Regulation of Apolipoprotein E Biosynthesis by cAMP and Phorbol Ester in Rat Ovarian Granulosa Cells*

(Received for publication, August 90, 1988)

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Apolipoprotein E (apoE) is synthesized by the liver and many peripheral cells. Rat ovarian granulosa cells synthesize and secrete apoE, and this apoE production is increased by agents that increase cellular cAMP. In these studies of granulosa cell apoE synthesis we have examined the effect of agents that stimulate various cell kinases, including protein kinases A, G, and C. The cell content of apoE mRNA was measured simultaneously. Cholera toxin (1.25 μg/ml), dibutyryl-cAMP (5 mg/ml), and forskolin (10^-4 M), all of which increase cellular cAMP, stimulate apoE accumulation in the medium 7–10-fold. On the other hand, dibutyryl-cGMP (20 mg/ml) has no effect on apoE synthesis or secretion. The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (100 ng/ml), a protein kinase C stimulator, increases apoE accumulation in the medium 8–10-fold, while 4α-phorbol 12,13-didecanoate, the inactive phorbol congener, has no such effect. The cAMP effect on apoE synthesis by granulosa cells is maximal at 48 h, while the phorbol ester effect is maximal at 72–96 h in culture. The data indicate that agents whose effects are mediated by activation of protein kinases A and C, but not G, stimulate granulosa cell apoE production. These effects on the amount of secreted apoE are temporally preceded by increases in the granulosa cell content of apoE messenger RNA. Together, these data suggest that the regulation of apoE production in the rat ovarian granulosa cell could involve transcriptional and post-transcriptional mechanisms.

ApoE is a 34,000-dalton glycoprotein which is found as a surface component of several classes of lipoproteins. ApoE plays a critical role in cholesterol metabolism, functioning as a ligand for the B/E receptor on peripheral cells and hepatocytes and the E receptor on hepatocytes (1). Binding of the lipoprotein allows delivery of cholesterol for membrane synthesis in all cells and also for steroidogenesis in steroidogenic cells (2).

apoE synthesis has been demonstrated in most extrahepatic tissues, including steroidogenic organs such as the adrenal, testis, and ovary. ApoE, newly synthesized by monkey adrenal and testicular tissue, represents the same percentage of total secreted proteins as it does in the liver (3). Macrophages (4) and smooth muscle cells (5) can also synthesize apoE in vitro, which suggests that these cells may contribute to the synthesis in extrahepatic tissues in vivo. However, the magnitude of apoE synthesis in extrahepatic tissues suggests that other cells are involved. For example, primary cultures of rat ovarian granulosa cells (6), astrocytes, and Schwann cells (7) synthesize and secrete apoE. The function and regulation of apoE synthesis and secretion in extrahepatic tissues is still unclear. Extrahepatically synthesized apoE could increase cholesterol uptake in peripheral cells via the low density lipoprotein receptor for which it serves as a ligand. Additionally, it could act to transport cholesterol within its immediate environment which in this case would be the ovarian follicle; granulosa cell-produced apoE could help deliver lipid to the oocyte. ApoE produced by granulosa cells could also interact with the surrounding theca cells to serve a paracrine function within the follicle, as suggested by Dyck et al. (8).

ApoE represents approximately 0.12% of the total protein synthesized in cultured rat ovarian granulosa cells. Primary cultures of rat ovarian granulosa cells have been used extensively to study hormone regulation and cell differentiation (9). The addition of the gonadotropin FSH to primary cultures of granulosa cells induces cell differentiation, the development of receptors for other gonadotropins (luteinizing hormone/human chorionic gonadotropin and prolactin) (10, 11), and stimulates the production of estrogens and progestins (12–14). Gonadotropins stimulate adenylate cyclase, and cAMP acts as the second messenger for gonadotropin action (9). FSH effects on steroidogenesis are reproduced by the addition of exogenous cAMP (as But-cAMP) (15–17) and by other agents which increase intracellular levels of cAMP, such as CT, phosphodiesterase inhibitors, and forskolin, which is a direct activator of adenylate cyclase (16, 18, 19).

We have previously shown that FSH stimulation of primary cultures of granulosa cells increases apoE secretion 2-fold above that by nonstimulated cells (6). The addition of But-cAMP or CT stimulates apoE secretion 8–10-fold. The CT stimulation of apoE secretion is both dose-dependent (maximum level of secreted apoE achieved at a CT concentration of 1.25 μg/ml) and time-dependent, with the amount of secreted apoE peaking at 48 h in culture and coming almost back to base line by 96 h. These results suggest a role for the cAMP-dependent kinase A in the control of rat ovarian apoE synthesis and secretion.

