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Voltage-dependent Anion-selective Channels VDAC2 and VDAC3 Are Abundant Proteins in Bovine Outer Dense Fibers, a Cytoskeletal Component of the Sperm Flagellum*

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Outer dense fibers (ODF) are specific subcellular components of the sperm flagellum. The functions of ODF have not yet been clearly elucidated. We have investigated the protein composition of purified ODF from bovine spermatozoa and found that one of the most abundant proteins is a 30–32-kDa polypeptide. This protein was analyzed by sequencing peptides derived following limited proteolysis. Peptide sequences were found to match VDAC2 and VDAC3. VDACs (voltage-dependent, anion-selective channels) or eukaryotic porins are a group of proteins first identified in the mitochondrial outer membrane that are able to form hydrophilic pore structures in membranes. In mammals, three VDAC isoforms (VDAC1, -2, -3) have been identified by cDNA cloning and sequencing. Antibodies against synthetic peptides specific for the three mammal VDAC isoforms were generated in rabbits. Their specificity was demonstrated by immunoblotting using recombinant VDAC1, -2, and -3. In protein extracts of bovine spermatozoa, VDAC1, -2, and -3 were detected by specific antibodies, while only VDAC2 and -3 were found as solubilized proteins derived from purified bovine ODFs. Immunofluorescence microscopy of spermatozoa revealed that anti-VDAC2 and anti-VDAC3 antibodies clearly bound to the sperm flagellum, in particular to the ODF. Transmission electron immunomicroscopy supported the finding that VDAC2 protein is abundant in the ODF. Since the ODF does not have any known membranous structure, it is tempting to speculate that VDAC2 and VDAC3 might have an alternative structural organization and different functions in ODF than in mitochondria.

Eukaryotic voltage-dependent anion channels (VDAC)[1] or porins are small pore-forming proteins first identified in outer mitochondrial membranes (1). Since then, VDAC proteins have been discovered in most eukaryotic organisms ranging from yeast (2) to animals (3, 4) to plants (5, 6). Functional characterization of these proteins has been mainly achieved by purified protein reconstitution into planar bilayer membranes (for a review see Ref. 7). Mitochondrial porin displays a rather unspecific single channel activity with a molecular exclusion for hydrophilic permeable molecules of ~3 kDa (4, 7). Interestingly, VDAC displays a very sensitive voltage dependence when assayed under *in vitro* conditions (8). At voltages close to 0 mV, the channel exists mainly in its fully open state. Upon application of voltages larger than 20 mV, it switches to subconducting states (9). In these subconducting states, VDAC not only has a reduced permeability but also a reversed selectivity. For instance, the channel allows the diffusion of negatively charged solutes such as succinate, malate, and ATP in the fully open state, but not after switching to a subconducting state that is cation selective (10). Therefore, it has been assumed that the voltage dependence might be the mechanism used by VDAC to regulate mitochondrial function.

Soon after the characterization of the VDAC protein and gene, molecular techniques permitted the discovery of homologous genes in several different eukaryotic organisms (11). In the yeast *Saccharomyces cerevisiae* it was shown that a second gene exists encoding a VDAC-like protein, termed VDAC2 by sequence homology (12). In human, rat, and mouse three different VDAC genes encoding distinctly expressed isoforms have been reported (11, 13). Human and mouse VDAC1 and VDAC2 isoforms display channel-forming activity *in vitro* and can complement VDAC-deficient yeast, while VDAC3 can only partially complement this defect (14). On the basis of transcriptional analysis, mammalian VDAC isoforms are expressed in a wide variety of tissues (11, 13); at the protein level the expression of specific VDAC isoforms was observed in specialized cells in the mammalian testes (15). There is only limited information regarding the physiological function of the alternative VDAC isoforms.

The role of mitochondrial VDAC1 (porin isoform 1) in apoptosis has also been investigated in recent years (16–18), and its participation in the permeability transition pore (PTP) has been postulated (19). A recent report focused on the VDAC2 isoform as an important component of the cell death pathway (20). Other unexpected information has come in the last few years following the reported possibility that VDAC may interact with other cellular proteins, especially those of the cytoskeleton (17, 21–23), and from the localization of the VDAC1 isoform to other cellular membranes in addition to the outer mitochondrial membrane. Thinnes *et al.* (24) were first to report the existence of VDAC (porin 31 HL) in the plasmalemma

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[1] The abbreviations used are: VDAC, voltage-dependent anion channel; PTP, permeability transition pore; ODF, outer dense fibers; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ECL, enhanced chemiluminescence; AS, antiserum; BSA, bovine serum albumin; mAb, monoclonal antibodies; MAP, microtubule-associated protein; KLC, kinesin light chain; DTT, dithiothreitol; NTA, nitrilotriacetic acid; ELISA, enzyme-linked immunosorbent assay.

TABLE I
Primers sequences used for amplification

Primers	Sequences
HVDAC1-ATG-Bam:	5′-AAA GGA TTC ATG GCT GTG CCA CCC ACG TAT
HVDAC1-TAA-Sal	5′-AAA TAG GTC TTA TGC TTG AAA TTC CAG TCC
HVDAC2-ATG-Sac	5′-TAG GAG CTC ATG TGT ATT CCT CCA TCA TAT
HVDAC2-TAA-Sal	5′-TAG GTC GAC TTA AGC CTC CAA CTC CAG
HVDAC3-ATG-Sac	5′-TAG GAG CTC ATG TGT AAC ACA CCA ACG TA
HVDAC3-TAA-Sal	5′-TAG GTC GAC TTA AGC TTC CAG TTC AAA TCC

of human lymphocytes. Most recently, VDAC was identified in the plasma membrane of human endothelial cells, and these investigators suggested that VDAC is the receptor for plasminogen Kringle 5 in these cells (25).

Currently, there is only scant information regarding the presence and the function of VDACs in mammalian spermatozoa. It has been demonstrated that male mice in which the VDAC3 gene was inactivated by targeted disruption in embryonic stem cells are infertile (26). The animals displayed normal sperm counts but exhibited markedly reduced motility due to structural defects in the sperm flagella. Recently, we reported the sequencing of the VDAC2 (porin-2) cDNA from bovine testis and demonstrated a high identity to the murine, rabbit, and human subtypes at both the nucleotide and amino acid levels (15). mRNA analysis revealed the expression of VDAC2 in the bovine testis; high levels of VDAC2 proteins were also found in spermatocytes, spermatids, and spermatozoa. In contrast, VDAC1 (porin-1) was exclusively localized to Sertoli cells (15).

