Calcium and Ca\(^{2+}\)-dependent signals play a crucial role in sperm motility and mammalian fertilization, but the molecules and mechanisms underlying these Ca\(^{2+}\)-dependent pathways are incompletely understood. Here we show that homozygous male mice with a targeted gene deletion of isoform 4 of the plasma membrane calcium/calmodulin-dependent calcium ATPase (PMCA), which is highly enriched in the sperm tail, are infertile due to severely impaired sperm motility. Furthermore, the PMCA inhibitor 5-(and-6)-carboxy eosin diacetate succinimidyl ester reduced sperm motility in wild-type animals, thus mimicking the effects of PMCA4 deficiency on sperm motility and supporting the hypothesis of a pivotal role of the PMCA4 on the regulation of sperm function and intracellular Ca\(^{2+}\) levels.

Successful fertilization requires the sperm to travel long distances and undergo capacitation prior to reaching the female egg. After reaching their target, the sperm must interact with the extracellular matrix of the egg, including proteins of the zona pellucida, and release acrosomal material. Calcium is considered to exert a function on most, if not all, of these processes. In this field, most of the work on Ca\(^{2+}\)-dependent pathways is incompletely understood. Here we show that homozygous male mice with a targeted gene deletion of isoform 4 of the plasma membrane calcium/calmodulin-dependent calcium ATPase (PMCA), which is highly enriched in the sperm tail, are infertile due to severely impaired sperm motility. Furthermore, the PMCA inhibitor 5-(and-6)-carboxy eosin diacetate succinimidyl ester reduced sperm motility in wild-type animals, thus mimicking the effects of PMCA4 deficiency on sperm motility and supporting the hypothesis of a pivotal role of the PMCA4 on the regulation of sperm function and intracellular Ca\(^{2+}\) levels.

The abbreviations used are: PMCA, plasma membrane calcium/calmodulin-dependent calcium ATPase; CE, 5-(and-6)-carboxy eosin diacetate succinimidyl ester; RT, reverse transcriptase.
Fig. 1. Sequence comparison of mouse PMCA4 with human and rat PMCA4. A, sequence comparison of PMCA4b splice variants of mouse, human, and rat revealed high homology (boxed) of the entire corresponding proteins as well as conservation of the C-terminal PDZ domain binding motif. B, sequence comparison of C termini (c term) of mouse, human, and rat PMCA4b splice variants demonstrating homology of PMCA4a variants.
Experimental Procedures

Cloning of Mouse PMCA4 Isoforms—Mouse PMCA4 isoforms were cloned by rapid amplification of cDNA ends from a mouse testis Marathon cDNA library (catalog number 7455-1, Clontech) using the Advantage® 2 PCR system (Clontech) and the following gene-specific primers: mouse PMCA4 forward, 5′-GTC TGA TCA TGT CTT TCC TCA CAG TTG-3′, and mouse PMCA4 reverse, 5′-GCC GGC CCT CTC GCA GAC CCA CT-3′. Polymerase chain reaction was performed as suggested by the manufacturer, and the resulting PCR fragments were cloned into pCR-XL-TOPO vector (Invitrogen) and subsequently sequenced with the standard T7 and M13 reverse primers. The resulting sequences were analyzed and aligned with DNAMAN 4.0 software (Lynnon BioSoft), and the transmembrane helices were predicted with HMMTOP Version 2.0 at www.enzim.hu/humtop (25). Mouse PMCA4b and -4a sequences have been deposited in GenBank® and the accession numbers are AY560885 and AY560886, respectively.

Northern Blotting and Immunofluorescence Stainings—To determine the expression pattern of PMCA4 in mouse testes, we hybridized the MessageMap™ Northern blot (each lane containing 2 μg of poly(A)+ RNA, Stratagene, catalog number 716900) with the 32P-dATP-labeled full-length PMCA4b cDNA according to the manufacturer’s protocol and reprobed the subsequently stripped membrane with a 32p-dATP-labeled β-actin probe. The subcellular distribution of PMCA4b was determined by immunofluorescence stainings. Isolated sperm were allowed to swim out from cauda epididymidis in sperm preparation buffer (MediCult, catalog number 10680060) for 15 min, and the suspension was streaked out on poly-lysine-coated slides, air-dried for 10 min, and fixed and stained as described previously (21). The PMCA4-specific polyclonal antisem was also described previously (26).

