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Follicle-stimulating Hormone Regulation of A-kinase Anchoring Proteins in Granulosa Cells*

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It has been well established that the biochemical and morphological changes during maturation of granulosa cells that are induced by follicle-stimulating hormone (FSH) occur through the elevation of intracellular cAMP and consequent activation of the cAMP-dependent protein kinase (PKA). In this report we show that FSH action alters the expression of A-Kinase Anchoring Proteins (AKAPs), which function to target the subcellular distribution of the type II PKA. Exposure of granulosa cells grown in primary culture with FSH and estradiol for 72 h resulted in the up-regulation of an 80-kDa AKAP and the RIIβ subunit of PKA, whereas cells grown in control medium containing only estradiol produced a time-dependent increase of a 140-kDa AKAP. RII overlays performed with [^{32}P]RIIα preferentially detected RII-binding bands of 80 and 95 kDa compared to blots probed with [^{32}P]RIIβ, suggesting that FSH may alter the subcellular location of PKA in an isoform-specific manner. FSH treatment causes a translocation of RIIα from the particulate to the cytosolic fraction coincident with the induction of the 80-kDa AKAP, which is also predominately cytosolic. These data suggest that FSH promotes a redistribution of the type II PKA holoenzyme through the selective induction of an RII isoform-specific AKAP.

The differentiation of granulosa cells (GC)[1] associated with the maturation of preantral to preovulatory follicles is regulated by the combined actions of the steroid hormone estradiol and the pituitary glycoprotein follicle-stimulating hormone (FSH) (1). Several biochemical and morphological changes are induced by FSH through the elevation of the intracellular second messenger, cAMP (2, 3). Upon synthesis by adenylylcyclase, cAMP diffuses or is transported to its site of action where four molecules bind each inactive cAMP-dependent protein kinase (PKA) holoenzyme. The kinase is activated by the release of two catalytic subunits (C) from the regulatory (R) subunit-cAMP complex. An array of PKA isozymes are expressed in mammalian cells, and genes encoding three C subunits (Cα, Cβ, and Cγ) and four R subunits (RIα, RIβ, RIIα, and RIIβ) have been identified (reviewed in Ref. 4). Two holoenzyme subtypes called type I and type II are formed by the combination of RI or RII with the C subunits (5, 6). At least 75% of the soluble PKA holoenzymes in GC of both preantral and preovulatory follicle-enriched ovaries are composed of RIIβ subunits, while the remaining PKA is composed of RIIα[2] (7). While the amount of C subunit activity does not appreciably change during GC differentiation (8), expression of RIIβ is increased upon exposure to estradiol and FSH and is decreased as a consequence of the luteinizing hormone (LH) surge (9). DEAE-cellulose and sucrose density gradient studies demonstrate that the hormonally induced RIIβ is not associated with C subunit (7). Since the subcellular location of PKA is directed by the R subunits (4), it has been proposed that the induction of RIIβ causes a redistribution of the kinase (10). This hypothesis is supported by indirect immunofluorescence studies, which suggest that the majority of RIIβ induced by FSH is directed to the cytoplasm of the cells (11).

Although PKA is a multifunctional enzyme with a broad substrate specificity, hormonal activation of the kinase must somehow permit a preferential phosphorylation of specific target substrates. It has been proposed that selective activation of compartmentalized pools of PKA co-localized with key substrates could provide a mechanism for hormone-specific effects (reviewed in Ref. 12). The type II PKA holoenzyme can be tethered to specific subcellular locations through interaction with specific AKAPs (13–16). In this study we show that exposure of granulosa cells to estradiol and FSH alters the subcellular distribution of the type II PKA. GCs treated with FSH undergo changes in the patterns of anchoring protein expression that include the induction of a mostly cytosolic 80-kDa AKAP, which selectively associates with RIIα.

