Separation of the Complexes Formed between the Regulatory and Catalytic Subunits of Cyclic Adenosine Monophosphate-Dependent Protein Kinase and Topoisomerase I Activity in Preovulatory Follicle-Enriched Immature Rat Ovaries

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Our previous studies have shown that the regulatory subunits of the type II form of cAMP-dependent protein kinase (R') present in soluble extracts of immature rat ovaries elute from diethylaminoethyl-cellulose as three separate peaks of activity, based on their association with the catalytic subunit (C) of this enzyme, as $R'_5C_5$, an apparent $R'_4C_5$, and $R'_6$. Based upon the existence of ovarian $R'$ in three different subunit arrangements, the large amount of C subunit-free $R'_6$ in this tissue, and a previous report which indicated that $R'^6$ exhibited intrinsic topoisomerase I activity, we determined whether DNA topoisomerase I activity was associated with any of these molecular complexes of the ovarian $R'$ subunits. Cyclic AMP-binding activities in soluble extracts of preovulatory follicle-enriched immature rat ovaries were separated by diethylaminoethyl-cellulose chromatography and sucrose density gradient centrifugation. Topoisomerase I activity co-sedimented with the apparent $R'_4C_5$ and $R'_6$, obtained from sucrose gradients but was not detected in fractions containing $R'_5C_5$. Upon cAMP affinity purification of the $R'$ derived from fractions containing $R'_4C_5$, $R'_5C_5$, and $R'_6$, respectively, no topoisomerase I activity could be detected in any fraction. Phosphorylation of the affinity purified $R'$ by the C subunit of beef heart cAMP-dependent protein kinase did not alter this result. These data indicate that none of the $R'$ subunits in soluble extracts of preovulatory follicle-enriched ovaries exhibit intrinsic topoisomerase I activity. (Molecular Endocrinology 3: 780–789, 1989)

INTRODUCTION

Many of the actions of LH and FSH in ovarian cells are mediated by cAMP (1). The only known mechanism by which cAMP effects cellular function is by activating cAMP-dependent protein kinases (2). Upon activation, these enzymes catalyze the phosphorylation of cellular proteins (unidentified in the ovary), thereby initiating a cascade of intracellular events which culminate in altered cellular functions, including steroidogenesis and receptor induction (3).

Cyclic AMP-dependent protein kinases are tetrameric holoenzymes composed of two regulatory (R) subunits and two catalytic (C) subunits. Upon the binding of cAMP to the R subunits, with a stoichiometry of 2 mol cAMP/mol R, the C subunits are freed and the enzyme is activated.

Two major classes of cAMP-dependent protein kinases are present in cells, type I and type II. The major distinguishing feature between the two classes of cAMP-dependent protein kinase is the cAMP-binding R subunit. R subunits of the type I enzyme (R) and type II enzyme (R') differ in their molecular weights, their ionic properties, their antigenicity, their ability to be autophosphorylated, their affinities for cAMP and the C subunit, and their amino acid sequences (4–11). Historically the two enzyme forms are separated by diethylaminoethyl (DEAE)-cellulose chromatography, their distinct elution position based upon the ionic properties of the R subunits (12). The more basic type I enzyme elutes with less than or equal to 0.1 M salt; the more acidic type II enzyme elutes with greater than or equal to 0.1 M salt.

Recently, two isoforms of R' have been shown to exist (13–16). The rat ovary contains both the R'' form
(M, = 54,000) and R\texttextsuperscript{14} form (M, = 51,000 and 52,000) (15). Richards and colleagues (16, 17) have shown that estradiol plus FSH selectively increases the transcription of R\texttextsuperscript{14} in granulosa cells. In fact, the highest quantities of R\texttextsuperscript{14} are found in granulosa cells from estrogen and FSH-primed rats (18). However, this increase in R\texttextsuperscript{14} subunit is not associated with a concomitant increase in C subunit activity or protein (19, 20), suggesting that R\texttextsuperscript{14} exists in the rat ovary free of C subunit activity (i.e. not as R\texttextsuperscript{14}C\texttextsubscript{2} but as R\texttextsuperscript{14}).