The presence of various cellular protein kinases in rat ovarian granulosa cells has been well established (9, 20, 21). Kinase A is activated via cAMP by FSH, But-cAMP, CT, or
forskolin (15-19, 22, 23). Kinase G is activated by BtzcGMP which increases the formation of luteinizing hormone receptors in these cells (21). Kinase C, a phospholipid-dependent and calcium-activated protein kinase, is activated by the phorbol ester 12-0-tetradecanoylphorbol 13-acetate (TPA), resulting in inhibition of FSH-stimulated cell differentiation, maturation, and steroidogenesis (24). However, phorbol ester does cause a modest increase in steroidogenesis when presented to cells by itself (24, 25).

In this study, we demonstrate that activation of kinases A and C, but not of kinase G, can stimulate independently apoE synthesis and secretion. We also present data on apoE messenger RNA levels whose changes parallel the changes in the levels of secreted apoE, following stimulation of both kinases A and C. These data suggest that the regulation of apoE synthesis may be at least partially at the transcriptional level.

**EXPERIMENTAL PROCEDURES**

**Materials—**Radioisotopes were purchased from Amersham Corp. BtzcAMP, BtzcGMP, TPA, 4a-PDD, cholera toxin, and androstenedione from Sigma. NADPH was purchased from Schleicher & Schuell. GeneScreen was obtained from DuPont New England Nuclear. McCoy's 5A medium, penicillin-streptomycin, and glutamine were obtained from Gibco. The rat apoE cDNA clone pALE124 was a gift from Dr. John Taylor (Gladdstone Foundation). The chicken β-actin cDNA clone was a gift from Dr. Elaine Fuchs (Department of Biochemistry and Molecular Biology, University of Chicago). Glutaraldehyde-fixed/heat-inactivated, protein A bearing Staphylococcus aureus (Cowan 1 strain) cells were prepared by the method of Kessler (26). The rabbit anti-rat apoE antiserum was prepared in New Zealand White rabbits by the method of Hay and Getz (27).

**Granulosa Cell Cultures—**Rat ovarian granulosa cells were isolated and cultured as described previously (28). Briefly, hypophysectomized immature female (Sprague-Dawley; 21 days old) rats were supplied by Johnson Laboratories (Bridgeville, IL). A subcutaneous Silastic capsule filled with diethylstilbestrol was placed in the rats at the time of hypophysectomy. They were maintained on physiologic saline and rat chow ad libitum and were killed by cervical dislocation 4-5 days later. Granulosa cells were isolated from the ovaries of these animals by puncturing the preantral follicles with an iris knife. Cells were washed and plated in Corning flasks (25 cm²) at 1 x 10⁶ cells/ml in serum-free McCoy's 5A medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10⁻² M androstenedione. Cells were cultured for various lengths of time (as indicated in the figure legends) with or without one of the following additives: forskolin, BtzcAMP, cholera toxin, BtzcGMP, TPA, or 4a-PDD. Four, eight, or sixteen hours prior to the harvest time the conditioned medium from primary cultures of rat granulosa cells or from mouse L cells was given to the cells after the wash because fresh medium was unable to maintain apoE production or steroidogenesis. Both types of conditioned medium were found to support apoE production and steroidogenesis to the level of nonwashed cells. The conditioned medium was prepared by incubating confluent flasks of mouse L cells with serum-free McCoy's 5A medium for 12-24 h immediately before use. The mouse L cells were examined to confirm that no apoE was produced under these culture conditions prior to placing the medium on the granulosa cells. The granulosa cells were then cultured to 32 h in the case of CT-treated cells or to 72 h in the case of TPA-treated cells.

**Immunoprecipitation—**After the labeling period, the medium was removed from the [³⁵S]methionine-labeled granulosa cells and centrifuged at 1000 x g for 10 min. We have shown previously that apoE represented a small percentage (0.12%) of the total proteins secreted by granulosa cells (6), so that relative changes in apoE secretion were measured by a representative immunoprecipitation assay (29). Proteins were removed by heating 50 µCi of [³⁵S]methionine-labeled medium with an antibody complex. The amount of rabbit anti-rat apoE antisera required was determined by immunoprecipitation of equal amounts of BtzcAMP-stimulated, [³⁵S]methionine-labeled medium with increasing amounts of antisera. The amount of antisera used for each immunoprecipitation assay is in excess of the minimum required to precipitate all the apoE in the aliquote of labeled medium employed. A second immunoprecipitation of the supernatant after the first immunoprecipitation yielded no further detectable apoE. All immunoprecipitations yielded a single band of appropriate molecular weight. It has been shown previously that a partial proteolysis map of this labeled band is identical to that of authentic apoE (6). Aliquots of medium containing equal amounts of trichloroacetic acid precipitable radioactivity (equal to or less than that used in the titration) were adjusted to 2% SDS and then boiled for 3 min. The sample was diluted 20-fold with 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100, pH 7.4 (TNET), so that the final concentration of SDS was less than 0.2%. Sodium dodecyl sulfate was added to the samples to pre-clear the media. The diluted sample was rotated at room temperature for 60 min and then centrifuged. The supernatant was then incubated overnight at room temperature with the rabbit anti-rat apoE antisera. The samples were then rotated with S. aureus for 60 min, centrifuged, and washed with TNET. The bound proteins were eluted by boiling for 2 min in 0.5 M NaOH for 30 min. The supernatant was analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Radiolabeled medium from rat hepatoma cells (FuSAH) (30) was simultaneously immunoprecipitated as a positive control. The relative amount of radioactivity incorporated into apoE in each sample was quantitated spectrophotometrically as described below.

