Adenovirus-Directed Expression of a Nonphosphorylatable Mutant of CREB (cAMP Response Element-Binding Protein) Adversely Affects the Survival, but Not the Differentiation, of Rat Granulosa Cells

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Although usually considered to be a constitutively expressed protein, in the primate ovary the expression of CREB (cAMP response element-binding protein) is extinguished after ovulation, and its loss is temporally associated with the cessation of proliferation of luteal cells and the ultimate commitment of the corpus luteum to undergo regression. To determine the cellular consequences of the loss of CREB expression, we expressed a nonphosphorylatable mutant of CREB (CREB M1) in primary cultures of rat granulosa cells using a replication-defective adenovirus vector. Expression of CREB M1 did not block granulosa cell differentiation as assessed by acquisition of the ability to produce estrogen and progesterone in response to FSH or forskolin. However, granulosa cells expressing CREB M1, but not adenovirus-directed β-galactosidase or enhanced green fluorescent protein, exhibited a 35% reduction in viability that was further reduced to 65% after stimulation with 10 μM forskolin. These results demonstrate that the trophic effects of cAMP (proliferation and survival) on ovarian granulosa cells are functionally separate from the effects of cAMP on differentiation and provide novel evidence that CREB may function as a cell survival factor in the ovary. The separation of signaling pathways that govern differentiation and survival in the ovary thereby provides a mechanism by which progesterone production, which is absolutely essential for the maintenance of pregnancy, can continue despite the cessation of proliferation of luteal cells and their commitment to cell death (luteolysis). (Molecular Endocrinology 13: 1364–1372, 1999)

INTRODUCTION

The ovarian cycle involves progressive stages of cell proliferation, differentiation, and cell death (1). As the ovarian follicle matures under the control of FSH, granulosa cells proliferate and differentiate into steroid-producing cells to form a mature Graafian follicle (2). Upon ovulation, the Graafian follicle is transformed into a corpus luteum whose production of progesterone under the influence of LH is absolutely required for the establishment and maintenance of pregnancy (3). An enigma in the understanding of ovarian function is although both FSH and LH, at least in part, exert their actions through the cAMP intracellular signaling system (4), the responses of granulosa cells and luteal cells to their respective trophic hormones differ. In particular, the actions of FSH on the follicle include both proliferation and stimulation of steroid production. However, after ovulation, luteal cells no longer proliferate and become committed to cell death but progesterone production by these cells remains highly responsive to LH (5, 6).

We demonstrated previously that the expression of the cAMP-dependent transcription factor CREB (cAMP response element-binding protein) is extinguished upon the luteinization of granulosa cells in the
monkey ovary and proposed that the loss of CREB signaling might subtract the expression of a subset of genes involved in the control of cellular proliferation and survival (7). We reasoned that the consequences of the loss of CREB in luteal cells could be determined by eliminating the CREB-signaling pathway in progenitor granulosa cells. To accomplish this, we used a recombinant adenovirus vector that directs the expression of a mutant of CREB (CREB M1) in which the activating phosphorylation site at serine 133 is mutated to alanine (8). Herein we show that while interfering with CREB signaling in granulosa cells does not inhibit their differentiation into steroid-producing cells, expression of CREB-M1 adversely affects their viability. These findings suggest that CREB may function as a molecular switch that governs cell survival in the ovary and support the rapidly emerging notion that CREB may function as a survival factor in a number of different cell types.

RESULTS
Recombinant Adenoviruses Direct \(\beta\)-Galactosidase Expression with High Efficiency in Primary Cultures of Rat Granulosa Cells

To determine the feasibility of using adenoviruses to direct the expression of recombinant proteins in granulosa cells, we employed an adenovirus vector (Ad \(\beta\)-gal) that directs the expression of a nuclear targeted \(\beta\)-galactosidase reporter gene under the control of a cytomegalovirus (CMV) promoter. Surprisingly, as shown in Fig. 1, Ad \(\beta\)-gal-directed \(\beta\)-galactosidase activity in primary cultures of immature rat granulosa cells was hormone responsive. In the absence of hormonal stimulation (panel A), \(\beta\)-galactosidase staining was weak. In contrast, incubation of granulosa cells with either 10 ng/ml human (h)FSH (panel B) or 10 \(\mu\)M forskolin (FSK) (panel D) resulted in a more intense staining of individual cells. The expression of \(\beta\)-galactosidase was ligand-dependent as immature granulosa cells that lack LH receptors did not respond to the addition of 10 ng/ml hLH (panel C).

