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# Voltage-Dependent Anion Channel Proteins in Synaptosomes of the Torpedo Electric Organ: Immunolocalization, Purification, and Characterization[1]

**Iris Shafir,[2] Wei Feng,[2,3] and Varda Shoshan-Barmataz[2]**

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In this study, we purified and characterized the voltage-dependent anion channel (VDAC) from the Torpedo electric organ. Using immunogold labeling, VDAC was colocalized with the voltage-gated $Ca^{2+}$ channel in the synaptic plasma membrane. By immunoblot analysis, five protein bands in synaptosomes isolated from the Torpedo electric organ cross reacted with two monoclonal anti-VDAC antibody. No more than about 7 to 10% mitochondrial contains could be detected in any synaptosomal membrane preparation tested. This was estimated by comparing the specific activity in mitochondria and synaptosomes of succinate–cytochrome-*c* oxidoreductase and antimycin-insensitive NADH–cytochrome-*c* oxidoreductase activities; mitochondrial inner and outer membrane marker enzymes, respectively. [$^{14}$C]DCCD (dicyclohexylcarbodiimide), which specifically label mitochondrial VDAC, labeled four 30–35 kDa protein bands that were found to interact with the anti-VDAC antibody. The distribution of the Torpedo VDAC protein bands was different among membranes isolated from various tissues. VDAC was purified from synaptosomes and a separation between two of the proteins was obtained. The two purified proteins were characterized by their single channel activity and partial amino acid sequences. Upon reconstitution into a planar lipid bilayer, the purified VDACs showed voltage-dependent channel activity with properties similar to those of purified mitochondrial VDAC. Amino acid sequence of four peptides, derived from VDAC band II, exhibited high homology to sequences present in human VDAC1 (98%), VDAC2 (91.8%), and VDAC3 (90%), while another peptide, derived from VDAC band III, showed lower homology to either VDAC1 (88.4%) or VDAC2 (79%). Two more peptides show high homology to the sequence present in mouse brain VDAC3 (100 and 78%). In addition, we demonstrate the translocation of ATP into synaptosomes, which is inhibited by DCCD and by the anion transport inhibitor DIDS. The possible function of VDAC in the synaptic plasma membrane is discussed.

**KEY WORDS:** VDAC; Torpedo; ion channels; ATP transport; porin.

## INTRODUCTION

Torpedo is a marine electric fish generating high-power currents used for stunning prey. The source of the electric current is the interplay of nicotinic acetyl-

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; Syn, synaptosomes; VDAC, voltage-dependent anion channel; PLB, planar lipid bilayer; NP-40, nonidet PN-40; PBS, phosphate-buffered saline; PMSF, phenylmethyl-sulfonyl fluoride; DIDS, 4,4′-diisothiocyanostilbene/2,2′-disulfonic acid; DCCD, dicyclohexylcarbodiimide; WRK, 1-ethyl-3-[3-dimethylanime]prophyl)-carbodiimide.

[2] Department of Life Sciences and the Zlotowski Center for Neuroscience, Ben Gurion University of the Negev, Beer Sheva, Israel.

[3] Current address: Department of Molecular Biosciences School of Veterinary Medicine University of California, Davis, Davis, California 95616-8741.



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choline receptor/cation channel and the voltage-gated $Cl^-$ channels operating in the plasma membrane of specialized cells in the electroplax organ (Miller and Whote, 1984). The voltage-gated $Cl^-$ channel has been extensively characterized at the single-channel level following reconstitution into planar lipid bilayers (Jentsch *et al.*, 1990), and has been cloned and functionally expressed (Galze *et al.*, 1991). Acetylcholine receptor from Torpedo has been isolated and well-characterized at the structural and functional levels (Edry-Schiller *et al.*, 1991). Electrophysiological analysis using voltage-clamp studies demonstrates the presence of other ionic channels in the plasma membrane of the electroplax cells, such as the voltage-dependent potassium channel (Meir and Rachamimoff, 1996), the calcium-permeable ion channel (Neumann *et al.*, 1996), and the $Na^+$ channel (Woodbuty, 1995).

In this study we demonstrate the presence of the voltage-dependent anion channel (VDAC), referred to as the mitochondrial porin, in the plasma membrane of synaptosomes derived from the electric organ of Torpedo *marmorata* and Torpedo *oscellata*.

VDAC from the mitochondrial outer membrane is a well-characterized protein. It has been purified and characterized following its reconstitution into a planar lipid bilayer (PLB) (Colombini, 1989, 1994; Mannella *et al.*, 1992). Due to its large pore, VDAC is presumed to play an important role as a controlled passage for adenine nucleotides and other metabolites to and from mitochondria. This has been recently shown by demonstrating direct ATP (Rostovtseva and Colombini, 1996, 1997) or various metabolites (Hodge and Colombini, 1997) transport via VDAC reconstituted into PLB.

Under the influence of transmembrane voltages, VDAC reconstituted into PLB undergoes transitions in its channel conductance and ion selectivity (Mannella *et al.*, 1992; Colombini, 1994). At low voltages ($< 10$ mV), the channel is in its highest conducting, anion-selective state, but at high voltages ($> 40$ mV), the channel is converted to a lower conducting state with higher selectivity to cations (Colombini, 1994). The molecular nature of the VDAC gating mechanism has yet to be resolved.

Five human cDNA clones have been identified to date showing 75–94% homology (Bureau *et al.*, 1992; Dermietzel *et al.*, 1994; Ha *et al.*, 1993; Blachly-Dyson *et al.*, 1994); however, only two proteins have been isolated and identified. The existence of multiple human porin has been demonstrated and interpreted as an indication for a variety of functions (Ha *et al.*, 1993).

Recently, VDACs were shown to be located in the plasmalemma of several cell types. They were found in transformed human B-lymphocytes (Reymann *et al.*, 1995; Thinnes, 1992; Jurgens *et al.*, 1994), in rat brain plasma membrane (Bureau *et al.*, 1992), and in bovine astrocytic plasma membrane (Dermietzel *et al.*, 1994). Moreover, the presence of VDAC in the SR of amphibian (Lewis *et al.*, 1994) and rabbit skeletal muscles (Jurgens *et al.*, 1995, Junankar *et al.*, 1995; Shoshan-Barmatz *et al.*, 1996; Shafir *et al.*, 1998) have been recently demonstrated.

In this paper, we demonstrate the presence of five anti-VDAC antibody cross-reacted protein bands in synaptosomes isolated from the Torpedo electric organ. We colocalized VDAC proteins with the voltage-gated $Ca^{2+}$ channel, purified, and characterized them by their single-channel activity, their labeling with [$^{14}$C]DCCD, and their partial amino acid sequence.

## MATERIALS AND METHODS

### Materials

BSA, Tris, Tricine, HEPES, NaCl, NP-40, DCCD, and spermine-agarose were purchased from Sigma Chemicals Co. DIDS was purchased from Molecular Probes, Oregon. Alkaline phosphatase-conjugated goat anti-mouse IgG was obtained from Promega and VDAC monoclonal antibodies (31HL, human, clone nos. 173/045 and 173/025) were obtained from Cal Biochem. Gold-coated (10 nm) anti-mouse IgG and [$\alpha$-$^{32}$P]ATP were purchased from Amersham. Gold-coated (5 nm) anti-rabbit IgG was obtained from Zymed Laboratories, California. Phosphatidylserine (PS) and Phosphatidylethanolamine (PE) were purchased from Avanti polar lipids (Alabaster, Alabama). Polyclonal antibodies against the $Ca^{2+}$ channel $\alpha_2$-subunit peptide (Leu$^{645}$-Tyr$^{856}$) conserved in rat brain, PC12 cells, and skeletal muscle $\alpha_2$ subunits (Wiser *et al.*, 1996), and were kindly provided by Prof. D. Atlas (Hebrew university, Israel). Nickel grids were purchased from Graticules, LTD, Tonbridge, England.

