cAMP compartmentalization in sperm physiology

Compartmentalization of Distinct cAMP Signaling Pathways in Mammalian Sperm*

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*Running title: cAMP compartmentalization in sperm physiology

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Keywords: Adenylate cyclase (adenylyl cyclase); Calcium imaging; Cell signaling; Cyclic AMP (cAMP); Heterotrimeric G proteins; Protein kinase A (PKA); Signal transduction; acrosome reaction; forskolin; sperm capacitation

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 Gs are detected in 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.

ABSTRACT

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. While the HCO₃⁻-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 Gs proteins; however, the presence of Gs or tmACs in mammalian sperm has been controversial. In this manuscript, we used Western blotting and cholera toxin-dependent ADP ribosylation to show Gs 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. While Gs 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, Gs reactivity is lost in acrosome reacted sperm, and forskolin is able to increase intracellular Ca²⁺ and induce the acrosome reaction. Altogether, these data suggest that cAMP pathways are compartmentalized in sperm, with Gs and tmAC in the head and Adcy10 and PKA in the flagellum.
INTRODUCTION
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 (2;3)). At least part of cAMP’s actions is mediated through the activation of protein kinase A (PKA). As demonstrated by the McKnight laboratory (4;5), mice lacking the sperm-specific PKA catalytic subunit alpha 2 (Cα2) are sterile and their sperm do not hyperactivate. In vertebrates, cAMP is synthesized by two types of adenylyl cyclases: a ubiquitous family of transmembrane adenylyl cyclases (tmACs) with 9 members (Adcy1 to 9) and soluble adenylyl cyclase encoded by a single gene (Adcy10 aka SACY or sAC), which is alternatively spliced into multiple isoforms (6-8).

Adcy10 was originally thought to be restricted to testis and sperm (9), but more recently it has been shown that different aspects of capacitation are regulated by the second messenger cAMP (reviewed in (2;3)). At least part of cAMP’s actions is mediated through the activation of protein kinase A (PKA). As demonstrated by the McKnight laboratory (4;5), mice lacking the sperm-specific PKA catalytic subunit alpha 2 (Cα2) are sterile and their sperm do not hyperactivate. In vertebrates, cAMP is synthesized by two types of adenylyl cyclases: a ubiquitous family of transmembrane adenylyl cyclases (tmACs) with 9 members (Adcy1 to 9) and soluble adenylyl cyclase encoded by a single gene (Adcy10 aka SACY or sAC), which is alternatively spliced into multiple isoforms (6-8).

Adcy10 was originally thought to be restricted to testis and sperm (9), but more recently it has been identified in other cell types (reviewed in (10)). 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 by 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 (αs and αolf), and negatively regulated by any of three inhibitory α subunits (αi1, αi2 and αi3). Both stimulatory and inhibitory α subunits can be post-translationally modified by bacterial toxins. This modification can be followed in vitro using NAD(125)P as substrate. While all Gαs subunits are ADP ribosylated by Pertussis toxin, Gαi and Gαolf 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 Gαi in mouse sperm (32;33). However, similar to the situation with tmACs, the presence of stimulatory Gαs 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 Gαs antibodies. Our results also indicate compartmentalization of those molecules involved in the cAMP pathway: while Gαi was localized to the anterior head, the PKA catalytic subunit was only found in the sperm flagellum. Interestingly, forskolin increased intracellular Ca2+ ([Ca2+]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 (db-cAMP), Sp-diastereomer of adenosine 3',5'-cyclic monophosphothiorate (Sp-cAMPS), 8-Br-cAMP, 3-isobutyl-1-methyl xanthine (IBMX), guanosine 3-phosphate (GTP), adenosine 3-phosphate (ATP), adenosine
diphosphate (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-[32P]] ([32P] NAD) was obtained from Perkin Elmer (Boston, MA). 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 (PY) monoclonal antibody (Clone 4G10) and anti-Gα, 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 (Franklin Lakes, NJ) and anti-phospho PKA substrates 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 Life Technologies Corporation (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 were placed in 500 µl of a modified Krebs-Ringer medium (non-capacitating Whitten’s HEPES-buffered (WH)) (35) (in mM: 100 NaCl; 4.4 KCl; 1.2 KH2PO4; 1.2 MgSO4; 5.4 glucose; 0.8 pyruvic acid; 4.8 lactic acid; 2.4 Ca2+; 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 NaHCO3 (15 mM). After 10 min, the sperm suspension was washed by addition of 1 ml of non-capacitating medium and centrifugation at 800 x g for 5 min at room temperature. Sperm were then resuspended (final concentration: 2 x 10⁸ 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 NaHCO3 were added and sperm were incubated at 37°C for at least one hour.

