Specific Testicular Cellular Localization and Hormonal Regulation of the PKIα and PKIβ Isoforms of the Inhibitor Protein of the cAMP-dependent Protein Kinase*

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We have previously demonstrated that there exist two distinct genes for the thermostable inhibitor protein of the cAMP-dependent protein kinase, PKIα and PKIβ (Van Patten, S. M., Howard, P., Walsh, D. A., and Maurer, R. A. (1992) Mol. Endocrinol. 6, 2114–2122). We have also shown that in the testis, at least eight forms of PKIβ exist, differing as a result of at least post-translational modification and alternate translational initiation (Kumar, P., Van Patten, S. M., and Walsh, D. A. (1997) J. Biol. Chem. 272, 20011–20020). We now report that in the testis, there is a unique cellular distribution of protein kinase inhibitor forms, with PKIβ being essentially (if not exclusively) a germ cell protein and PKIα being expressed primarily in Sertoli cells. Furthermore, there is a progressive change in the forms of PKIβ that are present within germ cells with development that is initiated in testis tubules and continues as the germ cells migrate through the epididymis. These conclusions are derived from studies with isolated cell populations and with the atlat germ cell-deficient mouse line, by in situ hybridization, and by following the developmental expression of these proteins in both testis and epididymis. We have also shown that follicle-stimulating hormone (FSH) can increase the expression of both PKIα and PKIβ. The FSH-regulated expression of PKIα in the Sertoli cell likely occurs via the normal route of second messenger signal transduction. In contrast, the FSH-dependent PKIβ expression must arise by some form of Sertoli cell-germ cell intercommunication.

The cAMP signal transduction pathway is central to the physiological function, development, and maturation of both the Sertoli and germ cells of the testis. cAMP is one of the key mediators of the actions of FSH, whose primary, nearly sole target in males is the Sertoli cell (1). The response of the Sertoli cell to FSH is complex and changes developmentally, affecting at least two critical phases of Sertoli cell development (2). In the rat, FSH is required for normal proliferation of Sertoli cells in utero and in the first 2 weeks of life, at which time their proliferation essentially ceases. FSH is essential for proper maturation of the Sertoli cell and is required for correct formation of the tight junctions responsible for the “blood-testis” barrier. FSH action on the Sertoli cell is also necessary for initiation of the first wave of spermatogenesis (2). cAMP also has a number of important functional roles in germ cells. In early spermatogenesis, it is a key regulator of transcription, acting via stimulation of PKA-dependent phosphorylation of the complex family of activator and repressor transcription factors, CREB, CREM, and CREMβ (3, 4). During the transit of sperm through the epididymis, an elevation of cAMP is one of the primary initiation signals for the acquisition of flagellar movement (5–7). One of the prominent proteins whose cAMP-dependent phosphorylation is correlated with the onset of sperm motility is a 56,000-Da protein, first called axokinin (8, 9), but subsequently identified as the PKA R1β subunit (10, 11). Later in germ cell function, cAMP appears to be a key mediator to induce capacitation (12).

Given the extensive role of cAMP-mediated phosphorylation in these essential processes of germ cell maturation, it is not surprising that there also exists some complexity of PKA itself. Essentially all of the different species of PKA subunits (R1α, R1β, R1γ, R1δ, Cα, and Cγ) are apparent in these cells, with key differences in their distribution among the distinct cell types and noted changes with cellular development (13–16). A complex pattern of PKI isoforms is also beginning to emerge. It is now established that the PKI isoforms, as first studied in skeletal muscle (17) and testis (18, 19), arise from two distinct genes (20, 21) and furthermore that at least eight forms of PKIβ exist that differ due to at least post-translational modification and alternate translational initiation (22, 23). In this report, we demonstrate that the PKIα and PKIβ isoforms are differentially localized to Sertoli and germ cells, respectively; that there is a progressive change in PKIβ isoform formation with germ cell development; that testis PKIα and PKIβ are both under hormonal/developmental regulation; and that Sertoli cell-germ cell communication likely plays an important role in the regulation of PKIβ expression.

EXPERIMENTAL PROCEDURES

Tissue and Animal Preparation—Unless indicated otherwise, for the tissues used for both Northern blot analysis of mRNA species and Western blot analysis of PKI isoforms, adult Harlan Sprague Dawley rats were sacrificed by decapitation; and immediately after dissection, the tissues were freeze-clamped with Wollenberger clamps precooled in liquid nitrogen, and the tissue was powdered. For studies of FSH dependence, immature male rats of the specified age were injected intraperitoneally with either 0.1 ml of phosphate-buffered-saline (PBS) containing 8 μg of ovine FSH (USDA-oFSH-19-SIAFP, National Hormone and Pituitary Program, NIDDK, National Institutes of Health, Bethesda, MD) or PBS alone. The tissues were removed from the animal at the indicated times after injection, and at least three separate animals were used for each data point.

