# Identification of cAMP-dependent protein kinase holoenzymes in preantral- and preovulatory-follicle-enriched ovaries, and their association with A-kinase-anchoring proteins

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Undifferentiated cells from preantral (PA) follicles respond to high levels of cAMP in a different manner than do differentiated cells from preovulatory (PO) follicles. We hypothesized that this differential response of PA and PO cells to cAMP could be due, in part, to either a difference in the profile of isoforms that comprise the cAMP-dependent protein kinase (PKA) holoenzymes and/or a difference in the interaction of PKA with A-kinase-anchoring proteins (AKAPs). To test these hypotheses, PKA activity, PKA holoenzymes, PKA subunits and AKAPs from PA and PO ovaries were compared. Soluble PKA holoenzymes and regulatory (R) subunits were separated by DEAE-cellulose chromatography and sucrose-density-gradient centrifugation. PKA R subunits were distinguished by photoaffinity labelling, autophosphorylation, size, isoelectric point and immunoreactivity. AKAPs were identified by RII subunit overlay assays and immunoreactivity. The results showed that extracts from PA and PO ovaries exhibited equivalent PKA holoenzyme profiles and activities, characterized by low levels of PKA type I (PKAI) holoenzyme and two distinct PKAII holoenzyme peaks, one containing only RII$\beta$ subunits (PKAII$\beta$) and one containing both PKAII$\beta$ and PKAII$\alpha$ holoenzymes. Both PA and PO ovarian extracts also contained PKA catalytic (C)-subunit-free RI$\alpha$, while only PO ovaries exhibited C-subunit-free RII$\beta$. Consistent with the elevated levels of C-subunit-free RII$\beta$ in PO cells, PKA activation in PO cells required higher concentrations of forskolin than that in PA cells. While extracts of PA and PO ovaries exhibited a number of similar AKAPs, including four prominent ones reactive with anti-AKAP-KL antisera (where AKAP-KL is an AKAP especially abundant in kidney and liver), cAMP–agarose affinity chromatography revealed two major differences in AKAP binding to purified R subunits. PO ovaries contained increased levels of AKAP80 (AKAP of 80 kDa) bound selectively to R subunits in DEAE-cellulose peak 2 (comprising PKAII$\beta$ and RI$\alpha$), but not to R subunits in DEAE-cellulose peak 3 (comprising PKAII$\alpha$, PKAII$\beta$ and RII$\beta$). PO ovaries also showed increased binding of R subunits to AKAPs reactive with anti-AKAP-KL antisera at 210, 175, 150 and 115 kDa. Thus in PO ovaries, unlike in PA ovaries, the majority of AKAPs are bound to R subunits. These results suggest that altered PKA–AKAP interactions may contribute to the distinct responses of PA and PO follicles to high levels of cAMP, and that higher cAMP levels are required to activate PKA in PO ovaries.

Key words: AKAPs, cAMP, granulosa cells, ovarian differentiation, PKA.

## INTRODUCTION

The differentiation of ovarian granulosa cells from a preantral (PA) to a preovulatory (PO) phenotype is dependent on follicle-stimulating hormone (FSH) and is mimicked by either relatively low or high levels of cAMP [1,2]. This differentiation response of PA granulosa cells is characterized by the induction of receptors for luteinizing hormone (LH) and prolactin, steroidogenic enzymes, inhibin and gap junction proteins [1,2]. Differentiation of PO granulosa cells into luteal cells, however, requires surge levels of LH and resulting high cAMP levels, and is characterized by the down-regulation of a number of genes that are induced by FSH in PA granulosa cells, including those for the LH and FSH receptors, cytochrome P450 aromatase, inhibin and gap junction proteins, as well as by the induction of other genes, such as that encoding the progesterone receptor [2]. Based on the distinct responses of PA compared with PO granulosa cells to cAMP, we hypothesized that these responses are mediated, at least in part, by different cAMP effectors, the cAMP-dependent protein kinases (PKAs).

The PKAs are tetrameric holoenzymes, each comprising a dimeric regulatory (R) subunit and two catalytic (C) subunits. PKA holoenzymes are classified as type I (PKAI) or type II (PKAII), depending on the R subunit. Several gene products of the R and C subunits have been identified, and include four C subunits (C$\alpha$, C$\beta$1, C$\beta$2 and C$\gamma$), two RI subunits (RI$\alpha$ and RI$\beta$) and two RII subunits (RII$\alpha$ and RII$\beta$) [3,4]. R subunits can be distinguished by antigenicity, molecular mass, isoelectric points and potential for autophosphorylation. RII isoforms will autophosphorylate; RI isoforms will not [4]. In rat tissues, the molecular masses of the R subunits are as follows: RI$\alpha$, 49 kDa [5]; RI$\beta$, 53 kDa [6]; RII$\alpha$, 54 kDa [7]; RII$\beta$, 51–52 kDa [8].

Abbreviations used: PA, preantral; PO, preovulatory; AKAP, A-kinase-anchoring protein; AKAP-KL, AKAP especially abundant in kidney and liver; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PMSG, pregnant mare serum gonadotropin; PKA, cAMP-dependent protein kinase; C subunit, PKA catalytic subunit; R subunit, PKA regulatory subunit; DTT, dithiothreitol; IEF, isoelectric focusing; PKI, PKA inhibitor peptide; StAR, steroidogenic acute regulatory protein.

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Studies in the present paper were designed to identify the R subunits associated with PKA holoenzymes in the ovaries of hypophysectomized rats, enriched in PA follicles, and in the ovaries of rats primed with pregnant mare serum gonadotropin (PMSG), enriched in PO follicles.

Alternatively, we hypothesized that the distinct responses induced by cAMP in PA compared with PO follicles are mediated, in part, by the binding of distinct PKAII isoforms to distinct A-kinase-anchoring proteins (AKAPs). It is well established that PKAIIs are tethered to intracellular structures via high-affinity binding to a family of AKAPs [9,10]. RII dimers bind with nanomolar affinity to AKAPs [11,12]. RII–AKAP complexes, in turn, are anchored through the targeting domain of the AKAP to cell-specific locations, including the actin cytoskeleton for AKAP-KL (an AKAP especially abundant in kidney and liver) [13], the plasma membrane for AKAP18 (AKAP of 18 kDa) [14] and AKAP79 [15–17], the nuclear matrix for AKAP95 [18], the centrosome/Golgi network for AKAP350 [19,20] and AKAP85 [21], the mitochondria for sperm sAKAP84 [22], the sarcoplasmic reticulum for AKAP100 [23,24], and the microtubules for microtubule-associated protein 2 [25]. At least 25 different AKAPs have been described, based largely on their ability to bind to $^{32}$P-labelled RII subunits *in vitro* in a solid-phase overlay assay [9]. The best studied AKAP is the bovine protein AKAP75, originally described by Rubin and co-workers [26]; the murine analogue has a molecular mass of 150 kDa (AKAP150), while the human homologue has a molecular mass of 79 kDa (AKAP79) [16,17,26,27]. AKAPs are proposed to optimize substrate phosphorylation by PKA by targeting a pool of PKA to a specific cellular site. For example, PKA-activated gene transcription in a thyroid cell line was inhibited when cells were transfected with a mutated AKAP lacking the N-terminal targeting domain [28]; PKA-dependent cell differentiation responses were restored in a mutant PC12 cell line on co-transfection with AKAP75 and PKA C subunit [29]; and PKA-catalysed phosphorylation and functional activation of the cardiac L-type $Ca^{2+}$ channel was restored by AKAP79 [30]. Restoration of cellular responses by transfection with AKAPs has consistently resulted in the relocation of a portion of the RII subunits from a cytosolic to a particulate fraction [14,16,28,29], suggesting that optimal PKAII responses are dependent on the cellular location of PKAII.

To determine whether distinct PKAII holoenzymes bind one or more AKAPs, we have evaluated the DEAE-cellulose elution profiles of AKAPs in soluble extracts of ovaries from hypophysectomized rats (enriched in PA follicles) and from PMSG-primed rats (enriched in PO follicles). We have used ovaries enriched in PA or PO follicles as models of undifferentiated and differentiated follicles respectively. While these ovaries contain a heterogeneous population of cells, the predominant tissue in these organs consists of follicle-enclosed granulosa cells [31,32]. Results show that, while expression of PKA holoenzymes and AKAPs 210, 175, 150 and 115 is not regulated, expression of AKAP80 (as previously reported [33,34]) and binding of these AKAPs to distinct pools of R subunits is increased on differentiation of ovaries from a PA to a PO phenotype.

