Signaling in Sperm: Toward a Molecular Understanding of the Acquisition of Sperm Motility in the Mouse Epididymis¹

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ABSTRACT

Sperm motility encompasses a wide range of events involving epididymal maturation and activation of biochemical pathways, most notably cyclic AMP (cAMP)-protein kinase A (PKA) activation. Following the discovery of guanine-nucleotide exchange factors (RAPGEFs), also known as exchange proteins activated by cAMP, we investigated the separate roles of PKA and RAPGEFs in sperm motility. RT-PCR showed the presence of *Rapgef3*, *Rapgef4*, and *Rapgef5*, as well as several known RAPGEF partner mRNAs, in spermatogenic cells. However, *Rapgef3* and *Rapgef4* appeared to be less abundant in condensing spermatids versus pachytene spermatocytes. Similarly, many of these proteins were detected by immunoblotting. RAPGEF5 was detected in germ cells and murine epididymal sperm. Indirect immunofluorescence localized SGK1, SGK3, AKT1 pT³⁰⁸, and RAPGEF5 to the acrosome, while PDPK1 was found in the postacrosomal region. SGK3 was present throughout the tail, while PDPK1 and AKT1 pT³⁰⁸ were in the midpiece. When motility was assessed in demembranated cauda epididymal sperm, addition of ATP and the selective ligand for RAPGEFs, 8-pCPT-2'-O-Me-cAMP, resulted in motility, but the sperm were unable to undergo hyperactivated-like motility. In contrast, when demembranated cauda epididymal sperm were incubated with ATP plus dibutyryl cAMP, sperm became motile and progressed to hyperactivated-like motility. However, no significant difference was observed when intact sperm were examined. GSK3 phosphorylation was altered in the presence of H89, a PKA inhibitor. Significantly, intact caput epididymal sperm became motile when incubated in the presence of extracellular ATP. These results provide evidence for a new pathway involved in endowing sperm with the capacity to swim.

epididymis, rodents (rats, mice, guinea pigs, voles), sperm, sperm maturation, sperm motility and transport

INTRODUCTION

The initiation of motility occurs in the epididymis and was initially thought to be controlled by a series of cyclic AMP (cAMP)-dependent phosphorylation reactions operating through protein kinase A (PKA) [1]. A critical factor in the production of cAMP for sperm physiology is soluble adenylyl cyclase (ADCY10); when the *Adcy10* gene is ablated by homologous recombination, the result is male infertility [2]. Although spermatogenesis in the testis appears to proceed undisturbed, the morphologically normal sperm are immotile. Motility can be partially rescued with membrane-permeable cAMP analogs, highlighting the importance of cAMP in this process [2, 3]. However, several other defects are still apparent in the presence of exogenous cAMP. Most importantly, the motile sperm are not able to fertilize eggs in vitro [3]. These sperm display a flagellar angularity, do not show the normal pattern of protein tyrosine phosphorylation under capacitating conditions, and cannot become hyperactive. A key objective in understanding how sperm become fertilization-competent is to define the role(s) of cAMP and its downstream targets.

It was initially thought that cAMP exerted its effect in sperm exclusively through PKA. When the sperm-specific catalytic subunit of PKA is eliminated by genetic ablation, sperm motility is still initiated; however, hyperactive motility and protein tyrosine phosphorylation in response to capacitation conditions does not occur [4]. Together with the finding that the lack of ADCY10 activity results in immotile sperm, these results indicate that cAMP is involved in both PKA-dependent and -independent pathways for sperm motility. In this regard, guanine-nucleotide exchange factors (RAPGEFs) also are activated by cAMP [5]. With the discovery of RAPGEFs, the cAMP-regulated sperm events that have been attributed to PKA-dependent signaling need to be reevaluated. These considerations lead us to the following hypothesis: The cAMP generated by ADCY10 is involved in a RAPGEF-regulated pathway that is independent of PKA signaling, leading to basal sperm motility.

We have developed and have begun testing a model in which cAMP generated by ADCY10 in the sperm tail leads to RAPGEF-mediated activation of AKT1 (also known as protein kinase B). The phosphorylation of glycogen synthase kinase 3 (GSK3) by AKT1 would result in sperm motility in the epididymis. Continued activation of ADCY10 and production of cAMP would phosphorylate PKA, leading to hyperactivated motility in the female reproductive tract. During the course of studies using detergent-modeled mouse sperm, we examined various agonists and antagonists of the pathway and their effects on caput epididymal sperm. When using intact caput epididymal sperm as control cells, we serendipitously discovered that treatment of these cells with extracellular ATP endowed them with the capacity to be motile.

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MATERIALS AND METHODS

Spermatogenic Cell Isolation

All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mixed germ cells were prepared from decapsulated testes of adult male mice (C57BL/6 retired breeders; Charles River Laboratories, Wilmington, MA) by sequential dissociation with collagenase and trypsin-DNase I [6]. To purify populations of pachytene spermatocytes, round spermatids, and condensing spermatids, the mixed germ cells were separated at unit gravity in a 2%–4% bovine serum albumin (BSA) gradient in Eagle Essential Medium with Earle Salts [7, 8]. Both the pachytene spermatocyte and round spermatid populations were at least 85% pure as determined by microscopic examination and differential counting with a hemocytometer. The condensing spermatid population was approximately 40%–50% pure, with the balance primarily being anucleate residual bodies and round spermatids.

