Hormonal Regulation of Nuclear Cyclic AMP-dependent Protein Kinase Subunit Levels in Rat Ovaries*

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Biochemical as well as immunochemical studies were carried out to quantitatively and qualitatively evaluate the hormonal regulation of nuclear cAMP-dependent protein kinase subunits in ovaries from estrogen-treated hypophysectomized rats. Photoaffinity labeling of nuclear extracts with 8-azido-[^32P]cAMP and electrophoretic analysis showed the existence of three variants of the regulatory subunit RI and of a 52,000-dalton RII variant (RII-52) in ovaries of estrogen-primed hypophysectomized rats. After follicle-stimulating hormone (FSH) stimulation, an additional variant of RII (RII-51, Mr = 51,000) was detected in nuclei. The cytosolic RII-54 variant (Mr = 54,000) could not be identified in nuclei by photoaffinity labeling. The FSH-mediated appearance of the nuclear RII-51 variant was accompanied by an approximate 2-fold increase of nuclear catalytic subunit activity.

Using quantitation by enzyme-linked immunosorbent assay, we identified a marked FSH-mediated increase of nuclear RII variant(s) and confirmed the increase of nuclear catalytic subunit levels. Furthermore, morphometric analysis of nuclear and cytoplasmic antigen density by immunogold electron microscopy demonstrated a cell-specific modulation by FSH of RII and C subunit density. In granulosa cells, both nuclear as well as cytoplasmic RI density was increased by FSH, whereas catalytic subunit density was increased in the nuclear area only. In thecal cells, FSH increased only the nuclear catalytic subunit density.

These results provide biochemical as well as immunochemical evidence for a cell-specific FSH regulation of nuclear RII and catalytic subunit levels which may be involved in the molecular events responsible for the FSH-mediated differentiation of the rat ovary.

Many of the actions of gonadotropic hormones in the ovary are mediated by cAMP through cAMP-dependent protein kinases (Marsh, 1975). Cyclic AMP-dependent protein kinase isoforms, through phosphorylation of specific regulatory cellular proteins, are believed to initiate a "built-in" program for action established during early differentiation and development. In the rat ovary, cAMP-binding and cAMP-dependent protein kinase activities increase progressively during postnatal development (Lamprecht et al., 1973; DeAngelo et al., 1975) and in response to gonadotropins administered to neonatal rats or adult hypophysectomized rats (DeAngelo et al., 1975). Analyses of changes of protein kinase isozymes have shown that ovarian cytosol of 6-day-old neonates contains largely type I cAMP-dependent protein kinase which changes to a predominantly type II protein kinase pattern in 34-day-old and older rats (Jungmann and Hunzicker-Dunn, 1978; Hunzicker-Dunn et al., 1984). The type II isozyme similarly increases in rat ovary nuclei as a function of postnatal development (Jungmann and Hunzicker-Dunn, 1978; Hunzicker-Dunn, 1982) but little is known about the hormonal regulation of ovarian nuclear protein kinase levels. Several experiments have suggested that ovarian nuclear cAMP-dependent protein kinase activity is acquired through a cAMP-mediated translocation of cytoplasmic protein kinase subunits (Jungmann et al., 1974; Spielvogel et al., 1977).

In the rat granulosa cell, the level of the cytosolic regulatory subunit RII increases as preantral follicles differentiate into antral follicles (Ratoosh and Richards, 1985; Richards and Rolfe, 1980; Richards et al., 1983, 1985; Darbon et al., 1984). Concomitant with the changes in RII levels, FSH and cAMP change the cAMP-dependent protein kinase substrate patterns (Richards et al., 1983; Halpern-Ruder et al., 1980). Since at least some of these cAMP-mediated events can be expected to involve a nuclear action of cAMP and cAMP-dependent protein kinase (Jungmann and Hunzicker-Dunn, 1978), the present studies were undertaken to identify protein kinase subunits RI, RII, and C in rat granulosa cell nuclei and to identify if the nuclear subunits levels are qualitatively and quantitatively modulated by FSH. The coordinated regulation of the nuclear subunits would suggest a regulatory action for the subunits at the nuclear level conceivably participating in regulating the genomic expression of FSH- and cAMP-inducible granulosa cell proteins.

