing hormone; GnRHa, [D-Ala^6, des-Gly^{10}]GnRH *N*-ethyl amide; hCG, human choriogonadotropin; OAG, 1-oleoyl-2-acetoyl-*sn*-3-glycerol; DOG, *sn*-1,2-dioctanoyl glycerol; PMA, phorbol 12-myristate 13-acetate.

a concentration of 1 mg/ml, and were diluted in medium just before use. 1-Oleoyl-2-acetoyl-*sn*-3-glycerol (OAG) and *sn*-1,2-dioctanoyl glycerol (DOG; Avanti Polar Lipids) were dissolved in Me_2SO at a concentration of 20 mg/ml and diluted with Me_2SO prior to addition to the culture medium. The final concentration of Me_2SO (0.5%) was added to all cell cultures, and did not affect FSH-induced cell differentiation.

Ovine FSH (NIH-oFSH-15) and hCG-CR123 were gifts from the National Pituitary Agency. [D-Ala^6, des-Gly^{10}]-GnRH *N*-ethylamide (GnRHa) was obtained from Peninsula Laboratories (San Carlos, CA). Cholera toxin and forskolin were from Calbiochem-Behring, and [γ-^{32}P]ATP was purchased from New England Nuclear.

Statistical analysis was performed by Student's *t* test or by analysis of variance, according to the experimental design.

RESULTS

The LH receptor content of cultured granulosa cells was increased 20-fold by FSH treatment during the 48-hr culture period (Fig. 1, *Top*). Such FSH-induced expression of LH receptors was completely inhibited by phorbol 12-myristate 13-acetate (PMA) in a dose-dependent manner with an IC_{50} of 0.05 ng/ml, corresponding to 0.16 nM PMA. The inhibition of LH receptor expression in cells cultured with FSH plus GnRHa is also shown in Fig. 1. At the concentrations used, PMA itself had no effect on cell viability or basal LH receptor expression and did not inhibit binding of labeled hCG when added to the receptor assay system. FSH-stimulated progesterone formation was suppressed in parallel with LH receptor formation, as shown in Fig. 1 (*Middle*). Production of cAMP during the 48-hr culture period was also reduced by PMA to the same extent as in cells treated with GnRHa, but not to the control level of unstimulated cells (Fig. 1 *Bottom*).

Microscopic examination of the cell cultures revealed that the characteristic morphological features of cell differentiation induced by FSH or 8-bromo-cAMP, with multilayered aggregation of the cells (17), was not observed when FSH-treated cells were exposed to PMA (1 ng/ml). Instead, there was marked flattening of the cytoplasms as in the case of undifferentiated control cells, with no evidence of cell aggregation.

The time course of the phorbol inhibitory effect was analyzed in cells cultured with FSH for 48 hr, with addition of PMA (1 ng/ml) at several time points after the initiation of culture. As shown in Fig. 2 (*Upper*), PMA completely inhibited LH receptor expression when the drug was added up to 36 hr after initiation of culture. This inhibitory pattern was identical with that produced by GnRHa in parallel cell cultures. The time course of inhibition of cAMP production by PMA paralleled that of LH receptor inhibition, as shown in Fig. 2 (*Lower*). Again, the effect of GnRHa was identical with that of PMA, suggesting that both ligands share a similar inhibitory mechanism of action. When the cells were treated with PMA for the first 4 hr, then washed three times and cultured with FSH for a further 44 hr, the inhibitory effect of PMA was not reversed (data not shown). Such a prolonged inhibitory response after brief initial exposure to the ligand is also observed in cells treated with GnRH agonists (5).

