Cellular location and age-dependent changes of the regulatory subunits of cAMP-dependent protein kinase in rat testis

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This study was undertaken to examine the expression and cellular location of the various cAMP-dependent protein kinase (PKA) subunits in different testicular cell types, using cDNA probes, isoenzyme-specific antibodies and activity measurements. Amounts of mRNA and protein were examined in cultured Sertoli cells, cultured peritubular cells, germ cells (pachytene spermatocytes, round spermatids), Leydig cell tumours as well as whole testes from rats of various ages. In Sertoli cells, there was a good correlation between the amount of mRNA and the respective immunoreactive proteins. In other types of cell, such as germ cells and Leydig tumour cells, this was not always the case. Large amounts of RII_β mRNA were found in Leydig tumour cells, whereas the amount of immunoreactive protein was low. Furthermore, large amounts of small-sized, germ cell-specific mRNAs for RI_α (1.7 kb) and RII_α (2.2 kb) were also found in the developing rat testis after 30 to 40 days of age, but the large amounts of mRNA were only partially reflected at the protein level. Pachytene spermatocytes and round spermatids were practically devoid of both RII_α and RII_β protein. During spermatid differentiation, there was a decrease in RI_α and an increase in RII_α protein. Cell specific distribution of the various PKA subunits in testicular cell types is described. In some types of cell, discrepancies between mRNA and protein were demonstrated, which clearly suggest cell specific differences in translational efficiencies for some of these mRNAs, particularly the small-sized mRNAs for RI_α and RII_α in meiotic and post-meiotic germ cells.

Introduction

Testicular function depends on FSH and LH stimulating Sertoli cells and Leydig cells, respectively. These hormones act via cAMP (Robison *et al.*, 1971; Means *et al.*, 1980). Cyclic cAMP is also involved in the regulation of sperm functions such as motility (Tash and Means, 1983; Tash *et al.*, 1984, 1986), epididymal maturation, capacitation (Visconti and Tezón, 1992) and the acrosome reaction (Hyne and Garbers, 1979; Garbers and Kopf, 1980). Most, if not all, of the effects of cAMP are mediated by the cAMP-dependent protein kinase (PKA) (Beebe and Corbin, 1986). Numerous investigations have thus focused on the role of cAMP and its effector enzyme, PKA in testis (Podesta *et al.*, 1976; Fakunding and Means, 1977; Horowitz *et al.*, 1984; Spruill *et al.*, 1984).

The cAMP-dependent protein kinase (E.C. 2.7.1.37) holoenzyme is a tetrameric complex consisting of a regulatory subunit dimer and two catalytic subunits (Corbin *et al.*, 1978). Several isoforms of PKA have been described. On the basis of their elution positions from DEAE cellulose columns, two major isozymes of PKA were first identified as type I and type II (Corbin *et al.*, 1975). These isozymes were shown to differ in the

properties of the R subunits (Fleischer *et al.*, 1976). Later, a whole family of PKA subunits, representing distinct gene products, were demonstrated. Four different regulatory subunits (RI_α, RI_β, RII_α and RII_β) but only one catalytic (C_α) subunit has so far been identified in rat tissues (Kuno *et al.*, 1987; Scott *et al.*, 1987; Sandberg *et al.*, 1988; Massa *et al.*, 1990; Landmark *et al.*, 1991; Wiemann *et al.*, 1991). Very small amounts of mRNA (4.7 kb) for another catalytic subunit, C_β, have also been demonstrated in rat testis using a human cDNA probe (Øyen *et al.*, 1990), but the corresponding protein has not been demonstrated. In addition, a cDNA for a third catalytic subunit of PKA (C_γ) was isolated from human testis (Beebe *et al.*, 1990). This subunit has not been detected in any other organ or species.

Studies based on ion-exchange chromatography and photo-affinity labelling have shown that there are two types of PKA in rat Sertoli cells (Spruill *et al.*, 1984; Nistico *et al.*, 1991), rat Leydig cells (Winters and Dufau, 1984), peritubular cells (Nistico *et al.*, 1991) and spermatozoa (Horowitz *et al.*, 1984, 1989; Atherton *et al.*, 1985; Tash *et al.*, 1986). These methods have certain limitations in that they do not separate the α and β forms of R or C. With the advent of cDNA probes, the distinction between α and β forms was available at the mRNA level (Øyen *et al.*, 1987a, 1988a; Skålhegg *et al.*, 1992a). Specificity at

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the protein level was achieved by the development of subunit specific antibodies (Skálhegg *et al.*, 1992b; Lohmann *et al.*, 1983). In the present report, mRNA and protein expression of all known isoforms of PKA has been studied in whole rat testis during development and in isolated testicular cells.

