

Switch of PMCA4 Splice Variants in Bovine Epididymis Results in Altered Isoform Expression during Functional Sperm Maturation*

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Ca^{2+} and Ca^{2+} -dependent signals are essential for sperm maturation and fertilization. In mouse sperm the plasma membrane Ca^{2+} -ATPase (PMCA) isoform 4 plays a crucial role in Ca^{2+} transport. The two major splice variants of PMCA4 are PMCA4a and PMCA4b. PMCA4a differs from PMCA4b in the mechanism of calmodulin binding and activation. PMCA4a shows a much higher basal activity and is more effective than PMCA4b in returning Ca^{2+} to resting levels. Knock-out mice carrying a PMCA4-null mutation are infertile because their sperm cannot achieve a hyperactivated state of motility. As sperm reach functional maturity during their transit through the epididymis, the expression of PMCA4a and 4b was assessed in bull testis and epididymis. Quantitative PCR revealed that PMCA4b is the major splice variant in testis, caput, and corpus epididymidis. In contrast, PMCA4a is the major splice variant in cauda epididymidis, whereas sperm are transcriptionally silent. Immunohistochemical staining using a new antibody against bovine PMCA4a located the PMCA4a to the apical membrane of the epithelium of cauda epididymidis, whereas testis, caput, and corpus epididymidis were negative. Western blotting of testis, epididymis, and sperm isolated from caput and cauda epididymidis showed a much higher level of PMCA4a in cauda epididymidis and sperm from cauda epididymidis compared with testis membranes and sperm from caput epididymidis. These findings suggest that PMCA4a is transferred to bovine sperm membranes in cauda epididymidis. This isoform switch may facilitate a higher calcium turnover in sperm necessary to traverse the female genital tract.

Ca^{2+} and Ca^{2+} -dependent signals are considered to exert many processes in sperm maturation and fertilization (1–3). The steep Ca^{2+} gradient between the intracellular and the extracellular space is maintained by effective Ca^{2+} -transporting enzymes. One of these enzymes is the plasma membrane Ca^{2+} -ATPase (PMCA).² Four isoforms of the PMCA

(PMCA1–4) have been identified, each encoded by a separate gene. By alternative splicing, >30 splice variants of the four PMCAs can be generated (4). The main splicing regions of the PMCA are located in the N- and C-terminal regions. Whereas PMCA1 and 4 are expressed ubiquitously, PMCA2 and 3 are restricted to a limited number of tissues (4, 5). The various PMCA isoforms and splice variants show developmental-, tissue-, and cell-specific patterns of expression. Studies on knock-out mice carrying PMCA1-, PMCA2-, or PMCA4-null mutations point to important *in vivo* functions of the different isoforms (6). *Atp2b1*^{-/-} mice are embryonic lethal, *Atp2b2*^{-/-} mice are ataxic and profoundly deaf (7–10). Homozygous mice with a targeted gene deletion of PMCA4 are infertile due to severely impaired sperm motility (7, 12). Null mice show normal spermatogenesis and mating behavior, but sperm of these animals cannot achieve a hyperactivated state of motility. Furthermore, PMCA4 is essential for achieving a low resting Ca^{2+} concentration in mouse sperm, whereas the contribution of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, mitochondrial uniporter, and the sarcolemmal Ca^{2+} -ATPase pump to this effect are minimal (11). Therefore, a pivotal role of PMCA4 in the regulation of sperm function and intracellular Ca^{2+} levels in sperm has been proposed (7, 12).

The epididymis is essential for sperm to acquire their fertilization capacity. Sperm maturation during transit through the epididymis from caput to cauda epididymidis is characterized by a change in the phospholipid and cholesterol composition of the sperm plasma membrane, modification of plasma membrane protein composition, and nuclear condensation of sperm (13).

In the present study, we analyzed the gene and protein expression of PMCA4 in bull testis, epididymis, and sperm because this PMCA isoform is believed to be essential for sperm fertilization capacity. We found that the two PMCA splice variants 4a and 4b are differentially expressed with a shift from 4b to 4a along the length of the epididymis from caput to cauda. This suggests that functional sperm maturation requires a shift in calcium pump expression from the slow PMCA4b to the faster PMCA4a isoform.

EXPERIMENTAL PROCEDURES

Preparation of Bull Tissue and Isolation of Caput and Cauda Sperm—Bovine testes and epididymides were obtained from the local slaughterhouse. Testes and epididymides were

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² The abbreviations used are: PMCA, plasma membrane Ca^{2+} -ATPase; qPCR, quantitative PCR; DIG, digoxigenin.

dissected immediately and frozen in liquid N₂ for subsequent expression analysis or fixed with Bouin's fixative in PBS (pH 7.4) for immunohistochemistry and *in situ* hybridization experiments. Isolation of sperm from caput, corpus, and cauda epididymidis was performed as described previously (14).

Total RNA Isolation and Reverse Transcription PCR—Total RNA from bull epididymidis (caput, corpus, and cauda), testis, seminal vesicle, heart, liver, and sperm was extracted using TriFAST Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. RNA was reverse transcribed as described (14). Fragments of PMCA4 and β -actin cDNA were amplified in a total volume of 25 μ l, containing 50 pmol of reverse and forward primers, 2 mM MgCl₂, 2.5 μ l of 10 \times PCR buffer, 0.25 unit of PlatinumTaq DNA polymerase (Invitrogen), 10 mM nucleotide mix (Promega, Madison, WI), and 1 μ l of cDNA. The primer sequences were as follows: PMCA4 forward, 5'-CAC CTT CGT GCT CAT GCA GC-3'; PMCA4 reverse, 5'-TGG AGT GAC CTC ACC ATC CAG-3' (631-bp and 812-bp fragments for PMCA4b and PMCA4a, respectively); β -actin forward, 5'-GGA CTT CGA GCA AGA GAT GG-3'; β -actin reverse, 5'-AGC ACT GTG TTG GCG TAC AG-3' (288 bp). The conditions for PMCA4 amplification were as follows: 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 60 s. For β -actin, 28 cycles were performed consisting of denaturation at 95 °C for 30 s followed by annealing at 60 °C for 30 s and elongation at 72 °C for 40 s. PCR products were electrophoresed on 1.5% agarose gels and eluted using the Qiaex kit (Qiagen) according to the manufacturer's protocol. PCR products were subsequently cloned into the pCR[®]II vector using the TA Cloning kit Dual Promoter (Invitrogen) and transformed into *Escherichia coli* strain inv-alpha F'. The insert was sequenced by MWG (Ebersberg, Germany) and the sequences were aligned using the BLAST program at the National Center for Biotechnology Information (NCBI).