**Fluorography—**Gels were impregnated with 1 M sodium salicylate (31) and exposed to Kodak X-Omat AR or AS film. The amount of radiolaabeled apoE in each sample was determined according to the method of Suissa (32). The bands of the autoradiogram itself were excised from the film and incubated in 1 M NaOH for 2-12 h to elute the silver grains from the film. The absorbance of the eluted grains was determined spectrophotometrically at 500 nm (32, 33). Film exposures of varying duration were quantitated to ensure linearity of film response to the apoE signal. Each experiment was performed with a single batch of primate cells. All experiments are the antecedent to the appropriate nonstimulated control from that experiment (i.e. from the same pool of cells) with reproducible levels of stimulation by each agent employed.

**Radioimmunoassay—**The amount of DHP and estrogen in the unextracted tissue culture medium was determined by previously described radioimmunoassay (25, 34). DHP is the primary steroid synthesized by these cells.

**Preparation and Analysis of RNA—**Total cellular RNA was isolated from the granulosa cells using the guanidinium thiocyanate/cesium chloride procedure of Chirgwin et al. (35). In each case the RNA was extracted from the same cells whose radiolabeled medium was used for immunoprecipitation. Physical integrity of RNA was assessed by agarose gel electrophoresis and subsequent Northern blot analysis. RNA was then stored as an ethanol precipitate. For Northern blot analysis, the RNA was denatured in 10% glyoxal, electrophoresed through a 1% agarose gel, and electrotransferred to GeneScreen. For slot blot hybridizations, aliquots of total RNA (0.3, 0.6, 0.9, 1.2, 1.5, and 1.8 µg) were individually denatured in 4.6 M formaldehyde, 1.1 M NaCl, and 0.11 M sodium citrate at 65°C for 15 min. The samples were diluted into 0.2 volume of 1.5 M NaCl containing 0.15 M sodium citrate and individually applied to nitrocellulose membranes using a slot blot template manifold (Schleicher & Schuell). Both slot blots and Northern blots were hybridized with cDNA probes, as described (36).

The autoradiograms of the slot blots were quantified by the method of Suisa (32). The bands were excised from the film and incubated in 1 M NaOH for 2 h to elute the silver grains from the film. The absorbance of the eluted grains was determined spectrophotometrically at 500 nm (32). The results were plotted and analyzed according to the method of Hay et al. (33) to ensure that the results fell within the linear and proportional range. Additionally, film exposures of
Cells were incubated for 48 h with increasing doses of forskolin from $10^{-3}$ to $10^{-4}$ M (Fig. 1). The lowest dose of forskolin that showed an effect on apoE secretion or DHP production was $10^{-5}$ M. This concentration of forskolin raised DHP slightly above base line and increased the level of secreted apoE 3-fold above nonstimulated cells. The addition of $10^{-4}$ M forskolin increased DHP production 14-fold and increased secreted apoE 7-fold above that of nonstimulated cells. Since androsterone is added to the culture medium, we were also able to measure a stepwise increase in estrogen production following forskolin stimulation ($10^{-6}$ to $10^{-4}$ M) (estrogen production is not measurable in the absence of exogenous androgen substrate). Cholera toxin (1.25 µg/ml), which was added as a positive control, caused the expected 8-fold increase in apoE secretion, as well as stimulating both DHP and estrogen synthesis. The data presented in Fig. 1 indicate that the direct activation of adenylate cyclase by forskolin results in increased granulosa cell apoE secretion and suggests a role for the cAMP-dependent protein kinase A in the control of apoE production.