Fig. 1. Effect of FSH, LH, and FSK on \(\beta\)-Galactosidase Expression in Primary Cultures of Rat Granulosa Cells Infected with Ad \(\beta\)-gal
Granulosa cells were cultured and exposed to Ad \(\beta\)-gal at a 1:100 dilution. Twenty-four hours after viral infection, the culture medium was removed and cells were exposed to medium alone (A), 10 ng/ml hFSH (B), 10 ng/ml hLH (C), or 10 \(\mu\)M FSK (D). After 48 h of exposure to stimulatory agents, cell cultures were stained for \(\beta\)-gal activity for 30 min.

Functional Characterization of Ad CREB M1

Figure 2 (top panel) illustrates a Western immunoblot using an antibody directed against the amino terminus of CREB on whole-cell lysates of granulosa cells that were infected with increasing amounts of the Ad CREB M1 adenovirus. Exposure of granulosa cells to increasing viral titers resulted in progressive increases in immunoreactive CREB expression as compared with noninfected cells. When cell lysates from noninfected granulosa cells were subjected to immunoprecipitation with an anti-CREB antibody and the immunoprecipitates exposed to protein kinase A (PKA) and \([\text{\textsuperscript{32}}P]\)-ATP \textit{in vitro}, intense phosphorylation of a 43-kDa protein was observed (Fig. 2, bottom panel). In contrast, immunoprecipitates of granulosa cells from Ad CREB M1-infected cells were weakly phosphorylated by PKA \textit{in vitro} as would be expected by the elimination of the PKA-dependent phosphorylation site on ser 133.

Ad CREB M1 Inhibits the Expression of CMV \(\beta\)-Galactosidase

To determine whether CMV-\(\beta\)-gal stimulation is CREB dependent, primary cultures of rat granulosa cells were infected with increasing amounts of Ad CREB M1 in the presence of a fixed amount of Ad \(\beta\)-gal (1:1000). Figure 3 shows that Ad CREB M1 inhibited FSH-stimulated CMV-\(\beta\)-galactosidase activity in a dose-dependent fashion, with the highest Ad CREB M1 concentration (1:100 dilution) completely inhibiting \(\beta\)-galactosidase expression. This concentration of Ad-CREB also inhibited \(\beta\)-galactosidase expression in response to 10 \(\mu\)M FSK and 0.5 mm 8-Br-cAMP (data not shown).
Effect of Ad CREB M1 on Granulosa Cell Steroidogenesis

To determine the effects of the CREB M1 mutant on granulosa cell function and the specificity of the Ad CREB M1 vector, we compared the steroidogenic responses and CMV-β-galactosidase expression of primary cultures of rat granulosa cells not infected by adenoviruses with those infected with Ad CREB M1 as well as those exposed to identical concentrations of an adenovirus that directs the expression of enhanced green fluorescent protein under the control of the CMV promoter (Ad EGFP). Consistent with previous results, both FSH and FSK stimulated β-galactosidase activity in noninfected cells and cells infected with the control Ad EGFP adenovirus. In contrast, β-galactosidase expression was completely abrogated in cells infected with the Ad CREB M1 virus (Fig. 4A). The observation that β-galactosidase activity was somewhat lower in Ad EGFP-infected cells in comparison to noninfected cells may be due to competition of the CMV promoters for endogenous transcriptional regulators, as both basal and FSK-stimulated β-galactosidase activities were reduced proportionately. The relative increases in β-galactosidase activity in response to FSK were comparable in cells not exposed to virus and those exposed to Ad EGFP (18-fold vs. 13-fold, respectively).