Real-time Quantitative PCR—Total RNA was isolated as described above ($n = 4$). 1 μ g of total RNA was reverse-transcribed using the High Capacity RNA-to-cDNA Master Mix according to the manufacturer's protocol (Applied Biosystems). Primer sequences were as follows: PMCA4 forward, 5'-GGC CAG GTC ATY TCY RCR ATA CCT AC-3'; PMCA4 reverse, 5'-AGC TCC ATC TCK GCA TGG TC-3'; PMCA4a reverse, 5'-CTC CCG TCT GGA ATK TGT TAA TTA C-3'; TaqMan probe, 6FAM-AAG GAY GCT GAG GGG CTG GA-BBQ (K = G/T; Y = C/T; R = A/G) (TIB Molbiol, Berlin, Germany). β -Actin was used for normalization (Assay ID Bt03279174_g1; Applied Biosystems). qPCR conditions were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s on an Applied BioSystems 7300HT thermocycler (Applied Biosystems). All samples were run in duplicate, and PCR was repeated at least twice. Relative expression was estimated using the $\Delta\Delta$ Ct method (15).

Labeling of Riboprobes by *In Vitro* Transcription—*In vitro* transcription and labeling of riboprobes for PMCA4 were performed as described (14).

***In Situ* Hybridization**—*In situ* hybridization was performed as described in detail by Wilhelm *et al.* (16). Briefly, deparaffinized tissue sections from testes and epididymides were

treated with 0.1 M HCl for 10 min and incubated with proteinase K (15 μ g/ml) for 30 min at 37 °C to increase probe accessibility. After incubation with 4% paraformaldehyde in PBS (pH 7.4) for 5 min, sections were rinsed twice in PBS. Thereafter, sections were acetylated and prehybridized in 50% deionized formamide, 50 mM Tris-HCl (pH 7.5), 25 mM EDTA, 20 mM NaCl, 250 μ g/ml tRNA, and 2.5 \times Denhardt's solution for 2 h at 45 °C. Hybridization was performed overnight at 45 °C in hybridization buffer, consisting of prehybridization buffer plus 10% dextran sulfate and DIG-labeled PMCA4 probe (5 ng/ μ l). *In situ* hybridization was detected after overnight incubation with an anti-DIG alkaline phosphatase-conjugated Fab fragment (diluted 1:500; Roche Diagnostics). Bound alkaline phosphatase was visualized by an enzyme-catalyzed color reaction utilizing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Promega) as chromogenic substrates.

Cloning of Bovine PMCA4a and 4b—Primers for cloning of the full-length bovine splice variants PMCA4a and 4b were as follows: PMCA4 cloning forward, 5'-GTT GAG GGG CTT GGT AAC AG-3'; PMCA4 cloning reverse, 5'-TGG AGT GAC CTC ACC ATC CAG-3'. The PCR conditions were: 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 180 s. Complete cDNAs of the two PMCA4 splice variants were amplified using the Accu Prime Polymerase (Invitrogen). The PCR products were gel electrophoresed and eluted using the Qiaex kit (Qiagen). The eluted products were cloned into TOPO XL vector (TOPO[®] XL PCR Cloning kit; Invitrogen) and transformed into chemically competent *E. coli* (XL Gold[®]). The insert was restricted by the endonucleases HindIII and SacI. Clones containing inserts of adequate size were sequenced twice by MWG (Ebersberg, Germany), and the sequences were aligned using BLAST at NCBI.

Expression of PMCA4a and 4b in Sf9 Cells and Microsomal Preparation—Expression of human PMCA4a and 4b in Sf9 cells was performed as described previously (17). Crude microsomal membranes from Sf9 cells, untransfected or transfected with PMCA4a or PMCA4b, were prepared as described for COS cells with some minor modifications (18). Quantification of the amount of PMCA in microsomal membranes was done as described (17).

Generation of a Polyclonal PMCA4a Antibody—The included exon of splice variant 4a was identified by cloning and sequencing. The resulting amino acid sequence of bovine PMCA4a was subsequently used to identify a peptide for immunization (Coring; Gernsheim, Germany): GQHLDVKHVPSSSYV (residues 1114–1128, GI: 284156666). This peptide sequence shows no homology to other proteins but is 93% identical to the human PMCA4a sequence. A polyclonal antibody directed against the peptide was raised in rabbits according to a standard protocol (Coring). The polyclonal antibody was affinity-purified on a Sepharose column coupled to the immunogenic peptide. Specificity of the antibody was tested by ELISA (CovAbtest; Coring) and Western blotting using human PMCA4a and 4b overexpressed in Sf9 cells as well as bovine brain membranes. A negative control was performed with the preimmune serum and showed no binding.

PMCA4 Splice Variant Switch during Bovine Sperm Maturation

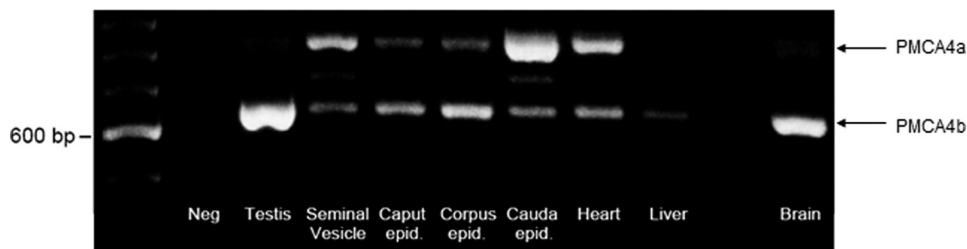


FIGURE 1. RT-PCR analysis of bovine tissues by using primers specific for PMCA4. The primers are flanking splice site C. The PCR product of 812 bp represents PMCA4a, and the PCR product of 631 bp corresponds to PMCA4b. The product at 724 bp is an artifact likely due to heteroduplex formation as noted previously (54). Bovine brain cDNA served as positive control. Negative controls were performed by omitting cDNA (Neg). Pronounced expression of PMCA4b was detected in testis, caput, and corpus epididymidis as well as in liver, whereas PMCA4a showed more prominent expression in seminal vesicle, cauda epididymidis, and heart.

