Purification and Characterization of Hormone-regulated Isoforms of the Regulatory Subunit of Type II cAMP-dependent Protein Kinase from Rat Ovaries*

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The regulatory subunit (R-II) of cAMP-dependent protein kinase type II is induced in rat ovarian granulosa cells by the synergistic actions of estradiol and follicle-stimulating hormone. The R-II from rat ovaries was compared with R-II from rat heart, rat brain, bovine heart, and bovine brain using immunological methods, S-Nα[32P]cAMP photoaffinity labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Three isoforms of R-II were identified in rat ovarian cell extract (R-II52, \( M_r = 54,000 \), R-II52, \( M_r = 52,000 \), R-II51, \( M_r = 51,000 \)), two isoforms of R-II in rat brain cell extract (\( M_r = 54,000, M_r = 52,000 \)), and one isoform of R-II in rat heart cell extract (\( M_r = 54,000 \)). Rat ovarian R-II52, heart R-II, and brain R-II (\( M_r = 54,000 \)) were recognized by antisera against rat heart R-II, whereas rat ovarian R-II52/R-II51 and rat brain R-II (\( M_r = 52,000 \)) were not. In contrast, an antisera raised against bovine heart R-II recognized all three isoforms of ovarian R-II as well as the lower molecular weight form of rat brain R-II. Ovarian types R-II52 and R-II51 but not R-II54 were increased selectively in granulosa cells by estradiol and follicle-stimulating hormone. In addition: 1) ovarian R-II52/R52 subunits were purified to homogeneity and shown to recombine with C subunit from bovine heart to form a cAMP-dependent protein kinase; 2) pure R-II52/R52 were not interconvertible to a higher molecular weight form by C subunit-dependent phosphorylation; 3) pure rat heart R-II (\( M_r = 54,000 \)) and ovarian R-II52/R52 exhibited distinct differences based on one- and two-dimensional peptide mapping; and 4) by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis pure R-II52 were resolved as three (rather than two) isoelectric variants which were clearly different from pure rat heart R-II54. Thus, the hormone-regulated form of R-II in rat ovarian granulosa cells appears to represent a gene product distinct from R-II54 in rat heart.

One mechanism by which some hormones achieve their physiological effects is by increasing cAMP for activation of cAMP-dependent protein kinases (types I and II) which differ in their molecular weights, affinities for cAMP and CAMP analogs (Corbin et al., 1982; Robinson-Steiner and Corbin, 1983), their antigenicity (Fleischer et al., 1976; Kapoor et al., 1979; Munby and Beavo, 1981; Lohmann et al., 1980), their ability to be autophosphorylated (Corbin et al., 1975; Hofmann et al., 1975; Rosen and Erlichman, 1975), and their amino acid sequences (Takio et al., 1984; Titani et al., 1984). Additionally, subclasses of R-II from various tissues and species have been identified (Erlichman et al., 1980; Lohmann et al., 1980; Robinson-Steiner et al., 1984; Stein et al., 1984) by their different apparent molecular weights (based on mobility in SDS-PAGE), by whether or not the R-II is converted to a slower migrating form upon phosphorylation, and by their antigenicity. In particular, a bovine neural form of R-II appeared to be a distinct form of R-II since it is composed of a protein doublet rather than the usual singlet even when fully phosphorylated (Erlichman et al., 1980; Lohmann et al., 1980). Recently, observations of microheterogeneity of R-II from many sources have led to the conclusion that the variability in R-II is greater than originally realized (Robinson-Steiner et al., 1984). Further studies are required to ascertain if these forms of R-II are truly different gene products and to determine the significance of multiple forms of R-II.