Materials and Methods

Sertoli and peritubular cells

Primary cultures of Sertoli cells and peritubular cells were prepared from testes of 19-day-old immature rats (Sprague-Dawley), and treated as described by Dorrington *et al.* (1975) and Øyen *et al.* (1988a). Experiments on Sertoli cells were started on day 4 after plating. Fresh medium was added 12 h before stimulation and changed at stimulation and after 24 h. The overall purity was estimated to be 90–95%. The peritubular cells were harvested when confluent, after 10–12 days of culture.

Preparation of germinal cell fractions

Germ cells (pachytene spermatocytes, round spermatids) were isolated from seminiferous tubules of 32-day-old rats. Germ cells enriched in elongating spermatids were obtained by using seminiferous tubules from 44-day-old rats. A cell preparation was obtained by consecutive collagenase, trypsin, DNase and mechanical treatment and separation by a BSA gradient using a Sta-Put unit (Øyen *et al.*, 1988a, b). Purity of the cell fractions was assessed as described by Øyen *et al.* (1987b) and was 85–90% for pachytene spermatocytes, and 80–85% for round spermatids of the total cell number. The impurities in these fractions were accounted for by Sertoli cells, interstitial cells and germinal cells at other stages of spermatogenesis.

Leydig cell tumour

Leydig cell tumour (H-540), 1 ml, disintegrated in 0.9% NaCl, was transplanted s.c. onto the back of 30–35-day-old Sprague-Dawley rats. After 2–3 weeks, the rats were killed by decapitation. The tumours were excised immediately and stored in liquid nitrogen until use (Erichsen *et al.*, 1984).

Whole testis preparation

Sprague-Dawley rats of different ages were decapitated. For the age study, 3–10 rats of each age were used. Testes were dissected free, removed, immediately frozen in liquid nitrogen and stored at -75°C .

RNA extraction and northern transfer

RNA extraction and northern analysis were performed as described by Maniatis *et al.* (1983) and Landmark *et al.* (1991). RNA was determined by measuring absorbance at 260, 280 and 310 nm, and quantification was based on the 260 nm reading.

Twenty micrograms of total RNA was loaded per lane. The RNA-agarose gels were stained with ethidium bromide before blotting to evaluate RNA amount and quality.

cDNA probes

The mouse RII_{α} probe was a 0.6 kb PstI fragment (provided by Stanley McKnight, Seattle, WA), entirely from inside the open reading frame. To probe for RII_{α} mRNA, a 0.7 kb rat cDNA probe (SacI-BglI fragment, all within the open reading frame) was used. RII_{β} mRNA was probed using a 1.5 kb rat cDNA containing the 3'-coding (1.2 kb) and non-coding (0.3 kb) region (Jahnsen *et al.*, 1986). For C_{α} , a 0.6 kb EcoRI fragment (mouse) was used which contained a 3'-coding region and about 160 base pairs of 3'-non-coding sequence (Uhler *et al.*, 1986).

Preparation of cell extracts

Cells were homogenized in PE buffer (10 mmol potassium phosphate l^{-1} , 1 mmol EDTA l^{-1} , pH 6.8) with 150 mmol NaCl l^{-1} (PEN) containing the protease inhibitors chymostatin, antipain, leupeptin and pepstatin (10 $\mu\text{g ml}^{-1}$ each). Before homogenization, Sertoli cells in culture dishes were washed with 3×5 ml Eagle's modified minimal essential medium, then reincubated in a large volume (30–50 ml) of media for 1 h to remove cAMP before cAMP-binding experiments. The culture dishes were cooled for 15 min on ice, washed with 3×5 ml PEN buffer and scraped into 0.5 ml PES containing protease inhibitors.

Whole testis and Leydig cell tumour tissue were fragmented while frozen and then homogenized in 8 volumes (w/v) of PEN buffer. Sta-Put-fractionated germ cells were homogenized in four volumes (w/v) of PEN buffer. Homogenization was performed by 15 plus 10 strokes with a tight-fitting Dounce homogenizer. Supernatants were prepared by centrifugation for 60 min at 100 000 g, at 4°C . Aliquots were frozen in liquid nitrogen and stored at -75°C . Protein concentration was determined by the method of Bradford (1976), using BSA as standard.

cAMP-binding, protein kinase activity and determination of R:C ratio

These assays were performed as described by Cobb and Corbin (1988). The levels of R were determined by specific [^3H]cAMP binding (assuming two cAMP binding sites per monomer, (Corbin *et al.*, 1986)).

