Excision of \textit{Trpv6} Gene Leads to Severe Defects in Epididymal Ca$^{2+}$ Absorption and Male Fertility Much Like Single D541A Pore Mutation*\textsuperscript{§}

Received for publication, November 28, 2011, and in revised form, February 20, 2012. Published, JBC Papers in Press, March 15, 2012, DOI 10.1074/jbc.M111.328286

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\textbf{Background:} The TRPV6\textsuperscript{D541A} pore mutation abrogates epididymal Ca$^{2+}$ absorption causing hypofertility in mice, raising the possibility of residual TRPV6\textsuperscript{D541A} channel activity.

\textbf{Results:} \textit{Trpv6} deletion reduces fertility parameters to the same extent as the D541A pore mutation.

\textbf{Conclusion:} The D541A pore mutation leads to complete inactivation of TRPV6 channels in epididymal epithelium.

\textbf{Significance:} Targeted mutations in mice help to understand the function of TRPV6 proteins in native systems.

Replacement of aspartate residue 541 by alanine (D541A) in the pore of TRPV6 channels in mice disrupts Ca$^{2+}$ absorption by the epididymal epithelium, resulting in abnormally high Ca$^{2+}$ concentrations in epididymal luminal fluid and in a dramatic but incomplete loss of sperm motility and fertilization capacity, raising the possibility of residual activity of channels formed by TRPV6\textsuperscript{D541A} proteins (Weissgerber, P., Krieb, U., Tsvilovskyy, V., Olausson, J., Kretz, O., Stoeger, C., Vennekens, R., Wissenbach, U., Middendorff, R., Flockerzi, V., and Freichel, M. (2011) \textit{Sci. Signal}. 4, ra27). It is known from other cation channels that introducing pore mutations even if they largely affect their conductivity and permeability can evoke considerably different phenotypes compared with the deletion of the corresponding protein. Therefore, we generated TRPV6-deficient mice (\textit{Trpv6}\textsuperscript{+/−}) by deleting exons encoding transmembrane domains with the pore-forming region and the complete cytosolic C terminus harboring binding sites for TRPV6-associated proteins that regulate its activity and plasma membrane anchoring. Using this strategy, we aimed to determine whether the TRPV6\textsuperscript{D541A} pore mutant still contributes to residual channel activity and/or channel-independent functions \textit{in vivo}. \textit{Trpv6}\textsuperscript{+/−} males reveal severe defects in fertility and motility and viability of sperm and a significant increase in epididymal luminal Ca$^{2+}$ concentration that is mirrored by a lack of Ca$^{2+}$ uptake by the epididymal epithelium. Therewith, \textit{Trpv6} excision affects epididymal Ca$^{2+}$ handling and male fertility to the same extent as the introduction of the D541A pore mutation, arguing against residual functions of the TRPV6\textsuperscript{D541A} pore mutant in epididymal epithelial cells.

The maintenance of body Ca$^{2+}$ homeostasis is essential for many vital functions including neuronal excitability, muscle contraction, and bone formation. Ca$^{2+}$ acquisition in the body occurs via trans- and paracellular transport processes across the continuous layer of epithelial cells. About 10 years ago, transcripts of the structurally closely related TRPV6 and TRPV5 were identified in the epithelia of the kidney (2), in the duodenum (3), and in placenta, pancreatic acinar cells, and other exocrine glands (4–7), and expression of the \textit{Trpv6} and \textit{Trpv5} cDNAs in HEK293 cells or other expression systems leads to the formation of cation channels with a high selectivity for Ca$^{2+}$ (3–5, 8). These channels exhibit many features possessed by Ca$^{2+}$ transporters in epithelial cells (6, 7): they mediate passive transport of Ca$^{2+}$ down the electrochemical gradient without energy consumption, and they are constitutively active (4, 5, 8). Accordingly, they were assumed to be epithelial Ca$^{2+}$ uptake channels.