EXPERIMENTAL PROCEDURES

Chemicals—8-Azido-[^32P]cAMP (specific activity, 90 Ci/mmol) was from International Chemical and Nuclear, Inc., Irvine, CA; [γ-[^32P]ATP (specific activity, 3000 Ci/mmol) and [2,8-[^3H]cAMP (specific activity, 32.3 Ci/mmol) were purchased from DuPont-New England Nuclear. Benzamidine was from Aldrich, phosphocellulose paper (P81) was from Whatman, nitrocellulose membranes were from Schleicher & Schuell. Enzyme-grade sucrose was obtained from Schwarz/Mann. Kempride (a synthetic heptapeptide, Leu-Ala-Ser-Leu-Gly), ATP, cAMP, N6,9-dibutyryl-cAMP, PMSF, EGTA, EDTA and all other analytical grade chemicals were purchased from Sigma. XRP-5 x-ray film was obtained from Eastman Kodak. Ovine FSH (NIH-oFSH-16) was from the National Pituitary Agency. Goat anti-rabbit immunoglobulin linked to 20-nm colloidal gold was from The American Society for Biochemistry and Molecular Biology, Inc.

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gold particles (GAR 20) was purchased from Janssen Pharmaceuticals, Beerse, Belgium. Animals—Immature female rats (Charles River, Sprague-Dawley), hypophysectomized at the age of 21 days, were purchased from the Hormone Assay Laboratories (Chicago, IL). Seven days after surgery, hormone treatments were initiated as follows: Rats were injected subcutaneously once daily for 4 days with 1.5 mg of 17beta-estradiol dissolved in 0.2 ml of propylene glycol and then twice daily for 2 days with 7.5 μg of ovine FSH (in 0.1 ml of phosphate-buffered saline) or with only 0.1 ml of saline. Rats were killed by cervical dislocation 12 h after the last injection.

Preparation of Nuclear Nonhistone Protein Extracts—Nuclear nonhistone protein extracts were prepared as described by us (Maizels and Jungmann, 1983) with slight modifications as follows: Ovaries were homogenized in nuclear isolation buffer (10 mM Tris, pH 7.4, 4 mM MgCl₂, 50 mM benzamidine, 0.5 mM PMSF, and 1.8 mM sucrose). The homogenate was layered onto a 2-ml cushion of nuclear isolation buffer and centrifuged at 105,000 g for 10 min. Preparation of Cytosol—Ovaries were homogenized in 10 mM Tris, pH 7.4, 4 mM MgCl₂, 50 mM benzamidine, 0.5 mM PMSF, and 0.35 M NaCl. Insoluble nucleohistone was removed by centrifugation at 105,000 × g for 10 min.

Cyclic AMP-dependent Protein Kinase Subunits and cAMP-binding activity were assayed as described in detail (Kuettel et al., 1985). Enzyme-linked Immunosorbent Assay—The enzyme-linked immunosorbent assay (ELISA) was described in detail (Schwoch and Hamann, 1982; Kuettel et al., 1985).

Nuclear CAMP-binding activity was significantly higher in nuclei isolated in hypertonic sucrose (1.8 M sucrose) buffer than in nuclei isolated in 0.32 M sucrose buffer (Maizels, 1983). With this method, the recovery of nuclei from the ovarian homogenate in 1.8 M sucrose buffer as determined from the DNA concentrations was between 55% and 60%. Evaluation of nuclear morphology and contamination of nuclei by secretory granules and cytosolic particles as well as measurement of marker enzyme activity (Laks et al., 1981) were indicative of "pure" nuclear preparations. The protein:DNA ratio of all nuclear preparations was between 2.3 and 2.0, typical of highly purified nuclei (Tata, 1974).