Figure 4B illustrates that estrogen production after 48 h exposure to FSH or FSK was comparable by cells not exposed to adenovirus and cells exposed to Ad EGFP. The responses to FSK were statistically significant (P < 0.05, n = 4 independent observations) when compared with untreated cells. Although cells exposed to Ad CREB M1 produced lower amounts of estrogen when compared with noninfected cells or Ad EGFP-infected cells, they retained their responsiveness to FSH and FSK (P < 0.05). For all groups, there was a net increase in estrogen concentration between samples collected at 24 and 48 h after exposure to FSH or FSK (data not shown). Identical results were observed with progesterone production (Fig. 4C).

Effect of Ad CREB M1 on Granulosa Cell Survival

Figure 5 illustrates the morphology of the granulosa cells from the study shown in Fig. 4. The top panel presents fluorescent microscopic analysis in the granulosa cells infected with Ad EGFP. Like that of the CMV-β-gal, the expression of the CMV promoter-directed EGFP was FSH and FSK responsive. The center panel illustrates the morphology of Ad EGFP-infected granulosa cells. Well defined monolayers were present in cells not exposed to stimuli as well as cells
exposed to FSH or FSK. The lower panel illustrates the morphology of granulosa cells exposed to Ad CREB M1. In the absence of hormone stimulation or in the presence of FSH, granulosa cells were present in well-defined monolayers. In marked contrast, Ad CREB M1-infected granulosa cells that were exposed to 10 μM FSK displayed pronounced morphological alterations that included profound cell clumping, which was associated with the detachment of the majority of the cells from the culture dish.

To determine whether the morphological changes observed in Ad CREB M1-infected cells were associated with a reduction in cell viability, granulosa cells were exposed to either medium alone, Ad EGFP, or Ad CREB M1 followed by incubation in the presence and absence of 10 μM FSK; cells were then assessed for viability at the indicated times after the addition of FSK as shown in Fig. 6. There were no significant differences in the viability of cells (P > 0.05) exposed to Ad EGFP compared with cells not exposed to virus either in the presence or absence of FSK. Although not apparent by visual examination, granulosa cells infected with Ad CREB M1 and not exposed to FSK exhibited a significant (P < 0.01) decline in viability at 48 and 72 h when compared with cells not infected with virus or cells infected with Ad EGFP. At both 48 h and 72 h, incubation of Ad CREB M1-infected cells with 10 μM FSK resulted in a further decrease in viability when compared with Ad CREB M1-infected cells not exposed to FSK (P < 0.01). The reduced production of estrogen and progesterone in Ad CREB M1-infected granulosa cells in response to FSK (Fig. 4, B and C) is thus likely due to a diminished number of viable cells in the culture.

Although expression of CREB M1 adversely affected survival of granulosa cells in primary culture, a comparable effect was not observed in established ovarian cancer cell lines. No alteration of cell morphology in response to 10 μM FSK was observed in Ad CREB M1-infected human ovarian cancer cell lines SKOV-3 or OV 1063. MTT viability assay (–FSK vs. +FSK) were 0.968 vs. 1.008 for SKOV-3 cells and 1.650 vs. 1.699 A490 units for OV 1063 cells (means of duplicate observations). In addition, no cytopathic effects in response to 10 μM FSK were evident in Ad CREB M1-infected primary cultures of rat Sertoli cells under conditions in which Ad CREB M1 completely blocked the FSK stimulation of a c-fos-CRE reporter gene (9).

**DISCUSSION**

Our results demonstrate that expression of a CREB M1 in ovarian granulosa cells adversely affects their survival but does not abolish their ability to differentiate into steroid-producing cells in response to agents that stimulate cAMP production. The separation of signaling pathways that govern differentiation (steroi-
Dogenesis) and survival in the ovary thereby provides a mechanism by which progesterone production, which is absolutely essential for the maintenance of pregnancy and the survival of the species, can continue despite the cessation of proliferation of luteal cells and their commitment to cell death (luteolysis). The observations that CREB may participate in a proliferation/survival pathway in the ovary is consistent with findings from a number of other cell systems. Expression of CREB M1 in transgenic animals results in proliferative defects of pituitary somatotrophs, T lymphocytes, and cardiac myocytes (8, 10, 11), and transfection of FRTL-5 thyroid cells with a different CREB mutant reduced thymidine incorporation in response to TSH (12). In addition, human somatotroph adenomas resulting from activating mutations of \( \Gamma \)s are associated with elevated levels of phosphorylated CREB (13).