DEAE-cellulose chromatography

Whatman DE52 columns (0.9×7.5 cm, 4.8 ml) were equilibrated with PE buffer (pH 6.8). Extracts for application to DEAE columns were prepared in PE buffer with 250 mmol sucrose l^{-1} (PES). Round spermatids (1.6 mg of soluble protein) from 32-day-old rats and a fraction of haploid cells containing spermatids at different stages of elongation from 44-day-old rats (2.9 mg soluble protein) were applied in a volume of 800 μl . Linear salt

gradients (0–0.4 mol NaCl l^{-1} , total volume 50 ml) were created with a Büchler Instruments 2 5070A gradient mixer. Each column was washed with 30 ml (about six bed volumes) PE buffer, before starting the gradient and fractions of 0.5 ml were collected. Kinase assays and cAMP binding assays were performed immediately following elution. The NaCl concentrations were determined by conductivity measurements.

Protein gel electrophoresis and western blotting

Denaturing polyacrylamide gel electrophoresis was performed using slab gels containing either 7.5% (for R subunits) or 10% (for C subunits) polyacrylamide in the separating gel and 4.5% polyacrylamide in the stacking gel (Laemmli, 1970; Landmark *et al.*, 1991). The samples were denatured by boiling for 2 min in a solution containing final concentrations of 1.4% SDS, 20% β -mercapto-ethanol and 0.05% bromophenol blue. Western blotting was performed as described by Towbin *et al.* (1988) and Landmark *et al.* (1991).

Radioimmunolabelling

Radioimmunolabelling of western blots using specific antibodies for the different R subunits was performed using ^{125}I -labelled IgG as described by Jahnsen *et al.* (1985). The specificities of the antibodies against rat RI $_{\alpha}$, RI $_{\beta}$ and C have been described by Lohmann *et al.* (1983). Affinity purified antisera against rat RI $_{\alpha}$ were provided by S. O. Døskeland (Institute of Anatomy, University of Bergen).

Immunoquantitation

RI and total RII were measured by immunoabsorption on protein A–Sepharose. Samples were equilibrated with [3H]cAMP, and antisera against RI or RII were added. Immunoabsorbed R was estimated by counting [3H]cAMP eluted by 1 mol acetic acid l^{-1} as described by Ekanger *et al.* (1988).

Materials

[3H]cAMP, 34 Ci $mmol^{-1}$, (TRK.498) and γ -[^{32}P]ATP (about 5000 Ci $mmol^{-1}$, PB 10 218) were purchased from Amersham (Bucks) and goat anti-rabbit [^{125}I]-IgG (7.6 $\mu Ci \mu g^{-1}$, NEX-155) was obtained from NEN Research (Boston, MA). *N*⁶-2'-*O*-dibutyryl adenosine 3'5' cyclic monophosphate (D-0627), adenosine triphosphate grade I (A-2383), adenosine 3'5' cyclic monophosphate (A-4137), aprotinin (A-6279), Triton X-100 (T-6878), Nonidet P-40 (N-3516), DNase (D-0876), collagenase (C-0130) and histone (calf thymus) Type II-AS were from Sigma (St Louis, MO). Pepstatin A (4039), leupeptin (4041), antipain (4062), chymostatin (4063) and Kemptide phosphate acceptor protein (8065) were purchased from Peninsula Laboratories (Belmont, CA). Agarose (5510 UA) was from Bethesda Research Laboratories (Gaithersburg, MD). Protein-A Sepharose CL-4B was from Pharmacia (Bromma). DEAE cellulose (DE52) was purchased from Whatman (Maidstone). Non-fat dry milk (Molico) was bought from Nestlé (Vevey). Bradford protein assay reagent (500-0006) was bought from Bio-Rad (Richmond,

Fig. 1. Specific binding of [3H]cAMP in extracts from whole testes. Testes were removed from rats at different ages (days), homogenized, and specific [3H]cAMP binding was determined in 100 000 g supernatants. Each point represents the mean of three experiments $\pm$ SEM.

CA). Penicillin was obtained from Apothekernes Laboratorium (Oslo), streptomycin from Heyl (Berlin) and fungizone from Novo Industries (E. R. Squibbs and Sons, Princeton, NJ). Fetal calf serum (011-06290), Eagles minimum essential medium (041-01090) and Hanks balanced salt solution without Ca^{2+} and Mg^{2+} (041-04140) were purchased from Gibco (Grand Island, NY). Sodium dodecyl sulfate (161-0301), acrylamide (161-0101), *N,N,N',N'*-methylene-bis-acrylamide (161-0201), *N,N,N',N'*-tetramethylenediamine (161-0800), ammonium persulfate (161-0700), tris(hydroxymethyl)aminomethane (161-0716), glycine (161-0718), bromophenol blue (161-0404) and Coomassie blue R250 (161-0400) were obtained from BioRad. All other chemicals were of analytical grade.