**RESULTS**

**Regulation of ApoE Synthesis and Steroidogenesis by Forskolin**—We have shown previously that agents which increase the concentration of granulosa cell CAMP (FSH, FSH and isobutylmethylxanthine, and BtζcAMP) also stimulate apoE secretion in a dose- and time-dependent fashion (6). To further determine the role of adenylate cyclase in the regulation of apoE secretion, we examined the effect of forskolin, a direct activator of adenylate cyclase (37), on apoE synthesis and steroidogenesis. Freshly isolated granulosa cells were incubated for 48 h with increasing doses of forskolin, from $10^{-8}$ to $10^{-4}$ M (Fig. 1). The lowest dose of forskolin that showed an effect on apoE secretion or DHP production was $10^{-5}$ M. This concentration of forskolin raised DHP slightly above base line and increased the level of secreted apoE 3-fold above nonstimulated cells. The addition of $10^{-4}$ M forskolin increased DHP production 14-fold and increased secreted apoE 7-fold above that of nonstimulated cells. Since androsterone is added to the culture medium, we were also able to measure a stepwise increase in estrogen production following forskolin stimulation ($10^{-6}$ to $10^{-4}$ M) (estrogen production is not measurable in the absence of exogenous androgen substrate). Cholera toxin (1.25 µg/ml), which was added as a positive control, caused the expected 8-fold increase in apoE secretion, as well as stimulating both DHP and estrogen synthesis. The data presented in Fig. 1 indicate that the direct activation of adenylate cyclase by forskolin results in increased granulosa cell apoE secretion and suggests a role for the cAMP-dependent protein kinase A in the control of apoE production.

**ApoE Synthesis Is Specifically Responsive to Stimulation of Kinases A and C**—Freshly isolated granulosa cells are responsive to stimulators of kinases A, G, and C in specific and different ways (9, 38). We examined the effectors of these three kinase classes on apoE synthesis and secretion. Freshly isolated granulosa cells were treated with one of the following: medium alone (nonstimulated), BtζcAMP (5 mg/ml), BtζcGMP (20 mg/ml), or cholera toxin (1.25 µg/ml) for 32 h, or with TPA (100 ng/ml) or 4α-PDD (100 ng/ml) for 72 h. These time points were chosen based upon data which are presented below. The results are shown in Fig. 2.

**BtζcAMP stimulated granulosa cell secretion of apoE 6-fold above that by nonstimulated cells at 32 h, while cholera toxin stimulated it 7.4-fold.** The base-line secretion of apoE by nonstimulated cells is very low until 72 h in culture when it is 3-fold above the earlier time points. The secreted apoE from TPA-stimulated cells is 11.2-fold above that of the 72-h nonstimulated cells (Fig. 2, top panel). BtζcGMP and 4α-PDD have no effect on apoE secretion. BtζcAMP, cholera toxin, and TPA also increased DHP synthesis, while BtζcGMP and 4α-PDD were without effect (Fig. 2, bottom panel). Cholera toxin rather than BtζcAMP was employed in all further studies aimed at increasing cAMP, because the response to the former stimulant was more reproducible.

These results suggest that both kinases A and C, but not kinase G, are involved in the regulation of apoE secretion in rat ovarian granulosa cells. The BtζcGMP (20 mg/ml) was without effect on DHP, secreted apoE and apoE mRNA at 0, 8, 16, 24, 32, 48, 72, and 96 h. BtζcGMP was also used at 5, 10, or 20 mg/ml at 32 h without effect. The lack of stimulation by BtζcGMP also indicates that the BtζcAMP effect cannot be ascribed to a nonspecific cyclic nucleotide or dibutyryl effect. Similarly, the stimulation of apoE synthesis by TPA, but not by 4α-PDD, suggests that its stimulatory effect is mediated by an influence on kinase C rather than being a nonspecific phorbol ester action on the granulosa cell apoE production. Although the effectors of kinase A and kinase C profoundly influence apoE synthesis and secretion, they do not have global effects on protein synthesis in ovarian granulosa cells. Incorporation of radioactive protein into protein increases by no more than 10-15% with either BtζcAMP, Ct, BtζcGMP, or TPA. This argues for a specific stimulation of a small subset of proteins which include apoE.

**Presence of ApoE Messenger RNA in Granulosa Cells**—We set out to establish whether changes in apoE secretion were correlated with changes in apoE mRNA. Freshly isolated granulosa cells were cultured either for 32 h with or without CT (1.25 µg/ml) or for 72 h with or without TPA (100 ng/ml). Total cellular RNA was examined by Northern blot analysis (data not shown). The same sized species of apoE mRNA was present in nonstimulated, CT, or TPA-treated cells, and it is the same size as that found in RNA extracted from livers of sham hypophysectomized female rats of the same age and species. Primer extension analysis also showed no difference between these RNA samples (data not shown).

To examine further the mechanism of the effect of the various kinase stimulators on apoE production, we compared cellular apoE mRNA levels by slot blot hybridization analysis following stimulation by the agents previously shown to promote DHP synthesis and apoE secretion (Fig. 2). Each RNA sample yielded a linear plot of signal with the amount of RNA loaded (Fig. 3). The relative changes in apoE mRNA in treated
follows. Nonstimulated cells cultured for the same time, while TPA caused a 9.1-fold increase in apoE mRNA in the presence of TPA was 5.2-fold higher in cells as compared to nonstimulated cells are shown in Fig. 2 (middle panel). ApoE mRNA levels were 2-fold higher in cells stimulated with Bt2cAMP and cholera toxin than in nonstimulated cells cultured for the same time, while TPA caused a 6-fold increment in apoE mRNA. The same RNA samples were analyzed by slot blot hybridization using a chicken β-actin cDNA probe (data not shown) to assess the specificity of apoE mRNA response. Actin is a constitutive cell protein. No changes in β-actin mRNA were noted with CT or TPA treatment at any of the times examined.

