Targeted Ablation of Plasma Membrane Ca\textsuperscript{2+}-ATPase (PMCA) 1 and 4 Indicates a Major Housekeeping Function for PMCA1 and a Critical Role in Hyperactivated Sperm Motility and Male Fertility for PMCA4*

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The relative importance of plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) 1 and PMCA4 was assessed in mice carrying null mutations in their genes (\textit{Atp2b1} and \textit{Atp2b4}). Loss of both copies of the gene encoding PMCA1 caused embryolethality, whereas heterozygous mutants had no overt disease phenotype. Despite widespread and abundant expression of PMCA4, PMCA4 null (\textit{Pmca4} \textsuperscript{-/-}) mutants exhibited no embryolethality and appeared outwardly normal. Loss of PMCA4 impaired phasic contractions and caused apoptosis in portal vein smooth muscle \textit{in vitro}; however, this phenotype was dependent on the mouse strain being employed. \textit{Pmca4} \textsuperscript{-/-} mice on a Black Swiss background did not exhibit the phenotype unless they also carried a null mutation in one copy of the \textit{Pmca1} gene. \textit{Pmca4} \textsuperscript{-/-} male mice were infertile but had normal spermatogenesis and mating behavior. \textit{Pmca4} \textsuperscript{-/-} sperm that had not undergone capacitation exhibited normal motility but could not achieve hyperactivated motility needed to traverse the female genital tract. Ultrastructure of the motility apparatus in \textit{Pmca4} \textsuperscript{-/-} sperm tails was normal, but an increased incidence of mitochondrial condensation indicated Ca\textsuperscript{2+} overload. Immunoblotting and immunohistochemistry showed that PMCA4 is the most abundant isoform in testis and sperm and that it is localized to the principle piece of the sperm tail, which is also the location of the major Ca\textsuperscript{2+} channel (CatSper) required for sperm motility. These results are consistent with an essential housekeeping or developmental function for PMCA1, but not PMCA4, and show that PMCA4 expression in the principle piece of the sperm tail is essential for hyperactivated motility and male fertility.

Plasma membrane Ca\textsuperscript{2+}-ATPases (PMCA)\textsuperscript{1} are highly regulated Ca\textsuperscript{2+} extrusion pumps that provide fine-tuning of intra-

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† The abbreviations used are: PMCA1 and PMCA4, plasma membrane Ca\textsuperscript{2+} ATPase isoforms 1 and 4 (\textit{Pmca1} and \textit{Pmca4} refer to the genes for each isoform); \textit{neo}\textsuperscript{R}, neomycin resistance; ES cells, embryonic stem cells; RT, reverse transcriptase; \textit{Pmca4} \textsuperscript{+/+} and \textit{Pmca1} \textsuperscript{+/+}, PMCA1 wild-type and heterozygous mutant mice, respectively; \textit{Pmca4} \textsuperscript{-/-}, cellular Ca\textsuperscript{2+} levels (1, 2). In mammals, they comprise a family of four distinct isoforms, each encoded by a separate gene, with PMCA1 and PMCA4 being expressed in most if not all adult tissues, and PMCA2 and PMCA3 having a very limited tissue distribution (3–7). Tissue- and developmental stage-specific alternative splicing of exons encoding two different regulatory domains leads to additional diversity, with the potential for as many as 32 protein variants (8, 9). Given the importance of cellular Ca\textsuperscript{2+} homeostasis and signaling, it seems likely that PMCA activity is necessary for both tissue-specific physiological functions and the “housekeeping” function of maintaining intracellular Ca\textsuperscript{2+} in a range compatible with cell viability.

PMCA2 and PMCA3, which are invariably coexpressed with another isoform, are likely to serve tissue-specific functions. In fact, studies of \textit{Pmca2} null mutants have shown that ablation of this isoform leads to profound hearing and balance problems (10–12) due to the loss of PMCA activity in stereocilia of sensory hair cells of the inner ear.