We previously observed that two phases of cAMP production occur during the induction of granulosa cell differentiation by FSH (5), an initial increase during the first several hours of culture being followed by a plateau until 18–24 hr of culture, with a second increase after 24 hr of culture. The typical biphasic pattern of cAMP production by FSH-treated cells is evident in Fig. 3, which shows that PMA did not affect the first phase but prevented the secondary increase in cAMP formation. This pattern of inhibition was again similar to that observed in GnRH-treated cells (5) and is largely confined to the secondary phase of cAMP production. Thus, the final

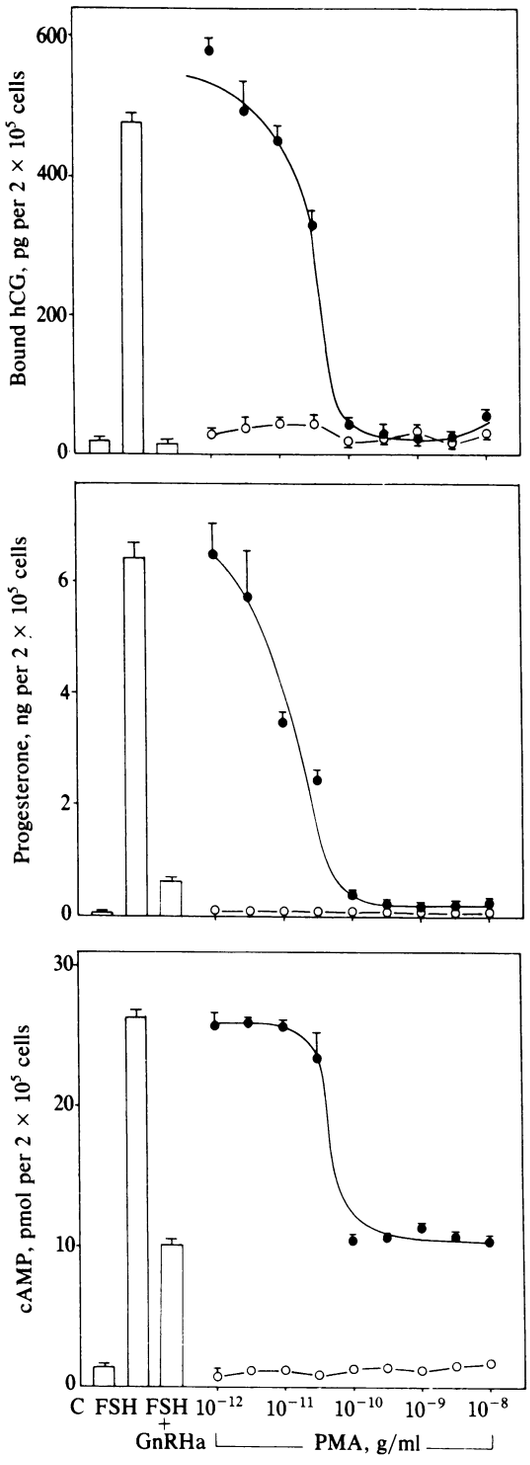

FIG. 1. Dose dependence of the inhibitory effect of PMA on granulosa cell differentiation. Granulosa cells (2 × 10^5) were cultured for 48 hr with (●) or without (○) FSH (100 ng/ml) and various amounts of PMA or GnRHa (10 nM). Media were collected for measurement of progesterone and cAMP levels, and cells were assayed for LH receptor content. In this and subsequent figures, each point represents the mean ± SEM of three determinations in at least two experiments. C, control.

cAMP level of cells treated with both PMA and FSH was reduced to 25–50% of that observed with FSH-treated cells, whereas LH receptor expression was suppressed to the control level (Fig. 1).

The effects of PMA on cytodifferentiation induced by other cAMP-mediated stimuli were also analyzed. Cholera toxin (0.1 μg/ml), forskolin (10 μg/ml), and 8-bromo-cAMP (5