Results

[3H]cAMP binding capacity

Extracts (100 000 g supernatants) were prepared from rat testes of the given ages and total R-subunit activity was assayed by specific [3H]cAMP binding (Fig. 1). Specific [3H]cAMP binding in 5-day-old rats was relatively low (approximately 5 pmol mg^{-1}). The total amount of regulatory subunit in testis showed little change between 10 and 30 days of age (6 pmol mg^{-1}). From 30 to 60 days there was an increase in [3H]cAMP-binding activity which reached a maximum of approximately 9.5 pmol mg^{-1} at 60 days.

Age-dependent variation in RI $_{\alpha}$ mRNA and protein

A mouse cDNA probe (0.6 kb), specific for the RI $_{\alpha}$ subunit, detected three mRNA bands of 3.2, 2.9 and 1.7 kb (Fig. 2a). The two larger mRNAs (2.9 and 3.2 kb) declined slightly after

Fig. 2. (a) Age-dependent changes in RI_{α} mRNA (northern) and protein (western) in rat testis. Total RNA was purified, separated on 1.5% agarose gels (20 μ g per lane), blotted to nylon filters, probed with 32 P-labelled cDNA for RI_{α} and exposed for autoradiography. Soluble proteins were extracted, separated on one-dimensional SDS-PAGE (7.5%, 4.5% stacking gel), electroblotted to nitrocellulose filters and probed with specific antisera. After radioimmunolabelling, autoradiograms were prepared. (b) Cell-specific distribution of RI_{α} mRNA (northern) and protein (western) in rat testis. Lane 1: unstimulated Sertoli cells from 19-day-old rats. Lane 2: Sertoli cells stimulated with dibutyryl cAMP (0.1 mmol l⁻¹) for 48 h. Lane 3: pachytene spermatocytes from 32-day-old rats. Lane 4: round spermatids from 32-day-old rats. Lane 5: peritubular cells. Lane 6: Leydig cell tumour. Lane 7: testis from 19-day-old rats. Lane 8: testis from 32-day-old rats. Std: purified rabbit skeletal muscle RI_{α} .

30 days of age, coinciding with a high expression of a 1.7 kb RI_{α} mRNA previously shown to be specific for pachytene spermatocytes and round spermatids (Øyen *et al.*, 1988b). Immunoreactive

RI_{α} protein (Fig. 2a, western) also increased after 20 days of age. The relative increase in RI_{α} protein appears to be much less than that for the 1.7 kb mRNA band.

Cell-specific expression of RI_{α} mRNA and protein

Three mRNAs (3.2, 2.9 and 1.7 kb) were detected in rat testis, but in various relative densities in the different cell types (Fig. 2b, upper panel). The two larger bands (2.9 and 3.2 kb) were primarily expressed in the somatic cells of the testis (Sertoli cells, peritubular cells and tumour Leydig cells) (Fig. 3), whereas the smaller 1.7 kb RI_{α} mRNA revealed high expression in pachytene spermatocytes (lane 3) and round spermatids (lane 4). The dramatic increase in the 1.7 kb mRNA for RI_{α} in whole testis from day 20 (lane 7) to day 30 (lane 8) reflects the increase in these types of germ cell during this period. RI_{α} mRNA and protein in Sertoli cells (lanes 1 and 2) were stimulated 2–3-fold by dibutyryl cAMP (0.1 mmol l^{-1}). Furthermore, immunoreactive RI_{α} was detected in all types of testicular cell. Greatest amounts were seen in Sertoli cells (lanes 1 and 2) and peritubular cells (lane 5). There was much less immunoreactive RI_{α} in germ cells, in spite of the fact that mRNA content for this subunit of PKA was highest in these cells (lanes 3 and 4). The standard is purified rabbit skeletal muscle RI, which migrates with an apparent molecular mass of 49 kDa.

Age-dependent variation in RII_{α} mRNA and protein

In whole testis, a single RII_{α} band of 0.6 kb was present up to 40 days of age (Fig. 3a). The intensity of this band decreased with age of the animal. From 40 days of age, high expression of a smaller 2.2 kb band was observed. For immunoreactive RII_{α} , a single protein band migrating at 54 kDa was detected at all ages (lower panel, Fig. 3a). In spite of the dramatic increase in the RII_{α} mRNA (2.2 kb) after 40 days of age, only a small increase in RII_{α} protein was observed.