The widespread distribution of PMCA1 and PMCA4 in adult tissues has led to the suggestion that these isoforms might play critical housekeeping functions (5, 13). If this were the case, then a null mutation in either isoform could cause a loss of viability of one or more cell types either in the embryo, with consequent embryolethality or structural malformations, or after birth. Alternatively, the loss of one or the other of these isoforms might be tolerated if the other isoform could provide sufficient compensation for its absence or if it served an entirely tissue-specific rather than a housekeeping function. In the latter case, one might expect to observe a physiological deficit, but not cell death. To begin assessing the relative importance of PMCA1 and PMCA4 with respect to both housekeeping and organ-specific functions, and to determine whether there is any redundancy in their functions, we developed and analyzed mice with null mutations in their genes.

The results suggest that PMCA1, but not PMCA4, is an essential housekeeping isoform; however, the data also showed that there were tissues in which PMCA4 was the more abundant isoform and that its absence, under some conditions, led to apoptosis. Another group recently reported that the loss of PMCA4 causes a sperm motility defect and male infertility (14). The current experiments confirm and extend those findings. Our data show that PMCA4 is the major isoform in sperm, that it is expressed specifically in the principle piece of the...
sperm tail, and that it is required for hyperactivated, but not non-hyperactivated, sperm motility. Interestingly, the Ca\(^{2+}\) channel that controls Ca\(^{2+}\) influx into the sperm tail is also restricted to the principle piece (15). Thus, the major Ca\(^{2+}\) influx and efflux mechanisms regulating Ca\(^{2+}\) in the sperm tail and hyperactivated motility are both located in the principle piece of the sperm tail.

**EXPERIMENTAL PROCEDURES**

**Preparation of Mutant Mice and Genotype Analysis—**Mice Pmca1 and Pmca4 genomic sequences from a 129/SvJ library were used to prepare targeting constructs. The PMKK vector (16) contained both the 5′ and 3′ homologous regions of the ES cells obtained. The 152-bp fragment derived from 129/SvJ strain mice, the initial mice analyzed were of a mixed 129/SvJ and Black Swiss background. For later studies the mice were bred onto the Black Swiss background. DNA from ES cells and tail biopsies were analyzed by Southern hybridization using Pmca genomic probes. For Pmca1, we used a 198-bp 3′ outside probe from exon 12 (codons 622–888) and a 196-bp 5′ inside probe from exon 9 (codons 380–445). For Pmca4, we used a 1.6-kb 5′ outside probe extending from codon 304 in exon 8 to codon 320 in exon 9 and a 1 kb 3′ inside probe extending from codon 670 in exon 13 to codon 708 in exon 14.

Electrophoresis and analysis of ES cells, blastocyst-mediated transgenesis, and breeding of male chimeras with Black Swiss mice, were performed as described previously (16). Because the ES cells obtained were derived from 129/SvJ strain mice, the initial mice analyzed were of a mixed 129/SvJ and Black Swiss background. For later studies the mice were bred onto the Black Swiss background. DNA from ES cells and tail biopsies were analyzed by Southern hybridization using Pmca genomic probes. For Pmca1, we used a 198-bp 3′ outside probe from exon 12 (codons 622–888) and a 196-bp 5′ inside probe from exon 9 (codons 380–445). For Pmca4, we used a 1.6-kb 5′ outside probe extending from codon 304 in exon 8 to codon 320 in exon 9 and a 1 kb 3′ inside probe extending from codon 670 in exon 13 to codon 708 in exon 14.

Genotyping of mice was performed by blot hybridization of tail (or spleen) DNA or by PCR using a combination of three primers that simultaneously amplify wild-type and mutant alleles. For Pmca1 genotyping, forward, (5′-GTCAGACACCTGAGCAGCCGC-3′) and reverse (5′-TCCCGGCTACAGAGGAGGACG-3′) primers, corresponding to sequences from the 5′ and 3′ ends of exon 10, respectively, amplified a 183-bp product (codons 457–517) from the wild-type allele. For the mutant allele, a reverse primer complementary to sequences in the phosphoglycerate kinase promoter (5′-GCATGGTCAGCTCCCTTGTTTGT-3′) and reverse (5′-GACGCCCGTATGGGCAGGAGAAG-3′) primers, derived from the 3′ and 5′ ends of intron 10 and intron 11, respectively, amplified a 335-bp product (codons 634–969). The apoptotic index was calculated by dividing the number of such cells by the total number of microns digitized times 100. Because the muscle thickness did not vary among groups, this provided a measure of smooth muscle cell death. Portal veins were also assessed by electron microscopy to confirm that the observed cell death was due to apoptosis rather than necrosis.