### Membrane Preparation

Rabbit or Torpedo skeletal muscle SR, rat liver mitochondria, and sheep brain microsomal membranes were prepared according to MacLenenn (1970), Ernster and Nordenbrand (1967), and Tamkun and Cat-

terall (1981), respectively. Torpedo fish (*marmorata* and *oscellata*) were captured in the eastern Mediterranean sea and held in oxygen-perfused sea water at 15°C. The fish were killed by chilling at 4°C and their electric organs excised; synaptosomes were prepared as described before (Michaelson and Sokolovsky, 1978). Protein concentration was determined according to Lowry *et al.* (1951).

### Immunogold Electron Microscopy

Synaptosomal membranes were fixed *in situ* for 30 min in 2% paraformaldehyde and 1% gluteraldehyde in phosphate-buffered saline (PBS), pH 7.4. Membranes were postfixed in osmium oxide for 10 min and embedded in propylene oxide–araldite mixture. After embedding was completed, ultrathin sections were cut and placed on nickel grids for immunogold studies, which were carried out essentially as described previously (Konstantinova *et al.*, 1995). In brief, for double-immunogold labeling, sections (0.08 μm) were incubated for 1 h with monoclonal anti-VDAC antibodies (diluted 1:500), washed (twice for 1 min) with PBS containing 1 mg/ml BSA (PBS/BSA), and incubated for 20 min with anti-mouse IgG coated with 10-nm gold particles (diluted 1:20). Similarly, the membranes were then introduced to polyclonal anti-$Ca^{2+}$ channel $\alpha_2$-subunit peptide antibodies (diluted 1:100), and to anti-rabbit IgG coated with 5-nm gold particles (diluted 1:10). Grids were stained with uranyl acetate and lead citrate and visualized by electron microscopy. All antibodies were diluted with PBS/BSA.

### ATP Transport

SR and synaptosomes (0.5 mg/ml) were assayed for ATP transport by incubation for 1 min at 30°C in a solution containing 0.1 M NaCl and 20 mM Tricine, pH 7.2 (Buffer A) and the indicated concentrations of [$\alpha$-$^{32}$P]ATP ($10^6$ cpm/nmol). The membranes were filtered through mixed-cellulose ester membranes (0.3 μm), washed immediately three times with 4 ml of buffer A containing 0.1 mM ATP, and the retained radioactivity was measured.

### Purification of VDAC

Synaptosomal VDAC was purified as described previously for SR membranes (Shoshan-Barmatz *et al.*, 1996). In brief, synaptosomal membranes (5 mg), were solubilized for 20 min at 0–4°C (at 2mg/ml) in a solution containing 1.3% NP-40, 10 mM Tris-HCl, pH 7.0, 3 mM DTT, 0.15 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin. After centrifugation at 44,000 × *g* for 30 min, the NP-40 extract was diluted with 10 mM Tris, pH 7.0, to a final concentration of 0.4% NP-40 and loaded (0.7 ml/min) onto a spermine–agarose column (1 × 4 cm), preequilibrated with 10 mM Tris-HCl, pH 7.0 and 0.4% NP-40 (buffer N). The loaded column was washed with buffer N and fractions containing the various VDAC-proteins were eluted with buffer A containing 50 mM NaCl. They were analyzed by SDS–PAGE and Western blotting using monoclonal anti-VDAC antibodies.

### Gel Electrophoresis and Western Blot Analyses

Analysis of the protein profile was performed using SDS–polyacrylamide slab gel electrophoresis (PAGE) with a discontinuous buffer system of Laemmli (1970), in 1.5-mm thick slab gels with the indicated acrylamide concentration. Gels were either stained with Coomassie brilliant blue or electrophoretically transferred onto nitrocellulose membranes according to Towbin *et al.* (1979). For immunostaining, membranes were blocked with 3% nonfat dry milk and 0.1% Tween-20 in Tris buffered-saline, incubated with the monoclonal anti-VDAC antibody (1:5,000), and then with alkaline phosphatase-conjugated anti-mouse IgG (1:10,000), as a secondary antibody. The color was developed (within 5 min) with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium. Molecular weight standards were: phosphorylase b, 97,000; bovine serum albumin, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000; trypsin inhibitor, 21,000; and lysosyme 14,000 (Bio-Rad). The relative amount of VDAC was determined by quantitative analysis of immunoblots. Various amount (1–20 μg) of the different membranal fractions were subjected to SDS–PAGE followed by immunoblotting with anti-VDAC antibody. Quantitative analysis of VDAC was performed by densitometric scanning of the blots with a computing densitometer (Molecular Dynamics) using Image Quant software provided by the manufacturer. A linear relationship between the density units and the amount of protein was obtained.

### Single-Channel Reconstitution and Analysis

VDAC channel reconstitution, recording, and analysis were carried out as described previously (Shoshan-Barmatz *et al.*, 1996; Shafir *et al.*, 1998; Hadad *et al.*, 1994). The single-channel data were obtained from purified VDAC reconstituted into a planar lipid bilayer (PLB). Synaptosomal VDAC(s) were purified from the Torpedo electric organ as described above. PLB was prepared from L-α-phosphatidylethanolamine (heart) and L-α-phosphatidylserine (brain) in *n*-decane (50 mg/ml) in a 5:3 ratio. Data from the amplifier were filtered at 1 KHz through a low-pass filter and digitized for computer storage. Further analysis was performed by pClamp (Axon Instruments) and Sigma Plot (Jandel Scientific). All experiments were conducted at 22–25°C in 1 M NaCl and 10 mM HEPES, pH 7.4.

## RESULTS

The protein profiles of rabbit skeletal muscle SR, rat brain microsomes, rat liver mitochondria, and Torpedo electric organ synaptosomal membranes are shown in Fig. 1A. The presence of protein bands that cross-reacted with two different monoclonal antibodies, prepared against the N-terminal part of VDAC1 in all these membranes, as detected by SDS–PAGE and Western blot analysis, is shown in Fig. 1B. Five Torpedo synaptosomal protein bands (labeled I to V) were found to cross-react with the VDAC-monoclonal antibody. In other membrane preparations, the anti-VDAC antibody cross-reacted with only two protein bands: a major protein with a molecular weight of 35 kDa and a minor one of 30 kDa. Since VDAC is highly resistant to proteases (De Pinto *et al.*, 1991a), and several protease inhibitors were present during the preparation of synaptosomes, the protein bands that cross-reacted with the anti-VDAC antibody may represent different isoforms of VDAC rather than degradation products. It should be noted that the separation between the various anti-VDAC antibody cross-reacted bands was not always obtained, but the Torpedo anti-VDAC antibody cross-reacted bands always run with higher mobility than the mammalian VDAC. It has been shown that in the absence of reducing agents (DTT or β-mercaptoethanol) mammalian VDAC runs in SDS–PAGE with higher mobility (De Pinto *et al.*, 1991b). This contributed to the cystine bridge present in the VDAC (De Pinto *et al.*, 1991b). We confirmed this observation for the mammalian VDAC, but no such effect was observed for Torpedo VDAC in SR or synaptosomal membranes (data not shown).