MATERIALS AND METHODS

Primary Granulosa Cell Cultures—Female Sprague-Dawley rats (Charles River Laboratories) were injected subcutaneously with estradiol 17β (1.5 mg) in 0.1 ml of propylene glycol daily at 26, 27, and 28 days of age to stimulate the development of preantral follicles (9). On day 29 rats were killed, the ovaries removed, and granulosa cells obtained from the follicles by modifications to the method of Bley *et al.* (17). The medium used for all procedures was Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12, 1:1), with 15 mM HEPES, 3.15 g/liter glucose, 100 IU penicillin G, and 100 μg/ml streptomycin. Following sequential incubations at 37 °C in 6 mM EGTA in DMEM/F-12 and 0.5 M sucrose in DMEM/F-12 (17), ovaries were returned to DMEM/F-12. GCs were extruded into the medium from individual follicles using 30-gauge needles and gentle pressure. Cells were pelleted at 100 × *g* for 15 min, counted using trypan blue, and plated at a density of approximately 1 × 10^6 cells/ml on plastic dishes (Falcon) coated with

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[1] The abbreviations used are: GC, granulosa cell(s); FSH, follicle-stimulating hormone; PKA, cAMP-dependent protein kinase; C and R subunits, catalytic and regulatory subunits, respectively; LH, luteinizing hormone; DMEM, Dulbecco's modified Eagle's medium; PKI, cAMP-dependent protein kinase inhibitor; hCG, human chorionic gonadotropin.

[2] M. Hunzicker-Dunn, personal communication.

0.5 mg/ml human plasma fibronectin (Sigma). Cultured cells were grown in a humidified atmosphere at 37 °C, 5% CO_2 with 10 nM estradiol 17β (in ethanol, final concentration 0.5%). After 20 h, the medium was changed to remove non-attached cells and estradiol plus FSH (50 ng/ml) (oFSH-16, NIDDK), hCG (50 ng/ml) (CR-125; Center for Population Research) or forskolin (10 μM) (Sigma) were added to individual plates. After incubation with hormone the medium was aspirated, cells were rinsed with phosphate-buffered saline and harvested by: (*a*) scraping into 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM Na_3VO_4, 80 mM β-glycerophosphate, 100 μg/ml pepstatin-A, 21 μM leupeptin, and 0.23 mM phenylmethylsulfonyl fluoride (18) followed by sonication for 1 min or (*b*) solubilized directly in SDS-polyacrylamide gel electrophoresis sample buffer and boiled 20 min. Representative media progesterone values for cells cultured with and without additions are reported in the legend to Fig. 1. Radioimmunoassays for progesterone were conducted by the P30 Center for Research on Fertility and Infertility.

Cellular Fractionation—Cells (in each dish) were rinsed with 5 ml of 50 mM Tris-HCl (pH 7.2), containing 0.15 M NaCl and 1 mM EDTA, then scraped and lysed at 4 °C in buffer A (20 mM HEPES, (pH 7.4) containing 20 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 1 mM EGTA, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 10 mM benzamidine (19). The lysed cell suspension was then homogenized in a Dounce homogenizer (Kontes Glass Co.), and the homogenate was centrifuged at 40,000 × *g* for 15 min, yielding a supernatant fraction (19). The pellet was resuspended in 1 ml of buffer A containing 0.2% Triton X-100 and sonicated for 1 min.

Kinase and cAMP Binding Assay—Kemptide kinase activity was measured in triplicate in sample aliquots (containing 20–40 μg of GC protein) using 71.5 μM Kemptide (Sigma) and 40 μM [γ-^{32}P]ATP in a final volume of 0.25 ml, as previously described (20). [^{3}H]cAMP binding activity was measured as previously described (20).

Expression and Purification of Recombinant RIIα and RIIβ—Recombinant RIIα was expressed in *Escherichia coli* and purified as previously described (21). A cDNA encoding murine RIIβ in pUC 18 was provided by Dr. G. S. McKnight (University of Washington). This plasmid was used as a template in a polymerase chain reaction using a Coy TempCycler to amplify a 1495-base pair fragment encompassing the coding region of RIIβ. Polymerase chain reaction primers were designed to create an *Nde*I site at the 5′ end (CGCGCATATGAGCATCGAGATCCC) and a *Bgl*II site at the 3′ end (CGCGAGATCTCATGCAGTGGGCTCAAC). The amplified product was digested with both restriction enzymes and ligated into the bacterial expression vector pET11c (Novagen). Expression and purification of RIIβ were by the same methods used for RIIα (21).