Crucial for Ca$^{2+}$ permeation through TRPV6 and TRPV5 channels is a single aspartate residue within the pore-forming loop of both proteins; replacing this aspartate residue at position 541 in mouse TRPV6 or at position 542 in rabbit TRPV5 by an alanine residue renders the channels impermeable to Ca$^{2+}$ (9). We studied the impact of this mutation \textit{in vivo} with a mouse model in which the D541A mutation of TRPV6 was introduced in the germ line by a gene targeting approach (1). Males homozygous for this single amino acid substitution (TRPV6\textsuperscript{D541A/D541A}) exhibited a severely impaired fertility and a large reduction of motility and fertilization capacity of sperm despite intact spermatogenesis. An increase in Ca$^{2+}$ in spermatozoa is an important signal to promote their...
motility, capacitation, and the acrosome reaction (10, 11), but *Trpv6* transcripts were not detectable in spermatozoa or in the germinal epithelium, and there was no evidence for a cell-autonomous impairment of sperm Ca\(^{2+}\) signaling (1). However, we identified *Trpv6* transcripts in the epididymal epithelium and TRPV6 proteins in the apical membrane of this epithelium and showed that the luminal Ca\(^{2+}\) concentration is increased by 10-fold in the caudal epididymal fluid of *Trpv6*\(^{D541A}\)/D541A males compared with that of wild-type mice. Additional measurements of Ca\(^{2+}\) uptake from the epididymal fluid from *Trpv6*\(^{D541A}\)/D541A mice into the epididymis revealed a reduction of uptake by 7–8-fold compared with fluid from wild-type animals (1). Apparently, the intact TRPV6 proteins are essential components of Ca\(^{2+}\) uptake channels in the epididymal epithelium that are responsible for the decrease of the Ca\(^{2+}\) concentration in the epididymal fluid along the epididymal duct, generating a luminal Ca\(^{2+}\) gradient with higher Ca\(^{2+}\) concentration in the caput portion (proximal segments) of the epididymal duct and lower Ca\(^{2+}\) concentrations in its caudal (distant) segments. It is well known that the composition of the epididymal fluid differs considerably among separate epididymal segments. This is caused by differences in secretory and absorptive activities of the epididymal epithelium (12–16), resulting in changes of the net water; the HCO\(_3\^-\) reabsorption; the secretion of proteins; concentrations of Na\(^+\), Cl\(^-\), and K\(^+\); and luminal acidification. Notably, the Ca\(^{2+}\) concentration decreases markedly in the epididymal fluid along the epididymal duct toward its distal segments, the cauda epididymis (14). It is this luminal Ca\(^{2+}\) gradient that is severely compromised by the TRPV6\(^{D541A}\) mutation, resulting in a dramatic but incomplete loss of sperm motility and fertilization capacity.

The direct electrophysiological recording of channel activity was not possible either from wild-type or from D541A mutant epididymal cells. Our own results and a survey of the literature on TRPV6 and its closest relative, TRPV5, revealed that direct recordings of TRPV6 and TRPV5 currents from acutely prepared primary cells expressing *Trpv6* or *Trpv5* transcripts have not been described. Instead, Ca\(^{2+}\) uptake measurements similar to those we performed (17) have been used to identify (10, 18) and characterize these channels in primary cells. Although Ca\(^{2+}\) uptake was reduced by 7–8-fold in *Trpv6*\(^{D541A}\)/D541A mice, we could not exclude residual channel activity of the properly expressed and trafficked, yet mutated TRPV6\(^{D541A}\) proteins, which might in addition serve channel-independent functions as structural and/or scaffolding components (19) of the epithelial plasma membrane. Thus, the TRPV6\(^{D541A}\) mutation might not be sufficient to identify all TRPV6-related functions, and it is conceivable that a complete loss of TRPV6 proteins leads to additional phenotypes. Similarly, the GluR8292618R pore mutation, although rendering GluR82 channels nominally impermeable to Ca\(^{2+}\) (20), did not cause the defects in synaptic plasticity of hippocampal neurons and motor coordination that were evoked by the complete inactivation of the GluR82 gene in mice (21). We therefore generated a second mouse line using a Cre-loxP-based gene targeting strategy to delete exons 13, 14, and 15 of the *Trpv6* gene that encode part of the fifth and sixth transmembrane-spanning domains, the pore linker in between, and the complete cytosolic C terminus. By deleting almost one-third of the protein-encoding DNA, we not only eliminated an essential part of the ion-conducting pore but also disrupted essentially the predicted structure of the protein. We now show that mice homozygous for this gene excision were viable, and our analysis reveals that the Ca\(^{2+}\) concentration in the caudal epididymal fluid and the motility, fertilization capacity, and viability of *Trpv6*\(^{-/-}\) sperm are affected to the same extent as in *Trpv6*\(^{D541A}\)/D541A mice. These results indicate that the TRPV6 deletion occurring in *Trpv6*\(^{-/-}\) mice does not further aggravate the phenotype observed in *Trpv6*\(^{D541A}\)/D541A mice, arguing against residual channel activity and against channel-independent scaffolding functions of the TRPV6\(^{D541A}\) protein that may influence epididymal Ca\(^{2+}\) absorption or fertilization capacity indirectly.

**EXPERIMENTAL PROCEDURES**

All animal experiments were performed in accordance with German legislation on the protection of animals and were approved by the local ethics committee. The generation of TRPV6\(^{-/-}\) mice and the maintenance of the mouse line as well as the experiments performed were approved (reference number K110/180-07; approved on December 10, 2002, December 28, 2004, and January 17, 2007) by the “Kreispolizeibehörde des Saarpfalz-Kreises, Deutschland” and was performed by M. F., V. F., P. W., U. K., J. O., S. B., K. F., and C. M. All these people got permission to perform the experiments described by the local ethics committee and “Kreispolizeibehörde des Saarpfalz-Kreises, Deutschland.” They all have a long-standing experience and authorization in the generation and phenotypic analysis of transgenic mice (e.g. Ref. 1 and references therein).