**Sperm Motility Assays—**As a preliminary assessment of sperm motility, female mice were mated with Pmca4+/− and Pmca4+/− male mice (n = 3 for each genotype). Oviducts were excised 4 h after copulation and examined for the presence of motile sperm and fertilized eggs. Sperm were isolated from epididymides of 8- to 12-week-old mice, were bred onto the Black Swiss background, and 18 were of the Black Swiss background. Portal veins were collected under varying conditions of euthanasia or anesthesia (CO2 and Avertin), fixation (perfusion, immersion), and after force measurements were conducted. The cell death response was measured by digitizing the length of the portal vein endothelium and counting the number of dense nuclei (those with highly condensed chromatin) in the underlying smooth muscle. The area of the cell was calculated by dividing the number of such cells by the total number of microns digitized times 100. Because the muscle thickness did not vary among groups, this provided a measure of smooth muscle cell death.

**RT-PCR Analysis of Mutant and Wild-type PMCA1 and PMCA4 mRNAs—**First strand cDNA was prepared from total RNA of mutant or wild type brain, kidney, stomach, and lung using oligo(dT) primers and reverse transcriptase. For Pmca1, the PCR primers corresponded to codons 380–388 from exon 9 and the reverse complement of codons 588–595 from exon 12. For Pmca4, the PCR primers corresponded to codons 322–330 from exon 9 and the reverse complement of codons 588–595 from exon 12. The PCR fragments were separated on a 5% agarose gel, visualized with ethidium bromide, and then subcloned and sequenced.

**Histological, Morphometric, and Ultrastructural Analysis of Portal Vein—**Light microscopy, cross-sections of portal veins were stained with toluidine blue or a trichrome stain (azure II, methylene blue, or basic fuchs). Sections for electron microscopy were stained with uranyl acetate and lead citrate. Portal veins were assessed in 20 Pmca4+/− and 19 Pmca4+/− mice. Twenty-one of the mice were of the original mixed 129/SvJ and Black Swiss background, and 18 were of the Black Swiss background. Portal veins were collected under varying conditions of euthanasia or anesthesia (CO2 and Avertin), fixation (perfusion, immersion), and after force measurements were conducted. The cell death response was measured by digitizing the length of the portal vein endothelium and counting the number of dense nuclei (those with highly condensed chromatin) in the underlying smooth muscle. The area of the cell was calculated by dividing the number of such cells by the total number of microns digitized times 100. Because the muscle thickness did not vary among groups, this provided a measure of smooth muscle cell death. Portal veins were also assessed by electron microscopy to confirm that the observed cell death was due to apoptosis rather than necrosis.

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**Sperm Ultrastructure—**After 90 min, the sperm from the hyperactivation assay were collected by centrifugation, resuspended in fixative, and prepared for electron microscopy as described above. The ratio of...
Orthodox and condensed mitochondria was determined by counting 346 and 566 mitochondrial profiles in the midpiece of the sperm tail in samples from Pmca4+/− and Pmca4−/− mice, respectively.

**Immunoblot and Immunohistochemical Analysis of Pmca4 in Sperm**—For immunoblot analysis, proteins in homogenates of both testes and sperm, each from three pairs of 10- to 12-week-old Pmca4+/+ and Pmca4−/− mice, were separated by reducing 7% polyacrylamide gel electrophoresis and transferred to nitrocellulose. Relative PMCA levels were determined using the 5F10 monoclonal antibody (Affinity Bioreagents) that recognizes all PMCA isoforms (21). For immunohistochemistry, sperm were placed on slides and allowed to air dry for 30 min. The tissues of testes and sperm, each from three pairs of 10- to 12-week-old Pmca4+/+ and Pmca4−/− mice, were fixed overnight in phosphate-buffered formalin at 4 °C, and when the resulting chimeric male mice were bred with Black Swiss females, germ line transmission of the mutant allele was achieved (Fig. 1B). Mating of heterozygous breeding pairs yielded live wild-type and heterozygous pups, in a 1:2 ratio, as shown by Southern blot (Fig. 1B, right panel) and PCR analysis (Fig. 1C) of genomic DNA.