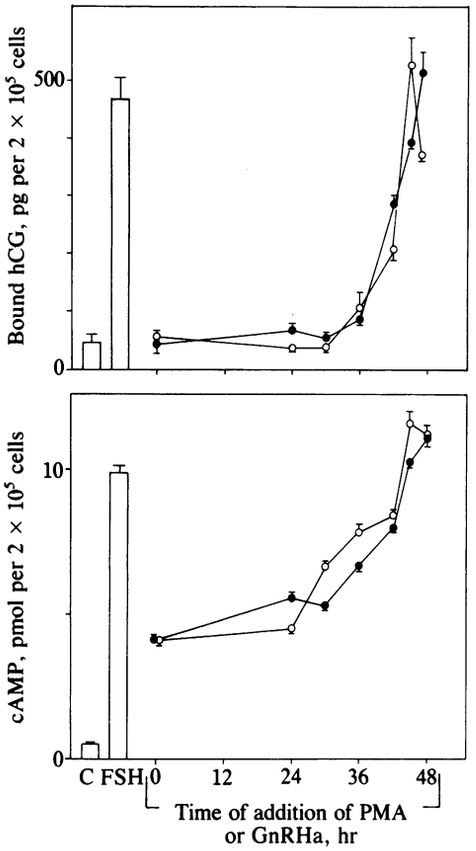

FIG. 2. Inhibitory effects of PMA (●) and GnRHa (○) added during granulosa cell culture. PMA (1 ng/ml) or GnRHa (10 nM) was added to FSH- (100 ng/ml) treated granulosa cells (2 × 10^5) at the indicated time points, and cells were harvested at 48 hr of culture for measurement of LH receptor content (*Upper*) and extracellular cAMP (*Lower*). C, control.

mM) stimulated LH receptor formation at 48 hr of culture to ≈50% of the level induced by FSH, consistent with the major role of cAMP in receptor induction. In the presence of PMA (1 ng/ml), these effects were completely abolished and LH receptor expression was suppressed to the basal level (Fig. 4 *Upper*). In the same cells, cAMP production induced by

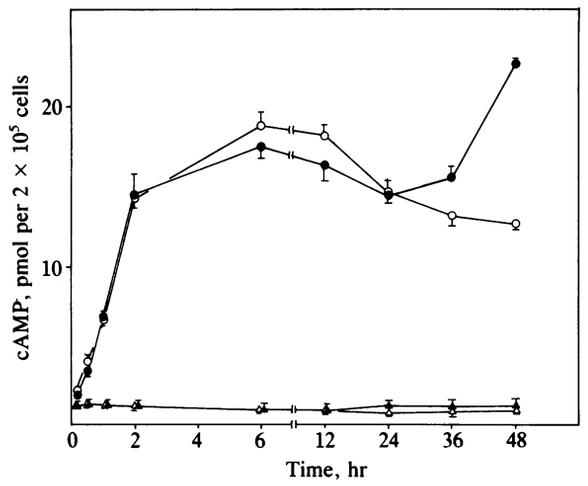

FIG. 3. Time course of the effect of PMA on FSH-stimulated cAMP production. Granulosa cells (2 × 10^5) were cultured with FSH (100 ng/ml) and/or PMA (1 ng/ml), and media were analyzed for cAMP content throughout the 48-hr incubation period. ●, FSH; ○, FSH and PMA; ▲, PMA; △, control.

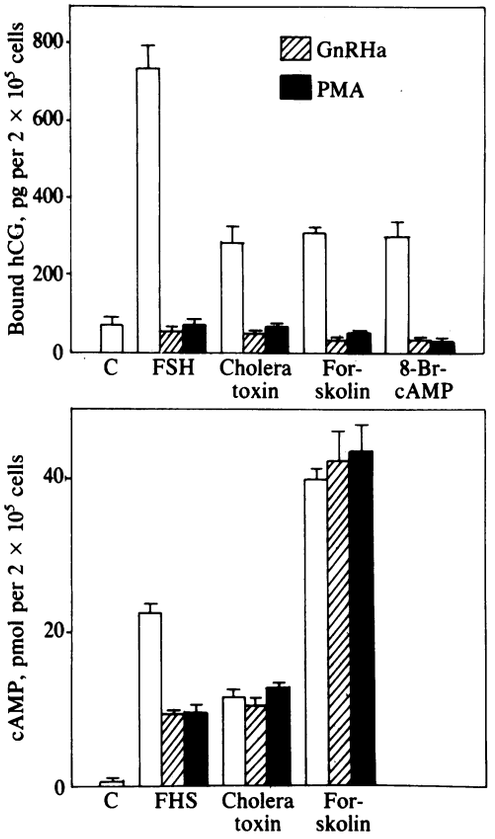

FIG. 4. Effects of PMA on LH receptor expression (*Upper*) and cAMP production (*Lower*). Granulosa cells (2 × 10^5) were cultured with FSH (100 ng/ml), cholera toxin (0.1 μg/ml), forskolin (10 μg/ml), or 8-bromo-cAMP (8-Br-cAMP) (5 mM) for 48 hr with or without PMA (1 ng/ml).

cholera toxin or forskolin was completely unaffected by PMA, indicating that the inhibition of FSH-induced LH receptor expression by PMA is not dependent on its concomitant attenuation of cAMP formation (Fig. 4 *Lower*).