In this study, we show for the first time the extramembranous localization of VDAC type 2 and type 3 in the ODF of the sperm flagellum. The sperm tail can be subdivided into two compartments: the midpiece, which contains the mitochondria surrounding the ODF and the axoneme, and the principal piece, which contains the fibrous sheath, ODF, and the axoneme. The ODF have a distinguishable, characteristic shape, and the structure is highly insoluble. Functionally, the ODF have been suggested to maintain the passive elastic structure and elastic recoil of the sperm flagellum needed for flagellar bending and possibly also to protect it from shearing forces during epididymal transport (27). Depending on the species, mammalian ODF consist of at least 14 polypeptides with apparent molecular masses of ~11–87 kDa (28, 29). We found evidence that high amounts of VDAC2 and VDAC3 are present in the ODF of the bovine sperm flagellum. The possible role of such pore-forming VDAC proteins in sperm ODF, which represents a non-membranous structure in mammalian spermatozoa, will be discussed.

EXPERIMENTAL PROCEDURES

Preparation of Outer Dense Fiber Proteins and Protein Sequencing—Bovine ejaculates were collected with the aid of an artificial vagina and samples were frozen in liquid nitrogen without dilution and stored at −80 °C. For the enrichment of ODF fractions, spermatozoa from about 50 ejaculates were used. ODF were purified by a previously published protocol (30, 31)

Purified bovine ODF were resuspended in SDS-sample buffer. The SDS-insoluble and -soluble proteins were separated by centrifugation for 15 min at 12,000 × *g*. The supernatant was subjected to preparative SDS-PAGE. Slab gels were composed of 10% (w/v) acrylamide and 0.21% (w/v) bisacrylamide. Solubilized ODF polypeptides were visualized by staining with Coomassie Brilliant Blue G250. The prominent broad band with an apparent molecular mass of about 30 kDa was excised and stored at −70 °C until use. Elution of 30-kDa ODF polypeptides from the polyacrylamide gel, concentration of protein with an agarose-based concentration gel system, and tryptic digestion was performed as described previously (32). HPLC of the resulting peptides and microsequencing were performed as described by Ungewickell *et al.* (33).

Preparation of Recombinant Human VDAC Isoforms—Primers used for the amplification of each human porin isoform were designed at the start and stop codons. The forward primers for VDAC2 and VDAC3 introduced a SacI restriction site, while the primer for VDAC1 introduced a BamHI site upstream of the amplified products. The reverse primers for the three VDAC isoforms introduced a SalI restriction site downstream of the amplified products. These primer sequences are listed in Table I. The template used for amplification of each human VDAC isoform was the corresponding cDNA.

The PCR reaction was performed according to a standard protocol in a volume of 50 μl. The final concentration of dNTPs was 0.2 mM each, $MgCl_2$ was 1.5 mM, and the primer final concentration was 0.5 μM each. The PCR products were cloned into the linear TOPO-TA vector (Invitrogen). The fragments released from the TOPO-TA vector after digestion with the proper restriction enzymes were ligated into the expression vector pQE30 (Qiagen) to obtain the plasmids pQE30HVDAC1, pQE30HVDAC2, and pQE30HVDAC3. These plasmids coded additionally for six histidines (His tag) upstream of the multiple cloning site containing the coding sequences. Transformed cells were spread on ampicillin- and kanamycin-containing agar plates. Positive clones were picked, and the plasmid DNA was extracted from a single colony, digested, checked on a 1% agarose gel for the correct fragment size, and finally sequenced to confirm product identity.

Heterologous Expression of Human VDAC Isoforms—An overnight culture of modified *Escherichia coli* M15 cells transformed with the appropriate plasmid in LB medium was diluted 1:20 in 50 ml of fresh medium and grown to a density of 0.6 ($OD_{600\ nm}$). One ml culture was removed to serve as the non-induced control, and 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to induce expression of recombinant VDACs. After a 5-h growth period, the cells were harvested by centrifugation. The cell pellet was resuspended in 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-HCl (pH 8) and incubated for 1 h at room temperature to allow the lysis of the inclusion bodies containing the recombinant protein.

The urea suspension was centrifuged for 20 min at 10,000 rpm in an Eppendorf centrifuge, and the supernatant was collected and loaded onto a Ni-NTA spin column (Qiagen) equilibrated with the same buffer. The column was washed with the same buffer at pH 6.3 to remove proteins weakly adsorbed to the Ni-NTA. Elution was performed with the same buffer at pH 4.5. All the samples were loaded onto a 12% SDS-PAGE to check protein induction and purity of the expressed proteins.

Generation of Antibodies against Subtype-specific Synthetic Porin Peptides—The sequences of the synthetic peptides used as antigens are shown in Table II. The sequences were either derived from VDAC2 or VDAC3 amino acid sequences that were identified in bovine sperm ODF protein proteolytic peptides (Fig. 1) and/or were deduced from cDNA clones coding for bovine VDAC1, -2, and -3. Accession numbers and amino acid residue numbers were as follows: VDAC1 Bos taurus (POR1_BOVIN) P45879: amino acids 271–282; VDAC2 Bos taurus Q9MYV7: amino acids 236–247; VDAC3 Bos taurus (POR3_BOVIN) Q9MZ13: amino acids 16–25 (Fig. 2). Selection and synthesis of VDAC peptides, as well as cross-linking of peptides to KLH, were performed as described (34). Affinity purification of anti-VDAC antibodies was performed using the respective synthetic VDAC peptides that were covalently linked to AminoLink columns (AminoLink Kit, Pierce, Bonn, Germany). Binding of synthetic peptides and antibody purification was performed according to the manufacturer's recommendations. Antibody binding activity and specificity of the antibody reaction were evaluated by ELISA as described previously (35).

Protein Extraction, Gel Electrophoresis, and Immunoblotting—Bovine ejaculates were washed in PBS to separate spermatozoa from the seminal plasma. The concentration of sperm cells were adjusted to 180 × 10^6/ml and suspended in 0.6 M NaCl in PBS to remove loosely attached seminal plasma proteins from the sperm surface. Following pre-extraction in 0.2% Triton X-100 in extraction buffer (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KCl, 2 mM DTT, 1 mM EDTA, 10 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl,

pH 7.4) for 1 h at 4 °C, the spermatozoa were centrifuged at 6,000 × *g* for 30 min. The pellet was resuspended in extraction buffer containing 2% Triton X-100 and incubated for 1 h at 4 °C. Finally, the sperm suspension was centrifuged at 6,000 × *g*, and the supernatant was stored at −80 °C until use.