Studies in my laboratory have evaluated the oligomeric complexes formed by the R and C protein kinase subunits present in ovarian cells, separating them by DEAE-cellulose chromatography. We have shown that R\texttextsuperscript{1} exists in soluble extracts of immature rat ovaries in three molecular associations (21): 1) as R\texttextsuperscript{1}C\texttextsubscript{2}, the tetrameric holoenzyme, eluting off a DEAE-cellulose as peak 2, the major peak of cAMP-stimulated protein kinase activity, and migrating on sucrose density gradient sedimentation with an S\texttextsubscript{20, w} of 7.4; 2) as an apparent R\texttextsuperscript{1}C\texttextsubscript{3}, based on its sedimentation position on sucrose density sedimentation analysis between R\texttextsuperscript{3}C\texttextsubscript{2} and R\texttextsuperscript{1}C\texttextsubscript{3}, eluting off DEAE-cellulose as peak 3a; 3) and as free R\texttextsuperscript{1}, eluting off DEAE-cellulose as peak 3b, the major peak of cAMP binding activity, and migrating on sucrose density gradient sedimentation with an S\texttextsubscript{20, w} of approximately 5.2. (These studies did not distinguish the different isoforms of R\texttextsuperscript{1} although C subunit-free R\texttextsuperscript{1} is predicted to be composed primarily of R\texttextsuperscript{1}. The functional significance of the multiple associations of R\texttextsuperscript{1} with and without the C subunit is not known.)

A recent study showed that rat liver R\texttextsuperscript{1} exhibited intrinsic topoisomerase activity (22). Topoisomerases are enzymes that alter the degree of DNA supercoiling (23–25) and are thought to be involved in the regulation of gene transcription (26). Because R\texttextsuperscript{1} is present in the ovary in association with C subunit as R\texttextsuperscript{1}C\texttextsubscript{2}, as an apparent R\texttextsuperscript{1}C\texttextsubscript{3}, and as C subunit-free R\texttextsuperscript{1} (21); because both R\texttextsuperscript{1} and R\texttextsuperscript{1} are expressed in ovarian cells (15); and because of the presence of a large amount of C subunit-free R\texttextsuperscript{1} in granulosa cells of the ovary, we determined whether any of these R\texttextsuperscript{1} forms exhibited topoisomerase I activity. In these studies, CAMP-dependent protein kinase is a soluble extract of PMSG-primed (preovulatory follicle enriched) immature rat ovaries were separated by DEAE-cellulose chromatography and sucrose density gradient centrifugation into the three molecular associations of R\texttextsuperscript{1}C\texttextsubscript{2}, the apparent R\texttextsuperscript{1}C\texttextsubscript{3}, and R\texttextsuperscript{1}. Although topoisomerase I activity was associated with the apparent R\texttextsuperscript{1}C\texttextsubscript{3} form and the R\texttextsuperscript{1} forms obtained from sucrose density gradient centrifugation, cAMP affinity purified R\texttextsuperscript{1} derived from these fractions exhibited no detectable topoisomerase I activity. While this manuscript was in preparation, Shabb and Graner (27) showed that affinity purified R\texttextsuperscript{1} from various tissues, including R\texttextsuperscript{1} from the rat granulosa cells, did not exhibit topoisomerase I activity and Sikorska et al. (28) reported that rat liver R\texttextsuperscript{1} did not exhibit intrinsic topoisomerase activity.

RESULTS

We previously reported that R\texttextsuperscript{1} in soluble extracts of immature rat ovaries eluted from DEAE-cellulose as three separate peaks of activity, based on its association or lack of association with the C subunit of CAMP-dependent protein kinase (21). Namely, R\texttextsuperscript{1} eluted as R\texttextsuperscript{1}C\texttextsubscript{2}, the tetrameric holoenzyme of CAMP-dependent protein kinase; as R\texttextsuperscript{1}C\texttextsubscript{3}, an apparent trimeric holoenzyme of CAMP-dependent protein kinase (based on its sedimentation position on sucrose density gradient analysis); and as R\texttextsuperscript{1}, C subunit-free R\texttextsuperscript{1} (21). The purpose of the present study was to determine if DNA topoisomerase activity was selectively associated with one or more of these molecular arrangements of ovarian R\texttextsuperscript{1}.

Preovulatory follicle- enriched ovaries from PMSG-primed immature rats were used, due to the increased levels of R\texttextsuperscript{1} in this tissue (20; Hunziker-Dunn, M., personal observation). A representative DEAE-cellulose elution profile of the cAMP-binding and CAMP-dependent protein kinase activity present in soluble extracts of preovulatory follicle-enriched ovaries is shown in Fig. 1A. R\texttextsuperscript{1} is present in DEAE-cellulose peaks 2, 3a, and 3b, identified by its molecular weight on photoaffinity labeling with 8-N\texttextsuperscript{2}P\texttextsuperscript{32}P-cAMP and by its susceptibility to autophosphorylation (Fig. 1B).