CREB was first identified as a nuclear protein that is phosphorylated by PKA and binds to the cAMP response element (CRE) on the somatostatin gene promoter and stimulates transcription (14). Since its identification as a PKA-dependent transcriptional activator, it has become apparent that CREB is a nuclear target of a number of intracellular signaling pathways including mitogen-activated protein kinase (15), p90RSK (16), and calmodulin kinases (17, 18) as well as the antiapoptotic Akt/PKB pathway (19). Given the wide diversity of inputs that may converge on CREB, it is not unreasonable to suggest that CREB may be involved in basic processes of cellular homeostasis including proliferation and cell death. Our current finding that expression of CREB M1 results in diminished viability of granulosa cells is consistent with other recent studies indicating that CREB may function as a survival factor. Thus, in addition to causing cell cycle

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**Fig. 5. Morphology of Granulosa Cell Monolayers**

Before removal of culture medium and collecting granulosa cells for assessment of \( \beta \)-galactosidase activity, cell cultures from the study presented in Fig. 3 were photographed. The top panel shows granulosa cells exposed to Ad EGFP and photographed with fluorescence microscopy (×200). The center and lower panels show bright field photographs of granulosa cells exposed to Ad EGFP and Ad CREB M1, respectively (×100). Identical results were observed in four independent experiments.

**Fig. 6. Effect of Ad CREB M1 and Ad EGFP on Granulosa Cell Viability**

Granulosa cells were cultured and exposed to either no virus, Ad EGFP, or Ad CREB M1 at 1:100 dilutions. Twenty-four hours after virus infection, cells were exposed to M199 alone or M199 containing 10 \( \mu \)M FSK. Cell cultures were assessed for viability by the CellTiter assay at 48 and 72 h after the addition of FSK. Results show means ± 1 SEM of four independent experiments.
arrest in T cells, the expression of a dominant-negative mutant of CREB in transgenic animals also resulted in apoptosis of these cells in response to agents that would normally stimulate activation (10). Likewise, expression of a dominant-negative mutant of CREB in human melanoma cells led to reduced tumorigenesis as well as an enhanced sensitivity of these cells to apoptosis (20). Whether the diminished viability of granulosa cells as assessed by the MTT assay seen in the current study is the result of apoptotic cell death is not known and will be the subject of future research.

During the course of our studies, we observed that the expression of β-galactosidase under the control of a CMV promoter was highly responsive to FSH, FSK, and cAMP stimulation. In retrospect, the responsiveness of the CMV promoter to cAMP is not surprising as it has been shown by others that the CMV promoter contains three cAMP response elements (CREs) and is stimulated by FSK (21). Using the hormonal dependence of CMV-β-galactosidase as a reporter for documenting the effectiveness of CREB M1, we demonstrated that FSH- and FSK-stimulated β-galactosidase expression was completely blocked by overexpression of the mutant CREB. Assuming that there are no differences between the ability of CREB M1 to inhibit the transcription of the extra-chromosomal CMV-β-galactosidase gene and endogenous genes, we would conclude that CREB-mediated expression of endogenous genes was dramatically compromised in these cells. Despite the apparent inhibition of CREB-dependent transcription, estradiol and progesterone production by CREB M1-expressing granulosa cells remained highly responsive to FSH and FSK. Although we have not measured mRNA levels for steroidogenic enzymes in the present study, it is well recognized that the acquisition of the ability of granulosa cells from immature rats to produce estrogen and progesterone is due to cAMP-mediated increases in mRNAs for aromatase (P450arom), cholesterol side chain cleavage (P450sccc), and 3β-hydroxysteroid dehydrogenase, 5–4 isomerase (3β-HSD) (22). We would therefore infer from our studies that those genes involved in progesterone and estrogen production are not CREB dependent. This is consistent with the findings of others that the promoter regions of P450sccc and 3β-HSD do not appear to contain CREs (23, 24) and that P450arom, which does contain a CRE-like sequence, also appears to be responsive to steroidogenic factor-1 (25). The separation of cAMP-regulated signaling pathways that control survival from those that control differentiated phenotype (steroidogenesis) in the ovary would serve to ensure that progesterone production, which is essential for the maintenance of pregnancy, continues even though trophic actions of cAMP are lost after ovulation.