Membrane Preparation, SDS-PAGE, and Western Blotting—Microsomal membranes of bovine brain, testis, epididymis, and sperm were prepared according to Sikdar *et al.* (19). Sperm originating either from caput or cauda epididymidis as well as testis and epididymidis (caput, corpus, cauda) were homogenized in homogenization buffer (25 mM imidazole (pH 7.5), 1 mM EDTA, 1 mM DTT, 250 mM sucrose, and the protease inhibitor CompleteTM; 1 tablet/50 ml; Roche Diagnostics) using a tissue homogenizer. After centrifugation for 10 min at $600 \times g$ and 4°C , the supernatant was collected, and the pellet was resuspended in homogenization buffer, homogenized, and centrifuged again as above. The pooled supernatants were spun at $12,000 \times g$, 4°C for 10 min. The resulting supernatant was centrifuged for 1 h at $100,000 \times g$ at 4°C . The pellet was resuspended in homogenization buffer, and protein concentration was determined as described previously (20). 30 μg (PMCA4) or 40 μg (PMCA4a) of sperm, testis, epididymis, or brain membrane protein or 1 μg of recombinant overexpressed human PMCA4a and 4b was separated as described (14, 21). After Western blotting transfer, membranes were incubated with the isoform-specific PMCA4 antibody (1:1,000 JA9 monoclonal; Dianova, Hamburg, Germany) or the newly generated splice variant specific polyclonal PMCA4a antibody (1:100) at room temperature overnight. After washing, membranes were incubated with enhanced chemiluminescence (ECL)-anti-mouse IgG horseradish peroxidase (POD) antibody or ECL-anti-rabbit IgG horseradish peroxidase (POD) antibody (1:2,000; Amersham Biosciences). The peroxidase reaction was visualized by ECL detection (Amersham Biosciences) according to the manufacturer's instructions.

Immunohistochemistry—Deparaffinized sections were rinsed twice in PBS, blocked in PBS containing 5% dried nonfat milk powder for 30 min, and incubated at 4°C overnight with the isoform-specific PMCA4 antibody (1:1,000 JA9) or the splice variant-specific PMCA4a antibody (1:500). Bound antibody was detected using the CSA kit (Dako, Hamburg, Germany) according to the manufacturer's instructions.

Statistics—qPCR values are presented as means \pm S.D. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $p < 0.05$.

RESULTS

Cloning of the Bovine PMCA Splice Variants 4a and 4b—The cDNA sequences of bovine PMCA4 splice variants were only partially known prior to this study. We therefore cloned and sequenced the bovine splice variants PMCA4a and 4b and deposited the complete sequences in the NCBI GenBank data base (GI:284156665, accession number, GU353069 for PMCA4a; GI:284156667, accession number GU353070 for PMCA4b). The bovine PMCA4 nucleotide sequences show 88–89% homology to the corresponding human (22, 23), 90% to the dog (24), and 82–83% to the rat (25, 26) and mouse (27) PMCA4 sequences.

Analysis of PMCA4 Transcripts in Bull Spermatogenic Cells and Epididymis Reveals Region-specific Expression of Splice Variants—To obtain information on the expression of the C-terminal splice variants of PMCA4 in bull testis and epididymis, we performed RT-PCR using primers flanking splice site C. Brain membranes were used as control tissue known to express PMCA4a and 4b. As shown in Fig. 1, PMCA4 is expressed in bull testis and all parts of the epididymis including caput, corpus, and cauda. Two major PCR fragments of 812 and 631 bp were observed which showed distinct distribution in different parts of the epididymis. Sequence analysis of the 631-bp PCR product confirmed that it corresponds to the PMCA4 cDNA sequence from position 2895–3525 representing the PMCA4b splice variant. The 812-bp PCR product matched with the cDNA sequence of PMCA4a and included an alternatively spliced exon of 181 bp in the C-terminal region. The PCR results show a switch of splice variant 4b to variant 4a from caput and corpus to cauda epididymis. Whereas PMCA4b is the quantitatively major splice variant in caput and corpus, PMCA4a predominates in cauda epididymis. To investigate this finding further, we cut the bovine epididymis into 19 pieces, each 1 cm in length (Fig. 2A). We then performed semiquantitative RT-PCR and confirmed that although PMCA4b dominates in caput and corpus epididymis, there is a distinct switch to splice variant 4a immediately beginning in cauda epididymis (Fig. 2, B and C).

The expression of PMCA4 and 4a transcripts in bull testis, epididymis, and sperm from caput and cauda epididymis was further analyzed by qPCR. Fig. 3 shows the expression level of all PMCA4 transcripts (Fig. 3A) and of PMCA4a-specific transcripts (Fig. 3B) relative to that in bovine brain. The highest expression of PMCA4 mRNA (including both splice