**Sperm and brain membrane purification.** The preparation of sperm and brain fractions was carried out as previously described (36). Briefly, mouse sperm (2 x 10⁸ cells) or brains were homogenized using 10 strokes with a Teflon dounce homogenizer in TE buffer (50 mM Tris-HCl, pH7.5, 1 mM EDTA) supplemented with protease inhibitors (Protease Inhibitor Cocktail (Roche) as indicated by the manufacturer, plus 0.4 mM Leupeptin, 0.4 mM Aprotinin, 0.1 mM Pepstatin, 0.3M Benzanidine and 0.32 mg/ml Calpains I and II inhibitor). After homogenization, the sample was sonicated 3 times for 15 sec on ice with 1 min intervals. Cell debris were pelleted (1,000 x g for 10 min at 4°C) and the supernatant was centrifuged at 10,000 x g for 10 min at 4°C. Again, the resultant pellet was saved and the supernatant was further centrifuged at 100,000 x 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.

### [32P] 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 MgCl2, 6 mM EDTA, 6 mM DTT, 60 mM thymidine, 6 mg/ml LBTI, 0.3 mg/ml aprotinin, 0.3 mg/ml Leupeptin, 6 mM p-amino benzamidine and 5 x 10⁻⁶ cpm /pmol [32P] NAD⁺ in a final volume of 30 µl. Reactions were carried out for 1 hr 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 hrs up to 3 days at -80°C.

**Immunoprecipitation of [32P]ADP-ribosylated Ga.** After the [32P] 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 hrs at 4°C with an anti-Gα polyclonal antibody or with normal rabbit serum at a final concentration of 0.01mg/ml. After

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this period, protein G Sepharose beads were added (10% v/v) and further incubated at 4°C for 2 hrs. After incubation, beads were washed with Triton X-100/PBS by centrifugation at 12,000 x g for 45 sec. 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. Afterwards, same membranes were used for Western blotting with 1 µg/ml anti-Gα, antibodies. Chemiluminescence could be detected in less than 5 min. At this time, the radioactivity from the 32P was negligible.

SDS-Page and immunoblotting. After incubation under different experimental conditions, sperm were collected by centrifugation, washed in 1 ml of phosphate-buffered saline (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 boiled again for 5 min. Samples were subjected to SDS-PAGE using 8-10% mini-gels; protein extracts equivalent to 1-2x10^6 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 (Biorad, Hercules, CA). Electrophoretic transfer of proteins to Immobilon (Bio-Rad) and immunodetection of tyrosine-phosphorylated proteins were carried out using PY monoclonal antibodies as described previously (37). Anti-Gα 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 Immuno Research Laboratories, Inc) 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 6 x 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 previously outlined (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 (AR) 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 AR.