## EXPERIMENTAL

### Materials

Ovine FSH (oFSH-20) was kindly provided by Dr. A. F. Parlow of the NIDDK National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Torrance, CA, U.S.A.). The following materials were purchased: [$\gamma$-$^{32}$P]ATP (ammonium salt; specific radioactivity 3000 Ci/mmol) from Dupont–New England Nuclear (Boston, MA, U.S.A.); 8-$N_3$-[$^{32}$P]cAMP (specific radioactivity 25–200 Ci/mmol) and [2,8-$^3$H]cAMP (sodium salt; specific radioactivity 15–40 Ci/mmol) from ICN Chemical and Radioisotope Division (Irvine, CA, U.S.A.); DEAE cellulose (DE-52) and P-81 cellulose phosphate paper from Whatman Inc. (Clifton, NJ, U.S.A.); enhanced chemiluminescence (ECL®) reagents, Rainbow molecular mass markers and Hybond TM-EL nitrocellulose membranes from Amersham Life Science (Arlington Heights, IL, U.S.A.); SDS/PAGE reagents from Bio-Rad (Richmond, CA, U.S.A.); and protein standards from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.) or Amersham. All other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), unless otherwise indicated.

### Preparation of soluble ovarian extracts

Sprague–Dawley rats (Charles River Laboratories) were obtained at 18–21 days of age and were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals at the Northwestern University Animal Care Facility. All protocols used were approved by the Northwestern University ACUC Committee. Rats at 21 days of age were hypophysectomized by Charles River Laboratories and were used 7–14 days after surgery. PO ovaries were obtained by injecting 24–26-day-old rats with 25 units of PMSG (subcutaneous); rats were killed $\sim$ 54 h later. Ovaries were quickly removed, cooled to 4 °C in an iced 10 mM potassium phosphate buffer, pH 7.0, dissected free of ovarian bursa, fat, and oviducts, weighed and homogenized (27-fold weight × volume) in buffer A (10 mM Tris/HCl, pH 7.0, 3 mM $MgCl_2$, 0.32 M sucrose, 10 $\mu$M EGTA and 25 mM benzamidine), buffer B (10 mM Tris/HCl, pH 7.0, 5 mM EDTA, 1 mM EGTA, 0.32 M sucrose, 5 $\mu$g/ml pepstatin, 5 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 50 $\mu$g/ml soybean trypsin inhibitor, 10 mM benzamidine and 10 $\mu$g/ml E-64 [27]) or buffer C [10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM $\beta$-glycerol phosphate, 1 mM sodium orthovanadate, 2 mM dithiothreitol (DTT), 40 $\mu$g/ml PMSF, 0.5 % Nonidet P40 and 0.1 % deoxycholate], as indicated elsewhere, using 15 strokes with a ground-glass homogenizer. Supernatant fraction (cytosol) was obtained by centrifuging the homogenate either at 105000 ***g*** for 70 min when using buffer A or for 30 min with buffer B, or at 17000 ***g*** for 5 min using buffer C. All procedures were conducted at 4 °C.

### DEAE-cellulose chromatography, protein kinase activity, cAMP binding assays and photoaffinity labelling

DEAE-cellulose chromatography was conducted as previously described [35]; fractions of 0.75 ml were collected into tubes containing 50 $\mu$l of a concentrated cocktail of protease inhibitors (buffer D) at the following final concentrations: 10 mM benzamidine, 2 mM DTT, 5 $\mu$g/ml pepstatin, 10 $\mu$g/ml leupeptin, 5 $\mu$g/ml aprotinin and 5 $\mu$g/ml soybean trypsin inhibitor. Protein kinase activity was determined [35] in the absence of cAMP or in the presence of either 0.5 $\mu$M cAMP or cAMP plus a saturating concentration of the heat-stable PKA inhibitor (PKI) [34,36] using 100 $\mu$g of protamine sulphate or 71.5 $\mu$M Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate.

cAMP RIAs and total [$^3$H]cAMP binding sites (see Table 1 and Figure 1) were determined as described previously, the latter by incubating the sample with 0.3 $\mu$M [$^3$H]cAMP for 30 min at 30 °C [35]. When indicated (see Table 2), [$^3$H]cAMP binding sites were determined by incubating the sample with 0.04 $\mu$M [$^3$H]cAMP for 60 min at 4 °C [37]. This R subunit assay yields equivalent relationships to the assay for total [$^3$H]cAMP binding

sites for R subunits present in PKA holoenzymes, but underestimates C-subunit-free R subunits, presumably due to the presence of endogenously bound cAMP, which requires higher [$^3$H]cAMP concentrations to be displaced completely. R subunits were photoaffinity-labelled with 8-$N_3$-[$^{32}$P]cAMP by incubation with 1 $\mu$M 8-$N_3$-[$^{32}$P]cAMP in the presence of 5 mM EGTA, 10 mM $MgCl_2$ and 1 mM ATP in the absence or presence of 0.1 mM cAMP for 30 min at 30 °C, followed by irradiation and denaturation for SDS/PAGE or trichloroacetic acid precipitation to concentrate proteins for isoelectric focusing (IEF) [35].

### Antibodies

The following antibodies were used. Anti-RII MAB87, kindly provided by Dr. Jack Erlichman (Albert Einstein College of Medicine, Bronx, NY, U.S.A.), is a monoclonal antibody generated against bovine brain RII which binds both RII$\alpha$ and RII$\beta$, but preferentially binds RII$\alpha$ [38]. Anti-AKAP79 was kindly provided by Dr. Charles Rubin (Albert Einstein College of Medicine); anti-ezrin was kindly provided by Dr. Heinz Furthmayr (Stanford University, Stanford, CA, U.S.A.). Affinity-purified anti-AKAP-KL is a polyclonal antibody made to a fusion protein containing amino acids 354–741 of AKAP-KL. An antibody against steroidogenic acute regulatory protein (StAR) was kindly provided by Dr. Dale B. Hales (University of Illinois School of Medicine at Chicago). Anti-AKAP95 is a polyclonal antibody generated against a recombinant fragment of AKAP 95 [18]. Anti-(PtdIns 3-kinase) and anti-AKAP100 were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-(PKA C$\alpha$ subunit), anti-AKAP220 [39] and anti-AKAP149 [40], which recognizes AKAP149 and sAKAP84 [22], were obtained from Transduction Laboratories (Lexington, KY, U.S.A.).

### Expression of recombinant RII$\alpha$, RII overlay assays and cAMP–agarose affinity chromatography

Recombinant murine RII$\alpha$ was expressed in *Escherichia coli* and purified as described previously by affinity chromatography on cAMP–Sepharose [41]. For solid-phase RII overlay assays, proteins were separated by SDS/PAGE and electrotransferred to Immobilon-P (Millipore Corp., Bedford, MA, U.S.A.), and blots were incubated with 0.5 $\mu$g of recombinant RII$\alpha$ that had been previously phosphorylated with the C subunit of PKA [18]. When indicated, blots were incubated in the presence of 0.4 $\mu$M Ht31 peptide (residues 493–515), which has been shown to competitively inhibit binding of RII to the protein on the blot [42]. Pooled and concentrated DEAE-cellulose fractions were added to cAMP–agarose (0.2 ml packed volume; Sigma) equilibrated in buffer E (10 mM Hepes, pH 7.0, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT, 10 $\mu$g/ml pepstatin, 10 $\mu$g/ml leupeptin, 5 $\mu$g/ml aprotinin, 10 $\mu$g/ml soybean trypsin inhibitor, 0.23 mM PMSF, 1 mM sodium vanadate and 10 $\mu$M isobutylmethylxanthine, pH 7.0) and incubated overnight at 4 °C on a Nutator [18]. The flow-through fraction ($\sim$ 1.0 ml) was collected by centrifugation at 5000 ***g*** for 5 min; an aliquot (0.1 ml) was diluted to 0.3 ml, mixed with 0.15 ml of 3-fold SDS STOP buffer, and heat-denatured. The cAMP–agarose pellet was then washed four times by sequential centrifugation with 4 ml (total) of buffer E, four times with buffer E containing 1 M NaCl and four times with buffer E containing 0.3 M NaCl, with collection of an aliquot (0.3 ml) of the final 1.0 ml wash. AKAPs were eluted from cAMP–agarose by incubation of the agarose pellet at room temperature for 30 min with 0.3 ml of 5 $\mu$M Ht31 peptide (493–515) in buffer E containing 0.3 M NaCl, with collection of eluate from agarose using a syringe followed by heat denaturation after addition of 3-fold SDS STOP buffer (0.15 ml). Remaining proteins bound to cAMP–agarose were eluted by boiling agarose in 0.45 ml of SDS STOP buffer.