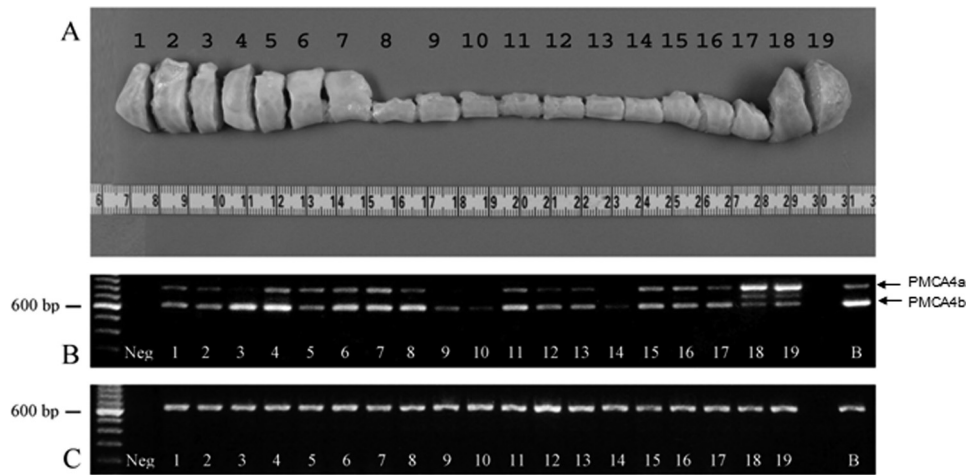


FIGURE 2. **Semiquantitative PMCA4 RT-PCR of bovine epididymis.** *A*, the bovine epididymis was cut into 19 pieces. *Pieces 1-5* represent caput, *pieces 6-17* represent corpus, and *pieces 18 and 19* represent cauda epididymidis. *B*, PMCA4 gene expression was analyzed by using primers flanking splice site *C*. Bovine brain RNA served as positive control (*lane B*). Negative controls were performed by omitting cDNA (*Neg*). The PCR product of 812 bp represents PMCA4a, and the PCR product of 631 bp represents PMCA4b. A splice variant switch from PMCA4b to 4a is observed from corpus (*piece 17*) to cauda epididymidis (*piece 18*). *C*, β -Actin PCR served as internal standard.

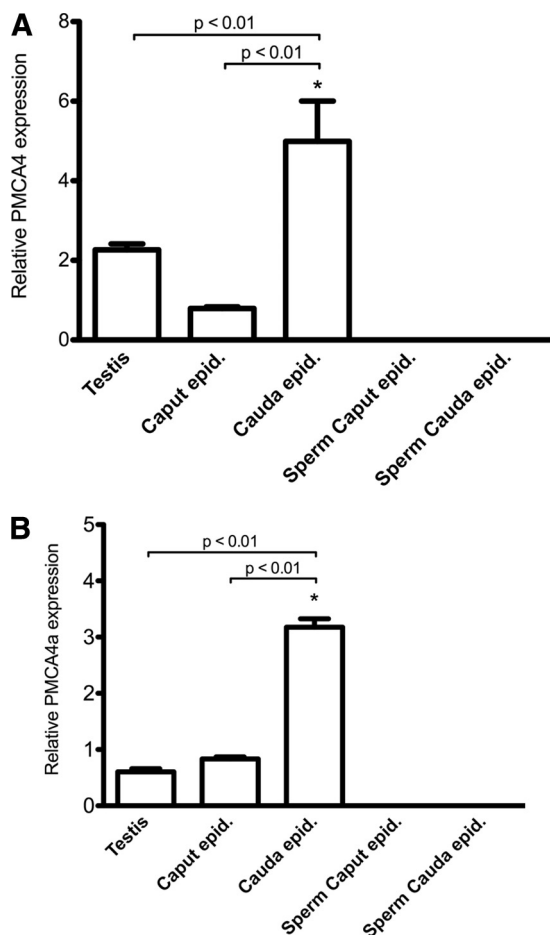


FIGURE 3. **Real-time qPCR to determine the relative levels of PMCA4 (A) and PMCA4a (B) transcripts in testis, caput epididymidis, cauda epididymidis, and sperm from caput and cauda epididymidis ($n = 4$).** Expression levels are shown relative to that in bovine brain set at 1.0. *A*, expression of PMCA4 transcripts is 4.7-fold higher in cauda epididymidis compared with brain ($p < 0.01$) and significantly higher than in testis and caput epididymidis ($p < 0.01$). PMCA4 transcripts are not detected in sperm from caput and cauda epididymidis. *B*, highest level of PMCA4a transcripts is observed in cauda epididymidis (3.2-fold compared with brain, $p < 0.01$), which is significantly higher than in testis and caput epididymidis ($p < 0.01$). PMCA4a transcripts are not expressed in sperm isolated from caput or cauda epididymidis.

variants 4a and 4b) is found in cauda epididymidis (4.7-fold higher compared with brain, $p < 0.01$). PMCA4 mRNA levels in cauda are significantly higher ($p < 0.01$) than in testis and caput epididymidis. In contrast, expression of PMCA4 in testis and caput epididymidis is not significantly different from brain (relative expression testis, 2.2; caput epididymidis, 0.8). No expression of PMCA4 transcripts is detected in sperm from caput and cauda epididymidis, demonstrating the transcriptional inactivity of sperm. Similar to total PMCA4, the highest level of PMCA4a transcripts is observed in cauda epididymidis (3.2-fold higher than in brain, $p < 0.01$; Fig. 3*B*). This level is significantly ($p < 0.01$) higher compared with testis and caput epididymidis where PMCA4a transcripts are lower than in the brain (relative levels 0.6 and 0.83, respectively). As expected, PMCA4a transcripts are not expressed in sperm isolated from either caput or cauda epididymidis (Fig. 3*B*).

To obtain information on the specific cell types that express the PMCA4, we performed *in situ* hybridization on paraffin-embedded sections. In the bovine testis, PMCA4 transcripts were expressed predominantly in spermatogonia and spermatocytes I (Fig. 4*A*). Leydig cells, Sertoli cells, and peritubular cells are negative for PMCA4 RNA. PMCA4 mRNA was also not detectable in later spermatogenic stages such as late spermatocytes or spermatids (Fig. 4*A*). In the epididymis, strong expression was found in the epithelium of principal and basal cells of caput, corpus, and cauda, whereas the stroma was negative (Fig. 4, *B-D*).