Protein Blot Procedures—Solid phase binding overlays were performed as previously described (21). Western blot analysis was performed by standard methods (22), and immune complexes were detected using an enhanced chemiluminescence kit (Amersham Corp.). RII isoform-specific antibodies were raised against peptides specific to murine RIIα (50–62, dilution 1:250) or murine RIIβ (39–51, dilution 1:5000) and produced by Bethyl Laboratories). Anti-human vinculin is a monoclonal antibody (Sigma, dilution 1:2000).

RESULTS

FSH and Forskolin Alter the Expression of AKAPs in Granulosa Cells—Treatment of GC with FSH and estradiol promotes altered cell morphology through a rearrangement of the cytoskeleton (2). If indeed changes in PKA compartmentalization accompany FSH-induced maturation of granulosa cells, we reasoned that FSH might alter PKA compartmentalization by regulating the RII-binding activity of specific AKAPs. To test this hypothesis, extracts from cells treated with FSH and other effectors were screened for AKAPs by the modified RII overlay procedure using [^{32}P]RIIα as a probe (Fig. 1*A*). Cells exposed to FSH and estradiol for 72 h showed distinct patterns of AKAP expression as compared to cells treated with estradiol alone (Fig. 1*A*, *lanes 1* and *2*). Following treatment with FSH, detection of RII-binding bands of approximately 75–80 kDa increased in intensity, whereas a prominent band of 140 kDa significantly decreased. The induced 52-kDa protein detected by RII overlay was also detected by immunoblotting with RIIβ-specific antiserum (data not shown), suggesting this band is an endogenous RIIβ dimerizing with the [^{32}P]RIIα probe. Control

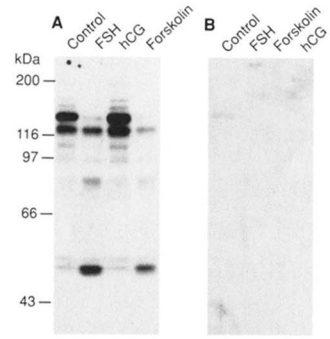

FIG. 1. **Hormone-induced changes in granulosa cell AKAP expression.** Total cell extracts (30 μg of protein/lane) were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to Immobilon membrane for analysis by the RII-binding overlay assay. [^{32}P]RIIα-binding bands (*panel A*) were detected by autoradiography. Control cell extracts (*lane 1*) were compared with extracts from granulosa cells treated for 72 h with FSH (*lane 2*), hCG (*lane 3*), and forskolin (*lane 4*). An identical filter (*panel B*) was probed with [^{32}P]RIIα in the presence 1 μM anchoring inhibitor peptide, which blocks RII/AKAP interaction. Media progesterone produced by GCs are as follows: control, <0.1 ng/ml; FSH, 36.5 ng/ml; hCG, <0.1 ng/ml; and forskolin, 16.9 ng/ml.

experiments demonstrated that all RII/AKAP interaction was specific, because the interaction was blocked when overlays were performed in the presence of 1 μM anchoring inhibitor peptide (Fig. 1*B*). We have shown previously that this peptide binds RII with nanomolar affinity and is a potent inhibitor of RII anchoring (23). Incubation with the anchoring inhibitor peptide also prevented detection of RII by the overlay method, presumably because it stabilized the soluble RII probe and blocked the formation of solid-phase RII-RII dimers.

Treatment of GC with forskolin, a pharmacological agent that activates adenylylcyclase, mimics the effect of FSH treatment on the pattern of AKAPs, suggesting that the cAMP-signaling pathway is involved in regulating these changes in the expression/activity of RII and specific AKAPs (Fig. 1*A*, *lane 4*). As an additional control, immature cells were treated with the LH analog, human chorionic gonadotropin (hCG). The pattern of AKAP expression was unchanged between the hCG-treated and control cells (Fig. 1*A*, *lane 3*). These results suggest that exposure to FSH or forskolin for 72 h causes the induction of RIIβ and at least two AKAPs around 75–80 kDa, which could be involved in the redistribution of the type II PKA in granulosa cells.