In addition to these apparent differences in the microstructure of R-II subunits from various tissues, there are tissue-specific differences in the content of R-II and its regulation. For example, the amount of R-II in rat preovulatory ovarian follicles (i.e. those capable of ovulating in response to the luteinizing hormone surge) in vivo is increased 10-fold by estradiol and FSH (Richards and Rolfe, 1980; Richards et al., 1983; Richards et al., 1984) while the content of the catalytic subunit (C) is unchanged (Richards et al., 1984). Studies done in vitro have further demonstrated that the rat granulosa cell type R-II is increased not only by estradiol and

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† The abbreviations used are: R-I and R-II, the regulatory subunit of type I and type II cAMP-dependent protein kinase, respectively; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; C, catalytic subunit of cAMP-dependent protein kinase; FSH, follicle-stimulating hormone; EGTa, ethylene glycol bis(β-aminoethyl)ether)-N,N,N',N''-tetraacetic acid.
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FSH but also by estradiol and cAMP (Darbon et al., 1984; Ratoosh and Richards, 1985).

The present report identifies the hormonally regulated R-I of rat ovarian follicles as a specific R-II isofrom distinct from rat heart R-II.

EXPERIMENTAL PROCEDURES

Materials—Cayogen bromide–activated Sepharose 4B was purchased from Pharmacia, 8-(6-aminohexylamino)-cAMP from P-L Biochemicals, nitrocellulose paper from Sartorius, [32P]ATP and 8-Н3[32P]cAMP from ICN, V-form microtiter plates from Greiner, and anti-rabbit-alkaline phosphatase from Miles. Transylol (aprotinin) was obtained from Bayer, bovine serum albumin and histone H-AS (from calf thymus) from Sigma, hemoglobin from Serva, gelatin from Merck, and XR-1 film from Kodak. cAMP, 5’-AMP, and cGMP were from Boehringer Mannheim.

Animals—Immature female rats were hypophysectomized on day 26 of age by Hormone Assay Laboratories or Johnson Laboratory, Chicago, IL, and shipped the following day. Three days after surgery the rats were treated with subcutaneous injections of either 1.5 mg of estradiol-17β once daily for 3 days (H-E: preovulatory follicles), or sodium tetrathionate or biotin (H-E-FSH: granulosa). Estradiol-17β was dissolved and administered in 0.2 ml of propylene glycol; FSH was administered in 0.1 ml of phosphate-buffered saline containing 0.1% gelatin.

Isolation of Rat Granulosa Cells—Granulosa cells were manually expressed from rat ovaries as described by Zeleznik et al. (1983) modified from that described by Towbin et al. (1975), and the supernatants were used for experiments.

Preparation of Soluble Extracts—Tissues were homogenized in 4 volumes of PEMT buffer (10 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 10 mM β-mercaptoethanol, 50 Kallikrein-inhibitor units/ml of Transylol), the homogenates were centrifuged at 30,000 x g for 30 min at 4 °C, and the supernatants were used for cGMP and cAMP experiments.

Purification of R(ovary) R-II—Regulatory subunit R-II was purified from 1320 ovaries (21 g of tissue) of hypophysectomized rats treated as described above. The purification was performed according to published procedures (Corbin et al., 1978) with the following modifications. Regulatory subunits were eluted from the 8-(6-aminohexylamino)-cAMP-Sepharose column by PEMT buffer containing 8 mM urea. After dialysis against PEMT buffer, regulatory subunit and degradation products were separated on a DEAE-cellulose column (1 X 6 cm) equilibrated in PEMT buffer. Regulatory subunit was eluted with a 0.03-0.3 M NaCl gradient in PEMT buffer (total elution volume, 400 ml), and 5-ml fractions were collected. Fractions containing R-II were pooled, dialyzed, and concentrated, yielding 120 μg of R-II.

To obtain a higher yield of R-II a second purification (23 g of tissue) was performed. The urea eluate from the 8-(6-aminohexylamino)-cAMP-Sepharose column was dialyzed, concentrated, and used without subsequent purification by the DEAE gradient step. Omission of the DEAE gradient resulted in a yield of R-II of about 1.5 mg which included R-II (63%), two degradation products (36%), and minor contamination by other proteins (1%) as revealed by one-dimensional SDS-PAGE and silver staining.

The R-II from rat heart, bovine heart, and bovine brain were purified further after the affinity column using the gradient elution from DEAE as described above.

Antiserum—The antiserum against bovine heart R-II and rat heart R-II and R-I used in this study were prepared as described previously (Lohmann et al., 1980; Walter et al., 1985).