Localization of PMCA4 and PMCA4a in Bull Spermatogenic Cells and Epididymis—To determine the expression and localization pattern of the PMCA4 splice variants, we first generated an antibody specific for PMCA4a, using a unique peptide located in the C-terminal region of the bovine PMCA (see “Experimental Procedures”). To demonstrate the specificity of this antibody, human PMCA4a and 4b, overexpressed in Sf9 cell membranes, as well as bovine brain membranes, were analyzed by Western blotting. As shown in Fig. 5*B*, the antibody recognizes human PMCA4a and a band of similar size

PMCA4 Splice Variant Switch during Bovine Sperm Maturation

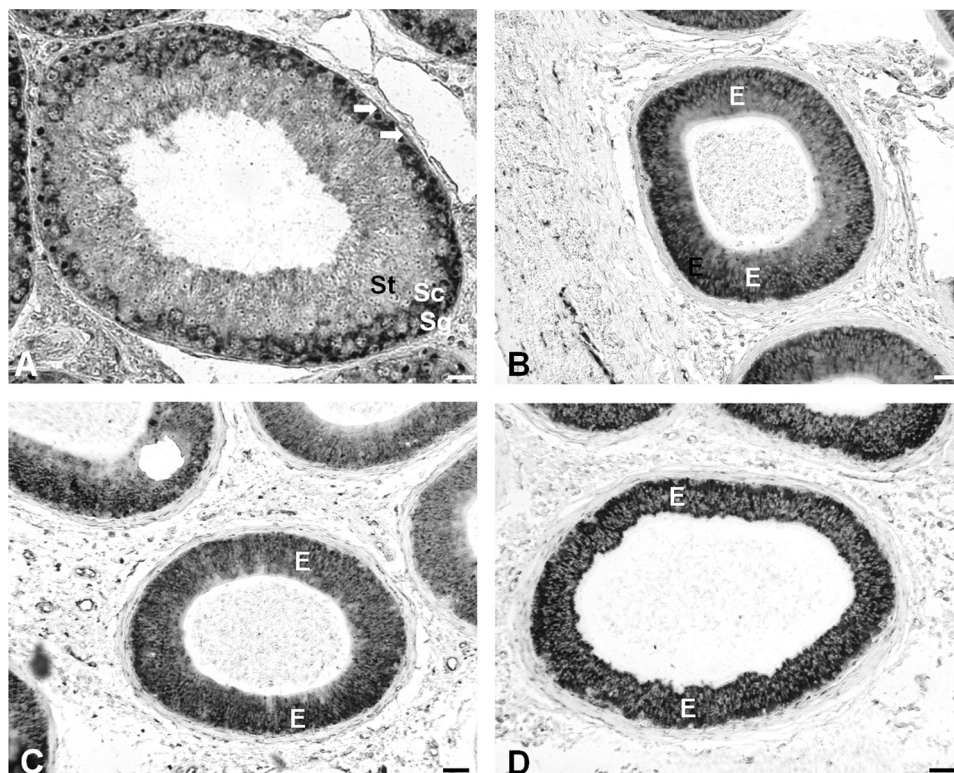


FIGURE 4. *In situ* hybridization to detect PMCA4 transcripts (all splice variants) in paraffin-embedded tissues. A, using the DIG-labeled PMCA4 antisense probes detecting all PMCA4 transcripts, strong expression of PMCA4 RNA was observed in bovine testis in spermatogonia (Sg) and primary spermatocytes (Sc). No expression was detected in later spermatogenic stages (St) and in Sertoli cells (arrows). B–D, in the epididymal caput (B), corpus (C), and cauda (D), strong PMCA4 mRNA expression was detected in all epithelial cells (E). Both the PMCA4 antisense probe of the 812-bp PCR fragment (corresponding to PMCA4a) and of the 631-bp PCR fragment (corresponding to PMCA4b) showed identical results. This is expected because the probe sequences are identical over most of their length; both probes therefore detect all PMCA4 transcripts combined. The figures displayed here represent the *in situ* hybridization using the 631 bp PCR-fragment probe. In negative controls, the DIG-labeled PMCA4 sense probe failed to label any of the tissues (data not shown). Scale bars, 25 μ m (A); 40 μ m (B–D).

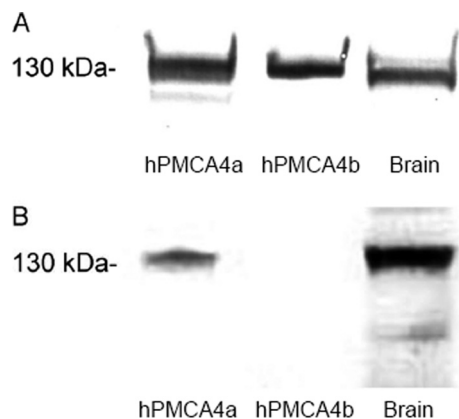


FIGURE 5. Western blotting analysis to demonstrate the specificity of the new polyclonal PMCA4a antibody. A, presence of PMCA4 protein was verified using the monoclonal pan-PMCA4 antibody JA9 that recognizes all splice variants of PMCA4. PMCA4 was detected in Sf9-cell membranes (1 μ g) overexpressing recombinant human PMCA4a and PMCA4b as well as in bovine brain membranes (30 μ g). B, PMCA4a was specifically detected by the new anti-PMCA4a antibody in membranes overexpressing recombinant human PMCA4a (1 μ g) and in bovine brain membranes (30 μ g), whereas human PMCA4b was negative (1 μ g).

(~130 kDa) representing bovine PMCA4a in membranes from bovine brain. By contrast, the antibody fails to react with human PMCA4b. Both the human PMCA4a and 4b splice variants and the bovine brain membranes showed positive signals with the monoclonal pan-PMCA4 antibody JA9 (Fig. 5A).