**Generation of TRPV6 Knock-out Mice**—The 5' and 3' homology arms of the targeting vector were amplified from genomic DNA of R1 ES cells using *Pfu* polymerase. The 5' homology arm was cloned 5' of a loxP site followed by the genomic sequence containing exons 13, 14, and 15; an FRT\(^{-}\) sequence-flanked PGK promoter-driven neomycin resistance gene cassette (neo), and a second loxP site. The sequence of the loxP site was inserted in the 16th intron of the *Ephb6* gene, which is oriented tail to tail with *Trpv6* in the mouse genome separated by 120 bp only. Accordingly, Cre-mediated excision of exons 13, 14, and 15 of the *Trpv6* gene leads to deletion of exons 17 and 18 of the adjacent *Ephb6* gene. The herpes simplex virus thymidine kinase cassette (HSVtk) and an eGFP cassette were introduced for negative selection. Gene targeting in R1 ES cells was performed as described (1). One of 327 double resistant colonies showed homologous recombination at the *Trpv6* locus as confirmed by Southern blot hybridization with a 5' and 3' probe external to the targeting vector and a neo probe. Germ line chimeras were obtained by injection of correctly targeted ES cell clone 6F11 into C57Bl/6 blastocysts. *Trpv6*\(^{-/-}\) mice on the mixed (129/SvJ × C57Bl/6N) background were compared with *Trpv6*\(^{-/-}\) mice on the *129Sv/J* background. All animal experiments were performed in accordance with German legislation on the protection of animals and were approved (reference number K110/180-07; approved on December 10, 2002, December 28, 2004, and January 17, 2007) by the “Kreispolizeibehörde des Saarpfalz-Kreises, Deutschland” and was performed by M. F., V. F., P. W., U. K., J. O., S. B., K. F., and C. M. All these people got permission to perform the experiments described by the local ethics committee and “Kreispolizeibehörde des Saarpfalz-Kreises, Deutschland.” They all have a long-standing experience and authorization in the generation and phenotypic analysis of transgenic mice (e.g. Ref. 1 and references therein).

3 The abbreviation used are: FRT, Flp recombinase target site; PGK, phosphoglycerol kinase.
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Trpv6+/D541A mice (ES cell clone 8E11b) by Cre-mediated excision of genomic sequences containing exons 13-15 and analyzed via computer-assisted sperm analysis. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and were approved by the local ethics committee.
Expression Analysis of Trpv6-encoding Transcripts—Poly-(A)* RNA (10 µg) from epididymis and testis of wild-type and Trpv6+/− mice was used for Northern blot analysis as described (17). Blots were hybridized with a randomly labeled cDNA probe comprising exons 13, 14, and 15 of the mTrpv6 cDNA (nucleotides 1741–2849; GenBankTM accession number AJ542487), and filters were exposed to x-ray films for 19 h. To analyze expression of Trpv6 transcripts in prostate, we prepared RNA from prostate using the RNeasy Mini kit (Qiagen) and performed one-step reverse transcription-PCR (RT-PCR; Invitrogen) using 20 ng of total RNA/reaction. The following intron-spanning primers were used: for amplification of a specific Trpv6 fragment comprising 258 bp: UK_V6_16 (5′-GTC TGG CAT CAG CCT CAG C-3′; exon 1) and UK_V6_33 (5′-CTC ACA TCC TTC AAA CTT GAG C-3′; exon 2); for amplification of full-length Trpv6 cDNA comprising 2312 bp: UK_V6_17 (5′-GAG GTT CGA GCC CAG TGT G-3′; located in the 5′-untranslated region of exon 1) and UK_V6_14 (5′-CTC GCA GGA TGA CCT TAG CTG-3′; located in the 3′-untranslated region of exon 15). RNA isolated from epididymis was used as a positive control.

Maternal, Weight Gain, and Fertility Analysis—Fertility analysis was done basically as described in Weissgerber et al. (1). In brief, adult male Trpv6+/− and Trpv6−/− mice were continuously housed with adult Trpv6+/− and Trpv6−/− females over a period of 16 weeks, and the number and size of litters as well as the genotype of offspring were recorded. Considering 3 weeks per pregnancy and assuming that the adult female mouse gets pregnant within the 1st week after the birth of the litter, four litters can be expected per mating under optimal conditions during this period of 16 weeks. In reality, we observed that each wild-type/wild-type mating produced slightly fewer numbers of litters, that is 3.375 litters in 16 weeks (27 litters per eight matings; see Ref. 1). All mating analyses described in this study and by Weissgerber et al. (1) were performed in our animal facility under identical conditions. For copulatory behavior analysis, the ratio of plug-positive females per mating was calculated. In addition, Cre activity also leads to the excision of the PGK-neor cassette so that the 3.7-kb neo probe signal detectable for the Trpv6−/− allele was inherited in a mendelian ratio, Trpv6+/− males and females at the age of 3–4 months were intercrossed, and offspring were analyzed with respect to the Trpv6 genotype.

Videomicroscopic Analysis of Sperm Motility—Capacitated spermatozoa were placed in a 100-µm chamber at 37 °C temperature on a slide warmer and videotaped using a DCR-SR90 Handycam (Sony) connected to an inverted microscope (AxioVision 40 CFL, Zeiss). Selected sequences were processed using Adobe PREMIERE software.