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1 The abbreviations used are: FSH, follicle-stimulating hormone; PKA, cAMP-dependent protein kinase; PKI, protein kinase inhibitor; PBS, phosphate-buffered saline; TPCK, t-1-tosylamido-2-phenylethyl chloromethyl ketone; kb, kilobase(s).
Isolated testis tubules were dissected as described by Parvinen and Ruokonen (24) using transillumination-assisted microdissection. Tubules were dissected into 2-mm segments starting from an interface pale zone (Stages IX–XI) through to the next dark zone (Stage VIII) and staged by the transillumination pattern. The segments were transferred to PBS containing 0.9% NaCl, 50 mM Na2HPO4, 1.0 mM MOPS (2-aminoethyl)benzenesulfonyl fluoride, 1 μM leupeptin, 2 mM benzamidine, 0.1 mM TPCK, and 20 milliunits/ml aprotinin. The tubular segments were homogenized with an Eppendorf Teflon homogenizer, and the proteins were then extracted and analyzed. The blots shown are representative of three experiments.

Cryopreserved epididymal sperm were obtained by microdissection and extraction as described by Moore et al. (25). The sperm were separated from the epididymal tissue fragments by first suspending the dissected tissue in PBS containing a protease inhibitor mixture of 2 μg/ml aprotinin, 1 mM EDTA, 10 μg/ml benzamidine, 0.28 mM TPCK, 2.1 μM leupeptin, and 1 mM (2-aminoethyl)benzenesulfonyl fluoride (PBS-PIC buffer); gently rocking the dishes for 30 min; filtering the aspirated supernatant through cheesecloth; and then collecting the sperm by centrifugation. The sperm were resuspended in fresh PBS-PIC buffer and washed twice by centrifugation and resuspension. Soluble protein was extracted by incubating the isolated sperm at 4 °C for 5 min in 1 mL/animal of PBS-PIC buffer containing 1% Triton X-100 and then removing sperm fragments by centrifugation at 600 g for 5 min. Epididymal tissue fragments were treated by extracting freeze-clamped powdered tissue in PBS-PIC buffer, heating for 10 min at 100 °C, and then removing insoluble/denatured material by centrifugation. Cultured Sertoli cells were prepared as described by Karl and Griswold (26), as an adaptation of the method originally described by Dorrington and Fritz (27). Total germ cells were isolated by the method of Stullard and Griswold (28), as adapted from Bellve et al. (12), and the enriched germ cell populations were obtained and characterized following the procedure of Grootegoed et al. (29) using unit gravity sedimentation. The at/at germ cell-deficient mice, as originally described by Handel and Eppig (30), and the heterozygous ut/ut mice, used for controls, were purchased from Jackson Laboratories (Bar Harbor, ME). All animal studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Northern Blot mRNA Analyses—The preparation of total RNA from powdered frozen testis, agarose gel electrophoresis, and procedures for blotting/probing were done exactly as described previously (21). The probes for PKβa and PKβb, prepared as described (21), correspond to base pairs 10–1000 and 1–945 of the rat cDNAs, respectively. Following autoradiography of the blots, quantitation of the mRNA was accomplished either by cutting out the bands of radioactivity corresponding to the appropriate mRNA (4.3-kilobase pair band for PKβa and 1.4-kilobase pair band for PKβb) and counting in a liquid scintillation counter or by scanning the blots using a Bio-Rad GS-250 molecular imager (for the experiment of Fig. 8).

Western Blot Analyses of PKI Isoforms—The procedures for Western blot analyses were identical to those used in the accompanying paper (22) and are not indicated elsewhere. Protein extracts were extracted in 1 mM EDTA, pH 7.0, containing 1.6 mM EGTA, 0.1 mM TPCK, and 0.28 mM TPCK, 2.1 μM leupeptin, and 0.5 mM (2-aminoethyl)benzenesulfonyl fluoride and heated-treated for 10 min at 100 °C, and insoluble material was removed by centrifugation. One-dimensional electrophoresis (SDS) and two-dimensional electrophoresis (isoelectric focusing) and SDS were performed as described (22) using anti-PKβa-(5–22)-amide and anti-PKβb-(5–22)-amide antisera, as noted.