Reverse-Transcription Polymerase Chain Reaction

RNA was prepared from spermatogenic cells using TRI Reagent (Sigma-Aldrich Corp., St. Louis, MO). Reverse transcription using 1 µg total RNA was performed using SuperScript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Products were amplified by denaturation at 98°C for 10 sec and 30 cycles of 98°C for 10 sec, 55°C for 30 sec, and 72°C for 1 min using the appropriate primers with Ex Taq DNA polymerase (Takara Co., Tokyo, Japan). The primers used were: *Rapgef3* Forward: 5'-GCT TGG AGT TCT TAG CCT TTG GG-3', *Rapgef3* Reverse: 5'-TGG TAT TTT CTC TCT CGG ATG AGG-3'; *Rapgef4* Forward: 5'-AGC AGA CAT CCT GTC TTC TCT CGG ATG AGG-3'; *Rapgef4* Reverse: 5'-GCC AAA GTC ATC TCC TTC GTG C-3'; *Rapgef5* Forward: 5'-AGT GTG CTG GTG CTG AAG AAA GTA G-3', *Rapgef5* Reverse: 5'-TCA TCA ACA GTG TGA CGG CG-3'; *Sgk1* Forward: 5'-CGC CAA GTC CCT CTC AAC AA-3', *Sgk1* Reverse: 5'-AAA ACT GCC CTT TCC GAT CAC-3'; *Sgk2* Forward: 5'-CCT CAA AGT CAT TGG CAA AGG G-3', *Sgk2* Reverse: 5'-GTC TCC TCA GGC TCT ACA CAT TCC-3'; *Sgk3* Forward: 5'-TTA TGG CTG AAC GCA ATG TGC-3', *Sgk3* Reverse: 5'-TCT CGG CAG TAA AAA GGA GGC-3'; *Akt1* Forward: 5'-GCA CAT CAA GAT AAC GGA CTT CG-3', *Akt1* Reverse: 5'-CTC ATA CAC ATC CTG CCA CAC G-3'; and *Pdpk1* Forward: 5'-TGG CAA CTA CGA CAA TCT CCT GAG-3', *Pdpk1* Reverse: 5'-CCT GTT AGG CGT GTG GAC AAA G-3'.

Antibodies for Immunoblot and Indirect Immunofluorescence Analyses

The primary antibodies used in this study were as follows, with catalog number, antibody description, company source, and dilution: AKT1 (44609; AKT/PKB; Biosource, Grand Island NY; 1:1000 for blot), AKT1 pS⁴⁷³ (9271; Phospho-Akt [Ser 472]; Cell Signaling, Danvers, MA; 1:500 for blot), AKT1 pT³⁰⁸ (9275; Phospho-Akt [Thr 308]; Cell Signaling; 1:1000 for blot), glycogen synthase kinase 3 (GSK3; 9331; Phospho-GSK-3α/β [Ser 219]; Cell Signaling; 1:1000 for blot), 3-phosphoinositide dependent protein kinase 1 (PDK1; 07707; PDK-1; Upstate, Billerica, MA; 1:2000 for blot, 1:200 for immunofluorescence), RAPGEF3 (95559; RAPGEF-3/EPAC-1; Novus, Littleton, CO; 1:1000 for blot), RAPGEF4 (25633; RAPGEF-4/EPAC-2; Santa Cruz, Santa Cruz, CA; 1:500 for blot), RAPGEF5 (68636; RAPGEF-5; Novus), serum and glucocorticoid-induced kinase (07315; SGK-1; Upstate; 1:10 000 for blot, 1:500 for immunofluorescence), and SGK3 (7949; SGK-3; Abgent, San Diego, CA; 1:300 for blot, 1:25 for immunofluorescence). The following secondary antibodies were used for immunoblot analysis: donkey anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare, Milwaukee, WI). Two secondary antibodies were used for indirect immunofluorescence analysis: Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L), and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Invitrogen).

Purification of Caput and Cauda Epididymal Sperm

Sperm were collected from the caput and cauda epididymides of male mice by cutting the epididymides and extruding the sperm at 37°C into phosphate-buffered saline (PBS; 1:500 for blot, 1:100 for immunofluorescence [pH 7.4]; 2.68 mM KCl, 136.09 mM NaCl, 1.47 mM KH₂PO₄, and 8.07 mM Na₂HPO₄, containing protease inhibitors). Caput epididymal sperm were purified by centrifugation at 400 × g for 20 min at room temperature through a 35% PureSperm 100 solution (MidAtlantic Diagnostics, Mt. Laurel, NJ) in PBS. Purified sperm were collected from the pellet, resuspended in PBS at 4°C, counted, and assessed for purity. Cauda epididymal sperm were collected by

centrifugation at 800 × g for 5 min at room temperature, resuspended in PBS at 4°C, and counted.

Sperm Incubation Media

Sperm were collected and incubated under two conditions as previously described [9]. Briefly, sperm were allowed to swim out from the caudae epididymides into 2 ml of modified Whitten medium (MW [pH 7.35]; 15 mM HEPES, 1.2 mM MgCl₂, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, 4.8 mM lactic acid hemi-calcium salt, and 5.5 mM glucose) at 37°C. Epididymal tissue was removed, and the sperm were washed at 100 × g for 1 min in a clinical centrifuge to remove any gross tissue debris. The sperm were resuspended in a final volume of 5 ml of MW then centrifuged at 500 × g for 8 min in a round-bottom tube. The resultant pellet was counted, assessed for motility, and diluted for use. In all cases, large-bore plastic transfer pipettes or large-orifice pipette tips were used to minimize damage to the sperm membranes. After collection and washing, sperm were incubated in MW or modified MW (MMW; MW with 10 mM NaHCO₃ and 3 mM 2-hydroxypropyl-β-cyclodextrin [2-OH-β-CD]) for 1 h in a 37°C water bath at a final concentration of 4 × 10⁶ sperm in 600 µl.

