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CASK interacts with PMCA4b and JAM-A on the Mouse Sperm Flagellum to Regulate Ca^{2+} Homeostasis and Motility¹

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Abstract

Deletion of the highly conserved gene for the major Ca^{2+} efflux pump, Plasma membrane calcium/calmodulin-dependent ATPase 4b (*Pmca4b*), in the mouse leads to loss of progressive and hyperactivated sperm motility and infertility. Here we first demonstrate that compared to wild-type (WT), *Junctional adhesion molecule-A* (*Jam-A*) null sperm, previously shown to have motility defects and an abnormal mitochondrial phenotype reminiscent of that seen in *Pmca4b* nulls, exhibit reduced ($P < 0.001$) ATP levels, significantly ($P < 0.001$) greater cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) and ~10-fold higher mitochondrial sequestration, indicating Ca^{2+} overload. Investigating the mechanism involved, we used coimmunoprecipitation studies to show that CASK (Ca^{2+} /calmodulin-dependent serine kinase), identified for the first time on the sperm flagellum where it co-localizes with both PMCA4b and JAM-A on the proximal principal piece, acts as a common interacting partner of both. Importantly, CASK binds alternatively and non-synergistically with each of these molecules via its single PDZ (PDS-95/Dlg/ZO-1) domain to either inhibit or promote efflux. In the absence of CASK-JAM-A interaction in *Jam-A* null sperm, CASK-PMCA4b interaction is increased, resulting in inhibition of PMCA4b's enzymatic activity, consequent Ca^{2+} accumulation, and a ~6-fold over-expression of constitutively ATP-utilizing CASK, compared to WT. Thus, CASK negatively regulates PMCA4b by directly binding to it and JAM-A positively regulates it indirectly through CASK. The decreased motility is likely due to the collateral net deficit in ATP observed in nulls. Our data indicate that Ca^{2+} homeostasis in sperm is maintained by the relative ratios of CASK-PMCA4b and CASK-JAM-A interactions.

Keywords

hyperactivated motility; flagellum's principal piece; calcium efflux; electron dense mitochondria

Introduction

Calcium homeostasis is essential for sperm motility (Publicover et al, 2007), and deletion of the gene encoding the highly conserved major mammalian Ca^{2+} efflux pump, *Pmca4b*, leads to loss of both progressive and hyperactivated sperm motility with resulting infertility in mice (Okunade et al, 2004; Schuh et al, 2004; Withers et al, 2006). PMCA4b is the prominent slice variant isoform that is predominantly expressed in the testis (Di Leva et al,

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2008) where only two of the four PMCA isoforms are present (Okunade et al, 2004). Although PMCA4b is ubiquitously expressed (Di Leva et al, 2008), the only deleterious effect of its deletion is male infertility (Okunade et al, 2004; Schuh et al, 2004; Withers et al, 2006). This may be due to an apparent compensation by other PMCA family members in organs other than the testis where PMCA4b comprises >90% of the two PMCAs present (Di Leva et al, 2008). Despite the physiological and pathological importance of PMCA4b in sperm, where it is the major Ca^{2+} clearance molecule in the mouse (Di Leva et al, 2008; Wennemuth et al, 2003), the mechanism of its regulation remains unknown, and its interacting partners unidentified. Further, unlike the case of other members of the PMCA family, the mechanism of PMCA4b's inactivation is unknown even in somatic cells (Zylinska and Soszynski, 2000), although its activation by PKC (Enyedi et al, 1996) is well-known. To date, there have been no investigations on the mechanism(s) by which the failure or disruption of Ca^{2+} efflux leads to sperm motility defects and infertility.

Recently we identified a novel sperm membrane protein, Junctional Adhesion Molecule A (JAMA) and have shown that *Jam-A* null sperm exhibit progressive and more severe hyperactivated motility defects (Shao et al, 2008). Since *Jam-A* null sperm show electron-dense and condensed mitochondria (Shao et al, 2008), a histopathological phenotype that is identical to that seen in *Pmca4b* null sperm (Okunade et al, 2004) where there is a >2-fold elevation of intracellular Ca^{2+} (Schuh et al, 2004), our observations suggest abnormal mitochondrial sequestration of Ca^{2+} and potential roles for JAM-A in Ca^{2+} clearance and sperm motility. Thus it appears likely that JAM-A may play a critical role in facilitating PMCA4b in fulfilling both of these roles in sperm.

While PMCA4b performs a housekeeping function by maintaining Ca^{2+} homeostasis in a variety of mammalian cells (Withers et al, 2006) it also functions as a modulator of signal transduction pathways (DeMarco and Strehler, 2001; Schuh et al, 2003). In brain and kidney, apart from various signaling events such as Ca^{2+} /calmodulin activation, phosphorylation, and activation by phospholipids (Carafoli, 1991), PMCA4b is known to be regulated by its ability to bind, via its C-terminus, to PDZ (PDS-95/Dlg/ZO-1) domains of the MAGUK (membrane-associated guanylate kinase) family of proteins (DeMarco and Strehler, 2001) such as CASK (Ca^{2+} /calmodulin-activated serine/threonine kinase) (Schuh et al, 2003).

Interestingly, in human epithelial cells, CASK associates via its PDZ domain with JAM-A (Martinez-Estrada et al, 2001) which is known to function as a signaling molecule (Naik et al, 2003). These associations suggest that CASK may be an interacting partner of both PMCA4b and JAM-A in sperm. Here we report for the first time the presence of CASK on the mammalian sperm flagellum and show that it co-localizes and physically interacts with both JAM-A and PMCA4b and that its interaction with the latter occurs at varying degrees, depending on the $[\text{Ca}^{2+}]_c$. Further, we demonstrate that CASK-PMCA4b interaction is increased in the absence of CASK-JAM-A interaction in *Jam-A* null sperm, resulting in decreased PMCA4b enzymatic activity and defective Ca^{2+} efflux, accompanied by reduced ATP levels. The latter may explain both the sperm motility and Ca^{2+} clearance defects observed in *Jam-A* null sperm.

Materials and Methods

Sexually mature C57BL/6 mice (Harlan Sprague-Dawley, Indianapolis, IN) and *Jam-A* null mice of the same background (Shao et al, 2008) were used in the investigations. Studies were approved by the Institutional Animal Care and Use Committee at the University of Delaware and were in agreement with the Guide for the Care and Use of Laboratory Animals published by the National Research Council of the National Academies, 8th ed.,

Washington, D.C. (publication 85-23, revised 2011). All chemicals were purchased from Fisher Scientific Co. (Malvern, PA), Sigma-Aldrich (St. Louis, MO) or Invitrogen (Carlsbad, CA), unless otherwise specified.

Antibodies

The two rat anti-mouse JAM-A monoclonal antibodies used in these studies, BV12 and H202-106, were obtained from Abcam Inc. (Cambridge, MA). Affinity purified goat polyclonal PMCA4 antibody raised against an N-terminal peptide of rat protein (Y-20; sc-22080) and the anti-human HSC 70 (heat shock cognate protein 70, B-6; sc-7298) mouse monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-rat CASK/Lin2 mouse monoclonal antibody (#75-000) which cross-reacts with both mouse and human was obtained from UC Davis/NINDS/NIMH NeuroMab Facility, Davis, CA. The immunogen for this antibody is a rat CASK fusion protein with amino acid residues 318-415 located between the CaM kinase and the PDZ domains. A second CASK Ab, a rabbit polyclonal against amino acids 353-459 of human CASK, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Normal goat, mouse, and rat IgGs were obtained from Sigma-Aldrich. Anti-SPAM1 antibody, used to detect SPAM1 as a loading control for an irrelevant sperm membrane protein, was a mouse anti-peptide antiserum (Zhang and Martin-DeLeon, 2003) custom-made by Zymed (South San Francisco, CA). Rabbit polyclonal anti-CATSPER antibody against the N-terminal 150 amino acids, kindly gifted by Dr. Dejian Ren (Ren et al, 2001), was used to determine the expression of CATSPER 1.