Of the several phorbol derivatives tested, only the biologically active esters, PMA and phorbol 12,13-dibutyrate, were able to suppress LH receptor expression in the FSH-stimulated granulosa cell (Fig. 5). The effects of phorbol esters are largely attributable to their ability to activate protein kinase C in a manner analogous to that of diacylglycerol (18). Since diacylglycerol would be produced during the stimulation of granulosa cell phospholipid turnover by agents such as GnRH (11–13), we examined the effects of the synthetic permanent diacylglycerols, OAG and DOG, on granulosa cell differentiation. As shown in Fig. 6, OAG completely inhibited LH receptor formation in FSH-stimulated cells, and it did so in a dose-dependent manner with an ID_{50} of 20 μg/ml. The other synthetic lipid, DOG, was a more potent inhibitory analog and it suppressed LH receptor formation with an IC_{50} of 2 μg/ml (not shown). In contrast, the nonpermanent diacylglycerol, diolein, had no inhibitory effect when added at concentrations up to 100 μg/ml.

The presence of cAMP during the entire period of culture appears to be essential for optimal granulosa cell differentiation (5, 19), and FSH-induced cAMP production was significantly decreased after 24 hr of culture with PMA (Fig. 3) or GnRH (5). To evaluate the relevance of this effect to the concomitant blockade of differentiation, cells were cultured with FSH (100 ng/ml) and PMA (1 ng/ml) or GnRHa (10 nM), and supplemented with cholera toxin (1 μg/ml), forskolin (10 μg/ml), or 8-bromo-cAMP (2 mM) after 30 hr to raise or maintain cAMP production during the remainder of the

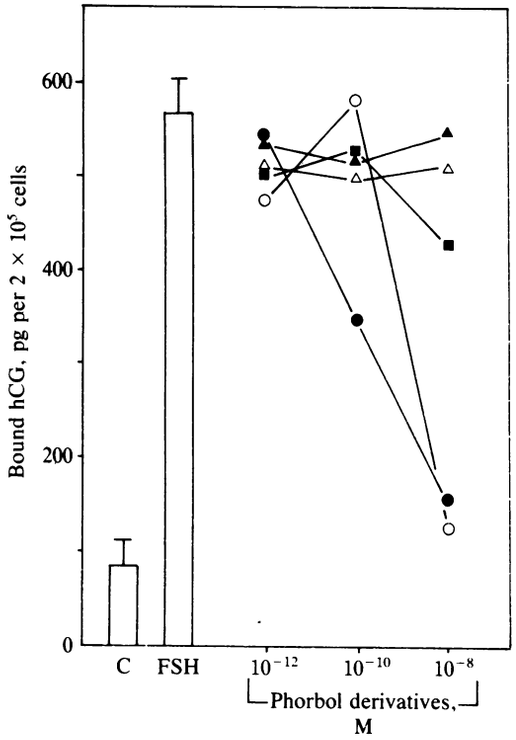

FIG. 5. Effects of phorbol derivatives on LH receptor formation. Granulosa cells (2×10^5) were cultured with FSH (100 ng/ml) and individual phorbol derivatives for 48 hr. For clarity, only the means of data for dose response were presented; SEMs were within 10% of the means. ○, Phorbol 12,13-dibutyrate; ▲, 4α-phorbol; △, 4β-phorbol; ■, 4α-phorbol 12,13-didecanoate; ●, PMA; C, control.

culture period. However, the inhibition of LH receptor expression by PMA was not reversed by addition of cAMP-increasing agents during the culture period (Table 1), indicating that the site of action of PMA (and GnRH) was beyond the level of adenylate cyclase, despite its ability to impair cAMP production.

