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Compartmentalization of Distinct cAMP Signaling Pathways in Mammalian Sperm*[S]◆

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Eva Wertheimer[‡§1], Dario Krapf[‡¶1], José L. de la Vega-Beltran[‖], Claudia Sánchez-Cárdenas[‖], Felipe Navarrete[‡], Douglas Haddad[‡], Jessica Escoffier[‡], Ana M. Salicioni[‡], Lonny R. Levin, Jochen Buck**, Jesse Mager[‡], Alberto Darszon[‖2], and Pablo E. Visconti[‡3]**

*From the [‡]Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003, the [§]Centro de Estudios Farmacológicos y Botánicos, Facultad de Medicina, Consejo Nacional de Investigaciones Cientificas y Técnicas-Universidad de Buenos Aires, Buenos Aires C1121ABG, Argentina, the [¶]Instituto de Biología Celular y Molecular de Rosario, Facultad de Ciencias Bioquímicas y Farmacéuticas, Consejo Nacional de Investigaciones Cientificas y Técnicas-Universidad Nacional de Rosario, Rosario S2002LRK, Argentina, the [‖]Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnologia-Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62250, México, and the **Department of Pharmacology, Weill Cornell Medical College, New York, New York 10021*

Background: cAMP is essential for the acquisition of sperm fertilizing capacity. The presence of transmembrane adenylyl cyclases (tmACs) in sperm remains controversial.
Results: tmAC activity and its activator G_s are detected in the sperm head.
Conclusion: Two cAMP synthesis pathways coexist in sperm and lead to capacitation.
Significance: Understanding capacitation is essential for improvement of assisted fertilization and for finding novel contraceptive targets.

Fertilization competence is acquired in the female tract in a process known as capacitation. Capacitation is needed for the activation of motility (*e.g.* hyperactivation) and to prepare the sperm for an exocytotic process known as acrosome reaction. Although the HCO_3^--dependent soluble adenylyl cyclase Adcy10 plays a role in motility, less is known about the source of cAMP in the sperm head. Transmembrane adenylyl cyclases (tmACs) are another possible source of cAMP. These enzymes are regulated by stimulatory heterotrimeric G_s proteins; however, the presence of G_s or tmACs in mammalian sperm has been controversial. In this study, we used Western blotting and cholera toxin-dependent ADP-ribosylation to show the G_s presence in the sperm head. Also, we showed that forskolin, a tmAC-specific activator, induces cAMP accumulation in sperm from both WT and *Adcy10*-null mice. This increase is blocked by the tmAC inhibitor SQ22536 but not by the Adcy10 inhibitor KH7. Although G_s immunoreactivity and tmAC activity are detected in the sperm head, PKA is only found in the tail, where Adcy10 was previously shown to reside. Consistent with an acrosomal localization, G_s reactivity is lost in acrosome-reacted sperm, and forskolin is able to increase intracellular Ca^{2+} and induce the acrosome reaction. Altogether, these data suggest that cAMP pathways are compartmentalized in sperm, with G_s and tmAC in the head and Adcy10 and PKA in the flagellum.

Freshly ejaculated mammalian sperm are not competent for fertilization. They acquire fertilizing capacity during their transit through the female tract in a process known as capacitation. This process includes the preparation for a physiologically induced acrosome reaction and for changes in the sperm motility pattern known as hyperactivation (1). Although the molecular basis of this process is not completely understood, it has been shown that different aspects of capacitation are regulated by the second messenger cAMP (reviewed in Refs. 2, 3). At least part of cAMP's actions is mediated through the activation of protein kinase A (PKA). As demonstrated by McKnight and co-workers (4, 5), mice lacking the sperm-specific PKA catalytic subunit $\alpha 2$ ($C\alpha 2$) are sterile, and their sperm do not hyperactivate. In vertebrates, cAMP is synthesized by two types of adenylyl cyclases as follows: a ubiquitous family of transmembrane adenylyl cyclases (tmACs)[4] with nine members (Adcy1–9) and soluble adenylyl cyclase encoded by a single gene (Adcy10 also known as SACY or sAC), which is alternatively spliced into multiple isoforms (6–8). Adcy10 was origi-

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[S] This article contains supplemental Movies 1–3.
[1] Both authors contributed equally to this work.
[2] To whom correspondence may be addressed: Dept. de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, México. Tel.: 52-777-329-1650; E-mail: darszon@ibt.unam.mx.
[3] To whom correspondence may be addressed: Dept. of Veterinary and Animal Sciences, University of Massachusetts, Integrated Sciences Bldg. 427S, 661 North Pleasant St., Amherst, MA 01003-9301. Tel.: 413-545-5565; Fax: 413-545-6326; E-mail: pvisconti@vasci.umass.edu.

[4] The abbreviations used are: tmAC, transmembrane adenylyl cyclase; FK, forskolin; ANOVA, analysis of variance; Adcy, soluble adenylyl cyclase; sZP, solubilized ZP; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; IBMX, 3-isobutyl-1-methyl xanthine; PNA, peanut agglutinin; EPAC, exchange proteins activated by cAMP.

nally thought to be restricted to testis and sperm (9), but more recently it has been identified in other cell types (reviewed in Ref. 10). In contrast to tmACs, Adcy10 is insensitive to either heterotrimeric G proteins or the natural bicyclic diterpene forskolin (FK); instead, it is regulated by HCO_3^- and Ca^{2+} ions (8, 11, 12). Pharmacological and genetic approaches have conclusively demonstrated that Adcy10 is necessary for male fertility (13, 14). Specifically sAC was shown to be required for sperm motility and capacitation (13, 14).

In contrast, the role and presence of tmACs in sperm have been a subject of controversy. The presence of tmACs in sperm has been investigated using different methodologies, including enzymatic assays (15), mass spectrometry (16), and mouse knock-out studies (17). Although many studies concluded that tmACs were present in mammalian sperm (17–21), an equally large number of studies could not find evidence of the presence of tmAC in these cells (22–28). In the one genetic study demonstrating a role for a tmAC in male fertility using Adcy3 null mice, it was unclear whether Adcy3 played a role in mature sperm or whether it contributed to fertility during spermatogenesis (17).

One defining property of tmACs is that they are regulated by members of the heterotrimeric G protein family. These complexes are composed of three subunits (α, β, and γ), each of which belongs to different subfamilies. Transmembrane ACs are positively modulated by either of two stimulatory α subunits (α_s and α_{olf}) and negatively regulated by any of three inhibitory α subunits (α_{i1}, α_{i2}, and α_{i3}). Both stimulatory and inhibitory α subunits can be post-translationally modified by bacterial toxins. This modification can be followed *in vitro* using NAD(^{32}P) as substrate. Whereas all Gα_i subunits are ADP-ribosylated by pertussis toxin, Gα_s and α_{olf} can be modified by cholera toxin (29–31). Experimental evidence from two independent laboratories (*i.e.* the Kopf and Garbers laboratories) conclusively demonstrated the presence of Gα_i in mouse sperm (32, 33). However, similar to the situation with tmACs, the presence of stimulatory Gα_s in mammalian sperm has been controversial with conflicting reports for (34) or against (26) its presence in these cells.

We now provide evidence that tmACs are present in mouse sperm by demonstrating that cAMP is generated in response to forskolin in sperm from *Adcy10*-null mice. In addition, we show that sperm contain a cholera toxin ADP-ribosylated substrate that can be immunoprecipitated with anti-Gα_s antibodies. Our results also indicate compartmentalization of those molecules involved in the cAMP pathway, whereas Gα_s was localized to the anterior head, the PKA catalytic subunit was only found in the sperm flagellum. Interestingly, forskolin increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) and induced the acrosome reaction in sperm incubated in conditions that support capacitation.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from the following sources: bovine serum albumin (BSA, fatty acid-free), sodium methanesulfonate, sodium gluconate, potassium gluconate, dibutyryl cyclic AMP, (S_p)-diastereomer of adenosine 3′,5′-cyclic monophosphothiorate ((S_p)-cAMPS), 8-bromo-cAMP, 3-isobutyl-1-methyl xanthine (IBMX), guanosine 3-phosphate (GTP), adenosine 3-phosphate (ATP), ADP, ionophore A23187, and forskolin were purchased from Sigma. Cholera toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). The nicotinamide adenine dinucleotide, [*adenylate*-^{32}P] ([^{32}P]NAD) was obtained from PerkinElmer Life Sciences. SQ22536 was purchased from RBI (Natick, MA). KH7 was previously described (13). Cyclic AMP analogs, forskolin, and inhibitors were prepared fresh the day of the experiment in either MilliQ water or DMSO depending on solubility. Anti-phosphotyrosine (Tyr(P)) monoclonal antibody (clone 4G10) and anti-Gα_s rabbit polyclonal antibody were purchased from EMD Millipore/Upstate Biotechnology, Inc., Lake Placid, NY. Anti-PKA catalytic subunit (PKAc) monoclonal antibody (Clone 5B) was from BD Transduction Laboratories, and anti-phospho-PKA substrate monoclonal antibody (Clone 100G7E) was from Cell Signaling Technology (Danvers, MA). Ionomycin was purchased from Alomone Labs (Jerusalem, Israel) and Fluo-4 AM was from Invitrogen.