Northern blot analysis revealed no differences in the apparent levels of the 5.5- and 7.5-kb PMCA1 mRNAs in various tissues of Pmca1+/− and wild-type mice (Fig. 1D), suggesting that the wild-type allele was up-regulated or that an mRNA lacking exon 10 might be expressed from the mutant allele. Such a small difference in size would not be detected by blot hybridization. RT-PCR analysis of total RNA from wild-type and Pmca1+/− mice confirmed that the donor site of exon 9 was spliced to the acceptor site of exon 11, thereby producing an RNA that lacked codons for amino acids 449–529 (Fig. 1E), which are essential for pump activity.

**Generation of Mice with a Null Mutation in the Pmca4 Gene**—A similar strategy was used for disruption of the Pmca4 gene (Fig. 2A). The region of exon 11 encoding amino acids 448–474, which includes the catalytic phosphorylation site (aspartic acid 465), was replaced with the neoβ gene. Chimeric males were bred with Black Swiss females, and germ line transmission was achieved (Fig. 2B). Mating of heterozygous mice yielded live wild-type, heterozygous, and homozygous mutant offspring (Fig. 2C) in a normal 1:2:1 Mendelian ratio. There was no apparent reduction in the levels of PMCA4 mRNA in Pmca4−/− tissues, nor was there a detectable difference in the size of the ~8.5-kb mRNA (Fig. 2D), suggesting that exon 11 might have been deleted during processing of the mRNA. RT-PCR analysis confirmed that exon 10 was spliced to exon 12, thereby deleting codons 439–519 (Fig. 2E).

There was no evidence of structural malformations or growth retardation among Pmca4−/− mice in utero. Breeding of ho-
mozygous mutants of each sex with wild-type mice showed that female Pmca4−/− mice were fertile; however, male Pmca4−/− mice of both the original mixed 129/SvJ and Black Swiss background and the pure Black Swiss background used for later studies were infertile. When the mutation was carried on the original mixed 129/SvJ and Black Swiss background, there was a significant incidence of adult Pmca4−/− mice with skin around the jaw that was raw from scratching. This phenotype correlated with enlarged submandibular lymph nodes. Like the apoptotic phenotype described below, however, this characteristic disappeared as the mice were bred onto the Black Swiss background. Otherwise, Pmca4−/− pups and adults of both sexes were indistinguishable from wild-type littermates in both appearance and behavior and appeared normal with respect to survival and growth. Analysis of tissues taken immediately after euthanasia revealed no histopathological evidence that the loss of PMCA4 causes cell death, as might have occurred if the corresponding allele. The locations of the diagnostic probes are indicated below the targeted allele. B, Southern blot analysis of ES cell DNA and tail DNA from offspring of Pmca4+/− matings using the indicated enzymes and probes. C, PCR genotyping revealed offspring of all three genotypes. D, RNA (15 μg/sample) was hybridized with a PMCA4 probe and a GAPDH probe (as a loading control). E, RT-PCR analysis of Pmca4+/− and Pmca4−/− mRNAs using primers from exons 9 and 12 (left). DNA sequence analysis revealed a 243-bp difference in size due to excision of exon 11 during splicing of the mutant mRNA (right).