For the solubilization of ODF proteins, purified ODF obtained as previously described were extracted under reducing conditions using a buffer consisting of 5% (w/v) SDS, 5% β-mercaptoethanol, 1 mM EDTA, 10 mM benzamidine, 20 mg/ml Trasylol, 2 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 7.2. Recombinant and solubilized sperm protein fractions were dissolved in SDS-containing sample buffer. Protein was assayed according to the method of Lowry (36) using bovine serum albumin as a standard. SDS-PAGE was performed according to Laemmli (37) in 12% (v/v) acrylamide (Bio-Rad).

Immunoblotting using antisera and affinity-purified antibodies was performed essentially as previously described (35). Nitrocellulose membranes containing transblotted protein bands were blocked with 5% (v/v) teleostean gelatin (Sigma) in TTBS (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20). Thereafter, filters were incubated for 1 h with affinity-purified antibodies at appropriate dilutions. After extensive washing, membranes were incubated with 1:3000-diluted peroxidase-conjugated protein A for 1 h. Specific binding of antibodies was visualized by the enhanced chemiluminescence Western blotting detection reagents (ECL; Amersham Biosciences) and subsequent exposure to x-ray film (Fuji Photo Film, Düsseldorf, Germany) as recommended by the supplier. The specificity of the reaction of the polyclonal antiserum AS P1/6 was determined by pre-absorption with the peptide employed as immunogen. The antiserum was preincubated at 1:200 dilution with 10 μg of P1 peptide/ml for 1 h at room temperature and subsequently assayed by immunoblotting.

Immunofluorescence—Bovine spermatozoa from fresh ejaculates were washed twice by centrifugation. After the second wash, the concentration of spermatozoa was adjusted to 20–30 × 10^6 cells/ml with PBS (pH 7.4). Subsequently, sperm cells were frozen/thawed three times using liquid nitrogen and a 38 °C water bath. Thereafter, spermatozoa were spread across a glass slide (covered with 0.5% gelatin, 0.05% potassium chromate (III) sulfate in H_2O), air-dried, and fixed for 3 min in a solution consisting of 60% (v/v) methanol, 30% (v/v) chloroform, and 10% (v/v) acetic acid (methacarn). Fixed spermatozoa were incubated with affinity-purified P2/45 anti-VDAC2 antibodies (15 μg/ml) in phosphate-buffered saline, pH 7.4, supplemented with 1% bovine serum albumin (PBS-BSA) or, as a control, with non-relevant rabbit IgG antibodies (15 μg/ml in PBS-BSA) for 1 h at room temperature. After washing, the cells were incubated with biotinylated goat anti-rabbit IgG antibodies (Sigma) (1:2000 dilution) for 30 min at room temperature. Specific binding of antibodies was visualized using fluorescein isothiocyanate-labeled avidin (Sigma) (1:400) for 30 min at room temperature. After washing, spermatozoa were mounted onto coverslips using Citifluor (Plano, Wetzlar, Germany) to avoid fading of fluorescence. Spermatozoa were examined for fluorescence using a Zeiss fluorescence microscope.

Another immunofluorescence study was performed with cryopreserved bovine spermatozoa after removal of the mitochondrial sheath to reveal ODF epitopes (29). Briefly, cryopreserved spermatozoa (40 × 10^6 cells) were thawed in a water bath at 39 °C for 30 s and washed 3 × 5 min with 2 ml of PBS by centrifugation at 300 × *g*. The pellet was incubated 2 × 15 min at 4 °C with 4 ml of extraction buffer (2% v/v Triton X-100, 50 mM Tris, pH 9.0, 1 mM EDTA, 2 mM DTT, 0,2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine). After each extraction, the sperm suspension was centrifuged at 1000 × *g* and 4 °C for 10 min. The last pellet was washed 2 × 10 min with 2 ml of 50 mM Tris-HCl (pH 9.0) by centrifugation at 1000 × *g* and 4 °C. The spermatozoa were resuspended in 200 μl of PBS and spread across a glass slide (see above). Subsequently, detection of antibody binding was visualized by immunofluorescence as described above using the 1:50 diluted AS P3/31 antiserum. As negative control the respective pre-immune serum was used.

Immunoelectron Microscopy—The preparation of bovine spermatozoa and preparations of ultrathin sections for immunoelectron microscopy has been described previously (38). Sections were exposed to antiserum AS P2/20 (15) (1:5 dilution in TBS) or the respective preimmune serum (1:5 dilution in TBS) for 1 h at room temperature. The specimens were subsequently incubated with goat anti-rabbit IgG antibodies conjugated with colloidal gold (particle size of 10 nm) (Bio Cell, Cardiff, UK) at a 1:10 dilution for 45 min. Finally, sections were stained for 20 min with 2% (w/v) uranyl acetate, viewed, and photographed with a Philips 201 G transmission electron microscope operated at 80 kV.

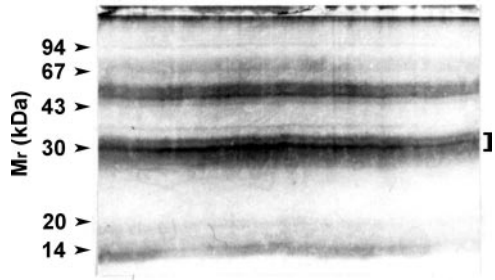

FIG. 1. **SDS-PAGE of bovine ODF proteins.** SDS-extracted bovine ODF proteins (450 μg) were separated by SDS-PAGE using a preparative comb. Solubilized ODF polypeptides were visualized by staining with Coomassie Brilliant Blue. The prominent 30–32-kDa band (*bracket*) was excised for subsequent digestion of proteins and peptide sequencing. The position of M_r standards is indicated on the *left*.

RESULTS

Sequencing of 30–32 kDa ODF Proteins—The polypeptides from purified ODF were separated by preparative SDS-PAGE under reducing conditions (Fig. 1). The enriched ODF fraction consisted of six main polypeptides with apparent molecular masses of 14, 28, 30, 32, and 50/52 kDa. In addition, some minor, well-focused and broad bands between 28 and 94 kDa were also visible.