To obtain a further separation of the different subunit associations of R\texttextsuperscript{1}, DEAE-cellulose fractions from peaks 2 and 3 (2a + 3b) were pooled and subjected to sucrose density gradient sedimentation. A representative result is shown in Fig. 2. A and B. The CAMP-dependent protein kinase and associated CAMP-binding activities from DEAE-cellulose peak 2 sedimented between fractions 8–16 with an S\texttextsubscript{20, w} of approximately 7.5 (Fig. 2A), appropriate for R\texttextsuperscript{1}C\texttextsubscript{2}. The CAMP binding activity sedimenting with the hemoglobin standard (fractions 19–23) represents C subunit-free R\texttextsuperscript{1} (Footnote 1) (Fig. 2A). In Fig. 2B, C subunit-free R\texttextsuperscript{1} from DEAE-cellulose peak 3 sedimented with hemoglobin (fractions 20–24) while the peak of cAMP-stimulated protein kinase activity and associated CAMP-binding activity from DEAE-cellulose peak 3 sedimented between fractions 15–18 with an S\texttextsubscript{20, w} of approximately 6.0 consistent with the existence of an R\texttextsuperscript{1}C trimeric form of CAMP-dependent protein kinase. Thus, R\texttextsuperscript{1} in extracts of preovulatory follicle-enriched ovaries, like that in immature rat ovaries, exists free of C subunit, as R\texttextsuperscript{1}C\texttextsubscript{2} and as an apparent R\texttextsuperscript{1}C\texttextsubscript{3}.

To determine if R\texttextsuperscript{1} in ovarian extracts exhibited topoisomerase activity, an aliquot from pooled fractions of the sucrose gradients containing R\texttextsuperscript{1}C\texttextsubscript{2}, R\texttextsuperscript{1}C\texttextsubscript{3}, and R\texttextsuperscript{1} were assayed for topoisomerase I activity. Topoisomerase I activity was detected by the appearance of the relaxed forms of DNA (0) migrating on agarose gels as intermediates between the supercoiled form of

\textsuperscript{1} This conclusion is based on the sedimentation position of the peak of cAMP-binding activity (~5.05) and on specific labeling with 8-N\texttextsuperscript{2}P\texttextsuperscript{32}P-cAMP (not shown; 21).
Fig. 1. Resolution of cAMP-Stimulated Protein Kinase and cAMP-Binding Activities in Soluble Extracts Prepared from Ovaries of PMSG-Treated Immature Rats

A. Cytosol (109 mg) prepared from ovaries of PMSG-primed immature rats was applied to a DEAE-cellulose column and protein kinases were eluted with a linear salt gradient. Aliquots of column fractions were assayed for protein kinase activity in the presence of 0.5 μM cAMP (●) and for cAMP binding activity (△), as described in Materials and Methods. Equivalent DEAE-cellulose elution profiles have been obtained with more than 15 separate experiments. A representative potassium phosphate gradient is shown. Based upon our previous designations (21), peak 2 represents a tetrameric type II holoenzyme of cAMP-dependent protein kinase, peak 3a represents an apparent trimeric type II holoenzyme of cAMP-dependent protein kinase, and peak 3b represents G subunit-free R₀. B. SDS-PAGE of autophosphorylated and 8-Ν₆[^32P]cAMP-labeled cAMP-dependent protein kinase R subunits. Lanes 1–8 show[^32P] incorporation; lanes 9–16 show 8-Ν₆[^32P]cAMP binding to R₀ in the absence (lanes 9, 11, 13, 15) and presence of 0.1 mM cAMP (lanes 10, 12, 14, 16). Autophosphorylation reactions were conducted in the absence of exogenously added cAMP as described in Materials and Methods. Aliquots of fractions from DEAE peak 1 (lanes 1, 2 and 9, 10), peak 2 (lanes 3, 4 and 11, 12), peak 3a (lanes 5, 6 and 13, 14) and 3b (lanes 7, 8 and 15, 16) are shown. The azido[^32P]cAMP analog labels R₀ subunits irrespective of their association with the C subunit of cAMP-dependent protein kinase. However, under the phosphorylation conditions used (i.e. in the absence of exogenously added cAMP), only R₀ associated with the C subunit of cAMP-dependent protein kinase in the holoenzyme form should be (autophosphorylated. C subunit-free R₀ in peak 3b should not be accessible to cAMP-dependent protein kinase-catalyzed phosphorylation. The phosphorylation of R₀ seen in lanes 7, 8 (peak 3b) must either reflect trailing cAMP-dependent protein kinase from peak 3a and/or phosphorylation of R₀ catalyzed by another kinase (35). Thus, 8-Ν₆[^32P]cAMP labeling studies more accurately reflect the amount of R₀ than the autophosphorylation data. Only R₀ and R₀ are specifically labeled by 8-Ν₆[^32P]cAMP. The identity of the various proteins phosphorylated in lanes 1–8 other than R₀ are not known.
Fig. 2. Separation of Rat Ovarian Type II Protein Kinases by Sucrose Density Gradient Sedimentation