### Sucrose-density-gradient centrifugation

Sucrose-density-gradient centrifugation was conducted by applying pooled, concentrated DEAE-cellulose peak fractions plus protein standards (haemoglobin, phosphorylase *b*) to 5.5–15 % (w/v) linear sucrose gradient and centrifuging at 200000 ***g*** for 20 h. Aliquots of the fractions collected were assayed for PKA (with Kemptide as substrate) and total [$^3$H]cAMP binding activities, and R subunits in selected fractions were photoaffinity labelled [35].

### Two-dimensional PAGE

IEF (pH 4–6.5) and SDS/PAGE of concentrated DEAE-cellulose fractions were performed as previously described [35]. For the results shown in Figure 7, immature rats received subcutaneous injections of 25 units of PMSG on day 26, and 25 units of human chorionic gonadotropin on day 28; rats were killed after 24 h. Ovaries were dissected, dropped into liquid $N_2$, and then homogenized using a Polytron instrument ($2 \times 15$ s bursts) in boiling buffer B (without sucrose). The mixture was placed in a boiling water bath and boiled for 20 min, then placed on ice for 10 min. Buffer D was added, and denatured proteins were pelleted by centrifugation at 5000 ***g*** for 10 min. Proteins in the supernatant were concentrated by trichloroacetic acid precipitation; the resulting pellet was washed with ethanol and then boiled in SDS STOP buffer (0.45 ml). A 10 $\mu$l aliquot was subjected to IEF using pH 3–10 Ampholines (Sigma) followed by SDS/PAGE [35]; proteins were transferred to Immobilon membranes and subjected to an RII overlay assay, and then reprobed with affinity-purified anti-AKAP-KL serum.

### Cell culture

Granulosa cells were isolated from the ovaries of 26-day-old rats and cultured ($6 \times 10^6$ cells/10 cm dish) in serum-free medium in the absence or presence of 50 ng/ml FSH for 72 h [34]. For determination of forskolin-dependent PKA activation, fresh medium (without FSH) was then added to the cells, which were then cultured for an additional 3 h. The cells were then treated with various concentrations of forskolin in 100 % DMSO (0.5 % DMSO final) for 10 min. Cells were rinsed, sonicated at 4 °C for 30 s in buffer F (20 mM Hepes, pH 7.4, 20 mM NaCl, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, 5 $\mu$g/ml pepstatin, 5 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 50 $\mu$g/ml soybean trypsin inhibitor, 10 mM benzamidine, 10 mM $MgCl_2$ and 0.2 % Triton X-100 [34]) and centrifuged for 1 min at 17000 ***g***. Kinase activity with Kemptide as substrate was measured in triplicate in supernatant fractions containing 32 $\mu$g of protein in the absence or presence of 0.5 $\mu$M cAMP and 2 $\mu$M PKI [43].

### Other methods

Protein was determined [44] using BSA as a standard. Differences between groups were assessed by unpaired Student's *t*-tests.

## RESULTS

### Separation of PKAs in ovarian extracts by DEAE-cellulose chromatography, and identification of R subunits

PKA activity in soluble extracts of PO ovaries was eluted from DEAE-cellulose as two peaks, 2 and 3A (Figure 1, lower panel), while a prominent peak of cAMP binding activity without a

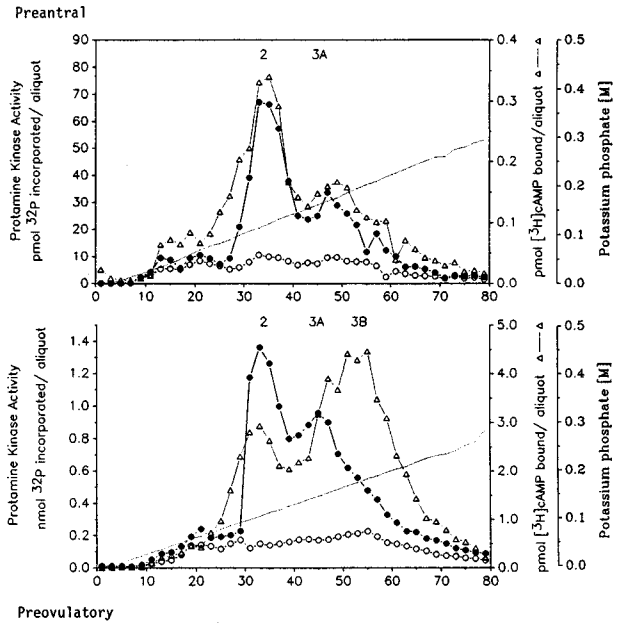


**Figure 1 Separation on DEAE-cellulose of PKAs present in soluble extracts of PA and PO ovaries**

Upper panel: PKAs in extract of ovaries from hypophysectomized rats, enriched in PA follicles. Tissue was homogenized in buffer A and 12 mg of protein was loaded on to a DEAE-cellulose column. Protein kinase activity was measured in 50 $\mu$l aliquots in the absence (○) and presence (●) of cAMP using protamine sulphate as substrate; [$^3$H]cAMP binding activity was measured in 100 $\mu$l aliquots (using 0.3 $\mu$M [$^3$H]cAMP) (△); measured potassium phosphate concentrations are indicated (dotted line). For details, see the Experimental section. Results are representative of more than five experiments. Lower panel: PKAs in extracts of ovaries from PMSG-primed rats, enriched in PO follicles. A sample of 28 mg of protein was loaded on to a DEAE-cellulose column. Other details were as described for the upper panel. Results are representative of more than five experiments.

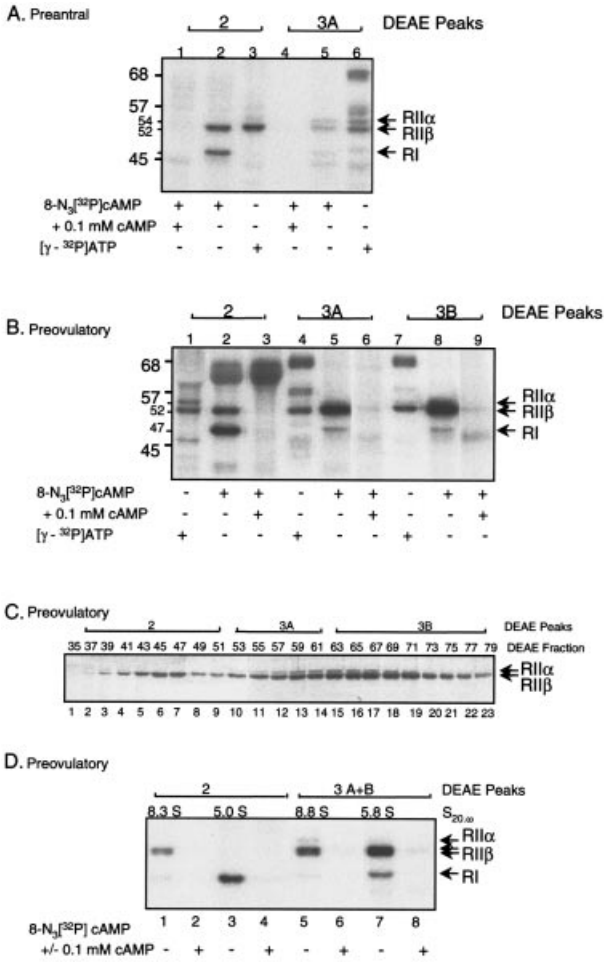


**Figure 2 Phosphorylation, photoaffinity labelling with 8-N$_3$[$^{32}$P]cAMP, and immunoreactivity of R subunits in DEAE-cellulose fractions**

For (**A**) and (**B**), DEAE-cellulose fractions from peaks 2 or 3 exhibiting maximal PKA activity were incubated with 8-N$_3$[$^{32}$P]cAMP in the absence or presence of 0.1 mM cAMP, as indicated. Proteins in reactions were separated by SDS/PAGE (10.5% acrylamide in the lower gel). Extracts were from PA (**A**) and PO (**B**) ovaries. Results in each panel are representative of more than five separate experiments. (**C**) Western blot using the anti-RII antibody MAB87, and showing reactivity with RII proteins in aliquots of DEAE-cellulose fractions derived from soluble extract of PO ovaries. Fractions were from a separate but equivalent DEAE-cellulose elution profile to that seen in Figure 1, lower panel. Results are representative of three experiments. (**D**) Identification of R subunits in sucrose-density-gradient fractions derived from DEAE-cellulose peaks of extracts of PO ovaries. Fractions from DEAE peaks 2 and 3 were pooled, concentrated and subjected to sucrose-density-gradient centrifugation to separate PKA holoenzymes from C-subunit-free R subunits. Aliquots of gradient fractions exhibiting maximal Kemptide kinase activity (at 8.3 and 8.8 S) or [$^3$H]cAMP binding activity (at 5.0 and 5.8 S) at the indicated sedimentation coefficient (based on migration of protein standards; see the Experimental section) were incubated with 8-N$_3$[$^{32}$P]cAMP in the absence or presence of 0.1 mM unlabelled cAMP, as indicated. DEAE fractions used in this experiment were also used in (**C**). Results are representative of five experiments. For details, see legend to Figure 1 and the Experimental section.

concomitant PKA peak of activity was eluted with higher salt concentrations (peak 3B). Although the PKA holoenzymes (peaks 2 and 3A) and the peak of C-subunit-free cAMP binding activity (peak 3B) in PO ovaries have been shown to contain RII subunits [37,45], the specific RII subtype ($\alpha$ or $\beta$) comprising each peak was not known.