The broad 30–32-kDa band was excised and digested with trypsin. Proteolytic peptides were separated by HPLC and subjected to microsequencing. As shown in Fig. 2, sequencing of peptides derived from the 30–32-kDa band yielded four amino acid sequences. Comparison with published amino acid sequences revealed identical or similar sequences to VDAC2 and VDAC3. The amino acid sequences of peptides 1 and 2 are in agreement with the bovine VDAC3 sequence (position 16–25). However, the first and the last amino acid residues differed from the published VDAC1, -2, and -3 sequences. The amino acid sequence of peptide 3 is identical to the sequence of all three isoforms (positions 218–223 of VDAC1). Peptide 4 is identical to the bovine VDAC2 amino acid sequence (positions 236–247).

To further identify the broad 30–32 kDa protein band from bovine ODF, Western blot analyses of extracted bovine sperm proteins were performed. Using antisera AS P1/6, AS P2/45, and AS P3/31, immunoblots with 0.2% Triton X-100 pre-extracted sperm proteins revealed that only traces of VDAC proteins could be identified (data not shown). Sperm proteins extracted in 2% Triton X-100 and an SDS extract of ODF were subjected to SDS-PAGE, blotted, and immunoreacted with a monoclonal antibody against the N-terminal end of human VDAC (mAb 4 anti-Porin 31HL, Ref. 39) and an antiserum generated by use of a synthetic VDAC1 peptide (AS P1/6). As a control, purified recombinant VDAC proteins were run on the same gels. As the blots clearly show (Fig. 3), AS P1/6 antibodies recognized a polypeptide of about 34 kDa in the Triton X-100 extract. No immunoreaction could be observed with the SDS extract of ODF or with the purified recombinant VDAC2 and VDAC3 proteins, while AS P1/6 strongly detected recombinant VDAC1 and Porin 31HL purified from human lymphocytes (39). Two additional, faint bands were also detected in the human lymphocyte VDAC preparation by AS P1/6, just above and below the broad band at 34 kDa. Also in the recombinant VDAC1 sample, two additional, unknown bands were detected. The specificity of the reaction was controlled by preincubating AS P1/6 with the synthetic VDAC1 peptide that was employed as an immunogen. When the peptide was preincubated with the antiserum, the interaction of AS P1/6 with immunoreactive proteins in the Triton X-100 extract of bovine spermatozoa were completely abolished, indicating that the polypeptides

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VDAC1_BOVIN     1 ------------AVVRPYADLGKSARDVFTKGYGFGLIKLDLKTKSENGLEFTSS
VDAC2_BOVIN     1 MATHGQNCARPMCIPPSYADLGKAARDIFNKGFGFGLVKLDVKTKSCSGVEFSTS
VDAC3_BOVIN     1 -----------MCNTPTYCDLGKAAKDVFNKGYGFGMVKIDLRTKSCSGVEFSTS
                                          SVFNKGYGFM
                                           (1)  (2)

VDAC1_BOVIN    44 GSANTETTKVTGSLETKYRWTEYGLTFTEKWNTDNTLGTEITVEDQLARGLKLTF
VDAC2_BOVIN    56 GSSNTDTGKVTGTLETKYKWCEYGLTFTEKWNTDNTLGTEIAIEDQICQGLKLTF
VDAC3_BOVIN    45 GHAYTDTGKASGNLETKYKICNYGLTFTQKWNTDNTLGTEISWENKLAEGLKLTL

VDAC1_BOVIN    99 DSSFSPNTGRKNAKIKTGYKREHINLGCDVDFDIAGPSIRGALVLGYEGWLAGYQ
VDAC2_BOVIN   111 DTTFSPNTGKKSGKIKSSYKRECINLGCDVDFDFAGPAIHGSAVFGYEGWLAGYQ
VDAC3_BOVIN   100 DTIFVPNTGKKSGKLKASYKRDCFSLGSNVDIDFSGPTIYGWAVLAFEGWLAGYQ

VDAC1_BOVIN   154 MNFETAKSRVTQSNFAVGYKTDEFQLHTNVNDGTEFGGSIYQKVNKKLETAVNLA
VDAC2_BOVIN   166 MTFDSAKSKLTRNNFAVGYRTGDFQLHTNVNDGTEFGGSIYQKVCEDLDTSVNLA
VDAC3_BOVIN   155 MSFDTAKSKLSQNNFALGYKAADFQLHTHVNDGTEFGGSIYQKVNEKIETSINLA

VDAC1_BOVIN   209 WTAGNSNTRFGIAAKYQIDPDACFSAKVNNSSLIGLGYTQTLKPGIKLTLSALLD
VDAC2_BOVIN   221 WTSGTNCTRFGIAAKYQLDPTASISAKVNNSSLIGVGYTQTLRPGVKLTLSALVD
VDAC3_BOVIN   210 WTAGSNNTRFGIAAKYKLDCRTSLSAKVNNASLIGLGYTQTLRPGVKLTLSALID
                            FGIAAKYQLDPTASISAK
                              (3)       (4)

VDAC1_BOVIN   264 GKNVNAGGHKLGLGLEFQA
VDAC2_BOVIN   276 GKSINAGGHKLGLPLELEA
VDAC3_BOVIN   265 GKNFNAGGHKVGLGFELEA
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FIG. 2. **Alignment of bovine VDAC sequences and localization of peptides derived from the 30–32-kDa ODF polypeptide.** Numbers indicate the sequences obtained by Edmann degradation of proteolytic peptides from the 30–32-kDa band. Sequences highlighted in *gray* were used to raise antisera in rabbits. *Boxed sequences* indicates peptide 3 that is present in all three VDAC isoforms. *Underlined* sequences represent the similarity of the sequenced peptide to the published sequence. The accession numbers of the proteins used in the alignment are as follows: VDAC1, P45879; VDAC2, Q9MYV7; VDAC3, Q9MZ13.

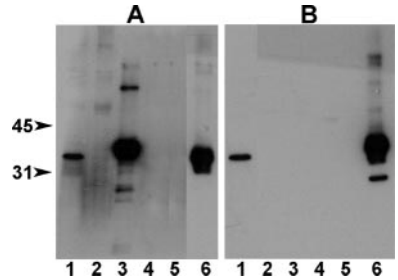

FIG. 3. **Immunoblotting of VDAC1 from bovine spermatozoa.** Triton X-100 extract of bovine spermatozoa (*lane 1*, 30 μg/lane), SDS extract of bovine ODF (*lane 2*, 20 μg/lane), recombinant VDAC1 (*lane 3*), VDAC2 (*lane 4*), and VDAC3 (*lane 5*) protein (each 1 μg/lane) and Porin 31HL purified from human lymphocytes (*lane 6*, 2 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated respectively with antiserum AS P1/6 (*panel A*, serum dilution 1:200) or with the monoclonal anti-N end VDAC1 mAb 4 (*panel B*, mAb dilution 1:200). Immunoblotting was performed as described under "Experimental Procedures." The position of M_r standards is indicated on the *left*.