To localize PMCA4 in bull spermatogenic cells and epididymis, we performed immunohistochemical staining on paraffin-embedded sections of bull testis and epididymis, using the monoclonal antibody JA9 against all PMCA4 splice variants and the new polyclonal antibody against PMCA4a (Fig. 6). A PMCA4 signal is detectable in later stages of spermatogenesis in bull testis. Round and elongated spermatids are strongly positive for PMCA4 (Fig. 6A). In contrast, spermatogonia are negative or only faintly positive for PMCA4. In caput and corpus epididymis, the peritubular cells show a stronger PMCA4 signal than the epithelial cells (Fig. 6, B and C). In cauda epididymis, the basolateral membranes of the principal epithelial cells are highly positive for PMCA4 (Fig. 6D). On the other hand, a strong signal with the splice variant specific PMCA4a antibody was only observed in cauda epididymis. Interestingly, PMCA4a staining is concentrated in the apical membranes of the principal cells in cauda epididymis (Fig. 6H). Testis, caput, and corpus epididymis are mostly negative for PMCA4a (Fig. 6, E–G).

Finally, we analyzed the distribution of PMCA4 in bull testis, epididymis, and epididymal spermatozoa by Western blotting using the monoclonal pan-PMCA4 antibody and the polyclonal PMCA4a antibody. As expected, PMCA4 is present in testis and all parts of the epididymis as well as in the membranes from caput and cauda sperm. A double band, representing PMCA4a (128 kDa) and 4b (133 kDa), is visible in

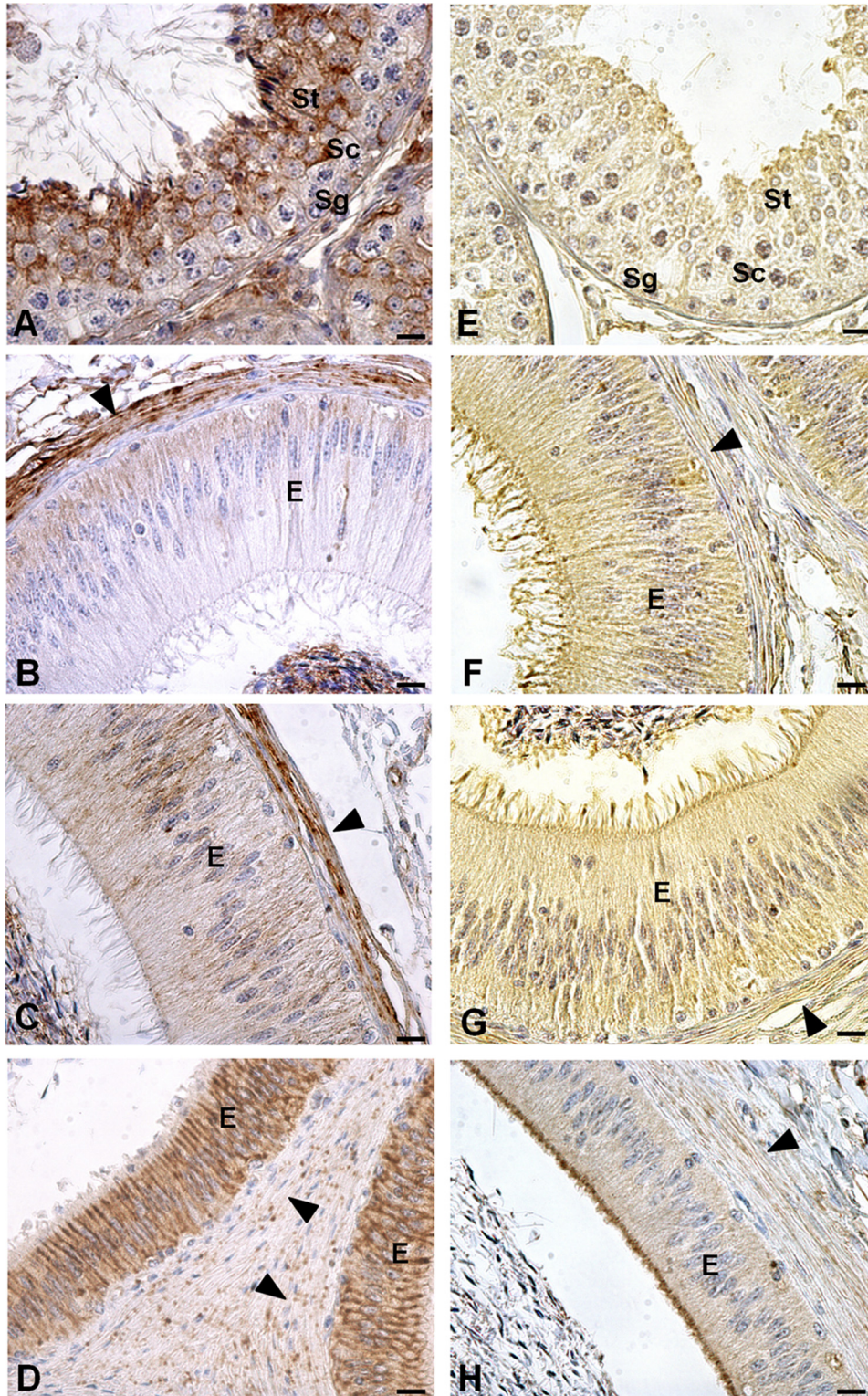


FIGURE 6. Immunohistochemistry on paraffin-embedded tissues using the monoclonal antibody JA9 against PMCA4 (A–D) and the new polyclonal antibody against PMCA4a (E–H). In bovine testis, PMCA4 immunoreactivity was detected in a stage-specific manner. PMCA4 is localized predominantly in spermatids (A). PMCA4a was not detectable in bovine testis at all (E), suggesting that all PMCA4 staining in A is due to PMCA4b. Epithelial cells of the epididymal caput (B and F) and corpus (C and G) showed only weak (B) or moderate (C) staining for PMCA4 and were negative for PMCA4a (F and G). In the epididymal caput and corpus, PMCA4 was more prominent in peritubular cells (arrowheads). In contrast, PMCA4 staining was highly positive in the basolateral plasma membranes of the epithelial cells of the cauda epididymidis and was faintly detected in peritubular cells (D). PMCA4a was localized in the apical membrane of the cauda epididymidis (H). Sections were counterstained with hematoxylin. Sg, spermatogonia; Sc, spermatocytes; St, spermatids; E, epithelial cells. Scale bars, 10 μ m.