Immunoblot Analysis

The spermatogenic and sperm cells were concentrated by centrifugation, washed in 1 ml of PBS, resuspended in sample buffer (62.5 mM Tris-HCl [pH 6.8], 1.67% SDS, and 10% glycerol), and boiled for 5 min. After centrifugation, the supernatants were recovered and saved. Protein concentrations were determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL), and then dithiothreitol (DTT) and bromophenol blue were added to final concentrations of 100 mM and 0.002%, respectively. The samples were boiled for 5 min, and protein samples (15 µg per lane) were separated by SDS-PAGE in 10% polyacrylamide gels [10]. The gels were then transferred to polyvinylidene difluoride membranes [11]. After the membranes were blocked with TBST (125 mM NaCl, 25 mM Tris-HCl [pH 8.0], and 0.1% Tween 20) containing 5% BSA, they were incubated with primary antibody for 1 h. After washing with TBST, the blots were incubated for 1 h with secondary antibody in 5% BSA in TBST and, after washing with TBST, the bound enzyme was developed with the ECL kit (GE Healthcare) according to the manufacturer's directions.

In the experiments for Figure 5, sperm were collected into MW as described above, then transferred to MW or MMW alone or containing one of the following additives or sets of additives: 25 µM H89; 1 mM dibutyl cAMP (dbcAMP); 1 mM dbcAMP and 25 µM H89; 1 mM 8-(4-chlorophenylthio)-2'-O-Me-cAMP (8pCPT); 1 mM 8pCPT and 25 µM H89; 1 mM N6-benzol-cAMP; or 1 mM N6-benzol-cAMP and 25 µM H89. After 1 h at 37°C, they underwent analysis by SDS-PAGE and immunoblotting with anti-GSK3.

Indirect Immunofluorescence Analysis

One million caput or cauda epididymal sperm, collected as described above, were attached to polylysine-coated cover slips for 15 min, fixed, and permeabilized with acetone/methanol (1:1) for 1 min at −20°C. After extensive washing with PBS, the cover slips were incubated at 37°C for 1 h with 10% goat serum in PBS (blocking solution). The cover slips were incubated overnight at 4°C with primary antibodies diluted in blocking solution. The following day, the cover slips were washed with PBS and incubated for 1 h at 37°C with the corresponding Alexa Fluor 488-conjugated secondary antibodies diluted in blocking solution. Following washing with PBS, the cover slips were mounted on slides using 15 µl of Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), observed with a Nikon Eclipse TE 2000-U inverted microscope (Nikon Instruments, Melville, NY), and photographed with a CFW-1610C digital FireWire camera (Scion, Frederick, MD) using the NIH ImageJ imaging software available online (<http://rsb.info.nih.gov/ij/>). Nomarski differential interference contrast micrographs were photographed in parallel with the fluorescence images. Negative controls using normal sera or secondary antibody alone were also used to check for specificity.

Demembration and Reactivation of Sperm Cells

Sperm were allowed to flow out from the caput and caudae epididymides into 2 ml of MW at 37°C as described above. Caput and cauda epididymal sperm were demembrated by placing 2.4 × 10⁶ sperm in 300 or 400 µl of MW, respectively, adding 450 µl of extraction solution, and gently mixing for 30 sec. The extraction buffer contained 200 mM sucrose, 25 mM glutamic acid, 25 mM KOH, 0.10% Triton-X, 20 mM HEPES [pH 7.9], and 1 mM DTT. Fifty microliters of the sperm-extraction mixture was then added to the reactivation

solution and gently mixed for 30 sec. The reactivation solution consisted of 200 mM sucrose, 25 mM glutamic acid, 25 mM KOH, 1 mM ethylene glycol tetraacetic acid, 4 mM MgSO_4 , 20 mM HEPES [pH 7.9], 0.01% polyvinyl alcohol, and 1 mM DTT. The reactivation solution either contained 3 mM ATP; 3 mM dbcAMP; 3 mM ATP and 3 mM dbcAMP; or 3 mM ATP and 50 μM 8pCPT. For experiments with intact sperm, cells were collected as described above; however, they were placed directly into reactivation solution containing the above additives, depending on the treatment.

The motility patterns of each treatment group were assessed using visual microscopy and computer-assisted semen analysis (CASA) as described below. Video recordings of sperm from each treatment were obtained using a Nikon Eclipse TE 2000-U inverted microscope (Nikon Instruments) and recorded with a DAGE-MTI DC330 camera (DAGE-MTI, Michigan City, IN). To ensure cells were indeed intact and viable, sperm from each treatment were stained with Hoechst 33258 (Sigma-Aldrich Corp.). Independent experiments were performed on pooled samples of caput and cauda epididymal sperm from five mice and replicated three times. Means and SDs were calculated. Statistical difference was determined with a Student *t*-test.