Experiments were thus designed to identify the specific RII subunits present in each PKA holoenzyme in extracts of PO ovaries, and to compare these results with the PKA holoenzyme composition of soluble extracts of PA ovaries from hypophysectomized rats. Like extracts from PO ovaries (Figure 1, lower panel), extracts from PA ovaries exhibited two peaks of cAMP-stimulated protamine kinase activity (Figure 1, upper panel). [$^3$H]cAMP binding activity was associated with each peak of kinase activity; however, the prominent peak of [$^3$H]cAMP binding activity detected in extracts from PO ovaries (Figure 1, lower panel, peak 3B) was absent from extracts from PA ovaries (Figure 1, upper panel). [$^3$H]cAMP binding activity was also eluted as a shoulder with a slightly lower salt concentration than that required to elute peak 2 in both sets of ovarian extracts. Both peaks of PKA activity (2 and 3A) were inhibited by more than 90% by PKI (results not shown).

In order to identify R subunits, aliquots of DEAE-cellulose peaks were incubated with the photoaffinity cAMP analogue 8-N$_3$-[$^{32}$P]cAMP or with [$\gamma$-$^{32}$P]ATP under conditions which lead to phosphorylation of RII within the PKAII holoenzyme (autophosphorylation). DEAE peak 2 from both PA and PO ovaries exhibited two protein bands which bound 8-N$_3$[$^{32}$P]cAMP, at 52 and 47 kDa (Figures 2A and 2B, lane 2); binding was blocked by inclusion of 0.1 mM cAMP (Figure 2A, lane 1; Figure 2B, lane 3). In addition, the 52 kDa band, but not the 47 kDa band, was phosphorylated on incubation with [$\gamma$-$^{32}$P]ATP (Figure 2A, lane 3; Figure 2B, lane 1). Based upon molecular mass, specific binding of 8-N$_3$[$^{32}$P]cAMP and autophosphorylation potential, these characteristics are consistent with identification of the

**Table 1 RIA-detectable cAMP in sucrose gradient fractions**

Results are means ± S.E.M. of (*n*) separate DEAE-cellulose and subsequent sucrose-density-gradient experiments. Results are expressed as pmol of cAMP measured by RIA in a 0.1 ml sample aliquot per mol of [$^3$H]cAMP bound to R subunits, detected by measuring total [$^3$H]cAMP binding sites in a separate 0.1 ml sample aliquot. For details, see the legend to Figure 1 and the Experimental section.

| DEAE peak | $s_{20,w}$ (S) | cAMP (pmol/pmol of [$^3$H]cAMP bound) |
|---|---|---|
| 2 | 8.0 | 0.05 ± 0.02 (3) |
| | 5.0 | 1.62 ± 0.21 (3) |
| 3 | 8.8 | 1.0 ± 0.14 (5) |
| | 5.8 | 1.94 ± 0.39 (4) |

47 kDa protein in DEAE peak 2 as RI$\alpha$, and of the 52 kDa protein as RII$\beta$ [4,8]. Consistent with the conclusion regarding the identity of the 52 kDa protein, an anti-RII antibody which reacts with both RII$\alpha$ and RII$\beta$ showed strong reactivity with the 52 kDa protein in DEAE peak 2 (Figure 2C).

DEAE-cellulose peak 3A derived from PA ovaries (Figure 2A) showed nearly equivalent binding of 8-$N_3$[$^{32}$P]cAMP to both the 54 and 52 kDa proteins, and minimal binding to the 47 kDa protein (lane 5); binding of 8-$N_3$[$^{32}$P]cAMP was blocked by 0.1 mM cAMP (lane 4), and both the 54 and 52 kDa proteins were phosphorylated under RII phosphorylation conditions (lane 6). These results for peak 3A from PA ovarian extracts are consistent with identification of the 54 kDa protein as RII$\alpha$, the 52 kDa protein as RII$\beta$ and the 47 kDa protein as RI$\alpha$. Generally equivalent results were obtained for DEAE peak 3A from extracts of PO ovaries; however, PO ovaries expressed markedly elevated levels of RII$\beta$, such that the contribution of RII$\alpha$ was partially obscured (see Figure 2B, lanes 4 and 5). That RII$\alpha$ is associated with DEAE peak 3A is evident from the RII Western blot (Figure 2C). DEAE peak 3B, which is present only in PO ovaries (see Figure 1), is composed of RII$\alpha$, RII$\beta$ and RI$\alpha$ (Figure 2B, lanes 7–9; Figure 2C), based on the characteristics described for the R subunits in DEAE peaks 2 and 3A.

Identification of the individual R subunits, labelled with either 8-$N_3$[$^{32}$P]cAMP or [$\gamma$-$^{32}$P]ATP and comprising each DEAE-cellulose peak, was confirmed by their relative migration on both IEF followed by SDS/PAGE and slab IEF. Results showed that RII$\beta$, which consists of multiple charge variants, in DEAE peaks 2 and 3A was focused to the same pI and exhibited an equivalent pattern of labelling both with 8-$N_3$[$^{32}$P]cAMP and on phosphorylation (results not shown). Therefore the elution of the two PKAII$\beta$ holoenzymes (DEAE peaks 2 and 3A) cannot be accounted for by distinct isoelectric points of the RII$\beta$ subunits.

Sucrose-density-gradient centrifugation combined with R subunit labelling with the cAMP photoaffinity analogue was used to identify R subunits that are associated with C subunits to form a holoenzyme; the latter will sediment with a high sedimentation coefficient (with phosphorylase *b* marker protein), whereas R subunits that are free of C subunits will sediment with haemoglobin marker protein (lower sedimentation coefficient). Sucrose-gradient fractions exhibiting peak cAMP-stimulated Kemptide kinase or [$^3$H]cAMP binding activities (results not shown) were incubated with 8-$N_3$[$^{32}$P]cAMP to label R subunits; unlabelled cAMP was added to alternate samples to show binding specificity. Figure 2(D) shows that DEAE peak 2 comprised a predominant PKAII$\beta$ holoenzyme, along with very low levels of PKAI$\alpha$ holoenzyme, sedimenting with phosphorylase *b* marker protein at a sedimentation coefficient of 8.3 S, and a relatively large amount of C-subunit-free RI$\alpha$ sedimenting with the haemoglobin marker protein at 5 S. Pooled DEAE peaks 3A and 3B also consisted of a predominant PKAII$\beta$ holoenzyme along with lower levels of PKAII$\alpha$ and apparent trailing PKAI$\alpha$ holoenzymes, all of which sedimented with phosphorylase *b* marker (at 8.8 S), and (sedimenting at 5.8 S with haemoglobin) C-subunit-free RI$\alpha$ and the majority of RII$\beta$ (also free of C). No C-subunit-free RII$\alpha$ from peak 3 was detected co-eluting with haemoglobin (in four separate experiments).

We also determined whether the C-subunit-free R subunits separated from holoenzymes by sucrose-density-gradient centrifugation contained bound cAMP. cAMP was measured by RIA in soluble trichloroacetic acid extracts of the sucrose-gradient fraction containing the maximal [$^3$H]cAMP binding activity, and expressed as pmol of cAMP per pmol of [$^3$H]cAMP bound to R subunits (Table 1). Near-stoichiometric levels of cAMP were bound to both C-subunit-free RI (from DEAE peak 2 and sedimenting at 5.0 S) and C-subunit-free RII (from DEAE peak 3 sedimenting at 5.8 S); minimal levels of cAMP were bound to the R subunits associated with PKA holoenzyme peak from DEAE peak 2 (sedimenting at 8.0 S), while 50% of the cAMP binding sites were saturated in PKA holoenzymes from DEAE peak 3 (sedimenting at 8.8 S).