Caudal sperm retrieval and evaluation

Immediately after CO₂ asphyxiation, the cauda epididymides of adult WT and *Jam-A* null mice were removed, placed in 500 μ l of pre-warmed human tubal fluid [HTF, with HEPES (InVitroCare, Frederick, MD)], finely minced, and allowed to stand for 10 min at 37°C. The dispersed sperm were collected with the supernatant, avoiding tissue debris. An aliquot of the sperm suspension was used to assess motility and concentration, using a hemocytometer, and the remaining sperm were washed twice in PBS (500 $\times$ g, 15 min) and the pellet containing cells reconstituted in appropriate media and used immediately for further experimentation.

Viability of cauda sperm was determined using eosin-nigrosin dye exclusion test as described (World Health Organization, 2010). Briefly, to a 50 μ l sperm suspension on a clean glass slide was mixed with an equal volume of eosin-nigrosin staining solution (0.67g eosin Y and 0.9 g NaCl were mixed in 100 ml of purified water with gentle heating, and 10 g of nigrosin added, mixed, boiled, cooled and filtered), and let stand for 30 seconds. The suspension was quickly smeared onto a clean glass slide, dried, mounted and examined using brightfield optics in a Zeiss microscope. The numbers of stained (dead) and unstained (live/viable) sperm were tallied using a laboratory counter and at least 200 cells were counted for each of the duplicate slides from each sample and percent viable cells calculated.

Intracellular ([Ca²⁺]_i) and cytosolic ([Ca²⁺]_c) Ca²⁺ concentrations

WT and *Jam-A* null sperm were either pre-incubated in HTF, a known capacitation medium, for 90 min at 37°C (Cap), or used immediately after retrieval without incubation, (Uncap), to load Fura-2/AM as outlined below. [Ca²⁺]_c, representing Ca²⁺ concentrations prior to Ca²⁺ release from mitochondrial stores, was measured by spectrofluorometry using a procedure adapted from an earlier report (Hirst et al, 1999). Cap and Uncap sperm were incubated with 3 μ M Fura-2/AM (Molecular Probes, Eugene, OR), a fluorescent Ca²⁺ indicator, in HBSS (Hanks balanced salt solution), supplemented with 0.5% Pluronic F-127 (Molecular Probes)

either in the presence or absence of Ca^{2+} , for 30 min in the dark at 37°C , with gentle shaking. The sperm were collected ($500 \times g$, 15 min) and the fluorescent indicator was completely de-esterified in the dark for an additional 30 min with HBSS alone at RT, with or without added Ca^{2+} . The cells were collected by centrifugation, rinsed in HBSS, resuspended in 2 ml HBSS ($2.5 \times 10^5/\text{ml}$) with or without Ca^{2+} , transferred to a 1 cm acrylic cuvette (Sarstedt), and emitted fluorescence measured, under gentle stirring at 37°C , in a spectrofluorometer (DeltaRam, Photon Technology International, Birmingham, NJ), for ~700 sec at 340 nm and 380 nm excitation and 510 nm emission wavelengths. The F340/380 ratios recorded at 1 sec intervals in a desktop computer were used to calculate $[\text{Ca}^{2+}]_i$ by comparison with Fura-2 free acid standards and a Fura-2 calibration kit (Molecular Probes, Eugene, OR) as previously reported (Zhang et al, 2006). Measurements were automatically corrected for autofluorescence by obtaining the fluorescence of cells not loaded with Fura 2/AM. Calibrations were performed at the beginning and at the end of each of the four independent experiments.

To assess $[\text{Ca}^{2+}]_i$ which includes $[\text{Ca}^{2+}]_c$ and Ca^{2+} that can be released from that sequestered in mitochondria, 2 μl of 2 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP, final conc. 2 μM), an uncoupler of mitochondrial oxidative phosphorylation, was directly pipetted into the cuvette containing 2 ml of sperm suspension at the end of ~10 min of recordings, by briefly interrupting the fluorometer before resuming measurements.

Single-cell Ca^{2+} Imaging

Following procedures described earlier (Wood et al, 2003), Cap and Uncap sperm layered on poly-D-lysine coated coverslips were loaded with 20 μM Fluo-4/AM (fluorescent Ca^{2+} indicator, Molecular Probes, Eugene, OR) and 0.5% Pluronic F-127 for 120 min in the dark. The cover-slips were then gently washed, placed face down in single-well Nalge nunc chambered system and sperm imaged at RT using a high-speed confocal microscope (LSM 5 DUO, Zeiss; 40 $\times$ oil Plan-Neofluor objective) equipped with an argon laser for excitation at 488 nm and 505 nm long-pass filter for emission. Only motile sperm that were attached at one or more points to the cover-slips were used for analysis.

Determination of sperm ATP levels

Adenosine 5'-triphosphate (ATP) estimation was performed for Cap and Uncap sperm, using a luciferase bioluminescence assay similar to that described earlier (Miki et al, 2004). For simulating capacitating conditions, HTF was replaced with M16 capacitation medium for at least 90 min or until analysis. Briefly, samples (4×10^6 sperm) were either assayed immediately (Uncap) after retrieval or after 90 min incubation at 37°C in M16 medium (Cap) in the presence or absence of CCCP. ATP was extracted from uncapacitated sperm immediately after recovery from the cauda in M16 medium. The samples were boiled in an extraction buffer for 1 min and the supernatant collected in duplicates for assay using Luciferase ATP Bio-luminescence Kit (Sigma). Reactions were assembled in shielded 96-well microtiter plates and read using a Victor² 1420 Multilabel Counter (Wallac, Turku, Finland). Three separate experiments were performed.

Measurement of mitochondrial membrane potential

Sperm were collected, as above, from the cauda of four WT and four *Jam-A* null mice and mitochondrial membrane potential assayed as described earlier (Tanphaichitr et al, 1984). Briefly, sperm were washed in D-MEM/F12 medium, and to a pellet containing 1×10^5 cells was added 100 μl of Rhodamine 123 (20 $\mu\text{g}/\text{ml}$) and incubated in the dark at 37°C for 10 min. At the end of incubation, 1 ml of D-MEM/F12 was added, the cells pelleted, washed in 1 ml of D-MEM/F12, and resuspended in ~100 μl of medium. An aliquot of the suspension was wet-mounted on a slide and examined under a Zeiss axioscope equipped

with a UV excitation source (HBO 100 W) and appropriate filter set and interfaced to a Macintosh desktop computer. Images were acquired with a charged coupled device-cooled camera (Photometrics, Tucson, AZ) and IPLab Spectrum software module, version 3.5 (Scanalytics, Fairfax, VA). A total of 200 sperm/ animal were screened for those with and without stained midpieces on the flagella. The difference in the number of stained cells in WT and null sperm was compared with Chi-square analysis. In a parallel set of samples, Rhodamine 123 fluorescence was quantified in ~50,000 sperm by flow cytometry, as described below.

Immunofluorescence (IF) and Flow cytometry

To identify the presence and localization of CASK, PMCA4b, JAM-A, and CATSPER in WT and null caudal sperm, IF was performed using modifications of the procedure published earlier (Shao et al, 2008). Briefly, freshly collected sperm were fixed in 1.5% paraformaldehyde for 1 hr at RT, washed twice in PBS, pelleted ($500 \times g$) and permeabilized (0.1% Triton X-100; Ricca Chemical Co., Arlington, TX) for 10 min at RT. The cells were then retrieved by centrifugation, washed once with PBS followed with blocking buffer (PBS containing 2% BSA) and re-suspended overnight in 400 μ l of blocking buffer at 4°C. They were then immunostained with diluted anti-CASK (1:500) or anti-PMCA4 (1:200) or anti-JAM-A (1:500) or anti-CATSPER 1 (5 μ g/ml) antibodies for 1 h. Controls were treated with appropriate isotype IgGs. Cells were then washed in PBS, collected by centrifugation ($500 \times g$ for 15 min), and treated with the appropriate secondary antibody (conjugated with FITC or Alexafluor 488 for detecting CASK, JAM-A, and CATSPER, and with Alexafluor 558 for detecting PMCA4b) for 30 min in the dark at RT. After washing, the cell suspension was smeared on slides and counterstained with DAPI before microscopic examination and imaging using the appropriate filter set on the Zeiss axioscope, or with the confocal microscope. To quantify and compare the amount of CASK and PMCA4b in WT and *Jam-A* null sperm, cells (~50,000/sample) treated for IF were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon ion laser at 488 nm excitation and with detectors for FITC.