Cell Electrophoresis—For one-dimensional SDS-PAGE, samples diluted in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glyceral, 5% β-mercaptoethanol, 0.001% bromophenol blue) were boiled for 2 min and then applied to gels (Laemmli, 1970) consisting of a 3.45% stacking gel and a 7.5, 7.5, or 8% separating gel as indicated in the text. The method of two-dimensional SDS-PAGE was performed as described by O’Farrell (1975) with the modifications described by Richards et al. (1983). Proteins were visualized by a polychromatic silver-staining procedure described by Sammons et al. (1984).

8-Н3[32P]cAMP Incorporation—Photoaffinity-labeling experiments were performed essentially as described by Walter and Greenard (1983). The reaction mixture (final volume 100 μl) contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 1 mM ATP, 1 mM isobutylmethylxanthine, 2 μM β-mercaptoethanol, 1 mM EDTA, 40 Kalli- krein-inhibitor units/ml Trasylol, 1 μM 8-Н3[32P]cAMP (500 Ci/ mmol), and either 500 ng of purified R-II or 100 μg of cell extract. Incubations were carried out for 60 min at 4 °C in the dark with or without 100 μM unlabeled cAMP to monitor specific labeling. Cova lent incorporation was accomplished by exposing the reaction mixture to a UV lamp (model SL 2507 from Ultra Violet Products, Inc.) for 10 min at a distance of 15 cm, after which 50 μ1 of 30% SDS-sample buffer followed by boiling for 2 min before the samples were subjected to one-dimensional SDS-PAGE.

Phosphorylation of Purified R-II Using Unlabeled ATP—Purified R-II (2 μg) was phosphorylated in the presence of 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 20 μM cAMP, 10 μM β-mercaptopethanol, 1 μg of purified bovine heart catalytic subunit, and 100 μM unlabeled ATP in a final reaction volume of 110 μl. The samples were incubated for 10 min at room temperature, and the reactions were terminated by adding 50 μ1 of 3× SDS-sample buffer, followed by boiling for 2 min before the samples were subjected to one-dimensional SDS-PAGE. For comparison, samples of nonphosphorylated R-II were prepared in a similar manner except that no Κ subunit was added.

Phosphorylation of Purified R-II or Cell Extracts Using [γ-32P] ATP—Phosphorylation was performed as above except that the reaction volume was reduced to 20 μl, and 10 μl [γ-32P]ATP (50 Ci/ mmol) was used instead of unlabeled ATP.

Radioimmunodlubeling on Nitrocellulose Paper (Immmuno- blots)—Purified R-II subunits or soluble cell extract proteins from various tissues were resolved by one-dimensional SDS-PAGE and transferred from gels to nitrocellulose paper by a procedure (Lohmann et al., 1983) modified from that described by Towbin et al. (1979), Burnette (1981), and Vaessen et al. (1981). For immunolabeling, the nitrocellulose sheets were incubated with 1 μg of 125I-Protein A/10 ml of blocking media as described (Lohmann et al., 1983). Labeled nitrocellulose sheets were dried and exposed to film for autoradiography.

ELISA of R-II Subunit—Competitive ELISA assays were performed as described previously (Lohmann et al., 1985). Microtiter plates were coated with 4 μg of purified rat heart R-II/ well. Antiserum against rat heart R-II was diluted 1:4000. Either purified R-II (from rat heart or rat ovary) or R-II in soluble extracts of these tissues were tested for competition with the stationary phase R-II for binding to antibody.

Reconstitution of cAMP-dependent Protein Kinase Activity Using Purified Ovarian R-II and Catalytic Subunit—Purified ovarian R-II (1 μg) was recombined with purified bovine heart catalytic subunit (0.3 μg) in a 100-μ1 reaction mixture containing 10 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 10 μM CAMP, 10 μM β-mercaptoethanol, 100 μg of histone II-AS, 70 μM [γ-32P]ATP (0.1 Ci/ mmol) of C and 100 ng of R-II in the absence or presence of 10 μM cAMP.