Indirect immunofluorescence. Sperm obtained by the swim-up method in WH medium were washed once, resuspended in PBS (1-2 x10^6 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 (4 washes 5 min each one) 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 4 washes in PBS, and slides mounted using Slow-Fade Light reagents (Molecular Probes, Eugene, OR). Epifluorescence microscopy was performed using a TE300 Eclipse microscope (60x) (Nikon). DIC 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 source of PKA, and with Kemptide (Sigma Co., St. Louis, MO) as a PKA specific substrate. To obtain the triton insoluble suspension, sperm (10^6 cells) obtained in Whitten’s HEPES-buffered as described above
were centrifuged and resuspended in Triton buffer (25 mM Tris–HCl, 150 mM NaCl, Roche EDTA-free protease inhibitor cocktail, 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 3x assay cocktail so that the final concentration of the assay components was 100 µM Kemptide, [γ-32P]ATP (3000 Ci/mmol) (2 x 10^6 cpm/assay), 100 µM ATP, 1% (v/v) Triton X-100, 1 mg/ml BSA, 10 mM MgCl2, 100 µM IBMX, 40 mM β-glycerophosphate, 5 mM p-nitrophenylphosphate, 10 mM Tris–HCl, pH 7.4, Roche EDTA-free protease inhibitor cocktail. 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. Forty µl of the resultant supernatant were then spotted onto phosphocellulose papers (2 x 2 cm) (Whatman P81). Phosphocellulose papers were washed 5 x 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 x 10^6 cells/ml) were incubated for 60 min in 1.2 ml of Whitten’s Media (WM) containing BSA/HCO3 (capacitated) and 100 µM IBMX. Suspensions were further incubated for 30 min in the presence of different concentrations of FK. Alternatively, adenylly 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 x g for 5 min, washed with 1 ml PBS, centrifuged again, and finally resuspended in lysis buffer (40 mM Hepes PH 7.4, 0.1 mM IBMX, Roche EDTA-free protease inhibitor cocktail), to a final volume of 30 ul. Samples were then boiled for 10 min to inactivate sperm enzymes and centrifuged for 5 min at 8,000 x g. Supernatants were saved and supplemented to 35 ul 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. 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 32P incorporated into the Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly, Sigma)-specific substrate was quantified as described (36) (see Figure 2A). This method was able to reproducibly detect cAMP concentrations ranging from 100 fmol to 20 pmol. In the absence of added cAMP, the basal value was 2.5 pmol of 32P 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 which ranges from ~10 fmol to 1 pmol of cAMP. A standard curve was run for each assay and the unknown cAMP concentrations 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 sec, layered over 75% Percoll and centrifuged at 725 x 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 x 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 and immediately incubated with the [32P]-ATP cocktail 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, centrifuged and the cAMP produced was measured in the remaining supernatant as described above.

Intracellular Ca2+ 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 NaHCO3 according to experimental capacitating conditions. Once loaded, sperm were immobilized on mouse laminin (100 µg/ml)-coated cover slips to allow recordings. Sperm were capacitated for 1 h and Ca2+ 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 60X/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 previously described (38), with 4 ms light excitation pulses. Fluorescence was captured with a Cool Snap camera (Photometrics USA) at 0.5 Hz or 1 Hz. Movies were processed using Image J (Version 1.38, National Institutes of Health). Fluorescence is expressed as f-f0/f0. 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 through 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 mouse were also tested. Total RNA was extracted using High Pure RNA Isolation Kit (Roche) 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 (Biorad). For RT-PCR, 20 ul PCR reactions were performed with RubyTaq polymerase (Affymetrix) using 2 ul of cDNA as template. All PCR reactions were performed in BioRad Thermocycler with 35 cycles of: 10 sec at 95°C, 15 sec at 60°C and 15 sec 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.

Statistical analysis. Data are expressed as the means ± s.e.m. In order to assume normal distribution, percentages were converted to ratios and all data subjected to the arcsine square root transformation. Statistical analyses were performed with the aid of Graph Pad Prism v6.01 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-ways ANOVA for multiple comparisons.

RESULTS

Gaα 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 Ga stimulatory subunits (αs or αolf) using a cholera toxin ADP ribosylation assay. Sperm and brain particulate membrane fractions were treated with cholera toxin in the presence of [32P]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. 1A, left panel). Western blotting of the same Immobilon P membrane with anti Gaα antisera revealed two bands overlapping the ADP-ribosylation signals (Fig. 1A, right panel). To confirm the identity of these bands as Gaα proteins, the anti Gaα antibodies were used to immunoprecipitate sperm and brain membrane extracts subsequent to [32P]ADP-ribosylation by cholera toxin (Fig. 1B). The doublet observed in each of these experiments suggests that two Gaα splicing variants (small ~45 KDa and large ~52 KDa) (29-31) are present in sperm (Fig. 1A & B, left panel). Alternatively, it remains possible that
one of the hands corresponds to G\textsubscript{olf}, another member of the G\textsubscript{α} family which is also a cholera toxin substrate and shares high sequence homology with G\textsubscript{α}.

**Forskolin stimulates cAMP levels in capacitated mouse sperm.** The finding of stimulatory G\textsubscript{α}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 forskolin (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 in Experimental Procedures. A representative calibration curve is shown in Figure 2A. Sperm were incubated in conditions that support capacitation for 1 hr followed by 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. 2B). 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. 2B). On the other hand, inclusion of the tmAC inhibitor SQ22536 (100 µM) (44) significantly reduced the FK effect (Fig. 2B). 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 hr 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. 2C). Controls were carried out in parallel using wild type mice from the same litter (Fig. 2C). 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 is able to respond to FK in sperm.