Generation of PMCA4-deficient Mice—A 12-kb genomic DNA fragment containing exons 2–4 of the mouse PMCA4 gene was isolated from a 129Sv mouse genomic library in the xFix™II vector (Stratagene). The targeting vector was generated by inserting the homologous BglII/KpnI and BspMII/NotI fragments into the pPNT vector (27). The targeting vector was electroporated into E11.1 embryonic stem cells. Neomycin- and ganciclovir-resistant clones (500 μg/ml G418 and 2 μM ganciclovir) were screened for homologous recombination by Southern blot using the external BamHI/BglII fragment of the mouse PMCA4 gene as a probe (Fig. 3A). This probe hybridizes to two 0.7-kb BglII fragment (endoogenous pmca4 allele) and a 3.3-kb fragment (disrupted allele). Embryonic stem cells that had undergone homologous recombination were injected into C57Bl/6 blastocysts, and the manipulated blastocysts were transferred into pseudo-pregnant foster females to test for germ line transmission of the targeted PMCA4 allele. PMCA4-deficient mice were obtained by appropriate inbreeding of heterozygous offspring and subsequent genotyping.

RT-PCR and Western Blotting—The absence of PMCA4 mRNA in PMCA4-deficient mice was tested by RT-PCR using the OneStep RT-PCR kit (Qiagen) and the following PMCA4-specific (not species-specific) primers: PMCA4 forward, 5′-CTG AGG AGG CTC ATGGAG C-3′, and PMCA4 reverse, 5′-CCG AAG GGC TGC TCT CTT C-3′. To test for the absence of PMCA4 at the protein level in sperm, isolated sperm from one cauda epididymis were collected by centrifugation and boiled for 5 min in 200 μl of Laemml sample buffer (Bio-Rad). 20 μl of each lysate was separated on a 10% SDS-PAGE, blotted onto a nitrocellulose membrane (Schleicher & Schuell), and the membranes were cut at the level of the 75-kDa protein marker band. The upper half was tested for PMCA4 expression using the PMCA4-specific monoclonal antibody JA9 (28) (NeoMarkers, 1:500 dilution), and the lower half was probed for actin (polyclonal goat anti-actin, Santa Cruz Biotechnology, catalog number sc-1616, 1:5000 dilution) to check for equal loading of the samples. Blocking and antibody incubations were done with 5% milk in phosphate-buffered saline (PBS) for 20 min. Signals were detected with specific horseradish peroxidase-labeled secondary antibodies and the ECL detection system (Amersham Biosciences).

Tissue Sections of Testis and Histological Stainings—Tissue sections were frozen and prepared as described previously (21) and stained with hematoxylin and eosin using a standard protocol. Cytological staining of mouse sperm was carried out using Spermastain™ (Stain Enterprises Inc.) according to the manufacturer’s instructions.

In Vivo Fertilization—Sperm were collected from cauda epididymis in sperm preparation buffer (MediCult) and capacitated in vitro for 2 h at 37 °C. Oocytes were prepared from C57Bl/6 females that had been synchronized with 10 units of pregnant mare serum gonadotropin (Sigma) and 10 units of human chorionic gonadotropin (Sigma) 48 and 14 h prior to oocyte collection. Eggs were flushed from oviducts in M2 medium (Sigma) and cultivated in M16 medium (Sigma) in 5% CO2 at 37 °C. In vitro fertilization capacity was tested as described previously (29). In brief, eggs were incubated with ~105 wild-type or ~105 PMCA4-deficient sperm for 24 h at 37 °C, and eggs that had divided to the two-cell stage were counted as indicative of successful fertilization.

Estimation of Sperm Motility and Measurement of Intracellular Calcium—Sperm were collected from cauda epididymis as described above. The supernatant containing sperm was decanted into a fresh tube, and the cells were left untreated at 37 °C for 30 min or loaded with the PMCA4 inhibitor (5- and 6-carboxyxyanide, diacetate succinimidyl ester (10 μM, Molecular Probes).