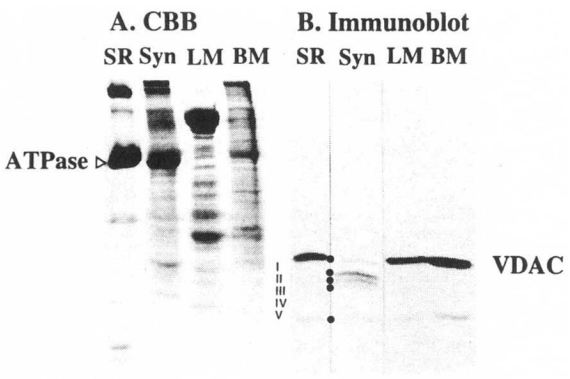


**Fig. 1.** Cross-reactivity of four Torpedo protein bands with the anti-VDAC antibody. Rabbit skeletal muscle SR membranes (SR), rat liver mitochondria (LM), sheep brain membranes (RB), and torpedo synaptosomes (Syn) were prepared as described previously (Wiser *et al.*, 1996; MacLennan, 1970; Ernster and Nordenbrand, 1967; Tamkun and Catterall, 1981). Membranes were subjected to SDS–PAGE (8–20% acrylamide, 40 μg protein/lane) and gels were either stained with Coomassie blue (A) or immunoblotted (B) using anti-VDAC monoclonal antibody (Calbiochem, Clone No. 173/045 or No. 173/025). ATPase, $(Ca^{2+} + Mg^{2+})$ATPase.

The distribution of the anti-VDAC antibody cross-reacted protein bands in the synoptosomes and in membranes derived from other Torpedo tissues was examined (Fig. 2). The distribution of two major VDAC protein bands (band II and III) in various membranal fractions isolated from Torpedo (determined by densitomeric scanning of several immunoblots) is summarized in Table I. The results show differential distribution, as reflected by the different ratio between band II and III in synaptosomes, mitochondria, brain, and sarcoplasmic reticulum.

Table I also summarizes the activities of succinate–cytochrome-*c* oxidoreductase, a mitochondrial inner membrane marker enzyme, and antimycin-insensitive NADH–cytochrome-*c* reductase, an enzyme present in the mitochondrial outer membrane and in endoplasmic reticulum membranes (Mannella, 1987) in various membranal fractions isolated from Torpedo tissues. The results indicate that the activity of the inner and outer mitochondrial membranes marker enzymes in synaptosomes is about 6.8 and 9.6% of their activity in the mitochondria-enriched fraction. This, together with the different ratio between the anti-VDAC antibody cross-reacted bands II and III, suggest that the synaptosomal VDAC-like protein is derived also from nonmitochondrial membranes.

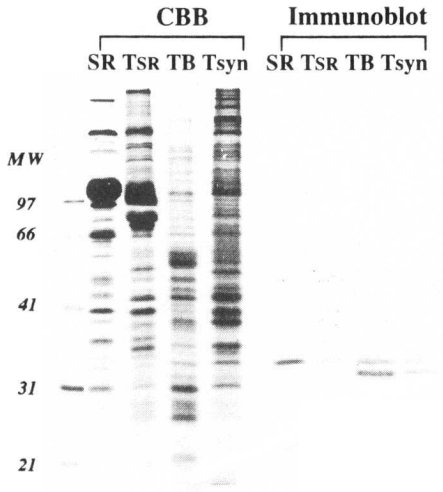


**Fig. 2.** Distribution of VDAC proteins in various Torpedo membranes. Synaptosomes (Syn), Torpedo skeletal muscle SR (TSR), and Torpedo brain homogenate (TB) (35 μg protein) were isolated from Torpedo, as well as rabbit skeletal muscle SR (SR), were subjected to SDS–PAGE (12% acrylamide, 30:0.1% acrylamide: bisacrylamide) followed by either Coomassie blue staining (CBB, left panel) or immunoblotted (right panel) using monoclonal anti-VDAC antibody. MW, molecular weight protein standard in kDa.

The location of VDAC in the synaptosomal membranes is demonstrated by electron microscopy of synaptosomes subjected to double-immunogold labeling using anti-VDAC and anti-$Ca^{2+}$-channel ($\alpha_2$-subunit peptide) antibodies. Binding of each antibody was followed by exposure to a secondary antibody coated with 10- and 5-nm gold particles for the anti-VDAC and anti-$\alpha_2$ subunit peptide ($Ca^{2+}$-channel) antibodies, respectively (Fig. 3A). The large dots, which represent anti-VDAC cross-reacted protein are present together with the small dots, which represent the voltage-gated $Ca^{2+}$-channel in the same membrane. VDAC-like protein and the $Ca^{2+}$ channel are either uniquely distributed at the membrane surface or form clusters (arrow heads). In some sections, a few classic mitochondria cross-section structures were observed in which anti-VDAC antibodies were bound (not shown). The cross-reactivity of anti-$Ca^{2+}$ channel $\alpha_2$-subunit antibodies with the Torpedo proteins in synaptosomal and SR fractions as well as with rabbit skeletal muscle Junctional SR is demonstrated in Fig. 3B. The different mobility of the Torpedo Junctional SR $\alpha_2$-subunit is not addressed in this study. Since the $Ca^{2+}$-channel is a protein marker of the synaptosomal plasma membrane, colocalization of both proteins on the same membrane suggests the presence of VDAC-like protein in the plasma membrane of the Torpedo synaptosomes.

[$^{14}$C]DCCD was shown to specifically label mitochondrial (De Pinto *et al.*, 1985, 1993; Nakashima, 1989; Nakashima *et al.*, 1986;) and SR (Shoshan-Barmatz *et al.*, 1996; Shafir *et al.*, 1998) VDAC. The labeling of synaptosomal membranes with [$^{14}$C]DCCD is shown in Fig. 4. At the concentration used, [$^{14}$C]DCCD specifically labeled only one band of 35 kDa, which was identified as VDAC using the anti-VDAC antibody (see Fig. 5). As expected for specific labeling, unlabeled DCCD prevented [$^{14}$C]DCCD labeling of VDAC. The hydrophilic carboxyl reagent (1-ethyl-3-[3-dimethylamino]propyl)carbodiimide (WRK), has no effect on [$^{14}$C]DCCD labeling of VDAC. Similar results were found for SR and mitochondrial VDAC, suggesting that the DCCD reacting carboxyl residue(s) is located in a hydrophobic region of the protein (Shafir *et al.*, 1998).

Using a new method developed in our laboratory for the purification of SR-VDAC (Shoshan-Barmatz

**Table I.** Distribution of Marker Enzymes and VDAC Bands in Various Membranal Fractions Isolated from Torpedo[a]

| Membrane preparations | Succinate–cytochrome-*c* reductase (nmol cytochrome *c* red./mg/min) | NADH–cytochrome-*c* reductase (nmol cytochrome *c* red./mg/min) | VDAC bands distribution band II/band III |
|---|---|---|---|
| Skeletal muscle mitochrondria | 129.6 ± 6 | 50 ± 3 | 1.5 ± 0.17 ($n$ = 4) |
| Brain microsomes | nd | nd | 1.0 ± 0.10 ($n$ = 2) |
| Skeletal muscle SR | 5.6 ± 0.4 | 93 ± 6 | 2.5 ± 0.36 ($n$ = 5) |
| Electric organ synaptosomes | 8.9 ± 0.8 | 4.8 ± 0.8 | 1.9 ± 0.20 ($n$ = 6) |

[a] Synaptosomes were isolated from the Torpedo electric organ, partially purified brain microsomes; mitochondria and SR were isolated from Torpedo skeletal muscle as described under the section on materials and methods. Succinate–cytochrome-*c* reductase (after osmotic shock) and antimycin-insensitive NADH–cytochrome reductase activities of the various membranal fractions were assayed as described previously (Mannella, 1987). The results are averages of four to six experiments. VDAC distribution was determined as described in the section on materials and methods. The numbers represent averge ± SE (number of experiments); nd, no data.