Mouse Sperm Preparation—Cauda epididymal mouse sperm were collected from CD1 retired male breeders (Charles River Laboratories, Wilmington, MA) or from Adcy10-C1 KO mice (13) and their WT littermates. All mice were sacrificed in accordance with IACUC guidelines. Minced cauda epididymis from each animal was placed in 500 μl of a modified Krebs-Ringer medium (noncapacitating Whitten's HEPES-buffered (WH)) (35) (in mM: 100 NaCl; 4.4 KCl; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 5.4 glucose; 0.8 pyruvic acid; 4.8 lactic acid; 2.4 Ca^{2+}; 20 HEPES, pH 7.4). This medium does not support capacitation unless supplemented with bovine serum albumin (5 mg/ml BSA, fatty acid-free) and $NaHCO_3$ (15 mM). After 10 min, the sperm suspension was washed by addition of 1 ml of noncapacitating media and centrifugation at 800 × *g* for 5 min at room temperature. Sperm were then resuspended (final concentration: 2 × 10^7 cells/ml) and diluted 10 times in the appropriate medium depending on the experiment performed. In experiments where capacitation was investigated, 5 mg/ml BSA and 15 mM $NaHCO_3$ were added, and sperm were incubated at 37 °C for at least 1 h.

Sperm and Brain Membrane Purification—The preparation of sperm and brain fractions was carried out as described previously (36). Briefly, mouse sperm (2 × 10^8 cells) or brains were homogenized using 10 strokes with a Teflon Dounce homogenizer in TE buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) supplemented with protease inhibitors (Protease Inhibitor Mixture (Roche Applied Science), as indicated by the manufacturer, plus 0.4 mM leupeptin, 0.4 mM aprotinin, 0.1 mM pepstatin, 0.3 M benzamidine, and 0.32 mg/ml calpains I and II inhibitor). After homogenization, the sample was sonicated three times for 15 s on ice with 1-min intervals. Cell debris was pelleted (1,000 × *g* for 10 min at 4 °C), and the supernatant was centrifuged at 10,000 × *g* for 10 min at 4 °C. Again, the resultant pellet was saved, and the supernatant was further centrifuged at 100,000 × *g* for 1 h at 4 °C. The final pellet, which contained the membrane fraction, was resuspended in sample buffer and used for SDS-PAGE and immunoblotting.

[^{32}P]ADP-ribosylation Assays—ADP-ribosylation was performed essentially as described (33), with minor modifications. Briefly, cholera toxin was activated at a concentration of 2

mg/ml by incubation for 30 min at 30 °C in media containing 100 mM DTT, 10 mg/ml BSA, 1% SDS, and 200 mM HEPES, pH 7.5. Cholera toxin assays contained 20 μg of either sperm or brain membrane protein, 6 mM ATP, 0.6 mM Gpp(NH)p, 30 mM $MgCl_2$, 6 mM EDTA, 6 mM DTT, 60 mM thymidine, 6 mg/ml lima bean trypsin inhibitor, 0.3 mg/ml aprotinin, 0.3 mg/ml leupeptin, 6 mM *p*-aminobenzamidine, and 5×10^4 cpm/pmol [^{32}P]NAD$^+$ in a final volume of 30 μl. Reactions were carried out for 1 h at 30 °C in the presence or absence of 333 μg/ml activated cholera toxin and were stopped by addition of Laemmli sample buffer. Each sample was subjected to SDS-PAGE using 10% polyacrylamide gels and transferred to PVDF membranes. Autoradiography was carried out using Kodak Biomax MS film (Rochester, NY) for 12 h up to 3 days at −80 °C.

Immunoprecipitation of [^{32}P]ADP-ribosylated Gα_s—After the [^{32}P]ADP-ribosylation assay, the solution was boiled in Tris saline buffer containing 1% SDS (25 mM Tris, pH 7.4, 150 mM NaCl, 1% SDS) for 5 min and then diluted 10 times with SDS-free Tris saline buffer. Samples were then incubated for 2 h at 4 °C with an anti-Gα_s polyclonal antibody or with normal rabbit serum at a final concentration of 0.01 mg/ml. After this period, protein G-Sepharose beads were added (10% v/v) and further incubated at 4 °C for 2 h. After incubation, beads were washed with Triton X-100/PBS by centrifugation at 12,000 × *g* for 45 s. Samples were then washed three times with PBS and finally boiled for 5 min in sample buffer. After centrifugation, eluates were subjected to SDS-PAGE, transferred to a PVDF membrane, and autoradiographed. Then the same membranes were used for Western blotting with 1 μg/ml anti-Gα_s antibodies. Chemiluminescence could be detected in less than 5 min. At this time, the radioactivity from the ^{32}P was negligible.

SDS-PAGE and Immunoblotting—After incubation under different experimental conditions, sperm were collected by centrifugation, washed in 1 ml of PBS, resuspended in Laemmli sample buffer without β-mercaptoethanol, and boiled for 5 min. After centrifugation, 5% β-mercaptoethanol was added to supernatants, and the mixture was boiled again for 5 min. Samples were subjected to SDS-PAGE using 8–10% mini-gels; protein extracts equivalent to $1–2 \times 10^6$ sperm were loaded per lane. In experiments using brain membrane extracts, 10 μg per lane were used. Each gel contained dual-prestained molecular weight standards (Bio-Rad). Electrophoretic transfer of proteins to Immobilon (Bio-Rad) and immunodetection of tyrosine-phosphorylated proteins were carried out using Tyr(P) monoclonal antibodies as described previously (37). Anti-Gα_s antibody was used at a concentration of 1 μg/ml and anti-pPKA substrate monoclonal antibody at 30 ng/ml. Immunoblots were developed with the appropriate secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch) and ECL chemiluminescence reagents. When needed, PVDF membranes were stripped at 60 °C for 15 min in 2% SDS, 0.74% β-mercaptoethanol, 62.5 mM Tris, pH 6.5, and washed six times for 5 min in T-TBS.

Acrosome Reaction Assay—Soluble zona pellucida (sZP) was prepared from homogenized ovaries of virgin female 60-day-old outbreed CD1 mice (Charles River Laboratories), and solubilized as outlined previously (38). A swim-up method was used to separate cauda epididymal sperm with >90% motility. The sperm suspension was incubated at 37 °C for 40 min under capacitating conditions. Acrosome reaction was induced after capacitation in a 50-μl aliquot by adding either different forskolin concentrations or calcium ionophore A23187 (15 μM final concentration) in the presence or absence of SQ22536. After further incubation of 30 min at 37 °C, sperm were fixed with 5% formaldehyde in PBS, then mounted on glass slides, and air-dried. Slides were stained with 0.22% Coomassie Blue G-250 in 50% methanol and 10% glacial acetic acid for 5 min, rinsed, and mounted with 50% (v/v) glycerol in PBS (38). At least 100 sperm were assayed per experimental condition to calculate the percentage of acrosome reaction.

Indirect Immunofluorescence—Sperm obtained by the swim-up method in WH medium were washed once, resuspended in PBS ($1–2 \times 10^5$ sperm/ml), and seeded on 8-well glass slides. After being air-dried, sperm were fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS (four washes at 5 min each), and permeabilized with 0.5% Triton X-100 for 5 min. Following permeabilization, sperm were treated with 10% BSA in PBS for 1 h at room temperature and then incubated with either the respective primary antibody (1:200) diluted in PBS containing 1% BSA or with the same concentration of the corresponding affinity-purified IgG; incubations were carried out at 4 °C overnight. After incubation, sperm were washed thoroughly with PBS and incubated with the corresponding Alexa 555-conjugated secondary antibody (1:200) diluted in PBS containing 1% BSA for 1 h at room temperature; these solutions also contained Alexa 488-conjugated peanut agglutinin (PNA) (1:100) for acrosomal staining. Incubation with the secondary antibody was followed by four washes in PBS, and slides were mounted using Slow-Fade Light reagents (Molecular Probes, Eugene, OR). Epifluorescence microscopy was performed using a TE300 Eclipse microscope (×60) (Nikon). Differential interference contrast images were taken in parallel and served as control for sperm morphology. Negative controls using either normal serum or secondary antibody alone were used to check for antibody specificity.

