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The Regulatory Subunit of the Type II cAMP-dependent Protein Kinase in Rabbit Ovaries is the RIIβ Isoform

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Based on RII autophosphorylation, photoaffinity labeling with 8-N_3[^{32}P]cAMP, and Western blot analysis we have identified the RII isoform found in rabbit corpora lutea as RIIβ. The RIIβ subunit found in rabbit corpora lutea differs from the RIIβ found in rat follicles and corpora lutea in that it migrates at M_r 52,500 on SDS-PAGE and shifts to M_r 53,000 when phosphorylated.

The cAMP-dependent protein kinase holoenzmye forms, type I and type II, are distinguished from one another by their R subunits, designated as RI and RII. RI and RII differ in their antigenic and ionic properties, amino acid sequences, and ability to be autophosphorylated (1-6). Two RI and two RII isoforms have been identified recently. RIα and RIIα have been shown to be ubiquituously expressed (7,8), whereas RIβ expression is limited to brain and testis (9,10) and RIIβ is specific for neural, adipose, and some endocrine tissues (11-17). We have identified the RI isoform in rabbit corpora lutea as RIα (18). In this communication we identify the RII isoform present in rabbit corpora lutea as RIIβ.

Materials and Methods

Materials-The following materials were purchased: [γ-^{32}P]ATP (3000 Ci/mmol) from DuPont-New England Nuclear; 8-N_3[^{32}P]cAMP (25-100 Ci/mmol), [2,8-^{3}H]cAMP (sodium salt, 15-40 Ci/mmol), and ^{125}I-protein A from ICN Chemical and Radioisotope Division; DEAE-cellulose (DE52) from Whatman; SDS-PAGE protein standards from Boehringer Mannheim Biochemicals; electrophoresis reagents from Bio-Rad; hCG from Serono. All other biochemical reagents were purchased from Sigma.

Animals-New Zealand White rabbits, retired breeders, were housed in air-conditioned quarters and allowed free access to water and a commercially pelleted

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Abbreviations: RI and RII, the regulatory subunits of cAMP-dependent protein kinases type I and type II, respectively; hCG, human chorionic gonadotropin; iv, intravenous(ly); 8-N_3[^{32}P]cAMP, 8-azidoadenosine 3':5'-mono[^{32}P]phosphate; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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food. To induce ovulation and corpora lutea formation, rabbits were injected with hCG (100 IU, iv). hCG-injected rabbits are referred to as pseudopregnant. Rabbits were killed at indicated times by cervical dislocation; ovaries were quickly removed and placed in iced homogenizing buffer A (10mM Tris-HCl, pH 7.0, 3mM $MgCl_2$, 25mM benzamidine, and 0.32M sucrose). Corpora lutea were then rapidly dissected.

Preparation of Soluble Extracts and Ion-exchange Chromatography-The preparation of soluble extracts from corpora lutea and subsequent DEAE-cellulose chromatagraphy have previously been described (19).

Protein Kinase Assay-Protein kinase activity was determined as described by Hunzicker-Dunn et al. (19) with the exception that 50μM [γ-^{32}P]ATP was used.

Photoaffinity labeling of RII-Aliquots (40μl) of column fractions were incubated with 2.0μM 8-N_3[^{32}P]cAMP in 10mM $MgCl_2$, 1mM ATP (to encourage RII autophosphorylation; indicated as MgATP) or 4mM EDTA (to discourage RII autophosphorylation) for 30 min at 30°C in the dark in the absence and presence of 0.1mM cAMP to determine specificity of photoaffinity labeling. Following irradiation, samples were denatured by boiling in a 3X "stop" solution (0.5 vol) for 5 min (20). Proteins were separated by SDS-PAGE, with 5% acrylamide stacking and 10.5% acrylamide running gels (21) at 50mA/gel. Gels were dried and autoradiographed at -70° using Kodak XRP-5 or XAR-5 film and DuPont intensifiying screens. Radioactively labeled bands were identified on autoradiographs as R subunits by their migration relative to protein standards and by loss of label in samples incubated with 0.1mM cAMP.

Autophosphorylation-RII phosphorylation was achieved by incubating 50μl aliquots of indicated DEAE-cellulose column fractions for 15 min at 37°C in a total volume of 67μl as previously described (22).