In Situ Hybridization—Rats of ages 5–60 days (5-day intervals) were killed using carbon dioxide asphyxiation followed by decapitation, and testes from animals were rapidly frozen on dry ice and stored at −80 °C until sectioned. Cross-sections of testes were cut to give transverse sections of the seminiferous tubules. Brains were sectioned sagittally to give a general overview of mRNA distribution. Sections (10 μm) were cut at −20 °C on a cryostat (Bright) and freeze-thawed onto gelatin/poly-L-lysine-subbed slides. Slides were stored at −80 °C until processed for in situ hybridization.

Vector and Probe Preparation—The full-length cDNAs for PKβa and PKβb are 1183 and 1359 base pairs, respectively (20, 21). PKβa cDNA was digested with HindIII/HinfI, gel-purified, treated with exonuclease DNA polynucleotidase to 3′-5′-ended, end-ligated (EcoR I/NorI1 cDNA fragment 1–364 in pBluescript). PKβb cDNA was digested with BamHI and gel-purified, and the backbone was religated (EcoRI/BamHI cDNA fragment 1–356 in pBluescript). This strategy removed poly(A) sequences present in the 3′-regions of both cDNAs. Riboprobes complementary to each isoform mRNA were generated from these constructs using in vitro transcription with T7 (PKβa) and T3 (PKβb) RNA polymerases and [35S]-UTP. Probes were labeled to a specific activity of 3–5 × 106 cpm/mmol.

Hybridization Procedure—In situ hybridization was performed as described previously (31) with modifications as noted in the fixation of tissues and hybridization temperatures. Briefly, sections of brain were post-fixed in 4% paraformaldehyde and were hydrated with PBS solution for 10 min, and sections of testis were fixed similarly for 1 h. Sections were then rinsed once in PBS and three times in 2× SSC. All solutions were treated with diethyl pyrocarbonate (0.02%). [35S]-Labeled cDNA probes were denatured by heating at 70 °C and added to hybridization buffer to give 10× counts/ml. Hybridization buffer (200 μl) was added to each slide to cover the sections, and hybridization was carried out overnight in sealed humid chambers at 45 °C. After hybridization, slides were rinsed in 2× SSC, treated with RNase A (30 μg/ml in 0.5 M NaCl, 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA, pH 8) for 60 min at 37 °C, and washed to a maximum stringency of 0.1× SSC at 55 °C for 60 min. Sections were then dehydrated in 50, 70, and 90% ethanol in 0.3 M ammonium acetate and air-dried. Sections were exposed to autoradiographic film (Amersham Hyperfilm β-max) for 10 days and then dipped in K5 nuclear emulsion (Ilford, Cheshire, United Kingdom), exposed at 4 °C for 2 weeks, developed, and counterstained with hematoxylin and eosin.

Control sections either were pretreated with RNase A (100 μg/ml) for 60 min at 37 °C prior to hybridization or were hybridized with a "sense" probe transcribed from the complementary strand of the same cDNA template. Sections with both RNA extracts with both probes, RNase pretreatment or sense probes gave no detectable signal.

Quantification of mRNA Expression by Silver Grain Number—mRNA levels at each age were determined by estimation of silver grain number overlying particular fields dependent on tissue. For PKβb mRNA in the testis, mature tubules are defined as those containing elongated spermatids. Immature tubules are those containing the stages of developing germ cells up to, but not including, elongated spermatids. Images of sections were captured on a Macintosh computer using a video camera attached to a Nikon Optiphot microscope at a magnification of ×200. Images were then exported to NIH Image (Version 1.52), where silver grains were counted using a threshold slice to detect silver grains. Area covered by silver grains was calculated as the pixel number in the field. Silver grain counts were counted per section, and background counts were subtracted before means were calculated. Values shown are means ± S.E.