Computer-Assisted Semen Analysis

For motility analysis, aliquots of each sperm suspension were analyzed using CASA (Hamilton-Thorne IVOS V12.2L; Hamilton-Thorne Research, Danvers, MA). At least 1000 sperm per sample were analyzed. For statistical analysis, frequencies of eight motion parameters—motility (%), VAP, VSL, VCL, ALH, BCF, STR, and LIN—were determined. The CASA analysis was performed as follows. Sperm aliquots (20 μl) were placed on a prewarmed, 20- μm -depth counting chamber slide (Leja Products, Nieuw-Vennep, The Netherlands) and allowed to sit on a 37°C slide warmer for 3 min. For the analysis of data, we used a recently developed, automated, quantitative method (CASANOVA) that objectively classifies five distinct motility patterns of mouse sperm using Support Vector Machines [12]. Hyperactivated-like motility was defined as motility with high-amplitude thrashing patterns and short trajectory distances. The criteria for detecting hyperactivated spermatozoa were $\text{VCL} > 180 \mu\text{m}/\text{sec}$, $\text{ALH} > 9.5 \mu\text{m}$, and $\text{LIN} < 38\%$ [13].

For motility studies, sperm were collected into MW as described above, then transferred to MW or MMW alone or MW or MMW containing one of the following additives or sets of additives: 25 μM H89; 1 mM dbcAMP; 1 mM dbcAMP and 25 μM H89; 1 mM 8pCPT; 1 mM 8pCPT and 25 μM H89; 1 mM N6-benzol-cAMP; or 1 mM N6-benzol-cAMP and 25 μM H89. CASA measurements were obtained every 15 min for 2 h.

RESULTS

Akt1, Pdpk1, Rapgef, and Sgk Genes Are Expressed in Spermatogenic Cells

Our rationale for performing these experiments derived from previous studies demonstrating that *Adcy10*-null mice produce infertile sperm that appear morphologically normal but are immotile [2], further supporting the concept that cAMP works through one or more signaling pathways to regulate motility. Historically, cAMP in sperm has been thought to work through PKA [1]; however, cAMP generated by ADCY10 may activate RAPGEFs. AKT1 is activated by RAPGEFs in a PDPK-specific manner [14]. The phosphatidylinositol-3' kinase (PI3K) pathway promotes phosphorylation of AKT1 at serine 422, which increases phosphorylation of threonine 256 by PDPK1. SGK contains these residues found in AKT1 that are phosphorylated by PI3K and PDPK1 [15]. SGK is a serine/threonine kinase that is regulated by phosphorylation. We propose that PDPK1 may activate AKT1 and/or SGK in the sperm motility pathway. Downstream of these events, the activation of AKT1 could phosphorylate and inactivate glycogen synthase kinase 3 (GSK3), which has been correlated with the initiation of motility of epididymal sperm [16]. To determine what components of these pathways may be present, RT-PCR was performed on spermatogenic cells. *Akt1*, *Pdpk1*, *Rapgef3*, *Rapgef4*, *Rapgef5*, *Sgk1*, *Sgk2*, and *Sgk3* mRNA levels were examined, and all were present in spermatogenic cells, except *Sgk2* (Fig. 1).

FIG. 1. Expression of mRNAs encoding various signaling molecules in spermatogenic cells. RT-PCR was performed on spermatogenic cells: pachytene spermatocytes (P), round spermatids (R), condensing spermatids (C), and mixed germ cells (MGC). One microgram of total RNA was used as the initial template for the RT-PCR reaction. The amplicons were then separated by agarose gel electrophoresis and stained with ethidium bromide. Shown are the signals for *Akt1* (423 bp), *Pdpk1* (467 bp), *Rapgef3* (594 bp), *Rapgef4* (527 bp), *Rapgef5* (497 bp), *Sgk1* (488 bp), *Sgk2* (456 bp), and *Sgk3* (475 bp) amplicons.

RAPGEFs and Proposed Pathway Members Are Located in Cauda Epididymal Sperm

Based on our results from RT-PCR, immunoblotting was performed. Antibodies against the proteins AKT1, AKT1 pS⁴⁷³, AKT1 pT³⁰⁸, PDPK1, RAPGEF3, RAPGEF4, RAPGEF5, SGK1, and SGK3 were used. AKT1 pS⁴⁷³ and AKT1 pT³⁰⁸ detect AKT1 when it is phosphorylated at serine 473 or threonine 308, respectively. Although our antibodies to RAPGEF3 and RAPGEF4 did not recognize any protein from caput or cauda epididymal sperm by immunoblotting, results from other laboratories have demonstrated the presence of these proteins in the acrosome and flagellum of sperm from human, hamster, mouse, horse, and pig [17–20]. The remaining proteins were detected by immunoblot (Fig. 2). RAPGEF5 was observed in pachytene spermatocytes, round spermatids, and condensing spermatids. We should note here that any proteins associated with the developing tail might artificially appear to decrease in spermatids, because these cells lose their flagella as a consequence of the proteolytic enzymes used to dissociate the seminiferous epithelium into a suspension of single germ cells.