One- and Two-dimensional Peptide Mapping—One-dimensional peptide mapping was performed as described by Cleveland et al. (1977). Purified rat heart and ovary R-II were run separately on an 8% one-dimensional SDS-PAGE and stained with Coomasie Blue. The bands of interest (approximately 3 μg of protein each) were cut out and rerun on a 15% one-dimensional SDS-PAGE gel after digestion of R-II in the stacking gel (3 cm) using 35 μg of Staphylococcus aureus V8 protease/well. The proteolytic fragments were visualized by polychromatic silver staining.

Tryptic fingerprinting of R-II subunits was performed using two-dimensional separation of radiolabeled peptides according to a procedure (Ross et al., 1985) modified from that of Elder et al. (1977). Pure R-II subunits were subjected to one-dimensional SDS-PAGE, then the gels were stained and destained, and the slices of gel containing the individual R-II subunits were cut out and washed with 50% methanol. The gel pieces were dried in a Speedvac and radioimmunoaassayed. Purified R-II (100 μg of cAMP) was preincubated with 100 μl of a suspension of 107 of E. coli cells, and the unbound fraction of the radiolabeled proteins with trypsin and two-dimensional separation of the resulting peptides by thin layer electrophoresis and chromatography were performed as described (Elder et al., 1977). Autoradiography of the thin layer sheets was performed at -70 °C using intensifying screens.
FIG. 1. Autoradiogram of 8-N$_2$[32P]cAMP incorporation into soluble extracts from rat brain, heart, and granulosa cell cytosol. Tissue extracts (100 µg of protein) were photoaffinity labeled in the absence (−) or presence (+) of 100 µM unlabeled cAMP and analyzed on a 7.5% one-dimensional SDS-PAGE gel. Granulosa cell extracts were prepared from hypophysectomized rats treated with estradiol (H-E) or estradiol plus FSH (H-E-FSH). Photoaffinity-labeled bands R-II$_{54}$, R-II$_{52}$, and R-II$_{51}$ refer to the different isoforms of the regulatory subunit of type II cAMP-dependent protein kinase in the rat ovary. R-I subunit is also shown.

Miscellaneous—Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

RESULTS

Identification of Different Isoforms of R-II in Rat Granulosa Cells

Soluble cell extracts of rat brain, heart, and ovarian granulosa cells were compared with respect to their content of proteins capable of incorporating the 8-N$_2$[32P]cAMP photoaffinity label (Fig. 1). These experiments were performed with 10 mM MgCl$_2$ and 1 mM ATP present to enable comparison of all R-II regulatory subunits in the phospho state, thus eliminating the variations in R-II mobilities observed on one-dimensional SDS-PAGE gels that are solely due to differences in phosphorylation state. The bands labeled with 8-N$_2$[32P]cAMP were shown to be specific cAMP-binding proteins because their labeling was prevented by the presence of excess unlabeled cAMP (Fig. 1). Rat brain extract showed two clear bands, $M_r$ 49,000 (R-I) and $M_r$ 52,000 (R-II), and a less distinct wider band above the 52,000 band. Rat heart contained R-II ($M_r$ 54,000) and R-I ($M_r$ 49,000). The designation of these as type I or II regulatory subunits was based not only on apparent molecular weight but also on their recognition on immunoblots by specific antibodies against the two different proteins (data not shown). Rat granulosa cell extracts contained several proteins labeled by 8-N$_2$[32P]cAMP. These have been designated R-II$_{54}$ ($M_r$ 54,000), R-II$_{52}$ ($M_r$ 52,000), R-II$_{51}$ ($M_r$ 51,000), and R-I ($M_r$ 49,000) based not only on the photoaffinity labeling observed in Fig. 1, but also on the distinct bands observed in the purified material (see below). Furthermore, the relative content of these R-II forms in granulosa cells changed in response to hormone treatments. Forms R-II$_{54}$ and R-II$_{52}$ were increased by estradiol and FSH (Figs. 1 and 2), whereas forms R-II$_{54}$ and R-I did not increase, but in many cases (as in Figs. 1 and 2) appeared to decrease.