TABLE II
Peptides sequences chosen for antibodies

Antibodies	Sequences[a]
AS P1/6	GHKLGLGLEFQA
AS P2/45	YQLDPTASISAK[4]
AS P3/31	SVFNK[1]GYGFM[2]
AS P_{common}	GHKVGLGFEL

[a] Peptides 1, 2, 3 (FGIAAK), and 4 derived from the 31-kDa ODF polypeptide.

were recognized specifically by antibodies directed against the corresponding hapten (data not shown).

The anti-N-terminal VDAC1 mAb 4 recognized a protein in the Triton X-100 extract with the same apparent molecular mass as that detected by AS P1/6 (Fig. 3*B*). As has been shown with AS P1/6, there was no reaction against proteins extracted from ODF. Monoclonal antibody 4 also reacted with Porin 31HL, which was the antigen used for its generation. Also here, the broad band detected by mAb 4 seems to consist of a major protein of about 34 kDa and two minor bands above and below. An additional band of about 28 kDa could represent a proteolytic fragment of VDAC1 as observed in De Pinto *et al.* (40). VDAC1 mAb 4 did not react with any of the three recombinant VDACs that bear a His-tag at the N-terminal end. This result indicates that the presence of a His tag in VDAC1 causes steric hindrance and thus prevents antibody recognition of the epitope.

Generation and Characterization of Antisera against VDAC1, -2, and -3 Synthetic Peptides—Antisera were generated in rabbits against synthetic peptides corresponding to specific VDAC1, -2 and -3 epitopes (Table II and Fig. 2). We were also interested in generating antibodies against synthetic peptides with amino acid sequences identical to the proteolytic peptides isolated from bovine ODF. Final selection of the synthetic peptides was based on their antigenicity as predicted by analysis of the primary structure.

The specificity of anti-VDAC peptide antibodies generated was determined by ELISA (data not shown). Antisera containing specific anti-VDAC peptide antibodies that yielded high titers were chosen for further evaluation of specificity and for affinity purification.

In Fig. 2, the position of the peptides used as antigens is indicated in an alignment of the three VDAC isoforms. The reactivity of anti-VDAC antisera with VDAC proteins was analyzed by immunoblotting (Fig. 4, *A–D*). Using recombinant VDAC proteins, Fig. 4 shows the specificity of affinity-purified anti-VDAC peptide antibodies. A 2% Triton X-100 extract from bovine spermatozoa and a 5% SDS extract from purified bovine ODF were applied to identify bovine VDAC proteins in spermatozoa.

As demonstrated by their reactivity with recombinant proteins (human VDAC1, VDAC2, and VDAC3 sequences fused with an N-terminal His tag), affinity-purified antibodies from three antisera contained specific antibodies for the three VDAC isoforms (Fig. 4, *A–D*, VDAC1, -2, and -3; *lanes 3*, *4*, and *5*, respectively). Affinity-purified P1/6 antibodies reacted only with recombinant VDAC1, a polypeptide with an apparent molecular mass of 34 kDa (Fig. 4*A*, *lane 3*). P2/45 antibodies recognized solely recombinant VDAC2 that displayed an apparent molecular mass of 33 kDa (Fig. 4*B*, *lane 4*), and P3/31 antibodies detected only the VDAC3 isoform, a protein with an apparent molecular mass of 32 kDa (Fig. 4*C*, *lane 5*). Antibodies from a fourth antiserum, P_{common}, reacted with all three VDAC isoforms (Fig. 4*D*). It is important to note that even after long exposure of films, specific anti-VDAC isoform antibodies did not cross-react with other VDAC proteins.

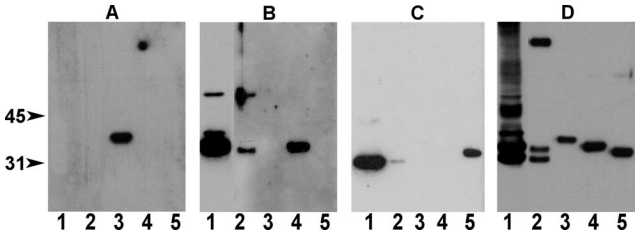

FIG. 4. **Characterization of antisera against VDAC1, -2, and -3 and identification of VDAC proteins in bovine spermatozoa as determined by immunoblotting.** Triton X-100 extract of bovine spermatozoa (*lane 1*, 30 μg/lane), SDS extract of bovine ODF, (*lane 2*, 20 μg/lane) and recombinant VDAC1 (*lane 3*), VDAC2 (*lane 4*), and VDAC3 (*lane 5*) proteins (each 1 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with affinity-purified antibodies P1/6 (*A*, 0.7 μg/ml), P2/45 (*B*, 1.3 μg/ml), P3/31 (*C*, 3.6 μg/ml) or P_{common} (D, 2.6 μg/ml). Immunoblotting was performed as described under "Experimental Procedures." The position of M_r standards is indicated on the *left*.

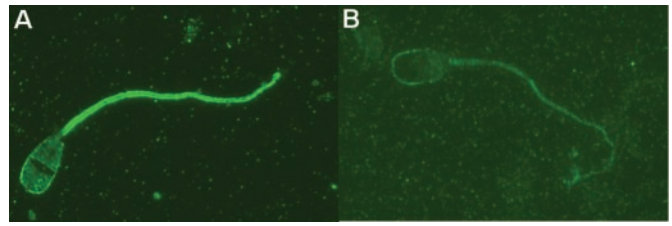

FIG. 5. **Immunolocalization of VDAC2 in the bovine spermatozoon.** Indirect immunofluorescence microscopy of methacarn-fixed bovine spermatozoa smears was performed. *A*, cells were treated with affinity-purified P2/45 antibodies (15 μg/ml). *B*, control experiments were performed with non-relevant rabbit IgG (15 μg/ml). Bound antibody was detected with biotinylated goat anti-rabbit IgG antibodies (dilution 1:2000) and fluorescein isothiocyanate-labeled avidin (dilution 1:400). A typical immunofluorescence pattern of bound anti-VDAC2 antibodies to the bovine spermatozoon is shown. Original magnification ×630.

Immunoblot analysis with affinity-purified antibodies indicates the presence of two VDAC isoforms, VDAC2 and VDAC3, in sperm protein fractions (Fig. 4, *A–D*, *lanes 1* and *2*).