FIG. 2. Immunoblotting of caput and cauda epididymal sperm extracts to detect signaling proteins. Caput epididymal sperm (CpS) and cauda epididymal sperm (CdS) were purified and extracted immediately for immunoblot analysis. Blots were probed with antibodies against AKT1, AKT1 pS⁴⁷³, AKT1 pT³⁰⁸, RAPGEF3, RAPGEF4, RAPGEF5, PDPK1, SGK1, and SGK3. Immunoblotting for RAPGEF3 and RAPGEF4 did not recognize any bands, so the data are not shown. RAPGEF5 was also examined in spermatogenic cells: pachytene spermatocytes (P), round spermatids (R), and condensing spermatids (C). Immunoblots for AKT1, AKT1 pS⁴⁷³, and AKT1 pT³⁰⁸ are displayed in **A**. Immunoblots for PDPK1 and RAPGEF5 are displayed in **B**. Immunoblots for SGK1 and SGK3 are displayed in **C**. The predicted size of each protein is as follows: AKT1, 60 kD; AKT1 pS⁴⁷³, 60 kD; AKT1 pT³⁰⁸, 60 kD; PDPK1, 60 kD; RAPGEF5, 68 kD; SGK1, 50 kD; SGK3, 55 kD.

SGK3, PDPK1, and AKT1 pT³⁰⁸ Are Present in the Sperm Flagellum

Indirect immunofluorescence using antibodies against AKT1, AKT1 pS⁴⁷³, AKT1 pT³⁰⁸, PDPK1, RAPGEF5, SGK1, and SGK3 were used on caput and cauda epididymal sperm. Indirect immunofluorescence localized AKT1 pT³⁰⁸, RAPGEF5, SGK1, and SGK3 to the acrosomal region, while PDPK1 was localized to the postacrosomal region of cauda epididymal sperm (Fig. 3). There was no difference in the localization of these proteins in caput or cauda epididymal sperm; therefore, we have not included images of sperm at both stages of epididymal maturation. SGK3 was found throughout the flagellum of cauda epididymal sperm, while AKT1 pT³⁰⁸ was present only in the midpiece of cauda epididymal sperm. Anti-PDPK1 detected the protein throughout the flagellum, although the staining was more intense in the midpiece. Antibodies to AKT1 and AKT1 pS⁴⁷³ did not detect the proteins in sperm by indirect immunofluorescence. It is possible that although these two antibodies recognized the protein in sperm by immunoblotting techniques, they were unable to recognize the proteins on sperm using indirect immunofluorescence because either these antibodies only work on denatured proteins or their epitopes were hidden in the native protein.

Incubation of Intact Caput Epididymal Sperm in Extracellular ATP Initiates Basal Motility

A selective activating ligand for RAPGEF3 and RAPGEF4, 8pCPT, discriminates between PKA and RAPGEF-mediated effects [21]; this reagent has been used in various systems, including sperm, and exhibits minimal effects on cell viability under conditions similar to those used in this study [19]. The effects of ATP, dbcAMP, and/or 8pCPT on intact and demembranated caput and cauda epididymal sperm motility patterns were evaluated (Table 1). As expected, when intact, cauda epididymal sperm were examined, the sperm displayed a sinuous movement throughout the flagellum (Table 1). In contrast, when motility was assessed in intact caput epididymal sperm, no movement was observed (0% of the whole sperm population as assessed by CASA). However, when extracellular ATP was added to intact caput epididymal sperm, the sperm became motile (12% of the whole sperm population as assessed by CASA), and a unique pattern was observed (Fig. 4 and Supplemental Videos 1 and 2, available online at www.biolreprod.org). The midpiece of these sperm remained rigid, while the distal principal piece displayed a sinuous motion. The addition of ATP to intact cauda epididymal sperm maintained the sinuous movement throughout the sperm flagellum (Table 1). Similarly, addition of dbcAMP or ATP plus 8pCPT to intact caput and cauda epididymal sperm resulted in the same sinuous motility pattern (Table 1).

FIG. 3. Immunofluorescence of signaling proteins in mouse sperm. Antibodies against SGK1 (A), SGK3 (B), PDPK1 (C), AKT1 pT³⁰⁸ (D), AKT1, and AKT1 pS⁴⁷³ were used to detect the proteins in cauda epididymal sperm. RAPGEF5 (E) was detected in caput (data not shown) and cauda epididymal sperm. No staining was observed with AKT1 or AKT1 pS⁴⁷³; therefore, the data are not shown. The panels to the right include the fluorescence and Nomarski images of negative controls using normal sera (IgG). Bars = 10 μ m (phase images).

TABLE 1. Effects of ATP, dbcAMP, and 8pCPT on intact or demembranated caput and cauda epididymal sperm motility patterns.

Condition	Sperm	ATP	dbcAMP	8pCPT	Motility pattern ^a
Intact	Caput				No movement
Intact	Cauda				Sinuous movement throughout flagellum
Intact	Caput	X			Rigid midpiece, sinuous movement of principal piece
Intact	Cauda	X			Sinuous movement throughout flagellum
Intact	Caput		X		Sinuous movement throughout flagellum
Intact	Cauda		X		Sinuous movement throughout flagellum
Intact	Caput	X		X	Sinuous movement throughout flagellum
Intact	Cauda	X		X	Sinuous movement throughout flagellum
Demembranated	Caput				No movement
Demembranated	Cauda				No movement
Demembranated	Caput	X			Rigid midpiece, sinuous movement of principal piece
Demembranated	Cauda	X			Rigid midpiece, sinuous movement of principal piece
Demembranated	Caput		X		No movement
Demembranated	Cauda		X		No movement
Demembranated	Caput	X	X		Rigid midpiece, sinuous movement of principal piece
Demembranated	Cauda	X	X		Fast movement progressing to hyperactivated-like motility
Demembranated	Caput	X		X	Sinuous movement throughout flagellum
Demembranated	Cauda	X		X	Sinuous movement throughout flagellum

^a Four patterns were observed and described: no movement, midpiece remained rigid while the distal principal piece displayed a sinuous motion, sinuous movement throughout the sperm flagellum, or fast movement throughout the flagellum with progression to hyperactivated-like motility.