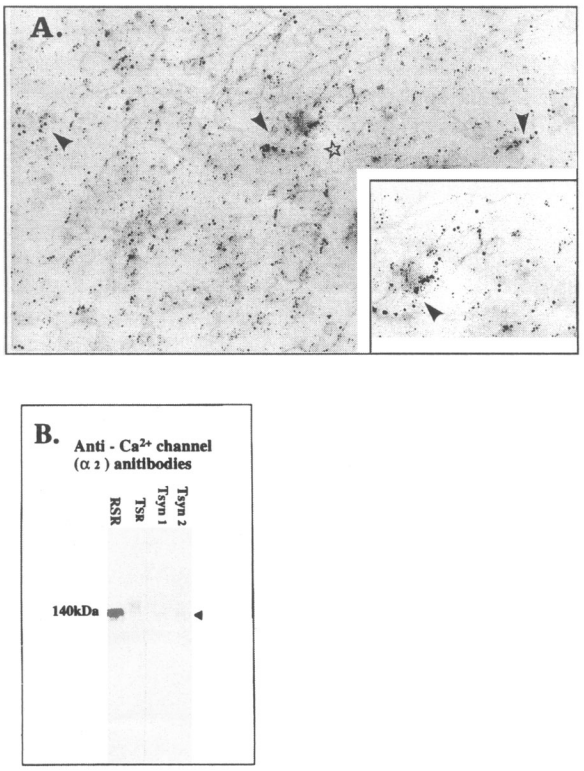


**Fig. 3.** Colocalization of VDAC and the $Ca^{2+}$ channel in synaptosomes. (A) Synaptosomal membranes were fixed in PBS, pH 7.4, containing 2% paraformaldehyde and 1% gluteraldehyde. Small pieces of the fixed membranes were embedded in propylene oxide–araldite mixture and ultrathin sections were cut and transferred to nickel grids. Sections were introduced to monoclonal anti-VDAC antibody (1:500)and to anti-mouse IgG coated with 10–nm gold particles (1:20). The same sections were then introduced to a polyclonal anti-$Ca^{2+}$ channel ($\alpha_2$-subunit peptide) antibodies (1:100) and to 5-nm gold-coated anti-rabbit IgG (1:10), and visualized by electron microscopy (magnification × 30,000). Inset shows an enlargement of the region indicated by star, and the arrows point to clusters of colloidal-gold immunolabeling for VDAC and $\alpha_2$-subunit $Ca^{2+}$ channel. (B) Immunoblot staining using polyclonal anti-$\alpha_2$ subunit peptide antibodies (1:1000) and alkaline phosphatase-conjugated protein A as secondary antibody. The 140 kDa band indicates the $Ca^{2+}$ channel $\alpha_2$-subunit.

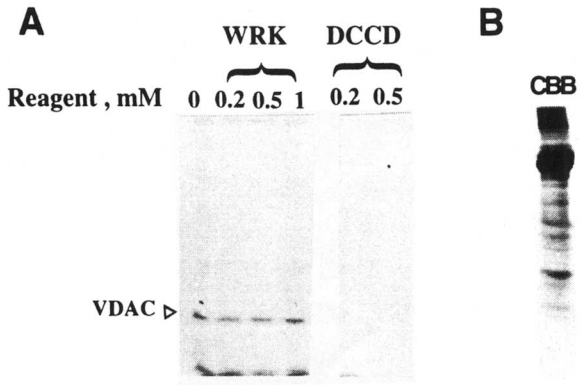


**Fig. 4.** Specific [$^{14}$C]DCCD labeling of synaptosomal VDAC proteins. Synaptosomal membranes were incubated with [$^{14}$C]DCCD (1 μM) in the absence and the presence of the indicated concentrations of WRK or unlabeled DCCD. The membranes were subjected to SDS-PAGE followed by Coomassie blue staining (CBB) and fluorography (A). A Coomassie-stained representative lane is shown in (B).

*et al.*, 1996), four [$^{14}$C]DCCD-labeled 30–35 kDa proteins were purified from synaptosomes and found to interact with anti-VDAC antibody. [$^{14}$C]DCCD-prelabeled synaptosomal membranes were solubilized with 1.3% NP-40 and the supernatant loaded onto a spermine–agarose column, from which the labeled 30–35 kDa proteins were eluted with 50 mM NaCl. The electrophoretic profile of the various fractions, stained with Coomassie blue (Fig. 5A), and their corresponding fluorogram (Fig. 5B) shows the purification of four [$^{14}$C]DCCD-labeled polypeptides, which cross-reacted with anti-VDAC antibody (Fig. 5C). A similar procedure is used to purify nonlabeled synaptosomal VDAC proteins (Fig. 6). Selected fractions obtained in this purification were subjected to SDS–PAGE, followed by either staining with Coomassie blue (Fig. 6A), or analyzed by immunostaining (Fig. 6B) using anti-VDAC antibody. A separation between two of the anti-VDAC antibody cross-reacted VDAC proteins, bands II (fractions 2–6) and III (fractions 23–26), is obtained.

To further identify the Torpedo protein bands cross-reacting with anti-VDAC antibody, the partially purified two major protein bands (II and III) were further purified by SDS–PAGE and subjected to partial amino acid sequencing. They were digested by endoproteinase Lys C, and the resulting peptides were separated by reverse phase high-performance liquid chromatography (HPLC). The sequences of four selected peptides derived from band II (peptides 1 and 2) and band III (peptides 3 and 4) are represented in Fig. 7, together with published sequences of peptides from VDAC1 (por1 human), VDAC2 (por2 human), and VDAC3 (brain mouse). Torpedo peptide 1 exhibits high homology to the human VDAC1 sequence (98%), to human VDAC2 (91.8%) (Kayser *et al.*, 1989), and to VDAC3 (90%) (Sampson *et al.*, 1996). This may suggest that this peptide is derived from either VDAC1 or VDAC2, while the differences might be the result of species difference. Torpedo VDAC peptide 2 shows lower homology to either VDAC1 (88.4%) or VDAC2 (79%) than peptide 1. It might be derived from VDAC1 or from a different VDAC isoform. Peptides 3 and 4