In the Triton X-100 extract, a strong immunoreaction with a polypeptide of about 33 kDa was observed with anti-VDAC2 P2/45 antibodies (Fig. 4*B*, *lane 1*). Additionally, two proteins with apparent molecular masses of 36 and about 60 kDa were detected. In ODF (Fig. 4*B*, *lane 2*), two immunoreactive proteins of 33 and about 60 kDa were visible. With the use of anti-VDAC3 P3/31 antibodies, a single, broad, 32-kDa VDAC3 protein band was visible in the Triton X-100 extract (Fig. 4*C*, *lane 1*); a faint but clear immunoreactive polypeptide with an identical apparent molecular mass was observed in the ODF protein fraction. Affinity-purified P1/6 antibodies did not detect VDAC1 protein, neither in bovine sperm Triton X-100 extract nor in the ODF protein fraction. It should be noted, however, that VDAC1 is present in bovine spermatozoa because it was immunostained by the antiserum AS P1/6 in Triton X-100-solubilized sperm proteins (Fig. 3). This can be explained by a lower titer of affinity-purified antibodies or by a loss of particular antibodies during the purification process. P_{common} antibodies mainly reacted with polypeptides of 32 and 33 kDa in Triton X-100 extracts, most probably representing VDAC3 and VDAC2, respectively, and with a 36-kDa polypeptide (Fig. 4*D*, *lane 1*). This protein might be VDAC1 or the 36-kDa polypeptide detected by P2/45 antibodies (Fig. 4*B*, *lane 1*). Several unknown, immunoreactive bands with apparent molecular masses above 36 kDa were also visible in this lane. In the ODF extract, P_{common} antibodies react with VDAC3 (32 kDa) and VDAC2 (33 kDa) and with a very high M_r polypeptide of unknown identity (Fig. 4*D*, *lane 2*).

Localization of VDAC Protein in the Sperm Flagellum using Immunofluorescence—Indirect immunofluorescence was carried out with smears of methacarn-fixed bovine spermatozoa. The cells were incubated with affinity-purified P2/45 antibodies. A typical immunofluorescence pattern of bovine spermatozoa is shown in Fig. 5. P2/45 antibodies exhibited specific staining of the midpiece and the principal piece of the flagellum (Fig. 5*A*). A bright staining throughout the tail without distinction of the mitochondria-containing midpiece and the principal piece was observed. Barely visible was a less intense but more patchy fluorescence pattern in the sperm head region with a non-reactive area at the equatorial segment. Using the methacarn fixation protocol, AS P3/31 and AS P1/6 antibodies did not show any immunofluorescence above background staining. It should be noted that the antibodies used in this study are directed against defined linear epitopes of not more than 12 amino acid residues. Binding of antibodies depends on the proper presentation of the antigenic epitopes; especially in immunohistochemical studies, the anti-peptide antibodies might not detect the protein because it is not sufficiently unfolded, and the particular epitope is not available for antibody binding. Control experiments with non-relevant rabbit IgG yielded a low background staining of the entire spermatozoon (Fig. 5*B*). In addition, almost no staining was detected with spermatozoa used in control experiments when the first antibody was omitted (not shown).

For further characterization of the structures detected by anti-VDAC antibodies in the bovine sperm flagellum, the cells were preincubated with 2% Triton X-100 prior to smear and fixation. This procedure made it possible to eliminate mitochondria from the sperm and expose the ODF in the midpiece region. The above mentioned effect of this procedure was documented by electron microscopy of Triton X-100-treated spermatozoa (not shown). For the detection of VDAC3 protein, AS P3/31 was used. As demonstrated in Fig. 6, *A* and *B*, the antibodies clearly bound to filamentous structures that most probably are the ODF. Fig. 6*A* shows that almost all Triton X-100-treated spermatozoa showed a disintegrated midpiece with strongly stained filament structures. At higher magnification, 9 filaments slipping out of the midpiece were able to be distinguished. No staining was detected in the principal piece. This result indicates that the epitope detected by the antibodies is, possibly due to the hindrance of sperm structures that cover the ODF in the principal piece, not available for antibody binding. This assumption is strengthened by the observation that, if the axoneme and fibrous sheath are removed by other detergents, AS 3/31 antibodies bind to the entire length of the ODF (data not shown). In control experiments with the respective pre-immune serum (Fig. 6, *C* and *D*), only a low background staining of the sperm tail was visible. Compared with AS P3/31-stained cells, no considerable differences in staining intensity of the sperm head was observed in the control smear.

It is important to mention that neither anti-VDAC1 nor anti-VDAC2 antibodies bound to ODF of spermatozoa that were treated with Triton X-100, indicating that the antigenic epitope was not available under this experimental condition.

Immunoelectron Microscopic Localization of VDAC2 in Bovine ODF—The ultrastructural appearance of VDAC2 protein in bovine flagella was investigated with immunoelectron microscopy (Fig. 7). In ultrathin sections of bovine spermatozoa, the sites of immunoreaction of AS P2/20 were found in the ODF and in the mitochondria-containing midpiece (Fig. 7, *A* and *B*). Plaque-like deposition of gold particles was observed in ODF of an obliquely cut section of the flagellum (Fig. 7*A*, *arrowheads*); a more circular pattern of gold particle deposition can be seen in mitochondria (M, *arrowheads*). As demonstrated at a higher