It was also important for our studies to ascertain that (a) nuclear protein kinase subunits were quantitatively recovered by this isolation procedure and (b) to assure that the subunits were of nuclear origin and not due to contaminations by dissociated cytosolic subunits or undissociated cytosolic holoenzyme. This is particularly critical, since the dissociated cytosolic catalytic subunit will artificially adhere to nuclei during their isolation (Keely et al., 1975). In contrast, the dissociated cytosolic regulatory subunits RI and RII do not exhibit artificial binding activity to nuclei.

To prevent artificial nuclear binding of the cytosolic catalytic subunit, Keely and co-workers (1975) have developed a procedure, consisting of isolation of nuclei in buffers containing isotonic concentrations of NaCl or KCl, which prevents artificial binding of cytosolic catalytic subunit to nuclei. Accordingly, we have isolated nuclei in 1.8 M sucrose buffer containing 0.15 M NaCl. On the other hand, since cytosolic RI and RII do not artificially adhere to nuclei and since inclusion of 0.15 M NaCl in the nuclear isolation buffer led to a considerable loss of nuclear RI and RII (Maizels, 1983), NaCl was deleted from the nuclear isolation buffer when RI and RII were determined.

To ascertain, however, that artificial nuclear binding of dissociated cytosolic RI and RII as well as of the undissociated holoenzyme did, indeed, not occur, the following experiments were carried out. Cytosol was UV-irradiated in the presence of the photoaffinity label 8-azido-[3H]cAMP without and with added nonradioactive cAMP (100 μM). The presence of nonradioactive cAMP prevents specific labeling of RI and RII but it does not prevent potential nonspecific labeling of cytosolic proteins other than RI and RII. After removal of excess free 8-azido-[3H]cAMP, an aliquot of radioactively labeled cytosol protein (about 25,000 cpm of 32P) was added to an equivalent amount of rat ovarian homogenate. Following isolation of nuclei in buffer containing 1.8 M sucrose but no NaCl, nuclei were extracted with 0.35 M NaCl and 32P radioactivity of the extracts was determined. In each case, less than 1% of the total amount of radioactivity added to the homogenate was recovered in the 0.35 M NaCl extracts (data not shown). There was no difference in the amount of radioactivity recovered regardless of whether the added cytosol had been photoaffinity-labeled in the presence or absence of non-
radioactive cAMP indicating that the radioactivity recovered in the nuclear 0.35 M NaCl extract was due to nonspecifically labeled cytosolic proteins and not to RI or RII. Furthermore, it is shown below (see Figs. 2 and 3) that cytosol but not nuclei contained the RII-54 electrophoretic variant of RII. From this selectivity of RII-54 compartmentation it can be concluded that artifactual binding of cytosolic regulatory subunits to nuclei does not occur.

We have already reported (Jungmann et al., 1974) that the protein kinase holoenzymes exhibit little or no binding affinity for calf ovary chromatin. After incubation of nuclei with varying amounts of either the type I or type II DEAE cellulose-purified holoenzymes and isolation of nuclei in 1.8 M sucrose buffer, catalytic subunit, RI, and RII concentrations in nuclear extracts were not significantly altered as compared to nuclei that had not been incubated with the holoenzymes. This indicates that contamination of nuclei with cytoplasmic holoenzyme was not a significant problem.

In order to evaluate the quantitative recovery of subunits from the nuclei of unstimulated and FSH-stimulated ovaries, nuclei were extracted successively with buffers containing increasing amounts of NaCl (see Table I). A concentration of 0.35 M NaCl was sufficient to solubilize about 95% of the total nuclear cAMP-binding and catalytic subunit activities. Subsequent extractions with buffers containing higher concentrations of NaCl did not solubilize appreciably more cAMP-binding and catalytic subunit activities. Therefore, in all subsequent experiments nuclear subunits were extracted with buffer containing 0.35 M NaCl.

As the data of Table I show, the recovery of catalytic subunit and cAMP-binding activities in nuclear NaCl extracts from both unstimulated as well as stimulated ovaries was quantitative and of similar efficiency. This is important to note, since it could be argued that the increased levels of subunits in nuclear extracts was due to a lower binding affinity of the subunits to nuclei after FSH treatment resulting in a higher quantitative recovery of the subunits. The data of Table I illustrate that this was not the case.