caput sperm and in tissue membranes of the epididymis (Fig. 7A). The signal for PMCA4a (lower band in Fig. 7A) is increasing from epididymal caput to cauda tissue, with the

strongest PMCA4a band in cauda epididymidis. In contrast, the PMCA4a signal in caput sperm membranes is weak (lower band in Fig. 7A). Interestingly, only a single PMCA4-positive

PMCA4 Splice Variant Switch during Bovine Sperm Maturation

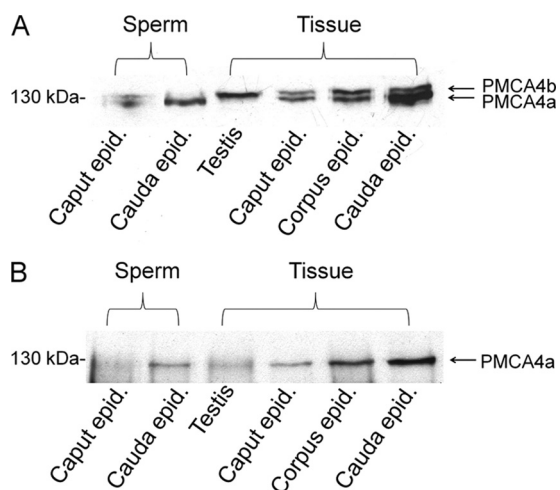


FIGURE 7. Western blotting analyzes of PMCA4 expression in bovine epididymal caput and cauda sperm membranes as well as in testis, epididymal caput, corpus, and cauda tissue membranes. 30 μ g of protein/lane was loaded in A, and 40 μ g of protein/lane was loaded in B. A, PMCA4 splice variants (4a and 4b) were detected using the monoclonal pan-PMCA4 antibody JA9. An immunoreactive double band corresponding to PMCA4b (~133 kDa) and PMCA4a (~128 kDa) was seen in epididymal caput sperm and in all epididymal tissue membranes. In contrast, in testis and in cauda sperm only a single band was visible (testis, ~133 kDa; cauda sperm, ~128 kDa). B, the presence of PMCA4a was demonstrated using the new polyclonal PMCA4a antibody. PMCA4a was only faintly detected in caput sperm and testis membranes as a diffuse signal but was robustly expressed in cauda sperm and in epididymal tissue where it increased strongly from caput to cauda epididymidis.

band is detectable in cauda sperm membranes and in testis. In testis, this is the more slowly migrating *upper band* corresponding to PMCA4b, whereas in cauda sperm, it is the *lower band* representing PMCA4a (Fig. 7A). To corroborate these findings, we used the new splice variant-specific PMCA4a antibody on a Western blot of the same sperm and tissue samples (Fig. 7B). As expected, the antibody detects a single band corresponding to the ~128 kDa band recognized by the pan-PMCA4 antibody. PMCA4a is virtually undetectable in caput sperm membranes and appears as a weak and diffuse signal in testis. In contrast, PMCA4a is clearly detected in all parts of the epididymis, showing the expected increase in signal strength from epididymal caput to cauda tissue membranes (Fig. 7B). PMCA4a is also readily detected in cauda sperm membranes, with a signal that is much stronger than in caput sperm membranes. Taken together, the above results suggest that sperm in the epididymis undergo a splice variant shift from PMCA4b to 4a as they are transferred from caput to cauda. Because sperm in their late developmental stages show little or no PMCA4 transcriptional activity (as is evident from the qPCR and *in situ* hybridization data; Figs. 3 and 4A), they likely acquire the PMCA4a isoform through a transcellular route from the epididymal cauda epithelium.

DISCUSSION

Isoform Switch from PMCA4b to PMCA4a in the Bovine Epididymis—PMCA4 appears to be essential for male fertility. PMCA4-knock-out mice are infertile. Sperm taken from these animals fail to achieve the hyperactivated motility needed to traverse the female genital tract (7, 12). When leaving the testis, their sperm are morphologically differentiated but func-

tionally immature. Spermatozoa obtain their fertilization capacity during their transit through the epididymis. During this epididymal maturation many biochemical characteristics of spermatozoa are modified, such as the phospholipid, cholesterol, and plasma membrane protein composition (28–30). Because of the relevance of the epididymis for sperm maturation and the importance of PMCA4 for sperm function, we analyzed PMCA4 gene expression and PMCA4 protein localization in bull epididymis. The PMCA4 gene transcripts are subject to alternative splicing (4). Splicing at the C-terminal site C generates variants 4a and 4b. PMCA4a contains an alternatively spliced exon of 178 nucleotides (human) or 181 nucleotides (cow), which causes a truncation of the open reading frame and results in a protein with a shorter C-terminal tail (31, 32). Using RT-PCR and qPCR, we found a splice variant switch from low PMCA4a and abundant PMCA4b in bovine caput and corpus epididymidis to low PMCA4b and abundant PMCA4a in cauda epididymidis. The PCR results were corroborated on the protein level by Western blotting experiments. An increase in the PMCA4a protein level was detected from epididymal caput to cauda. *In situ* hybridization revealed the presence of PMCA4 transcripts mainly in the epithelial cells of all parts of the epididymis. Interestingly, immunohistochemistry showed little staining for PMCA4 in epithelial cells of the caput and corpus epididymidis (Fig. 6, B and C), indicating inefficient translation of the mRNA or rapid turnover of PMCA4 in these parts of the epididymis epithelium. In contrast, in cauda epididymidis both total PMCA4 as well as PMCA4a protein were readily detected in epithelial cells. Whereas the pan-antibody against all PMCA4 splice variants (JA9) stained primarily the basolateral plasma membranes of principal cells of cauda epididymidis, PMCA4a was prominently detected in the apical membrane of principal cells by the new bovine PMCA4a-specific antibody. It is not clear why the apical membrane was not also prominently labeled by the JA9 antibody, although it is possible that the N-terminal recognition site of JA9 on PMCA4 (20) may be inaccessible in the apical membrane. It is interesting to note that in the rat, PMCA4 shows a different distribution in the epididymal parts. PMCA4 was not detected in rat cauda epididymidis but instead localized in the basolateral membrane of epithelial cells in the caput epididymidis (14).