A number of potential mechanisms exist by which the elimination of CREB-mediated signaling in granulosa cells may adversely affect cell proliferation and survival. CREB may act as a transcription factor that directly regulates genes involved in these processes. One candidate gene is proliferating cell nuclear antigen (PCNA), an auxiliary factor for DNA polymerase δ, which is required for both replicative and repair DNA synthesis, as both the murine and the human PCNA gene promoters contain obligatory CREs (26, 27). Indeed, in the primate ovary, the expression of both PCNA and CREB cease upon luteinization (7). A second possible candidate gene is cyclin D2, which has been shown to be FSH and FSK responsive in rat granulosa cells (28, 29). Interestingly, ovarian function in the cyclin D2 knockout mouse is similar to that seen in our current study as granulosa cell differentiation (steroidogenesis), is disrupted (28). It is not yet known, however, whether expression of the cyclin D2 gene is CREB dependent. Alternately, CREB could also act by controlling the expression of other transcription factors that regulate genes involved in proliferation and survival such as c-fos and C/EBPβ, both of which are CREB-dependent (30, 31). In this regard it is noteworthy that the C/EBPβ knockout mouse is infertile due to defects in the luteinization process (32).

In our current study, pronounced morphological disruptions and reduced granulosa cell viability were most apparent when Ad CREB M1-infected cells were stimulated by FSK. This observation is analogous to those obtained with other cells in which the expression of inactive CREB mutants resulted in diminished cell viability. Thus, Jean et al. (20) did not observe marked cytotoxic effects of a CREB mutant on melanoma cells unless the cells were treated with thapsigargin, which elevates intracellular free Ca2+ concentrations. Likewise, Barton et al. (10) did not observe pronounced cell death in T cells that express a nonphosphorylatable CREB mutant unless the cells were presented with activating stimuli. As FSK has been shown to elevate intracellular free Ca2+ in granulosa cells (33), it is possible that the elimination of CREB-mediated signaling allows other pathways that are antagonistic to cell proliferation and survival to exert dominance, possibly by increasing their ability to interact with limited amounts of nuclear coactivators (34). In this regard, previous studies by Aharoni et al. (35) demonstrated that treatment of granulosa cells from rat preovulatory follicles with very high concentrations of FSK (50 μM) promoted apoptosis but, like that seen in our study, did not inhibit steroid production. As we did not observe any cytotoxic effects of a lower concentration (10 μM) of FSK in noninfected cells or cells infected with either Ad βgal or Ad EGFP (Figs. 1D, 5C, and 6), it appears that the loss of CREB signaling rendered granulosa cells more sensitive to adverse effects of FSK, as would be expected if competition exists between CREB and other antagonistic signaling pathways.

A caveat to the interpretation of our current results is the assumption that the cytotoxic effect of CREB M1 on granulosa cells is due specifically to the competition of this mutant protein with endogenous CREB for binding to CREs within the regulatory regions of CREB-responsive genes. However, it remains a possibility that overexpression of CREB M1 in granulosa
cells may squelch the activity of other signaling pathways. For example, in addition to forming homodimers, CREB is also able to form heterodimers with other members of the bZip family of transcription factors, such as ATF-1 (36). Thus, an alternate explanation for our findings could be that the effects of CREB M1 could indirectly be mediated through sequestration of ATF 1 or other transcription factors. Although we cannot rule this out, our previous studies with the primate ovary failed to detect, by Southwestern analysis, any CRE-binding proteins other than CREB in luteal cells (7). In addition, CREB has been shown to interact in a phosphorylation-independent manner with components of the TFIID complex (37).