Kinetics of AKAP Expression— Control and FSH-treated GCs were harvested at times ranging from 0 to 72 h and analyzed by the RII overlay procedure. When compared to untreated controls, treatment with FSH increased detection of the 75–80-kDa RII-binding bands (Fig. 2). No changes were detected in the level of the 140-kDa RII-binding band from the FSH-treated cells, but surprisingly, the intensity of this band increased in control cells grown in primary culture for times between 24 and 72 h (Fig. 2).

FSH induces RII Isoform-specific AKAPs—FSH and estradiol treatment of granulosa cells causes a 10–15-fold induction of the RIIβ mRNA (9) and increases the intracellular concentration of the protein. Because RIIα and RIIβ are believed to preferentially associate with distinct anchoring proteins (14), we reasoned that FSH might induce the expression of isoform-specific AKAPs, which could contribute to a differential localization of type II holoenzymes. To test this hypothesis, identical blots containing total protein extracts from control and FSH-treated granulosa cells were screened for RII-binding proteins by the overlay procedure using either [^{32}P]RIIα or [^{32}P]RIIβ probes. In an effort to insure that isoform-specific patterns of

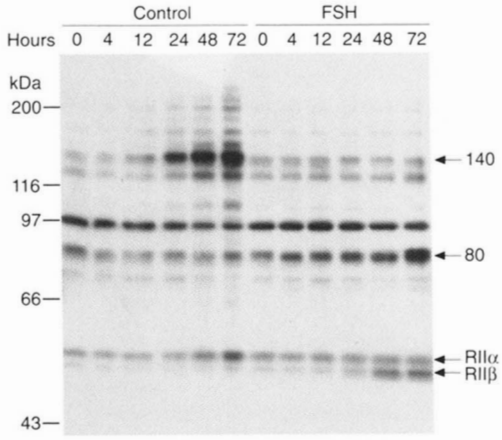

FIG. 2. **Time-dependent changes in AKAPs upon hormone treatment.** Total cell extracts (30 μg of protein/lane) from control or FSH-treated cells were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. The proteins were then transferred to Immobilon membrane for analysis by the RII overlay assay. The duration of treatment with individual effectors is indicated above each lane.

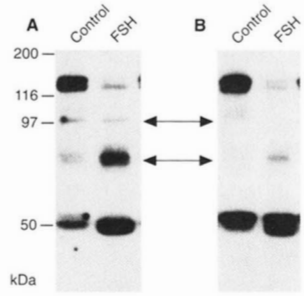

FIG. 3. **Selective interaction of RII isoforms with AKAPs.** Total cell extracts (30 μg of protein/lane) from control and FSH-treated (72 h) granulosa cells were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. Proteins were then transferred to Immobilon membrane for analysis by the RII overlay assay using either [^{32}P]RIIα (*panel A*) or [^{32}P]RIIβ (*panel B*) as a probe. RII binding was detected by autoradiography. Proteins that preferentially interact with RIIα are indicated.

RII-binding were not artifactual, identical filters were incubated with RII probes of the same specific activity. Overlays performed with [^{32}P]RIIα specifically detected RII-binding bands of 75–80 and 95 kDa (Fig. 3*A*), whereas the same bands were barely detectable on filters probed with [^{32}P]RIIβ (Fig. 3*B*). Furthermore, the 75–80-kDa RII binding bands were the only AKAPs specifically induced in response to FSH treatment (Fig. 3*A*). All RII binding was blocked in the presence of 1.0 μM anchoring inhibitor peptide (data not shown). These results suggest that FSH induces 75–80-kDa AKAPs that preferentially associates with RIIα.