Effect of FSH on Ovarian Nuclear Catalytic and Regulatory Subunit Levels—The data of Table II show that the specific activities of the subunits were lower in nuclear extracts as compared to cytosol. FSH stimulation led to a 2–3-fold increase of both the specific cAMP-binding and catalytic subunit activities in nuclei. In the cytosol, on the other hand, FSH stimulation led only to an increase of the specific cAMP-binding but not of the catalytic subunit activity in agreement with previously published data (Richards and Rolfe, 1980; Darbon et al., 1984). Taking the protein concentration in nuclei and homogenate and a recovery of nuclei of 55% into consideration, it was calculated that about 2.7% of the total cellular catalytic subunit activity was present in nuclei from unstimulated ovaries. FSH stimulation increased this percentage to 4.7%. Similarly, FSH increased the nuclear cAMP-binding activity from 4.5% to 11.9% of the total cellular activity.

A more detailed study of the changes of nuclear catalytic subunit levels is illustrated in Fig. 1. FSH treatment of estrogen-primed hypophysectomized rats for 1 or 12 h and 2 days, increased catalytic subunit activity of nuclear extracts as compared to untreated controls (Fig. 1, panels A and B). Maximal increase of kinase activity was observed after 1 day of FSH treatment (see Fig. 1, panel B). Phosphorylation of endogenous nuclear substrates was relatively low (Fig. 1, panel A) and did not contribute significantly to the level of phosphorylation seen with Kemptide as substrate. The priming of hypophysectomized rats with 17β-estradiol without subsequent FSH treatment did not affect nuclear catalytic subunit activity. Saturating concentrations of heat-stable protein kinase inhibitor led to an approximate 50% inhibition of the total nuclear protein kinase activity in unstimulated ovaries and approximately 75% inhibition of the total kinase activity in FSH-stimulated ovaries (data not shown). This indicates that the FSH-mediated increase of nuclear protein kinase activity was primarily due to an increase of catalytic subunit activity and not to a modulation of other nuclear cAMP-independent protein kinase activities.

Estimation of the nuclear regulatory subunit levels by the [3H]cAMP-binding assay allows measurement of the total sum of RI and RII, but this method does not discriminate between the two subunits. Since FSH markedly increased nuclear [3H]cAMP binding activity (see Table II), we evaluated the effect of FSH on the patterns of nuclear regulatory subunits by photoaffinity labeling with 8-azido-[32P]cAMP. For comparative purposes, cytosolic RI and RII were also photoaffinity labeled and identified. After electrophoresis and autoradiography several types of photoaffinity-labeled polypeptides were reproducibly observed (Fig. 2). The regulatory subunit RI is represented by a single band of an apparent molecular weight, Mr = 49,000 (Rubin and Rosen, 1975; Nimmo and Cohen, 1977; Krebs and Beavo, 1979; Flockhart and Corbin, 1982; Schwartz and Rubin, 1983, 1985). The electrophoretic patterns of RII, on the other hand, are heterogeneous and differ in nuclear extracts and cytosol. The nuclear RII subunit appears to consist of a 52,000-dalton...
concentration in ovarian nuclear extracts from estrogen-primed un-stimulated (●) and estrogen-primed FSH-stimulated (2 days) (▲) hypophysectomized rats. Activity measurements were done in triplicate in the presence of 50 μM cAMP using Kemptide as the substrate. Endogenous nuclear phosphorylating activity was assessed in the absence of Kemptide: ○, −FSH; △, +FSH. Panel B, comparison of ovarian nuclear protein kinase activity in unstimulated estrogen-primed (day 0) and in estrogen-primed hypophysectomized rats stimulated with FSH for the time periods indicated. Protein kinase activity is expressed as percentage of activity determined in unstimulated rats ovaries (set at 100%). The results are expressed as the arithmetic mean ± S.E. of three separate groups of rats.