Addition of RAPGEF Activator to Demembranated Sperm Does Not Alter Basal Motility

The scope of this particular experiment was to examine the effects of ATP, dbcAMP, and 8pCPT on demembranated sperm. Demembranated caput or cauda epididymal sperm exhibited no motility in the absence of exogenously added ATP (Table 1). When ATP was added to demembranated caput or cauda epididymal sperm, the midpiece of these sperm remained rigid, while the distal principal piece displayed a sinuous motion similar to the pattern seen when ATP was added to intact caput sperm. In the absence of ATP, no movement was observed with the addition of dbcAMP to demembranated caput or cauda epididymal sperm. When demembranated caput epididymal sperm were incubated with ATP plus dbcAMP, the sperm displayed a pattern similar to that seen with the addition of ATP alone. However, the addition of ATP plus dbcAMP to demembranated cauda epididymal sperm resulted in sperm that became motile, with a fast movement throughout the flagellum, and progressed to hyperactivated-like motility characterized by a high-amplitude, asymmetrical beating pattern of the sperm tail. In contrast, with the addition of ATP plus 8pCPT, demembranated caput and cauda epididymal sperm became motile, displaying a sinuous movement throughout the

flagellum, but were unable to undergo hyperactivated-like motility.

RAPGEF Activator Has No Effect on Motility of Intact Sperm

We next explored the effects of the addition of dbcAMP, 8pCPT, N6-benzol-cAMP, and H89 on cauda epididymal sperm. The purpose of these experiments was to test if cAMP analogs that are nonspecific (dbcAMP), RAPGEF-specific (8pCPT), or PKA-specific (N6-benzol-cAMP) can facilitate motility in the presence or absence of a PKA inhibitor (H89). In all cases, motility decreased over time. No difference was observed between treatments or between mediums (sperm maintained in MW versus MMW; Fig. 5). Data are not shown for motility assessed in sperm maintained in MW.

GSK3 Phosphorylation Decreases in the Presence of H89

Immunoblotting was used to assess the phosphorylation status of GSK3 with or without dbcAMP, 8pCPT, N6-benzol-cAMP, and H89. The antibody detects GSK3 in its inactive state (phosphorylated at serine 21 of GSK3 α and serine 9 of GSK3 β). Despite the treatment (dbcAMP, 8pCPT, or N6-benzol-cAMP), the levels of phosphorylation of GSK3 α (upper band) and GSK3 β (lower band) were elevated in sperm (Fig. 6). However, when the PKA inhibitor H89 was included in the media, the levels of phosphorylation decreased in both GSK3 α and GSK3 β . No differences were seen between sperm incubated in MW (data not shown) and MMW. Freshly isolated cauda epididymal sperm displayed a more intense level of phosphorylation in GSK3 β compared to that in GSK3 α , similar to sperm maintained in MMW.

DISCUSSION

What are the molecular mechanisms governing the acquisition of motility by sperm after they are formed in the testis? This question originates in the seminiferous tubule where, aside from some almost imperceptible flexing, the newly formed sperm are not motile. After spermiation, the sperm released into the lumen of the seminiferous tubule are transported by bulk fluid flow into the rete testes, through the efferent ducts, and into the initial segment of the epididymis.

FIG. 4. Extracellular ATP stimulates caput epididymal sperm to become motile. Sperm were recovered from the caput epididymis and incubated in MW medium in the absence (A, still image from Supplemental Video 1) or presence (B, still image from Supplemental Video 2) of extracellular ATP. Caput epididymal sperm freshly isolated from the caput epididymis were completely immotile (Supplemental Video 1). However, caput epididymal sperm placed into medium containing ATP display a flagellar beat (Supplemental Video 2). Bars = 10 μ m.

FIG. 5. CASA analysis of sperm motility in the presence of various agents. The motility of cauda epididymal sperm with or without H89 in the presence of no additional agent (A), 8pCPT (B), dbcAMP (C), or N6-benzol-cAMP (D) was assessed using CASA. The percent motility is displayed along the y-axis. The time points are displayed along the x-axis. CASA measurements were taken every 15 min for 2 h. Independent experiments were performed on pooled samples of caput and cauda epididymal sperm from five mice and replicated three times. Means and SDs were calculated. Statistical difference was determined with a Student *t*-test.

From there, the immotile sperm are transported into the caput epididymis, through the corpus epididymis, and, finally into the cauda epididymis where they are stored, awaiting ejaculation. As sperm transit through the epididymis and then reside in the cauda epididymis, they gain the ability to become progressively motile once they are placed into an appropriate environment (e.g., ejaculation into the female reproductive tract following coitus or incubation in an appropriate laboratory medium).

FIG. 6. Phosphorylation states of GSK α and GSK β in the presence of various agents. SDS-PAGE and immunoblotting with an antibody against the phosphorylated states of GSK α and GSK β were performed on cauda epididymal sperm after 1 h in MMW. Eight treatments were performed: MMW without additives (MMW), H89, dbcAMP, dbcAMP + H89, 8pCPT, 8pCPT + H89, N6-benzol-cAMP (N6Benzol), and N6Benzol + H89. Freshly isolated cauda epididymal sperm (CdS) from the epididymis was also included on the blot.