Measurement of PKA Activity—PKA activity was measured as described (36), using the sperm Triton-insoluble fraction as the source of PKA and with Kemptide (Sigma) as a PKA-specific substrate. To obtain the Triton-insoluble suspension, sperm (10^8 cells) obtained in Whitten's HEPES buffered as described above were centrifuged and resuspended in Triton buffer (25 mM Tris-HCl, 150 mM NaCl, EDTA-free protease inhibitor mixture (Roche Applied Science), pH 7.4, 1% Triton X-100). Suspensions were incubated on ice for 30 min and then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in the same buffer and saved for PKA activity assays. For determination of PKA activity, 10 μl of the Triton-insoluble sperm suspension was mixed with 10 μl of the same buffer containing known or unknown amounts of cAMP and kept on ice for no more than 30 min. The PKA enzymatic assay was started by adding 10 μl of 3× assay mixture so that the final concentration of the assay components was 100 μM Kemptide, [γ-^{32}P]ATP (3,000 Ci/mmol) (2×10^6 cpm/assay), 100 μM ATP, 1% (v/v) Triton X-100, 1 mg/ml BSA, 10 mM $MgCl_2$, 100 μM IBMX, 40 mM β-glycerophosphate, 5 mM *p*-nitrophenyl phosphate, 10 mM

Tris-HCl, pH 7.4, EDTA-free protease inhibitor mixture (Roche Applied Science). Samples were then incubated for 30 min at 37 °C. The reactions were stopped by adding 10 μl of 40% TCA, cooled on ice for 20 min, and centrifuged at room temperature for 3 min at 10,000 × *g*. Thirty μl of the resultant supernatant were then spotted onto phosphocellulose papers (2 × 2 cm) (Whatman P81). Phosphocellulose papers were washed five times for 5 min in 5 mM phosphoric acid with agitation, air-dried, placed in vials with 2.5 ml of scintillation fluid (ICN; EcoLite), and subjected to liquid scintillation counting.

Preparation of Sperm Extracts for cAMP Concentration Measurements—Sperm (5 × 10^6 cells/ml) were incubated for 60 min in 1.2 ml of Whitten's Media (WM) containing BSA/HCO_3 (capacitated) and 100 μM IBMX. Suspensions were further incubated for 30 min in the presence of different concentrations of FK. Alternatively, adenylyl cyclase inhibitors SQ22536 and KH7 were added after the initial 45 min, incubated for 15 min, and then supplemented with FK for 30 additional min. In all treatments, 1% DMSO concentration was kept constant. Suspensions were then centrifuged at 180 × *g* for 5 min, washed with 1 ml of PBS, centrifuged again, and finally resuspended in lysis buffer (40 mM HEPES, pH 7.4, 0.1 mM IBMX, EDTA-free protease inhibitor mixture (Roche Applied Science)), to a final volume of 30 μl. Samples were then boiled for 10 min to inactivate sperm enzymes and centrifuged for 5 min at 8,000 × *g*. Supernatants were saved and supplemented to 35 μl final volumes with lysis buffer. These samples were used for cAMP measurement as described below. Boiled supernatants did not contain any measurable PKA activity. When the EIA kit for cAMP determinations was used (see below), reactions were stopped with 0.1 M HCl instead of boiling, as recommended by the manufacturer.

Measurement of cAMP Levels—Intracellular sperm cAMP concentrations were determined using two different assays as follows: a PKA activity assay and an ELISA-based assay. For the PKA activity assay, the sperm Triton X-100-insoluble fraction was used as source of PKA. The amount of ^{32}P incorporated into the Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly, Sigma)-specific substrate was quantified as described (see Fig. 2*A*) (36). This method was able to reproducibly detect cAMP concentrations ranging from 1×10^{-13} M to 2×10^{-11} M. In the absence of added cAMP, the basal value was 2.5 pmol of ^{32}P incorporated into the Kemptide/30 min/10^6 sperm. This value was stimulated ~10 times in the presence of 20 pmol of cAMP. A cAMP concentration curve, using known concentrations of cAMP, was performed in parallel for each cAMP assay, and the PKA activity values obtained were used to generate a standard curve correlating PKA activity with cAMP concentrations on a logarithmic scale. This plot was then used to determine the sperm intracellular cAMP concentration by interpolation. This assay was used recently for human sperm (39). Alternatively, cAMP levels were measured using the direct cyclic AMP ELISA kit (Enzo Life Sciences/Biomol, Farmingdale, NY). This assay has an increased sensitivity that ranges from ~10 fmol to 1 pmol of cAMP. A standard curve was run for each assay, and the unknown cAMP concentrations were obtained by interpolation as recommended by the manufacturer.

Measurement of PKA Activity and cAMP Synthesis in Sperm Tail and Head Fractions—Separation of capacitated sperm heads and tails was performed by sonication as described (40) in WM. Briefly, sperm samples were sonicated on ice for 15 s, layered over 75% Percoll, and centrifuged at 725 × *g* for 15 min at 37 °C. The topmost interface between the WM and Percoll, consisting of sperm tails and the pellet, containing sperm heads, were separately collected and washed twice in WM by centrifugation (2,500 × *g* for 5 min). Sperm tail and head fractions were then used to measure PKA activity and their ability to synthesize cAMP. For the PKA activity assay, the respective fractions were supplemented or not with 100 μM FK, immediately incubated with the [^{32}P]ATP mixture containing Kemptide, and analyzed as described above for whole sperm. To evaluate the role of FK on cAMP synthesis in each fraction, the respective fraction was incubated for 30 min in the presence or absence of 100 μM FK. After this period, the sample was boiled for 10 min and centrifuged, and the cAMP produced was measured in the remaining supernatant as described above.

Intracellular Ca^{2+} Imaging—Cauda epididymal motile sperm from mice were collected by swim-up in WM medium at 37 °C for 15 min. Motile cells were incubated with 2 μM Fluo-4 AM and 0.05% pluronic acid in WM medium supplemented or not with BSA and $NaHCO_3$ according to experimental capacitating conditions. Once loaded, sperm were immobilized on mouse laminin (100 μg/ml)-coated coverslips to allow recordings. Sperm were capacitated for 1 h, and Ca^{2+} imaging was performed before, during, and after FK or sZP stimulation, in a system consisting of a Nikon Diaphot 300 inverted microscope with a Plan Apo ×60/1.40 oil Nikon objective. Ionomycin (10 μM) was added at the end of each experiment as a vitality control. Fluo-4-loaded sperm were excited with a stroboscopic LED-based fluorescence illumination system as described previously (38), with 4-ms light excitation pulses. Fluorescence was captured with a Cool Snap camera (Photometrics) at 0.5 or 1 Hz. Movies were processed using Image J (Version 1.38, National Institutes of Health). Fluorescence is expressed as $f - f_0/f_0$. Those sperm cells that showed an increase of fluorescence higher than twice the standard deviation of the basal fluorescence were considered as "responsive cells."

Analysis of Mouse Adcy Expression by Reverse Transcription-PCR—Analysis of *Adcy* (1–10) expression during testis development was carried out using mouse testis total RNA isolated from mice of 7, 14, 21, 24, 28, and 48 days of age; sperm cells, eye, and brain tissue from adult mice were also tested. Total RNA was extracted using High Pure RNA isolation kit (Roche Applied Science), including a DNase digestion treatment to remove genomic DNA. RNA (500 ng) was reverse-transcribed in a 30-μl reaction volume using the iScript cDNA synthesis kit (Bio-Rad). For RT-PCR, 20-μl PCRs were performed with RubyTaq polymerase (Affymetrix) using 2 μl of cDNA as template. All PCRs were performed in Bio-Rad Thermocycler with 35 cycles of 10 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C, with a final 2-min extension at 72 °C. PCR products were electrophoresed in 1.5% agarose gel with ethidium bromide and visualized on a Syngene gel imaging system. Intron spanning primers are listed in Table 1.

TABLE 1
Intron spanning primers used for analysis of *Adcy* expression by RT-PCR

Transcript	Forward primer	Reverse primer
Adcy1	ATTGAGAACAGCCTCGGAGA	ACTGAGCTCCAGGACAAGGA
Adcy2	GACTTCTGCTTTCCCTGCTG	TATGGCTTCGCACATATCCA
Adcy3	TACCCAGCTGTCCTCTGCTT	CGTACGAGATGGCCTCTACC
Adcy4	GTGTCCACCTCCACTCCACT	CACCAGCCACAGCAGAAGTA
Adcy5	CCTACCTCAAGGAGCACAGC	CCAGGGTGATGGTGAATACC
Adcy6	ATCCACATCACTCGGGCTAC	GGCAGAGATGAACACAAGCA
Adcy7	ACTCTGGGTGTGTCCTTTGG	GCTTTGTGCATCAGACAGGA
Adcy8	GAGAACCAAAGACAGGAGCG	AGTCACCATTGAGGCAATCC
Adcy9	CATATCTGAGGCCACTGCAA	GTGCTGGTCTTAGGCTCGTC
Adcy10	GGCTGTTGCTATTGCACTGA	CTGTGGTGGTCGAGGTTTTT
Tssk4	TGGAACTGTCTATGAGGCATACT	CCAAAGCCATCTGGGAGAA
Actin β	GGCCCAGAGCAAGAGAGGTATCC	ACGCACGATTTCCCTCTCAGC

Statistical Analysis—Data are expressed as the means ± S.E. To assume normal distribution, percentages were converted to ratios, and all data were subjected to the arcsine square root transformation. Statistical analyses were performed with the aid of GraphPad Prism Version 6.01 software, using the parametric *t* test for simple comparisons and either the Tukey test following one-way ANOVA or the Bonferroni post tests after two-way ANOVA for multiple comparisons.