Miscellaneous Methods—Whole organ preparation, histological analysis, and Ca2+ measurements in the epididymal fluid were done as described (1). Statistical Analysis—Data are presented as mean ± S.E. of n independent experiments unless otherwise stated. The Origin 7.0 software (OriginLab) was used for statistical analysis. Significance was assessed with the two-sample Student’s t test (p < 0.05 for significance) unless stated otherwise. Offspring frequency in the mating analysis and weight gain were analyzed using analysis of variance. The occurrence of different genotypes in the inheritance analysis of the Trpv6− allele was analyzed using a two-tailed χ2 test (GraphPad Software).

RESULTS

Generation and Characterization of Trpv6−/− Mice—We used a Cre-loxP-mediated gene targeting strategy in embryonic stem cells to generate Trpv6-deficient mice (Fig. 1, a and b). We confirmed homologous recombination in Trpv6−/− ES cells and Cre-mediated excision of exons 13, 14, and 15, which encode part of transmembrane domain 5, transmembrane domain 6, the pore-forming region in between, and the adjacent amino acids forming the complete intracellular C terminus of TRPV6, in Trpv6+/− and Trpv6−/− mice by Southern blot analysis (Fig. 1, c and d). In parallel, mice heterozygous for the Trpv6− allele (Trpv6+/−/L2F2) were bred with FlpeR (129S4/SvJaeSor-Gt (ROSA)26Sortm1(FLP1) Dym/j) mice (18) to remove the neo* cassette and to produce mice with a conditional Trpv6+/+L2F2 allele (Fig. 1, e and f). Northern blot analysis revealed specific 2.9-kb Trpv6 transcripts expressed in placenta.
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FIGURE 2. Segregation of Trpv6 allele is normal, but Trpv6/−/− males are hypofertile despite normal copulatory behavior. a, segregation analysis from 477 offspring derived from 62 litters and 16 Trpv6/−/−/− matings. b and c, weight gain analysis in wild-type (black), Trpv6/+/− (green), and Trpv6/−/− (red) male (b) and female (c) mice. Body weight was recorded weekly between 1 and 21 weeks of age. Six to seven male and nine to 10 female wild-type mice, 19–27 male and 17–18 female Trpv6/−/−/− mice, and nine male and six to seven female Trpv6/−/−/− mice were analyzed. *, p < 0.05, d, offspring analysis from matings between wild-type (+/+), Trpv6/+/− (+/−), and Trpv6/−/− (−/−) mice. The number of matings, the cumulative mating time of all matings with mice of a given genotype, the total number of offspring, the ratio between the number of litters and number of matings, and the observed and expected number of litters are indicated. §, redrawn from Weissgerber et al. (1); *, p < 0.001 versus wild-type/wild-type matings and p < 0.001 versus male (♂) × female (♀) −/− (type 1) matings; $, not statistically different: type 2 versus type 3 (p = 0.17) matings and type 3 versus type 4 (p = 0.29) matings. e, analysis of copulatory behavior by vaginal plug frequency in timed matings of wild-type (black) and Trpv6/−/− (red) males with wild-type females. n, total number of analyzed matings; *, p > 0.5. f, averaged number of isolated embryos from matings with wild-type (black) and Trpv6/−/− (red) males. n, numbers of analyzed vaginal plug-positive females; *, p < 0.01. Data are presented as mean ± S.E.
and epididymis but not in testis from wild-type mice (Fig. 1g), supporting our previous finding that TRPV6 is not expressed in testes. After Cre-mediated excision, TRPV6 transcripts were no longer detectable in epididymis of TRPV6”/” mice (ES cell clone 6F11) and of an independent TRPV6”/” mouse line (ES cell clone 8E11b; generated from TRPV6””/” mice (1)).

**FIGURE 3. Decreased motility and fertility of spermatozoa isolated from Trpv6”/” mice.** a–e, computer-assisted sperm analysis of sperm isolated from the cauda epididymis of TRPV6”/” mouse line (ES cell clone 6F11) and of an independent TRPV6”/” mouse line (ES cell clone 8E11b; generated from TRPV6””/” mice (1)).

**Average motility (a), progressive motility (b), path velocity (velocity average path (VAP)) (c), track velocity (velocity curvilinear (VCL)) (d), and linear velocity (velocity straight line (VSL)) (e) of spermatozoa from wild-type (black; n = 8) and TRPV6”/” (red; n = 6) mice 90 min after capacitation; *p < 0.001.**

**In vitro fertilization experiments.** Averaged fraction of fertilized eggs incubated with spermatozoa from wild-type (black) and TRPV6”/” (red) mice; *p < 0.001; n indicates the number of analyzed eggs. Data are presented as mean ± S.E.

**Impaired Motility and Fertilization Capacity of Sperm from Trpv6”/” Mice—** Videomicroscopic analysis of capacitated sperm from wild-type mice (see supplemental Movie S1, wild-type sperm) indicated forced beating and progressive movement. In contrast, most of the TRPV6”/” sperm were immotile with impaired movement and bending in the tail region (see supplemental Movie S2, TRPV6”/” sperm). Computer-assisted sperm analysis of capacitated sperm isolated from the cauda epididymis revealed that the number of motile sperm from TRPV6”/” mice (Fig. 3a) and their progressive motility were significantly decreased compared with sperm from wild-type mice (Fig. 3, a and b). Even within the small population of TRPV6-deficient sperm that showed progressive motility (Fig. 3, c–e), all speed parameters were significantly reduced, demonstrating that the TRPV6 deletion critically affected sperm motility. The analysis of two, independent TRPV6”/” mouse lines, which we generated from the TRPV6””/” mice (1), confirmed these results (Fig. 3, a–e).