Relative Levels of PMCA1 and PMCA4 mRNAs in Mouse Tissues—It seemed possible that the loss of PMCA1 null mutants during embryogenesis and the relatively normal appearance of PMCA4 null mutants even through adulthood could reflect major differences in expression levels of the two pumps. To assess the relative levels of PMCA1 and PMCA4 mRNA in mouse tissues, RT-PCR analysis was performed using primers from exons 9 and 12 (Fig. 3A). The resulting fragments were then digested with restriction enzymes that recognize only one or the other of the two fragments and analyzed by agarose gel electrophoresis (Fig. 3B). Only PMCA1 mRNA was detected in liver, but all other tissues contained mRNA for both isoforms. Brain, intestine, kidney, lung, and stomach had higher levels of PMCA1 than PMCA4 mRNA. In contrast, aorta, portal vein, urinary bladder, diaphragm, seminal vesicles, and testes had higher levels of PMCA4 than PMCA1 mRNA. Many of the tissues examined had less than a 2-fold difference between the two isoforms, consistent with the relative abundance of PMCA1 and PMCA4 mRNAs observed in human tissues (13).
activity to elicit the original phenotype. PMCA4 and PMCA1 mutants were crossed to obtain Pmca4−/−Pmca1+/− mice on the Black Swiss background. Portal vein contractility was impaired, with mutant portal veins exhibiting weak to moderate phasic contractions (Fig. 4C). The tension-time integral for Black Swiss Pmca4−/−Pmca1+/− portal veins was sharply reduced relative to that of both wild-type portal veins and Black Swiss Pmca4+−/− portal veins, although it was not as severely affected as Pmca4−/− portal veins from the original mixed background strain.

Portal veins normally remain viable and exhibit phasic contractions for many hours after being placed in the organ bath. Thus, the absence of phasic contractions observed in the initial experiments was consistent with the possibility that PMCA4 plays a specialized role in portal vein contractility; however, histological and ultrastructural analysis revealed that portal vein smooth muscle cells from Pmca4−/− mice of the original mixed background were susceptible to apoptosis. Cell death was not apparent in fresh portal veins of either genotype (Fig. 5A), but was significant in Pmca4−/− portal veins after 4 h of contractility experiments in vitro (Fig. 5, E and F). The number of apoptotic smooth muscle cells per 100 μm along the length of the portal vein wall was 8.1 ± 1.6 and 0.12 ± 0.12 for Pmca4−/− and Pmca4+/− mice, respectively (p < 0.0001). The increase in apoptotic cells in Pmca4−/− smooth muscle was confirmed by ultrastructural analysis of the smooth muscle cells (Fig. 5, G and H). After 4 h in the organ bath, the appearance of Pmca4−/− cells looked like that of fresh tissue, while the structure of Pmca4−/− cells had deteriorated dramatically. Mitochondria were swollen, contained dense follicular inclusions, and became re-positioned to places near the plasma membrane. In addition, cytoplasmic smooth membranes virtually disappeared, as did pinocytotic vacuoles from the apical membrane. Smooth muscle filaments lost their organized distribution in

**Fig. 3.** Analysis of the relative levels of PMCA1 and PMCA4 mRNAs in mouse tissues. A, strategy for RT-PCR analysis. RNA was reverse transcribed and amplified by PCR using forward (FP) and reverse (RP) primers that yield ~1 kb fragments corresponding to the same region of the PMCA1 (upper) and PMCA4 (lower) mRNAs. Digestion with Clai cleaved the PMCA1 product into ~0.4-kb and ~0.6-kb fragments; digestion with BglI cleaved the PMCA4 product into two ~0.5-kb products. B, the amplified uncut (U), BglI-digested (B), Clai-digested (C), and Clai/BglI double-digested (CB) products were separated by agarose gel electrophoresis and visualized with ethidium bromide. The full-length products in the BglI-digested and Clai-digested samples correspond to PMCA1 and PMCA4 mRNAs, respectively.
with apoptosis and, depending on the strain, was due to the loss of PMCA4 either alone or in combination with PMCA1 haploinsufficiency.

**Infertility in Male Pmca4<sup>−/−</sup> Mice Is Due to the Failure of Hyperactivated Sperm Motility**—Analysis of testis sections revealed no histopathology or indications that spermatogenesis was abnormal. To determine whether Pmca4<sup>−/−</sup> sperm were capable of traversing the female genital tract, female mice were mated with either wild-type or Pmca4<sup>−/−</sup> males. Sperm were present in the uteri of all of the females and were highly motile in those mated with Pmca4<sup>−/−</sup> males but were non-motile in those mated with Pmca4<sup>−/−</sup> males. Oviducts from mice mated with Pmca4<sup>−/−</sup> males also contained highly motile spermatozoa, which were often clustered around the eggs, and over half of the eggs were already fertilized. In contrast, only a few non-motile sperm were observed in the oviducts of mice mated with Pmca4<sup>−/−</sup> males, and none of the eggs were fertilized.