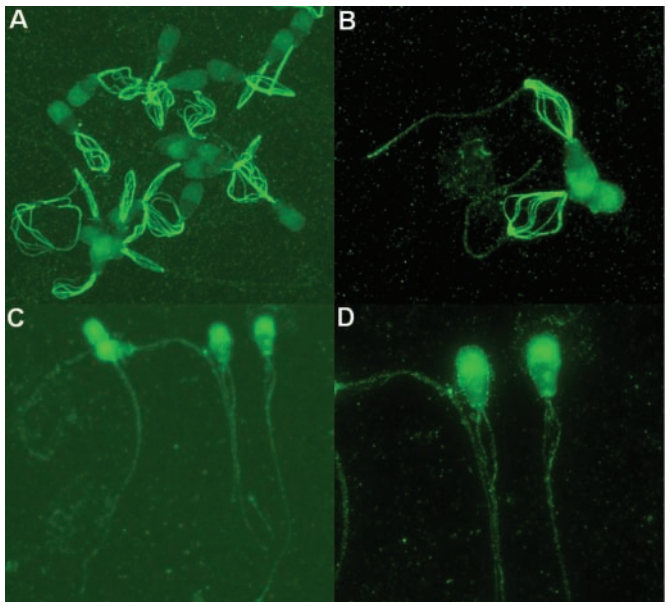

FIG. 6. **Immunolocalization of VDAC3 in Triton X-100-pretreated bovine spermatozoa.** Indirect immunofluorescence microscopy of Triton X-100-treated bovine spermatozoa smears was performed. *A* and *B*, cells were treated with AS P3/31 antibodies (dilution 1:50). *C* and *D*, control experiments were performed with the respective preimmune serum (dilution 1:50). Bound antibody was detected with biotinylated goat anti-rabbit IgG antibodies (dilution 1:2000) and fluorescein isothiocyanate labeled avidin (dilution 1:400). A typical immunofluorescence pattern of bound anti-VDAC3 antibodies to the bovine sperm ODF is shown. Original magnification: *A* and *C*, ×400; *B* and *D*, ×630.

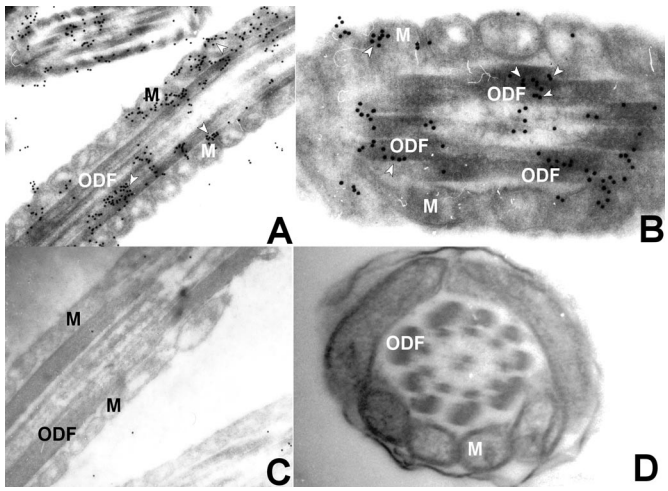

FIG. 7. **Transmission electron micrographs of ultrathin sections of bovine spermatozoa incubated with anti-VDAC2 antiserum AS P2/20.** Specifically bound antibodies were detected by immunogold labeling (dilution of gold-labeled anti-rabbit antibodies 1:10). *A*, oblique and transversal sections of a bovine flagellum incubated with anti-VDAC2 antiserum AS P2/20 (dilution 1:5). *M*, mitochondrion; *ODF*, outer dense fiber. Original magnification was ×8910. *B*, transversal section of a bovine flagellum incubated with anti-VDAC2 antiserum AS P2/20 (dilution 1:5). *M*, mitochondrion; *ODF*, outer dense fiber. Original magnification was ×17690. *C* and *D*, control experiments were performed with the respective preimmune serum (dilution 1:5; original magnifications: *C* ×8910 and *D* ×17690.

magnification in Fig. 7*B*, a transverse section of a sperm tail midpiece revealed immunoreactive material in or close to the ODF (*arrowheads*) as well as in mitochondria (M, *arrowheads*). Only a few randomly disposed gold grains were observed in control sections that were incubated with the respective preimmune serum (Fig. 7, *C* and *D*). It should be noted that AS P2/20 antibodies no longer reacted with their antigen when spermatozoa were fixed in a way conducive to better preserved subcellular structures. Only AS P2/20 was applicable for immunoelectron microscopic studies because the other anti-VDAC antibodies presented in this study did not recognize the protein (also when different cell fixation protocols were used).

DISCUSSION

In our search to identify the protein composition of bovine ODF, we extracted ODF proteins and sought to investigate the nature of one of the most abundant proteins in our preparation, an as yet unidentified 30–32-kDa polypeptide. By limited proteolysis of this protein band excised from the gel, we identified amino acid sequences matching a VDAC/porin cDNA present in the public databases. This was the first clue for the presence of VDAC(s) in the ODF. Interestingly, in our experimental data we identified peptides with amino acid sequences present both in VDAC2 and VDAC3 but not VDAC1. In a recent study, we reported the localization of VDAC1 and 2 isoforms in the bovine testis and in ejaculated bovine spermatozoa (15). However, the subcellular localization of VDAC isoforms in spermatozoa had not been investigated. To obtain further evidence for the presence of VDAC proteins in ODF, we generated antibodies specific for VDAC subtypes 1 (P1/6), 2 (P2/45), and 3 (P3/31) as well as an antiserum against all three VDAC proteins (P_{common}). To our knowledge, this is the first report of the generation of truly monospecific antibodies for all three VDAC isoforms. Monoclonal and polyclonal anti-VDAC antibodies have been previously reported (39), however, in many instances these antibodies were generated against VDAC1 or VDAC3 as antigens, and their specificity often has been not sufficiently established.

We used our set of highly specific anti-VDAC antibodies to investigate the subcellular distribution of their respective antigens in bovine sperm. First, we demonstrated by immunoblotting that all three VDAC subtypes are present in a 2% Triton X-100 bovine sperm extract. By electron microscopy we observed that the Triton extraction yields remnants of spermatozoa that still contained ODF but lack acrosomal, plasmalemmal, intracellular, and mitochondrial structures (data not shown). Interestingly, affinity-purified antibodies against the VDAC1 synthetic peptide did not detect VDAC1 in the 2% Triton X-100 sperm extract although the respective antiserum clearly reacts with a 34-kDa polypeptide; the specificity of the reaction obtained with the antiserum was demonstrated by successful pre-absorption of the antibodies with the VDAC1 peptide. This discrepancy cannot be resolved completely but might be explained by a lower titer of the affinity-purified antibodies or by a selection of a subpopulation of antibodies (*e.g.* clones with higher and lower affinity or reacting with different VDAC1 peptide epitopes) through affinity purification.

In SDS extracts of purified bovine ODF, however, VDAC subtype-specific antibodies detected solely VDAC2 and VDAC3 proteins. These data are in agreement with our finding that only VDAC2 and VDAC3 peptide sequences could be identified in the 30–32-kDa protein band that was most abundant in the purified bovine ODF. This is the first biochemical evidence to date for the presence of pore-forming VDAC proteins in ODF, a cellular structure that presumably does not contain membranous components.