To analyze which tmACs might have a role in sperm, we analyzed all Adcy isoforms 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 Adcy 6, 7, 8 and 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 non-capacitating conditions (Fig. 4A). On the other hand, addition of cAMP permeable agonists (dbcAMP, 8-BrcAMP) induced phosphorylation of PKA substrates as well as phosphorylation of tyrosine residues (Fig. 4B and C) in non-capacitating conditions.

**Compartmentalization of different cAMP signaling events.** Results described above raised a key question regarding the signaling pathways leading to sperm capacitation: if cAMP permeable analogues are able to promote PKA activation and tyrosine phosphorylation, why is FK stimulation of tmACs unable to do likewise? Since 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 G\textsubscript{α} antibodies labeled only the anterior head (Fig. 5A) suggesting that the tmAC-dependent cAMP pathway is restricted to sperm heads. Double staining with peanut agglutinin (PNA), a lectin that recognizes the acrosomal content and is lost when the acrosome reaction occurs, revealed that anti-G\textsubscript{α} staining is lost in acrosome-reacted sperm. This experiment suggests that G\textsubscript{α} is present in the plasma membrane overlaying the acrosome and that this protein is lost during the acrosome reaction.
The localization of $G_\alpha$, 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 non-capacitated sperm cells using antibodies against the PKA catalytic subunit. These antibodies recognized a single band at ~42 KDa which is the expected molecular weight of the PKA catalytic subunit (Fig. 5B). When used for Immunofluorescence, these antibodies indicated that PKA localized exclusively to the flagellum compartment (Fig. 5C).

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 previously described (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. 6A). Consistent with the differential PKA localization, PKA activity was only detected in the anterior head, we hypothesized that tmACs and PKA. To measure PKA activity, cAMP levels were directly assessed in each fraction in the presence or absence of 100 µM FK. While FK significantly increased cAMP levels in the head fraction, cAMP levels in the tail fraction were unchanged by this compound (Fig. 6B). 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 $G_\alpha$, 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. 7A). 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$^{2+}$]), concentrations, sperm were loaded with Fluo 4 to measure the effect of FK on [Ca$^{2+}$], in single cells. Solubilized zona pellucida (sZP), which was used as control, induced Ca$^{2+}$ increase in about 50% of the sperm population (Fig. 7B and Supplemental movie 1). Similarly, 50 µM FK induced an increase in [Ca$^{2+}$], in more than 50 % of the sperm population (Fig. 7C and Supplemental movie 2). The FK-induced increase in [Ca$^{2+}$], was blocked significantly by SQ22536 at a concentration of 100 µM (Fig. 7D and Supplemental movie 3). The number of responsive cells is shown in Fig. 7E.

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). Due to 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: the tmACs (Adcy1 to 9) and the soluble adenylyl cyclase (Adcy10) (6). Adcy10 and tmACs are regulated by different pathways. Adcy10 is insensitive to GTP analogues 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).