Cell-specific expression of RII_{α}

The RII_{α} 6.0 kb mRNA was detected in various amounts (Fig. 3b). The peritubular cell fraction (lane 5) had the greatest amount; intermediate amounts were found in cAMP-treated Sertoli cells (lane 2) and Leydig cells (lane 6). RII_{α} mRNA was below the level of detection in unstimulated Sertoli cells (lane 1), pachytene spermatocytes and round spermatids from 32-day-old rats (lanes 3 and 4). Further exposure of this filter resulted in a clear RII_{α} (6.0 kb) signal also in unstimulated Sertoli cells, which shows a slight increase to lane 2. This increase is not reflected at the protein level (Landmark *et al.*, 1991). The same cell fractions were examined with respect to RII_{α} protein (Fig. 3b). The standard is RII purified from rat brain. Large amounts of immunoreactive RII_{α} were found in whole testis (lanes 7 and 8), Sertoli cells (lanes 1 and 2) and peritubular cells (lane 5). In tumour Leydig cells (lane 6), a smaller immunoreactive RII_{α} protein was observed, probably representing a proteolytic degradation product of RII_{α} (lane 6). No immunoreactive RII_{α} was observed in either of the germ cell fractions (lanes 3 and 4).

Age-dependent variation of RII_{β} mRNA and protein

A single band of RII_{β} mRNA (3.2 kb) was observed before 40 days of age (Fig. 4a). After 40 days of age, variable amounts

of a smaller RII_{β} mRNA (1.9 kb) were observed. There was a discrepancy between RII_{β} mRNA and immunoreactive protein in that maximum amounts of RII_{β} mRNA were seen at 30 days of age, an age at which there is little RII_{β} protein. After 40 days of age slightly less RII_{β} mRNA and protein were observed.

Cell-specific expression of RII_{β} mRNA and protein

Abundant RII_{β} mRNA was found in stimulated (0.1 mmol l^{-1}) Sertoli cells (lane 2) and in tumour Leydig cells (lane 6). Significant RII_{β} mRNA signals were also present in peritubular cells (lane 5), the fraction of germ cells containing round spermatids (lane 4) and in whole testis from 20- and 30-day-old rats. The 1.9 kb band was most clearly visible in tumour Leydig cells (lane 6). There was a remarkable difference in RII_{β} content between unstimulated (lane 1) and stimulated (lane 2) Sertoli cells. The increase in RII_{β} mRNA after dibutyryl cAMP stimulation was more than 50-fold. On the western blot, (Fig. 4b, lower panel), the same cell fractions were probed with a specific anti- RII_{β} antiserum. A single band migrating with an apparent molecular mass of 52 kDa was detected. Whereas the cAMP stimulated Sertoli cells (lane 2) gave a clear RII_{β} signal, immunoreactive RII_{β} could not be detected in the unstimulated Sertoli cells. Unstimulated Sertoli cells (lane 1), pachytene spermatocytes (lane 3), round spermatids (lane 4) and peritubular cells were practically devoid of RII_{β} protein. In contrast to the abundance of RII_{β} mRNA in tumour Leydig cells, there was relatively little RII_{β} protein.

Age-dependent variation of C_{α} mRNA and protein

When the northern filter was probed with C_{α} cDNA, a single mRNA at 2.4 kb was detected (Fig. 5a). This mRNA increased from 30 days of age and onwards. The anti-C antiserum (Fig. 5a, lower panel) detected a band with an apparent molecular mass of 40 kDa. Whereas there was an abrupt increase in C_{α} mRNA between 20 and 30 days of age, the increase in C_{α} protein was observed from day 40 and onwards.

Cell-specific expression of C_{α} mRNA and protein

C_{α} mRNA was present in all of the cell fractions examined (Fig. 5b); the greatest amount was found in the germ-cell fractions (lanes 3 and 4), Leydig cell tumour and in whole testis from 30 days of age (lane 8). C_{α} mRNA content of Sertoli cells (lanes 1 and 2), peritubular cells (lane 5) and 20-day-old testis was somewhat lower. In general, C_{α} protein showed a similar variation and highest amounts of immunoreactive protein were seen in the germ-cell fractions (lanes 3 and 4). Neither C_{α} protein nor mRNA in Sertoli cells were influenced by cAMP treatment.

Expression of RI_{α} and RII_{α} mRNA and protein during germ cell differentiation

As shown above (Fig. 2b), significant amounts of RI_{α} mRNA (1.7 kb) and protein were observed in pachytene spermatocytes (PS) and round spermatids (RST), isolated from 32-day-old rats.