We performed *in vitro* fertilization experiments to analyze the ability of TRPV6”/” sperm to fertilize oocytes. Therefore, isolated mature eggs from wild-type females were incubated with capacitated wild-type or TRPV6”/” sperm, and the successful fertilization (indicated by the development of two-cell stage embryos) was examined after 24 h. 161 of 238 eggs were fertilized by wild-type sperm, but only 18 of 265 eggs were fertilized by sperm derived from TRPV6”/” mice (Fig. 3f).

**The reproductive tracts of wild-type and TRPV6”/” males were macroscopically examined and showed no obvious differences (Fig. 4a).** No morphological differences were observed in histological sections of the testes from TRPV6”/” and wild-type
males (Fig. 4b); all cell types that can be observed during spermatogenesis, such as spermatogonia, spermatocytes, and spermatids, were apparent. In sections through the epididymis, we observed that the lumen of caput (Fig. 4c) and cauda (Fig. 4d) epididymis were filled with sperm. Additionally, microscopic analysis with higher magnification demonstrated that there were no differences between the morphology of the epididymal epithelium in Trpv6+/− mice (Fig. 4, c and d, lower panels) and wild-type mice (Fig. 4c) and Trpv6+/− mice. Eosin-nigrosin staining of sperm (Fig. 4e) revealed that the number of viable Trpv6+/− sperm isolated from the caput epididymis was 62.53 ± 4.71% (1219 sperm analyzed from four mice) and from the cauda epididymis was 6.66 ± 2.84% (n = 4; 3003 sperm analyzed). In our previous study, we have shown that the reduction of the number of eosin-negative sperm during the epididymal passage was also more than 10-fold in Trpv6+/−/+ mice but only 2-fold in wild-type mice (1).

As shown in Fig. 1g, Trpv6 transcripts were not detectable in testes from wild-type mice. Also, no defects in spermatogenesis were observed in the testes of Trpv6+/− mice. Taken together, these results suggest (like in the Trpv6+/−/+ mice) a secondary cause of the impaired viability of cauda epididymal sperm in Trpv6+/− mice. We have shown that the TRPV6 and TRPV6D541A proteins are apparently expressed in the apical membrane of epididymal epithelial cells (1) and the lack of TRPV6 expression in epithelial cells from Trpv6+/−/+ mice (see Fig. 1g and Ref. 1). A luminal Ca²⁺ gradient along the epididymal duct has been described (14) with luminal Ca²⁺ being lower in the cauda than in the caput, and we had found that luminal Ca²⁺ is markedly increased in the cauda from Trpv6+/−/+ mice when compared with the Ca²⁺ levels in wild-type mice. Therefore, we analyzed whether the Ca²⁺ concentration of the intraluminal fluid in the cauda epididymis in Trpv6+/−/+ mice is affected to the same extent using ion-selective microelectrodes.
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Enlarged ducts of the ventral, dorsal, and lateral lobes of the prostate where Trpv6 is also expressed in epithelial cells as in epididymal epithelial cells (1). In Trpv6D541A/D541A mice, the quantities of antigen in the prostate were drastically reduced. Ca2+ concentrations were abnormally high in the caudal fluid of Trpv6−/− males; and Ca2+ uptake by the epididymal epithelium was drastically impaired. Ca2+ precipitations were not identified in the epididymis but were found in the caudal fluid of wild-type males. Macrophages were detectable as white rigidifications in epididymis and prostate sections (Fig. 6b). In contrast to the findings in the epididymis, the ventral, dorsal, and lateral lobes of the Trpv6−/− prostate showed Ca2+ precipitations, and Ca2+ precipitations were macroscopically detectable as white rigidifications (Fig. 6b). The ducts were enlarged and showed a loss of epithelial infoldings into the lumen (Fig. 6b, middle panel).

**DISCUSSION**

In this study, we showed that deletion of part of the transmembrane domains including the pore-forming region and the entire C terminus of TRPV6 proteins led to hypofertility in Trpv6−/− males. A systematic breeding analysis revealed that the number of offspring in matings with homozygous Trpv6−/− males was markedly diminished regardless of the genotype of the females, whereas their copulatory behavior as well as the morphology of testis and epididymis was unaffected. However, the motility, fertilization capacity, and viability of sperm derived from cauda epididymis of Trpv6−/− males were drastically reduced; Ca2+ concentrations were abnormally high in the caudal fluid of Trpv6−/− males; and Ca2+ uptake by the epididymal epithelium was drastically impaired. Ca2+ precipitations were not identified in the epididymis but were found in the caudal fluid of wild-type males (0.18 ± 0.02 mM; n = 8; Fig. 5a). Next, we measured 45Ca2+ uptake in the cauda epididymis. Fig. 5b shows a 9-fold decrease in the ratio of 45Ca/51Cr in the epididymis from Trpv6−/− mice compared with wild-type littermates, indicating a severe defect in Ca2+ absorption.