On the other hand, tmACs are regulated both positively and negatively by heterotrimeric $G_\alpha$, and $G_\alpha$, protein complexes, respectively. The heterotrimeric G protein family consists of a $G_\alpha$-subunit and a $G_{\beta\gamma}$-dimer. In the resting state, the $\alpha$-
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 tmACs respectively (57). G_s can be stimulated by cholera toxin through ADP-ribosylation of the α_s-subunit, a property shared with G_i. 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 G_s or G_i in a particular cell type (29-31). On the other hand, α_i-subunits are Bordetella pertussis toxin’s substrates; analogous to cholera toxin, pertussis toxin catalyzes ADP-ribosylation of α_i-subunits. 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 G_s in mammalian sperm, independent work from Kopf’s (33) and Garbers’s (32) groups clearly showed the presence of this protein in sperm. Regarding G_i, ADP-ribosylation assays were also used to investigate the presence of this G protein in mammalian sperm. Similarly to the G_s case, Hildebrandt et al. (26) were not able to detect a cholera toxin ADP-ribosylation substrate in dog sperm. However, Fraser’s group (34) identified a faint 45 kDa ADP-ribosylated protein in whole sperm extracts. In the same work (34), anti G_i antibodies gave a positive signal by Western blots. However, also using anti G_i antibodies, Merlet et al. (58) concluded that the α_i-subunit is not present in human sperm. Finally, Spehr et al. (16) reported the presence of the α_i-subunit as well as other molecules of the tmACs cascade using a shot-gun tandem mass spectrometry proteomic approach in human sperm. Despite this body of work, the presence of G_i remained controversial. In this manuscript, we used[^2] ADP-ribosylation of purified membrane fractions to demonstrate the presence of G_i in mouse sperm. In addition, anti G_i-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 G_i 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 G_i variants (59-64) or to post-translational modifications. Immunofluorescence experiments using Western blot-validated anti-G_i antibodies labeled the anterior acrosome of mouse sperm but failed to stain the flagellum. Consistent with this observation, anti-G_i antibody staining was lost in acrosome-reacted sperm. Due to sequence similarities, it is difficult to discriminate between G_i and G_i variants: 1) both can be ADP-ribosylated in the presence of cholera toxin; 2) both are likely to be recognized by the anti G_i antibody that we used; and 3) once activated, both can stimulate tmACs. Interestingly, similar to G_s, G_i 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. G_i 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. While 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 analogues as well as with mastoparan, a wasp venom toxin known to activate G proteins. In addition, in the present work, the effect of FK was blocked by SQ22536, a compound known to inactivate tmACs in several cell types with an IC90 that varies depending on the tmAC isoform but not by the specific AdCyt10 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 Adcy 6, 7, 8 and 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 postmeiotic 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 or are present in testicular germ cells. In regards to reproductive phenotype, only Adcy10- (13) and Adcy3-null mice (17) 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 knockout mice is not known. Knockout mice for Adcy1, 5, 6 and 8 are fertile; however, it is important to consider that tmACs have overlapping functions in many cells making 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 knockout 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 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 which contains a putative consensus sequence for cAMP binding (71). As mentioned, immunofluorescence localization of Ga\(_{\alpha}\)-subunit suggests that the tmAC signaling pathway is present in 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’s lab (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, 8-pCPT-AMP is able to induce the acrosome reaction in these species at concentrations which 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 the present 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\(^{2+}\)], 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. On the other hand, Adcy10 KO mice are not deficient
cAMP compartmentalization in sperm physiology

in 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 Gs activation of tmAC(s) in sperm. This work constitutes the basis towards 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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50. Rall, TW. and Sutherland, EW. (1958) J. Biol. Chem. 232, 1065-1076

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FOOTNOTES
*This work was supported by NIH HD38082 and HD44044 (to P.E.V.); CONACyT-Mexico, (49113 to AD), DGAPA/UNAM (IN211809 and IN225406 to AD); NIH HD059913 and GM62328 (to J.B. and L.R.L.); PICT-ANPCyT-Argentina (2011-0540 to DK); and a postdoctoral fellowship from the National Council of Science and Technology (CONICET) to EW (2012-2263).

Drs. Levin and Buck own equity interest in CEP Biotech which has licensed commercialization of a panel of monoclonal antibodies directed against sAC.

The abbreviations used are: ADCY, soluble adenylyl cyclase; FK, forskolin; tmAC, transmembrane adenylyl cyclase.

FIGURE LEGENDS

FIGURE 1. Stimulatory Gαs proteins are present in mouse sperm. A, Mouse sperm and brain membranes were purified by differential centrifugation and assayed for in vitro [32P] ADP ribosylation using pre-activated cholera toxin (CTX) as described in Methods. After the reaction was completed samples were subjected to SDS-PAGE, transferred to PVDF membranes and exposed for autoradiography. Two bands were detected at 45 and 52 KDa that correspond to the molecular weight of Gαs (left panel). Western Blots using a polyclonal anti-Gαs 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-Gαs antibody was carried out on both sperm and brain membrane extracts that had been previously subjected to cholera toxin [32P] ADP ribosylation (Gαs IP). 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-Gαs antibodies. IgG (light chain) in IP samples are also shown.

