Deletion of Type IIα Regulatory Subunit Delocalizes Protein Kinase A in Mouse Sperm without Affecting Motility or Fertilization*

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Kimberly A. Burton‡, Barbara Treash-Osio‡, Charles H. Muller§, Elizabeth L. Dunphy‡, and G. Stanley McKnight¶

From the Departments of ‡ Pharmacology and § Urology, University of Washington School of Medicine, Seattle, Washington 98195-7750

Cyclic AMP stimulates sperm motility in a variety of mammalian species, but the molecular details of the intracellular signaling pathway responsible for this effect are unclear. The type IIα isozyme of protein kinase A (PKA) is induced late in spermatogenesis and is thought to localize PKA to the flagellar apparatus where it binds cAMP and stimulates motility. A targeted disruption of the type IIα regulatory subunit (RIIα) gene allowed us to examine the role of PKA localization in sperm motility and fertility. In wild type sperm, PKA is found primarily in the detergent-resistant particulate fraction and localizes to the mitochondria-containing midpiece and the principal piece. In mutant sperm, there is a compensatory increase in RIIα protein and a dramatic relocalization of PKA such that the majority of the holoenzyme now appears in the soluble fraction and colocalizes with the cytoplasmic droplet. Unexpectedly the RIIα mutant mice are fertile and have no significant changes in sperm motility. Our results demonstrate that the highly localized pattern of PKA seen in mature sperm is not essential for motility or fertilization.

In sperm, cAMP levels are elevated when adenylyl cyclase is activated by bicarbonate (12–14) or by calmodulin and Ca2+ (15, 16). The activation of the PKA holoenzyme occurs when cAMP binds to the regulatory subunit of PKA and causes the dissociation of the catalytic (C) subunit. The PKA holoenzyme is designated either type I or type II, depending on whether it contains RI or RII, and in mammalian sperm, both are expressed. In mouse postmeiotic spermatids RII predominates (17), with RIIα mRNA and protein being highly expressed in mature elongating spermatids (18, 19). Greater than 50% of the RII holoenzyme remains in the detergent-resistant tail fraction of mature sperm, suggesting that the majority of the RII holoenzyme is firmly attached to the flagellum (20). Although conflicting data exist on whether the RIIα subunit is present in the epididymal sperm head, both RIIα and C subunits are abundant in the midpiece and principal piece of the flagellum (21–23). These results suggest that type II PKA is the primary isoform of PKA in mature sperm and that it is tightly anchored to the particulate fraction of the sperm flagellum.

Recently it was found that PKA anchoring proteins (AKAPs) bind RII subunits with high affinity and that AKAPs also bind other signaling molecules such as calcineurin and protein kinase C (24). Multi-enzyme complexes tethered by AKAPs may position protein kinases and phosphatases near their organelle-bound substrates to promote the rapid and selective phosphorylation and dephosphorylation of target proteins. Several AKAPs have been characterized in mouse sperm including S-AKAP84 (also identified as d-AKAP1), which has been localized to the mitochondria of immature sperm but is lost during maturation (25), and AKAP82, which has been localized to the fibrous sheath of mature sperm in mouse (26). Recently, both S-AKAP84/d-AKAP1 and AKAP82 (also identified as the fibrous sheath component 1 (FSC1)) were shown to bind the RIIα subunit in addition to RII subunits (27, 28). The binding affinity of S-AKAP84/d-AKAP1 for RIIα is nearly 25-fold greater than for RIIα (27), suggesting that S-AKAP84/d-AKAP1 preferentially binds RIIα in cells expressing both RIIα and RII. In contrast, a region in AKAP82/FSC1 (domain B) was observed to bind RIIα exclusively (28), suggesting that in sperm, RIIα is bound to S-AKAP84/d-AKAP1 and AKAP82/FSC1, and RIIα is bound to AKAP82/FSC1. By using a synthetic peptide that mimics the amphipathic helix RII binding motif in AKAPs (29), the association between RII subunits and AKAPs can be disrupted (30). This synthetic peptide also blocks motility of bovine sperm (31). Together these findings have led to the proposal that PKA is anchored to AKAPs by RIIα in the flagellum and that this interaction is required for sperm motility.