Western Blot Analysis-SDS-polyacrylamide gels with prestained standards (Diversified Biotech) were electrophoretically transferred to Nytran™ membranes (0.2μm, Schleicher and Schuell) for 1h at 0.9A using a Hoeffer Transphor apparatus. Blots were blocked for 30 min at 42°C in PBS containing 5% Carnation non-fat dry milk and incubated overnight in the same buffer containing a 1:50 dilution of anti-RII antisera. Blots were then washed twice for 30 min in PBS containing 0.15% Triton X-100, incubated with goat anti-rabbit IgG antibody, and washed again by the procedure of Burnette (23). Antigen-antibody complexes were detected with ^{125}I-protein A (0.5μCi per lane). Blots were washed as described above with the addition of a final 30 min wash in PBS alone, dried, and autoradiographed as described above.

Results and Discussion

Separation of cAMP-dependent Protein Kinases in Rabbit corpora lutea by DEAE-cellulose chromatography-Previously we demonstrated the presence of both type I and type II cAMP-dependent protein kinases in rabbit corpora lutea by DEAE-cellulose chromatography (18; Fig. 1). The R subunits associated with the kinase peaks eluting from DEAE-cellulose were identified by RII autophosphorylation and photoaffinity labeling. Based on elution location, kinase peak 1 represents type I cAMP-dependent protein kinase, kinase peak 2 represents a cAMP-independent protein kinase, and kinase peak 3 represents type II cAMP-dependent protein kinase.

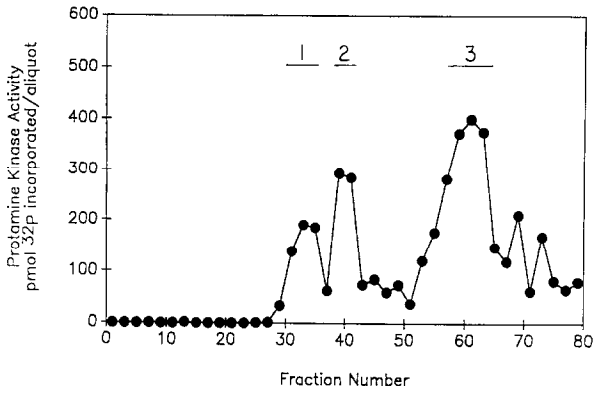

Fig. 1. **DEAE-cellulose elution profile of cAMP-dependent protein kinases in soluble 6-day old corpora lutea extracts.** Protamine kinase activity present in column fractions as determined in the presence (•) of 0.45μM cAMP. 15.5 mg of total protein loaded onto column.

Identification of RIIβ by Autophosphorylation, Photoaffinity labeling and Immunoreactivity-In contrast to rat RIIα, which exhibits a M_r 54,000 that shifts to 56,000 when autophosphorylated, rat RIIβ exhibits a M_r 51-52,000, does not significantly shift M_r when autophosphorylated, and has distinct antigenic reactivity compared to RIIα (19, 24-26). In order to identify the RII isoform present in rabbit corpora lutea as an RIIα or RIIβ, peak 3 fractions from the DEAE-cellulose elution profile of soluble 6-day old corpora lutea extracts (Fig. 1) were subjected to autophosphorylation, photoaffinity labeling, and Western blot analysis. Photoaffinity labeling was done using 8-N_3[^{32}P]cAMP in the presence of MgATP (*i.e.*, RII autophosphorylation conditions) or EDTA (*i.e.*, conditions in which phosphorylation should not occur). A protein band at M_r 53,000 was labeled with 8-N_3[^{32}P]cAMP in the presence of MgATP (Fig.2, lane7). When the incubation was conducted in the presence of EDTA, bands at M_r 52,500 and 53,000 were photoaffinity labeled (Fig.2, lane 8). Neither of these bands was labeled in the presence of 0.1mM cAMP (Fig.2, lane 6). A protein band migrating with M_r 53,000 was phosphorylated in column fractions representing peak 2a when these fractions were incubated with [γ-^{32}P] ATP under RII autophosphorylation conditions (Fig. 2, lanes 1-5). For Western blot analysis, samples were incubated at 37°C for 15 min in the presence and absence of MgATP, and MAB87, a monoclonal antibody generated against bovine brain RII which binds both RIIα and RIIβ but preferentially binds RIIα (17), was used for this analysis. When incubations were conducted in the absence of MgATP, two bands migrating with M_r 52,500 and 53,000 were detected (Fig.3A, lane 4), whereas when incubations were conducted in the presence of MgATP, only the band at M_r 53,000 was detected (Fig.3A, lane 3). In Fig.3A both M_r 52,500 and 53,000 are clearly seen, but the RII-52 (RIIβ) standard was not detected. When the experiment was repeated (Fig. 3B), the RIIβ standard was detected but the distinction between M_r 52,500 and 53,000 was not as clear. Protein bands at M_r 54,000 in rabbit extracts (lanes 3-4) were not detected with MAB87. Confirmation of the identity of the RII isoform was obtained using RII-