RESULTS

Gα_s Is Present in Mouse Sperm—To investigate the presence of the tmAC signaling complex in sperm, we first analyzed whether these cells contained at least one of the Gα stimulatory subunits (α_s or α_{olf}) using a cholera toxin ADP-ribosylation assay. Sperm and brain particulate membrane fractions were treated with cholera toxin in the presence of [^{32}P]NAD$^+$, separated by PAGE, transferred to Immobilon-P, and analyzed by autoradiography. We detected two ADP-ribosylated bands in the presence of cholera toxin in both sperm and brain membrane fractions (Fig. 1*A*, *left panel*). Western blotting of the same Immobilon P membrane with anti-Gα_s antisera revealed two bands overlapping the ADP-ribosylation signals (Fig. 1*A*, *right panel*). To confirm the identity of these bands as Gα_s proteins, the anti-Gα_s antibodies were used to immunoprecipitate sperm and brain membrane extracts subsequent to [^{32}P]ADP-ribosylation by cholera toxin (Fig. 1*B*). The doublet observed in each of these experiments suggests that two Gα_s splicing variants (small ~45 kDa and large ~52 kDa) (29–31) are present in sperm (Fig. 1, *A* and *B, left panel*). Alternatively, it remains possible that one of the bands corresponds to Gα_{olf}, another member of the Gα_s family that is also a cholera toxin substrate and shares high sequence homology with Gα_s.

Forskolin Stimulates cAMP Levels in Capacitated Mouse Sperm—The finding of stimulatory Gα_s in sperm could indicate that in addition to Adcy10 these cells might also contain tmACs. To investigate this possibility, we incubated capacitated sperm with the diterpene FK, a compound known to stimulate tmACs without activating Adcy10 activity (6, 41–43). Cyclic AMP levels were quantified using a PKA activation assay as described under "Experimental Procedures." A representative calibration curve is shown in Fig. 2*A*. Sperm were incubated in conditions that support capacitation for 1 h followed by an additional 30-min incubation in the absence or presence of FK. To prevent cAMP degradation, the phosphodiesterase inhibitor IBMX was present during the final 30-min incubation. FK induced a concentration-dependent cAMP accumulation (Fig. 2*B*). Inclusion of the Adcy10-specific inhibitor KH7 (50 μM) (13) did not affect the FK-induced increase in cAMP levels, indicating that this atypical adenylyl cyclase is not involved in the FK effect (Fig. 2*B*). However, inclusion of the tmAC inhibitor SQ22536 (100 μM) (44) significantly reduced the FK effect (Fig. 2*B*). To further discriminate the contribution of Adcy10 from the FK-induced cAMP increase, cAMP levels were measured in sperm from *Adcy10*-null mice. These sperm were incubated for 1 h in capacitating media supplemented with 100 μM IBMX and further exposed or not to 50 μM FK for 30 min. In the absence of FK, cAMP accumulation was beneath the lower detection limit of the assay; however, in the presence of FK (50 μM), cAMP increased >10-fold above the lower limit of detection (Fig. 2*C*). Controls were carried out in parallel using wild type mice from the same litter (Fig. 2*C*). In WT sperm, basal cAMP accumulation was detectable, and cAMP accumulation was significantly elevated due to FK. Altogether, these data indicate that an adenylyl cyclase different from Adcy10 was able to respond to FK in sperm.

To analyze which tmACs might have a role in sperm, we analyzed all Adcy isoform transcripts by RT-PCR in different testicular developmental stages as well as in mature sperm. Positive controls for each of the isoforms were tested in parallel. Even when most of the Adcys were found in different stages of testis development, only the tmAC Adcy6–9 and the sAC Adcy10 were found in mature sperm (Fig. 3).

Effect of Forskolin on Phosphorylation Events Associated with Sperm Capacitation—Capacitation is associated with the activation of a cAMP/PKA-dependent signaling pathway leading to phosphorylation of Ser/Thr residues in PKA substrates followed by up-regulation of protein tyrosine phosphorylation (1, 45). It was previously shown that cAMP synthesized by sAC could stimulate phosphorylation of a number of PKA substrate proteins, so we asked whether a FK-induced increase in cAMP could up-regulate similar phosphorylation events in sperm. However, addition of FK did not increase phosphorylation in sperm incubated in either capacitating or noncapacitating conditions (Fig. 4*A*). However, addition of cAMP-permeable agonists (dibutyryl cyclic AMP and 8-bromo-cAMP) induced phosphorylation of PKA substrates as well as phosphorylation of tyrosine residues (Fig. 4, *B* and *C*) in noncapacitating conditions.

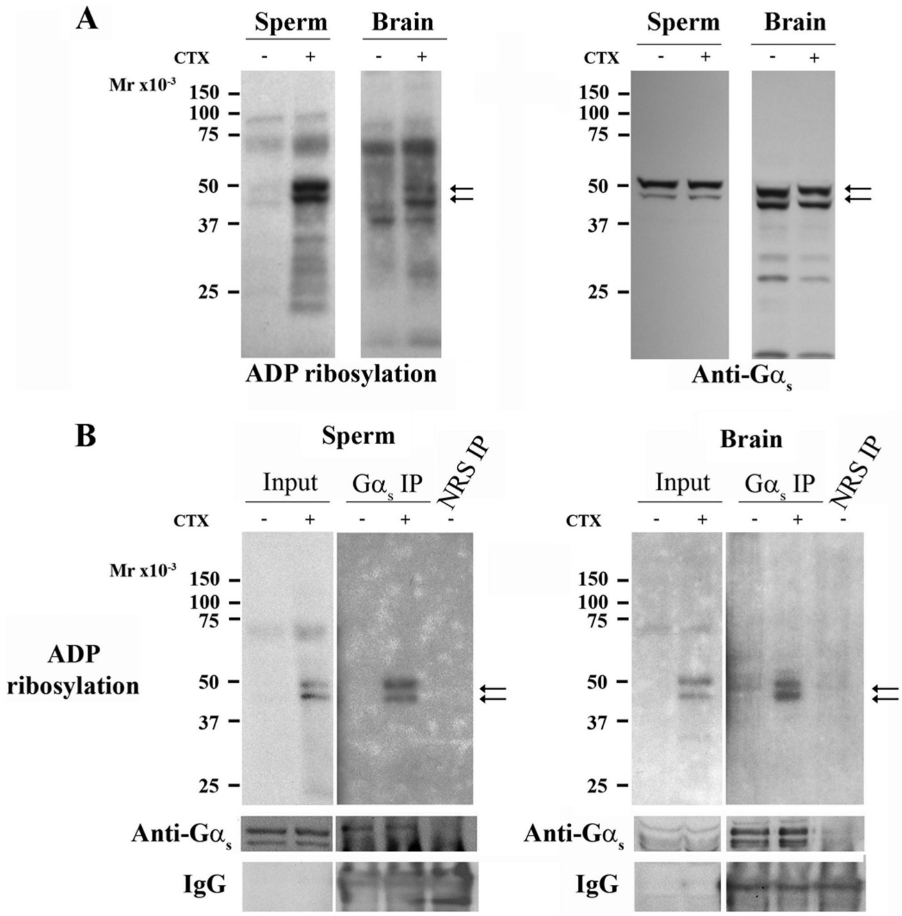

FIGURE 1. **Stimulatory Gα_s proteins are present in mouse sperm.** *A,* mouse sperm and brain membranes were purified by differential centrifugation and assayed for *in vitro* [^{32}P]ADP-ribosylation using pre-activated cholera toxin (*CTX*) as described under "Experimental Procedures." After the reaction was completed, samples were subjected to SDS-PAGE, transferred to PVDF membranes, and exposed to autoradiography. Two bands were detected at 45 and 52 kDa that correspond to the molecular weight of Gα_s (*left panel*). Western blots using a polyclonal anti-Gα_s antibody were performed on the same membranes after autoradiography, and a doublet was detected at the same molecular weight as the autoradiography (*right panel*). *B,* immunoprecipitation using the same anti-Gα_s antibody was carried out on both sperm and brain membrane extracts that had been previously subjected to cholera toxin [^{32}P]ADP-ribosylation (Gα_s immunoprecipitation). Immunoprecipitation controls were performed with normal rabbit serum (*NRS IP*). Immunoprecipitated samples were subjected to SDS-PAGE, transferred to PVDF membranes, exposed for autoradiography, and subsequently used for Western blot with anti-G_{α}s antibodies. IgG (light chain) in immunoprecipitation samples are also shown.

Compartmentalization of Different cAMP Signaling Events— Results described above raised a key question regarding the signaling pathways leading to sperm capacitation. If cAMP-permeable analogs are able to promote PKA activation and tyrosine phosphorylation, why is FK stimulation of tmACs unable to do likewise? Because it is now accepted that cAMP signaling is compartmentalized (46), one possibility consistent with these observations is that distinct molecules involved in the cAMP pathway localize to different sperm compartments. Anti-Gα_s antibodies labeled only the anterior head (Fig. 5*A*) suggesting that the tmAC-dependent cAMP pathway is restricted to sperm heads. Double staining with PNA, a lectin that recognizes the acrosomal content and is lost when the acrosome reaction occurs, revealed that anti-Gα_s staining is lost in acrosome-reacted sperm. This experiment suggests that Gα_s is present in the plasma membrane overlaying the acrosome and that this protein is lost during the acrosome reaction.