SDS-PAGE and Western blot analysis

For determining relative CASK and CATSPER1 expression in WT and null sperm, proteins were extracted from freshly collected sperm and Western blot analysis performed as previously described (Aravindan et al, 1996; Zhang and Martin-DeLeon, 2003). Briefly, cells were homogenized manually (using a mortar and pestle) in a solubilization buffer [62.5 mM Tris-HCl, 10% glycerol, 1% SDS, and 1 mM phenylmethyl-sulphonyl fluoride (PMSF) as a protease inhibitor] at pH 6.8 and 4°C. The suspensions were centrifuged at $14,000 \times g$ for 10 min at 4°C, the solubilized protein supernatant collected and protein concentration determined using a biocinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). About 30 μ g of solubilized proteins were resolved by SDS-PAGE under reducing conditions [99°C for 4 min in the presence of 100 mM dithiothreitol (DTT)], transferred onto nitrocellulose membranes (Sambrook and Russell, 2001), probed with the mouse monoclonal anti-CASK or the anti-rabbit CATSPER or anti-goat PMCA4b antibodies and detected using alkaline phosphatase-conjugated anti-rat or anti-rabbit or anti-goat secondary antibodies, respectively, and proteins visualized using the Western Breeze Chemiluminescent Immunodetection Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

To assess the amount of protein loaded, blots were stripped (Restore Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL) and re-probed sequentially with rabbit polyclonal mouse anti-SPAM1 antiserum, an irrelevant sperm membrane protein, and mouse anti-human HSC70 (heat shock cognate protein 70, a housekeeping protein) monoclonal antibodies. The appropriate secondary antibodies were conjugated to AP. To quantify the

relative expression of CASK, densities of the protein bands were analyzed by densitometry (FluorChem 8800, Alpha Innotech Corp., San Leandro, CA), and the ratio of CASK:HSC70 plotted.

Co-immunoprecipitation

To detect associations between JAM-A and CASK, and CASK and PMCA4 in sperm, coimmunoprecipitation studies were undertaken using sperm protein extracts, containing protease inhibitor Complete™ (Roche Diagnostics, Indianapolis, IN), prepared in the solubilization buffer, as described above. To 100 µl of sperm extract containing 125 µg proteins, 0.5 ml of immunoprecipitation (IP) buffer (25 mM Tris, 150 mM NaCl, pH 7.2) and either rat monoclonal anti-JAM-A (1:300), mouse monoclonal anti-CASK (1:300) or goat anti-PMCA4 (1:400) antibodies were added and incubated overnight at 4°C in a vertical rotator. Controls were set up with protein extracts incubated with the appropriate IgG at the same concentrations as the primary antibodies and processed identically. The antigen-antibody complexes were pulled down using Protein A resin beads (Thermo Scientific, Rockford, IL) or PureProteome Protein G magnetic beads (Millipore Corp. Billerica, MA). Following centrifugation ($2,500 \times g$, 5 min), the supernatant was discarded, and the pellet washed and electrophoresed (12% SDS-PAGE) under reducing conditions (864 mM 2-mercaptoethanol, 5 min at 99°C) and processed according to standard protocols (Sambrook and Russell, 2001). Bands were visualized using the Western Breeze Chemiluminescent Immuno-detection Kit, as above.

Mg²⁺-dependent Ca²⁺-ATPase activity in sperm membrane

Total membranes, prepared from caudal sperm pooled from 4 mice/experiment, were capacitated in HTF medium at 37°C for 90 min. Sperm were homogenized in lysis buffer [25 mM maleate pH 7.4, 1 mM EDTA, 0.5 mM DTT, 250 mM sucrose and protease inhibitor Complete™] using a mortar and pestle. The homogenate was centrifuged for 10 min at $14,000 \times g$ at 4°C, and the supernatant subjected to ultracentrifugation at $100,000 \times g$ for 60 min at 4°C to yield microsomes. The pellet was re-suspended for 15 min at 4°C in 100 µl of homogenization buffer containing 1.5% n-octyl-β-d-glucopyranoside as described (Post et al, 2010), and protein concentration was determined using the BCA kit mentioned above. Ca²⁺-ATPase activity was determined as described (Kosk-Kosicka, 1999) for the enzymatic hydrolysis rate of ATP via quantitation of the resulting inorganic phosphate (P_i), as a function of time. The assay was performed at 37°C for 30 min using 5 µg microsomal protein and 60 mM ATP. Duplicate reactions were terminated with Lin-Morales reagent and the P_i measured colorimetrically as a complex of molybdovanadate, using a spectrophotometer with the absorbance at 350 nm. Absorbance was converted into P_i using a calibration curve of known concentrations of K₂HPO₄. Experiments were performed in triplicate and the mean activity expressed as µmol/P_i/mg/h.

Real-Time RT-PCR

Relative expression of *Pmca4* and *Cask* in adult testis was determined in WT and *Jam-A* nulls (n=6). Gene expression reactions were set up in triplicates in an Applied Biosystems' TaqMan 7900HT sequence detection system. TaqMan® Universal PCR Master Mix with AmpErase UNG, and TaqMan gene expression assays for β-*Actin* (endogenous reference control) and *Pmca4* and *Cask* (targets) were purchased (Applied Biosystems, Foster City, CA). Reactions, including non-template controls, were set up on ABI PRISM® 384-Well Clear Optical Reaction Plate using 20 ng of each cDNA in a 20 µl reaction volume. An initial activation of AmpliTaq gold was performed at 90°C for 10 min, followed by 40 cycles of denaturing (95°C for 15 sec) and annealing/extension (60°C for 1 min). Amplified transcripts were quantified using the comparative Ct and ΔΔCt method, and data analyzed using the Sequence Detection Systems (SDS) Software.

Statistical analyses

The statistical significance of differences in $[Ca^{2+}]_c$ levels between WT and *Jam-A* null sperm, either Cap or Uncap, with and without CCCP, as well as with or without Ca^{2+} in the media during fluorescence loading and data acquisition, obtained from spectrofluorometric plots, were analyzed for each experiment by one-way Mann-Whitney Rank Sum Test. At least 3 experiments were conducted for each study. The values of ATP levels from 3 separate experiments were analyzed using paired *t*-test to determine significance of differences between WT and the null sperm. Differences in sperm viability among groups and PMCA4b activity between WT and *Jam-A* nulls were compared using Student's *t*-test. Chi square analysis was used to determine significance in the difference in frequencies of null and WT sperm that were stained with Rhodamine 123.

Results

Jam-A null sperm exhibit increased $[Ca^{2+}]_i$ and altered homeostasis

Analyses of the steady-state cytosolic Ca^{2+} concentration $[Ca^{2+}]_c$ indicated it to be highly significantly ($P < 0.001$) greater in nulls than in WT for both Uncap (uncapacitated) and Cap (capacitated) sperm (Fig. 1a). Incubation for 90 min in capacitation (Ca^{2+} -enriched) medium indicated significant ($P < 0.001$) Ca^{2+} accumulations in both WT and *Jam-A* null sperm (Fig. 1a), consistent with onset of capacitation (Visconti et al, 1995). In these experiments, in order to ascertain *in situ* concentrations of Ca^{2+} , Fura-2 loading buffer was maintained devoid of Ca^{2+} . However, when Ca^{2+} was added during the 30 min Fura-2 loading period (Fig. 1b), although $[Ca^{2+}]_c$ increased significantly ($P < 0.001$) in all groups as expected, the increase was greatest in Cap nulls, attaining levels of >350 nM compared to the ~ 200 nM observed in the corresponding WT sperm. Although the viability of sperm from both WT and nulls reduced during treatments the decreases were similar in both groups under all conditions (Fig. 1c), indicating that it did not contribute to the observed differences. Western and IF analyses revealed that the expression of, CATSPER 1, a subunit of the major Ca^{2+} influx protein, was unaltered in the nulls (Fig. 2), suggesting that the increased $[Ca^{2+}]_c$ was unlikely due to increased influx and that other factors may be involved. These observations provided the first direct evidence that the increased $[Ca^{2+}]_c$ in nulls is due to compromised efflux activity.