However, such an interaction with the general transcription factor apparatus would be expected to globally suppress transcription, which would be inconsistent with our findings that CREB M1 did not block granulosa cell differentiation. Finally, it is important to note that cytopathic effects of CREB M1 expression were not observed in either ovarian epithelial cancer cells (as presented in Results) or primary cultures of rat Sertoli cells (9). The latter is significant in view of the fact that Sertoli cells, which are the testicular homologs of granulosa cells, do not require FSH-mediated cAMP signaling for their survival in vivo (38).

In summary, our current results suggest that CREB may function as a molecular switch that governs cell proliferation and survival in the ovary, results that are in keeping with other recent observations that CREB may function as a general regulator of cell proliferation and survival (8–13, 19, 20). Extrapolation of these observations to the in vivo state suggests that the loss of CREB expression that occurs in the primate corpus luteum could be directly causal to the cessation of proliferation of luteal cells and, ultimately, the cell death that occurs during luteal regression.

The recent report that phosphorylated CREB is absent from nuclei of rat luteal cells indicates that an impairment in CREB signaling also occurs in rodents (39). Although the downstream targets of CREB that may participate in cell proliferation and survival are not known, the ability to rapidly and effectively interfere with CREB-mediated signaling in primary cell cultures with replication-defective adenovirus vectors provides a novel approach to address this important problem.

**MATERIALS AND METHODS**

**Construction of the CREB M1 Recombinant Adenovirus**

The adenovirus shuttle vector pACsk.2CMV CREB M1 was constructed by excising a 2.2-kb fragment containing the CREB M1 coding region and the SV40 polyadenylation signal from the plasmid pRSVCMB1 M1 (8) by digestion with EcoRI. The CREB M1 insert was ligated in frame into the multiple cloning site of the adenovirus shuttle plasmid pACsk.2CMV (gift of Dr. Joseph Alcorn). The methods for generating and propagating recombinant adenoviruses are described in detail elsewhere (40). In brief, 10 μg of the plasmid pACsk.2CMV CREB M1 were cotransfected with 10 μg of the plasmid pJM17, a plasmid containing a circularized adenovirus type 5 (variant dl309) genome (40) into the human embryonic kidney cell line 293 (41) using a calcium phosphate transfection system according to the manufacturer’s instructions (Life Technologies, Inc., Gaithersburg, MD). After transfection the cells were maintained in DMEM containing 4.5 g/liter glucose (Life Technologies, Inc., Gaithersburg, MD) and 10% FBS at 37°C in 5% CO2 for 13 days at which time the cells exhibited viral cytopathic effect. Cells and tissue culture supernatants were collected, frozen on dry ice and thawed three times, and then centrifuged (1000 × g, 4°C, 10 min) to remove cellular debris. Aliquots of virus stocks were diluted 50- and 100-fold in lysis solution [0.1% SDS, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA] and incubated for 10 min at 56°C in a shaking water bath. The absorbance of the samples was measured at 260 nm, and the value obtained was used to calculate virus content using the equation 1.0 absorbance units = 1.1 × 1011 virus particles/ml (42). Adenoviruses were propagated by infecting 293 cells with approximately 109 particles/ml in tissue culture medium without serum. Infected cells were incubated until they exhibited nearly complete cytopathic effect and processed as above. Virus stocks were prepared to a concentration of 6 × 1012 particles per ml as described above and diluted for use as indicated in Results.

**Granulosa Cell Culture and Adenovirus Infection**

All procedures were approved by the Magee-Womens Research Institute Institutional Animal Use and Care Committee. Immature female rats (20 or 25 days old) were purchased from Taconic Farms, Inc. (Germantown, NY) and were housed under standard husbandry conditions. For studies shown in Fig. 2, animals received a subcutaneously placed 1-cm SILASTIC capsule containing crystalline diethylstilbestrol (Sigma Chemical Co., St. Louis, MO) on day 20 of age and were killed on day 25 of age. The remainder of the studies used granulosa cells from untreated 22- to 25-day-old rats. Granulosa cells were collected from the ovaries by puncturing follicles with a 25-gauge hypodermic needle, and cells were expressed into Medium 199 (M199; Life Technologies, Inc.) containing 10% FBS. Granulosa cells were seeded into 6-well (– 105 cells per well) or 24-well (– 2 × 105 cells per well) tissue culture plates and allowed to attach overnight. The next morning, medium and unattached cells were removed and the granulosa cell monolayers were exposed to adenoviruses in M199 without protein supplements for 2 h at 37°C with occasional rocking. Medium was replaced with fresh M199 containing 1 mg/ml BSA (Sigma Chemical Co.). Twenty-four hours after exposure to adenoviruses, medium was removed and replaced with M199 plus BSA also containing 10 ng/ml testosterone (Sigma Chemical Co.) alone or with addition of human FSH (AFP 4161B; 3205 IU FSH/mg < 225 IU LH/mg) or FSK (Sigma Chemical Co.).