Overall sperm motility was estimated using the medeaLAB CASA 4.2 system (Erlangen) optimized for mouse sperm. Morphology and tracking threshold upper levels were set to: red 255, green 90, and blue 255, respectively. Additionally, all possible form parameters were de-activated (e.g. flagellum detection, color, and others), and area and form factor filters were kept unchanged. In addition to the classification of sperm motility, average path velocity, progressive velocity, and track speed were calculated with statistical analysis of raw motility data. To estimate intracellular Ca2+ levels in capacitated sperm, they were prepared in sperm preparation buffer (MediCult) as described above, capacitated for 30 min, and loaded with 10 μM Fluo-4-AM for 30 min at 37 °C. The cells were subsequently washed, counted (Coulter counter), and resuspended at a concentration of 1 × 106 cells/ml in sperm prep.
aration buffer supplemented with 2 mM CaCl₂. Changes in fluorescence were recorded with an excitation wavelength of 494 nm and emission wavelength of 516 nm with a PerkinElmer Life Sciences LS 50 B fluorescence photometer using the time drive protocol of the FL Winlab 2.00 software. Intracellular Ca²⁺ levels after the capacitation period were calculated based on a given dissociation constant \( K_D \) (Ca²⁺/H₁₁₀₀₁). For calculation of intracellular calcium levels, maximum fluorescence was induced with 50 \( \mu \)M calcium ionophore A₂₁₈₇, and minimal fluorescence was estimated in the presence of 2 mM EDTA in sperm preparation buffer.

RESULTS AND DISCUSSION

Full-length cDNA of mouse PMCA4b splice variant and the C terminus of mouse PMCA4a variant were cloned from a testis cDNA library by rapid amplification of cDNA ends (5' and 3' rapid amplification of cDNA ends). Comparison of predicted protein sequences with human (30) and rat (31) PMCA4b and -4a revealed a high degree of homology (Fig. 1). Mouse PMCA4b contains 10 predicted transmembrane domains, forming a pore in the plasma membrane, which is a typical feature of PMCs. The C terminus of mouse PMCA4b harbors a typical PDZ domain binding motif (amino acid sequence: ... ETPV), most presumably mediating specificity of binding to certain PDZ domains, as shown previously for other human PMCA b splice variants (17–21, 32).

However, the molecular function of this C-terminal PMCA4a motif remains unclear. Although previously shown to be expressed in several organs (14), multiple tissue Northern blot analysis revealed prominent expression of the PMCA4 messenger RNA in mouse PMCA4-deficient mice. The upper panel demonstrates lack of PMCA4 protein in isolated sperm, and in the lower panel, equal loading of proteins was demonstrated by detection of actin in the sperm lysates.

A polyclonal antibody directed against the N-terminal part of PMCA4 and cross-reactive with mouse PMCA4 (26) was used to determine subcellular localization of PMCA4 protein in wild-type mouse sperm. The protein was expressed in the principal piece of the sperm tail, the flagellar apparatus propelling the spermatozoon forward, and to a lesser extent to the sickle-shaped mouse sperm acrosome region (Fig. 2B). Interestingly, the recently described sperm-specific calcium channel CatSper was also localized in the principal region of the tail, and its gene deletion leads to severely reduced sperm motility and male infertility, thus underlining the important role of Ca²⁺ signaling in sperm motility (5).

To study the physiological function of PMCA4 in vivo, we disrupted the PMCA4 gene in embryonic stem cells by homologous recombination (Fig. 3A). The second exon and part of the third exon were replaced by the neomycin resistance cassette. Following homologous recombination, embryonic stem cells were injected into blastocysts and implanted into pseudo-preg-
PMCA4 Is Required for Sperm Motility and Male Fertility

Fig. 4. Morphology and function of PMCA4−/− testis and PMCA4-deficient sperm. A, hematoxylin/eosin staining of testis sections did not show histological differences between PMCA4−/− and wild-type (WT) littermate controls. KO, knock-out. B, no obvious cytological differences were observed in bright field microscopy of previously stained, isolated sperm (∼63 objective, oil immersion, SpermacStain® staining). C, no significant differences in in vitro fertilization capacity of wild-type (upper panel) and PMCA4-deficient sperm (middle panel) were observed. PMCA4-deficient sperm were able to attach to empty zona pellucida, suggesting normal acrosome reaction (lower panel). D, example of one recording of change in intracellular calcium in response to 50 μM Ca2+ ionophore A23187. PMCA4-deficient sperm (red) showed an elevated resting intracellular Ca2+ concentration [Ca2+]i and approximately the same maximal Ca2+ concentration in response to ionophore A23187 as the wild-type control sperm (blue). To estimate intracellular calcium, similar recordings in the presence of EDTA have been made (not shown), and the intracellular free calcium levels have been calculated. Time in seconds, relative intensity = relative intensity of fluorescence.