Based upon these findings we hypothesized that gene-targeted disruption of the RIIα subunit of PKA would produce infertile male mice as a consequence of having sperm with compromised motility. However, unexpectedly the RIIα-defi-
cient male mice are fertile, and both the curvilinearity velocity and percent motility of mutant sperm are not significantly different from that of wild type sperm. Furthermore, we show that a compensatory increase in RIIo protein in mutant sperm correlates with a change in the fractionation and localization of PKA such that the majority of the holoenzyme is now found in the soluble fraction and cytoplasmic droplet. We conclude from these studies that anchored PKA is not essential for sperm motility.

EXPERIMENTAL PROCEDURES

Animals—Wild type and RIIα mutant males were generated as described previously (32). Animals used in this study were 75, 87.5, or 97% C57BL/6 with the remaining genetic background as 129 SvJ. Female C57BL/6 mice used in this study were purchased from Jackson Laboratories (Bar Harbor, ME).

Western Blot Analysis—The cauda epididymides from adult wild type and mutant mice were removed, minced, and placed in phosphate-buffered saline (PBS). Sperm were allowed to swim out for 20 min at 37 °C. The sperm suspension was diluted in sample buffer to a final concentration of 63 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 5% glycerol, and 0.05% bromphenol blue and sonicated. DNA concentration was determined using a Hoechst dye fluorescence and was used to adjust for equal sample loading on 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. The blots were blocked overnight with 5% bovine serum albumin and 0.1% Tween 20 and then probed with affinity purified polyclonal antibodies to RIIα, Cα, RIIβ, or RIIγ. After washing and incubation with horseradish peroxidase-conjugated secondary antibody, immunoreactivity was visualized with the Amersham Pharmacia Biotech ECLTM system.

Cell Fractionation—Cauda epididymal sperm were allowed to swim out in buffer A, which contained 12 mM KH2PO4, 58.5 mM NaCl, 4.8 mM KCl, 1 mM MgCl2, 5 mM glucose, and 50 mM Tris-HCl, pH 7.4. Volumes were adjusted to provide equal concentrations (determined with a hemacytometer) of sperm from wild type and mutant mice. After gentle washing of the couplet fraction (above), equal volumes of sperm suspensions from wild type and mutant sperm were loaded on 10% polyacrylamide gels, and PKA subunits were visualized as described above by Western blot.

Immunocytochemistry—Sperm were recovered from the cauda epididymides as described above using PBS. Sperm were allowed to settle and attach on glass slides for 30 min then fixed in 4% paraformaldehyde and 0.05% gluteraldehyde in phosphate buffer overnight at 4 °C. Tissues were washed in 20% sucrose followed by 60% sucrose, both in phosphate buffer overnight at 4 °C. Tissues were then frozen/thawed three times in liquid nitrogen and placed on ice for 30 min. These extracts were clarified (40,000 × g for 15 min), and the supernatant was taken as the soluble fraction. The pellet was washed once by dispersal and sedimentation as above and then resuspended in the same volume of fortified buffer A as used for the supernatant fraction (above). Equal volumes of fractions from wild type and mutant sperm were loaded on 10% polyacrylamide gels, and PKA subunits were visualized as described above by Western blot.

Fertility Assessment—In this study, 10–15-week-old wild type and mutant mice and 8-week-old C57BL/6 females were used. Two females were placed with each male. Females were checked daily for vaginal plugs as an indication that mating had occurred. Cohabitation continued either for 5 days at which point the females were removed for 2 days or until mating was found. Matings continued until each male had successfully mated with at least four females. All mated females were euthanized 14 days after the discovery of the plug and the number of live and reabsorbed fetuses were counted. Pregnancy rate was calculated as the proportion of matings that produced pregnancy. Litter size was counted from implantations. The proportion of live fetuses was determined from the ratio of live fetuses to total implantations.

Statistical Analysis—Unpaired t tests were performed when comparing wild type and mutant groups.

RESULTS

The initial characterization of mice that contain a targeted deletion of the RIIα gene was described in a previous report.
(32). It was shown that the RIα protein is absent in all tissues examined including the testis and that these mice are viable and healthy.