Subcellular Fractionation of Granulosa Cells—Previous reports have shown that FSH treatment of granulosa cells increases the concentration of cytoplasmic RIIβ but not RIIα (9). Our studies show that FSH induces RIIα-specific AKAPs of 75–80 kDa, which could function to target RIIα, increasing its concentration within a distinct cellular compartment. To test this hypothesis, we monitored the distribution of PKA activity, cAMP binding, and AKAP levels in soluble and particulate fractions isolated from FSH-treated and control granulosa cells. PKA activity was defined as Kemptide phosphorylation that could be inhibited by PKI 4–24 peptide in the presence of cAMP; the relative concentration of R subunit was monitored by [^{3}H]cAMP binding. FSH treatment caused an increase of 17% in the soluble PKA activity (Table I) and a corresponding decrease of 58% in the particulate fraction without significantly altering total PKA activity. Furthermore, FSH caused a 4.6-fold increase in soluble [^{3}H]cAMP-binding activity (Table II), which is consistent with the known effect of FSH to induce free RIIβ subunit.

Equal concentrations of protein (30 μg) prepared from soluble and particulate fractions of control and FSH-treated granulosa cells (Fig. 4*A*) were analyzed for AKAPs using the RII overlay procedure (Fig. 4*B*). The 75–80-kDa RIIα-specific anchoring proteins were located predominantly in the cytosolic fraction of hormone-treated cells (Fig. 4*B*). FSH induction of the 75–80-kDa AKAPs were concomitant with a translocation of particulate RIIα to the cytosolic fraction (Fig. 4*C*). Western blot analysis with isoform-specific peptide antiserum showed that RIIα was equally distributed between the cytosolic and particulate fractions of control cells but was predominantly in the cytosolic fraction of cells treated with FSH (Fig. 4*C*). In contrast, RIIβ was almost exclusively soluble in control cells and showed a marked induction following FSH treatment (Fig. 4*D*). Because vinculin is known to be down-regulated in response to FSH treatment (24), its expression was monitored using human anti-vinculin antibody as an index of FSH effects on cultured granulosa cells (Fig. 4*E*). These results suggest that FSH may function to sequester the type IIα PKA holoenzyme in the cytosolic fraction through induction of RIIα-specific AKAPs.

TABLE I
Subcellular fractionation of PKA activity

	PKA activity		
	+cAMP	+cAMP + PKI	PKA activity[a]
	pmol/min/mg		
Control			
Supernatant	307 ± 5.7	29.1 ± 5.0	298 ± 10.6
Pellet	182 ± 8.4	27.8 ± 1.4	146 ± 7.2
FSH			
Supernatant	362 ± 5.7	20.9 ± 0.5	348 ± 6.1
Pellet	93 ± 3.5	17.3 ± 5.2	61 ± 7.8

[a] PKA activity = cAMP activity − (cAMP + PKI activity).

TABLE II
Subcellular fractionation of cAMP-binding proteins

	Activity
	pmol [^{3}H]cAMP/mg
Control	
Supernatant	3.95 ± 0.38
Pellet	1.61 ± 0.18
FSH	
Supernatant	18.06 ± 0.84
Pellet	1.39 ± 0.07

DISCUSSION

Exposure of granulosa cells grown in primary culture to FSH and estradiol alters the expression pattern of specific AKAPs resulting in the up-regulation of an 80-kDa AKAP and the RIIβ subunit of PKA. Analysis of data from several experiments strongly suggests that 80-kDa AKAP band induced by FSH most likely represents more than one anchoring protein in the molecular mass range of 75–80 kDa. At this time, it is unclear if these bands are distinct proteins or multiple size forms of the same protein that arise from posttranslational modification. Treatment with forskolin, a pharmacological agonist of adenylylcyclase, also increases detection of the 75–80-kDa AKAPs, implicating the involvement of the cAMP-signaling pathway in the maturation process. Either FSH or cAMP can induce expression of RIIβ protein 10–30 fold in granulosa cells (9). We have confirmed these observations by Western blot analysis with RII isoform-specific antisera, showing that RIIβ is signifi-