In view of this finding, and because the initial increase in cAMP formation was not blocked by PMA, we also examined the effects of FSH and PMA on cAMP-dependent protein kinase. When granulosa cells were incubated for 20 min with or without FSH (100 ng/ml) and PMA (10 ng/ml), the cytosolic protein kinase A activity ratio [(activity without cAMP)/(activity with cAMP)] was increased by FSH from 0.38 to 0.65, but PMA was without effect whether added alone or in the presence of FSH. In contrast to its lack of effect on protein kinase A, PMA caused a rapid and marked change in the cytosolic activity of protein kinase C, which began to decrease within 1 min and remained at a low level for up to 60 min during continued exposure to the phorbol ester (Fig. 7).

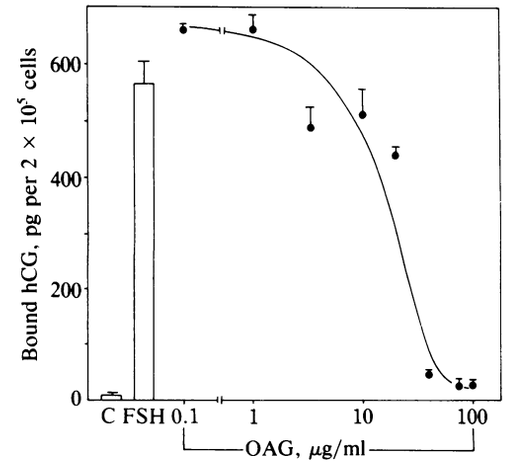

FIG. 6. Inhibitory effect of OAG on LH receptor expression. Granulosa cells (2×10^5) were cultured with FSH (100 ng/ml) and different concentrations of OAG for 48 hr. C, control.

Table 1. Effects of addition of 8-bromo-cAMP, cholera toxin, or forskolin at 30 hr of culture on LH receptors measured at 48 hr

Initial addition	Addition at 30 hr of culture			
	None	8-Bromo-cAMP	Cholera toxin	Forskolin
FSH	568 ± 19	364 ± 10	391 ± 5	313 ± 8
FSH/GnRHa	14 ± 2	11 ± 1	15 ± 2	13 ± 2
FSH/PMA	13 ± 1	16 ± 4	18 ± 1	14 ± 2

Cells (2×10^5) were cultured with FSH (100 ng/ml), FSH/GnRHa (10 nM), or FSH/PMA (1 ng/ml). At 30 hr, 8-bromo-cAMP (2 mM), cholera toxin (1 μg/ml), or forskolin (10 μg/ml) was added, and cells were subjected to LH receptor assay at 48 hr of culture. Results are expressed as pg of ^{125}I-labeled hCG bound per 2×10^5 cells. The basal level of bound ^{125}I-labeled hCG in unstimulated cultures was 10 ± 3 pg per 2×10^5 cells.

DISCUSSION

These results demonstrate that the potent phorbol ester, PMA, and two permanent diacylglycerols, OAG and DOG, completely inhibit cAMP-dependent granulosa cell differentiation *in vitro*. Since the cellular actions of these compounds are largely mediated by activation of protein kinase C, it appears that the calcium- and phospholipid-dependent enzyme is a major inhibitory modulator of granulosa cell maturation. The suppressive effect of PMA on FSH-induced LH receptor expression was accompanied by a decrease in cAMP and progesterone production from 24 to 48 hr of culture. However, the phorbol ester did not reduce cAMP production in cells incubated with cholergen or forskolin. Since maintenance of increased cAMP levels by the addition of choleragen, forskolin, or 8-bromo-cAMP did not prevent or reverse the suppression of LH receptor expression by PMA, this effect of the phorbol ester is independent of its inhibitory action on FSH-induced cAMP production. However, the induction of LH receptor formation *in vitro* is known to be dependent on the continuous presence of FSH or cAMP-producing agents (5, 19), and a change in the