Taken together, these results establish that DEAE-cellulose peak 2 from PO ovarian extracts represents a PKAII$\beta$ holoenzyme plus C-subunit-free RI$\alpha$ containing bound cAMP. Peak 3A also represents predominantly PKAII$\beta$ holoenzyme and a second co-eluting PKAII$\alpha$ holoenzyme present at markedly lower levels, both of which appear to be 50% saturated with cAMP. DEAE peak 3B consists primarily of C-subunit-free RII$\beta$

**Table 2 Quantification of PKA and R subunit activities on DEAE-cellulose chromatography of soluble ovarian extracts**

PKA activity for each peak is expressed per mg of protein loaded on to the DEAE-cellulose column. To calculate the area under each peak, kinase activity (pmol of $^{32}$P incorporated/10 min) in 50 $\mu$l aliquots of odd-numbered column fractions comprising a peak of kinase activity (extrapolated to form a symmetrical peak) was summed, then divided by the amount of protein (mg) loaded on to the DEAE-cellulose column. Because activities for each peak were summed, then divided by protein load, values for individual fractions do not necessarily match with this summary of values. Numbers in parentheses refer to the number of separate experiments. cAMP-binding activity for the indicated DEAE-cellulose peak 3B is expressed per mg of protein loaded on to the DEAE-cellulose column. To calculate the area under each peak, cAMP binding activity (fmol of [$^3$H]cAMP bound) in 100 $\mu$l aliquots of odd-numbered column fractions comprising a peak of cAMP binding activity (extrapolated to form a symmetrical peak) was summed, then divided by the amount of protein (mg) loaded on to the DEAE-cellulose column. Results are means ± S.E.M. of (*n*) separate experiments. Significance of differences between PA and PO ovaries: $^*P < 0.001$.

| | PKA activity (pmol/min per mg) | | |
|---|---|---|---|
| Ovarian tissue | Peak 2 | Peak 3A | C-subunit-free [$^3$H] cAMP binding activity in peak 3B (fmol/mg) |
| PA | 56.2 ± 15.0 (7) | 38.4 ± 8.8(7) | < 1.0 (6) |
| PO | 85.1 ± 19.7 (8) | 62.6 ± 15.9(8) | 180.7 ± 44.9* (8) |

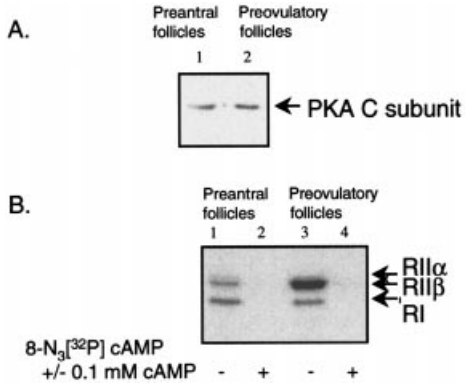


**Figure 3 PKA R and C subunits in extracts of PA and PO ovaries**

(**A**) Soluble detergent extracts were prepared, as described in the Experimental section, by homogenizing ovaries enriched in PA follicles (lane 1) or PO follicles (lane 2) in buffer C (which contains 0.5% Nonidet P40 and 0.1% deoxycholate). Extracts (50 $\mu$g of protein) were separated by SDS/PAGE, transferred to Immobilon and probed with antibodies to PKA C subunit. Results are representative of three experiments. (**B**) Soluble extracts, prepared as described in the Experimental section by homogenizing in buffer A, from PA ovaries (lanes 1 and 2) or PO ovaries (lanes 3 and 4) were incubated with 8-$N_3$[$^{32}$P]cAMP in the absence or presence of 0.1 mM cAMP, as indicated. The protein load was 30 $\mu$g for lanes 1 and 2, and 35 $\mu$g for lanes 3 and 4. Results are representative of three experiments.

subunits, as well as unexpectedly high levels of C-subunit-free RI$\alpha$, both of which appear to be saturated with cAMP, based on the stoichiometry of cAMP binding to R subunits.

PKA and C-subunit-free R subunit activities were estimated in DEAE-cellulose peaks from extracts of PA and PO ovaries. The area under each peak was estimated by summing PKA or [$^3$H]cAMP binding activities comprising each DEAE peak and dividing that value by the total soluble protein loaded on to each column (Table 2). The PKAII$\beta$ holoenzyme activity in DEAE peak 2 was not significantly different ($P > 0.05$) between ovaries enriched in PO compared with PA follicles relative to total ovarian protein, and PKAII$\alpha$ plus PKAII$\beta$ holoenzyme activities in DEAE peak 3 were not significantly different ($P > 0.05$) between the two stages of ovarian differentiation. Consistent with this result, PKA C subunit protein in PA compared with PO ovaries was unchanged on follicular differentiation (Figure 3A). These results prove, as surmised in earlier studies with granulosa cells [31,46], that the majority of RII$\beta$ that is induced in response to differentiation of PA to PO follicles [8,31,46] (as seen in Figure 3B as 8-$N_3$[$^{32}$P]cAMP binding to soluble R subunits from PA and PO ovaries) is eluted on DEAE-cellulose chromatography of soluble extracts of PO ovaries in a C-subunit-free state (Table 2, peak 3B).

### Elution from DEAE-cellulose of AKAPs from PA- compared with PO-follicle-enriched ovaries

It is generally believed that PKAs are eluted from DEAE-cellulose based upon the average charge of the holoenzyme, which is composed of relatively basic C subunits and more acidic R subunits. RII$\beta$ is slightly more basic than RII$\alpha$ [6,8,35], so PKAII$\beta$ holoenzymes would be expected to be eluted on anion-exchange chromatography with a slightly lower salt concentration than PKAII$\alpha$ holoenzymes. Indeed, in Ras-transformed NIH 3T3 fibroblasts, recombinant PKAII$\beta$ was eluted as a single peak of activity just preceding the peak of recombinant PKAII$\alpha$ at a salt concentration corresponding to DEAE-celluose peak 3A [47]. It was therefore surprising that PKAII$\beta$ was eluted as two separate peaks of activity (peaks 2 and 3A; see Figures 1 and 2C). As PKAIIs bind with nanomolar affinity to a family of AKAPs [9,10], we considered the possibility that the elution positions of the two PKAII$\beta$ peaks from DEAE-cellulose were affected by their association with one or more AKAPs. To begin to assess this possibility, we determined the elution from DEAE-cellulose of proteins that bind $^{32}$P-RII, using a solid-phase RII overlay assay. Shown in Figure 4 is the elution from DEAE-cellulose of AKAPs (Figures 4A and 4B) and the corresponding PKA activity (Figures 4E and 4F) from soluble extracts of PA and PO ovaries. The $^{32}$P-RII–AKAP interaction was blocked when Immobilon membranes were incubated with $^{32}$P-RII in the presence of 0.4 $\mu$M anchoring inhibitor peptide Ht31 (Figures 4C and 4D), a competitive inhibitor of the RII–AKAP interaction [42]. This demonstrates that all of the $^{32}$P-RII binding proteins seen in Figures 4(A) and 4(B) are AKAPs.

Soluble extracts of PA ovaries exhibited AKAPs at 270, 210, 175, 150, 115 and 95 kDa which were selectively eluted from DEAE-cellulose with increasing salt concentrations (Figure 4A). An equivalent class of AKAPs was detected in total extracts of immature granulosa cells incubated for 72 h with vehicle (Figure 5, lane 1) and in detergent extracts from PA ovaries (Figure 5, lane 3), except for a prominent AKAP at 160 kDa. Consistent with evidence that AKAP160 is localized to a pellet fraction [34], this protein was not detected in soluble PA ovarian extracts (Figure 4).

PO ovaries exhibited a profile of AKAPs equivalent to that seen in PA ovaries, except for the reduced expression of AKAP270 and the prominent expression of AKAP80 in PO ovaries (compare Figures 4A and 4B with Figure 5, lanes 1 and 2 versus lanes 3 and 4). AKAP80 was eluted from PO, but not PA ovarian extracts (Figures 4A and 4B) with DEAE peak 2 (compare Figures 4E and 4F). Based on our identification of PKAII holoenzyme elution positions (see Figures 1–3), these results indicate that AKAP80 is co-eluted with the PKAII$\beta$ holoenzyme in DEAE peak 2. The AKAP at 65 kDa in Figure 4(B) is believed to represent proteolytic breakdown of a larger AKAP(s), most probably AKAP80, since it was not detected either in freshly isolated extracts (Figure 5, lanes 2 and 4) or in PA extracts (Figure 4A).

The various ovarian AKAPs that were eluted from DEAE-cellulose at distinct salt concentrations could be eluting based on their association with either PKAs or other proteins [40], or based on their own charge. In order to identify which AKAPs were specifically bound to PKAs, R-subunit-bound AKAPs were purified using cAMP–agarose affinity chromatography. Fractions from DEAE-cellulose peaks 2 or 3 were pooled, concentrated and applied to cAMP–agarose. Non-specific proteins and AKAPs not bound to R subunits in ovarian extracts either did not bind to cAMP–agarose and were eluted in the flow-through (Figures 6A and 6B, lanes 1 and 5), or were removed with 1 M NaCl washes, as shown by the absence of AKAPs from the 'final wash' fraction (Figures 6A and 6B, lanes 2 and 6). The competitive anchoring inhibitor peptide Ht31 was then utilized to specifically displace RII-subunit-bound AKAPs, but not RII subunits (Figures 6A and 6B, lanes 3 and 7). Remaining proteins bound to cAMP–agarose were eluted by boiling agarose in SDS STOP buffer (lanes 4 and 8).