Pooled DEAE-cellulose fractions from peak 2 (A) and from peak 3 (B) were concentrated and subjected to sucrose density gradient centrifugation. Aliquots of gradient fractions were assayed for protein kinase activity in the presence of 0.5 μM cAMP (●) and for cAMP binding activity (○). Hgb refers to the sedimentation position of the hemoglobin standard. Equivalent sucrose density gradient profiles have been obtained with more than 10 separate experiments. Aliquots from pooled sucrose gradient fractions were assayed for topoisomerase I activity (C), by their ability to convert supercoiled pBR322 plasmid DNA (I) into its relaxed form (II), as described in Materials and Methods. The plasmid also contained a small amount of the nicked form (III). The negative control (lane 1) contained the reaction mixture without the nuclear extract, i.e., in the absence of any source of topoisomerase I. The positive control (lane 2) contained the reaction mixture and 5 U purified calf thymus topoisomerase I (as defined by BRL, where 1 U is the amount of enzyme required to convert 1 μg of form I to form II pBR322 DNA in 30 min at 37°C). Experimental sample additions to the reaction mixtures included the following: a 9-μl aliquot (0.04 pmol [3H]cAMP bound) of fractions nos. 8–15 pooled from the sucrose gradient shown in A (derived from DEAE-cellulose peak 2) incubated in the absence (lane 6) and presence of 0.15 mM cAMP (lane 3); a 9-μl aliquot (0.05 pmol [3H]cAMP bound) of fractions nos. 12–17 pooled from the sucrose gradient shown in B (derived from DEAE-cellulose peak 3) incubated in the absence (lane 7) and presence of 0.15 mM cAMP (lane 4); and a 9-μl aliquot (0.29 pmol [3H]cAMP bound) of fractions nos. 21–25 pooled from the sucrose gradient shown in panel B (derived from DEAE-cellulose peak 3) incubated in the absence (lane 8) and presence of 0.15 mM cAMP (lane 5). Generally equivalent results were obtained in additional experiments. Specifically, aliquots of sucrose gradient fractions obtained from DEAE-cellulose peak 2 did not exhibit topoisomerase activity (in the absence or presence of cAMP, or after autophosphorylation of R2), while aliquots of sucrose gradient fractions obtained from DEAE-cellulose peaks 3a and 3b consistently exhibited topoisomerase activity (but only in the presence of exogenously added cAMP in two of three experiments).
DNA (I) and the nicked form (II) in conjunction with a reduction of the supercoiled DNA form (I). As shown in Fig. 2C, R_2^\text{5}C (lanes 4 and 7) and R_1^\text{5} (lanes 5 and 6) but not R_1^\text{5}C (lanes 3 and 6) exhibited topoisomerase I activity. Lane 1 is a negative control and contains no source of topoisomerase I, while lane 2 is a positive control and contains 5 U purified calf thymus topoisomerase I. The association of topoisomerase activity with sucrose gradient fractions containing enriched preparations of R_2^\text{5}C and R_1^\text{5}, but not with fractions containing R_2^\text{5}C, was consistently seen in two additional experiments. When topoisomerase activity was measured in each fraction of the sucrose gradient profile of DEAE-cellulose peak 3 (Fig. 3A), the topoisomerase activity clearly aligned with the peak protein kinase activity (R_2^\text{5}C; Fig. 3B).