**Regulation of ApoE Synthesis and Secretion by Cholera Toxin**—We have shown previously that Bt2cAMP stimulation of apoE secretion and of steroidogenesis is concentration-dependent (6). The response to different concentrations of cholera toxin was also examined. Cells were treated with 0.125, 1.25, and 5 μg/ml cholera toxin (Fig. 4). The amount of labeled secreted apoE, apoE mRNA, and DHP increased progressively up to a concentration of 1.25 μg/ml CT. The results from cells treated with 5 μg/ml CT were highly variable. We therefore employed 1.25 μg/ml CT for further experiments.

The time dependence of cholera toxin stimulation was examined (Fig. 5). The amount of DHP accumulation increased linearly throughout the 96 h. ApoE mRNA began to increase at 16 h of culture in the presence of 1.25 μg/ml of cholera toxin. The apoE mRNA peaked at 32 h, declined 50%, and returned to baseline by 96 h. The level of secreted apoE did not begin to increase until 24 h with the peak at 48 h. CT stimulation caused a 7-8-fold increase in apoE secretion above nonstimulated cells at 48 h. Both the level of apoE mRNA and secreted apoE in nonstimulated (control) cells did not differ at 0, 8, 16, 24, 32, or 48 h; thus, the 32-h nonstimulated cells are an appropriate control. Further experiments were harvested at 32 h in order to measure secreted apoE and apoE mRNA from the same flasks.

**Regulation of ApoE Synthesis and Secretion by TPA**—The concentration dependence of TPA stimulation on ApoE secretion and cellular apoE mRNA content was also explored. Granulosa cells were cultured with increasing concentrations of TPA (0.1, 1, 10, 100, and 1000 ng/ml) for 72 h. The results are shown in Fig. 6. The amount of secreted apoE was maxi-
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Cholera Toxin pQ/ml
FIG. 4. Cholera toxin dose response. Freshly isolated granulosa cells were cultured in serum-free medium or in serum-free medium containing cholera toxin (0.125, 1.25, or 5 \( \mu \)g/ml) for 32 h. After 24 h, an aliquot of medium was removed and analyzed by radioimmunoassay for DHP, and the cells were labeled for the remaining 8 h with 50 \( \mu \)Ci/ml \([^{35}S]\)methionine. The medium apoE and apoE mRNA were analyzed as described under "Experimental Procedures" and were each normalized to the 32-h nonstimulated control which was set equal to 1. The results represent the average of two experiments between which the variation in measurements was \( \pm 10\% \), except in the case of the highest dose (5 \( \mu \)g/ml) where all three parameters varied \( \pm 40\% \). NS, nonstimulated cells.

In order to compare cholera toxin and TPA stimulation of apoE secretion and apoE mRNA content we examined the time course of the TPA stimulation. Nonstimulated (control) cells did not show a significant measurable change in the level of apoE mRNA at any of the time points but did show an increase in secreted apoE at 72 h (3-fold) and at 96 h (4-fold) as compared to any of the earlier time points. As shown in Fig. 7, apoE mRNA begins to increase after 32 h in culture in the presence of TPA and continues to increase throughout the 96-h culture period. The level of secreted apoE only begins to increase with 48 h of culture in the presence of TPA and remains similarly elevated at 72 and 96 h of culture. All TPA-induced levels of secreted apoE are compared to the amount of apoE secreted by nonstimulated control cells at the same time point (which is set equal to 1). TPA causes a slight stimulation of DHP production which slowly increases throughout the 96 h, a much lower stimulation than is observed with cAMP.

The activation of protein kinase C by phorbol ester is thought to depend upon its action as a homologue of diacylglycerol. Diacylglycerol is the physiologic activator of protein kinase C. Dioctanoylglycerol, which is the largest soluble diacylglycerol available, was tested for an effect on apoE. Freshly isolated granulosa cells were incubated in serum-free medium or serum-free medium containing one of the following: TPA (100 ng/ml) or dioctanoylglycerol (0.3, 3.0, 30, and 300 mM) for 72 h. Three millimolar (=1 \( \mu \)g/ml) dioctanoylglycerol was the only dose which stimulated all three parameters measured. It stimulated DHP production 65% as much as TPA did. Secreted apoE and apoE mRNA were both induced to a level which was 50% of the stimulation achieved with TPA. Since physiologic diacylglycerols have longer acyl chains, it is possible that the lower magnitude of the exogenous dioctanoylglycerol response is related to the nonphysiologic length of its acyl chains and possible differences in the stability of the exogenous and endogenous diacylglycerol.