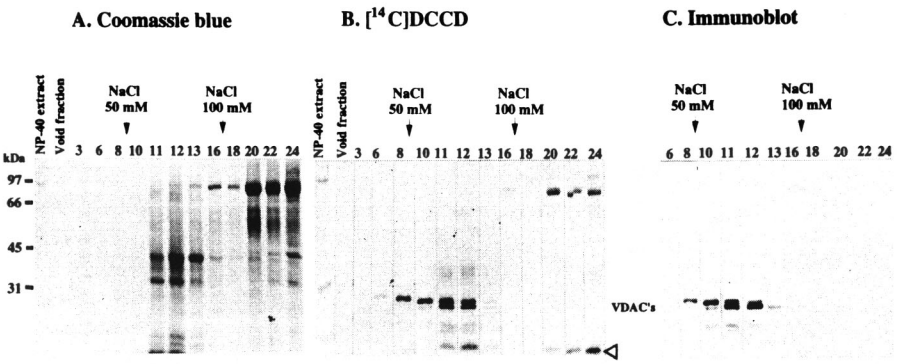


**Fig. 5.** Purification of [$^{14}$C]DCCD-labeled VDAC proteins. Synaptosomal membranes (5 mg) prelabeled with [$^{14}$C]DCCD (8 μM) were solubilized with 1.3% NP-40 (NP-40 extract). VDAC proteins were purified using a spermine–agarose column as described in the section on materials and methods. [$^{14}$C]DCCD-labeled VDAC proteins were eluted with 50 mM NaCl. Samples were subjected to SDS–PAGE followed by either Coomassie blue staining (A), fluorography (B), or immunoblot (C). The arrow indicates a [$^{14}$C]DCCD-labeled protein band that did not react with anti-VDAC antibody.

show 100% similarity and high homology (77.8 and 100%, respectively) to brain VDAC3 (Sampson *et al.*, 1996). Comparison of peptide maps, obtained by $V_8$ digestion of bands II, III, and mitochondrial VDAC were very similar (not shown).

Mitochondrial VDAC was shown to mediate the transport of nucleotides across the mitochondrial outer membrane (Rostovtseva and Colombini, 1996, 1997). We have recently demonstrated similar activity for the rabbit skeletal muscle SR-VDAC (Shoshan-Barmatz *et al.*, 1996). Examination of the transport of [α-

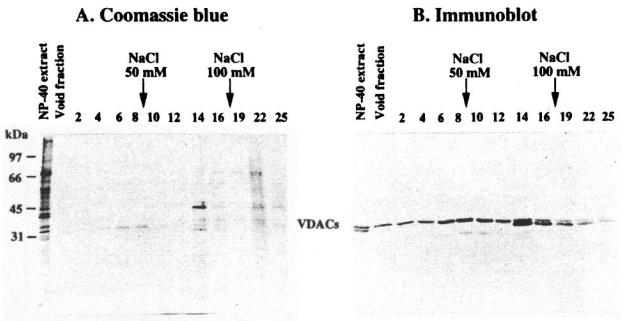


**Fig. 6.** Purification of synaptosomal VDAC proteins on a spermine–agarose column. Synaptosomes (5 mg) were solubilized with NP-40 (1.3%, 2 mg protein/ml), as described in the section on materials and methods. Following centrifugation (44,000 × *g* for 30 min), the supernatant (NP-40 extract) was applied to a spermine–agarose column from which VDAC proteins were eluted with buffer N or with buffer N containing 50 mM or 100 mM NaCl. Selected fractions were analyzed by SDS–PAGE and either stained with Coomassie blue (A) or immunoblotted using monoclonal anti-VDAC antibody (B).

| | | |
|---|---|---|
| Torpedo peptide - 1 | 1 | K G Y G F G L V K 9 |
| VDAC 1 (por1, human) | 19 | K G Y G F G L **I** K 27 |
| VDAC 2 (por2, human) | 46 | K G **F** G F G L V K 54 |
| VDAC 3 (brain, mouse) | 19 | K G Y G F G **M** V K 27 |
| Tropedo peptide - 2 | 1 | K S A R D I F A K 9 |
| VDAC 1 (por1, human) | 11 | K S A R D **V** F A K 19 |
| VDAC 2 (por2, human) | 38 | K **A** A R D I F **N** K 46 |
| Tropedo peptide - 3 | 1 | K V G M G F E F E 9 |
| VDAC 3 (brain, mouse) | 274 | K V G **L** G F E **L** E 282 |
| Tropedo peptide - 4 | 1 | K A X D F Q L 7 |
| VDAC 3 (brain, mouse) | 173 | K A A D F Q L 180 |

**Fig. 7.** Amino acid sequences of peptides derived from Torpedo VDACs. Partially purified VDAC was subjected to SDS–PAGE and two major Torpedo VDAC protein bands (labeled II and III in Fig. 1) were cut out of the gel and sequenced (in the Biology Center, Technion, Israel). The protein bands were digested by endoproteinase Lys C. The resulting peptides were separated by reverse-phase HPLC on an $C_{18}$ column and four of the peptide peaks were sequenced as described (Hewick *et al.*, 1981). The obtained sequences, in a single-letter amino acid code, are presented with the published sequences of human VDAC1 and VDAC2 (Kayser *et al.*, 1989) and brain mouse VDAC3 (Sampson *et al.*, 1996).

$^{32}$P]ATP into synaptosomes in comparison to its transport into rabbit skeletal muscle SR membranes revealed that the transport of ATP into synaptosomes and into the SR lumen is similar with respect to concentration dependence. In both membranes the transport of ATP is increased with increasing ATP concentration (Fig. 8A).

The effects of the anion transport inhibitor DIDS on the transport of [$\alpha$-$^{32}$P]ATP into synaptosomes and SR lumen are demonstrated in Fig. 8B. ATP transport in both SR and synaptosomes is inhibited similarly by DIDS. The effect of DCCD, a carboxyl-modifying reagent, known to bind specifically to mitochondrial VDAC (DePinto *et al.*, 1985, 1993; Nakashima, 1989; Nakashima *et al.*, 1986), on ATP transport is presented in Fig. 8C. DCCD inhibited ATP transport into the SR more than in synaptosomes.

The channel activity of the two purified protein bands, identified as VDAC by their amino acid sequence and their cross-reactivity with the anti-VDAC antibody, was reconstituted into a PLB (Fig. 9). Both reconstituted VDAC proteins (fraction 2 and fraction 25 in Fig. 6) demonstrate a voltage-dependent channel activity (Fig. 9). At symmetric solutions (1 M NaCl), when the voltage was changed from zero to $-30$ mV, the current first increased to the maximal opening level of the channel due to the greater driving force, but, soon afterward, decayed to a steady subconductance current level as the channel closed to a stable low-conducting state. Similar results were obtained following voltage change from zero to $-40$ mV. Both purified proteins exhibit similar voltage-dependent channel activity, typical of VDACs from other tissues reconstituted into PLB (Colombini, 1989; Shoshan-Barmatz *et al.*, 1996; Benz, 1994). The voltage dependence of the Torpedo VDACs channel activity is similar to that of the mitochondrial VDAC (Fig 9C).

## DISCUSSION

The existence of VDAC in extramitochondrial membranes has been reported by several groups (Bureau *et al.*, 1992; Dermietzel *et al.*, 1994; Ha *et al.*, 1993; Reymann *et al.*, 1995; Thinnes, 1992; Jurgens *et al.*, 1994, 1995; Lewis *et al.*, 1994; Junankar *et al.*, 1995; Shoshan-Barmatz *et al.*, 1996; Shafir *et al.*, 1998). We have recently demonstrated the presence of VDAC in skeletal muscle SR membranes (Shoshan-Barmatz *et al.*, 1996; Shafir *et al.*, 1998). In this study, the presence of VDAC proteins in synaptosomal membranes prepared from the electric organ of the Torpedo fish, their purification, characterization, and localization are presented.