The localization of Gα_s to the anterior head suggests that FK-induced increase in cAMP synthesis is limited to this compartment. Because FK-stimulated cAMP synthesis did not induce PKA activation, PKA localization was then evaluated in noncapacitated sperm cells using antibodies against the PKA catalytic subunit. These antibodies recognized a single band at ~42 kDa, which is the expected molecular mass of the PKA catalytic subunit (Fig. 5*B*). When used for immunofluorescence, these antibodies indicated that PKA localized exclusively to the flagellum compartment (Fig. 5*C*).

These experiments suggest that FK stimulation of tmACs did not elicit an increase in PKA-dependent phosphorylation due to the differential localization of tmACs and PKA. To further investigate this hypothesis, sperm heads and tails were obtained from capacitated sperm as described previously (40). As control, capacitated whole-cell sperm extracts were also used. Each fraction was evaluated for its ability to respond to FK in two complementary assays. First, PKA activity was measured using Kemptide as substrate (Fig. 6*A*). Consistent with the differential PKA localization, PKA activity was only detected in the sperm tail fraction. Also in agreement with the lack of Gα_s in the tail, addition of 100 μM FK did not elevate the basal PKA activity in the flagellum. Second, and complementary to measuring PKA activity, cAMP levels were directly assessed in each fraction in the presence or absence of 100 μM FK. Whereas FK significantly

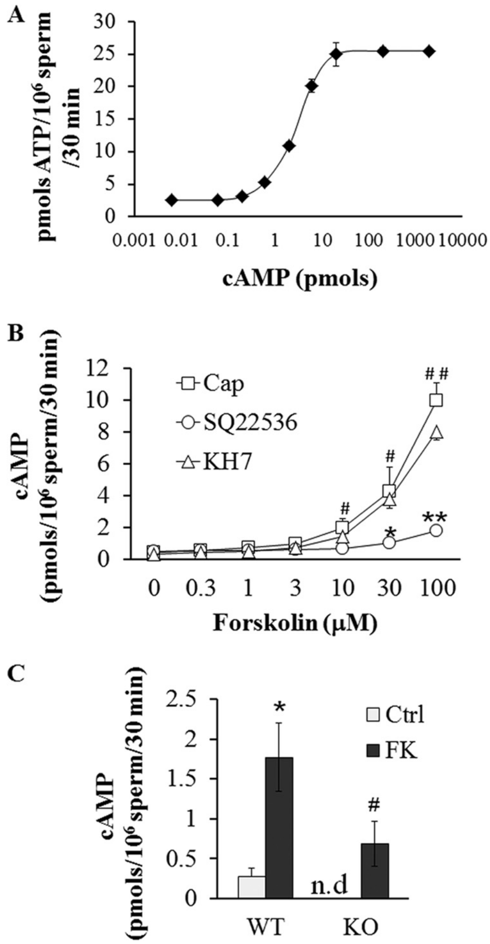

FIGURE 2. **Forskolin increases cAMP levels in capacitated mouse sperm.** *A*, representative cAMP concentration curve is shown, using known concentrations of cAMP. This curve was conducted in parallel for each independent assay. This method reproducibly detected cAMP concentrations ranging from 100 fmol to 20 pmol. In the absence of added cAMP, the basal value was 2.5 pmols of ^{32}P incorporated into the Kemptide/30 min/10^6 sperm. *B*, sperm were incubated for 60 min in capacitating medium and further exposed to either 50 μM of KH7 or 100 μM SQ22536 for 15 min. Sperm samples were then treated with different concentrations of FK for 30 min as indicated. Each sample was processed for indirect quantification of cAMP using a PKA activity assay as described under "Experimental Procedures." Two-way ANOVA followed by Bonferroni post tests were used to compare replicate means ($n = 5$). *, $p < 0.05$, and **, $p < 0.01$, when compared with capacitated sperm at same FK concentration. #, $p < 0.05$, and ##, $p < 0.01$, when compared with capacitated sperm with no FK. *C*, both WT and Adcy10 KO-capacitated sperm were exposed to 50 μM FK, and cAMP levels were analyzed using ELISA. Data represent mean ± S.E. $n \geq 4$. Values for Adcy10 KO sperm before FK stimulation were below the detection limits and named in the graph as "nondetectable" (*n.d.*) (74). A paired *t* test was used to compare WT control *versus* WT + FK (*, $p < 0.05$), and a one-sample *t* test was used to determine whether the mean of KO + FK was different from the hypothetical value "0" (#, $p < 0.05$). *Ctrl*, control.

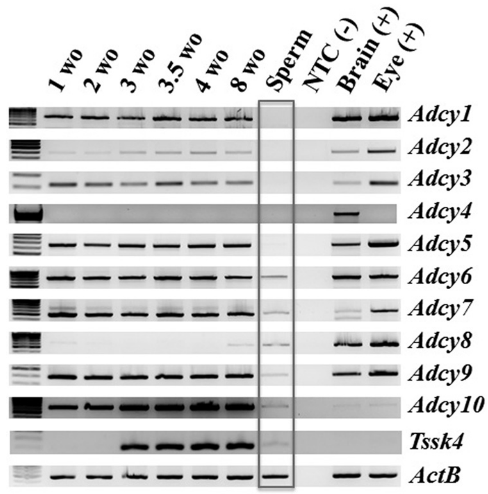

FIGURE 3. **Different *Adcy* isoforms are expressed in mouse sperm and during testis development.** Total RNA from sperm and testis of different developmental stages was isolated and used for RT-PCR experiments with isoform-specific intron-spanning primers. Eye and brain RNA were used as positive controls (+), and the absence of genomic contamination in the RNA samples was confirmed with reverse transcription negative controls (no reverse transcriptase) for each experiment (*NTC*(−)). Each isoform varies its pattern of expression during testis development. The expression of Adcy6–10 in sperm is observed.

increased cAMP levels in the head fraction, cAMP levels in the tail fraction were unchanged by this compound (Fig. 6*B*). Altogether, these experiments are in agreement with the hypothesis that different cAMP pathways are compartmentalized in sperm.

Forskolin Stimulates the Acrosome Reaction in Capacitated Mouse Sperm—Considering that cAMP has been shown to stimulate the acrosome reaction (47, 48) and that $G\alpha_s$ localized to the anterior head, we hypothesized that tmAC-dependent cAMP synthesis in this region might play a role in the regulation of the sperm acrosome reaction. To evaluate this possibility, measurements of the exocytotic acrosome reaction were conducted using Coomassie Blue staining analyses. When sperm were incubated in conditions that support capacitation, FK induced a concentration-dependent increase in the percentage of sperm undergoing the acrosome reaction (Fig. 7*A*). This effect was blocked in the presence of SQ22536. As expected, this inhibitor did not impair acrosomal exocytosis triggered by calcium ionophore A23187. Because the acrosome reaction is mediated by an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations, sperm were loaded with Fluo-4 to measure the effect of FK on $[Ca^{2+}]_i$ in single cells. sZP, which was used as control, induced Ca^{2+} increase in about 50% of the sperm population (Fig. 7*B* and supplemental Movie 1). Similarly, 50 μM FK induced an increase in $[Ca^{2+}]_i$ in more than 50% of the sperm population (Fig. 7*C* and supplemental Movie 2). The FK-induced increase in $[Ca^{2+}]_i$ was blocked significantly by SQ22536 at a concentration of 100 μM (Fig. 7*D* and supplemental Movie 3). The number of responsive cells is shown in Fig. 7*E*.

DISCUSSION

Since its discovery by Rall and Sutherland in the 1950s (49, 50), cAMP has been shown to be essential for the regulation of many cell signaling pathways. In mammalian sperm, cAMP has been reported to be involved in the regulation of several capacitation-associated processes such as motility hyperactivation (5), hyperpolarization of the sperm plasma membrane (51, 52), and the increase in protein tyrosine phosphorylation (45). Because of its relevance for cell processes, cAMP levels are tightly regulated both temporally and spatially by different types of adenylyl cyclases and cAMP phosphodiesterases (53–55). Two families of adenylyl cyclases are responsible for cAMP synthesis in animals as follows: the tmACs (Adcy1–9) and the