**Table II**

Cyclic AMP-binding and catalytic subunit activities in nuclei and cytosol of rat ovaries stimulated with FSH

| Specific cAMP-binding activity | Specific catalytic subunit activity |
|-------------------------------|-----------------------------------|
| Nuclei                        | Cytosol                           |
| pmol cP[32P]/AMP bound/mg of protein | pmol 32P incorporated/ min/mg protein |
| −FSH                          | +FSH                              |
| 455 ± 69                      | 1215 ± 134                       |
| 1.5 ± 0.21                    | 6.3 ± 0.58                       |
| +FSH                          | +FSH                              |
| 1404 ± 137                    | 2250 ± 266                       |
| 3.4 ± 0.32                    | 7.6 ± 0.81                       |
| +FSH/−FSH                     |                                   |
| 3.1 ± 0.41                    | 1.86 ± 0.28                      |
| 2.3 ± 0.35                    | 1.2 ± 0.22                       |

In contrast, ovarian cytosol from hypophysectomized rats exhibited the RI-54 form but lacked RI-52 and RI-51 before FSH stimulation (see Fig. 2). FSH stimulation resulted in the appearance of cytosolic RI-52 and -51 confirming previous reports that cytosolic RI-52 and -51 are increased by FSH (Richards and Rolfe, 1980; Richards et al., 1983, 1984; Jahnsen et al., 1985; Darbon et al., 1984). The binding of 8-azido-[32P]cAMP to RI and RI was completely inhibited in the presence of 10^{-4} M nonradioactive cAMP (not shown) demonstrating the specificity of 8-azido-cAMP binding.

To determine if the variant forms of RI could also be distinguished on the basis of charge, regulatory subunits were analyzed by two-dimensional SDS-polyacrylamide gel electrophoresis after photoaffinity labeling. For comparative purposes, the two-dimensional electrophoretic pattern of cytosolic regulatory subunits from FSH-treated ovaries is shown in Fig. 3, panel C. As previously reported (Jahnsen et al., 1984), several regulatory subunit variants can be identified in the cytosol. Although we were not able to achieve a better resolution, several other isoelectric RII variants, identified as RII-51, RII-51.5, and RII-52, are visible on the autoradiograph in addition to the RII-54 variant. In ovarian nuclear extracts from FSH-treated rats, RII was also resolved into several isoelectric variants (Fig. 3, panel A) conceivably identical with the cytosolic RII-51, -52, and -51.5 forms. As on the one-dimensional gel (Fig. 2), the RI-54 variant could not be identified in the nuclear extracts. The autoradiograph density pattern of nuclear RII from untreated rat ovaries is considerably less dense particularly at the more basic pI values (Fig. 3, panel B). However, the lack of resolution of the spots does not allow a precise identification of the variants. Comparison of the relative autoradiograph densities in panels A and B of Fig. 3 confirms the FSH-mediated increase of photo labeling of RII-51, -52 seen in Fig. 2. The distribution of labeled spots on the two-dimensional gels (Fig. 3, panels A, B, and C) shows that the RI subunit exhibits charge heterogeneity, since three isoelectric variants can be identified. The RI variants have previously been observed after two-dimensional gel analysis of rat ovary cytosol (Jahnsen et al., 1986). The photoaffinity labeling of RI and RII seen in Fig. 3, panels A, B, and C, was specific for cAMP-binding sites, since photoaffinity labeling of RI and RII was prevented by the addition of excess competing cAMP to the reaction mixtures (Fig. 3, panel D).