To analyze the various forms of R-II further, immunoblots were made using two different antibodies (Fig. 2). Antibody made against rat heart R-II recognized R-II of $M_r$ 54,000 in rat brain and heart but only the uppermost band (R-II$_{54}$) of ovarian granulosa cell R-II (Fig. 2A). However, an antibody made against bovine heart R-II recognized not only R-II$_{54}$ in all tissue extracts, but also forms R-II$_{52}$ and R-II$_{51}$ in rat ovarian granulosa cells and a lower $M_r$ 52,000 R-II in rat brain (Fig. 2B). Note that as with photoaffinity labeling (Fig. 1), forms R-II$_{52}$ and R-II$_{51}$ were increased in granulosa cells treated with estradiol and FSH (lane 4), whereas the R-II$_{54}$ was more prominent in granulosa cells exposed to estradiol alone (lane 3).

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Purification—To permit further characterization of the hormone-regulated form of granulosa cell R-II, the subunit was purified from ovaries of hypophysectomized rats treated with estradiol and FSH. When R-II was eluted from a cAMP-analog Sepharose affinity column and resolved by one-dimensional SDS-PAGE, several bands were stained with Coomassie Blue (Fig. 3). The uppermost prominent bands were identified as R-II$_{52}$ and R-II$_{51}$ according to the similarity of their mobilities with those of R-II$_{54}$ and R-I from rat granulosa cell extracts (Fig. 1) and recognition by antibodies (Figs. 2 and 6). The lower bands ($M_r$, 41,000 and 34,000) appeared to be proteolytic fragments based on subsequent analyses (see Fig. 4). In the first purification of ovary R-II, these bands were partially removed by elution of the R-II from a DEAE-cellulose column with a salt gradient (data not shown). However, this resulted in substantial loss of R-II. Therefore, in the second purification (Fig. 3) the R-II was not purified further beyond the affinity column. From 29 g of...
analyzed on a 7.5% one-dimensional SDS-PAGE gel prior to autoradiography of purified R-I1 (500 ng each) from bovine brain purified from several tissues. Shown are autoradiograms of N3[32P]~AMP incorporation this preparation contained 63% R-I1 scanning of the Coomassie Blue-stained gel indicated that proteins were autophosphorylated with [y3'P]ATP and catalytic subunit (Fig. 4B). The unphosphorylated and phosphorylated R-I1 forms were directly compared in Fig. 4. Photoaffinity labeling and phosphorylation of R-II purified from several tissues. Shown are autoradiograms of 8-N3[32P]cAMP incorporation (A) and [γ-32P]ATP phosphorylation (B) of purified R-II (500 ng each) from bovine brain (lane 1), rat ovary (lane 2), rat heart (lane 3), and bovine heart (lane 4). Samples were analyzed on a 7.5% one-dimensional SDS-PAGE gel prior to autoradiography.

ovarian tissue, 1.5 mg of R-II was obtained. Densitometric scanning of the Coomassie Blue-stained gel indicated that this preparation contained 63% R-II (M, 52,000 and 51,000) and 36% breakdown products. When higher concentrations (20 µg) of purified ovarian R-II were analyzed by one-dimensional SDS-PAGE and Coomassie Blue staining, R-II54 was also present as a minor band above R-I152 and 54,000 in the dephosphorylated state to 53,000 which does not shift was observed. Rat brain and bovine heart R-I1 showed a small but distinct shift under phosphorylation conditions. In contrast, phosphorylation of purified ovarian R-I151 did not cause a significant shift in electrophoretic mobility.