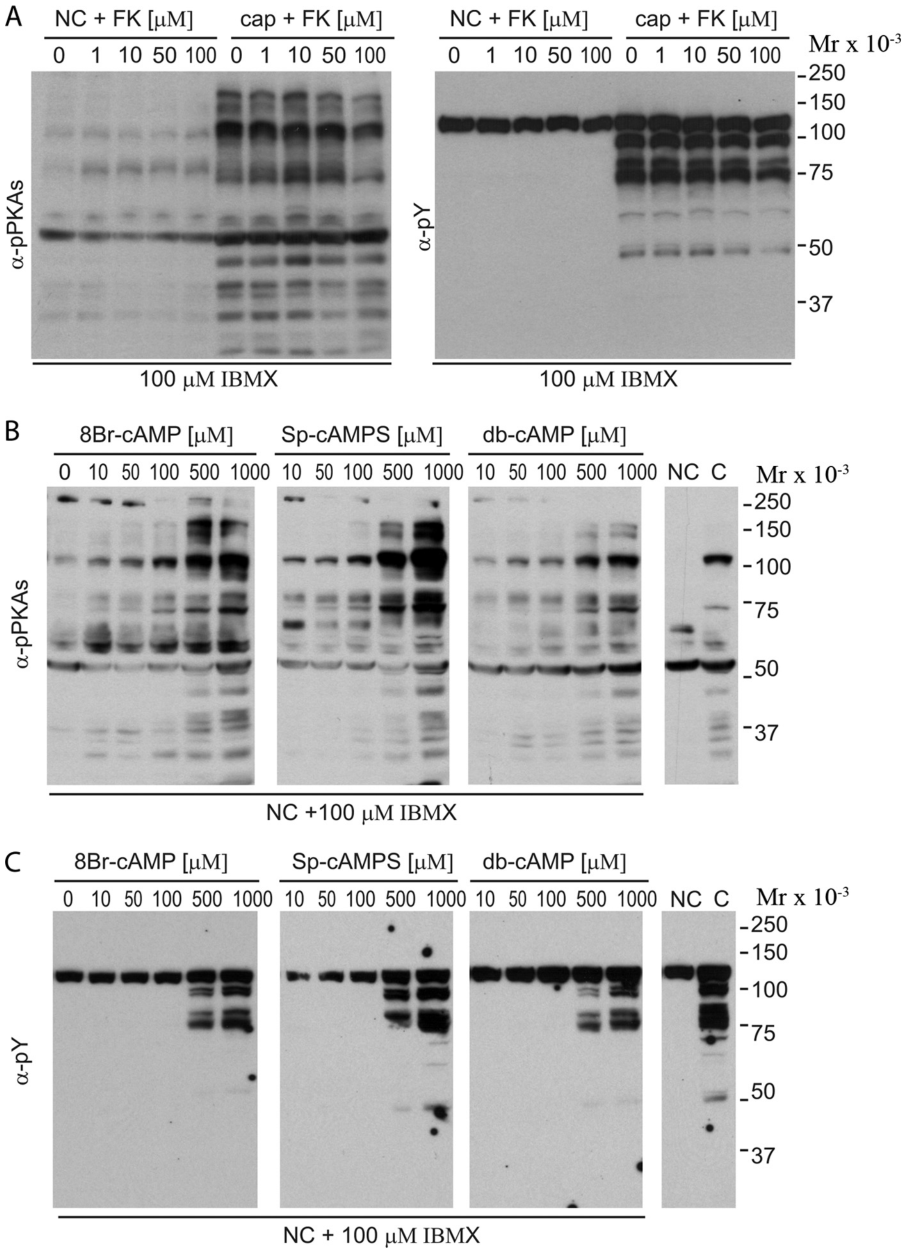

FIGURE 4. **FK has no effect on capacitation-associated phosphorylation events.** *A,* sperm were incubated under noncapacitating (*NC*) and capacitating (*cap*) conditions in the presence of increasing concentrations of FK for 60 min. Protein extracts were analyzed by Western blotting with anti-Tyr(P) (*αpY*) and anti-phospho-PKA-substrate antibodies as described under "Experimental Procedures." *B* and *C,* sperm were incubated in media without HCO_3^- supplemented with 100 μM IBMX and with increasing concentrations of 8-bromo-cAMP (*8-Br-cAMP*), (S_p)-cAMPS, or dibutyryl cyclic AMP (*db-cAMP*). Samples were processed for Western blotting with anti-pPKAs (*B*), stripped, and further processed with anti-Tyr(P)antibodies (*C*). All Western blots are representative of experiments repeated at least three times.

soluble adenylyl cyclase (Adcy10) (6). Adcy10 and tmACs are regulated by different pathways. Adcy10 is insensitive to GTP analogs or to FK. This atypical cyclase is activated by HCO_3^- (11, 56), and it is specifically blocked by KH7 (13). Pharmacological and genetic experiments have conclusively demonstrated the essential role of Adcy10 for sperm function (13, 14, 45).

However, tmACs are regulated both positively and negatively by heterotrimeric Gα_s and Gα_i protein complexes, respectively. The heterotrimeric G protein family consists of a Gα subunit and a $G_{\beta\gamma}$-dimer. In the resting state, the α subunit is bound to GDP; upon activation, it exchanges GDP for GTP, undergoes a conformational change, and activates downstream protein targets. The name for the stimulatory (G_s) and inhibitory (G_i) G proteins was originally coined for the ability of their α subunit to stimulate or to inhibit tmAC, respectively (57). G_s can be stimulated by cholera toxin through ADP-ribosylation of the α_s subunit, a property shared with G_{olf}. Together with the stimulation of tmACs activity, the cholera toxin-mediated α_s subunit ADP-ribosylation has been used as a signature assay to demonstrate the presence of either Gα_s or Gα_{olf} in a particular cell type (29–31). However, α_i subunits are *Bordetella pertussis* toxin substrates; analogous to cholera toxin, pertussis toxin catalyzes ADP-ribosylation of α_i subunits.

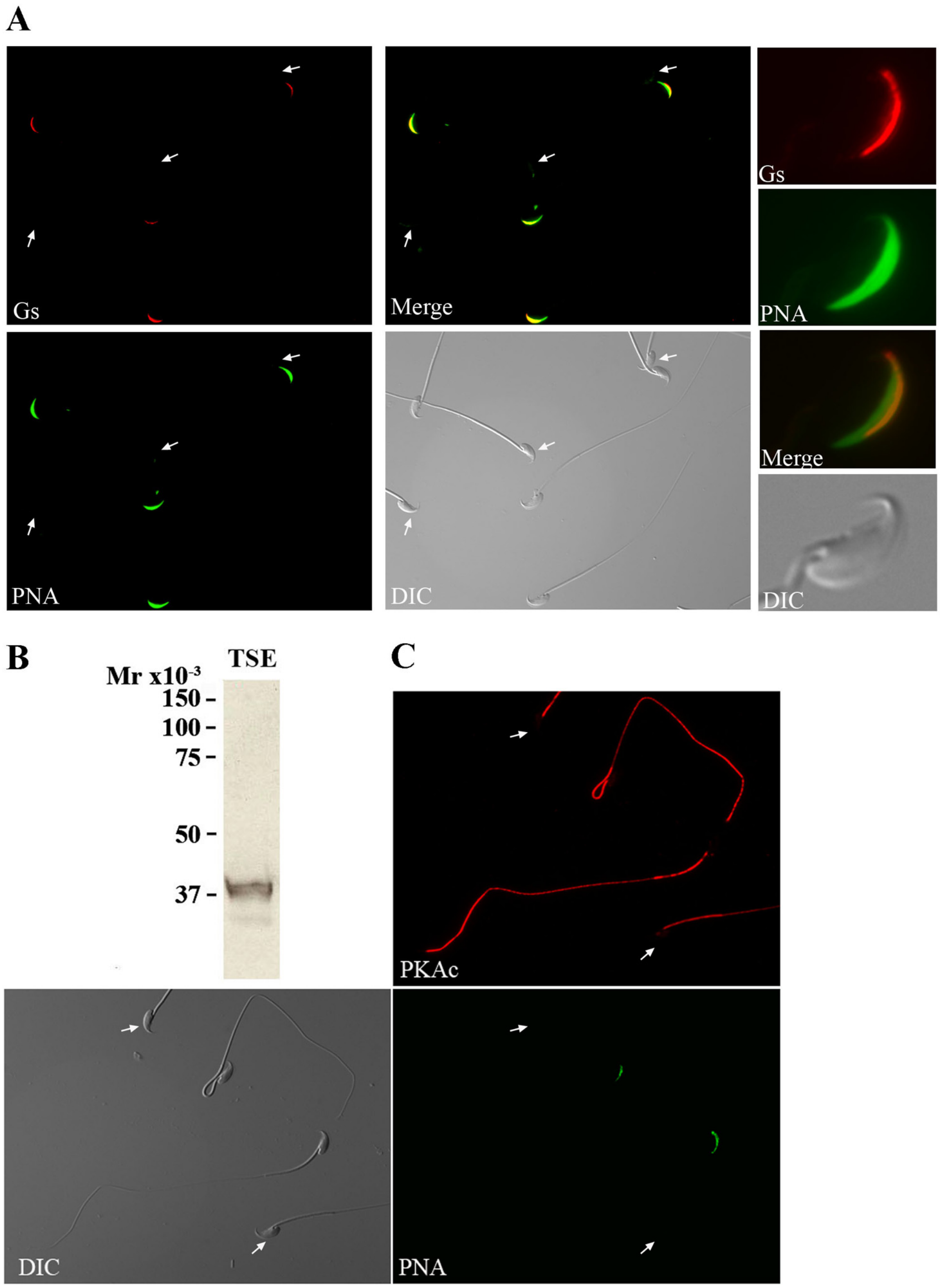

FIGURE 5. **Gα_s and the PKA catalytic subunit localized to different sperm compartments.** *A*, for immunofluorescence assays, mouse sperm were air-dried, fixed, permeabilized, and probed with a polyclonal anti-Gα_s antibody. PNA was used to follow the acrosomal status. Anti-Gα_s (*red*) and PNA (*green*) labeling showed that Gα_s is lost during acrosomal reaction (merged color panels). Differential interference contrast (*DIC*) microscopy was used to control for sperm morphology. Images are representative of at least three experimental replicates. *B*, immunodetection of the catalytic subunit of PKA by Western blotting. Total sperm extracts (*TSE*) were subjected to SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting with anti-PKA catalytic (*PKAc*) subunit antibodies. Only one band of the expected molecular weight is observed. *C*, immunolocalization of cPKA in mature sperm. Immunofluorescence was carried out in fixed and permeabilized sperm as detailed under "Experimental Procedures." Anti-cPKA staining of whole sperm shows the presence of PKA only in the sperm flagellum.