**Quantitation of Protein Kinase Subunits in Nuclear Nonhistone Protein Extracts by Enzyme-linked Immunosorbent Assay**—An enzyme-linked immunosorbent assay was used to quantitate FSH-mediated changes of nuclear protein kinase subunit levels. The competition curves obtained in a representative ELISA experiment are shown in Fig. 4. For the RII and C subunits, significantly less nuclear protein from FSH-
stimulated ovaries was needed to achieve the same degree of inhibition of antibody-antigen binding seen with nuclear protein from unstimulated ovaries. A similar ELISA experiment showed no difference of nuclear RI subunit levels in stimulated and unstimulated ovaries. These experiments indicate a selective increase of RII and C after FSH stimulation but not of RI. Table III summarizes the ELISA data from several independent experiments. We observed an approximate 2-fold increase of the C and RII content in ovarian nuclei from FSH-treated rats as compared to untreated rats.

| Subunit | Subunit concentration (pmol/mg non-histone protein) | FSH/control |
|---------|---------------------------------------------------|-------------|
| RI      | -FSH: 15.7 ± 1.9; +FSH: 17.1 ± 2.1                | 1.1 ± 0.07  |
| RII     | -FSH: 10.5 ± 1.9; +FSH: 28.6 ± 3.5                | 2.7 ± 0.15  |
| C       | -FSH: 44.4 ± 4.1; +FSH: 93.6 ± 9.8                 | 2.1 ± 0.19  |

Effect of FSH on Protein Kinase Subunit Density in Granulosa and Thecal Cells Determined by Immunogold Electron Microscopy—We have recently developed a post-embedding immunocolloidal gold electron microscopy technique which allows the ultrastructural localization and morphometric

**FIG. 3.** Photoaffinity labeling and two-dimensional SDS-polyacrylamide gel electrophoresis of nuclear and cytosolic regulatory subunits. Nuclear ovarian nonhistone protein extracts (100 μg of protein each) from estrogen-primed FSH-stimulated (panel A) and estrogen-primed unstimulated (panel B) hypophysectomized rats were photoaffinity labeled and analyzed by two-dimensional SDS-polyacrylamide gel electrophoresis prior to autoradiography. For comparison, panel C shows the autoradiograph of photoaffinity-labeled cytosolic RI and RII from estrogen-primed FSH-stimulated hypophysectomized rats. Panel D shows the autoradiograph of ovarian nuclear protein from estrogen/FSH-stimulated rats after photoaffinity labeling in the presence of 100 μM nonradioactive cAMP. IEF, isoelectric focusing; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

**FIG. 4.** ELISA of the regulatory (RI and RII) and catalytic (C) subunits of cAMP-dependent protein kinase in ovarian nuclear extracts from estrogen-primed unstimulated (○) and estrogen-primed hypophysectomized rats stimulated with FSH for 2 days (●). A/Ao = absorbance measured in the presence of competing antigen relative to that measured in the absence of competing antigen (i.e. 100% absorption).

**Table III**

Quantitation of nuclear cAMP-dependent protein kinase subunits in rat ovaries by enzyme-linked immunosorbent assay

Hypophysectomized rats were primed with 17β-estradiol and subsequently injected with FSH for 2 days as described under "Experimental Procedures." Nuclear non-histone protein was isolated and analyzed for protein kinase subunits by ELISA as described in Fig. 4. Absolute values were calculated by comparison of the competition curves shown in Fig. 4 with the parallel curves obtained with standard highly purified preparations of rat liver RI, RII, and C. Results reported are the mean ± S.E. of three experiments.
It has been shown (Keely et al., 1975) that due to its charge properties the catalytic subunit, but not the regulatory subunits, will bind artifactually to nuclei after cell disruption. When measuring nuclear catalytic subunit activity, it becomes necessary, therefore, to isolate nuclei under conditions, e.g. inclusion of 0.15 M NaCl or KCl in the isolation buffer, which prevent the nonspecific nuclear binding of the catalytic subunit. In contrast, since the regulatory subunits do not exhibit the artificial binding behavior, it is not necessary to include isotonic NaCl or KCl in the nuclear isolation buffer when measuring nuclear regulatory subunit levels. In fact, we and other investigators have shown that the presence of 0.15 M NaCl in the isolation buffer leads to a marked loss of nuclear regulatory subunits and other nuclear nonhistone proteins (Maizels, 1983; Comings and Tack, 1973). It became necessary, therefore, to isolate nuclei by two slightly differing procedures (isolation buffer with and without 0.15 M NaCl) depending on which subunits were determined. Additionally, through the use of high molarity sucrose in the nuclear isolation buffers, we have optimized the isolation procedure in such a way that a loss of inherent nuclear subunits was minimized.