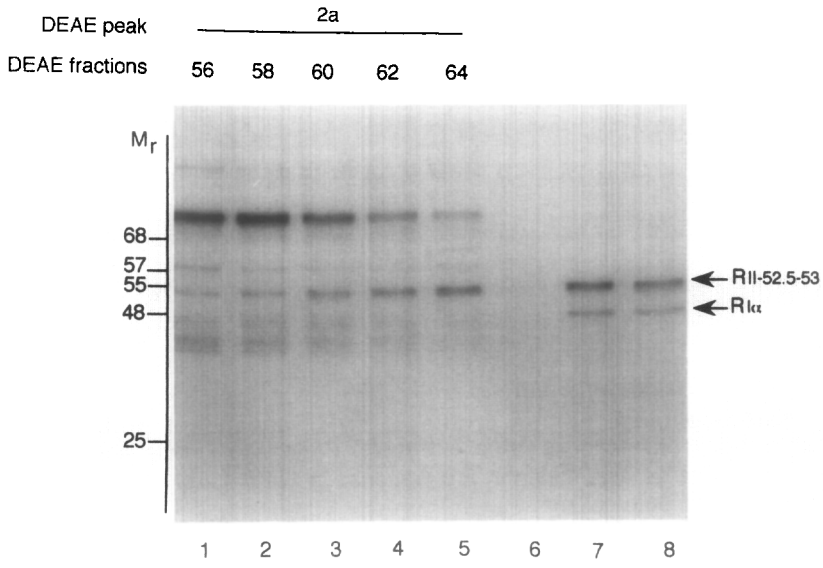

Fig. 2. **Identification of RII by photoaffinity labeling and autophosphorylation of fraction aliquots from a DEAE-cellulose elution profile of soluble 6-day old corpora lutea extracts.** In *lanes 1-5*, even-numbered fractions corresponding to cAMP-dependent kinase peak 3 from the DEAE-cellulose elution profile in Fig. 1 were incubated with [γ-^{32}P]ATP under autophosphorylation conditions. *Lane 6* represents photoaffinity labeling of DEAE fraction 61 with 1μM 8-N_3[^{32}P]cAMP in the presence of 0.1mM cAMP. *Lane 7* represents photoaffinity labeling of DEAE fraction 61 in the absence of 0.1mM cAMP and in the presence of MgATP (RII autophosphorylation conditions). *Lane 8* represents photoaffinity labeling of DEAE fraction 61 in the absence of 0.1mM cAMP and in the presence of 4mM EDTA. M_r values were calculated by linear regression of the relative migration position of the following protein standards: BSA, pyruvate kinase, glutamate dehydrogenase, fumarase, and ovalbumin. Molecular weights (M_r X 10^{-3}) of select protein standards are indicated at *left*. *Arrow* at *right* indicates phosphoproteins at M_r 52,500-53,000, as well as photoaffinity labeled RII-52.5,53 and RIα.

51,52 antisera which was raised against rat brain RII-52 and preferentially binds RII-51 and RII-52 (RIIβ) over RII-54 (RIIα; 11). RII-51,52 antisera immunoreacted strongly with a M_r 53,000 protein band present in pooled peak 2a fractions from a 7-day old corpora lutea column (Fig. 4, lane 2). Reactivity of this antisera with RIIα was not detected unless a long exposure was used (Fig. 4, lane 1). These results demonstrate that the RII isoform present in rabbit ovarian tissues is RIIβ based upon its immunoreactivity and size.