When sperm were isolated from the epididymides of wild-type or Pmca4<sup>−/−</sup> males and maintained for 3 h under conditions that do not cause hyperactivated motility, the low amplitude flagellar beating characteristic of sperm that have not undergone capacitation was apparent in both genotypes, and the percentage of motile sperm did not differ significantly between the two genotypes (Fig. 6). In the uterus, however, sperm undergo capacitation and exhibit hyperactivated motility that is characterized by vigorous, high amplitude, whiplash movements of the sperm tail. To determine whether hyperactivated motility was impaired, sperm were subjected to conditions that induce hyperactivated motility in vitro. Sperm of both genotypes initially displayed the motility pattern characteristic of epididymal sperm in vitro. By 90 min, most of the Pmca4<sup>−/−</sup> sperm were non-motile, and the few remaining sperm exhibited only weak motility; in contrast, Pmca4<sup>−/−</sup> sperm exhibited normal hyperactivated motility (Table I). These results suggest that Pmca4<sup>−/−</sup> sperm stored in the epididymus are viable and motile but are unable to achieve hyperactivated motility.
determine whether there might be structural differences that might account for this failure, ultrastructural analysis was performed.

**Sperm Ultrastructure**—Electron microscopic analysis of sperm that had been subjected to 90 min of in vitro hyperactivation revealed no differences in structure between the two genotypes other than the percentage of condensed mitochondria (Fig. 7). Longitudinal and cross-sections of the middle piece and cross-sections of the principle piece of the sperm tail revealed normal architecture for the mitochondrial sheath, outer dense filaments, and fibrous sheath. The axoneme had the normal arrangement of two inner singlet and nine outer doublet microtubules, and outer and inner dynein arms and radial spokes were visible. The only apparent abnormality was a significant difference in the number of condensed mitochondrial profiles (Fig. 7, middle panels). After 90 min under hyperactivation conditions, 34% of the mitochondrial profiles in the middle piece of Pmca4−/− sperm tails were in a condensed configuration, whereas only 19% of the mitochondrial profiles in Pmca4+/+ sperm tails were condensed.

**Immunoblot and Immunohistochemical Analysis of PMCA4 in Sperm**—Western blot analysis of sperm proteins using a monoclonal antibody that identifies all mammalian PMCA isoforms showed that total PMCA protein in Pmca4−/− sperm is less than 10% of that in wild-type sperm (Fig. 8, upper left). Similar analyses of testes homogenates showed that PMCA levels in Pmca4−/− testes are ~25–30% that of the wild-type (Fig. 8, lower left). Because only PMCA1 and PMCA4 are expressed in testes, the remaining PMCA protein represents PMCA1. When immunostaining of sperm was performed using a PMCA4-specific monoclonal antibody, Pmca4−/− sperm exhibited intense fluorescence in the principal piece of the sperm tail and faint staining in the interior of the mid-piece (Fig. 8, right). Faint staining was also observed in the head but is obscured by the DAPI (blue) staining of the nucleus.

**DISCUSSION**

Despite widespread and abundant expression of PMCA4 in mouse tissues, PMCA4 null mutants exhibited no evidence of developmental defects, growth retardation, or reduced viability, and histological studies of fresh tissues from adult mutants revealed no evidence of cell death. If there had been any cell types in which PMCA4 was essential for cell viability, it would have indicated that any Ca2+ extrusion mediated by PMCA1 or other isoforms in those cells was not sufficient to maintain viability and that PMCA4 therefore served an essential housekeeping role. These observations negate the hypothesis that PMCA4 plays an essential housekeeping role in the strictest sense of being required for survival of at least some cell types in vivo. In contrast to Pmca4 null mutants, mice lacking PMCA1, which is expressed throughout the embryo (22), did not survive to birth, thereby showing that PMCA1 is needed for embryonic development. If PMCA activity is needed to maintain intracellular Ca2+ at a level compatible with cell viability, then PMCA1 clearly serves an important housekeeping function, because cell survival occurs even when PMCA4 has been ablated, thereby leaving PMCA1 as the only isoform in most cell types.