Constantinou et al. (22), reported that only the auto-phosphorylated form of R^\text{5} which contained bound cAMP exhibited topoisomerase activity. The inclusion of cAMP in our topoisomerase assay yielded inconsistent results. In the experiment shown in Fig. 2, cAMP did not affect the presence or absence of topoisomerase activity associated with the different R^\text{5} forms (lanes 6–8 — exogenous cAMP, lanes 3–5 + 0.15 mC cAMP). In two other experiments (not shown), R_2^\text{5} and R_2^\text{C} exhibited topoisomerase activity only when cAMP was included in the assay. It should be noted that R_2^\text{5} isolated off sucrose gradients contains bound cAMP (as measured by RIA 1.9 pmol cAMP/pmol [3H]cAMP bound; Hunzicker-Dunn, M., personal observation); however, due to the overlap of free R_2^\text{5} into the kinase peak, we do not know if R_2^\text{5} contains bound cAMP.

Results so far have demonstrated that DNA topoisomerase I activity is consistently associated with sucrose density gradient fractions enriched with R_2^\text{5}C and usually with R_1^\text{5}, but never with sucrose gradient fractions enriched with R_2^\text{C}. We next determined whether topoisomerase I activity was intrinsic to purified R^\text{5}. To this end, R^\text{5} was affinity purified using cAMP-Sepharose affinity chromatography from pooled sucrose gradient fractions enriched with R_2^\text{5}C and R_1^\text{5}, and R_2^\text{5}. Purification to homogeneity was not achieved, as evidenced by the presence of three lightly stained bands in addition to R^\text{5} on Coomassie stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (not shown). However, the prominence of R^\text{5} is seen on the autoradiogram (Fig. 4A) in which R^\text{5} derived from R_2^\text{5}C (lane 1), R_2^\text{C} (lane 2), and R_2^\text{5} (lane 3) was phosphorylated using exogenous beef heart C subunit. No phosphorylation was detected when exogenous C subunit was omitted. A longer exposure of the gel (10 days with an intensifying screen) did not reveal the presence of any additional phosphorylated protein bands. To determine if this affinity purified R^\text{5} exhibited intrinsic topoisomerase activity, aliquots of the samples were assayed for their ability to relax supercoiled DNA. Results are shown in Fig. 4B. No topoisomerase I activity was detected (lanes 1–12; lane 13 is a negative control and contains no source of topoisomerase I; lane 14 is a positive control and contains 5 U purified calf thymus topoisomerase I). Since R^\text{5} was eluted with 8 mm cAMP from the affinity column, these R^\text{5} preparations are presumed to contain bound cAMP. It is unlikely that cAMP at this high concentration is hydrolyzed, especially since cAMP bound to R subunits is resistant to phosphodiesterases (23). In a second experiment, we evaluated the effect of the phosphorylation of R^\text{5} (isolated as described and in the same concentration as in Fig. 4) on the topoisomerase activity. Affinity purified R^\text{5} was preincubated under phosphorylation conditions (see Materials and Methods) in the absence and presence of beef heart catalytic subunit of cAMP-dependent protein kinase, and then assayed for topoisomerase activity. No topoisomerase activity was detected (not shown).

CONCLUSIONS

The R subunit of the type II forms of cAMP-dependent protein kinase, R^\text{5}, in soluble extracts of immature rat ovaries elutes from DEAE-cellulose as three separate peaks, based on its association with the C subunit of cAMP-dependent protein kinase (21), as R_2^\text{5}C, R_2^\text{5}C, and R_2^\text{5}. The purpose of the studies reported in this manuscript was to determine if DNA topoisomerase I activity was associated with any of these molecular complexes of the R^\text{5} subunits. The rationale for this series of experiments was based on an earlier report that rat liver R^\text{5} exhibited intrinsic topoisomerase activity (22). We felt it was reasonable to determine if topoisomerase I activity was selectively associated with one of the subunit arrangements of R^\text{5} present in ovarian cells, based upon the unusually high concentration of R^\text{5} in preovulatory follicle granulosa cells (18), approximately 50% of which is not associated with cAMP-dependent protein kinase subunits (21; Fig. 2B) and for which no function is known.

Our results show that R^\text{5} also exists in extracts of preovulatory follicle-enriched immature rat ovaries (as in extracts of immature rat ovaries), as R_2^\text{5}C, as an apparent R_2^\text{5}C, and as R_2^\text{5}. Indeed, the extremely large amount of C subunit-free R^\text{5} activity present in this tissue extract can be seen from the data presented in Fig. 2B, especially when one compares the amount of cAMP-binding activity associated with the cAMP-dependent kinase activity sedimenting with sucrose gradient fractions 15–18 with that sedimenting with fractions 20–23.