The RIIβ subunit present in rabbit ovarian tissues is unique in that it migrates at M_r 52,500 on SDS-polyacrylamide gels and shifts to M_r 53,000 upon autophosphorylation. RIIβ in rat corpora lutea migrates on SDS-polyacrylamide gels at M_r 52,000 and does not shift its M_r on phosphorylation (19). In rat granulosa cells, the initial product of RIIβ mRNA translation is a protein with M_r 51,000. Later, RII-51 is converted to RII-52. Both forms of RIIβ in rat granulosa cells are autophosphorylated, but autophosphorylation does not cause a shift in the M_r of either form (24-26). In other tissues in which RIIβ has been identified (rat corpora lutea, 19; rat granulosa

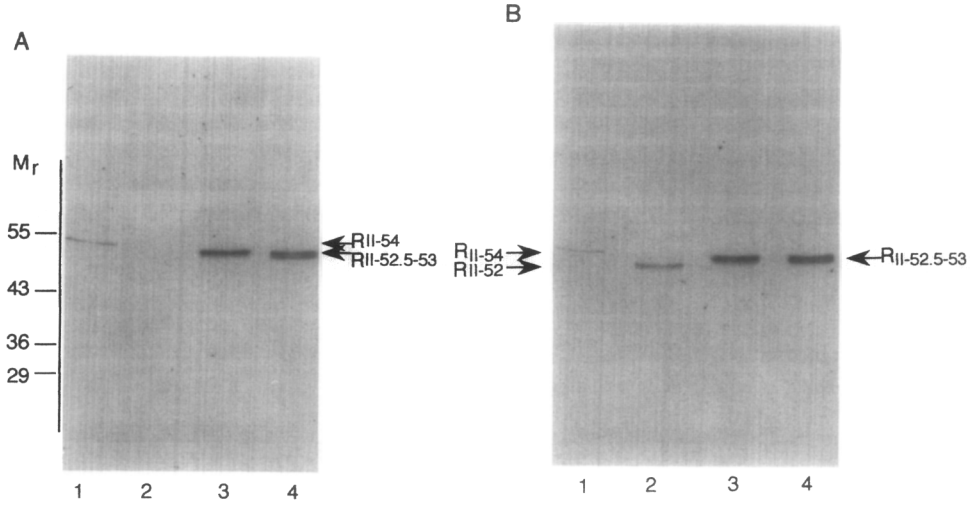

Fig. 3. **Identification of RII by Western blot analysis using MAB87.** Western blot protocols are as described in "Materials and Methods." DEAE-cellulose fraction 61 representing cAMP-dependent kinase peak 3 of elution profile in Fig. 1 was incubated for 15 min at 37°C in the presence and absence of 10mM $MgCl_2$, 10μM ATP (RII autophosphorylation conditions). *Lane 1* represents RII-54 (RIIα) from rat heart. *Lane 2* represents RII-52 (RIIβ) from peak 2 (RIIβ holoenzyme peak) of a rat granulosa cell DEAE-cellulose elution profile. *Lane 3* represents fraction 61 incubated under autophosphorylation conditions. *Lane 4* represents fraction 61 incubated in the absence of 10mM $MgCl_2$, 10μM ATP. *Arrows* indicate RII-54 and RII-52.5,53. *A and B* represent two different Western blots containing the same samples with the exception of the RII-52 (RIIβ) standard. RII-52 from peak 3 (catalytic subunit-free RIIβ peak) of a rat granulosa cell DEAE-cellulose elution profile was used as a standard in Fig. 3B.

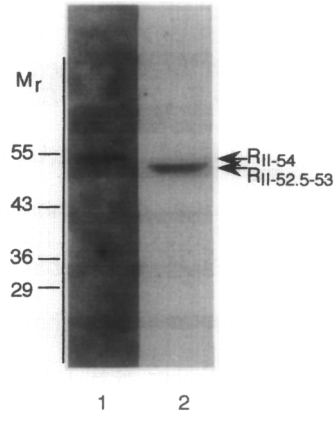

Fig. 4. **Identification of RII by Western blot analysis using an antibody specific for RII-51,52.** Pooled column fractions from a DEAE-cellulose elution profile of soluble rabbit 7-day old corpora lutea extracts were subjected to Western blot analysis using RII-51,52 antisera provided by Dr. J.S. Richards, Baylor College of Medicine, Houston, Texas. *Lane 1* represents RII-54 (RIIα) standard from rat heart. *Lane 2* represents pooled column fractions from a DEAE-cellulose elution profile of soluble rabbit 7-day old corpora lutea extracts. Lanes 1 and 2 represent different exposures of the same gel (9 and 3 days, respectively). Molecular weights (M_r X 10^{-3}) of select prestained protein standards are indicated at *left*. Location of RIIβ at M_r 52,500-53,000 is indicated by *arrow* at *right*.

cells, 12, 24; rat testis, 27), RIIα is also present and elutes with RIIβ as a complex holoenzyme(s). However, in rabbit corpora lutea, RIIα was not detected.

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