Compensation in RIα Mutant Sperm—To examine the effect of a genetic deletion of RIα on the expression of PKA subunits in sperm, we performed a Western analysis on sperm samples from wild type and mutant mice. As shown in Fig. 1, the mutant sperm are completely deficient in RIα protein. However, RIIα protein levels are elevated severalfold compared with wild type sperm. There is no apparent change in Ca protein content of mutant sperm. RIIβ and RIIβ protein were not detected in wild type or mutant sperm (data not shown). The compensatory increase in the expression of RIα is most likely the result of its increased association with C subunit that is no longer bound to RIα. We have observed a similar increase in RII subunit expression in the RIIβ mutant mouse and determined that the RIα subunit has an increased half-life presumably as a consequence of its sequestration in the holoenzyme complex (38). The lack of a difference in C subunit content of mutant and wild type sperm suggests that the association with RIα prevents a change in C subunit levels. In summary, the loss of RIα in mutant sperm results in a compensatory increase in RIα with no change in C subunit levels.

Particulate Association of PKA in Sperm—The RIα and RIIα isoforms differ in their affinity for various anchoring proteins (AKAPs). Therefore we anticipated that a shift from the expression of RIα in wild type sperm to RIα in mutant sperm would alter the intracellular localization of PKA. An examination of the partitioning of PKA subunits into the detergent-soluble and detergent-insoluble fraction (Fig. 2) confirms this prediction. In wild type sperm, RIIα and Cα protein are found in both the detergent-soluble and detergent-insoluble fractions. RIIα protein content in both fractions is much lower. With longer exposure times these immunoblots show that RIIα protein is present in equivalent amounts in both fractions of sperm (data not shown). By contrast, in mutant sperm RIIα protein is absent in both fractions, and RIIα and Cα proteins are detected primarily in the detergent-soluble fraction. These results suggest that in mutant sperm, PKA is no longer bound to the detergent-resistant structures but is found primarily in the soluble fraction.

Immunolocalization of PKA Subunits in Mutant Sperm—To confirm the differences in PKA distribution within mutant and wild type sperm, as indicated by cell fractionation, we examined the immunolocalization of PKA subunits in sperm (Fig. 3). As shown in Fig. 3c, RIα immunoreactivity was found in the flagellum of wild type sperm. The staining was greater in the midpiece and in the distal portion of the principal piece. No RIα immunoreactivity was detected in the mutant sperm (Fig. 3d). Fig. 3b shows that RIα immunoreactivity was undetectable in wild type sperm. However, in mutant sperm (Fig. 3c), RIα immunoreactivity was strong in the cytoplasmic droplet. Cα and RIα were similarly distributed along the flagellum (with higher staining in the midpiece and in the end of the principal piece) in wild type sperm. Interestingly, in mutant sperm, Cα and RIα were colocalized. Cα immunoreactivity was limited to the cytoplasmic droplet (Fig. 3f) for the vast majority of cells. In less than 10% of the mutant sperm, Cα staining was also faintly detectable in the midpiece (data not shown). Immunocytochemistry performed on wild type and mutant sperm in the absence of primary antibody produced samples with no staining (data not shown).

The colocalization of RIα and C subunit suggests that the type I PKA holoenzyme (RIα-Cα) is found primarily in the cytoplasmic droplet of mutant sperm. The density gradient studies in Fig. 4 support this interpretation. Cytoplasmic droplets were separated from whole sperm by a discontinuous sucrose gradient, and protein extracts from both were separated by SDS-polyacrylamide gel electrophoresis and probed for the presence of PKA subunits. It is important to note that the gradient separation is only partially successful with a considerable level of cytoplasmic droplet containing sperm still present in the whole sperm fraction but only a few sperm contaminating the droplet fraction. For wild type sperm, RIα and C subunits were found primarily in the whole sperm fraction with only low levels appearing in the cytoplasmic droplet fraction. RIα protein was not detected in either fraction. For mutant sperm, C and RIα protein were present in both the cytoplasmic droplet and whole sperm fractions. In summary, by both immunocytochemical and cell fractionation studies, we find that PKA is no longer anchored to the flagellum but is redistributed to the cytoplasmic droplet.

Fertility and Sperm Motility in RIα Mutant Mice—The unchanged levels of Cα and enhanced expression of RIα in sperm of mutant mice might allow PKA to fulfill some of its normal functions in sperm. However, the loss of anchoring of PKA to the flagellum suggested that deficits in motility (and therefore fertility) would occur. We examined this hypothesis by comparing the fertility of wild type and mutant males. These mice were mated with females until four plugged females were identified. On day 14 of gestation, plugged females were euthanized, and litter size was measured. Results are shown in Table I. There was no significant difference between wild type and mutant males in length of time to plug four females, pregnancy rate, litter size, or percentage of live fetuses.