We have shown that DNA topoisomerase I activity cosediments with R_2^\text{5}C and, usually, with R_2^\text{5}, but not with R_2^\text{5}C on sucrose density gradient centrifugation. However, topoisomerase I activity is not associated with any of the forms of rat ovarian R^\text{5} eluted from the cAMP-affinity resin. R^\text{5} eluted by cAMP-affinity chromatography exhibits many functional aspects of R^\text{5} R-subunit activity. It binds cAMP, based on its ability to be eluted from the affinity resin with cAMP, it is auto-phosphorylated by the cAMP-dependent protein kinase catalytic subunit (as shown in Fig. 4A), and it reasso-
Fig. 3. Cosedimentation on Sucrose Density Gradient Centrifugation of Topoisomerase I Activity with Protein Kinase Activity Derived from DEAE-Cellulose Peak 3

A. Sucrose gradient sedimentation analysis of pooled and concentrated fractions from DEAE-cellulose peak 3. Aliquots of gradient fractions were assayed for protein kinase activity in the presence of 0.5 μM cAMP (●) and for cAMP binding activity (▲). B. Aliquots (10 μl) of gradient fractions were assayed for topoisomerase I activity, as described in the legend to Fig. 2C (in the absence of exogenous cAMP). The numbers below the gel lanes represent sucrose gradient fraction numbers. (The band that appears above form II is due to a dimeric component of this form).

A specific function for R₄ separate from its ability to inhibit the C activity of cAMP-dependent protein kinase towards exogenous substrates thus remains unknown. This question is of particular interest in preovulatory ovarian cells, where so much of the cAMP binding activity exists as C subunit-free R₄.
MATERIALS AND METHODS

Materials

Biochemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. [γ-32P]ATP, ammonium salt (SA > 4000 Ci/mmol), 8-Br-cAMP (SA, 25–100 Ci/mmol), and 2,6,9-Br-cAMP, sodium salt (SA, 15–40 Ci/mmol) were purchased from ICN Chemical and Radioisotope Division (Irvin, CA). DEAE-cellulose (DE 52) was obtained from Whatman Inc. (Clifton, NJ). SDS-PAGE protein standards were from Boehringer Mannheim Biochemicals (Indianapolis, IN), and electrophoresis reagents were purchased from Bio-Rad (Richmond, CA). Millipore filters (HAWP 205, 0.45 μm pore size) and ultratransfer units (CX-10 and CX-30) were from Millipore Corporation (Bedford, MA). Negatively supercoiled pBR322 was purchased from Promega Biotech (Madison, WI); calf thymus topoisomerase I was purchased from Bethesda Research Laboratories (Gaithersburg, MD), and 8- (6-aminohexylamino)-cAMP- Sepharose 4B was obtained from Pharmacia LKB Biochemicals (Piscataway, NJ). Final concentrations are indicated throughout.

Animals

Weaning-aged female rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA), as Sprague-Dawley CD outbreeds and were housed as previously described (30). Prewashed and freeze-dried enteral enemas were obtained by injecting 24 to 28 day-old-rats with 25 IU PMSG, sc. Rats were killed between 1100 and 0200 h, 66–68 h after the injection. Ovaries were quickly removed, cooled to 4 °C in an ice-cold buffer consisting of 10 mM TRIS-HCl, 3 mM MgCl2, 0.32 mM sucrose, 10 μM EGTA, 25 mM benzamidin, pH 7.0 (buffer A), and dissected free of ovarian bursa, oviducts, and extraneous tissues.

Preparation of Soluble Extracts

Ovaries were homogenized in 5 ml buffer A using 15 strokes with a ground glass homogenizer. A supernatant fraction (cytosol) was obtained by centrifuging the homogenate at 105,000 × g for 70 min. All procedures were conducted at 4°C.

DEAE-cellulose Chromatography

Cytosol containing 100–200 mg protein was applied to a column (2 × 8 cm) of DEAE-cellulose. The column was washed with 10 mM potassium phosphate buffer, pH 7.0, until eluate was free of material that absorbed at 280 nm. Protein kinases were eluted with a linear salt gradient between solutions of 10 mM to 1 M phosphate, pH 7.0, containing 100 μM EGTA and 25 mM benzamidine in a total volume of 212 ml. Fractions containing approximately 0.75 ml each were collected in tubes containing EGTA and phenylmethylsulfonyl fluoride such that final concentrations were 1 mM and 0.5 mM, respectively. Fifty-microliter aliquots of odd fraction were assayed for protein kinase activity and 100-μl aliquots were assayed for [3H]cAMP binding activity. No phosphotransferase or cAMP-binding activities were detected in the column flow through or wash eluates. Remaining details are as previously described (21).