RESULTS

Developmental Expression of PKI Isoforms in Rat Testis—Previous studies, based upon Northern blot analyses (21), have indicated that a major developmental shift occurs with testis PKI isoforms. This is also seen by Western blot analysis (Fig. 1, a and b). In the neonate testis, only the PKβa protein isoform is evident, reaching a maximum level by days 15–20 post-birth, then declining to the much lower rat adult level. In contrast, none of the isoforms of PKβb are detectable until days 20–25, when first the low molecular mass forms, PKβ70 and PKβ78, develop. This is followed by the appearance by days 35–45 of the higher molecular mass species, PKβX and PKβY (Fig. 1, a and c). (The designation used for the PKβ forms, as based upon their currently established characteristics (22), is depicted in Fig. 1d). The developmental profile of the phosphoforms of each of the PKβ isoforms is coincident with that of their counterpart nonphosphorylated species (Fig. 1e), suggesting that development regulates the protein species present, but not their phosphorylation. In the adult rat testis, there is a slight predominance of the higher (PKβX and PKβY) versus lower (PKβ70 and PKβ78) molecular mass species (55% versus 45%, as based upon Western blot staining intensity (22)) and a greater than 98% predominance of total PKβ versus total PKα (as based upon determination of inhibitory activity following separation by DEAE chromatography (26)). The profile of change observed for PKI isoform expression is consistent with these two forms being located in different cell types. In the immature rat testis, the predominant cell type from neonate to 20 days of age is the Sertoli cell. After 20 days of age, as sexual maturation ensues, germ cells proliferate and Sertoli cells cease proliferating, and the percentage mass of the testis derived from Sertoli cells markedly decreases. These distinctive
changes in the Sertoli composition of the testis mirror the developmental profile exhibited by PKIα, suggesting that it may be primarily a Sertoli cell product. Results observed with PKIβ are in noted contrast. Germ cells do not begin to differentiate until day 20 with the appearance of pachytene spermatocytes, followed by round spermatids by about day 30 and elongated spermatids by about day 40. The profile of PKIβ development therefore suggests that it is primarily a germ cell protein and further that the higher molecular mass PKIβ species may be constituents of the more developed spermatocyte.

A parallel can also be noted between the appearance of the higher molecular mass species of PKIβ protein species (shown here, Fig. 1) and that of a lower molecular mass PKIβ mRNA (as identified previously (21)). In all rat tissues examined other than testis, only a single 1.4-kb PKIβ message is evident (21). In the testis, the 1.4-kb PKIβ message first becomes evident by day 20, coincident with the first appearance of PKIβ protein, but by day 30, a second smaller 0.7-kb PKIβ message is detected (21), the timing of which is coincident with the first appearance of PKIβ-X and PKIβ-Y proteins (Fig. 1). In the adult testis, both sizes of PKIβ messages are abundant (21), as are also both the lower and higher molecular mass forms of PKIβ protein (Fig. 1). Alternate (and smaller) mRNA species are evident for several germ cell proteins, including, in the cAMP signal transduction pathway, the CREM transcriptional regulator (32) and PKA subunits R1α, R1β, R2β, and Cα (14). It has been suggested that the reason for the shift to smaller messages may be their greater stability. As with the CREM message, a shorter form of PKIβ message, possibly as a result of an alternate polyadenylation site at base pairs 664–671, would have eliminated two or more destabilizing 3′-downfield “AUUUA” elements (20, 33).

Cellular Localization of PKIα and PKIβ Isoforms in the Testis—The specific localization of the PKIβ and PKIα isoforms to germ cells and Sertoli cells, respectively, has been specifically examined by in situ hybridization. The 35S-labeled cRNA probes specific for each PKI form were generated as described in under “Experimental Procedures.” PKIβ mRNA expression, as evidenced by in situ hybridization, was undetectable in the early neonate (>20 days), became well evident by postnatal day 30, and by day 55 was notably abundant (Fig. 2, a–c). When first detectable, PKIβ mRNA expression was quite uniform across the entire testis section, with only one or two tubules showing more pronounced mRNA levels (Fig. 2b, arrows). By day 55, however, it was quite apparent that some tubules showed very high levels of PKIβ expression (arrowheads), and others showed much lower amounts (Fig. 2c). Microautoradiography of testis sections confirmed these findings. As illustrated in Fig. 2d, silver grains clearly delineated the individual seminiferous tubules and were highly concentrated in them, and there was a much more intense signal is some tubules than in others. This observed variation between tubules clearly became more accentuated with increasing age and increasing testicular maturity and therefore suggested that the level of PKIβ mRNA expression might be related to the stage of the seminiferous cycle. In any given cross-section of testis, the different tubules would contain germ cells at different stages of development (34). Examination of the germ cell types in those tubules with high levels of PKIβ mRNA expression showed that they were indeed at a later developmental stage (Fig. 3). A high signal