Despite the indication that mutant mice have normal fertility, we reasoned that more subtle defects in sperm function might be revealed by a comparison of motility parameters for wild type and mutant sperm. Sperm were incubated in vitro either in a medium that supports capacitation or in a simpler medium that was supplemented with permeant cAMP analogues. Fig. 5 (a and b) shows that in the capacitating medium, swimming speed and the proportion of motile sperm were not significantly different for mutant and control animals, and similar results were obtained when motility was examined in a simpler, bicarbonate-free medium (Fig. 5, c and d). The percentage of sperm that were hyperactivated was no different between wild type and mutant sperm in either medium (data
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FIG. 3. Immunolocalization of PKA subunits in sperm. Immunocytochemistry was performed on wild type (Wt, a–c) and RIIα mutant (d–f) sperm. Sperm were incubated with an antibody to either RIIα (a and d), RIIα (b and e), or Caα (c and f). Arrowhead, sperm head; arrow, cytoplasmic droplet.

FIG. 4. C subunit in cytoplasmic droplets of RIIα mutant sperm. Western blot comparing levels of PKA subunits in whole sperm (Sperm) and cytoplasmic droplets (Droplets) separated by discontinuous sucrose gradient. Antibodies to RIα and Caα were used on wild type (+/+ and mutant (−/−) sperm and droplets. A representative experiment is shown. Similar results were obtained in two other experiments.

TABLE I

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<tr>
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<th>Male fertility in wild type and RIIα knockout mice</th>
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<td>Weeks to plug 4 females</td>
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<tr>
<td>Wild type (n = 10)</td>
<td>4.4 ± 0.5%</td>
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<tr>
<td>Knockout (n = 11)</td>
<td>4.1 ± 0.3%</td>
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not shown). The effects of both Sp-cAMP in stimulating and Rp-cAMP in inhibiting sperm motility were small or absent, respectively, indicating that once initiated, sperm motility is independent of PKA activity. In summary, these results indicate that mutant male mice are fertile and that mutant sperm motility is not significantly different from that of wild type.

Acrosome Reaction of RIIα Mutant Sperm—Although most attention has been directed to the role of PKA in the control of sperm motility, some reports indicate that sperm cAMP content increases under conditions that promote spontaneous acrosome reaction (3). Therefore we also considered the possibility that alterations in the composition and localization of PKA in mutant sperm might alter this measure of sperm exocytotic function. However, we observed no difference in the proportion of wild type and mutant sperm that had undergone the acrosome reaction (wild type, 36 ± 4%; knockout, 32 ± 3%). These results indicate that RIIα-deficient sperm are not significantly different than wild type sperm when comparing the ability of the sperm to release acrosomal contents.

DISCUSSION

Despite much study, the intracellular signaling pathways that govern sperm motility have not been defined. Considerable attention has focused on the cAMP system because it was shown that drugs that elevate cAMP (phosphodiesterase inhibitors) lead to increased motility. Until recently, the only pathway for cAMP action in sperm was assumed to be PKA, and this assumption was bolstered by the observed induction of the RIIα regulatory subunit late in spermatogenesis and the expression of several high affinity PKA anchoring proteins associated with either the mitochondria (S-AKAP84/β-AKAP1) or the fibrous sheath (AKAP82/FSC1). However, the recent demonstrations of a cyclic nucleotide-gated ion channel in sperm and of the cAMP-mediated guanine nucleotide exchange factors in testes provide additional pathways by which cAMP might act to stimulate motility. With these possibilities in mind, we have investigated the potential role of PKA in stimulating sperm motility. Our approach was to produce a mutation in the major sperm regulatory subunit gene, RIIα, that eliminates its expression. This study shows that male mice lacking RIIα protein are fertile and that the motility of sperm from mutant mice is not impaired.

RIα subunit levels are dramatically elevated in mutant sperm compared with levels in wild type sperm. This compensation by RIα has been observed in other tissues of RIIα mutants (32) and RIIβ mutants (39). The increase in RIIα levels in
sperm from RIIa mutants most likely results from increased protein stabilization of RIα in a holoenzyme complex that serves to protect the sperm from unregulated C subunit activity (38). As a consequence of the increased levels in RIα subunit, C subunit is bound in the stable holoenzyme complex and its levels are therefore not altered in mutant sperm.