The majority of the AKAPs in extracts of PA ovaries did not bind to cAMP–agarose (Figure 6A, lane 1) and were eluted with the flow-through fraction. Relatively low levels of AKAPs 210, 175, 150 and 115 were eluted with Ht31 on cAMP–agarose chromatography of DEAE-cellulose peaks 2 and 3 (Figure 6A, lanes 3 and 7). Thus the majority of AKAPs in PA ovaries that

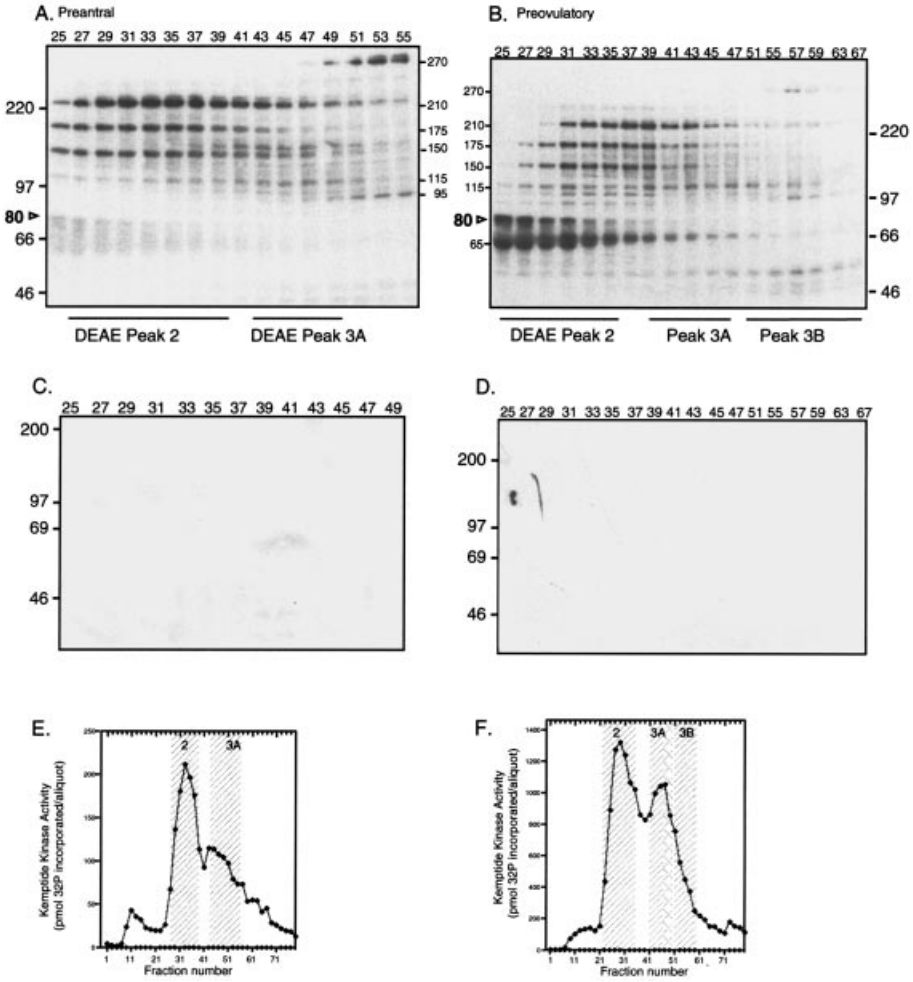


**Figure 4 AKAPs in DEAE-cellulose fractions of soluble extracts of PA and PO ovaries**

DEAE-cellulose chromatography was conducted as described in the legend to Figure 1, except that ovaries were homogenized in protease-inhibitor-enriched buffer B. The resulting elution pattern of PKA activity is shown in (**E**) and (**F**). Proteins in aliquots of DEAE-cellulose fractions were separated by SDS/PAGE (8% acrylamide) and transferred to Immobilon for RII overlay assays. (**A**) Autoradiogram showing the binding of $^{32}$P-RII$\alpha$ to proteins in DEAE-cellulose fractions (75 $\mu$l aliquot) from ovaries of hypophysectomized rats (6 mg of protein loaded on to DEAE-cellulose). DEAE fraction numbers are indicated at the top of the gel (see **E** for reference). Results are representative of two experiments. (**B**) Autoradiogram showing the binding of $^{32}$P-RII$\alpha$ to proteins in DEAE-cellulose fractions (50 $\mu$l aliquots) from PO ovaries (58 mg of protein loaded on to DEAE-cellulose). Details as for (**A**); see (**F**) for reference. Results are representative of two experiments. In (**C**) and (**D**), Immobilon, containing samples equivalent to or identical with respectively those used in (**A**) and (**B**), was probed with $^{32}$P-RII$\alpha$ in the presence of 0.4 $\mu$M Ht31.

are capable of specifically binding $^{32}$P-RII in an *in vitro* overlay assay (as shown in Figure 4) are not bound to R subunits following tissue extract preparation and DEAE-cellulose chromatography, based on their presence in the flow-through fraction on cAMP–agarose chromatography. This conclusion is reinforced by the fact that the flow-through lanes in Figure 6 reflect a 30-fold dilution of the volume eluted from cAMP–agarose. These results suggest that the PKAII holoenzymes eluted as DEAE-cellulose peaks 2 and 3 from PA ovaries were complexed with only a small fraction of the AKAPs that were present in the ovarian extract and co-eluted from DEAE-cellulose (see Figure 4A). AKAPs 210, 175, 150 and 115 in PA ovaries are therefore eluted from DEAE-cellulose based primarily either on their intrinsic charge or as a result of their association with proteins other than RII.

In contrast with the results with PA ovarian extracts, the majority of AKAPs in PO ovaries were bound to R subunits. A greater percentage of AKAPs 210, 175, 150 and 115 in soluble extracts of PO ovaries bound to R subunits and were eluted with Ht31, especially from peak 3 (Figure 6B; compare lanes 1 and 5 with lanes 3 and 7). Binding of these AKAPs to R subunits was associated with a subtle shift (towards peak 3) in the elution position of these AKAPs from DEAE-cellulose (compare equivalent fraction numbers in peaks 2 and 3A in Figures 4A and 4B). However, the most striking difference between the AKAPs bound to R subunits in extracts of PA compared with PO ovaries concerned AKAP80. All of the AKAP80 was bound to R subunits in DEAE-cellulose peak 2, and not to R subunits in DEAE peak 3, and most was eluted from cAMP–agarose with Ht31 (Figure 6B, lane 3). These results reveal that follicle maturation not only results in the increased expression of AKAP80, which selectively binds presumably to PKAII$\beta$ in DEAE-cellulose peak 2, but also promotes the binding of AKAPs 210, 175, 150 and 115 primarily to PKAIIs in DEAE peak 3. The basis for the exclusive binding of AKAP80 apparently to PKAII$\beta$ in DEAE peak 2 and not to PKAII$\beta$ or to C-subunit-free RII$\beta$

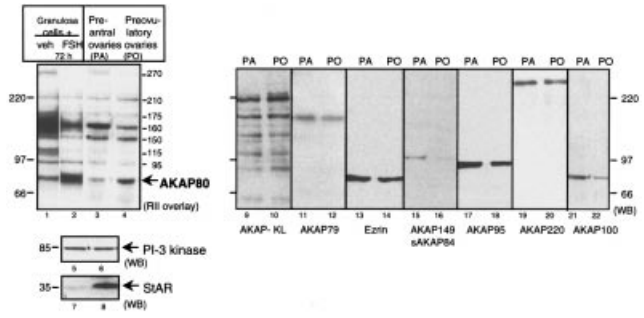


**Figure 5 AKAPs in detergent extracts of PA- and PO-follicle-enriched ovaries**

Soluble detergent extracts were prepared as described for Figure 3(A) from ovaries containing PA or PO follicles; samples were separated by SDS/PAGE, transferred to Immobilon and probed with $^{32}$P-RII$\alpha$ for RII overlay assays (lanes 3 and 4), as described in the Experimental section, or for Western blots (WB) with the indicated antibodies (lanes 5–22). Results in Figure 3(A) and in this Figure were obtained with the same samples and are representative of three experiments. For comparison, AKAPs in total cell extracts of immature granulosa cells incubated with or without FSH for 72 h are shown (lanes 1 and 2; veh, vehicle). Methods for granulosa cell culture and preparation of total cell extracts were as previously described [34]. Molecular masses of Rainbow standards are shown at the left for lanes 1–4 and at the right for lanes 9–22; molecular masses of AKAPs were calculated by linear regression from standards and are shown at the right of lanes 4 and 22.

in peak 3 (see Figures 2B–2D to compare levels of RII$\beta$ among DEAE-cellulose peaks 2, 3A and 3B) is not clear, especially since AKAPs bind *in vitro* with equal affinity to PKAII and RII dimers [16], and since PKAII$\beta$ in DEAE peak 2 is indistinguishable from that in DEAE peak 3, based on R subunit size, pI, autophosphorylation potential (see Figure 2) and cAMP affinity [37].