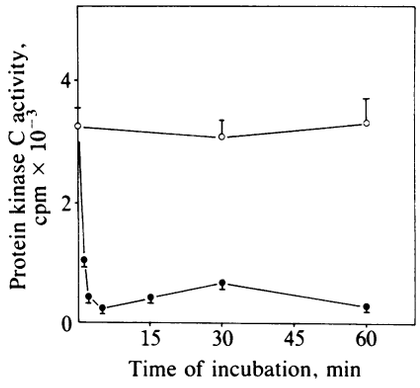

FIG. 7. Cytosolic protein kinase C activity in granulosa cells incubated with FSH (100 ng/ml) with (●) or without (○) PMA (10 ng/ml). After incubation, cells were sonicated and cytosols obtained by centrifuging at 30,000 × *g* for 30 min were assayed for protein kinase C activity.

production or action of cAMP at any point during culture impairs the process of differentiation.

PMA has been reported to suppress or to have no effect on protein kinase A activity in individual cell systems (20, 21). In the rat granulosa cell, PMA did not affect the activity ratio of protein kinase A at a dose (10 ng/ml) that was fully active in suppressing LH receptor expression. However, PMA is known to activate protein kinase C directly, often with a decrease in cytosolic enzyme activity without inducing phospholipid turnover (14), and this mechanism appears to be primarily responsible for many of the cellular actions of PMA (14, 15). Phorbol esters are also known to decrease the number of peptide hormone receptors in several tissues, including sites for epidermal growth factor, thyrotropin releasing hormone, and somatostatin in pituitary cells (22). Thus, the inhibition of FSH-induced cAMP production by PMA during the second day of culture could be due to a PMA-mediated decrease in FSH receptors or to an inhibitory effect on the receptor-coupled adenylate cyclase system. The delayed decrease in cAMP formation may contribute to the suppression of LH receptor expression, but the activation of protein kinase C by PMA and diacylglycerol is clearly capable of inhibiting granulosa cell differentiation via mechanisms that are independent of the protein kinase A system. Several reports have demonstrated that adenylate cyclase activity can be potentiated by protein kinase C (23, 24), but such an interaction is unlikely to apply in the immature granulosa cell, in which the initial time course of cAMP production was not influenced by PMA.

Phorbol esters have been found to have both stimulatory and inhibitory effects on cellular differentiation, mostly in cell lines from hematopoietic malignancies (25–27). However, the effects of PMA were reversible in hematopoietic cell lines but not in granulosa cells. Since granulosa cells obtained from estrogen-treated immature rats have the potential to differentiate in 36–48 hr, this system can be employed to analyze the role of protein kinase C during hormonally regulated cellular differentiation, when its effects are inhibitory rather than stimulatory.

In recent reports, phorbol esters have been shown to exert variable effects on steroidogenesis in gonadal cells. In the testicular Leydig cell and the immature granulosa cell, PMA inhibited steroidogenesis *in vitro* (28), whereas in mature rat granulosa cells (29) and human luteal cells (30) the effects of PMA were predominantly stimulatory. Also, PMA has been shown to promote translocation of protein kinase C in human luteal cells (30). Our findings suggest that such actions of phorbol esters on ovarian steroidogenesis are components of the global effects of protein kinase C activation on all aspects of granulosa cell differentiation. In the immature granulosa cell, these include reduction of cAMP production and suppression of LH receptor expression and morphological maturation, as well as inhibition of steroidogenic responses.

The similarity of action of PMA to that of GnRHa, together with the abilities of GnRH agonists to promote phospholipid turnover in the ovary (11–13) and translocation of protein kinase C activity in pituitary gonadotrophs (31), suggest that activation of protein kinase C could mediate the inhibitory action of GnRHa on granulosa cell differentiation. It is clear that activation of protein kinase C by phorbol esters or diacylglycerols initiates an inhibitory mechanism that prevents hormone-induced maturation of the rat granulosa cell, and that this process is largely independent of changes in cAMP production. The developmental program of the granulosa cell is influenced by both protein kinases A and C, but the effects of the protein kinase C pathway are predominantly inhibitory in the immature granulosa cell.

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