In sperm, ADP-ribosylation by either pertussis or cholera toxins was used previously to investigate the presence of these G protein subunits in mammalian sperm. Although originally Hildebrandt *et al.* (26) were not able to find evidence of Gα_i in mammalian sperm, independent work from Kopf *et al.* (33) and Bentley *et al.* (32) clearly showed the presence of this protein in sperm. Regarding Gα_s, ADP-ribosylation assays were also used to investigate the presence of this G protein in mammalian sperm. Similarly to the Gα_i case, Hildebrandt *et al.* (26) were not able to detect a cholera toxin ADP-ribosylation substrate in dog sperm. However, Baxendale and Fraser (34) identified a faint 45-kDa ADP-ribosylated protein in whole sperm extracts. In the same work (34), anti-Gα_s antibodies gave a positive signal by Western blots. However, also using anti-Gα_s antibodies, Merlet *et al.* (58) concluded that the α_s subunit is not present in human sperm. Finally, Spehr *et al.* (16) reported the presence of the α_s subunit as well as other molecules of the tmAC cascade using a shotgun tandem mass spectrometry proteomic

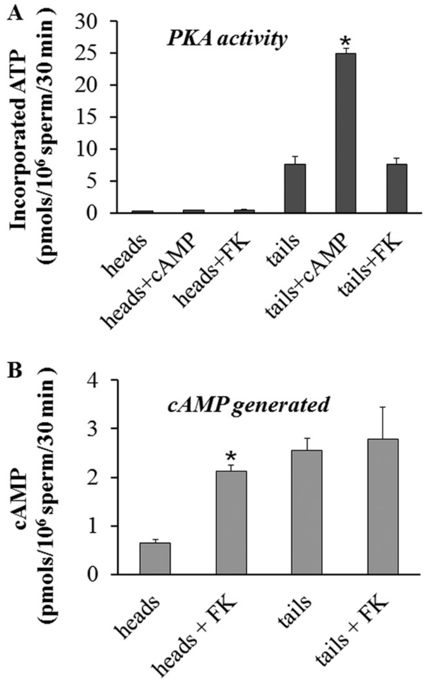

FIGURE 6. **PKA activity is only observed in sperm tails, whereas FK-induced cAMP production is detected in sperm heads.** *A,* head and tail fractions from capacitated sperm were separated as described under "Experimental Procedures" and assayed for PKA activity using [^{32}P]ATP and Kemptide as substrate in the presence of either 1 mM cAMP or 100 μM FK. Data represent mean ± S.E. of five independent experiments performed in triplicates; two-way ANOVA followed by Bonferroni post tests were used to compare replicate means. *, $p < 0.001$ when compared with tails without cAMP addition. *B,* head and tails fractions from capacitated sperm were treated with 100 μM FK and processed for cAMP quantification using PKA activity assays as described under "Experimental Procedures." Data represent mean ± S.E. of five independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni post tests were used to compare replicate means. *, $p < 0.05$ when compared with heads without FK addition.

approach in human sperm. Despite this body of work, the presence of Gα_s remained controversial. In this study, we used [^{32}P]ADP-ribosylation of purified membrane fractions to demonstrate the presence of Gα_s in mouse sperm. In addition, anti-Gα_s subunit antibodies recognized bands at the same molecular weight as the cholera toxin substrates by Western blots. The two ~45-kDa ADP-ribosylation substrates were immunoprecipitated from sperm membrane extracts with anti-Gα_s antibodies. These results were performed in parallel using brain membrane extracts as positive controls, and similar results were obtained. A slight difference in molecular weight between brain and sperm membrane preparations might be attributed to either differential spliced Gα_s variants (59–64) or to post-translational modifications. Immunofluorescence experiments using Western blot-validated anti-Gα_s antibodies labeled the anterior acrosome of mouse sperm but failed to stain the flagellum. Consistent with this observation, anti-Gα_s antibody staining was lost in acrosome-reacted sperm. Because of sequence similarities, it is difficult to discriminate between Gα_s and Gα_{olf} subunits. 1) Both can be ADP-ribosylated in the presence of cholera toxin. 2) Both are likely to be recognized by the anti-Gα_s antibody that we used. 3) Once activated, both can stimulate tmACs. Interestingly, similar to Gα_s, Gα_{olf} has been reported to be present in human testis (65).

Although essential for the tmAC signaling pathway, finding the stimulatory G proteins in sperm does not directly imply that tmACs are present in these cells. Gα_s proteins are able to modulate other signaling molecules, including Csk, Src, and Hck (66, 67), and their presence in sperm might be related to other functions different from activation of tmACs. Initially shown by Seamon and Daly (43), the diterpene FK activates almost all tmACs and is able to increase cAMP levels in most cell types. It is not surprising then that FK has been a useful tool to evaluate the presence of tmACs in sperm. Similar to the case of G_s, different results have been reported concerning the use of FK. Although several groups failed to detect cAMP elevation when the sperm were exposed to FK (8, 13, 22, 27, 28, 68), others have shown that FK increased cAMP levels in sperm (15, 17, 34). Consistent with these last studies, Leclerc and Kopf (20, 69) reported that FK significantly increased adenylyl cyclase activity in membrane fractions of capacitated mouse sperm. The same authors observed regulation by GTP analogs as well as with mastoparan, a wasp venom toxin known to activate G proteins. In addition, in this study, the effect of FK was blocked by SQ22536, a compound known to inactivate tmACs in several cell types with an IC_{50} value that varies depending on the tmAC isoform but not by the specific AdCy10 inhibitor KH7 (44).

Finally, we have performed RT-PCR for each member of the adenylyl cyclase superfamily in testis of mice from different ages and in sperm from adult mice. Although Sertoli and Leydig cells are present in all stages, their contribution is known to decrease in an age-dependent manner. At 7 days, only pre-meiotic spermatogonia contribute to mRNA expression; at 14 days, most of the germ cells are meiotic spermatocytes, and at 21 days, only postmeiotic spermatids are detected. In sperm, we were able to detect Adcy6–9, plus the already known sAC Adcy10. From these, the Adcy8 expression pattern indicates that this enzyme is expressed postmeiotically and that it is developmentally regulated during spermatogenesis. Because sperm are transcriptionally and translationally inactive, those mRNA transcripts found in isolated mature sperm correspond to transcripts that are present during spermiogenesis in post-meiotic spermatids. However, we cannot discard that other tmACs that are expressed in different stages of germ cells remain in the sperm after translation has occurred in testicular germ cells. In regard to the reproductive phenotype, only *Adcy10*-null (13) and *Adcy3*-null (17) mice have been reported to be sterile. *Adcy7* knock-out shows partial prenatal and complete postnatal lethality (70), and null mice for *Adcy2* and *Adcy9* are not available. Therefore, the reproductive phenotype for these knock-out mice is not known. Knock-out mice for *Adcy1*, *-5*, *-6*, and *-8* are fertile; however, it is important to consider that tmACs have overlapping functions in many cells making it difficult to pinpoint the specific isoforms involved in a given process. Altogether, these data are consistent with the presence of tmACs in mouse sperm.

To further rule out Adcy10 contribution to the FK effect, sperm from Adcy10 knock-out mice were used to analyze cAMP accumulation in the presence of FK. The advantage of this strategy is that the activity of a transmembrane cyclase was not underestimated by Adcy10 activity. If the effect of FK was indirectly mediated by Adcy10, then the effect of FK should not