Luminal fluids of the caudal epididymal duct contained ~11-fold higher concentrations of Ca2+ in Trpv6−/− mice (2.0 ± 0.3 mM; n = 8) compared with wild-type mice (0.18 ± 0.02 mM; n = 8; Fig. 5a). The number of analyzed epididymides are as follows: wild-type (n = 10; five animals) and Trpv6−/− (red) (n = 10; five animals) mice. The ratio of 45Ca/51Cr activity accumulated in cauda epididymis was determined 30 min after intratubular administration of the isotopes; *, p < 0.00001. Data are presented as mean ± S.E.
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**TABLE 1**

Body weight analysis in male Trpv6^{D541A/D541A} and Trpv6^{-/-} mice

Body weight of mutant mice was normalized to their wild-type littermates; no significant difference was observed between Trpv6^{D541A/D541A} and Trpv6^{-/-} mice (p > 0.05) at all time points. The number of mutant and corresponding wild-type mice is indicated (n).

| Age (weeks) | Wild type^{a} | Male Trpv6^{D541A/D541A} | Male Trpv6^{-/-} | Wild type^{a} |
|-------------|---------------|--------------------------|------------------|---------------|
| n | Normalized | n | Normalized | n | Normalized |
| 4 | 28 | 100 | 14 | 103 ± 4 | 9 | 97 ± 8 |
| 8 | 28 | 100 | 14 | 103 ± 2 | 9 | 100 ± 5 |
| 12 | 28 | 100 | 14 | 105 ± 2 | 9 | 99 ± 5 |
| 16 | 28 | 100 | 14 | 104 ± 2 | 9 | 104 ± 4 |

^{a} The number of wild-type littermates of the group of Trpv6^{D541A/D541A} mice.

**TABLE 2**

Body weight analysis in female Trpv6^{D541A/D541A} and Trpv6^{-/-} mice

Body weight of mutant mice was normalized to their wild-type littermates; no significant difference was observed between Trpv6^{D541A/D541A} and Trpv6^{-/-} mice (p > 0.05) at all time points. The number of mutant and corresponding wild-type mice is indicated (n).

| Age (weeks) | Wild type^{a} | Female Trpv6^{D541A/D541A} | Female Trpv6^{-/-} | Wild type^{a} |
|-------------|---------------|--------------------------|------------------|---------------|
| n | Normalized | n | Normalized | n | Normalized |
| 4 | 16 | 100 | 18 | 92 ± 4 | 6 | 102 ± 5 |
| 8 | 16 | 100 | 19 | 94 ± 2 | 7 | 103 ± 4 |
| 12 | 16 | 100 | 19 | 96 ± 3 | 7 | 96 ± 2 |
| 16 | 16 | 100 | 19 | 95 ± 2 | 7 | 97 ± 3 |

^{a} The number of wild-type littermates of the group of Trpv6^{D541A/D541A} mice.

**TABLE 3**

Offspring analysis from matings with Trpv6^{D541A/D541A} and Trpv6^{-/-} males

No significant difference was observed between matings with Trpv6^{D541A/D541A} and Trpv6^{-/-} mice (p > 0.05). The number of matings for each setup is indicated (n). ND, not determined.

| Mating | Compared with | Male Trpv6^{D541A/D541A} x female Trpv6^{D541A/D541A} | Male Trpv6^{-/-} x female Trpv6^{-/-} |
|--------|--------------|-------------------------------------------------|--------------------------------------|
| n | % | n | % | n | % |
| Male Trpv6^{+/+} x female Trpv6^{+/+} | 11 | 100 | 7 | 1.29 ± 1.29 | ND |
| Male Trpv6^{+/+} x female Trpv6^{+/-} | 3 | 100 | 5 | ND | 0.00 ± 0.00 |
| Male Trpv6^{+/-} x female Trpv6^{+/-} | 8 | 100 | 7 | 1.25 ± 1.25 | 5 | 0.00 ± 0.00 |

| Mating | Compared with | Male Trpv6^{D541A/D541A} x female Trpv6^{-/-} | Male Trpv6^{+/-} x female Trpv6^{+/-} |
|--------|--------------|---------------------------------|------------------------------------|
| n | % | n | % | n | % |
| Male Trpv6^{+/+} x female Trpv6^{+/-} | 11 | 100 | 7 | 1.94 ± 1.35 | ND |
| Male Trpv6^{+/+} x female Trpv6^{+/-} | 5 | 100 | ND | 7 | 3.46 ± 1.85 |
| Male Trpv6^{+/-} x female Trpv6^{+/-} | 8 | 100 | 7 | 1.87 ± 1.30 | 7 | 3.74 ± 2.00 |