Storage of spermatozoa is the main function of cauda epididymidis, whereas caput and corpus epididymidis are more involved in sperm maturation (13, 30). Potentially, these different functions require altered cellular Ca^{2+} handling. In epithelial cells predominantly involved in reabsorption, such as those of the intestine and distal tubules of the kidney, PMCA is generally enriched in the basolateral membrane (33–35). In contrast, in secretory epithelial cells such as pancreatic acinar cells, salivary gland cells, epithelial cells of the lactating mamma, and in epithelial cells of the rat coagulating gland, PMCA is largely concentrated in the apical membrane (36–39). The principal cells of the epididymis combine reabsorptive and secretory functions (40). Therefore, the localization of PMCA4 (mostly PMCA4b) in the basolateral plasma membrane and of PMCA4a in the apical membrane in cauda epididymidis is likely of functional relevance.

Consequences of Differential PMCA4 Splice Variant Localization for Sperm Maturation in the Epididymis—Earlier studies of our group have shown that the Ca^{2+} -ATPase activity increases significantly from epididymal caput sperm to epididymal cauda sperm (41). It has also been shown in mice that PMCA4 is the major isoform in testis (90%) and is located in the principal piece of sperm tail (7). In bovine sperm we could show that PMCA4 localizes to the mid piece of caput and cauda sperm (42). Using *in situ* hybridization and immunohistochemistry to study the gene expression and distribution of PMCA4 protein in bovine germ cell stages, we found clear evidence that PMCA4 mRNA expression is restricted to spermatogonia and early primary spermatocytes. In contrast, PMCA4 protein appeared in later stages of spermatogenesis, especially in round and elongated spermatids. The regional difference between PMCA4 mRNA expression in the basal compartment beneath the blood-testis barrier of the germinal epithelium, and localization of PMCA4 protein in the adluminal compartment of the germinal epithelium has also been noted in rat testis (14).

Our RT-PCR and qPCR results clearly show that in bull testis, splice variant PMCA4b is the predominant PMCA4 isoform. This is corroborated by Western blotting experiments, where a prominent band is visible with a size corresponding to PMCA4b using the pan-PMCA4 antibody. In contrast, only a diffuse signal is seen when the newly generated PMCA4a splice variant-specific antibody is used. Accordingly, no PMCA4a protein is detected by immunohistochemistry in bull testis germ cell stages, and therefore, the positive signal in spermatids using the PMCA4 antibody is largely due to splice variant 4b. Using Western blotting analysis to determine the PMCA4 splice variants in epididymal spermatozoa, we found a much higher amount of PMCA4a in sperm from cauda compared with those from epididymal caput. This implies that sperm in testis and caput contain primarily splice variant 4b whereas variant 4a is enriched in sperm from cauda epididymidis. Taken together, the data suggest that the increase in splice variant 4a from caput to cauda sperm occurs during the maturation process in the epididymis. But how is this accomplished in sperm cells? It is known, that sperm contain a certain amount of mRNA (43). Our qPCR studies, however, demonstrate that sperm collected from caput as well as cauda epididymidis express neither PMCA4a nor 4b mRNA transcripts. The apical localization of PMCA4a in epididymal cauda epithelium may be relevant in this context. During the maturation process in the epididymis, a transfer of proteins originating from the epididymal epithelium to spermatozoa is thought to occur (44, 45). The vehicles of transfer are presumably the so-called epididymosomes, which are secreted in an apocrine manner by the epididymal epithelium (44, 46). These vesicles have been found in mice (47), humans (48), and also in bulls where the glycosylphosphatidylinositol-anchored protein P25b plays an important role in the transfer process (49). Overall, this transfer improves sperm motility, zona pellucida binding, and the ability of the sperm to fuse with the egg plasma membrane (13, 28–30, 46). It is thus likely that PMCA4a is transferred from

the apical membrane of the cauda epithelium to the sperm to enrich their membrane with PMCA4a, which is presumably beneficial for sperm function.

What may be the functional consequences of the PMCA4 splice variant shift during sperm maturation in the epididymis? Alternative splicing at splice site C affects the regulation of PMCA by calmodulin and phosphorylation as well as differential interaction with anchoring and signaling proteins (4, 50, 51). Inclusion of the exon in PMCA4a effectively alters the C-terminal half of the calmodulin binding domain and changes the reading frame of the C-terminal tail. Consequently, PMCA4a differs from PMCA4b in the mechanism of calmodulin binding and activation (17, 52, 53). The maximal apparent rate constant for calmodulin activation of PMCA4a is almost twice that for PMCA4b, whereas the rates of activation for both isoforms show similar dependence on Ca^{2+} . The inactivation of PMCA4a by calmodulin removal is also faster than for PMCA4b and is much less dependent on Ca^{2+} than inactivation of PMCA4b (17). As a consequence of decreased autoinhibition, PMCA4a shows a higher basal activity, and its faster activation rate makes it more effective than PMCA4b in returning Ca^{2+} to resting levels after a transient Ca^{2+} spike. Thus, the physiological significance of the observed increase of PMCA4a in sperm during maturation in the cauda epididymidis may be to endow them with a mechanism for handling a higher calcium turnover, which might be a prerequisite to transfer the sperm in a hyperactivated state. This is in accordance with the findings by Sanchez-Luengo *et al.* (41) which showed that the Ca^{2+} -ATPase activity in bovine sperm increases dramatically during epididymal transit.

CONCLUSIONS

In this study we show for the first time that PMCA4 undergoes a splice variant shift in bovine epididymis from PMCA4b in caput and corpus epididymidis to PMCA4a in cauda epididymidis. This is paralleled by a similar shift in PMCA4 splice variant abundance in sperm during epididymal transit. Because epididymal sperm express neither PMCA4a nor 4b mRNA transcripts, the cauda sperm likely acquire PMCA4a via cell-to-cell transfer of PMCA4a from the apical membrane of the epithelium of the cauda epididymidis. This potential cell-to-cell transfer of epididymosomes is under current investigation.

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