Fig. 3. (a) Age-dependent changes in RII α mRNA (northern) and protein (western) in rat testis. Twenty micrograms of total RNA was loaded onto each lane, and the resulting filter was probed with 32 P-labelled cDNA for RII α and exposed for autoradiography. Soluble proteins were extracted, separated on one-dimensional SDS-PAGE (7.5%, 4.5% stacking gel), electroblotted to nitrocellulose filters and probed with specific antisera. After radioimmunolabelling, autoradiograms were prepared. (b) Cell-specific distribution of RII α mRNA (northern) and protein (western) in rat testis. Lane 1: unstimulated Sertoli cells from 19-day-old rats. Lane 2: Sertoli cells stimulated with dibutyryl cAMP (0.1 mmol l^{-1}) for 48 h. Lane 3: pachytene spermatocytes from 32-day-old rats. Lane 4: round spermatids from 32-day-old rats. Lane 5: peritubular cells. Lane 6: Leydig cell tumour. Lane 7: testis from 19-day-old rats. Lane 8: testis from 32-day-old rats. Std: purified rat ovary RII.

In contrast, RII α mRNA (2.2 kb) and protein were not detected (Fig. 3b). The 1.7 kb RI α mRNA was already present at 20 days of age (Figs 2a, 3a), whereas the 2.2 kb RII α mRNA first appeared at day 40. At this age the spermatids have begun to elongate. We therefore examined mRNA and protein for

RI α and RII α in pachytene spermatocytes and round spermatids in cells isolated from rats of 32 and 44 days of age. From 44-day-old rats, a fraction rich in elongating spermatids was also isolated. Levels of immunoreactive RI α in PS and RST were higher in cells from 44-day-old rats than in cells from

Fig. 4. (a) Age-dependent changes in RII β mRNA (northern) and protein (western) in rat testis. Twenty micrograms of total RNA was loaded onto each lane, and the resulting filter was probed with ^{32}P -labelled cDNA for RII β and exposed for autoradiography. Soluble proteins were extracted, separated on one-dimensional SDS-PAGE (7.5%, 4.5% stacking gel), electroblotted to nitrocellulose filters and probed with specific antisera. After radioimmunolabelling, autoradiograms were prepared. (b) Cell-specific distribution of RII β mRNA (northern) and protein (western) in rat testis. Lane 1: unstimulated Sertoli cells from 19-day-old rats. Lane 2: Sertoli cells stimulated with dibutyryl cAMP (0.1 mmol l^{-1}) for 48 h. Lane 3: pachytene spermatocytes from 32-day-old rats. Lane 4: round spermatids from 32-day-old rats. Lane 5: peritubular cells. Lane 6: Leydig cell tumour. Lane 7: testis from 19-day-old rats. Lane 8: testis from 32-day-old rats. Std: purified rat ovary RII.

32-day-old rats, but revealed a clear decrease in elongating spermatids (ES) (Fig. 6). In contrast, neither RII α mRNA nor protein were detected in germ cells (PS, RST) from 32-day-old rats (Fig. 7). Coinciding with increasing RII α mRNA (2.2 kb) in elongating spermatids (ES), immunoreactive protein could also be detected by western analysis (Fig. 7, lower panel).

Separation of type I and type II protein kinase in germ cells by DEAE cellulose chromatography

Total [^3H]cAMP binding and phosphotransferase activity were measured in the eluate from DEAE cellulose columns of cytoplasmic extracts from round spermatids isolated from

Fig. 5. (a) Age-dependent changes in C_α mRNA (northern) and protein (western) in rat testis. Twenty micrograms of total RNA was loaded onto each lane, and the resulting filter was probed with ^{32}P -labelled cDNA for C_α and exposed for autoradiography. Soluble proteins were extracted, separated on one-dimensional SDS-PAGE (7.5%, 4.5% stacking gel), electroblotted to nitrocellulose filters and probed with specific antisera. After radioimmunolabelling, autoradiograms were prepared. (b) Cell-specific distribution of C_α mRNA (northern) and protein (western) in rat testis. Lane 1: unstimulated Sertoli cells from 19-day-old rats. Lane 2: Sertoli cells stimulated with dibutyryl cAMP (0.1 mmol l^{-1}) for 48 h. Lane 3: pachytene spermatocytes from 32-day-old rats. Lane 4: round spermatids from 32-day-old rats. Lane 5: peritubular cells. Lane 6: Leydig cell tumour. Lane 7: testis from 19-day-old rats. Lane 8: testis from 32-day-old rats. Std: purified C from bovine heart.

seminiferous tubules of 32-day-old rats (Fig. 8a). These two activity plots have near identical shapes, showing a peak of activity eluting at $50 \text{ mmol salt l}^{-1}$. This is interpreted as a type I kinase, i.e. the binding may be referred to as RI_α . Very low activities were measured at higher salt concentrations, and there was almost no evidence of RII [^3H]cAMP binding or phosphotransferase activities in the region.