**TABLE 4**

Analysis of copulatory behavior and in vivo fertilization rate

For analysis of copulatory behavior, the ratio of plug-positive wild-type females per mating with male Trpv6^{D541A/D541A} and Trpv6^{-/-} mice, respectively, was normalized to the ratio obtained from matings with wild-type females with wild-type males. Corresponding normalization was performed to compare the efficiency of in vivo fertilization assessed by microscopic analysis of flushed embryos from plug-positive wild-type females with males of the indicated genotype. No significant differences were observed between matings with Trpv6^{D541A/D541A} and Trpv6^{-/-} mice (p > 0.05). The number of matings for each experiment is indicated (n).

| Analysis of | n | % | n | % |
|-------------|---|---|---|---|
| Copulatory behavior | 64 | 100 | 49 | 95.39 ± 17.21 |
| In vivo fertilization | 15 | 100 | 11 | 2.75 ± 2.75 |

Ca^{2+} deposits in the prostate cannot account for the massive elementary changes observed in caudal sperm of Trpv6^{-/-} mice because these sperm are located upstream of the prostate and were never exposed to prostate secretions at this stage. Nevertheless, it cannot be fully excluded that cell-autonomous alterations in epithelial cells of the prostate contribute at least to some extent to the drastically reduced rate of offspring found in our mating analysis.

All functional deficits in the male reproductive tract that were found in Trpv6^{-/-} mice are virtually identical to those we have found in Trpv6^{D541A/D541A} mice and to those that have been described (1), suggesting that both the Trpv6 gene excision (this study) and the specific D541A pore mutation (1) lead to a complete inactivation of TRPV6 channels. This conclusion is supported by a detailed comparison of the findings obtained with either mouse line. Development of body weight was not different between Trpv6^{-/-} mice and Trpv6^{D541A/D541A} mice (Tables 1 and 2). The fertility rate of homozygous mutant males (Trpv6^{-/-} or Trpv6^{D541A/D541A}) was assessed in matings with either homozygous (Trpv6^{-/-} and Trpv6^{D541A/D541A}) or heterozygous (Trpv6^{+/+} and Trpv6^{+/-}) females and normalized to that obtained using Trpv6^{+/-} males. Apparently, the reduction in fertility rate
was not different between mice with either \textit{Trpv6} mutation (Table 3). Also, no differences were observed in copulatory behavior or the rate of embryos isolated from plug-positive females of matings with males homozygous for either \textit{Trpv6} allele.

**TABLE 5**

Analysis of sperm motility and viability

Original data of different experiments in \textit{Trpv6}^D541A/\textit{D541A} and \textit{Trpv6}^-/- mice were normalized to the corresponding wild type. No significant difference was observed between \textit{Trpv6}^D541A/\textit{D541A} and \textit{Trpv6}^-/- mice (\(p > 0.05\)) except for overall motility. The number of analyzed animals is indicated (\(n\)). VAP, velocity, average path; VSL, velocity, straight line; VCL, velocity, curvilinear; IVF, \textit{in vitro} fertilization.

| Analysis of sperm | Wild type | \textit{Trpv6}^D541A/\textit{D541A} | \textit{Trpv6}^-/- |
|------------------|-----------|-------------------------------|------------------|
| Motility         | 6 100 6   | 7.32 ± 1.57                   | 6 30.35 ± 9.55   |
| Progressive motility | 6 100 6   | 6.18 ± 0.98                   | 6 10.42 ± 6.33   |
| VAP              | 6 100 6   | 68.26 ± 3.69                  | 6 72.24 ± 5.63   |
| VSL              | 6 100 6   | 83.06 ± 5.64                  | 6 76.23 ± 4.29   |
| VCL              | 6 100 6   | 64.09 ± 3.14                  | 6 69.08 ± 5.51   |
| IVF              | 5 100 5   | 14.47 ± 2.92                  | 5 11.25 ± 4.43   |
| Vitality caput   | 4 100 4   | 93.62 ± 1.36                  | 4 95.84 ± 7.23   |
| Vitality cauda   | 4 100 4   | 11.22 ± 2.32                  | 4 22.20 ± 9.48   |

\(\ast p = 0.04\).

**TABLE 6**

Comparison of defects in \textit{Ca}^{2+} homeostasis in cauda epididymis of \textit{Trpv6}^D541A/\textit{D541A} and \textit{Trpv6}^-/- mice

Absolute values of different experiments in \textit{Trpv6}^D541A/\textit{D541A} and \textit{Trpv6}^-/- mice were normalized to the corresponding wild type and compared with each other. No significant difference between \textit{Trpv6}^D541A/\textit{D541A} and \textit{Trpv6}^-/- mice was observed (\(p > 0.05\)).

| Wild type | \textit{Trpv6}^D541A/\textit{D541A} | \textit{Trpv6}^-/- |
|-----------|----------------------------------|------------------|
| \([\textit{Ca}^{2+}]_{\text{intr}}\)| 29 0.19 100 28 1.9 1028 8 2.0 1123 |                   |
| \textit{Ca}^{2+} uptake       | 16 100 16 10 13 10 11               |                   |