The absence of a readily apparent disease phenotype for PMCA4 null mutants was surprising, because estimates of the relative levels of PMCA1 and PMCA4 mRNAs in human tissues suggested that both isoforms were expressed at similar levels, differing by 3-fold at most in the tissues tested (13). With the exception of liver, estimates of their relative expres-
The studies of PMCA4-deficient portal vein smooth muscle show that, even in cell types in which PMCA4 is normally the most abundant isoform, PMCA1 can maintain the viability of cells in which PMCA4 has been ablated. This conclusion, however, requires some qualification. The absence of PMCA4 in portal vein smooth muscle from mice of the mixed 129/SvJ and Black Swiss background led to loss of phasic contractions. Although this initially suggested a specialized role for PMCA4 in the regulation of portal vein contractility, histological and ultrastructural analysis demonstrated that the smooth muscle was undergoing apoptosis. This was an in vitro phenomenon, however, because fresh tissue did not exhibit evidence of apoptosis. The portal vein contractility and apoptosis phenotype was lost when the mice were placed on the Black Swiss background but was largely restored by the addition of a null mutation in one copy of the Pmca1 gene. These results show that the Pmca1 gene can function as a modifier locus in PMCA4 null mutants and indicate that the apoptosis and loss of phasic contractions was due to an overall reduction in Ca\(^{2+}\)-extrusion capacity rather than the loss of a specialized PMCA4-mediated function. Thus, it appears that PMCA4 is the major isoform in some tissues but that PMCA1 can compensate for its absence, at least in vivo. On the basis of the current data, however, it is unclear whether there is sufficient redundancy of function between the two isoforms to allow PMCA4 to maintain viability of certain cell types if PMCA1 were ablated. Tissue-specific knockouts of PMCA1 will be needed to test this possibility.

The most likely explanation for the apoptosis phenotype is that the absence of PMCA4 leads to increased cytosolic Ca\(^{2+}\) and endoplasmic reticulum Ca\(^{2+}\) stores, conditions which contribute to both mitochondrial Ca\(^{2+}\) overload and activation of Ca\(^{2+}\)-dependent signaling and proteolytic processes involved in apoptosis (reviewed in Ref. 23). Overexpression of PMCA4 has been shown to reduce the incidence of apoptosis (24), and cleavage of PMCA2 and PMCA4 by caspases, which eliminates their activity, contributes to apoptosis (25). The basis for the mouse strain-dependent difference in susceptibility of Pmca4-/- portal vein smooth muscle to apoptosis is unclear but could be due to variations in the expression of any of a large number of pro-apoptotic or anti-apoptotic factors or differences in Ca\(^{2+}\) handling that affect susceptibility to cellular or mitochondrial Ca\(^{2+}\) overload. Regardless of the underlying cause(s) of the strain-dependent difference, our results provide direct evidence that a reduction in Ca\(^{2+}\) extrusion capability, while surprisingly well tolerated in vivo, can cause apoptosis.

In a recent study, Neyses and colleagues showed that the loss of PMCA4 causes a sperm motility defect and male infertility (14). In the current study, the motility defect was shown to be restricted to hyperactivated motility. When the epididymus-derived sperm were not subjected to in vitro capacitation, the motility patterns and percentage of motile sperm were essentially the same for both genotypes. This suggests that Pmca4-/- sperm remain viable under conditions that do not lead to activation of Ca\(^{2+}\) influx, such as during storage in the epididymus. This conclusion is consistent with the observation that Pmca4-/- sperm are fertilization-competent, an important issue addressed in the other study (14). The axoneme, outer dense fibers, and fibrous sheath, which form the major structural components of the sperm tail and are either directly or indirectly involved in sperm motility (26), were normal in Pmca4-/- sperm. Given the absence of ultrastructural abnormalities in the motility apparatus, the failure of hyperactivated motility is likely due to defective Ca\(^{2+}\) extrusion during capacitation, consistent with the ~2.5-fold elevation in intracellular Ca\(^{2+}\) in Pmca4-/- sperm (14). An increased incidence of condensed mitochondria has been observed in ram spermatozoa treated with the Ca\(^{2+}\) ionophore, A23187 (27). Thus, the increased percentage of condensed mitochondria in the Pmca4-/- sperm tail provides histopathological evidence of Ca\(^{2+}\) overload.