Antibody Recognition of Ovary R-II—The recognition of purified R-II from rat ovary and other tissues by two different antibodies is compared in Fig. 6. Of the pure R-IIs (50 ng each) transferred to nitrocellulose, only rat heart R-II was recognized well by the antibody made against rat heart R-II (Fig. 6A). Bovine heart R-II was recognized less well, bovine brain and surprisingly rat ovary, not at all. Antibody against bovine heart R-II recognized all the different types of R-II, although rat ovary R-II the least well (Fig 6B). This antiserum also poorly recognized the Ms = 53,000 form of bovine brain R-II which is apparently the special neural form of R-II. On longer exposure to the film, rat ovarian R-II50 and R-II51 were visualized (Fig. 6C). These results contrast with the substantially higher immunolabeling of R-II which was observed in the granulosa cell extracts in Fig. 2B, lanes 3 and 4. Photoaffinity labeling of R-II in granulosa cell extracts was also greater than that in other tissues (Fig. 1). These results combined with the immunoblots and silver-stained two-dimensional SDS-PAGE gels (see below, Fig. 8) suggest that the content of R-II in granulosa cell extracts is markedly higher than that in rat brain or rat heart. Thus, the higher content of R-II in ovarian tissue appears to compensate for the poorer recognition of the granulosa cell R-II form by the anti-bovine heart R-II serum. The band (R-II54) labeled by 32P-Protein A. C is a longer exposure of part of the autoradiogram seen in B (rat ovary and rat heart lanes).
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Ovarian tissue (Fig. 7). The antiserum against rat heart R-II recognized purified rat ovarian R-II very poorly. However, this same antiserum detected approximately 4 ng of R-II/100 μg of whole ovary soluble extract. This would be expected if the purified ovarian R-II consisted of primarily R-II15 and R-II150 (Figs. 3–6), whereas the soluble extract of whole ovarian tissue contained in addition a protein (R-I154) which was antigenically similar to rat heart R-II (Fig. 2). In comparison, rat heart extract contained approximately 8 ng of R-II/100 μg of protein (Fig. 7), and rat brain extract contained 16 ng of R-II/100 μg of protein (data not shown), corresponding to about 1.5 and 3 pmol/mg of protein, respectively, similar to the amounts which have been determined by photoaffinity labeling (Walter et al., 1977, 1978).

Holoenzyme Reconstitution—To be certain that the cAMP-binding proteins purified from rat ovary were indeed regulatory subunits, their ability to recombine with C subunit (from bovine heart) to form a CAMP-stimulated protein kinase was examined. The data obtained clearly indicated that kinase activity could be inhibited by the ovarian R-II forms and restored by cAMP treatment of the holoenzyme. Protein kinase activity measured as pmol of 32P incorporated per min was 32.2 ± 0.4 for C subunit alone, 7.6 ± 0.3 for C recombined with ovary R-II, and 36.6 ± 3.0 for C recombined with ovary R-II and stimulated by cAMP.

Two-dimensional SDS-PAGE—To determine if the different isoforms R-II15, R-II150, and R-II151 could also be distinguished on the basis of charge, purified rat heart and ovarian isoforms were analyzed by two-dimensional SDS-PAGE and silver staining (Fig. 8). The purified ovarian isoforms (panel C) were observed to be slightly more basic than the purified rat heart R-II (panel D) when R-IIs of the two tissues were combined and analyzed together (panel E). Furthermore, R-II15 and R-II151 were resolved as three rather than two spots suggesting that rat ovarian R-II consists not only of R-II15 and R-II5 but also an intermediate form R-III15. Three spots (Fig. 8, arrows in panel A) present in granulosa cell extracts analyzed by two-dimensional SDS-PAGE and silver staining were also identified as the ovarian R-II forms based on their 8-N6[32P]cAMP incorporation and phosphorylation by [γ-32P]ATP as described previously (Richards et al., 1983). In contrast neither R-II in rat heart extract (Fig. 8, panel B) nor R-II5 in rat granulosa cell extract (Fig. 8, panel A) was observed by silver staining due to their lower abundance.

One- and Two-dimensional Peptide Maps—Since many of the foregoing results indicated that the rat ovary R-II had properties distinct from R-II derived from other sources, differences in their primary structures were investigated using one- and two-dimensional peptide mapping. In Fig. 9 rat ovary R-II is compared with heart R-II from the same species. One-dimensional peptide maps using S. aureus V8 protease as described by Cleveland et al. (1977) indicated that the peptide

![Fig. 7. Measurement of R-II by ELISA.](image)

![Fig. 8. Two-dimensional SDS-PAGE of R-II.](image)

![Fig. 9. One-dimensional peptide mapping of R-II.](image)
fragments generated from rat heart R-II (lane 1) were very different from those of both rat ovary R-II52 (lane 2) and R-II54 (lane 3). Differences between the peptide of R-II52 and R-II54 were not demonstrable by this method.