The results of this study demonstrate that the cAMP-dependent protein kinase subunits RI, RII-51, -52, and C are present in rat ovary nuclei and that the nuclear levels of RII-51, -52, and C, but not of RI and RII-54, are regulated by FSH in estrogen-primed hypophysectomized rats. These data were experimentally obtained through measurements of the nuclear cAMP-binding and catalytic subunit activities, through qualitative evaluation of nuclear regulatory subunits by photoaffinity labeling, and through quantitation of nuclear subunits by two different immunochemical methods. Whereas the immunochemical methods do not distinguish between nuclear RII variants, analysis of photoaffinity-labeled regulatory subunits by one- and two-dimensional gel electrophoresis identified at least two RII variants in nuclei, RII-51 and -52, and possibly a third variant exhibiting a slightly more basic isoelectric point than RII-51 and -52.

The unique feature of the RII-51,-52 variants is that their nuclear level is hormonally regulated, whereas that of RII-54 is not. In fact, we were not able through photoaffinity labeling to identify the cytosolic RII-54 variant in nuclei. This finding is of particular interest because it has previously been demonstrated that only ovarian cytosolic RII-51 and -52, but not RII-54, are hormonally regulated in estrogen/FSH-treated hypophysectomized rats (Jahnsen et al., 1985). Thus, the presence of RII-51 and -52 in nuclei and their hormonal regulation suggests a selective nuclear regulatory action for these subunits. Based on peptide mapping analysis and cDNA structure analysis, Jahnsen et al., (1985, 1986) have demonstrated a structural similarity between RII-51 and RII-52 and have suggested that RII-52 may be a post-translational modification of RII-51. RII-54, on the other hand, is structurally distinct from RII-51.

It should be noted that the FSH-mediated increase of nuclear and cytosolic specific subunit activities was markedly higher in nuclei than in cytosol (see Table II). Furthermore, the catalytic subunit activity increased only in nuclei but not in cytosol. This indicates that the nuclear changes did not merely mirror any changes of subunit activity in the cytosol and argues against a change of nuclear subunit levels due to contamination of nuclei by a small, constant fraction of cytoplasmic subunits. It also suggests a selective increase of subunits in nuclei, a process which is regulated separately from the modulation of cytosolic subunits.

2 E. T. Maizel and R. A. Jungmann, unpublished observations.
The immunocolloidal gold electron microscopy method provided us with a valuable complementary analytic approach for the study of nuclear subunits. The method circumvents cell rupture and the ensuing potential for either artifactual loss of nuclear components or binding of cytosolic proteins to nuclei during nuclear isolation. Furthermore, it allowed a semi-quantitative evaluation of protein kinase subunits and a selective analysis of granulosa as well as thecal cells. In general agreement with the ELISA and biochemical data, the immunocolloidal gold method indicated that RI, RII, and C were localized in the nuclear and cytoplasmic areas of granulosa and thecal cells (Fig. 5). RI staining density was not altered by FSH. In contrast, FSH treatment resulted in a selective, cell-specific effect on nuclear RII and C levels. While FSH increased the RII density in the nuclear and cytoplasmic areas of granulosa cells (Fig. 5, panel A), RII density was unchanged in thecal cells (Fig. 5, panel D). On the other hand, catalytic subunit density was increased only in the nuclear but not cytoplasmic areas of granulosa and thecal cells (Fig. 5, panels B and E). The reason why FSH action requires modulation of nuclear RII-51, -52, and C in granulosa cells but only of nuclear C in thecal cells remains to be elucidated. Under the experimental conditions the protein kinase subunits are relatively resistant to the destructive potential of fixation, dehydration, and embedding. However, direct quantitative comparison between nuclear and cytoplasmic subunit density should only be done with caution because possible differences of the binding affinity of the subunits to nuclear substructures and extranuclear regions, and a possible preferential loss of cytoplasmic antigen during tissue processing, may make these comparisons imprecise. Additionally, because of differing antibody-antigen affinities, a quantitative comparison of the labeling densities of the various subunits is not feasible.