### Characterization of the Torpedo Synaptosomal Membrane VDAC(s)

Five Torpedo synaptosomal protein bands were found to cross-react with two different anti-VDAC

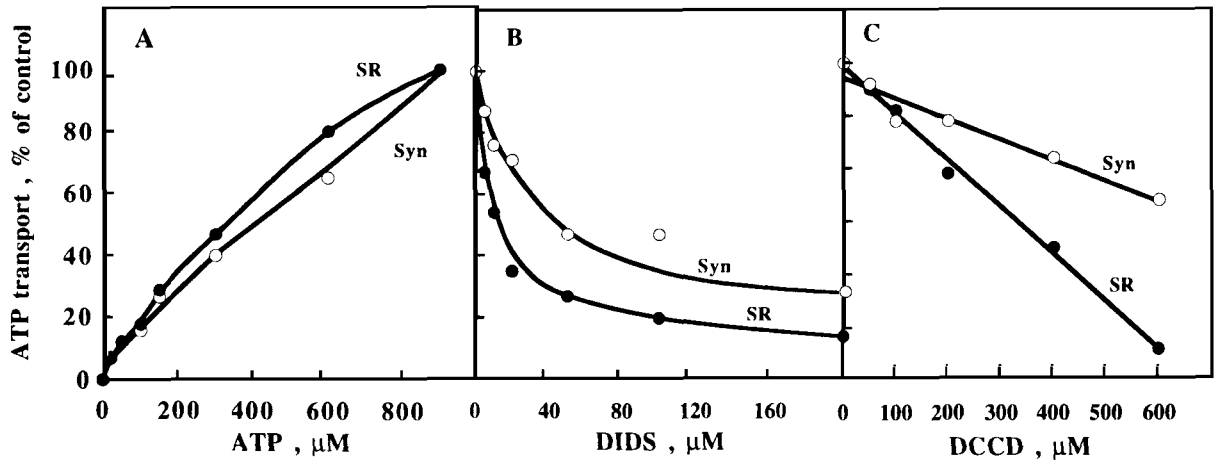


**Fig. 8.** ATP transport into SR and synaptosomal membranes. (A) SR and synaptosomal membranes (Syn) (0.5 mg/ml) were incubated with different concentrations of [$\alpha$-$^{32}$P]ATP for 1 min at 30°C in a solution containing 0.1 M NaCl and 20 mM Tricine, pH 7.2 (buffer A). Membranes were then filtered on mixed-cellulose ester filters (0.3 μm), washed, and the retained radioactivity measured. Prior to ATP transport, the membranes were preincubated with the indicated concentrations of DIDS for 10 min at 30°C in buffer A (B) or with DCCD for 10 minutes at 24°C in a solution containing 20 mM Mes, pH 6.4, and 0.1 M NaCl (C). Control activity (100%) were for SR and Syn, respectively: (A) 2.3 and 2.2 nmol/mg/min; (B) and (C) 1.2 and 0.7 nmol/mg/min. The results are representative experiments from six similar experiments.

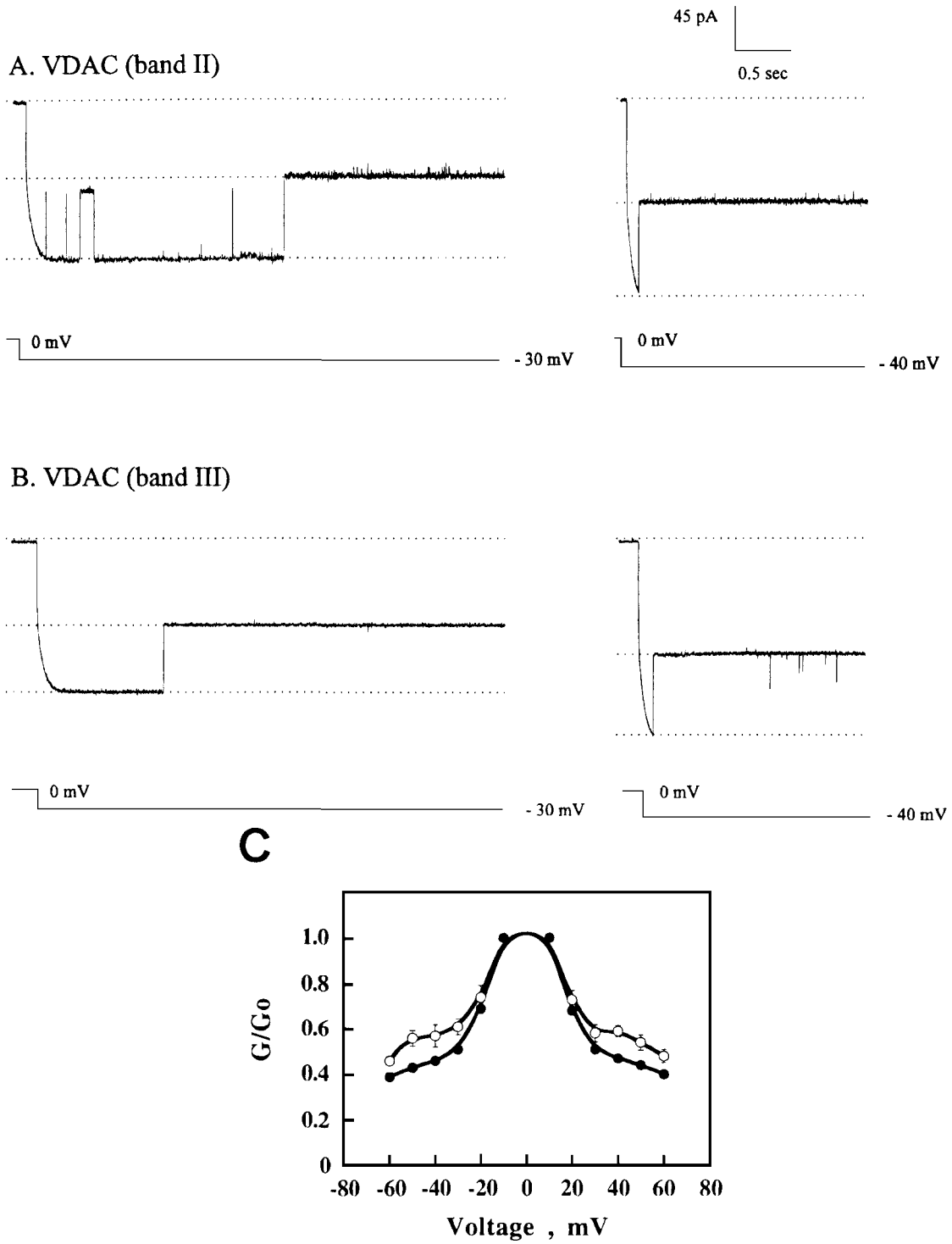


**Fig. 9.** Channel activity of the purified synaptosomal VDACs. The two major purified synaptosomal VDAC proteins were reconstituted into a PLB, as described in section on materials and methods. The activity of a single VDAC channel was recorded at the indicated holding potentials. The voltage-dependence channel activity of the VDAC (band II) and of VDAC (band III) is presented in (A) and (B), respectively. In C, the voltage-dependence channel activity of VDAC isolated from rat liver mitochondria (●) and from Torpedo synaptosomes (O) is presented as the ratio between the conductance (G), at a given voltage, divided by the conductance at −10 mV (Go)(n = 8).

monoclonal antibodies prepared against the N-terminal of VDAC1 (Babel *et al.*, 1991 (Fig. 1). For comparison, membranes isolated from different tissues of the Torpedo and other animal species were separated by SDS–PAGE and analyzed for their cross-reactivity with the anti-VDAC antibody. In SR isolated from rabbit skeletal muscle, rat liver mitochondria, and sheep brain microsomes, only two protein bands were found to cross-react with the anti-VDAC antibody (Figs. 1 and 2). VDAC is highly resistant to proteases (De Pinto *et al.*, 1991a), and the skeletal muscle SR VDAC was found to be resistant to 8 out of 9 proteases examined (data not shown). Nevertheless, several protease inhibitors were included in the preparation of all membranes. It is, therefore, likely that these VDAC protein bands represent VDAC-like proteins rather than degradation products.