One mechanism proposed to control the initiation and regulation of sperm motility is the cAMP-dependent phosphorylation of target proteins operating through PKA [1]. This concept was initially introduced by Hoskins et al. [22], who demonstrated that the rise in cAMP levels is associated with initiation of flagellar movement. This was correlated with the increase in the ratio of enzyme activity of cAMP-dependent protein kinase in cauda epididymal sperm to those from the caput epididymis. Hoskins et al. [23] then showed that motility can be induced in immature spermatozoa from the bovine caput epididymis by treatment of the sperm with cAMP phosphodiesterase inhibitors and seminal plasma. The phosphodiesterase inhibitors presumably work by increasing the intracellular cAMP levels. Inhibition of phosphatase activity, specifically protein phosphatase 1 gamma 2, by okadaic acid and calyculin A initiates motility in bovine caput epididymal sperm without the addition of exogenous cAMP [24, 25]. This inhibition resulted in an enhancement of the phosphorylated state at the activation loop of the PKA catalytic subunit in the principal piece and midpiece of mouse sperm [26]. The addition of a cell-permeable cAMP analog, such as dbcAMP, to caput epididymal sperm causes a similar stimulation of motility (Table 1) [27, 28]. From these experiments, the conclusion can be drawn that an increase in the intracellular level of cAMP within sperm is a component of the acquisition of motility; however, since seminal plasma can also have a stimulatory effect on caput epididymal sperm, extracellular factors may play a role in the development of normal motility parameters. Further support of a role for cAMP was obtained from studies with mice that had the soluble adenylyl cyclase gene (*Adcy10*)

ablated [2, 3]. *Adcy10*-null sperm were morphologically normal but immotile; motility was restored by the addition of a membrane-permeable cAMP analog. However, the sperm remained infertile and did not appear to undergo capacitation [3]. On the other hand, the ablation of the sperm-specific catalytic subunit of PKA, *Cα2*, resulted in cauda epididymal sperm that can swim spontaneously in vitro [4]. Together, these results suggest that ADCY10 governs the initiation of motility in the epididymis by a non-PKA-dependent pathway.

To address a pathway for the regulation of sperm motility that does not involve PKA, we considered the possibility that cAMP may work through exchange proteins activated by cAMP (EPACs), formally known as RAPGEF3 and RAPGEF4. *Rapgef3* and *Rapgef4* mRNAs are present in spermatogenic cells, as we have confirmed here for the mouse (Fig. 1). RAPGEF4 was recently shown to be present in the midpiece of the tail of stallion and mouse sperm, whereas RAPGEF3 was confined to the acrosome and equatorial segment in stallion sperm and the acrosome of mouse sperm [17, 29]. Previously, an antibody that binds to a region that is conserved between RAPGEF3 and RAPGEF4 was found to react with the acrosome of human sperm [19]. Thus, it appears that there may be some variability between species concerning the localization of RAPGEF4. For completeness, we also examined RAPGEF5 (also called Repac and MR-GEF), a homologue of RAPGEF3 and RAPGEF4 that lacks the cAMP-dependent regulatory sequences and constitutively activates both Rap1 and Rap2 [30]. This protein had never been studied in sperm before. We found that RAPGEF5 was associated with the acrosomal region of mouse sperm (Fig. 3E).

After demembration with Triton X-100, sperm were not motile. When these demembrated sperm were incubated with ATP, motility was reactivated in the form of a sinuous motion in the distal region of the flagellum, while the midpiece of these sperm remained rigid (Table 1). This motion was observed in caput and cauda epididymal sperm. In this regard, when detergent-modeled cauda epididymal sperm were activated in the presence of ATP and dbcAMP, a hyperactivated-like motility was seen. These results are consistent with the conclusions made by Nolan et al. [4], who showed that functional PKA is essential for sperm hyperactivation. In contrast, the addition of 8pCPT and ATP to the reactivation medium resulted in demembrated cauda epididymal sperm whose tails displayed sinuous movement along the full length of the flagellum, but it did not result in a hyperactivated-like motility. From these experiments, it appears that 8pCPT, if it interacts with RAPGEF4 in the midpiece of the flagellum, does not alter motility patterns of demembrated mouse sperm, suggesting that the RAPGEFs may be involved in other processes (Table 1).

It is well established that serine phosphorylation of GSK3 increases significantly in sperm during their passage through the epididymis and is associated with the acquisition of motility [25]. GSK3 is a critical downstream element of the PI3K/AKT1 cell survival pathway, and GSK3 activity can be inhibited by AKT1-mediated phosphorylation at serine 21 of GSK3α and serine 9 of GSK3β [31, 32]. Upstream of GSK3 in somatic cells, PI3K generates specific phosphoinositides critical for the activation of PDK1. This enzyme phosphorylates and activates AKT1 and SGKs, which are serine/threonine kinases that phosphorylate and inactivate GSK3 [33]. Kim et al. [34] localized AKT1 to the mouse sperm acrosomal region and midpiece, as we have shown here for the phosphorylated form AKT1 pT³⁰⁸ (Fig. 3D). The specific phosphorylation of threonine 308 by PDK1 activates AKT1 and is facilitated by the interaction of AKT1 with 3-

phosphoinositides [35, 36]. *Akt1*^{-/-} males are subfertile owing to decreased numbers of sperm and severe motility defects in the sperm that are recovered from the epididymis [34]. Because sperm motility has been correlated with phosphorylation and dephosphorylation of specific proteins [1], we determined whether there were differences in the phosphorylation status of AKT1 in caput and cauda epididymal sperm (Fig. 2). Our results at this point have been inconclusive, and this issue will be examined more thoroughly in future experiments. Prior to our study, SGKs had not been examined in sperm. With RT-PCR, we showed that *Sgk1* and *Sgk3* mRNAs, but not *Sgk2*, were present in spermatogenic cells (Fig. 1). In addition, SGK1 and SGK3 were present in sperm (Fig. 2). The finding that SGK3 is present in the flagellum opens up the possibility that both AKT1 and SGK3 may be targets for PDK1, at least in the midpiece of the sperm tail.