The PKA isozyme shift from a predominantly type II PKA, as seen in wild type sperm (17, 20), to a type I PKA in mutant sperm correlates with a change in the intracellular distribution of the C subunit. In wild type sperm, a major fraction of RIα and C subunits is found in the Triton-insoluble pellet. By immunocytochemistry, these subunits are localized in the flagellum with highest levels in the midpiece. This pattern of RIα and C staining has been observed by others (22, 23, 40). In mutant sperm, RIα and C subunits are found primarily in the Triton-soluble fraction and, by immunocytochemistry and density gradient experiments, in the cytoplasmic droplet. The fractionation studies demonstrate that the detergent-soluble type I PKA in mutant sperm is not anchored to the flagellum and is, therefore, more readily released into the Triton-soluble fraction. Recent biochemical studies have shown that RIα is capable of binding to a subset of the AKAPs identified in sperm including AKAP82/FSC1, which forms part of the fibrous sheath surrounding the flagellum (28), and SAKAP84b/α-AKAP1, which localizes to mitochondria (27) but is found only in immature sperm and is lost during maturation (25). The relative affinity of FSC1 for RI and RII has not been measured, but the apparent dissociation of PKA from the flagellum in RIα mutant sperm suggests that the interaction of RI with FSC1 is not strong enough to localize the holoenzyme in vivo. S-AKAP84b/α-AKAP1 binds to RI with about a 25-fold weaker affinity compared with RII, but this may be sufficient to localize PKA to mitochondria during the elongating spermatid stage. As the sperm mature, S-AKAP84b/α-AKAP1 is lost, and we would not expect to see mitochondrial localization in mature sperm of an RI-containing PKA holoenzyme.

Although RIα is not the major regulatory subunit expressed in mature sperm, it is present in low but detectable amounts in both the pellet and Triton-soluble fractions of wild type sperm. However, by immunocytochemistry, this subunit was not detected in wild type sperm. This result is not in agreement with previous reports in which RIα was found throughout the entire sperm (8, 41) and suggests that our assay is not sensitive enough to detect small amounts of RIα. This raises the possibility that small but undetectable amounts of RIα may be associated with the flagellum of RIα mutant sperm. If PKA exists as a holoenzyme in RIα mutant sperm, then the C subunit should also be detected along its flagellum. However, in the majority of RIα mutant sperm, C subunit was found in the cytoplasmic droplet, a localization that was dramatically different from that observed in wild type sperm flagellum. Although C and RIα subunits were not detected along the flagellum in RIα mutant sperm, it is possible that a small amount of PKA is associated with the flagellum and stimulates sperm motility.

Our demonstration that the majority of PKA is not anchored to the flagellum in RIα mutant sperm with no deleterious effects on sperm motility does not support the findings of Vijayaraghavan et al. (31), who showed that the motility of bovine and primate sperm was inhibited by a cell-permeant peptide (stearated H31) that blocks the association of PKA with AKAPs and presumably prevents the phosphorylation of key PKA substrates. This discrepancy suggests either a species difference in the dependence of sperm motility on anchored PKA or a nonspecific effect of stearated H31. Indeed, if stearated H31 blocked the ability of PKA to phosphorylate those substrate proteins required for motility, then it would be predicted that stearated PKI, an inhibitor of PKA activity, would likewise block motility. However, in this same study, stearated PKI did not block motility, which leads one to question the specificity of these stearated peptides.

RIα knockout mice are fertile. Although the sample size in this fertility study is too small to detect small differences in pregnancy rate (42), this study, as well as ongoing successful breeding of the mutant mice for 3 years, demonstrates that the RIα protein is not required for successful reproduction. This result is supported by our findings that there is no significant deficit in sperm motility or in the ability of mutant sperm to undergo the acrosome reaction. It is conceivable, however, that subtle deficits in fertilization capability are present in RIα mutant sperm that would be revealed by a sperm competition assay, as was shown with acrosin knockout mouse sperm (43), but this awaits further study.

Our findings clearly indicate that RIα is not essential for sperm motility and fertilization. In addition the loss of anchored PKA along the flagellum, as a result of the compensation by RIα, does not observably affect normal sperm function. The presence of PKA primarily in the cytoplasmic droplet of motile mutant sperm raises the possibility that PKA is not required for motility of mature sperm and that other targets of cAMP action are mediating its effects. It remains to be determined, however, whether PKA is anchored in earlier stages of spermatogenesis in the RIα mutant by the interaction of RIα with AKAPs that are expressed in immature sperm, such as S-AKAP84b/α-AKAP1, and whether this localization is essential for the maturation of sperm.

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