A closer microscopical examination of testes and sperm revealed no histological differences in testes architecture and no morphological differences in sperm of PMCA4−/− mice and their wild-type littermates (Fig. 4, A and B). In vitro fertilization assays were performed to test the ability of PMCA4-deficient sperm to fertilize eggs. 38% (10 of 24) of eggs incubated with capacitated wild-type sperm and 35% (11 of 30) of eggs incubated with PMCA4-deficient sperm reached the two-cell stage after 24 h (example in Fig. 4C). PMCA4-deficient sperm were also able to bind to empty zona pellucida, suggesting that these sperm undergo the normal acrosome reaction (example in Fig. 4C). To gain a first insight into the regulation of intracellular calcium of PMCA4-deficient sperm after capacitation, we have estimated the intracellular calcium after preparation and 60-min capacitation of sperm from cauda epididymides from PMCA4-deficient mice in comparison with sperm of their wild-type littermates. Assuming a Kd(Ca2+) of 345 nM for Fluo4, the average intracellular calcium concentration of wild-type sperm was found to be 157 nM, and the intracellular calcium in knockout sperm was 370 nM (example of one recording given in Fig. 4D, in total n = 15, p < 0.05). This underlines the previously suggested pivotal role of the PMCA in the regulation of basal calcium levels and calcium clearance in sperm (33), but detailed analyses of the regulation of intracellular free calcium in different compartments of sperm have to be made to understand the functions of the PMCA4 in the fertilization process.

As a consequence of the genetic manipulation, an obvious difference in the motility of mutant and wild-type sperm was observed. Sperm were classified by standard clinical tests for sperm motility disorders with a computer-aided sperm analysis system; a large number of PMCA4-deficient sperm were immotile (68%), displayed extremely low directed progressive motility (14%), or showed no directed movement (18%) as compared with sperm of wild-type littermates (7% immotile, 73% progressive motility, 20% no directed motility, examples of recordings in Fig. 5, A and B). Analysis of main motility parameters showed that average path velocity, progressive velocity, and track speed were severely impaired in PMCA4-deficient mice (Fig. 5D).

If deletion of PMCA4 leads to strongly impaired sperm motility, it should conceptually be possible to mimic this effect by...
using the cell-permeable PMCA inhibitor 5-(and-6)-carboxyeosin diacetate succinimidyl ester (CE). 10 μM CE severely reduced the motility of wild-type sperm (36% immotile, 30% progressive motility, 34% no directed motility, examples of recordings in Fig. 5C), providing additional evidence for the importance of PMCA activity in the regulation of sperm motility. CE in the concentration range of 10–20 μM has been shown previously to be an effective, specific inhibitor of the plasma membrane calcium pump (34–36).

In conclusion, we describe a highly specific form of male infertility in animals with a deletion of the PMCA4 gene. The latter is crucial to sperm motility but not to in vitro fertilization capacity. The PMCA inhibitor 5-(and-6)-carboxyeosin diacetate succinimidyl ester mimics the effect of gene deletion on sperm motility. Therefore, it may act as an ideal lead compound for the development of contraceptive drugs and has a number of chemically active side groups that can potentially be used for modification, for example to enhance accumulation in the testis or seminal vesicles and reduce potential side effects. Little is known about the toxicity of 5-(and-6)-carboxyeosin diacetate succinimidyl ester, but the parent compounds eosin and fluorescein are extensively used as a colorant in cosmetics and for diagnostic purposes, respectively, and therefore, they represent a promising class of lead compounds for further contraceptive drug development. Conceptually, our findings may open the way to a novel form of non-hormonal contraception. Furthermore, our findings make PMCA4 a candidate gene for the analysis of genetic and environmental causes of male infertility, an increasing problem globally (37, 38).

Acknowledgments—We are grateful to Ulrich Walter for generous support and Ricardo Benavente for helpful hints, to Elvira Rohde for injection of embryonic stem cells, and to Heidi Runknagel and Nicola Roethlein for excellent technical assistance.

REFERENCES
PMCA4 Is Required for Sperm Motility and Male Fertility