While it has previously been shown that the FSH-mediated increase of cytosolic RII is due to increased RII protein synthesis and elevated RII mRNA levels (Richards and Rolfs, 1980; Richards et al., 1983, 1984; Darbon et al., 1984; Ratoosh and Richards, 1986; Jahnsen et al., 1986; Ratoosh et al., 1987; Hedlin et al., 1987), it is of interest to consider the molecular mechanism of modulation of nuclear RII and C. Since granulosa cells proliferate rapidly during follicular development and gonadotropin stimulation (Hirshfield, 1985), it is possible that nuclei acquire elevated levels of CAMP-dependent protein kinase in the form of its undissociated holoenzyme or as dissociated subunits at the time of meiosis and chromatin assembly. However, because the addition of CAMP at various concentrations to ovarian nuclei fails to stimulate protein kinase activity (Jungmann and Kranias, 1977), we consider the presence of undissociated holoenzyme in nuclei unlikely. Also, the increase of nuclear RII levels after hormonal stimulation can not be explained by the presence and activation of nuclear holoenzyme. Based on an evaluation of all experimental data, elevation of nuclear subunit levels is best explained by either a FSH-mediated packaging of dissociated subunits into chromatin at the time of meiosis or, alternatively, as a consequence of translocation of subunits from the extranuclear space into the nucleus. This conclusion is strongly supported by findings that quantitation of nuclear subunit levels by two independent immunochromical methods, using antisera which interact efficiently with both the dissociated as well as undissociated subunits in the holoenzyme form, demonstrates a net increase of immunoreactive nuclear RII and C antigen.

Furthermore, while there is a distinct potential for an artifactual association of subunits to nuclear structures during cell homogenization, we have carefully assessed and controlled the nuclear isolation procedure to avoid such artifacts. In fact, our experimental findings do not support the notion of a nonspecific modulation of nuclear subunits. The selective nuclear increase of C and RII-51 and -52 but not of RI or RII-54, and the general agreement between the biochemical, ELISA, and immunocytochemical data argue strongly for a specific modulation and against a nonspecific binding and translocation of subunits.

The previous findings that different forms of RII accumulate in estrogen/FSH-stimulated ovaries (Richards and Rolfs, 1980; Jahnsen et al., 1985; 1986) and our demonstration of a modulation of nuclear levels of RII-51, -52, and C suggest that certain variant forms of RII as well as C are selectively required in the nucleus during ovarian differentiation. While the exact biological roles of the nuclear subunits remain to be determined, the degree of selective nuclear localization and the magnitude of modulation point to a complex nuclear control mechanism. The presence of catalytic subunit in the nucleus, leading to a CAMP-mediated phosphorylation and conceivable functional modification of nuclear proteins, is most likely of considerable functional consequence resulting in the CAMP-mediated control of nuclear events such as the induction of gene products involved in ovarian differentiation and the ability of granulosa cells to undergo luteinization.

No function of the regulatory subunits at the nuclear level has so far been identified. A report from our laboratory that topoisomerase activity is associated with rat liver regulatory subunit RII (Constantinou et al., 1985) suggests a functional role for RII at the DNA level. However, recent studies in our laboratory have shown that ovarian topoisomerase I activity is not increased after FSH stimulation of estrogen-primed rats. Such a FSH-mediated increase would be expected if RII-51 and/or RII-52 possessed topoisomerase activity. Thus, further studies are needed to elucidate the function of ovarian RII variants at the nuclear level. However, because RII-51, -52, and C levels are modulated in nuclei, it is probable that FSH, through CAMP and CAMP-dependent protein kinase, mediates some of its effects in developing preovulatory follicles at the level of the genome through regulation of specific gene expression.

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FSH Regulation of Ovarian Nuclear Protein Kinase Subunits

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