Inhibitor Studies—The above results indicate that the effects of both CT and TPA on accumulation of apoE in the medium and apoE mRNA in the ovarian granulosa cells are not immediate but involve a lag of 16–24 and 32 h, respectively. Studies of this lag period in the stimulated cells will be presented sequentially. To ascertain whether newly synthesized RNA or protein intermediates are required for the cAMP stimulatory effect on apoE synthesis, CT-treated cells were exposed during the lag period to cycloheximide or actinomycin D, inhibitors of protein or RNA synthesis, respectively. Cells
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Fig. 6. TPA dose response. Freshly isolated granulosa cells were cultured in serum-free medium or in serum-free medium containing TPA (0.1, 1.0, 10, or 100 ng/ml) for 72 h. After 64 h, an aliquot of medium was removed and analyzed by radioimmunoassay for DHP, and the cells were labeled for the remaining 8 h with 50 μCi/ml [35S]methionine. The medium apoE and apoE mRNA were analyzed as described under "Experimental Procedures" and were each normalized to the 72-h nonstimulated control which was set equal to 1. The results represent the average of two experiments between which the variation in measurements was ±10% except in the case of the highest dose where all three parameters varied ≥20%. NS, nonstimulated cells.

were incubated with either inhibitor for 4 or 8 h, after which it was removed by washing and the incubation was continued to 32 h in conditioned medium containing CT. Conditioned medium was required since replacement with fresh medium, even with CT added, abrogated the CT stimulation of apoE. Preliminary experiments demonstrated that replacement of the medium with mouse L cell (fibroblast) conditioned medium was as effective as conditioned medium from ovarian granulosa cells in maintaining steroidogenesis and apoE synthesis, while fresh medium was unable to maintain steroidogenesis or apoE secretion. The granulosa cells attach to the flask slowly over the first 20 h in culture; thus, the cultures cannot be washed in the first 20 h without significant cell loss. The cells were treated with the inhibitors either from 16 to 20 h (4 h) or 12 to 20 h (8 h) in culture in order to avoid washing the cells prior to 20 h in culture. Control experiments indicated that 5 μg/ml cycloheximide reversibly inhibited protein synthesis by 90% and that as long as conditioned medium was used for replacement after washing this dose of cycloheximide could be removed from the culture with restoration of the rate of total protein synthesis (80–100%). Actinomycin D (5 μg/ml) inhibited incorporation of [3H]uridine into nucleic acid by 70% and inhibited protein synthesis by >80%. After removal of the actinomycin D protein synthesis was restored to 50–60% of the level observed in cells that had not been exposed to the antibiotic. Regardless of the history of the cells, for the experiments involving CT, apoE mRNA was measured at 32 h of culture and apoE synthesis and secretion was measured between 28 and 32 h.

As shown in Fig. 8, treatment of CT-stimulated cells with sublethal concentrations of cycloheximide (5 μg/ml) from 16 to 20 h decreased the apoE mRNA to the level present in nonstimulated cells while the secreted apoE was decreased 40%. However, treatment with the same concentration of cycloheximide from 12 to 20 h decreased the secreted apoE and the apoE mRNA (examined at 32 h) to the level of the nonstimulated cell. We also found that treatment with actinomycin D (5 μg/ml) from 12 to 20 h inhibited apoE mRNA (measured at 32 h) to the level of the nonstimulated cell. Treatment of CT-stimulated cells with actinomycin D from 16 to 20 h inhibited the induction of apoE mRNA in two experiments, but in a third experiment it caused a slight stimulation of the apoE mRNA. This variability is probably due to small differences in the developmental schedule and the timing of critical intermediate synthesis among primary cultures. The figure represents the average of two experiments in which the actinomycin D had an inhibitory effect on the apoE mRNA when exposed over the 16–20-h time interval. These results suggest that the CT-stimulated response requires protein synthesis between 12 and 16 h. Presumably one or more regulatory intermediates
maximal doses of actinomycin D (5 μg/ml) from 12 to 24, 24 to 36, or 36 to 48 h in culture and then harvested at 72 h. In this case, no secreted apoE was detected at 72 h after the 4-h [35S]methionine pulse. Similarly, the apoE mRNA was not increased by TPA after any of these incubations with actinomycin D (data not shown). This raises the possibility that the new synthesis of one or more macromolecules may be required to mediate the TPA induction of apoE expression at 72 h.