A more distinctive tryptic fingerprint of the peptides of each R-II was obtained by trypsin digestion of radioiodinated R-II followed by resolution of the peptide fragments using a two-dimensional system of electrophoresis and chromatography on thin layer cellulose sheets. The results, shown in Fig. 10, clearly demonstrated major differences in the peptides generated from rat heart R-II (A) and rat ovary R-II (B). This was further emphasized when a mixture of the two proteins was analyzed together (Fig. 10C).

**DISCUSSION**

In the present study we have purified and characterized the hormone-regulated isoforms (R-II52 and R-II54) of the regulatory subunit of the type II cAMP-dependent protein kinase present in rat ovarian granulosa cells. Several properties of these R-II forms identified them as regulatory subunits. They were able to bind cAMP analogs (either a cAMP analog on a Sepharose affinity column or the photoaffinity label, 8-N3[32P]cAMP); they were recognized, although poorly, by antibody against R-II; and they formed a CAMP-dependent protein kinase with C subunit.

However, by several criteria, ovarian R-II52 and R-II54 seemed to be isoforms which could be distinguished from R-II of several other tissues using immunological, electrophoretic, and peptide analyses. Antibodies against rat heart R-II did not recognize purified ovarian R-II52 or R-II54 (by ELISA or immunblot), and antibodies against bovine heart R-II recognized them poorly. The molecular weights (52,000 and 51,000) of ovarian R-II isoforms on one-dimensional SDS-PAGE were low compared to those of R-II from several other tissues. The isoelectric points of ovarian R-II52 and R-II54, determined from the isoelectric focusing dimension of two-dimensional SDS-PAGE, were slightly more basic than those of R-II from rat heart. Furthermore, phosphorylation of ovarian R-II52 and R-II54 by ATP and catalytic subunit did not cause a significant shift in either the isoelectric points or the molecular weight (decreased mobility) of these isoforms.

Peptide fragments generated from pure ovary R-II52 and R-II54 by the Cleveland method were clearly distinct from the fragments of pure rat heart R-II (Fig. 9). Two-dimensional tryptic fingerprint analyses of the rat heart and ovary R-II indicated that they have few peptides in common (Fig. 10). All of these results considered together demonstrate unique characteristics of the ovary R-II and suggest that this R-II isoform may represent a different gene product than the other R-II forms studied.

Although the rat heart and rat ovary R-II are apparently quite different, no differences between the two forms of ovary R-II (R-II52 and R-II54) were observed by one-dimensional peptide mapping (Fig. 9). These forms were not interconvertible by phosphorylation by catalytic subunit of the cyclic AMP-dependent protein kinase. We are presently investigating whether these forms perhaps differ from one another due to phosphorylation by some other protein kinase or due to other post-translational modifications.

In addition to forms R-II52 and R-II54, extracts of rat ovaries also contained R-II54 and R-I. On the basis of molecular weight, antibody recognition, and one-dimensional peptide maps (data not shown) ovarian R-II54 seemed similar (if not identical) to the form of R-II in rat heart. Photoaffinity labeling, immunolabeling, and silver staining of two-dimensional SDS-PAGE gels indicated that the content of both R-II54 and R-I in granulosa cell extracts is much less than that of isoforms R-II52 and R-II54. In addition, R-II54 and R-I appeared to decrease in cells treated with estradiol and FSH. This may explain why these forms were only detected when 20–30 µg of purified subunits obtained from estrogen and FSH-treated rat ovaries were examined by one-dimensional SDS-PAGE and Coomassie Blue staining.

As mentioned under “Results,” proteolytic fragments of apparent M, 42,000 and 34,000 were present in the eluate of the CAMP-Sepharose affinity column. Photoaffinity labeling of granulosa cell extract also revealed specific CAMP binding to R-I (M, 49,000) as well as lower molecular weight fragments (Fig. 1). However, none of our preparations contained a M, 50,000 fragment which could be characterized as a fragment of R-II. Therefore, the M, 50,000 CAMP-binding protein reported by Darbon et al. (1984) most likely represents R-I which 1) is present in granulosa cells isolated from ovaries of estradiol-treated immature rats prior to and during culture (Ratoosh and Richards, 1985), 2) is present in granulosa cells isolated from ovaries of hypophysectomized rats (Richards and Rolfe, 1980), and 3) can be shown by two-dimensional SDS-PAGE to have a much more basic isoelectric point, specific for R-I (Richards and Rolfe, 1980).