Single cell Ca^{2+} imaging with Fluo-4 (Fig. 3) displayed the subcellular location of $[Ca^{2+}]_c$ and confirmed the increases in nulls, over WT, observed in Fig. 1. Nulls had greater fluorescence intensity in the midpiece, reflecting mitochondrial sequestration of Ca^{2+} for both Uncap (Fig. 3a, b) and Cap (Fig. 3c, d) sperm. Fluorescence in the head was minimal in both Uncap groups. Upon capacitation, WT sperm (Fig. 3c) exhibited increased fluorescence in the head, and a decrease in the midpiece. Null sperm, on the other hand, showed Ca^{2+} staining in the head with marked accumulation in the crescent-shaped intraacrosomal store consistent with earlier observations (De Blas et al, 2002), while continuing to exhibit intensely staining midpieces reflecting greater mitochondrial Ca^{2+} accumulation (Fig. 3d).

The abnormal mitochondrial sequestration of Ca^{2+} in the nulls was unequivocally confirmed by assaying $[Ca^{2+}]_c$ following its release by carbonyl cyanide *m*-chlorophenylhydrazine (CCCP). The difference in the amount of Ca^{2+} released in the cytosol from WT and null sperm was significantly ($P < 0.001$) elevated in the latter. Representative results from one of three separate experiments showing the release of mitochondrial Ca^{2+} into the cytosol are presented in Fig. 4. In response to CCCP, the Ca^{2+} released showed $\sim 50\%$ increase over the baseline concentrations in WT sperm, whereas it was elevated by $\sim 500\%$ in null sperm within 90 sec of exposure. Moreover, the elevation in $[Ca^{2+}]_c$ was punctuated in nulls by intermittent high amplitude fluctuations, suggesting partial, incomplete and intermittent

Ca^{2+} clearance and implicating the efflux mechanism in nulls as the candidate for further investigation.

ATP is reduced in Jam-A null sperm despite normal mitochondrial function

Since both sperm motility and the Ca^{2+} efflux pump are ATP-driven, we assayed ATP levels in both Uncap and Cap sperm, WT and null, in the presence and absence of CCCP, an uncoupler of mitochondrial membrane potential. *In situ* ATP concentrations ([ATP]) were not affected by the state of capacitation in either WT or null sperm (Fig. 5a), as reported earlier (Miki et al, 2004). However, compared to WT, nulls exhibited 45% and 40% decreases ($P < 0.0001$) in [ATP] in Uncap and Cap sperm, respectively (Fig. 5a). As expected, addition of CCCP to WT Cap sperm resulted in a highly significant decrease in steady-state [ATP] (-31.0% ; $P < 0.0001$), due to the uncoupling of mitochondrial oxidative phosphorylation. Unexpectedly, CCCP elicited a highly significant increase ($+39\%$; $P < 0.001$) in [ATP] over that seen in untreated Cap null sperm, and this was significantly higher ($+21\%$; $P < 0.05$) than that in similarly treated WT sperm (Fig. 5a). Despite the increase in CCCP-induced [ATP] in nulls, the levels remained significantly lower (-27% ; $P < 0.05$) than those in WT.

To ascertain whether the abnormal mitochondrial phenotype reported earlier (Shao et al, 2008) and the excess Ca^{2+} detected in the midpiece (where the mitochondria reside) might compromise mitochondrial function in nulls, we studied their ability to bind Rhodamine 123, an indicator of mitochondrial potential (Tanphaichitr et al., 1984). The staining intensity of the midpieces of the flagella varied from sperm to sperm (Fig. 5b). Microscopic analysis revealed that significantly ($P < 0.001$) more nulls were stained with the dye than WT (Fig. 5e). Further, within the nearly 28% increase in stained nulls there was a subpopulation that exhibited uncharacteristically brighter fluorescence (Fig. 5c). The findings from the microscopic analysis were confirmed and quantified by flow cytometry which showed in nulls undiminished mitochondrial potential that was even higher in a subset of the population (Fig. 5d), confirming microscopic analysis. Despite an overall decrease in ATP levels in nulls, these data indicated uncompromised mitochondrial function and normal potential for oxidative phosphorylation and ATP generation in nulls.

CASK is expressed on sperm flagella and co-localizes with PMCA4b and JAM-A

Because mitochondrial function was undiminished in nulls and there was a lack of difference in the levels and patterns of expression of CATSPER 1 subunit of the influx channel in WT and nulls, we focused on investigating PMCA4b's efflux function in order to decipher the reasons of abnormal Ca^{2+} accumulations in nulls. Since both JAM-A and PMCA4b harbor PDZ binding motifs in their C-termini (Schuh et al, 2003; Martinez-Estrada et al, 2001), we identified PDZ domain-containing CASK (Hata et al, 1996) as a potential candidate that may interact with either or both JAM-A and PMCA4b and participate in the maintenance of Ca^{2+} homeostasis. Indirect immunofluorescence (IF) revealed that all three proteins co-localize on the proximal principal piece, while PMCA4b (Fig. 6a-c) and JAM-A (Fig. 6j,k) are present on the plasma membrane over the acrosome on the head with JAM-A on the midpiece as well (Fig. 6j,k), confirming our earlier findings (Shao et al, 2008). PMCA4b's localization is similar to earlier reports (Wennemuth et al, 2003; Okunade et al, 2004) and is identical for WT (Fig. 6a,b) and *Jam-A* nulls (Fig. 6c) in its distribution and quantity, as assessed by flow cytometry (Fig. 6i). Further, qRT-PCR analysis (Supplementary data) did not show significant differences in the *Pmca4b* mRNA transcript expression between the two groups.

Using CASK monoclonal antibodies, we localized CASK for the first time to the proximal principal piece of the flagellum (Fig. 6e, f). The pattern of its localization indicated maximal

expression proximally, where the staining bordered on the midpiece, but a gradual loss of staining intensity was evident distally. Controls treated with mouse IgG₁ to determine the specificity of the anti-CASK and anti-PMCA4b staining were negative (Fig. 6d, g, h). Contrary to an earlier study using polyclonal antibodies raised against human CASK (Burkin et al, 2004), CASK was not detected on the sperm head in the present study. The difference in these results could be due to several factors. These include the species and protein residues used for the immunogen, polyclonal versus monoclonal nature of the antibodies, and the location of epitopes with respect to the proteolytic cleavage site of CASK during epididymal transit (Burkin et al, 2004). Additionally, the expression of CASK in murine sperm and its localization primarily on the proximal principal piece of the flagellum is conserved in humans (Aravindan and Martin-DeLeon, unpublished), underscoring its evolutionary significance in sperm function.

CASK associates with PMCA4b and JAM-A

Since CASK, PMCA4b and JAM-A co-localize on the mouse sperm membrane over the principal piece, we next investigated whether or not they physically interact. When anti-JAM-A antibody was used to immunoprecipitate JAM-A in protein lysates from WT sperm, and the precipitate probed with anti-CASK antibody in Western analysis, CASK co-immunoprecipitated with JAM-A (Fig. 7a, lane 3). As a control, isotype mouse IgG was used (Fig. 7a, lane 2) instead of anti-JAM-A antibody for immunoprecipitation (IP). The lysate ('Total Proteins', TP) used for IP also showed the presence of CASK which was absent from the IgG control (Fig. 7a). The 50 kDa 'non-specific' band seen in total proteins and the IgG control is heavy chain IgG released during sperm retrieval from mouse caudal epididymal minces and unavoidably detected because the antibody is a mouse monoclonal. In a non-quantitative reciprocal co-IP assay (Fig. 7b) using anti-CASK antibody and subsequent probing with anti-JAM-A antibody in a Western revealed the presence of the 35 kDa JAM-A band in both Uncap (Fig. 7b, lanes 1-3) and Cap (Fig. 7b, lanes 4-6) sperm, confirming that the two proteins mutually associate *in situ*.