Sucrose Density Gradient Centrifugation

DEAE-cellulose column fractions were pooled and concentrated approximately 10– to 15-fold with Millipore immersible ultratransfer unit. A 200-μl to 300-μl aliquot of the concentrated sample and 50 μl standard (15 mg/ml hemoglobin) were centrifuged in a Beckman SW 40 Ti rotor at 40,000 rpm for 18 h in 5.5–12% sucrose gradients containing 3 mM β-mercaptoethanol, 10 μM EGTA, and 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation, 400-μl fractions (~32) were collected and aliquots of each fraction were assayed for protein kinase activity (50 μl) in the presence of cAMP in a 15-min reaction and for cAMP binding activity (100 μl), as described below. Additional details are as previously described (21).

Protein Kinase and [3H]cAMP Binding Assays

Phosphotransferase activity was determined in the presence of 0.5 μM cAMP using 100 μg protein and 38 μM [γ-32P]ATP, as previously described (21), unless otherwise indicated. [3H]cAMP binding activity was determined according to the method of Corbin et al. (31), with modifications, in a final volume of 150 μl in a buffer consisting of 50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM MgCl2, 25 mM benzo- midine, 0.5 mM methylisobutyl xanthine, 0.17 mg/ml histone, 0.7 mM NaCl, and 0.3 μM [3H]cAMP, incubating 5 min at 30°C, then 1 h at 4°C. Fiftypicogram of total binding sites are detected with this assay (Hunzicker-Dunn, M., personal observation). Quantification of label is on Millipore filters.

Identification of Rα

To photoflat labelling the regulatory subunit, aliquots of column fractions were incubated with 1.0 μg 8-Br-cAMP in the presence of 10 mM MgCl2 and 1 mM ATP to encourage Rα phosphorylation, thereby eliminating variations in Rα mobility on SDS-PAGE gels that are due to a difference in the auto-phosphorylation state) for 60 min at room temperature in the absence of cAMP to determine dephosphorylated proteins. In the presence of Rα, cAMP, as described by Hunzicker-Dunn et al. (21). Proteins were separated by SDS-PAGE, with a 5% acrylamide stacking gel and an 8% or 10% acrylamide running gel (32). Radioactively labeled bands on autoradiographs were identified as Rα and Rβ by their migration relative to protein standards and by the abolition of label in samples incubated with 0.1 mM cAMP.

To phosphorylate Rα, 50-μl aliquots of column fractions were incubated 15 min at 37°C in a total volume of 67 μl containing 5 μCi 10 μM [γ-32P]ATP, 50 mM Tris-HCl, pH 7.0, and 10 mM MgCl2 in the absence of added cAMP (21). To phosphorylate to affinity purified Rα, 75 μl sample and 2.5 μl (as defined by Sigma) of bovine heart cAMP-dependent protein kinase C subunit (freshly diluted from a lyophilized powder) were incubated 15 min at 37°C in a total volume of 110 μl containing 5 μCi 10 μM [γ-32P]ATP, 50 mM Tris-HCl, pH 7.5, and 10 mM MgCl2. Glycerol was then added to a final concentration of 20%, and the sample was quick frozen and stored overnight at −70°C. On the following day, 40 μl of a 3-fold SDS-PAGE stop solution (33) were added, samples were boiled 3 min, and subjected to SDS-PAGE. For samples to be assayed for topoisomerase I activity, an equivalent set of reactions were performed up to the addition of glycerol, omitting the [γ-32P]ATP. Identification of phosphorylated protein bands on SDS-PAGE as Rα was by migration relative both to protein standards (33) and to 8-Br-cAMP-labeled Rα.