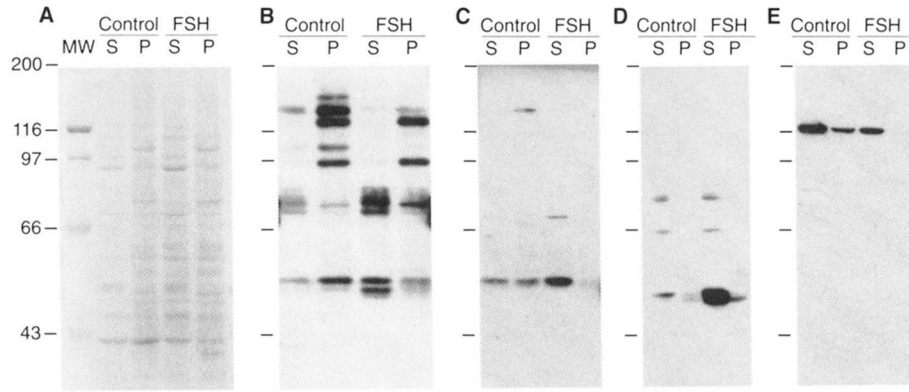

FIG. 4. **Subcellular fractionation of AKAPs and FSH-induced translocation of RIIα to the cytosol.** Cytosolic and particulate fractions of control and FSH-treated granulosa cells were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to Immobilon membranes. Identical blots were either stained with Coomassie Brilliant Blue (*panel A*) or probed with RII overlay procedure (*panel B*), antisera specific for RIIα (*panel C*) or RIIβ (*panel D*), or monoclonal antibodies against vinculin (*panel E*). Soluble (*S*) and particulate (*P*) fractions from control and treated cells are indicated above each lane.

cantly induced by FSH or cAMP (Fig. 4*D*) while RIIα levels remain relatively constant in control and treated cells.[3] While the mechanism that regulates RIIβ expression is not completely understood, recent evidence has shown that the nucleotide sequence 5′ to the RIIβ gene lacks a consensus cAMP-responsive element (25). The identity of the FSH-inducible anchoring protein is unknown as it does not react with antisera we have generated to five different AKAPs. Future studies are planned to isolate cDNA clones for this molecule and study its mode of regulation by FSH.

Overlay and subcellular fractionation data show that RIIα preferentially associates with a 95-kDa AKAP and the 75–80-kDa AKAPs (Fig. 3). While isoform-specific differences in RII/AKAP interaction have previously been inferred (14), we show that RIIα selectively binds an AKAP that is up-regulated by FSH. The identification of isoform-specific AKAPs provides an increased level of sophistication to the PKA-anchoring model as it identified a potential mechanism to target a specific type II PKA isoform to definite subcellular sites.

Subcellular fractionation data suggest that FSH-induced differentiation of GCs is accompanied by a translocation of RIIα to the cytosol. Hormonal induction of an isoform-specific AKAP, such as the 80-kDa protein, could mediate this translocation. This could occur by three mechanisms: *de novo* synthesis of anchoring proteins, translocation of already existing AKAP/PKA complexes, or post-translational modification of AKAPs (thereby increasing their affinity for PKA). In each case, the net effect would be to target the type IIα PKA to sites where it can preferentially phosphorylate selected substrates in response to cAMP. Intracellular redistribution of PKA might adapt granulosa cells for surges in cAMP concentrations that occur later in the maturation process. Since maximal detection of the 80-kDa AKAP occurred 72 h after exposure to FSH (Fig. 2), we prefer the model involving *de novo* protein synthesis. Additionally, this time scale is coincident with the morphological changes that are observed in maturing granulosa cells (2).

In summary, FSH causes the up-regulation of a cytosolic 80-kDa AKAP in granulosa cells which selectively binds to RIIα. The detection of this anchoring protein is coincident with a translocation of RIIα to the cytosol. In contrast, overexpression of AKAP 75 artificially targets the type II PKA to the particulate fraction (26). Accordingly, we propose that FSH effects the subcellular location of the type II PKA holoenzymes by translocation of RIIα and induction of RIIβ synthesis. Both events change the subcellular targeting of the type II kinase.

[3] D. W. Carr and J. D. Scott, unpublished observation.

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