Immunoprecipitation of PMCA4b from sperm lysates using anti-PMCA4b antibody followed by Western blotting with anti-CASK antibody showed the presence of the ~60 kDa CASK (Burkin et al, 2004), indicating that these two proteins also physically associate *in situ* (Fig. 7c). An isotype-specific IgG antibody was used as a control. CASK could also be detected in the total protein lysate on Western analysis (Fig. 7c, lane 1). The reciprocal co-IP using anti-CASK antibody and subsequent probing with anti-PMCA4b antibody revealed the presence of the ~130 kDa PMCA4b band for both Uncap and Cap sperm (Fig. 7d, lanes 3 and 5, respectively), confirming the mutual association between these two proteins. With equal amounts of proteins loaded in lanes 3 and 5 (Fig. 7d), more CASK-PMCA4b binding occurs in Uncap than Cap sperm, as densitometry revealed that the band intensity is decreased by approximately one-half in the latter. Our results show for the first time that CASK interacts with both JAM-A and PMCA4b in sperm.

CASK is over-expressed and mis-localized in *Jam-A* null sperm

Based on the evidence for the interactions of CASK with both PMCA4b and JAM-A, we reasoned that the absence of JAM-A in the nulls might affect the expression of its interacting partner. Immunofluorescence, used to analyze the expression pattern and localization of CASK and PMCA4b in *Jam-A* null and WT sperm, showed both sperm types to have the identical pattern of PMCA4b expression (Fig. 6a-c). However, in *Jam-A* null sperm the localization of CASK and its staining intensity indicated considerable over-expression throughout the population (Fig. 8a, b), compared to WT (Fig. 8c, Fig. 6e, f). Unlike WT, in nulls CASK expression on the principal piece of the flagella extends not only both distally and proximally but also encompasses the full length of the midpiece, terminating at the base

of the head. Flow cytometry confirmed the IF results for the elevated levels of CASK in nulls (Fig. 8f), revealing up to ~6-fold increase in expression over WT.

Western analyses of lysates from WT sperm showed the presence of the ~60 kDa CASK in sperm in all samples and in one sample there was an accompanying ~100 kDa isoform (Fig. 8g). The ~60 kDa CASK is the epididymally processed and truncated protein present in sperm and the ~100 kDa unprocessed isoform is reported to be present predominantly in the testis (Burkin et al, 2004). Moreover, lysates from *Jam-A* nulls exhibited an over-expression of the ~60 kDa CASK band (Fig. 8g) quantified to be up to ~6-fold (Fig. 8h), over WT, when normalized to HSC70 (a housekeeping protein), confirming the IF and flow cytometric results. As the PDZ domain in the untruncated ~100 kDa CASK is located at the center of the molecule and because it is required for the binding of JAM-A and PMCA4b which we show here for the ~60 kDa sperm-specific isoform, our results are consistent with PDZ-mediated binding (Funke et al, 2005). Since qRT-PCR (Supplementary data) revealed that *Cask* mRNA is not substantially increased (at least to the level of the protein) in nulls, factors other than transcriptional upregulation account for the over-expression of CASK.

Increased CASK-PMCA4b binding is associated with decreased PMCA4b enzymatic activity

Since in WT proteins a quantitative assay revealed that CASK-PMCA4b interaction is 2-fold greater in Uncap versus Cap sperm (Fig. 7d), we further analyzed it in both WT and nulls. In all three replicate co-IPs using anti-PMCA4 antibodies on proteins from capacitated sperm, the levels of CASK-PMCA4b interaction were elevated in nulls. In a representative gel seen in Fig. 9a (upper panel), there was ~34% increase in the amount of CASK that was immunoprecipitable with PMCA4b antibody in nulls (lane 4), compared to WT (lane 2). The lysate from WT proteins showed the presence of ~60 kDa CASK in the Western blot (lane 1) and an appropriate IgG was used as controls (lanes 3, 5) for the co-IPs. When the membrane in Fig. 9a was re-probed in a Western with anti-PMCA4 antibodies, the elevated CASK-PMCA4b interaction in nulls was confirmed, as the PMCA4b band was increased by 64% in nulls (Fig. 9a, lower panel). The absence of a PMCA4b band for total protein lysate in lane 1, may reflect the low abundance of PMCA [0.1-0.01% of total membrane proteins (Di Leva et al, 2008)] and/or the sensitivity of the antibody.

Reciprocally, when anti-CASK antibodies were used to co-IP PMCA4b in proteins from Cap sperm, the elevation of CASK-PMCA4b interaction in nulls was re-confirmed (Fig. 9b, upper panel). In WT a PMCA4b band could not be detected in the Western, as was the case for the lysate for the total protein (Fig. 9b), although one was seen for nulls. However, a re-probe of the membrane with anti-CASK antibodies showed not only a CASK band that was increased in density in nulls, re-confirming the elevated CASK-PMCA4b interaction, but also a band for WT and the total protein lysate. While together these results conclusively show that when Ca^{2+} levels are high in Cap sperm more PMCA4b is bound to CASK in nulls than in WT, it also shows that at high $[\text{Ca}^{2+}]_c$ the majority of CASK in WT is unbound to PMCA4b, and therefore bound to JAM-A or is free.

As plasma membrane Ca^{2+} -ATPase activity was shown to be higher in caudal Cap versus Uncap bovine sperm proteins (Post et al, 2010) and to be essentially the function of PMCA4 (Di Leva et al, 2008), we analyzed this activity in microsomes from capacitated null and WT sperm. Although *Pmca4b* mRNA (Supplemental data) and protein are essentially unaltered in *Jam-A* null sperm (Fig. 6i), Mg^{2+} -dependent Ca^{2+} -ATPase activity in capacitated null sperm was significantly decreased ($P < 0.01$; Fig. 9c), compared to WT. This indicates that the increase in CASK-PMCA4b binding in nulls has functional consequences with respect to PMCA4b's reduced enzymatic activity and the elevated $[\text{Ca}^{2+}]_c$ levels.

Discussion

In this study we report a novel mechanism by which Ca^{2+} homeostasis is maintained in mammalian sperm: we show here that CASK, identified for the first time on the sperm flagellum, interacts alternately and non-synergistically with JAM-A and PMCA4b via its PDZ domain, and the amount of its binding to PMCA4b is dependent on Ca^{2+} levels and determines the level of PMCA4b's enzymatic and efflux activity. First, using both quantitative and qualitative approaches, we provide direct evidence that *Jam-A* null sperm have increased Ca^{2+} levels not only in the cytosol but also sequestered in the mitochondria. For WT Cap sperm, steady-state $[\text{Ca}^{2+}]_c$ was ~200 nM, within the range previously reported for normal mouse sperm (Schuh et al, 2004; Wennemuth et al, 2003) and other animal cells (Di Leva et al, 2008). In *Jam-A* nulls, $[\text{Ca}^{2+}]_c$ were significantly elevated in both Uncap and Cap sperm (Fig. 1a), compared to WT, attaining levels of ~350 nM when analyzed in Ca^{2+} -containing medium (Fig. 1b).