The identification of these protein bands as VDAC is based on the following: (1) Purification by the same procedures as used to purify mitochondrial or SR VDAC. (2) Specific labeling with [$^{14}$C]DCCD with high affinity as the labeling of identified VDACs. (3) Cross-reactivity with two different monoclonal anti-VDAC antibodies. (4) Amino acid sequences of four peptides derived from two of the bands are highly homologous to sequences present in VDAC1, VDAC2 or VDAC3 isoforms. (5) Upon reconstitution into a PLB, the Torpedo synaptosomal purified proteins show voltage-dependent channel activity with properties similar to those of VDAC.

The suggestion that the distinct protein bands cross-reacted with anti-VDAC antibody represent VDAC isoforms and not degradation products is supported by the different ratio between bands II and III in several membranes derived from various tissues of the Torpedo fish, i.e., electric organ synaptosomes, skeletal muscle SR and mitochondria, and brain homogenate (Fig. 2 and Table I). Furthermore, partial amino acid sequencing of four peptides, derived from two of the anti-VDAC cross-reacted bands (II and III), shows that one of the sequenced peptides has high homology to sequences present in VDAC1 and VDAC2 (98 and 91.8%, respectively), while the other peptide shows relatively high, but lower than that of peptide 1, homology to VDAC3 and to both VDAC1 and VDAC2 (88.4 and 79.1%, respectively); peptides 3 and 4 shows high homology to VDAC3 (Fig. 7). If indeed, the last two peptides represent VDAC3, then this is the first demonstration of the presence of VDAC3 protein. Mouse VDAC1 and VDAC2, but not VDAC3, when expressed in yeast lacking the endogenous VDAC, were found to complement the phenotypic defect associated with this mutant, suggesting an alternative physiological function of the VDAC3 protein (Sampson *et al.*, 1997).

Assuming that the different anti-VDAC antibody cross-reacted bands represent different VDAC isoforms, then their differential distribution in the various Torpedo tissues may suggest that they play a different role in the various tissues. In comparison with the amino acid sequence of VDAC from human B lymphocytes, rat brain VDAC was found to have an extra 13 amino acids at the N-terminus, suggesting a specific tissue or cellular location (Bureau *et al.*, 1992). The two human genes *HVDAC 1* and *HVDAC 2* appear to encode at least three different proteins with distinct amino termini (Ha *et al.*, 1993; Yu *et al.*, 1995). Amino-terminal sequences have been demonstrated to target many proteins to appropriate subcellular compartments. The presence of different VDACs that differ primarily by distinct $NH_2$-terminal extensions may lead to the targeting of each isoform to a specific cellular location. The complete identification of the various VDAC protein bands as different isoforms awaits their cloning and sequencing, which will enable the development of specific antibodies to identify each protein band and to determine its location.

VDAC is considered to be the main passage of adenine nucleotides through the mitochondrial outer membrane. Recently, transport of ATP via VDAC reconstituted into PLB was demonstrated (Rostovtseva and Colombini, 1996, 1997). We have recently shown the involvement of VDAC in ATP transport into skeletal muscle SR (Shoshan-Barmatz *et al.*, 1996). The transport of ATP into synaptosomes is demonstrated here (Fig. 8). The anion transport inhibitor, DIDS, and the carboxyl-modifying reagent DCCD were found to inhibit ATP transport into the synaptosomal membranes, while the transport was not affected by atractyloside, an inhibitor of the ATP/ADP exchanger (Palmieri *et al.*, 1993). DCCD, which is known to specifically bind to VDAC (Shoshan-Barmatz *et al.*, 1996; Shafir *et al.*, 1998; De Pinto *et al.*, 1985, 1993; Nakashima, 1989; Nakashima *et al.*, 1986), has been shown to inhibit mitochondrial and SR-VDAC channel activity, as well as ATP transport into the SR (Shoshan-Barmatz *et al.*, 1996; Shafir *et al.*, 1998). [$^{14}$C]DCCD was found to label the synaptosomal VDAC protein bands (Figs. 4 and 5). DIDS was found to completely inhibit both the VDAC channel activity and ATP transport in the SR (Shoshan-Barmatz *et al.*, 1996) and VDAC was purified on DIDS-immobilized column

(Thinnes *et al.*, 1994). Thus, the inhibition of ATP transport into synaptosomes by DCCD and DIDS implies the involvement of VDAC in the transport of ATP into the synaptosomes. The function of the transported ATP is not addressed in this study. Synaptic vesicles from the electromotor tissue of Torpedo *marmorata* have been shown to contain molar quantities of ATP (Dawdall *et al.*, 1974), which is released upon stimulation along with acetylcholine. Thus, it is possible that VDAC is the pathway for ATP release in the nerve terminals.

### Localization of the VDAC in Synaptosomal Membranes

As summarized above, the presence of VDACs in synaptosomes prepared from the Torpedo electric organ is demonstrated here by several means. These synaptosomal VDACs do not represent only mitochondrial contamination since the specific activities of the antimycin-insensitive NADH–cytochrome-*c* reductase, a mitochondrial outer membrane marker, or of succinate–cytochrome-*c* reductase, a mitochondrial inner membrane marker enzyme, were only 10 and 6.8% of those of mitochondria-enriched fraction isolated from Torpedo skeletal muscle (Table I). Furthermore, the ratio between two of the anti-VDAC antibody cross-reacted bands is different in mitochondria and synaptosomes (Table I). The colocalization of VDAC and the voltage-gated $Ca^{2+}$ channel, a plasma membrane marker, was demonstrated by double-immunogold labeling using anti-VDAC and anti-$Ca^{2+}$ channel ($\alpha_2$-subunit) antibodies (Fig. 3). The results clearly indicate the presence of VDAC at the synaptosomal surface membrane. The gold particles are uniquely distributed at the membrane surface but also form clusters in a chainlike structure (Fig. 3, arrow heads) in which both anti-VDAC and anti-$Ca^{2+}$ channel antibodies are present. This finding suggests the existence of specific VDAC-rich regions in the synaptosomal membrane and that VDAC may be a putative synaptic plasma membrane protein. The function of VDAC(s) in the synaptic plasma membrane is not known. VDAC function, however, may be related to its properties as a channel with large pore size (3 nm) (Krasilnikov *et al.*, 1996), which conduct both cations and anions with large size (up to 6000 dalton) (Colombrini, 1980). The identification of VDAC function in synaptosomes requires further studies.