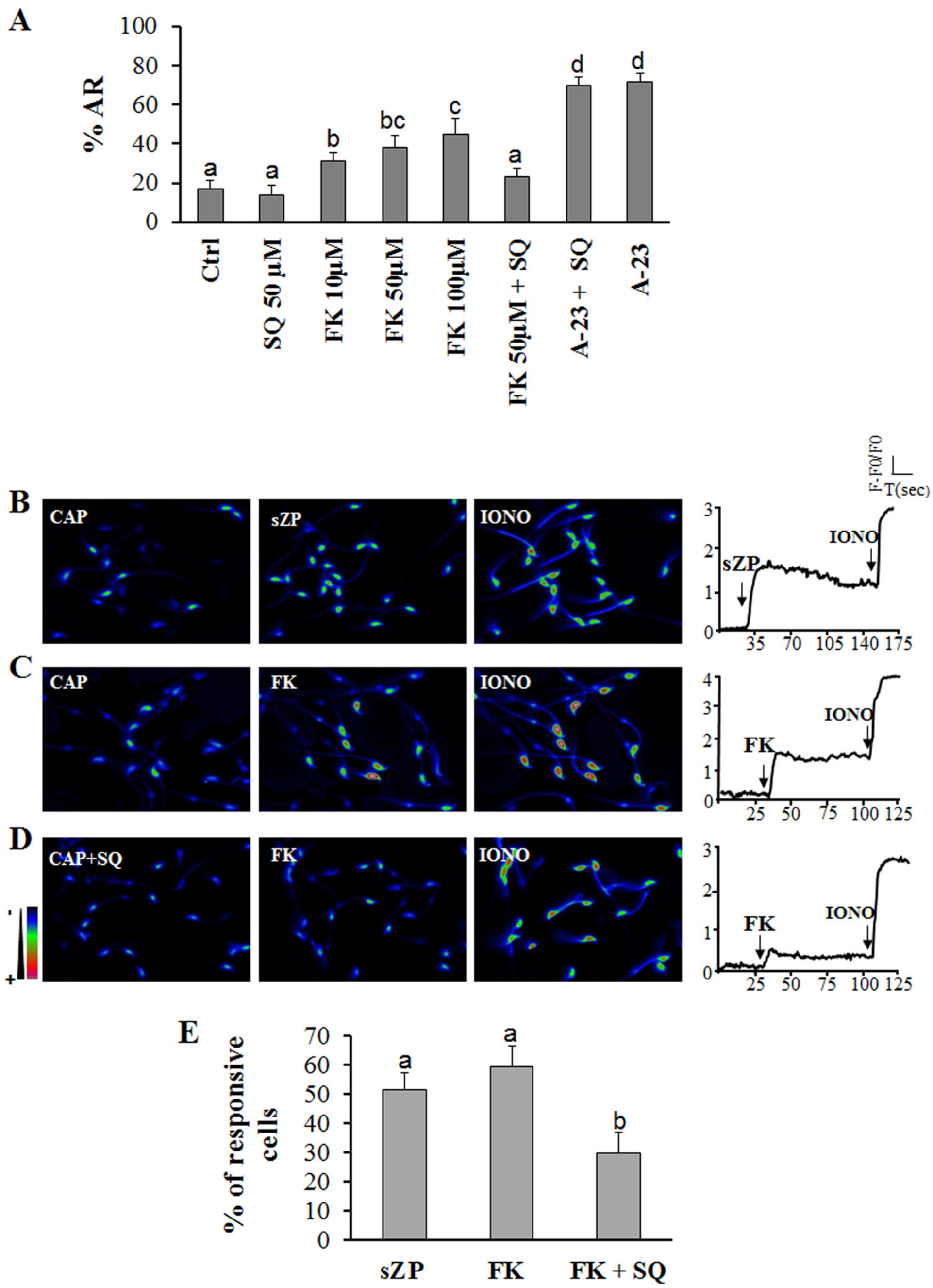

FIGURE 7. **FK promotes acrosome reaction, and $[Ca^{2+}]_i$ increases in capacitated mouse sperm.** *A,* FK induces acrosome reaction. Mouse sperm were incubated under capacitating conditions for 1 h and then further incubated for 30 min in the absence or presence of the tmAC inhibitor SQ22536. Subsequently, either FK or calcium ionophore A23187 (*A-23*) was added, and sperm were incubated for an additional 30 min. Acrosomal status was assessed as explained under "Experimental Procedures." Percentage of acrosome-reacted sperm (*%AR*) was calculated based on seven independent experiments run in triplicate. *B,* pseudocolored fluorescence images illustrating $[Ca^{2+}]_i$ levels before (*CAP*) and after addition of sZP to capacitated mouse sperm. The $[Ca^{2+}]_i$ increase induced subsequently by 10 μM ionomycin (*IONO*) is also shown as a positive control. The *right panel* shows representative $[Ca^{2+}]_i$ traces of sperm subjected to the conditions described above. *Arrows* indicate agonist application. *C,* fluorescence images of $[Ca^{2+}]_i$ levels before (*CAP*) and after addition of 50 μM FK (*FK*) to capacitated mouse sperm. *D,* fluorescence images of $[Ca^{2+}]_i$ of capacitated mouse sperm incubated for 30 min with the tmAC inhibitor SQ22536 (*CAP+SQ*) and after the addition of FK. *E,* percentage of sperm displaying $[Ca^{2+}]_i$ increases in response to ZP compared with those responsive to FK alone or after incubation with SQ22536. The number of cells analyzed for each condition is ZP = 121, FK = 197, FK + SQ = 176 (each experimental condition was performed in at least four independent experiments). Tukey's multiple comparison statistics tests were performed for both acrosome reaction and $[Ca^{2+}]_i$ experiments. Means of groups that have *different letters* differ significantly ($p < 0.01$).

be observed. However, FK significantly increased cAMP levels in sperm from Adcy10$^{-/-}$ mice. The extent of the FK response was difficult to quantify because basal cAMP levels in *Adcy10* KO were below the lower detection limit. Altogether, these data indicate that the FK-induced increase in cAMP levels is Adcy10-independent.

Among the best characterized cAMP molecular targets are PKA, EPAC, and cyclic nucleotide-gated channels. In sperm, we can add to this list the testis-specific Na^+-H^+ exchanger that contains a putative consensus sequence for cAMP binding (71). As mentioned, immunofluorescence localization of Gα_s subunit suggests that the tmAC signaling pathway is present in

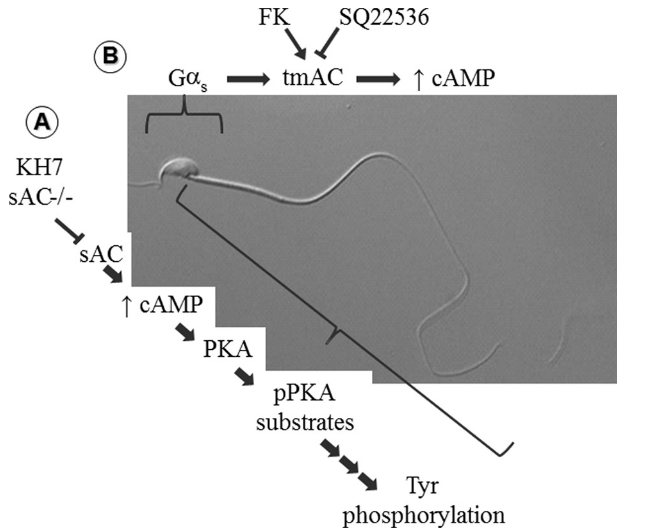

FIGURE 8. **Proposed model for the spatial distribution of cAMP in sperm functions.** *A,* Adcy10 mediates activation of flagellum cAMP-dependent pathways such as the activation of PKA is upstream of the increase in tyrosine phosphorylation. *B,* Gα_s is in the head and activates a tmAC that increases cAMP levels that would be necessary to activate acrosome reaction by FK. Inhibition of tmAC by SQ22536 blocks the FK-induced acrosome reaction. The cAMP effector in the head is not PKA. One possible cAMP target in the sperm head is the G protein exchange factor called EPAC (48).

the sperm plasma membrane surrounding the anterior acrosome. Our results also indicate that the PKA catalytic subunit is only present in the sperm flagellum, in agreement with a recent proteomic analysis by Aitken and co-workers (72). Consistent with this differential localization, FK did not activate PKA-dependent phosphorylation in mouse sperm. After head and tail separation, FK did not increase cAMP levels in the flagellum fractions but was able to induce cAMP accumulation in the head fractions. Altogether, these results suggest that cAMP-dependent pathways are compartmentalized in the sperm. Our working model (Fig. 8) postulates that tmACs are present in the sperm head, and Adcy10 is present in the sperm flagellum.

What is the function of tmACs in the sperm? In addition to the well established function in capacitation, cyclic AMP has been proposed to have a role in the sperm acrosome reaction (47, 48, 69, 73). In human sperm, the effect of cAMP agonists on the acrosome reaction has been attributed to the stimulation of EPAC (48). Consistent with this hypothesis, EPAC has been shown to be present in the head of both human and mouse sperm; in addition, EPAC-selective cAMP analog, 8-pCPT-AMP, is able to induce the acrosome reaction in these species at concentrations that do not stimulate PKA in live sperm (47, 48). EPAC is a Rap1A exchange factor, and in permeabilized human sperm, this small GTP-binding protein has been shown to stimulate the acrosome reaction downstream of cAMP (47, 48). In this study, we provide evidence showing that FK induces the acrosome reaction in capacitated mouse sperm and that the FK effect is blocked in the presence of SQ22536. Moreover, FK was also capable of elevating $[Ca^{2+}]_i$ in capacitated sperm but not in sperm incubated in conditions that do not support capacitation. Consistent with the hypothesis that FK targets a tmAC, the FK effect was blocked by SQ22536. Moreover, Adcy10 KO mice are not deficient in the ZP-stimulated acrosome reaction (13), which suggests that at least in mouse sperm Adcy10 does not mediate this event.

Overall, our results point toward a spatially and temporally controlled mechanism mediated by G_s activation of tmAC(s) in sperm. This work constitutes the basis toward the understanding of key questions in the regulation of sperm signaling pathways, many of which remain unsolved despite many years of research in the field. In this context, the clarification of the role of the tmACs and G proteins in sperm warrants further investigation. A clear understanding of how capacitation is regulated will benefit future experimental approaches aiming to increase the fertilizing ability of the sperm population, as well as the design of rational contraceptive approaches.

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