Elevated Ca^{2+} may result from increased influx or decreased efflux. Although TRPC2 channels in the acrosome are Ca^{2+} permeable, with transient influx that is triggered by ZP3 in capacitated sperm (Jungnickel et al, 2001), an alteration in their expression would not account for the elevated Ca^{2+} levels seen here in the Uncap null sperm. The lack of differences between WT and nulls in the distribution and level of expression of CATSPER1, a subunit of the major Ca^{2+} influx protein associated with hyperactivated motility (Ren et al, 2001), together with our finding that PMCA4b's enzymatic activity is significantly reduced upon increased binding to CASK indicate that the observed accumulation of $[\text{Ca}^{2+}]_i$ in nulls is a consequence of reduced efflux.

PMCA4b is the major efflux molecule in mouse sperm (Wennemuth et al, 2003), being approximately 3-fold more efficient than the Na^+ - Ca^{2+} exchanger (NCX) and the mitochondrial Ca^{2+} uniporter (MCU), which are the other two clearance mechanisms operative in murine sperm where SERCA is nonfunctional (Wennemuth et al, 2003). Moreover the elevated levels of $[\text{Ca}^{2+}]_c$ was shown not to be due to defective clearance by the MCU, as increased amounts of Ca^{2+} were sequestered in the mitochondrial stores of nulls (Fig. 3). That these could be specifically released (Fig. 4) is unequivocal evidence that condensed mitochondrial phenotype indeed reflects Ca^{2+} overload and that the MCU in nulls is operating normally to clear cytosolic Ca^{2+} into the mitochondrial store (Wennemuth et al, 2003). Importantly, since Ca^{2+} stored in the mitochondria is ultimately removed from the cell via PMCA4b's activity (Wennemuth et al., 2003), the chronically elevated Ca^{2+} levels in nulls are consistent with reduced activity of PMCA4b.

Decreased ATP is consistent with reduced motility and elevated Ca^{2+} levels in *Jam-A* nulls

Significantly lower ATP levels were observed in *Jam-A* null sperm and may be directly related to defective motility and decreased PMCA4b's activity in these sperm. Defective motility may result from decreased availability of ATP for dynein-ATPase that is required for motility (Mann and Lutwak-Mann, 1981; Gibbons, 1996; Inaba, 2003). It is known that less ATP is required for progressive motility than for hyperactivated motility (Ho et al, 2002). Thus the more severe effect of the loss of JAM-A on hyperactivated, than on progressive, motility (Shao et al, 2008) is consistent with low ATP levels as a causative factor in the motility defects in *Jam-A* nulls. Interestingly, ATP levels were unexpectedly increased in nulls after CCCP treatment. Since depolarization of mitochondrial membrane potential by CCCP abolishes mitochondrial ATP production (Wennemuth et al, 2003), this increase in [ATP] in nulls, of necessity, must derive from glycolysis which is the primary energy pathway in sperm metabolism (Miki et al, 2004), likely due to the activation of Ca^{2+} -sensitive glycolytic enzymes in response to the observed (Fig. 4) elevated release of Ca^{2+} into the cytosol (Mukai and Okuno, 2004).

Although [ATP] was significantly decreased in *Jam-A* nulls, the mitochondrial membrane potential (Tanphaichitr et al, 1984) remained undiminished, indicating that mitochondrial oxidative phosphorylation was not affected by the large amount of Ca^{2+} sequestered therein, as previously reported (Wennemuth et al, 2003). Since PMCA4b is an ATP-driven pump (Di Leva et al, 2008; Gunaratne et al, 2006), the low [ATP] in *Jam-A* nulls may be responsible for both the abnormal Ca^{2+} clearance and the defective motility. At least two possibilities, singly or in combination, may cause the low steady-state [ATP] observed in nulls, either of which would mandate a critical role for JAM-A in Ca^{2+} clearance and motility in sperm. In neurons CASK acts as a constitutively active CaM kinase which binds ATP for its auto-phosphorylation and phosphorylation of its binding targets (Mukherjee et al, 2008), and this may also be the case in sperm. If so, its elevated levels in *Jam-A* nulls may, at least in part, account for a net deficit in ATP due to over-utilization.

Secondly, decreased ATP in nulls may result from down-regulation of ATP synthesis as an adaptation to reduced activity of ATP-driven PMCA4b. In other words, low ATP levels may be an adaptation to decreased usage due to the observed reduced activity of PMCA4b (Fig. 9c). Supporting evidence for the latter is seen in *CatSper*-deficient sperm which lack Ca^{2+} entry and hyperactivated motility, with a mandatory decrease in Ca^{2+} efflux activity associated with lower ATP levels (Xia et al, 2007). Besides, treatment of mouse and sea urchin sperm with carboxyeosin, which blocks ATP-binding of PMCA4b, is known to increase Ca^{2+} levels and mimic the motility defects seen in *Pmca4b*-deficient sperm (Schuh et al, 2004; Gunaratne et al, 2006). However since CASK is known to bind the transcription factor Tbr1, and the complex is subsequently transported into the nucleus to activate transcription of specific genes (Funke et al, 2005), the possibility that its over-expression in nulls could lead to aberrant expression of a gene(s) involved in ATP production cannot be ruled out.

Co-localization of PMCA4b, JAM-A, and CASK

No significant expression of CASK was detected in the head of the sperm, unlike that reported in an earlier study using polyclonal antibodies raised against human CASK (Burkin et al, 2004). We have used this polyclonal Ab and have been unable to localize CASK on either the sperm head or flagellum (data not shown). Based on this observation and the absence of CASK on the sperm head when its levels are increased 6-fold in *Jam-A* nulls, we conclude that its localization is restricted to the flagellum. Thus the distribution of CASK, PMCA4b and JAM-A on the proximal principal piece of the flagellum is spatially similar. It is significant that all three molecules, as well as CATSPER (Ren et al, 2001; Quill et al, 2001), the major influx channel associated with hyperactivated motility, co-localize on the proximal principal piece, suggesting potential interacting relationships.

It is interesting that both PMCA4b (Fig. 6a-d) and JAM-A (Fig. 6j-m) (Shao et al, 2008) also co-localize in regions where CASK is absent, suggesting that they may perform functions both dependent on, and independent of, the involvement of CASK. It is noteworthy that the plasma membrane over the acrosome where PMCA4b and JAM-A are present, as well as the proximal principal piece, are rich in lipid rafts (Sleight et al, 2005; Travis et al, 2001), similar to that reported in plasma membranes of synaptosomes in cerebellum (Sepulveda et al, 2006) and endothelial cells in the tight junctions of the blood-brain barrier (Dodelet-Devillers et al, 2009) where these molecules have been speculated to participate in coordinating cell signaling events. In addition, JAM-A is also present on the midpiece (Fig. 6j-m) (Shao et al, 2008), where CASK and PMCA4b are absent. Because CATSPER channel-mediated Ca^{2+} entry is known to trigger tail-to-head propagation of Ca^{2+} (Xia et al, 2007), a role for JAM-A independent of both CASK and PMCA4b in facilitating this process in normal sperm, but not in *Jam-A* nulls, remains a possibility requiring further investigation.

CASK is an Interacting partner of both PMCA4b and JAM-A in Sperm

Our studies reveal that in sperm CASK is a common interacting partner of JAM-A and PMCA4b, via a PDZ interaction (Fig. 7). Both PMCA4b and JAM-A were found to co-precipitate using CASK antibody, reciprocally. Although either PMCA4b or JAM-A has been known to interact individually with scaffold proteins via their PDZ domain-binding sequence in other cell types (Martinez-Estrada et al, 2001; Schuh et al, 2003; Ebnet et al, 2004; Di Leva et al, 2008), both of these interactions have not been shown to occur together within the same cell. Our results show for the first time that CASK interacts with both JAM-A and PMCA4b in sperm. CASK is a large (~105 kDa) scaffold protein (Funke et al., 2005), long considered a 'pseudokinase' (Boudeau et al, 2006) until recently (Mukherjee et al, 2008; Kannan and Taylor, 2008), that is processed in sperm to a ~60 kDa isoform (Burkin et al, 2004). It contains a class II PDZ domain (Martinez-Estrada et al, 2001; Schuh et al, 2003; Di Leva et al, 2008), which is known to exhibit varying levels of specificity and a certain degree of promiscuity (Staudinger et al, 1997; Hata et al, 1996; Torres et al, 1998; Straight et al, 2000), and engages both class I and II ligands (Xia et al, 1999; Funke et al, 2005) which are found on the C-termini of PMCA4b (Thr¹²⁰³-Pro¹²⁰⁴-Val¹²⁰⁵) (Funke et al, 2005) and JAM-A (Phe²⁹⁸-Leu²⁹⁹-Val³⁰⁰) (Martinez-Estrada et al, 2001). Thus both proteins share a terminal valine that mediates their binding to the single PDZ site of CASK. Our findings indicate that in sperm CASK functions promiscuously, with respect to its interaction with PMCA4b and JAM-A.