![Cell-specific PKI Isoform Regulation in Rat Testis](image_url)
level of expressed PKIβ mRNA (Fig. 3b) was coincident with tubules having an extensive number of elongated spermatids (Fig. 3a), and in these tubules, a high density of silver grains ringed the inner area of the tubule colocalized with the abundant elongated spermatids. At higher magnification, a specific localization of dense silver grains over elongated spermatids is very clearly evident (Fig. 3, c and d). The profile of PKIβ developmental expression was quantitated by silver grain count, with the data reported as pixel number per tubule, and was determined over the full age range of 5–60 days of development (Fig. 4a). Prior to day 45, the counts reported in Fig. 4a for PKIβ are for the full complement of tubules. At postnatal day 45 and after, silver grain counts were determined separately for those tubules that contained elongated spermatids and those that did not. Elongated spermatid presence was assessed by visual inspection of stained tissue slices. PKIβ mRNA was undetectable in testes from rats of <20 days of age. Low levels of expression were detectable from days 25 to 35, following which PKIβ mRNA showed a rapid increase in expression level. After 40 days of age, the separate counts of those tubules that contained elongated spermatids (Fig. 4a, closed bars) and those without (hatched bars) clearly documented that the marked elevation in PKIβ mRNA with development was associated with the later stage tubules that contained elongated spermatids. Those tubules that did not contain elongated spermatids expressed PKIβ mRNA at a much reduced level. These more immature tubules (i.e. with no elongated spermatids) showed a level of expression similar to that found in younger animals. The overall pattern of developmental change observed by the in situ studies is identical to that observed by Western blot analyses of PKIβ protein (Fig. 1) and also to that previously reported for PKIβ mRNA determined by Northern blotting (21). The full complement of data clearly demonstrate that PKIβ is a germ cell protein whose expression is highest in the later stages of the seminiferous cycle.

The pattern of PKIα expression, as evidenced by in situ hybridization, was quite different from that of PKIβ. PKIα exhibited a uniform distribution over the entire testis (Fig. 3, e and f). Such a pattern is most consistent with PKIα being primarily in Sertoli cells. It is notably different from the germ cell pattern displayed by PKIβ (Fig. 3, a–d), and were PKIα to have been primarily localized to Leydig and/or peritubule myoid cells, a much more defined nontubular pattern would have been expected. The changes in PKIα were quantitated by silver grain count. PKIα was present at readily detectable levels even at the earliest time point examined (5 days) and then increased slowly and, following a peak of expression at about day 30, diminished somewhat to the adult level (Fig. 4b). This pattern is overall quite similar to that observed by Western blot analyses (Fig. 1). Because the PKIβ protein levels by Western blot analyses are reported for an identical amount of total testis protein applied to the gel, whereas the in situ results are reported per tubule, they differ in the day that the maximum level was observed (day 20 versus day 30) and the degree to which the level then subsequently declined. The total set of
data, however, are fully consistent. Developmentally, rat Sertoli cells differentiate from the supporting cells and proliferate until about days 15–20 (2, 34), at which point proliferation ceases, and they remain at a fairly constant number thereafter. Germ cells, in contrast, represent only a very small fraction of the total testis tubule until day 20, when very active proliferation is initiated such that by day 60 they constitute ~95% of testicular mass and testis protein. The increase in PKα observed by both Western blotting (Fig. 1) and in situ hybridization (Fig. 4) between days 5 and 20 is likely associated with the increasing number of Sertoli cells. Between days 20 and 30, the continuing increase identified by in situ hybridization per tubule is most likely due to a continuing increase in total tubular PKα mRNA per Sertoli cell. Since this is occurring during a period of very active germ cell proliferation, the amount of PKα per mg of total testis protein (i.e., as detected by Western blotting) declines. From day 30 on, the total PKα per tubule appears to drop only slightly (as evidenced by the in situ hybridization studies); however, with the massive increase in total testis protein due to germ cell proliferation, the amount of PKα per total testis protein markedly diminishes.

Further documentation that the testis PKα and PKβ isoforms have a different cellular distribution is supported by studies with at/at germ cell-deficient mice. These mice are homozygous for the recessive atrichosis mutation (at/at) and are characterized by having small testes essentially devoid of germ cells, but with apparently normal Sertoli cells (30). Homozygous mutants are easily distinguished as they are nearly hairless. Littermates, which are not homozygous for the mutant (at/wt or wt/wt), are phenotypically normal (normal testis size, mature sperm present in abundance, normal hair growth). The abundance of PKI forms in the testis and cerebellum of at/at mice was examined by Western blotting (Fig. 5). In testis extracts, PKα is present at similar if not somewhat higher levels in the germ cell-deficient mice compared with controls (Fig. 5a). This clearly indicates that this isoform of PKI is expressed in cells other than germ cells. The slightly higher level of PKα apparent in the germ cell-deficient animals is as might be expected. Equal amounts of total testis protein were loaded onto each gel lane; in the germ cell-deficient animals, Sertoli cells would constitute a higher percentage of the total tissue and protein of the testis. In contrast to the results observed with PKα, a very marked difference is observed with the expression of PKβ in the at/at mice. PKβ isoforms are clearly evident in the testes of control mice, but undetectable in the testes of germ cell-deficient animals (Fig. 5b). These data support the conclusion that PKβ in the testis is predominantly (if not exclusively) a germ cell protein. There was no detectable difference between the control and at/at mice in the level of either PKα or PKβ in the cerebellum, a tissue rich in both species. Thus, the PKβ gene itself is not defective in the at/at mutant, and the altered profile in the testis is a direct consequence of the germ cell deficiency. The SDS gel profile for the control mice suggests that, compared with rats, mice have a less complex pattern of PKβ isoforms. Both PKβ-70 and PKβ-70-X isoforms are prominent in the mouse cerebellum and testis, but there is little to no PKβ-Y.