DEAE cellulose chromatography of cytoplasmic extracts from a haploid germ cell fraction containing both round spermatids (50%) and elongating spermatids (45%) from 44-day-old rats revealed a different picture (Fig. 8b). Two peaks of [^3H]cAMP binding and phosphotransferase activity were clearly present, one eluting at $50 \text{ mmol NaCl l}^{-1}$ (type I) and the other at approximately $200 \text{ mmol NaCl l}^{-1}$ (type II). Quantitative

Fig. 6. RI_{α} mRNA (northern) and protein (western) in pachytene spermatocytes (PS), round spermatids (RST) and elongated spermatids (ES), isolated from 32- and 44-day-old rats. Twenty micrograms of total RNA was loaded onto each lane, and the resulting filter was probed with ^{32}P -labelled cDNA for RI_{α} and exposed for autoradiography. Soluble proteins were extracted, separated on one-dimensional SDS-PAGE (7.5%, 4.5% stacking gel), electroblotted to nitrocellulose filters and probed with specific antisera. After radioimmunolabelling, autoradiograms were prepared.

Fig. 7. RII_{α} mRNA (northern) and protein (western) in pachytene spermatocytes (PS), round spermatids (RST) and elongated spermatids (ES), isolated from 32- and 44-day-old rats. Twenty micrograms of total RNA was loaded onto each lane, and the resulting filter was probed with ^{32}P -labelled cDNA for RII_{α} and exposed for autoradiography. Soluble proteins were extracted, separated on one-dimensional SDS-PAGE (7.5%, 4.5% stacking gel), electroblotted to nitrocellulose filters and probed with specific antisera. After radioimmunolabelling, autoradiograms were prepared.

estimates by 'immunoimmobilization assay' revealed a 40% decrease in RI_{α} (from 13.7 to 8.5 pmol mg^{-1}), combined with a sixfold increase in RII_{α} (from 0.3 to 1.9 pmol mg^{-1}). The $RI:RII$ ratio changed from 46 in 32-day-old rats to 4.5 in 44-day-old rats.

Discussion

This report surveyed the distribution of PKA isoforms of both mRNA and protein during development in the testis and in isolated testicular cells (Sertoli cells, peritubular cells, Leydig cells and germ cells). Additional information was obtained by stimulation of Sertoli cells with cAMP and by purifying germ cells at different stages of differentiation.

The changes in amounts of R and C subunits in extracts of whole rat testis during development may be due to hormonal influences, cellular differentiation or altered cellular composition of the testis. During development in rats, there is an exponential increase in the number of germ cells from 15–25 days of age, resulting in a relative dilution of somatic cells. There is a concurrent differentiation of the germ cells through the meiotic divisions, and after 40 days of age elongating spermatids appear. Changes observed at these ages may, to a great extent, be attributed to changes in the cell composition in the testis. It should not be taken for granted that mRNA/protein content of cultured cells reflects the situation *in vivo*. However, our results on cell-specific expression for each of the subunits are compatible with the developmental changes observed and the known alteration in cell composition in rat testis of various ages (Claussen *et al.*, 1978).

The appearance of small mRNAs in whole testis have previously been reported for RI_{α} (1.7 kb) (Øyen *et al.*, 1987b) and RII_{α} (2.2 kb) (Øyen *et al.*, 1988b) and these mRNAs are formed by the use of alternative polyadenylation site signals (Øyen *et al.*, 1990). The cell specificity studies reported here convincingly demonstrate that these bands are germ-cell specific.