The existence of multiple isoforms of R-II in the rat ovary is additional evidence of the microheterogeneity recently described (Robinson-Steiner et al., 1984) for the regulatory subunit of cAMP-dependent protein kinase. These multiple forms do not appear to be explained as proteolytic fragments either by our studies or those of others (Robinson-Steiner et al., 1984). In particular, the poor recognition of R-II52 and R-II54 (in contrast to good recognition of R-II54) by a polyclonal antibody against rat heart R-II (Fig. 2A) would seem unlikely to occur in the case of R-II degradation, since this antibody recognizes other known (Kreb's and Beavo, 1973; Potter and Taylor, 1979; Rannels and Corbin, 1979; Weber and Hilz, 1979) R-II breakdown products of approximate M, 39,000 and 16,000.

Recent reports have demonstrated at least two forms of R-
II in rat brain (Mr, 54,000 and 52,000) (Panter et al., 1981; Strocchi et al., 1984), in Friend erythroblastic cells (Mr, 54,000 and 52,000) (Schwarz and Rubin, 1985), and in GH3 rat pituitary cells (Mr, 53,000 and 51,000) (Febbro et al., 1985). In rat brain, these forms were detected using two-dimensional SDS-PAGE and were shown to be localized mainly to smooth microsome and plasma membrane fractions (Strocchi et al., 1984). Our data (Figs. 1 and 2) distinguish two immunologically different forms of R-I1 (Mr, 54,000 and 52,000) in a soluble extract of rat brain as well. Of particular interest is the 52,000 form in rat brain which showed immunological similarities with R-IIa and R-IIb from the rat ovary and also did not shift its electrophoretic mobility significantly upon phosphorylation (data not shown). The two forms of R-I1 (Mr = 54,000 and 52,000) in Friend erythroblastic cells (Schwarz and Rubin, 1985) were shown to have different peptide maps, were not interconverted by phosphorylation, and were regulated selectivity during differentiation and in response to cAMP. Specifically, the relative synthesis of R-I1a, but not R-I1b, was increased 12-fold by 8-bromo-cAMP (Schwarz and Rubin, 1985). Whether or not R-I1 in rat brain (Mr, 52,000), Friend erythroblastic cells (Mr, 52,000), rat GH3 pituitary cells (Mr, 51,000), and rat ovaries (Mr = 51,000 and 52,000) represent a similar gene product remains to be determined.

Robinson-Steiner et al. (1984) have recently classified R-I1 from several sources into subclasses based on their apparent molecular weights and whether their mobilities shift on one-dimensional SDS-PAGE after phosphorylation. According to their results, rat heart R-I1 is classified as an R-I1 form that did not migrate slower on one-dimensional SDS-PAGE after phosphorylation. However, using our assay conditions, phosphorylation of rat heart R-I1 did result in a detectable mobility shift. It will be necessary to optimize assay conditions for the future study of small shifts in electrophoretic mobilities of proteins because their detection is highly dependent on the sensitivity of the separation procedure. Two-dimensional SDS-PAGE is an attractive alternative.

The unique feature of the ovary isoforms (Mr, 52,000 and 51,000) is that their content can be hormonally increased in neuroblastoma-glioma hybrids (Walter et al., 1979; Lohmann et al., 1983) and Friend erythroblastic cells (Schwarz and Rubin, 1983). In these tissues, as in the rat granulosa cells (Darbon et al., 1984; Richards et al., 1984; Ratoosh and Richards, 1985), the selective changes in the regulatory subunits appear to be regulated by cAMP and associated with differentiation.

In the future it will be important to determine 1) which of the different R-I1 isoforms are different gene products, 2) the functional significance of isoforms, and 3) how these isoforms are involved in cAMP mediation of estrogen and FSH stimulation of granulosa cell differentiation.

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