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## REFERENCES

Babel, D., Walter, G., Gotz, H., Thinnes, F. P., Jurgens, L., Konig, U., and Hilschmann, N. (1991). *Biol. Chem. Hoppe-Seyler* **372**, 1027–1034.
Benz, R. (1994). *Biochim. Biophys. Acta* **1197**, 167–196.
Blachly-Dyson, E., Baldini, A., Litt, M., McCabe, E. R. B., and Forte, M. (1994). *Genomics* **20**, 62–67.
Bureau, M. H., Khrestchatisky, M., Heeren, M., Zambrowicz, E. B., Kim, H., Grisar, T. H., Colombini, M., Tobin, A. J., and Olsen, R. W. (1992). *J. Biol. Chem.* **267**, 8679–8684.
Colombini, M. (1980). *J. Membr. Biol.* **53**, 79–84.
Colombini, M. (1989). *J. Membr. Biol.* **111**, 103–111.
Colombini, M. (1994). *Current Topics Membr.* **42**, 73–101.
Dawdall, M. J., Boyne, A. F., and Whittaker, V. P. (1974). *Biochem. J.* **140**, 1–12.
Dermietzel, R., Hwang, T. K., Buettner, R., Hofer, A., Dotzler, E., Kremer, M., Deutzmann, R., Thinnes, F. P., Fishman, G., Spray, D., and Siemen, D. (1994). *Proc. Natl. Acad. Sci. U. S.* **91**, 499–503.
De Pinto, V., Tommasino, M., Benz, R., and Palmieri, F. (1985). *Biochim. Biophys. Acta* **813**, 230–242.
De Pinto, V., Al-Jamal, J. A., Benz, R., Geuchi, G., and Palmier, F. (1991). *Eur. J. Biochem.* **202**, 903–911.
De Pinto, V., Prezios, G., Thinnes, F. P., Link, T. A., and Palmier, F. (1991). *Biochemistry* **30**, 10191–10200.
De Pinto, V., Aljamal, J. A., and Palmier, F. (1993). *J. Biol. Chem.* **268**, 12977–12982.
Edry-Schiller, J., Ginsburg, S., and Rachamimoff, R. (1991). *J. Physiol. (London)* **439**, 627–647.
Ernster, L., and Nordenbrand, K. (1967). *Methods Enzymol.* **X**, 86–94.
Galzi, J. L., Revah, F., Bessis, A., and Changeux, J. P. (1991). *Annu. Rev. Pharmacol.* **31**, 37–72.
Ha, H., Jajek, P., Bedwell, D. M., and Burrows, P. D. (1993). *J. Biol Chem.* **268**, 12143–12149.
Hadad, N., Abramson, J. J., Zable, T., and Shoshan-Barmatz, V. (1994). *J. Biol. Chem.* **269**, 24864–24869.
Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981). *J. Biol. Chem.* **256**, 7990–7997.
Hodge, T., and Colombini, M. (1997). *J. Membr. Biol.* **157**, 271–279.
Jentsch, T. J., Steinmeyer, K., and Schwarz, G. (1990). *Nature* **348**, 510–514.
Junankar, P. R., Dulhunty, A. F., Curtis, S. M., Pace, S. M., and Thinnes, F. P. (1995). *J. Muscle Res. Cell Motility* **16**, 595–610.
Jurgens, L., Ilsemann, P. Kratzin, H. D., Hesse, D., Eckart, K., Thinnes, F. P., and Hilschamann, N. (1994). *Biol. Chem. Hoppe-Seyler* **375**, 315–322.
Jurgens, S. L., Kleineke, J., Brdiczka, D., Thinnes, F. M., and Hilschmann, N. (1995). *Biol. Chem. Hoppe-Seyler* **376**, 685–689.
Kayser, H. Kratzin, H. D., Thinnes, F. P., Gotz, H. Schmidt, W. E., Eckart, K., and Hilschmann, N. (1989). *Biol. Chem. Hoppe-Seyler* **370**, 1265–1278.
Konstantinova, S. A., Mannella V. P., Skulachev, V. P., and Zorov, D. B. (1995). *J. Bioenerg. Biomembr.* **27**, 93–99.

Krasilnikov, O. V., Carneiro, C. M. M., Yuldasheva, L. N., Campos-de-Carvalho, A. C., and Nogueira, R. A. (1996). *Brazil. J. Med. Biol. Res.* **29**, 1691–1697.
Laemmli, U. K. (1970). *Nature* **227**, 680–685.
Lewis, T. M., Roberts, M. I., and Bretag, A. H. (1994). *Neurosci. Lett.* **181**, 83–86.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.
MacLennan, D. H. (1970). *J. Biol. Chem.* **247**, 4508–4518.
Mannella, C. A. (1987). *Methods Enzymol.* **148**, 453–464.
Mannella, C. A., Forte, M., and Colombini, M. T. (1992). *J. Bioenerg. Biomembr.* **24**, 7–19.
Meir, A., and Rachamimoff, R. (1996). *J. Neurophysiol.* **75**, 1858–1870.
Michaelson, D. M., and Sokolovsky, M. (1978). *J. Neurochem.* **30**, 217–230.
Miller, C., and Whote, M. M. (1984). *Proc. Natl. Acad. Sci. U.S.* **81**, 2772–2775.
Nakashima, R. A. (1989). *J. Bioeneg. Biomembr.* **21**, 461–469.
Nakashima, R. A., Mangan, P. S., Colombini, M., and Pedersen, P. L. (1986). *Biochemistry* **25**, 1015–1021.
Neumann, E., Weber, J., and Schuerholz, T. (1996). *Arch. Physiol. Biochem.* **104**, 731–744.
Palmieri, F., Indiveri, C., Bisaccia, F., and Kramer, R. (1993). *J. Bioenerg. Biomembr.* **25**, 525–535.
Reymann, S., Flörke, H., Heiden, M., Jakob, C., Stadtmüller, U., Steinacker, P., Lalk, V. E., Pardowitz, I., and Thinnes, F. R. (1995). *Biochem. Mol. Med.* **54**, 75–87.
Rostovtseva, T., and Colombini, M. (1996). *J. Biol. Chem.* **271**, 28006–28008.
Rostovtseva, T., and Colombini, M. (1997). *Biophys. J.* **72**, 1954–1962.
Sampson, M. J., Lovell, R. S., Davison, D. B., and Craigen, W. J. (1996).*Genomics* **36**, 192–196.
Sampson, M. J., Lovell, R. S., and Craigen, W. J. (1997). *J. Biol. Chem.* **272**, 18966–18973.
Shoshan-Barmatz, V., Hadad, N., Feng, W., Shafir, I., Orr, I., Varsanyi, M., and Heilmeyer, M. G. (1996). *FEBS Letter* **386**, 205–210.
Shafir, I., Feng, W., and Shoshan-Barmatz, V. (1998). *Eur. J. Biochem.* **233**, 627–636.
Thinnes, F. P. (1992). *J. Bioenerg. Biomembr.* **24**, 71–75.
Thinnes, F. P., Florke, H. Winkelbach, H., Stadtmuller, U., Heiden, M., Karabinos, A., Hesse, D., Kratzin, H. D., Fleer, E., and Hilschmann, N. (1994). *Biol. Chem. Hoppe-Seyler* **375**, 315–322.
Tamkun, M. M., and Catterall, W. A. (1981). *Mol. Pharmacol.* **19**, 78–86.
Towbin, H., Staehelin, T., and Gordon, J. (1979). *Proc. Natl. Acad. Sci. U.S.* **76**, 4350–4354.
Wiser, O., Trus, M., Tobi, D., Halevi, S., Giladi, E., and Atlas, D. (1996). *FEBS Lett.* **379**, 15–20.
Woodbuty, D. J. (1995). *Mol. Memb. Biol.* **12**, 165–171.
Yu, W. H., Wolfgang, W. P., and Forte, M. (1995). *J. Biol. Chem.* **270**, 13998–14006.