**Discussion**

Ovarian granulosa cells are steroidogenic cells, whose synthesis of steroid hormone and apolipoprotein E can be regulated hormonally. In this report, we provide data on several hormonal mediators that influence both of these products. Here previous observations are extended. In previous work it was demonstrated that FSH, probably mediated by cAMP as a second messenger, increases the amount of apoE secreted by these cells (6). BtCAMP, which binds the regulatory subunit of adenylate cyclase, increases apoE secretion even more than FSH does (6). Cholera toxin, which stimulates adenylate cyclase by preventing the inactivation of the G protein (39), increases apoE secretion almost as much as the BtCAMP (6). We now show that forskolin, which is only known direct activator of adenylate cyclase (37), stimulates apoE secretion in a dose-dependent fashion. This confirms our conclusion that manipulation of the intracellular cAMP concentration probably activates protein kinase A can regulate the expression of apoE secretion in rat ovarian granulosa cells.

Since ovarian granulosa cells also contain functional kinases G and C, we treated the cells with activators of these kinases to see if they would exhibit similar or contrasting effects on apoE synthesis and secretion. Our results demonstrate that effectors of kinases A and C but not of kinase G increase apoE synthesis and secretion.

To examine the mechanism of the regulation of granulosa cell apoE production, the levels of apoE mRNA were measured in stimulated cells. The changes in secreted apoE are reflected by qualitatively similar changes in apoE mRNA levels with elevations in apoE mRNA invariably preceding changes in apoE synthesis and secretion in each case (Figs. 5 and 7). These data suggest that, in these cases, transcriptional control may be involved in regulating the apoE synthesis and secretion.

The quantitative changes in the amount of apoE mRNA in the cells in comparison with apoE production and accumulation in the medium over a 4- or 8-h [35S]methionine labeling pulse are not totally concordant. In both the cAMP system and in the phorbol ester system, the apoE accumulation changes more with stimulation than does the cell content of apoE mRNA. ApoE secretion probably reflects apoE synthesis because there is no change in the relative proportion of intracellular apoE. CT leads to a maximal 2-3-fold increment in apoE mRNA but a 7-10-fold increment in apoE accumulation. The quantitative difference is not as great for TPA stimulation. The apoE mRNA increases 5-fold and the secreted apoE increases 8-12-fold. Furthermore, careful examination of the CT (Fig. 5) and TPA (Fig. 7) time dependence data, especially at the later time points, indicates that incremental or decremental changes in apoE mRNA are not invariably accompanied by concordant changes in apoE accumulation in the medium. These quantitative discrepancies could be accounted for by more than one effect of kinase A or C activation. Thus, in addition to probable transcriptional activation of the apoE gene, kinase A activation and to a lesser
extensive kinase C activation, may also influence the translational efficiency of apoE mRNA, or the stability of apoE, or the re-uptake of secreted apoE. With the information presented in this report it is not possible to distinguish among these possibilities.

Both CT (increased cAMP) and TPA induce apoE secretion several fold but with a significant lag of 16 and 32 h, respectively. The influence of inhibitors of protein and RNA synthesis during these lag periods suggests that new macromolecular synthesis, in addition to the synthesis of apoE mRNA and apoE, is required to mediate the stimulatory effects of CT and TPA. The cycloheximide sensitivity of the induction of apoE mRNA by CT or TPA indicates that these effectors cannot be operating directly on the apoE gene, or on pre-existing transcriptional activators (40). That these effects are demonstrable before any elevation of either apoE mRNA or apoE protein synthesis and accumulation is evident, argues for the synthesis of mediators rather than for direct effects on the synthesis of apoE mRNA and apoE itself. The nature of these potential mediators is not presently known although the likelihood is that they are transcriptional activators. Both CT, via cAMP elevation, and TPA are capable of activating phosphokinases A and C, respectively. The lag period and the influence of inhibitors of macromolecular synthesis might suggest that the substrate(s) for these phosphokinases may not be present in optimal amounts in nonstimulated ovarian granulosa cells. Their synthesis might be required for the increased production of apoE. Not one but a cascade of phosphoproteins might be involved in this process. This model, that the induction of apoE requires the synthesis of an intermediate protein, is similar to the model proposed by Simpson et al. (41) for the regulation of other cAMP-induced proteins which also have a lag period that is cycloheximide-sensitive.

The detailed effects of CT and TPA suggest that, although their mechanisms of action may share general features, they promote distinct activation cascades. The time course of the effects of CT and TPA on apoE production are quite distinct. Also the effect of optimal concentrations of TPA is greater than that of optimal concentrations of cAMP effectors. This phenomenon, the later peak of the TPA effect and the minimal effect on DHP all suggest that TAMP is unlikely to be the mediating effector for TPA. Also the fact that exogenous diocanoylglycerol stimulates apoE production, albeit less effectively than TPA, argues that the phorbol ester is working by activation of protein kinase C. However, although the CT and TPA effects probably involve distinct mediators, preliminary evidence suggests that these two activation cascades do interact with one another. Thus, the presence of a phosphodiesterase inhibitor, isobutylmethylxanthine, which has no direct effect itself on apoE production, shifts to an earlier time the influence of TPA on ovarian granulosa cell apoE synthesis. Preliminary results have shown that in the presence of TPA and isobutylmethylxanthine elevated apoE mRNA and apoE secretion is demonstrable at 32 h, while in the presence of TPA alone these changes are not yet detectable at that time point.