As mentioned above, motility can be induced in bovine caput epididymal spermatozoa by the addition of cAMP phosphodiesterase inhibitors or bovine seminal plasma to the incubation medium [23]. Significantly, we have found that the addition of extracellular ATP to the sperm from the mouse caput epididymis also stimulated a sinuous motility in these sperm. We, and others, have previously demonstrated beneficial actions of extracellular ATP on ejaculated human sperm [37–40]. In our earlier studies, we demonstrated that extracellular ATP has no effect on acrosomal exocytosis or protein tyrosine phosphorylation associated with capacitation of human sperm, but it does significantly alter several motility parameters, causing increased curvilinear velocity and percentage of human sperm exhibiting hyperactivation in samples from healthy donors [39]. Extracellular ATP has similar effects on sperm selected with poor motility and sperm that had been thawed following cryopreservation. Previously, we examined mouse cauda epididymal sperm and found that extracellular ATP (ATPe) treatment significantly enhances the rates of in vitro fertilization [41]. As was observed in the human, ATPe does not increase the percentage of mouse sperm undergoing spontaneous acrosomal exocytosis, nor does it change the pattern of protein tyrosine phosphorylation normally observed in capacitated sperm. However, ATPe alters cauda epididymal mouse sperm motility parameters, causing both noncapacitated and capacitated sperm to swim faster and straighter. The levels of hyperactivated sperm are the same under capacitating conditions regardless of the presence of ATPe. Two distinct P2 purinergic receptor inhibitors block the ATPe-induced rapid increase in the level of intracellular calcium, demonstrating that these receptors have an ionotropic action in sperm.

Navarro et al. [42] have provided evidence that one possible target for the ATPe effect is the P2X2 purinergic receptor, which they found to be associated with a cation-nonselective current located in the midpiece of spermatozoa and activated by external ATP. These investigators suggest that this receptor is involved in an increase in intracellular Ca²⁺ mediated by ATPe. In examining *P2rx2*^{-/-} mice, they observed slightly faster swimming with 1 mM ATPe, but they found that hyperactivated motility is not significantly different between wild-type and *P2rx2*^{-/-} sperm. These results agree with our previous studies suggesting that P2 purinergic receptors regulate the basal motility rates but are not associated with hyperactivated motility [41].

Our demonstration that ATPe stimulates the immotile caput epididymal sperm to become motile is significant and complements the recent demonstration that the cauda epididymis secretes ATPe into the lumen [43]. It is also possible that ATPe is the uncharacterized low-molecular-weight factor from cauda epididymal fluid that stimulates calcium uptake by

FIG. 7. Proposed model of RAPGEF involvement in sperm motility. We propose that RAPGEFs act to initiate basal motility cascade through the PDPK1-AKT1-GSK3 pathway of protein kinases. After sperm are released from the seminiferous tubules, they are immotile but are carried by fluid flow into the epididymis, eventually accumulating in the cauda epididymis. Here they encounter ATPe secreted by the cauda epididymal epithelium. ATPe, acting through purinergic receptors, causes a calcium influx that stimulates adenylyl cyclase ADCY10. The resulting cAMP causes a cascade of events, leading to the eventual acquisition of basal motility.

bovine caput epididymal sperm [44]. As a result, we propose the model depicted in Figure 7 for a signaling pathway modulating basal sperm motility. Although incomplete, this model provides a framework for the design of future experiments. In our proposed model, purinergic receptors on the sperm bind ATPe secreted by the epididymal epithelium, and through the ionotropic action of the purinergic receptor in sperm newly arrived into the cauda epididymis, calcium enters the cell. In turn, calcium activates ADCY10, leading to an increase in cAMP in the sperm. The cAMP binds to RAPGEFs and PKA to cause downstream events. GSK3 is inactivated by phosphorylation to initiate basal motility. We observed that when the PKA inhibitor, H89, was included in the media, the level of GSK3 phosphorylation was altered, indicating an active state of the kinase (Fig. 6). H89 is also known to have inhibitory effects on AKT1 [45]. These results suggest that PKA and/or AKT1 act upstream of GSK3 to modulate its function; therefore, we hypothesize that the altered phosphorylation levels in GSK3 were due to the inhibition of AKT1. There is also the possibility that SGK1 and/or SGK3 substitute(s) for AKT1 in this pathway of regulation. Furthermore, we propose that the cAMP produced by ADCY10 activates RAPGEFs in addition to PKA. The activation of RAPGEFs leads to a phosphatidylinositol 3-kinase-dependent AKT1 activation [14]. Downstream of these events, the activation of AKT1 could phosphorylate and inactivate GSK3 [16]. These events would then culminate in basal motility. These studies provide new information concerning the acquisition of sperm motility during the course of epididymal maturation and identify potential members of a signaling cascade that are compartmentalized in the flagellum. The pathway proposed here provides a framework for future experiments to elucidate the specific roles of each pathway member.

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