**Immunoblot Analysis of Lysates from AdCREB M1-Infected Primary Rat Granulosa Cell Cultures**

Granulosa cells were harvested by scraping into ice-cold PBS followed by centrifugation (16,000 × g, 4°C, 10 min). Pelleted cells were resuspended in Western lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 20 μg/ml phenylmethylsulfonylfluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml Pepstatin A, 10 mM Microcytin LR), lysed by sonication, and processed for anti-CREB immunoblotting as described previously (7) using a rabbit anti-rat CREB antibody directed against the first 205 amino acids of CREB (43) and probed with an anti-rat CREB antibody directed against amino acids 206-425 of CREB (43).
Immunoprecipitation and in Vitro PKA Phosphorylation

Ad CREB M1-infected and control granulosa cells were harvested in ice-cold RIPA buffer (150 mM NaCl, 10 mM Tris-Cl, pH 7.5, 0.1% (wt/vol) SDS, 1% (vol/vol) NP-40) supplemented with protease and phosphatase inhibitors (20 μg/ml phenylmethylsulfonylfluoride, 0.5 μg/ml leupeptin, 10 mM microcystin LR, 200 μM sodium vanadate). Immunoprecipitation of CREB was performed using the antibody directed against the amino-terminal region of CREB as described above, and in vitro phosphorylation of immunoprecipitates by PKA were performed as described previously (43).

β-Galactosidase Assays

Identification of β-galactosidase-expressing cells in granulosa cell cultures was assessed by histochemistry using X-gal as substrate (42). Granulosa cell monolayers were fixed in 2% paraformaldehyde for 60 min, washed three times in PBS, and incubated in Xgal solution containing 2 mM K₄Fe(CN)₆, 2 mM K₃Fe(CN)₆, 1 mM MgCl₂, and 1 mg/ml Xgal. Quantification of β-galactosidase activity was performed by fluorometric analysis (44). Granulosa cells were harvested by scraping into ice-cold PBS, and centrifuged at 16,000 × g, 4 °C, 10 min) to pellet the cells. The PBS was removed, and the cell pellets were frozen on dry ice and then stored at −80 °C until use. Cell pellets were thawed on ice with the addition of 100 μl β-galactosidase assay buffer (150 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 25 mM NaCl, 10 mM 2-mercaptoethanol). Identical volumes of cell extract were added to react a reaction mixture containing 150 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 25 mM NaCl, 10 mM 2-mercaptoethanol, 150 mM 4-methylumbelliferyl β-galactoside, and 1 mg/ml BSA and incubated at 30 °C for 30 min, after which reactions were stopped by the addition of 0.5 ml of 0.25M glycine (pH 10.65). Three hundred microliters of each assay were transferred to a 96-well dish, and fluorescence was measured using an excitation wavelength of 340 nm and emission wavelength of 460 nm.

Steroid Production

Estradiol and progesterone concentrations of culture medium were determined by RIAs as described previously (45).

Cell Viability Assay

Viability of granulosa cells was assessed using the CellTiter assay (Promega Corp., Madison WI) in which a tetrazolium salt is bioreduced into a formazan by metabolically active cells. Assays were performed according to the manufacturer’s direction and were terminated 4 h after the addition of substrate, at which time the production of the formazan, measured by recording the absorbance at 490 nm, was linear as a function of time.

Statistics

Results were assessed for statistical significance by ANOVA followed by comparison of group means with Duncan’s multiple range analyses (46).

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