FIGURE 2. Forskolin increases cAMP levels in capacitated mouse sperm. A, 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 fmole to 20 pmole. In the absence of added cAMP, the basal value was 2.5 pmole of [32P] 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 in 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 to capacitated sperm at same FK concentration. # p<0.05 and ## p<0.01 when compared to 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.M. n≥4. Values for Adcy10 KO sperm before FK stimulation were below the detection limits and named in the graph as “non-detectable” (n.d) (74). A paired t-test was used to compare WT control vs WT+FK (* p<0.05) and a one-sample t-test was used to determine if the mean of KO+FK was different from the hypothetical value “0” (# p<0.05).

FIGURE 3. Different Adcy isoforms are expressed in mouse sperm and during testis development. Total RNA from sperm and testis of different developmental stages were 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 Adcy 6, 7, 8, 9 and 10 in sperm is observed.

FIGURE 4. Forskolin (FK) has no effect on capacitation-associated phosphorylation events. A, Sperm were incubated under non-capacitating (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-pY and anti-phosphoPKA-substrate antibodies as described in Experimental Procedures. B and C, Sperm were incubated in media without HCO₃⁻ supplemented with 100 µM IBMX and with increasing concentrations of 8Br-cAMP, Spc-cAMPS or db-cAMP. Samples were processed for Western blotting with anti-pPKAs (B), stripped and further processed with anti-pY antibodies (C). All Western blots are representative of experiments repeated at least three times.

FIGURE 5. Gₐs 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-Gₐs antibody. Peanut agglutinin (PNA) was used to follow the acrosomal status. Anti-Gₐs (red) and PNA (green) labeling show that Gₐs is lost during acrosomal reaction (merged color panels). 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 (cPKA) 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 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 in Experimental Procedures. Anti-cPKA staining of whole sperm shows the presence of PKA only in the sperm flagellum.

FIGURE 6. PKA activity is only observed in sperm tails, while FK-induced cAMP production is detected in sperm heads. A, Head and tail fractions from capacitated sperm were separated as described in experimental procedures and assayed for PKA activity using ³²P-ATP and kemptide as substrate in the presence of either 1 mM cAMP or 100 µM FK. Data represent mean ± S.E.M. 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 to 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 in experimental procedures. Data represent mean ± S.E.M. of five independent experiments performed in triplicates; two-way ANOVA followed by Bonferroni post tests were used to compare replicate means. * p<0.05 when compared to heads without FK addition.

FIGURE 7. FK promotes acrosome reaction and [Ca²⁺]i increases in capacitated mouse sperm. A, FK induces acrosome reaction. Mouse sperm were incubated under capacitating conditions for 1h 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) were added and sperm were incubated for additional 30 min. Acrosomal status was assessed as explained in experimental procedures. Percentage of acrosome-reacted sperm (%AR) was calculated based on 7 independent experiments run in triplicate. B, Pseudocolored fluorescence images illustrating [Ca²⁺]i levels before (CAP) and after addition of solubilized ZP (sZP) to capacitated mouse sperm. The [Ca²⁺]i increase induced subsequently by 10 µM ionomycin (IONO) is also shown as a positive control. The right panel are representative [Ca²⁺]i traces of sperm subjected to the conditions described above. Arrows indicate agonist application. C, Fluorescence images of [Ca²⁺]i, levels before (CAP) and after addition of 50 µM FK (FK) to capacitated mouse sperm. D, Fluorescence images of [Ca²⁺]i, levels before (CAP) and after addition of 50 µM FK (FK) to capacitated mouse sperm. E, percentage of sperm displaying [Ca²⁺]i increases in response to ZP compared to 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 4 independent experiments). Turkey’s multiple comparison
statistics tests were performed for both acrosome reaction and [Ca\(^{2+}\)] experiments. Means of groups that have different letters differ significantly (p<0.01).

**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,** G\(_s\) is in the head and activates a tmAC that increases cAMP levels which would be necessary to activate acrosome reaction by forskolin (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 (GEF) called EPAC (48).
Table 1. Intron spanning primers used for analysis of *Adcy* expression by RT-PCR

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Figure 1; Wertheimer et al.
Figure 2; Wertheimer et al.
Figure 3; Wertheimer et al.
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Figure 4; Wertheimer et al.
Figure 5; Wertheimer et al.
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Figure 6: Wertheimer et al.

A	PKA activity

B	cAMP generated
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Figure 7; Wertheimer et al.

A

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B

CAP  | sZP  | IONO

C

CAP  | FK   | IONO

D

CAP+SQ | FK   | IONO

E

% of responsive cells

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