\(\text{FIGURE 7. Model of architecture of wild-type TRPV6 proteins, TRPV6}^{D541A/\textit{D541A}}\) proteins, and potential proteins produced from the \textit{Trpv6-null} (\textit{Trpv6}^-/-) allele. \(a\), TRPV6 exhibits the typical topology of all members of the \textit{transient receptor potential} potential family with six transmembrane regions and a short domain between transmembrane segments 5 and 6 forming the channel pore. The N-terminal region of wild-type TRPV6 contains at least five (possibly six) ankyrin repeats (5) and a binding site for calmodulin (CaM) (32). In the C-terminal tail of TRPV6, there are binding sites for calmodulin (23), Na\(^+/\text{H}^{+}\) exchanger regulatory factor 4 (NHERF4) (24), Ras-related protein Rab-11A (Rab11a) (25), and S100A10-annexin 2 (22). \(b\), replacement of aspartic acid at position 541 in the pore region of TRPV6 (Asp-541) eliminates \textit{Ca}^{2+} conductivity of TRPV6 channels. This TRPV6\(^{D541A}/\textit{D541A}\) pore mutant protein is properly expressed and trafficked to the plasma membrane (1) and contains all binding sites for the TRPV6 interaction partners that anchor TRPV6 channel proteins in its native environment. \(c\), proteins produced in cells homozygous for the \textit{Trpv6-null} allele (\textit{Trpv6}^-/-) lack part of transmembrane domain 5, the pore region, transmembrane domain 6, and the entire C-terminal tail due to the genetic ablation of exons 13, 14, and 15. Even if such truncated TRPV6 proteins are stable, the C-terminal binding sites for TRPV6-associated proteins are missing.
ilar to that in Kir3.2−/− mice (26, 27), whereas the spontaneous seizure activity observed in Kir3.2−/− mice is not evoked by the Kir3.2 G156S mutation. In another report, the Q618R mutation in the GluR subunit of the neuronal glutamate receptor resulted in significant changes in the expression of this protein in the brain. Additionally, inactivation of the GluR subunit was found to affect the function of TRPV5 and TRPV6 channels in the epididymal epithelium.

In addition to the fertility analyses in the TRPV6D541A mice and TRPV6−−/− mouse lines, there are also no differences in their gross appearance including growth. However, they differ significantly from a TRPV6-deficient mouse line generated by Bianco et al. (28). In this mouse line, the genomic sequences including exons 9–15 of the Trpv6 gene as well as exons 15–18 of the adjacent Ephb6 gene were deleted and replaced by a neomycin resistance cassette. In our TRPV6−−/− mouse line, exons 17 and 18 of the Ephb6 gene were deleted, whereas in the TRPV6D541A/mouse line (1), all exons of the Ephb6 gene were unchanged. Additionally, inactivation of Ephb6 does not affect fertility as shown in two independent Ephb6−−/− mouse lines (29, 30). The TRPV6−−/− mice described by Bianco et al. (28) exhibit significant growth retardation, alopecia in 80% of all mice homozygous for the targeted allele, and impaired fertility in males but also in females (which was not characterized). Differences in growth, hair coat, or fertility of females were not observed in our TRPV6−−/− or TRPV6D541A/mouse lines (this study and Ref. 1). The reason for the observed discrepancy with our TRPV6-deficient mouse line is not known, but differences may be due to deletion of either exons 15–18 of the Ephb6 gene in TRPV6−−/− mice described by Bianco et al. (28) or exons 17 and 18 in our mouse line. Additionally, disposition of the promoter-driven neomycin resistance cassette, which might affect expression of the adjacent Trpv5 gene, or differences in the genetic background between the mouse lines cannot be excluded. The highly Ca2+-selective TRPV5 channels resemble TRPV6 in many aspects as they are also constitutively active and exhibit many features possessed by Ca2+ transporters in epithelial cells (6, 7). TRPV5 plays a critical role in Ca2+ reabsorption in the kidney epithelium of collecting ducts, but TRPV5−−/− mice show no defect in fertility (31). Obviously, the defect in Ca2+ uptake in the epididymal epithelium induced by deletion of the Trpv6 gene could not be compensated for by the nearest relative, TRPV5, demonstrating that TRPV6 is essential for the posttesticular sperm maturation process.

In summary, we demonstrated that TRPV6−−/− mice show phenotypic changes in the male reproductive tract equal to those in TRPV6D541A/mice, corroborating the crucial role of TRPV6 in the epididymis for the development of fertilization capacity of sperm. We conclude that TRPV6 proteins are essential components of Ca2+-conducting channel complexes in the apical membrane of epididymal epithelial cells and are responsible for decreasing the Ca2+ concentration of the intraluminal fluid in the cauda epididymis. Furthermore, the results obtained with the TRPV6D541A mice underscore the finding in TRPV6D541A/mice that appropriate regulation of intraluminal Ca2+ concentration in the epididymal duct is essential for the production of fertilization-ready spermatozoa during the epididymal passage. Together with the phenotype analysis of TRPV6D541A/mice, our results argue for the conclusion that the D541A pore mutation leads to a complete inactivation of TRPV6 channels in the epididymal epithelium.

Acknowledgments—We thank S. Buchholz, C. Matka, T. Volz, K. Fischer, S. Schmidt, and S. Tasch for expert technical assistance.

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