The most dense bands that appeared on the autoradiograms in this study were the small mRNA bands that are associated with germ cells. It is not known to what extent the various

Fig. 8. DEAE cellulose chromatography of cytoplasmic extracts from round spermatids of (a) 32-day-old and (b) 44-day-old rats. Extracts were applied to DEAE-cellulose columns (Whatman DE52) and eluted by a linear NaCl gradient (0–400 mmol NaCl l⁻¹). Fractions of 1 ml were collected, and every second sample was assayed for [³H]cAMP binding (●) and phosphotransferase activity (○).

mRNA species detected by specific cDNA probes contribute to the protein end product. All the mRNA bands indicated, including the shortest, have a length that may encompass the entire open reading frame. Comparison with similar western filters indicates that these can all be translated. On the cell specificity filters, the high molecular weight mRNA bands are found in Sertoli cells, and protein products are simultaneously present. The small mRNAs for RI_α and RII_α appear to be translated in that the amount of protein for these subunits increased on the age filters (Figs 2a and 3a) and the only mRNA bands that increased were the small bands. The increase in total [³H]cAMP binding can therefore largely be attributed to the translation of small forms of RI_α and RII_α mRNAs. There is, however, a clear discrepancy between the vast increases in signal intensities for the RI_α (1.7 kb) and RII_α (2.2 kb) mRNA on the age studies and the changes in their respective protein content. On the cell specificity filter for RI_α, the protein bands detected by western analysis revealed similar variation in intensity to the two larger (for RI_α: 3.2, 2.9 kb) mRNA bands, but not to the small-sized mRNA (1.7 kb). This result may indicate a lower translational efficiency of the low versus the high molecular weight mRNAs.

It is not known what purpose the large amounts of small mRNA species serve in germinal cells. One possibility is that

the small mRNAs for RI_α and RII_α are specifically adapted to the transcription/translation machinery of the germ cells. They may also represent a storage form of these mRNAs to be translated in elongating spermatids after transcription has stopped.

An association between the regulatory subunit RII_α and mammalian sperm flagellum has been reported by Horowitz *et al.* (1989) and Conti *et al.* (1983). In this report, early stages of germ cell development (pachytene spermatocytes, round spermatids) contain virtually only RI_α, thus making these classes of germ cell the most RI-specific cells known. The decrease in RI_α and the increase in RII_α during spermatid elongation (this study and Conti *et al.*, 1983) indicate that these subunits of PKA may have distinct functions in germ cells and spermatozoa. However, phosphorylation by PKA of axonin (probably RII_α) is not only required for sperm motility (at a stage when transcriptional activity has terminated) but is also sufficient for activation of motility (Tash *et al.*, 1986).

RI_α and RII_β are both stimulated by cAMP in Sertoli cells at the mRNA and protein level (Øyen *et al.*, 1987a; Landmark *et al.*, 1991). In spite of this they show completely different changes during development at both the mRNA and protein levels. The reason for this is probably the different cellular location and the fact that amounts of RII_β mRNA and protein are much more influenced by FSH and cAMP than is RI_α. The age-dependent decline in RII_β protein during development is most likely a result of the relative increase in germ cells during this period. The increase in RI_α protein during the same period strongly indicates that the germ cell specific (1.7 kb) mRNA is translated (see above).

In previous studies, in which type I and type II PKA kinase activities were measured in Sertoli cells, the response to cAMP treatment has been interpreted as an increase in type I PKA (Nistico *et al.*, 1991). However, if one separates the type II PKA into RII_α and RII_β, the modest increase in type II kinase after dibutyryl-cAMP stimulation can be attributed to the appearance of RII_β, which is not detectable in unstimulated cells (Landmark *et al.*, 1991). Thus, the appearance of a new subunit of PKA is easily overlooked.

With the cell specificity filter used in this study, RII_α mRNA was below the level of detection in unstimulated Sertoli cells. The presence of RII_α in these Sertoli cells is readily shown by a longer time of exposure. Furthermore, RII_α mRNA was stimulated by cAMP in Sertoli cells. However, a similar increase could not be demonstrated at the protein level. A reason for this may be that free R (and C) subunits are degraded faster than holoenzyme subunits (Flockhart *et al.*, 1982). Thus, an increase in protein following cAMP stimulation will be observed only if the increase in translation exceeds the accelerated degradation. The peritubular cells had the highest amount of 6.0 kb mRNA for RII_α, but the protein content on western analysis was of similar intensity to that of Sertoli cells and whole testis. This result may indicate that there are cell-specific differences in translational efficiencies or in R subunit stability. In Leydig cell tumour in this study the RII_α protein was apparently partly degraded, whereas RI_α and RII_β were not. This may indicate that RII_α is more susceptible to proteolytic cleavage.

The multiplicity of PKA subunits, with cell-specific distribution and differential regulation (Cadd and McKnight, 1990; Lonnerberg *et al.*, 1992) strongly indicates that different functions of cAMP may be mediated via distinct isomeric forms of

PKA (Øyen *et al.*, 1990; Scott, 1991; Lonnerberg *et al.*, 1992). There is experimental evidence for this contention in other cell systems (Skålhegg *et al.*, 1992b; Lanotte *et al.*, 1991).

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