Northern blot analyses of isolated testis cell populations also support the conclusion of distinct cellular localization for the PKα and PKβ isoforms. The 4.3-kb PKα mRNA was readily detectable in isolated Sertoli cells (Fig. 6a, first lane), but undetectable in either a total germ cell population or enriched fractions of round or elongated spermatids (Fig. 6c). In contrast, no PKβ message was apparent in the isolated Sertoli cell
PKI Isoforms during Their Transit through the Epididymis and during Late Stage Germ Cell Development in the Testis—Studies of the PKI constituents of the epididymis and its constituent sperm both support and extend the conclusions of the distribution profile of PKI isoforms in germ cells. PKI was readily detectable in epididymal extracts, which contained both the epididymal tissue and sperm, but was absent in the isolated epididymal sperm (Fig. 7, a and b), thus indicating that PKI was a constituent of the cells of the epididymis, but not of their content sperm cells. This is similar to what is observed for the testis, with PKI being a constituent of the nurse cells that support the germ cells, but not of the germ cells themselves. PKI is abundant in extracts of the caput and cauda regions of the epididymis (containing both the epididymal tissue and sperm cells) and also in the epididymal sperm cells themselves isolated from these epididymal regions (Fig. 7, c and d). Clearly from these data, PKI is a constituent of the epididymal sperm; whether or not it is also a component of the epididymal tissue itself was not evaluated. The profile of PKI isoforms that is observed is of note. In both the caput epididymal extracts and the sperm isolated from these extracts, the primary forms present are PKI-\(\alpha\)-X and a form labeled “PKI-\(\beta\)-Z” that migrates at a slightly higher apparent molecular mass than PKI-\(\beta\)-Y. Minimum levels of PKI-\(\beta\)-Y and PKI-\(\beta\)-70 are evident. There is a very notable change in the PKI composition as the sperm transit the epididymis. In the cauda epididymal extracts and the sperm isolated from this region, the predominant PKI species is PKI-\(\beta\)-Z, with a marked reduction in the level of PKI-\(\beta\)-X.

We have further examined the nature of PKI-\(\beta\)-Z. Incubation of both caput and cauda sperm extracts with alkaline phosphatase resulted in the elimination of PKI-\(\beta\)-Z from the Western blot and the formation of PKI-\(\beta\)-Y (Fig. 8). This would indicate that PKI-\(\beta\)-Z is a phosphorylated form of PKI-\(\beta\)-Y. It is, however, different from the phospho form of PKI-\(\beta\)-Y denoted in Fig. 1d since it migrates in a different location. Presumably, PKI-\(\beta\)-Z either represents a multiple phosphorylated form of PKI-\(\beta\)-Y or is phosphorylated in a different site.

Given the pattern of PKI-\(\beta\) changes observed in sperm during

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**Fig. 6.** Northern blot analysis of PKI isoform distribution in isolated Sertoli and germ cells. The isolation of cell populations, culture conditions, and the procedures and probes for Northern blot analyses were performed as described under “Experimental Procedures.” RNA loading and RNA integrity were assessed by staining with methylene blue (lower section of each panel). a and b, mRNA levels in isolated Sertoli cells and the effects of hormonal treatments. a, PKI-\(\alpha\); b, PKI-\(\beta\). Where noted, the cultured Sertoli cells were incubated for 24 h with 25 ng/ml FSH, 5 mg/ml insulin, 0.1 mg/ml retinol, 200 ng/ml testosterone, 5% serum, 0.1 mM dibutyryl cAMP, or 0.16 mM phorbol 12-myristate 13-acetate (PMA). c and d, mRNA levels in isolated germ Cells. b, PKI-\(\alpha\); d, PKI-\(\beta\).

**Fig. 7.** Isoforms of PKI in rat epididymal extracts and epididymal sperm. The caput and cauda regions of the epididymis were obtained by microdissection, and total epididymal tissue (i.e., epididymis plus sperm) was analyzed by extracting the freeze-clamped powdered tissue (a and c). In addition, sperm were isolated from freshly dissected epididymal sections by the washing procedures as described by Moore et al. (25) and under “Experimental Procedures” (b and d). One-dimensional Western blot analyses were then undertaken as described under “Experimental Procedures” and in Ref. 22. a and b, detection with anti-PKI-\(\alpha\)-(5–22)-amide antisera; c and d, detection with anti-PKI-\(\beta\)-(5–22)-amide antisera.