To rule out any possibility of mitochondrial protein contamination in purified ODF and to assess the subcellular localization of VDAC isoforms, we performed immunohistochemical studies. Immunofluorescence microscopy clearly showed that the VDAC2 protein is present in the sperm tail. Immunotransmission electron microscopy gave additional evidence for the finding that VDAC2 proteins are abundant in ODF. On the other hand, studies with detergent-treated spermatozoa that

are devoid of mitochondria revealed that anti-VDAC3 antibodies clearly bind to structures that most probably represent ODF.

ODF are subcellular components exclusively present in mammalian spermatozoa. Biochemical and immunological analyses have revealed that bovine ODF contain sperm keratins (31). Presently it is thought that the ODF mainly consists of cytoskeletal proteins and lacks membranes. Hence, this is the first biochemical evidence to date for the presence of VDAC isoforms in ODF and the first observation of such proteins in a non-membranous compartment.

There are examples of VDAC proteins with apparently distinct properties. In striking contrast with the yeast porin YVDAC1, the second porin isoform in yeast, YVDAC2, does not form channels in reconstituted systems although it functionally complements growth defects associated with the loss of YVDAC1 (12). These results indicate that YVDAC2 may not normally form a channel and suggest that YVDAC2 mediates other cellular functions. Xu *et al.* (14) expressed mouse VDAC isoforms in a *S. cerevisiae* strain lacking the major yeast VDAC gene. Subsequently, VDAC proteins were purified from mitochondria, and their functional features were characterized. In this work, VDAC3 showed different permeability properties in comparison with VDAC1 and 2 (14). Upon reconstitution of the purified mitochondrial VDAC3 into liposomes, an increase in the permeability of the vesicles was recorded. In contrast, VDAC3 was not able to form channels in planar lipid bilayers, most likely because it did not insert into the membrane (14).

A number of previous reports suggest that VDAC functions are modulated by and interact with cytoskeletal proteins. Microtubule-associated proteins (MAPs) bind *in vitro* to specific sites on the mitochondrial outer membrane of purified brain mitochondria and mediate formation of cross-bridges between these cellular organelles and microtubules (3). MAP2 and VDAC can be coextracted from outer mitochondrial membranes. Furthermore, MAP2 induces changes in the outer membrane environment and partially prevents the extraction of VDAC from the outer membrane. These observations suggest an association between VDAC and the membrane domain involved in the cross-linking between microtubules and mitochondria (21, 41).

Further evidence for the relationship between VDAC1 and microtubules was reported by Schwarzer *et al.* (42). Using VDAC1 as bait protein in two-hybrid screening and overlay assays, interaction of VDAC1 with the dynein light chain Tctex 1 was demonstrated. Tctex 1, as a component of a dynein motor complex, could be involved in the transport mechanism along the cytoskeleton. Tctex proteins are also present in the axoneme of mouse spermatozoa (43). Tctex 1 and 2 proteins, assembled with the inner and outer dynein arms, respectively, are essential components involved in the phenomenon of transmission ratio distortion, which is a form of meiotic drive of mouse *t* haplotypes involving dysfunction of both flagellar inner and outer dynein arms but not requiring the cytoplasmic isoenzyme (43). Because ODF are closely associated with the axoneme that contains the dynein light chain Tctex 1 and microtubules, VDAC proteins could play a role in the regulation of sperm motion or sperm tail structural integrity through an interaction with Tctex proteins or MAPs. Another kinesin, kinesin light chain 3 (KLC3), has been demonstrated to be associated with ODF. Interestingly, its binding partner is one of the major ODF proteins, ODF1, indicating that KLC3 interacts with cytoskeletal proteins in a microtubule-independent fashion (44). It is important to note that kinesins cannot be strictly assigned to the microtubular system.

The hypothesis that VDAC protein function in spermatozoa is associated with the maintenance of flagellar cytoskeletal integrity and function is strengthened by a recent publication demonstrating that mice lacking VDAC3 are healthy but produce immotile sperm and are thus infertile (26). The sperm count of VDAC3-deficient mice was not decreased but structural defects were evident in two-thirds of the axonemes of epididymal spermatozoa. In testicular sperm, the defect was only rarely observed. The normal structures found in spermatids suggest that the defect develops with maturation of spermatozoa and initiation of sperm motility in the transition from the testes to the epididymis. Despite the reports that depletion of VDAC3 leads to axonemal defects, the precise function of VDAC3 protein in sperm ODF remains to be elucidated.

Evidence exists for ATP transport and an ATP binding site in VDAC (45, 46). Thus, a hypothesis emerging from our data could be that the highly abundant VDAC proteins present in ODF might be involved in the maintenance and adaptation of ATP levels in the sperm flagellum. This might include ATP transport from the mitochondria in the sperm midpiece to the very distal dynein ATPases in the principal piece. Anaerobic production of ATP in the bovine sperm tail has been shown but in swine, anoxic conditions lead to an immediate loss of sperm progressive motility (47). Thus, permanent availability of mitochondrial energy to dynein ATPases in the form of ATP seems to be mandatory for sperm forward motility. Because of its close localization to the energy-demanding axoneme, the ODF could serve as a storage battery that constantly supplies the axoneme with energy and immediately provides high levels of ATP when it is needed. It is now intriguing to speculate that, considering the high abundance of VDAC2 and VDAC3 in bovine ODF and their capacity to gate and to bind ATP, these polypeptides might be involved in trafficking and channeling of ATP to the most distal dynein ATPases in the principal piece and/or in protecting ATP from premature hydrolysis. The data presented in this study suggest that both VDAC2 and VDAC3 are abundant in ODF and thus might be involved in the regulation of sperm motility. Because the presence of membranes that could host pore-forming polypeptides is highly improbable, we propose from our data that VDAC2 and VDAC3 might be organized in different structures and perform other functions in ODF than in cell membranes. The spermatozoon is a highly differentiated cell with independent compartments, *i.e.* sperm head, midpiece, and principal piece. Plasma membrane components of the sperm head seem to be discontinuous with plasma membrane components of the sperm tail. Thus, it is interesting to note that, in our hands, VDAC isoforms are apparently localized in distinct compartments. ODF contain VDAC2 and 3 but are apparently devoid of VDAC1. VDAC2 is also present in mitochondria (Fig. 7), while we do not have direct evidence for a likely presence of VDAC1 and 3 in mitochondria. The most abundant VDAC isoform in sperm cells seems to be VDAC2. We are presently working on its purification from sperm extracts.

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Voltage-dependent Anion-selective Channels VDAC2 and VDAC3 Are Abundant Proteins in Bovine Outer Dense Fibers, a Cytoskeletal Component of the Sperm Flagellum

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