Immunohistochemical studies using an antibody that identifies all splice variants of PMCA4 showed that PMCA4 is largely restricted to the principle piece of the sperm tail, with only faint staining in the head. Because immunoblot analyses showed that >90% of the PMCA protein in sperm is PMCA4, with the rest being PMCA1, these results suggest that PMCA protein expression in other parts of the sperm is far less than in the principle piece. Schuh et al. (14) identified PMCA4 in both the principle piece and, to a lesser but significant degree, in the acrosomal region of the sperm head. The latter observation is at odds with our data and with that of Wennemuth et al. (27), in which the pan-specific 5F10 antibody detected high levels of PMCA protein in the principle piece, with little evidence of expression in other parts of the tail or head. The basis for this difference is unclear but may be related to the antibodies employed (21, 28).

The role of Ca\(^{2+}\) as a signaling molecule controlling sperm motility and fertilization capability is well established (29), and a recent study showed that PMCA activity is the major mechanism for Ca\(^{2+}\) extrusion (27). The expression of PMCA4 at high levels in the principle piece of the sperm tail is particularly interesting given the demonstration that the Ca\(^{2+}\) channel (termed CatSper) responsible for the Ca\(^{2+}\)-stimulated Ca\(^{2+}\) influx required for hyperactivated sperm motility is restricted to the principle piece (15). Genetic ablation of CatSper eliminated hyperactivated sperm motility and caused a severe reduction in Ca\(^{2+}\) concentrations in both the tail and head of the sperm (15). This indicated that CatSper is the major Ca\(^{2+}\) influx mechanism during capacitation. With the localization of PMCA4 (which accounts for >90% of the total PMCA in sperm) primarily to the principle piece, it is now clear that both the major Ca\(^{2+}\) influx and efflux mechanisms involved in hyperactivated motility are localized to this segment. The middle piece of the sperm tail, in which the plasma membrane is separated from the axoneme by the mitochondrial sheath, lacks these major influx and efflux mechanisms. Such an arrangement may allow optimal control of Ca\(^{2+}\) concentrations around the motility apparatus, while limiting exposure of the mitochondria to high levels of Ca\(^{2+}\). Ca\(^{2+}\) concentrations in other regions of the sperm would likely be limited by the concentrations maintained in the principle piece, and any excess Ca\(^{2+}\) passing into the middle piece would presumably be buffered by the mitochondria.

In summary, of the two relatively ubiquitous PMCA isoforms, only PMCA1 is essential for life and for embryonic development. This suggests that PMCA1 is the major housekeeping isoform, although PMCA4 undoubtedly contributes to this function in some cell types. The normal appearance and behavior of PMCA4 null mutants, PMCA1 heterozygous mutants, and even double mutants lacking both copies of the Pmca4 gene and carrying only a single copy of the Pmca1 gene suggest that both embryonic and adult tissues have sufficient reserve capacity for plasma membrane Ca\(^{2+}\) extrusion to withstand most perturbations. Nevertheless, the results of our portal vein studies show that PMCA deficiency, involving either isoform, can contribute to apoptosis under certain conditions. The only in vivo phenotype caused by the loss of PMCA4 identified so far was male infertility. The requirement of both the CatSper Ca\(^{2+}\) channel and PMCA4 for hyperactivated motility...
and the expression of both proteins in the principle piece of the sperm tail indicate that they serve as the major $Ca^{2+}$ influx and efflux mechanisms required for hyperactivated sperm motility.

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Targeted Ablation of Plasma Membrane Ca\(^{2+}\)-ATPase (PMCA) 1 and 4 Indicates a Major Housekeeping Function for PMCA1 and a Critical Role in Hyperactivated Sperm Motility and Male Fertility for PMCA4


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