Since both PMCA4b and JAM-A cannot bind to CASK simultaneously or cooperatively, it is imperative that they bind sequentially and non-synergistically. In WT sperm where CASK-PMCA4b interactions were analyzed quantitatively in Cap and Uncap sperm we detected increased interaction in Uncap sperm where Ca^{2+} is low, compared to Cap sperm where it is high (Fig. 7d), indicating that the level of binding depends on the $[\text{Ca}^{2+}]_c$. Thus in WT sperm both CASK-JAM-A and CASK-PMCA4b interactions are required for homeostasis, with CASK-PMCA4b interaction favored at low $[\text{Ca}^{2+}]_c$ (Fig. 7d).

Upregulation of CASK, increased CASK-PMCA4b interaction, and decrease in PMCA4b activity in *Jam-A* nulls

Based on the evidence for the interactions of CASK with both PMCA4b and JAM-A, we reasoned that the absence of one of the proteins might affect the expression of an interacting partner. Thus we analyzed the expression of PMCA4b and CASK in *Jam-A* null sperm and showed the latter to be upregulated. Using three complementary approaches (IF, flow cytometry and Western with densitometric analysis), we show up to a ~6-fold increase of CASK in nulls and its considerable mis-localization on the flagella. The increase in the levels of CASK appears to result from its stabilization since there was no significant increase in transcription of *Cask* mRNA, in the absence of JAM-A in nulls (Supplementary data).

In reciprocal co-IPs (Fig. 9a, b) we re-confirmed the detection of increased CASK-PMCA4b interactions (Fig. 7) in Cap nulls, compared to WT. Whether or not this increased CASK-PMCA4b binding in the absence of CASK-JAM-A binding in nulls results from, or in, the elevated levels of CASK, it indicates that these interactions are competitive and that CASK-JAM-A interaction promotes efflux. Further, in Cap WT sperm the decreased CASK-PMCA4b interaction (Fig. 7d) or its failure of detection (Fig. 9b) suggests that at high $[\text{Ca}^{2+}]_c$ CASK-JAM-A binding predominates, and likely facilitates PMCA4b's efflux activity. Importantly, the increased CASK-PMCA4b interaction that accompanied decreased enzymatic activity of PMCA4b in Cap *Jam-A* null sperm, with the resulting elevation of $[\text{Ca}^{2+}]_c$, suggests that this interaction in WT is associated with cessation of efflux activity.

Thus our findings lead to the conclusion that CASK-PMCA4b interaction physiologically inactivates PMCA4b at low Ca^{2+} to maintain homeostasis.

Although PMCA4b is known to bind a number of Ca^{2+} -dependent proteins and modulate their functions including calcineurin (Holton et al, 2007; Wu et al, 2009) and neuronal nitric oxide synthase (nNOS) (Schuh et al, 2001), this is the first report of its own efflux function being regulated by an interaction with another molecule, CASK. Moreover, unlike those interactions reported for other cells, we report here for the first time that the CASK-PMCA4b interaction occurs in conjunction with CASK-JAMA interaction within the same cell, in tandem, to effect a single physiological function, namely, modulation of PMCA4b efflux activity and maintenance of Ca^{2+} homeostasis.

Unlike the case with other MAGUKs (Funke et al, 2005) that have scaffolding functions exclusively, our results show that CASK is a potent modulator of Ca^{2+} efflux pump in sperm. Despite being long known as a 'pseudokinase' (Caruana, 2002; Montgomery et al, 2004; Hsueh, 2006), the recent finding that the CaM Kinase domain of CASK (Funke et al, 2005) is an active and potent kinase capable of serine-phosphorylation (Mukherjee et al, 2008; Kannan and Taylor, 2008) suggests that novel CASK-mediated mechanisms involving phosphorylation of either or both of these proteins may also play a part in the functional regulation of the efflux pump and Ca^{2+} homeostasis. As both PMCA4b and JAM-A have targets for serine phosphorylation in their C-termini (DeMarco and Strehler, 2001; Schuh et al., 2003; Martinez-Estrada et al., 2001; Funke et al., 2005), they are likely to be phosphorylated by CASK as occurs for at least one target that it recruits, neurexin (Mukherjee et al, 2008). Evidence from our lab indicates that CASK is conserved in human sperm where it not only interacts with JAM-A, but the CASK-bound JAM-A is also phosphorylated (Aravindan and Martin-DeLeon, unpublished), implying a cell-signaling function for JAM-A (Ebnet et al, 2004) in sperm. We predict that PMCA4b may also be similarly phosphorylated following its recruitment by CASK.

Conclusions

We conclude that the sperm motility defects associated with disruption of *Jam-A* (Shao et al, 2008) are accompanied by an ATP deficit and elevated $[\text{Ca}^{2+}]_i$ levels, due to decreased efflux resulting from diminished activity of the major efflux pump, PMCA4b. In sperm we have shown that CASK is an interacting partner of both PMCA4b and JAM-A and that in the absence of JAM-A in nulls CASK-PMCA4b binding is increased, accompanied by elevated levels of CASK. The resulting decreased PMCA4b enzymatic activity indicates that CASK regulates PMCA4b's activity by direct binding. Our results also suggest that JAM-A positively, but indirectly, regulates PMCA4b by sequestering CASK under conditions of elevated Ca^{2+} . The findings here revealing the physiological inactivation of PMCA4b by CASK in sperm are likely to be applicable to other cell types and tissues. As PMCA4b is also the principal Ca^{2+} extrusion molecule in human cells (Enyedi et al, 1996) and since JAM-A (Shao et al, 2008) and CASK (Aravindan and Martin-DeLeon, unpublished) are conserved in their expression and localization in human sperm, our findings suggest similar interactions and mechanism of regulation of Ca^{2+} homeostasis and motility in humans. Thus further studies in this area could have implications for understanding the role of several novel genes involved in human infertility with our now expanded " Ca^{2+} toolkit".

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Jam-A null sperm exhibit chronic overload of cytosolic Ca^{2+} . Spectrofluorometric analyses indicate significant increases in $[\text{Ca}^{2+}]_c$ levels in *Jam-A* null sperm, compared to WT, under all conditions studied: in the absence (a) or presence (b) of Ca^{2+} during Fura-2 loading and analysis as well as following pre-incubation in a Ca^{2+} -enriched capacitation medium (Cap) or in its absence (Uncap). Pre-incubation for 90 min induced Ca^{2+} uptake in both the groups, however nulls have a significant ($P < 0.001$, ANOVA) accumulation not seen in WT. Viabilities of the cells (c) immediately after collection (time 0) and following the de-esterification step prior to measurements showed similar changes among groups in the gradual decline over time. The color-coding on the bars and graphs in (c) applies to all 3 panels.

Figure 2.

CATSPER 1, a subunit of the sperm-specific calcium influx channel, is equally expressed in WT and *Jam-A* null sperm. The immunolocalization of CATSPER 1, green fluorescence on the proximal principal piece (PP) of the flagella, was identical in WT (a, c) and in nulls (b, d) and similar to that described by others⁴. Sperm heads are counterstained with DAPI (c-d). e, f, inverted grayscale images of c and d, respectively, to visualize the integrity and non-fluorescent midpieces and head (H) of the sperm. Western blots of CATSPER 1 in WT and null mouse sperm lysates (g, top panel) indicated that the protein expressions in the two groups were similar when calibrated using the expression of HSC70, a house-keeping protein, as loading control (g, middle panel), and when quantified using densitometry (g, lower panel). This is a representative blot of duplicate trials.