### Identification of AKAP-KL in ovarian extracts

To begin to identify ovarian AKAPs, we compared the migration positions, on SDS/PAGE of detergent extracts of PA and PO ovaries, of AKAPs for which antibodies are available with those of the proteins that bound $^{32}$P-RII$\alpha$. For reference, we showed that PtdIns 3-kinase, an unrelated protein, was not regulated, while StAR was induced with follicular differentiation (Figure 5, lanes 5–8). Affinity-purified anti-AKAP-KL antiserum reacted with proteins at 210, 175, 150, 115 and 90 kDa which co-migrated on SDS/PAGE with corresponding AKAPs in ovarian extracts (Figure 5, compare lanes 3 and 4 with lanes 9 and 10). AKAP79 (Figure 5, lanes 11 and 12), which in murine tissues has a molecular mass of $\sim$ 150 kDa [17,48], and AKAP95 (lanes 17 and 18) [18] migrated at molecular masses equivalent to AKAPs in ovarian extracts at 175 and 95 kDa respectively. Ezrin [49] (lanes 13 and 14) and AKAP100 [24] (lanes 21 and 22) migrated at $\sim$ 80 kDa, but were not up-regulated in PO ovaries and thus are not likely to correspond to AKAP80 (which is up-regulated with follicular differentiation). These results suggest that the following ovarian AKAPs could correspond to the following known AKAPs: 95 kDa ovarian AKAP to AKAP95; 210, 150 and 115 kDa ovarian AKAPs to AKAP-KL; and 175 kDa ovarian AKAP to AKAP-KL or AKAP79.

Based on these results, cAMP–agarose eluates of DEAE-cellulose peaks from PO ovaries were evaluated for the presence of AKAP79, AKAP-KL, ezrin and AKAP100 immunoreactive proteins. No ezrin or AKAP100 immunoreactive proteins were detected in the Ht31 eluate from DEAE peak 2 (results not shown), confirming that the ovarian AKAP80 does not correspond to ezrin or AKAP100. AKAP79 immunoreactive protein was detected exclusively in the flow-through fraction of DEAE peak 3 (results not shown) and therefore does not correspond to the elution of AKAP175 with Ht31 (see Figure 6B, lane 7). However, as shown in Figure 6(B) (lower panel), affinity-purified

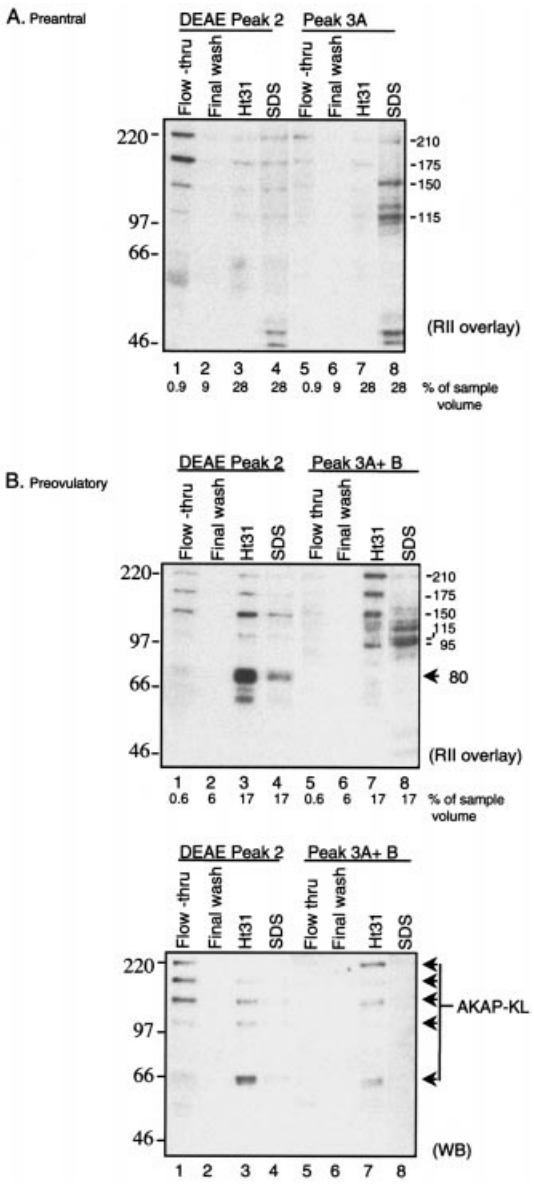


**Figure 6 Binding of AKAPs to R subunits purified by cAMP–agarose chromatography of PKA holoenzymes in extracts of PA- and PO-follicle-enriched ovaries separated by DEAE-cellulose chromatography**

Fractions from the indicated DEAE-cellulose peaks were pooled, concentrated and incubated with cAMP–agarose. Following collection of cAMP–agarose flow-through ('Flow-thru'; $\sim$ 1 ml) containing proteins that did not bind to cAMP–agarose, the agarose was washed with low- and high-salt buffers to remove non-specifically bound proteins. The 'Final wash' (1.0 ml) was collected, and then specifically bound AKAPs were eluted off R subunits bound to cAMP–agarose with Ht31 (5 $\mu$M). The remaining proteins bound to cAMP–agarose were eluted by boiling the agarose in SDS STOP buffer. The percentage of the sample volume loaded on to SDS/PAGE is shown below the RII overlay panels. Autoradiograms show binding of $^{32}$P-RII$\alpha$ (RII overlay) to proteins eluted with cAMP–agarose affinity purification of R subunits in the indicated DEAE-cellulose peaks of PA (**A**) or PO (**B**) ovarian extracts. Results are representative of two experiments. Fractions eluted from cAMP–agarose derived from PO ovaries were also probed in Western blots (WB) with antiserum to AKAP-KL.

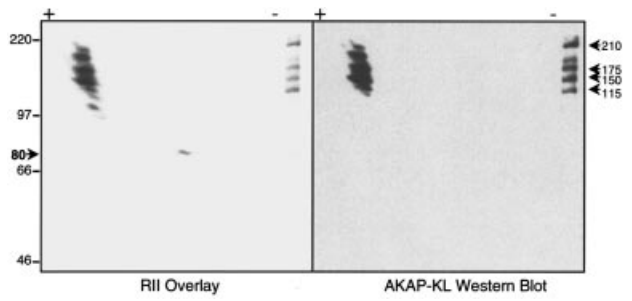


**Figure 7 Two-dimensional PAGE of ovarian AKAPs**

Left panel: autoradiogram of two-dimensional PAGE (tube IEF; slab SDS/PAGE) showing binding of $^{32}$P-RII (RII overlay) to proteins in an ovarian extract prepared from rats primed with PMSG and then human chorionic gonadotropin, as described in the Experimental section. Expected pH gradients ranged from ~ 10.0 to 3.0 at the bottom of the gel; + and − designations mark the boundaries of the IEF tube. Right panel: Western blot of the same sample probed with anti-AKAP-KL antiserum. The results are representative of a single experiment. The lane at the right of each panel contains ~ 1 $\mu$l of the ovarian extract subjected to SDS/PAGE only.

anti-AKAP-KL serum reacted with AKAPs at 210, 175, 150, 115 and 65 kDa that appear to correspond to those eluting in the flow-through and Ht31 fractions from DEAE peaks 2 and 3 (Figures 6A and 6B, lanes 1, 3 and 7). Anti-AKAP-KL antibody did not react with a protein at 80 kDa, but instead it reacted with a lower-molecular-mass protein which exhibited only faint RII binding activity (Figure 6B, lane 3). That AKAPs 210, 175, 150 and 115 in the DEAE peaks 2 and 3 indeed correspond to AKAP-KL was shown by the correspondence of AKAP-KL immunoreactivity and $^{32}$P-RII binding activity on IEF/SDS/PAGE of an ovarian extract (Figure 7). AKAP-KL is especially abundant in kidney and lung (hence its name), is primarily anchored to the actin cytoskeleton in those tissues, is acidic (pI ~ 5), and consists of at least six isoforms with molecular masses ranging from 133 to 105 kDa [13]. These results also show that ovarian AKAP80 does not correspond to any of the known AKAPs identified so far.