Topoisomerase Assays

The relaxation of negatively supercoiled pBR322 DNA was examined in a total volume of 20 μl containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM dithiothreitol, 0.1 mM NaN3, 30 μg/ml BSA, 1 μg DNA, and sample or standard, as indicated. The reactions were started by the addition of DNA. After a 30-min incubation at 37°C, the reactions were terminated by the addition of 4 μl 0.1 mM NaEDTA, 0.5% SDS, 60% sucrose, 0.05% bromphenol blue, and 0.05% xylene cyanol. The reaction products were analyzed by electrophoresing half of the sample volume on 0.8% agarose gels in TBE buffer (89 mM Tris-borate, pH 8.2, 2 mM EDTA) at 5 V/cm for 3 h. Alternatively, electrophoresis, the gels were stained with 1 μg/ml ethidium bromide, destained in 1 mM
Fig. 4. Absence of Topoisomerase Activity in Affinity Purified R1 Subunits

Cytosol (170 mg) prepared from ovaries of PMSG-primed immature rats was applied to a DEAE-cellulose column and protein kinases were eluted with a linear salt gradient (as shown in Fig. 1). Fractions from peaks 2 and 3 were pooled, concentrated, and subjected to sucrose density gradient centrifugation (as described in Fig. 2). Peak protein kinase activity fractions from the sucrose gradient obtained from DEAE-cellulose peak 3 (1.9 ml) and peak 3a (1.6 ml) and peak cAMP-binding activity fractions from the sucrose gradient obtained from DEAE-cellulose peak 3b (1.3 ml) were pooled, respectively, and R1 was purified from the three samples by cAMP affinity chromatography, eluting R1 with 0.65 ml of buffered cAMP (8 μM), as described in Materials and Methods. (Thus, based on volumes, R1 was concentrated 2- to 3-fold on affinity purification. This conclusion is substantiated by the amount of 32P incorporated into R1 detected on SDS-PAGE before and after affinity purification [not shown]). A, Autoradiogram of an 8% SDS-PAGE gel of the affinity purified phosphorylated R1 subunits derived originally from DEAE-cellulose peak 2 (lane 1, 1.5 μg protein), peak 3a (lane 2, 1.5 μg protein), and peak 3b (lane 3, 2.0 μg protein). The gel was exposed to x-ray film for 3 days with an intensifying screen. B, Aliquots (4 μl, lanes 1, 3, 5, 7, 9 and 11; and 8 μl, lanes 2, 4, 6, 8, 10 and 12) of affinity purified R1 derived from DEAE-cellulose peak 2 (lanes 1–4), peak 3a (lanes 5–8) and peak 3b (lanes 9–12) were assayed for topoisomerase I activity, as described in the legend to Fig. 2C. Samples were preincubated at 37 C for 15 min in the presence (lanes 1, 2, 5, 6, 9, 10) or absence (lanes 3, 4, 7, 8, 11, 12) of 50 mM TRIS-HCl, pH 7.5, 10 μM ATP, 10 mM MgCl2 (in the absence of exogenous C subunit); glycerol was added to 20% final concentration, and samples were frozen at −70 C for less than 24 h before their assay for topoisomerase I activity. In lane 13 the source of topoisomerase I was omitted from the reaction mixture (negative control), and lane 14 contains 5 U calf thymus topoisomerase I (positive control). Equivalent results (i.e. the absence of detectable topoisomerase activity) were obtained in a second experiment (starting with 109 mg ovarian cytosol protein) in which affinity purified R1 obtained from DEAE-peaks 2, 3a, and 3b (after sucrose density gradient centrifugation) were preincubated under phosphorylation conditions (as described above) in the presence and absence of 2.5 U exogenous beef heart C subunit (Sigma).
Affinity Purification of Rβ

Rβ was purified from pooled fractions obtained after sucrose density gradient centrifugation by the method of Dils et al. (29), by affinity chromatography on 8-β-aminohexylamino-cAMP-Sepharose 4B (Pharmacia, type 3). Rβ was eluted with 8 μM cAMP in a buffer containing 0.1 M NaCl, 10 mM potassium phosphate, 20 mM benzamidazole. Glycitol was added to the eluate to a final concentration of 20%. Samples were quick frozen and stored overnight at −70°C or subjected to the phosphorylation reaction described under Identification of Rβ. SDS-PAGE of the affinity purified Rβ demonstrated that three additional Coomassie stained bands were visible, in addition to Rβ, between Mr 60,000-68,000 (not shown). However, phosphorylation reactions in the absence of exogenous C subunit yielded no labeling, and phosphorylation in the presence of exogenous C subunit yielded phosphorylation of only Rβ (as shown in Fig. 4B).

Various Other Methods

Protein was determined according to the technique of Lowry et al. (34), using crystalline BSA as a standard.

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