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Preparations (Fig. 6, b and d), but both the 1.4- and 0.7-kb PKI-\(\beta\) mRNA messages were readily detected in the total germ cell population as well as in each of the enriched germ cell fractions (Fig. 6d). In total, the data of Figs. 1–6 show that in the testis, PKI is predominantly (if not exclusively) a germ cell protein, whereas PKI is a constituent of the Sertoli cell. We have yet to evaluate the possible presence of the PKI species in the other cell types of the testis. (The remaining data presented in Fig. 6 are discussed below.)
their transit through the epididymis, a further evaluation was undertaken of the possible changes that might be occurring prior to this time point during the later stages of germ cell development in the testis. Individual seminiferous tubules were separated from the interstitial tissue by manual dissection (35) and then separated into the different defined stages of tubular development by transillumination-assisted microdissection (24, 36). The PKIβ isoforms were determined by Western blot analysis.

FIG. 9. Isoforms of PKIβ in staged segments of rat seminiferous tubules. Individual seminiferous tubules, separated from the interstitial tissue by manual dissection (35), were cut into 2-mm slices using transillumination-assisted microdissection (24), starting from an interface pale zone (Stages IX–XI; first lane) through to the next dark zone (Stage VIII; last lane). The different defined stages of tubular development were determined by the transillumination pattern (36). PKIβ isoforms were determined by Western blot analysis.

is a greater abundance of PKIβ-Y and possibly the PKIβ-Z form, as also identified in the epididymal sperm.

Thus, overall, there appears to be a continuum of PKIβ developmental change in isofrom type with germ cell maturation starting in the testis and continuing with transit through the epididymis. The progression of developmental change observed for the testis (Figs. 1 and 7–9) showed initially the presence of the lower molecular mass PKIβ-70 and PKIβ-78 species (days 20–30), followed by the appearance of ever increasing amounts of the higher molecular mass PKIβ-X and PKIβ-Y forms, concordant with the increasing maturation of the spermatocyte. In most developed germ cells of the testis and in the epididymis, the higher molecular mass species of PKIβ are most prominent, and with progression through the epididymal tract from caput to cauda, there is a continuing change, with the predominant appearance of the phospho-PKIβ-Z species (Fig. 7, c and d; and Fig. 8).

Hormonal Regulation of Testis PKIα and PKIβ—Previous studies by Means and co-workers (18, 37–39) have shown that PKI expression in the testis is regulated by FSH based upon measurements of PKA inhibitory activity. The primary site of action of FSH in the male is the Sertoli cell (1, 2, 40). The elucidation that there are two distinct genetic forms of PKI, PKIα and PKIβ (20, 21), which also have distinctive cellular distribution in the testis (Figs. 1–6), prompted an extended investigation of this FSH-dependent control of PKI expression.

Sexually immature male rats of 14 days of age were injected intraperitoneally with FSH, using a protocol similar to that of the initial studies (18, 38), and PKI isoform expression was monitored by Northern blot analyses. At 14 days of age, the
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level of circulating endogenous FSH in the rat is at a nadir, having been high at birth, declining shortly thereafter, and then increasing again by days 25–30 (41). Northern blot analyses demonstrated that the expression of both PKIα and PKIβ is stimulated by FSH in the testes of rats of this age (Fig. 10). For each, there was a prompt response resulting in a 3–5-fold increase in message by 8–12 h. The level of the PKIβ message then remained quite rapidly to control levels, whereas that for PKIα remained elevated for a longer period. Exploration of this FSH-dependent sensitivity of PKI expression was also undertaken at other prepubertal ages. With PKIα, the greatest increases in response to FSH occurred during the period (days 12–18) when there were also the highest levels of endogenous protein in the absence of FSH treatment (Fig. 11). The decrease in FSH responsiveness with age may reflect a decrease in capacity for PKIα synthesis. In contrast to what was observed with PKIα, the greatest response of PKIβ to injected FSH occurred when the endogenous control level of proteins was very low (days 14–16). Later (days 18–20), the response to injected FSH was dampened, coincident with an elevated level of endogenous PKIβ. It would appear likely that FSH is a major regulator of PKIβ expression and that the increase in the level of transcript seen with development is a consequence of both the beginning presence of spermatocytes and the concordant increase in circulating levels of endogenous FSH.