Both kinases A and C have been implicated in the regulation of gene expression in other systems, so transcriptional activation of the apoE gene via the influence of these phosphokinases is surely feasible. The transcription of the rat phosphoenolpyruvate carboxykinase (42), human prolactin (43), human proenkephalin (44), rat somatomstatin (45), and human chorionic gonadotropin alpha-subunit (46, 47) genes have been demonstrated to be regulated by cAMP. The 5′-upstream region of all five of these genes contains closely related 15-base pair sequences which have been postulated as a cAMP regulatory region. The core motif of this region (the consensus sequence), TGACTCA, has been shown to bind a 43-kDa phosphoprotein (48) that has been termed the cAMP regulatory protein. While the upstream region of the rat apoE gene does not contain a sequence that is identical to this cAMP regulatory region, there are sequences with approximately 75% homology (6 of 8 nucleotides identical) to the cAMP regulatory regions just described (49). There are two other regions of 87% homology (7 of 8 nucleotides identical) to this cAMP regulatory region in the rat apoE gene; one is at the end of the third intron and the other is in the first intron on the noncoding strand.

The cAMP regulatory protein differs from another protein, AP-1, which binds to a similar motif, TGACTCA, both in size and in the fact that AP-1 responds to phorbol ester but not cAMP (50, 51). The consensus sequence for AP-1 binding is not present in the upstream region of the rat apoE gene, but it is found in the first intron. We are currently investigating that region and other highly homologous sequences which are found in the gene.

Two other transcriptional activator proteins that respond to phorbol ester, AP-2 and AP-3, have been described recently (52–54). AP-2 is a cell type-specific transcriptional activator which binds to control elements of a large number of genes, including human growth hormone, c-myc, and SV40. AP-2 is responsive to cAMP elevation in addition to responding to phorbol ester induction. The consensus sequence to which this protein binds is found in the upstream region of the rat apoE gene at position +98 and is a potential activating sequence which may help explain the results presented here. We are currently investigating whether it is utilized in the apoE gene of rat ovarian granulosa cells.

AP-3 is also a cell type-specific transcriptional activator which responds to phorbol ester but not to elevated cAMP. It has been studied primarily using the SV40 enhancer as a model and demonstrates a cycloheximide-resistant activity in HeLa TK- cells (54). The DNA binding site of AP-3 also has homologous regions in the rat apoE gene.

From the studies reported in this paper and previously (55, 56), it is clear that the expression of the apoE gene is subject to multiple controls, involving hormones and nutrients, which may operate in different regions, in a variety of cell systems and contexts. It remains to be established precisely how each of these mechanisms function and how they interact in a given physiologic milieu.

The physiologic role of apoE in the ovary is not clear. Since the role of apoE may vary between species we must be cautious about combining facts from different species to generate a unified hypothesis. Nevertheless, there are distinct physiologic mechanisms of regulation, as shown here, which may interact or act independently. This issue is complicated in the ovary because it is an organ whose constituent cells exhibit constantly changing functional abilities. These changes occur in response to outside regulators such as gonadotropins and to a whole host of internal regulatory factors. With these limitations in mind, several likely roles for apoE in the ovary may be suggested on the basis of the available data.

One possible role is the modulation of cholesterol uptake by ovarian cells. This may be particularly important in the ovarian follicle since granulosa cells, follicular fluid, and the oocyte are separated from the blood vessels by the follicle basement membrane. A second possible role is as a carrier for other lipids, such as phospholipid, within the ovary. A third possible role could be as an autocrine or paracrine regulator within the ovary. These effects could be independent of lipid
transport and perhaps even of the B/E receptor. An example of this could be the recently described inhibitory effect of apoE bearing lipoproteins on the induction by luteinizing hormone of the enzymes necessary for androgen production in rat theca cells (8). There are other examples whereby apoE may alter cell function independent of its binding to the low density lipoprotein receptor. For example, apoE inhibits mitogen-stimulated lymphocyte proliferation even in the absence of functional B/E receptors (57, 58).

In summary, apoE production is regulated by increased intracellular cAMP and by activation of protein kinase C. The physiologic role of apoE in the ovary may include cholesterol (and other lipid) transport and autocrine and/or paracrine effects that are independent of the B/E receptor. Further studies are needed to analyze these possibilities.

Acknowledgment—We would like to thank Edward Rafalski for his technical assistance.

REFERENCES