Figure 3.

Confocal images of Fluo-4 (Ca^{2+} indicator) stained sperm reveal elevated levels of Ca^{2+} sequestration in mitochondrial and acrosomal stores in capacitated *Jam-A* nulls, not seen in WT. In the absence of pre-incubation under capacitating conditions, WT (a) and null (b) sperm show the presence of Ca^{2+} in the midpiece (MP) where mitochondria are located, just below the head (H), with far more intense staining in nulls. After incubation under capacitating conditions, Ca^{2+} is seen in the heads of both WT and nulls. However the MP is only minimally stained in WT (c) while in the nulls (d), both the MP and the head are intensely stained with the acrosomes showing distended crescent-shaped acrosomal stores (Ac).

Figure 4.

Differential release of mitochondrial Ca^{2+} by CCCP confirms abnormal sequestration in nulls. Depolarization of mitochondrial membrane potential by the addition of CCCP, after 10 min of Ca^{2+} measurements, released a dramatically elevated amount of Ca^{2+} (500% over baseline) in capacitated nulls, compared to WT ($P < 0.001$; 50% over baseline), reflecting abnormally high levels sequestered in the mitochondria. The high amplitude spikes of $[\text{Ca}^{2+}]_c$ also suggested partial and intermittent clearance of Ca^{2+} by the extrusion pump, PMCA4b, without attaining normal levels in nulls.

Figure 5.

Steady-state ATP levels in *Jam-A* null sperm were significantly reduced, compared to WT, despite their undiminished mitochondrial membrane potential. **a)** ATP levels were significantly ($P<0.0001$) lower in both Uncap and Cap nulls, compared to WT. Depolarization of mitochondrial membrane potential by CCCP resulted in a significant ($P<0.001$) decrease in ATP by abolishing the contribution from mitochondrial oxidative phosphorylation in WT, but in nulls a significant ($P<0.001$) increase was detected (identical asterisks depict significant differences between bars). Rhodamine 123 staining in the midpiece of the flagellum (**b**, **c**) showed variation in both WT and null sperm. When the

fluorescence intensities on WT and null sperm were quantified by flow cytometry (**d**), fluorescence was undiminished in nulls and a subpopulation showed more intense staining than that on WT. Also significantly (* = $P < 0.01$) more nulls had greater fluorescence when sperm were analyzed blindly using light microscopy (**e**).

Figure 6.

Immunofluorescence localizes PMCA4b (**a-d**) and CASK in mouse (**e-h**) sperm primarily on the principal piece of the flagellum. WT sperm showed localization of PMCA4b (red) in the proximal principal piece (PP) as well as in the acrosome (Ac) in some cells (**a,b**), as did those from *Jam-A* nulls (**c**), and differences in the pattern of expression were not discernible between the two groups. 'MP' and 'A' indicate midpiece and annulus, respectively. **d**, IgG control revealed that the staining is specific in a-c. **e-f**, CASK (green) is localized in the PP in a manner identical to that of PMCA4b, the fluorescence tapering off into the proximal region and absent in the midpiece. IgG controls did not show specific staining in the tail (**g**) and a phase contrast of this image is seen in (**h**). Sperm heads are counterstained blue with DAPI. Although the intensity of the fluorescence for PMCA4b in *Jam-A* null sperm in **c**) appeared to less than that in WT, flow cytometric quantitation of the fluorescence in **i** showed virtually no difference (at least 30,000 cells analyzed). **j,k** show JAM-A staining in the proximal principal piece, (arrowed), the midpiece, and over the acrosome in the head. IgG controls are in **d, g**, and **l** showing specificity of the Abs and phase contrast (**h**) and grayscale inverted image (**m**) revealed the integrity of the cells.

Figure 7.

Reciprocal Co-immunoprecipitations (co-IP) of CASK with JAM-A and with PMCA4b from mouse sperm lysates indicate physical association *in situ*. Note that since the CASK Ab used is a mouse monoclonal and since IgG was released in the cauda minces in sperm retrieval, IgG bands are detected in the two Westerns that were probed with CASK Ab (a and c). **a, b**, Reciprocal co-IP of CASK and JAMA. **a**, lane 1: Western blot of CASK in total protein (TP) preparation showed the presence of not only the truncated (~60 kDa) but also a minor full-length (~100 kDa) protein. IP from lysates with either rat IgG (control, lane 2) or rat JAM-A Ab (lane 3) showed the presence of the ~60 kDa CASK only in the latter. **b**, Reciprocally, when Uncap (lanes 2,3) and Cap (lanes 5,6) sperm proteins were immunoprecipitated (IP) with either mouse IgG₁ isotype (lane 2,5) or anti-CASK antibody (lane 3,6) and subsequently probed with JAM-A antibody in a Western blot, the ~35 kDa JAM-A was detected only in lanes 3 and 6. Total protein is seen in lanes 1 and 4. **c, d**, Reciprocal Co-IP of CASK and PMCA4b from sperm lysates. In **c** the ~60 kDa CASK was detectable both in TP (lane 1) and PMCA4b IP proteins using goat PMCA4b Ab (lane 3) but not in goat IgG IP (lane 2). The slower migrating band on the gel is non-specific. In **d** total protein is seen in lane 1 and IgG₁ controls in lanes 2 and 4, while lanes 3 and 5 show protein from Uncap and Cap sperm with identical amounts of proteins loaded, respectively, after co-IP with CASK.

Figure 8.

CASK is over-expressed and mis-localized in *Jam-A* null sperm. Immunofluorescence and confocal microscopy demonstrate that CASK is uniformly elevated with brilliant staining in null sperm throughout the population (**a**). In nulls it is expressed in a manner that extends from the base of the head into the distal end of the tail (**b**), compared to WT (**c**) where it is restricted to the proximal principal piece (PP). The annulus (A) and midpiece (MP) are marked. **d**, **e**, Null sperm treated with IgG (control) and imaged with phase contrast, respectively. **f**, flow cytometry showed a six-fold over-expression of CASK in the majority of nulls, compared to WT; mouse IgG₁ isotype was used as a non-specific control for WT. At least 30,000 sperm from each sample were analyzed. Western blot analysis (**g**) revealed confirmation of an ~ 6-fold over-expression of CASK in nulls (lanes 3, 4) compared to WT (lanes 1, 2) when quantified using densitometry (**h**). Lower panels, loading controls, show an irrelevant mouse sperm membrane protein, SPAM1, and the housekeeping HSC-70 protein, both detected after stripping CASK and re-probing the membrane.

Figure 9.

Increased PMCA4b-CASK binding is associated with reduced activity of PMCA4b in *Jam-A* nulls. **a**) Total lysates used for IP (TP, lane 1) (40 μg proteins) show CASK. Co-IP of 125 μg proteins in the lysate with anti-PMCA4 Ab (Ln 2, 4) or IgG (Ln 3,5) show a 34% increase of CASK binding in nulls over WT. Re-probing of the membrane shows a 64% increase in PMCA4b associated with the increased binding of CASK in nulls. **b**) Reciprocal Co-IP of 125 μg lysate with anti-CASK antibodies reveals increased CASK-PMCA4b interaction in nulls (Ln 4) compared to WT (Ln 2) where a PMCA4b band is not seen, as is the case in the IgG controls (Ln 3,5) and the TP (Ln 1) (50 μg) in the upper panel. However, re-probing of the membrane not only confirmed the elevated CASK-PMCA4b interaction (lower panel), but also shows the presence of CASK in TP and the WT co-IP where it was ~11% less than in nulls. **c**) Mg^{2+} -dependent Ca^{2+} -ATPase assays of microsomes from Cap sperm reveal that PMCA4b's enzymatic activity is significantly ($P < 0.01$) reduced in nulls.