Hormone-dependent regulation of PKIα was also demonstrated with cultured Sertoli cells (Fig. 6a). FSH treatment resulted in a 1.6-fold increase in PKIα transcript level, an effect also apparent with dibutyryl cAMP treatment, likely reflective of the mechanism of FSH action. An increase in PKIα mRNA in the isolated Sertoli cells was also observed in response to insulin and serum treatment (~2-fold), whereas testosterone diminished the level of endogenous transcript to ~50%. Phorbol ester was without apparent effect. It is clear the PKIα is under the control of a range of hormonal effectors, and further investigation is warranted. Consonant with its absence in the Sertoli cell, none of these effectors resulted in a discernible level of PKIβ transcript (Fig. 6b).

FIG. 11. Developmental sensitivity of FSH-induced changes in PKI expression. Rats of between 10 and 20 days of age, as indicated, were injected with either FSH or PBS alone; and at the indicated times following injection, the testes were removed, RNA was extracted, and Northern blots were performed (applying 5 μg of RNA/lane). The conditions for each of these procedures are further defined under “Experimental Procedures.” Duplicate blots were probed for PKIα (upper panel) and PKIβ (lower panel). Bands on these blots corresponding to these two forms of PKI were detected and quantitated using a Bio-Rad GS-250 molecular imager and analyzed using PhosphoAnalyst software. Each bar represents the average signal from three individual animals (±S.E.). Shaded bars, FSH treatment; closed bars, control treatment.

DISCUSSION

These data add another layer of complexity to the already intricate picture of the role of cAMP in the transduction of events in developing germ and Sertoli cells and subsequent germ cell maturation. From extensive studies that have been undertaken by a variety of approaches including studies with isolated germ cell populations, testis from different developmental ages, and microdissected testis fractions to identify germ cells at different stages of development, Jahnsen and co-workers (13–15, 42) have demonstrated that the PKA subunits Rα, Rβ, Rγ, Rδ, and Ca exhibit cell- and stage-specific differential patterns of expression. These results are likely reflective that each of the PKA subunits has specific roles at different stages of spermatogenesis and in the different cell types. The cAMP-responsive transcription factors that are key for germ cell development exhibit a pattern of even greater complexity than that observed for the PKA subunits. Alternate transcript processing of the separate genes for CREM and CREB occurs, leading to both activators and repressors of cAMP-regulated transcription, and as with the PKA subunits, which form of these factors is present at which time is very cell-
and developmental stage-specific (3, 32, 43, 44). We now observe with PKIβ an intricate pattern of expression, especially evident by the profile of forms that evolve with germ cell maturation. These forms arise as a consequence of covalent modification and alternate translation (22). Left to be unraveled is the unique function/activity that each of the different PKIβ forms manifests, and only from that knowledge will an understanding be derived of why the transitions among the forms may be important for the process of germ cell maturation. What is apparent is that the potential for detailed and specific regulation within the cAMP signal transduction cascade for the regulation of germ cell function is immense. There is a growing body of evidence that one key function of PKI is in the trafficking of the PKA catalytic subunit (45–47). Whether each of the multiple forms of PKIβ has this as its function and/or manifests some other key regulatory role remains to be resolved, and such information is critical to our understanding of the role that PKIβ plays in germ cell maturation. As we have now demonstrated (Figs. 1–9), PKIα and PKIβ are also specifically segregated between nurse cells and germ cells, respectively, in both the testis and epididymis, and the specialized role of each of these isoforms awaits elucidation.

The transcriptional regulation of both CREM and PKIβ is FSH-dependent (Ref. 32 and Figs. 10 and 11). However, in the male, only Sertoli cells have been established as FSH-sensitive; germ cells are not directly regulated by FSH as they do not contain FSH receptors (1, 40). To accomplish the FSH-dependent transcription. A model for such is presented schematically in Fig. 12. Candidates for the message that is now demonstrated (Figs. 1–9), PKIβ plays in germ cell maturation. As we have achieved and the alternate translation (22). Left to be unraveled is the unique function/activity that each of the different PKIβ forms manifests, and only from that knowledge will an understanding be derived of why the transitions among the forms may be important for the process of germ cell maturation. What is apparent is that the potential for detailed and specific regulation within the cAMP signal transduction cascade for the regulation of germ cell function is immense. There is a growing body of evidence that one key function of PKI is in the trafficking of the PKA catalytic subunit (45–47). Whether each of the multiple forms of PKIβ has this as its function and/or manifests some other key regulatory role remains to be resolved, and such information is critical to our understanding of the role that PKIβ plays in germ cell maturation. As we have now demonstrated (Figs. 1–9), PKIα and PKIβ are also specifically segregated between nurse cells and germ cells, respectively, in both the testis and epididymis, and the specialized role of each of these isoforms awaits elucidation.

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