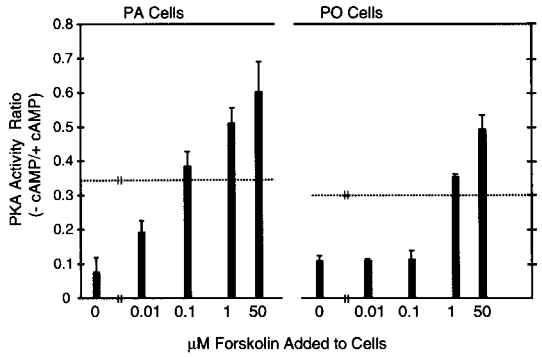


**Figure 8 Effects of forskolin on PKA activation in PA and PO granulosa cells**

Granulosa cells were cultured for 72 h in vehicle (PA cells) or 50 ng/ml FSH (PO cells). Cells were then exposed to FSH-free medium for 3 h, and then treated for 10 min with the indicated concentrations of forskolin. Kemptide kinase activity was measured in a supernatant fraction (buffer F) in the absence and presence of 0.5 $\mu$M cAMP and PKI. PKI-insensitive kinase activity was subtracted from Kemptide kinase activities. Results are shown as the PKA activity ratio (1.0 reflects total dissociation of R and C subunits), are means ± S.E.M. of triplicate determinations, and are representative of two separate experiments. The $ED_{50}$ of the PKA activity ratio is indicated by the dotted line.

### PKA activation in PA and PO granulosa cells

We reported previously that FSH-stimulated differentiation of PA to PO follicles resulted in a partial redistribution of PKA activity and RII$\alpha$ subunits from a pellet to a soluble fraction (in extracts prepared in buffer B without sucrose and with DTT), resulting in a 17 % increase in PKA activity in a soluble fraction and a corresponding 58 % decrease in the pellet fraction [34]. We were interested to determine whether follicular maturation was also associated with an altered sensitivity of PKA to activation. The sensitivity of PKA to activation by forskolin-stimulated cAMP production was compared in PA and PO granulosa cells. Results showed that ~ 10-fold more forskolin was required in PO compared with PA granulosa cells to promote half-maximal activation of PKA (Figure 8).

## DISCUSSION

Differentiation of ovarian follicular cells from a PA to PO phenotype is dependent on FSH and is mimicked by relatively low, tonic levels of cAMP, as well as by higher levels of cAMP induced by forskolin [1,2]. Differentiation of PO follicular to luteal cells is dependent on surge concentrations of LH, resulting in robust cAMP production and accompanied by down-regulation of a number of genes characteristic of PO follicular cells, as well as the up-regulation of others [1,2]. Based on the distinct response of PA compared with PO follicles to high concentrations of cAMP, we hypothesized that different PKA holoenzymes are expressed in PA than in PO follicles.

Our results showed that PA and PO ovaries exhibited equivalent PKA holoenzyme profiles on elution from DEAE-cellulose, consisting of a prominent PKAII$\beta$ holoenzyme and a second PKAII peak composed of apparently co-migrating PKAII$\alpha$ and PKAII$\beta$ holoenzymes. Very low levels of PKAI$\alpha$ were detected, and RI$\beta$, readily distinguished by its very basic migration on two-dimensional gel electrophoresis [6], was absent from ovarian extracts. PKA holoenzyme activities, relative to protein loaded on to the DEAE columns, were also not significantly different between ovaries enriched in PA compared with PO follicles, consistent with earlier studies [31,46] showing that PKA catalytic activity was not increased with follicular differentiation. Thus the very distinct responses induced by cAMP in these two tissues cannot be accounted for by the presence of distinct PKA holoenzymes.

Alternatively, we hypothesized that the distinct responses induced by cAMP in PA compared with PO follicles could be mediated in part by the binding of PKAIIs to distinct AKAPs, resulting in the targeting of selected PKAII pools to specific cellular locations and thus to specific substrates. It is well established that PKAII enzymes can be tethered to specific intracellular sites via their interactions with a family of AKAPs [9,10]. In a variety of cellular models, the cellular location of PKAII is regulated by its association with AKAPs [16,29] and appropriate targeting of PKAII to an AKAP is necessary for optimal phosphorylation of substrates by the freed C subunit upon PKA activation [28,30,40]. As shown by immunocytochemical and subcellular fractionation techniques, anchored PKAIIs and their AKAPs are most commonly localized to particulate fractions of the cell, including the actin cytoskeleton, nuclear matrix, Golgi membranes and microtubules, and are not readily solubilized into the cytosolic cellular fraction [10,40]. However, in ovarian cells, both PKAs and the majority of

AKAPs in PA and PO cells were localized to a cytosolic compartment [31,34].

Our results reveal striking differences in the binding profiles of AKAPs to the R subunits comprising DEAE peaks 2 and 3 in PA compared with PO ovaries. With the exception of AKAP80, which was increased with follicular differentiation *in vitro* [34] and *in vivo* (see Figures 4 and 5), and of AKAP270, which was decreased with follicular differentiation (see Figures 4 and 5), qualitative AKAP expression was the same in PA and PO ovarian extracts. However, R subunits from PA ovaries bound only a subset of the AKAPs present (based on AKAP elution with Ht31), while R subunits in PO ovaries bound the majority of the AKAPs present, including those corresponding to AKAP80 and to AKAP-KL at 210, 175, 150 and 115 kDa (see Figure 6). These results suggest that the binding of AKAPs to R subunits is regulated with differentiation from the PA to the PO ovarian phenotype.

Increased AKAP binding to R subunits in PO cells predicts a redistribution of R subunits to a different subcellular fraction. Consistent with this prediction, we previously reported a partial redistribution of PKA activity from a pellet to a soluble fraction and an equivalent shift in RII$\alpha$ subunits [34]. In the present report, we additionally show that follicle maturation results in a decreased sensitivity of PKA in PO cells to activation by forskolin-stimulated rises in intracellular levels of cAMP. This result could reflect a redistribution of PKA bound to AKAPs in PO cells to a location more distant from the site of cAMP generation. This result is not seen when PKA activation by exogenous cAMP is evaluated in soluble ovarian extracts (M. Hunzicker-Dunn, unpublished work; [50]), perhaps reflecting saturation of C-subunit-free R subunits with cAMP (see Table 1).

Ovarian cells, unlike many other cells [51], exhibit very unequal molar ratios of R and C subunits (see Figure 2D). PO cells contain not only C-subunit-free RI$\alpha$, but also RII$\beta$. The large amount of free RI is unexpected, due to its reported instability in the absence of C subunits [51]. It is possible that the presence of C-subunit-free RI$\alpha$ subunits reflects preferential activation of the type I holoenzyme [52] and the resulting association of C subunits with available RII subunits [51]. Based on the levels of PKAI$\alpha$, PKAII$\beta$ and RI$\alpha$ detected in PO cells (see Figure 2D, lanes 1 and 3), this interpretation requires 'tonic' activation of $> 90\,\%$ of PKAI$\alpha$ in these cells. The large excess of R subunits with constant levels of C subunits means that C subunits are never in excess, and that their reassociation is favoured. Our results show that release of C subunits from holoenzyme in PO cells and the resulting differentiation to the luteal phenotype requires higher concentrations of cAMP, as occurs with the LH surge. Perhaps higher cAMP concentrations are required as a result of the 'buffering capacity' of the R subunits to strictly limit development of the luteal cell phenotype.

In summary, our results demonstrate that, with differentiation of PA to PO follicles, R subunits in DEAE-cellulose peak 2 bound predominantly to AKAP80 and the 150 kDa AKAP-KL, while R subunits in DEAE peak 3 bound AKAP-KL of 210, 175 and 150 kDa. It is likely that, in addition to binding PKAIIs, these AKAPs bind other signalling molecules, such as other protein kinases or phosphatases, as has been shown for AKAP79 and microtubule-associated protein 2 [40].

These results comprise the first report showing preferential association of AKAPs with selected pools of R subunits, comprising DEAE-cellulose peaks 2 (an RII$\beta$ PKA holoenzyme and C-subunit-free RI$\alpha$), 3A (co-eluting RII$\alpha$ and RII$\beta$ PKA holoenzymes) and 3B (C-subunit-free RII$\beta$). Not only is AKAP80 increased with follicular differentiation, but we have shown for the first time that the binding of an equivalent cohort of AKAPs identified as AKAP-KL to RII subunits is also regulated with follicular differentiation.

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