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Mouse VDAC Isoforms Expressed in Yeast: Channel Properties and Their Roles in Mitochondrial Outer Membrane Permeability

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Abstract. The channel-forming protein called VDAC forms the major pathway in the mitochondrial outer membrane and controls metabolite flux across that membrane. The different VDAC isoforms of a species may play different roles in the regulation of mitochondrial functions. The mouse has three VDAC isoforms (VDAC1, VDAC2 and VDAC3). These proteins and different versions of VDAC3 were expressed in yeast cells (*S. cerevisiae*) missing the major yeast VDAC gene and studied using different approaches. When reconstituted into liposomes, each isoform induced a permeability in the liposomes with a similar molecular weight cutoff (between 3,400 and 6,800 daltons based on permeability to polyethylene glycol). In contrast, electrophysiological studies on purified proteins showed very different channel properties. VDAC1 is the prototypic version whose properties are highly conserved among other species. VDAC2 also has normal gating activity but may exist in 2 forms, one with a lower conductance and selectivity. VDAC3 can also form channels in planar phospholipid membranes. It does not insert readily into membranes and generally does not gate well even at high membrane potentials (up to 80 mV). Isolated mitochondria exhibit large differences in their outer membrane permeability to NADH depending on which of the mouse VDAC proteins was expressed. These differences in permeability could not simply be attributed to different amounts of each protein present in the isolated mitochondria. The roles of these different VDAC proteins are discussed.

Key words: Electrophysiology — Insertion — Gating — NADH — Liposomes

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Introduction

The mitochondrial channel, VDAC, also known as the mitochondrial porin, is a major protein in the mitochondrial outer membrane. It forms the major pathway for metabolite flux across this membrane. VDAC has been found in all eukaryotic species and its properties are highly conserved (Colombini, 1989).

The proposed physiological functions of VDAC are numerous and supported to varying degrees by experimental evidence. A growing body of evidence indicates that VDAC is involved in the regulation of metabolite flow into and out of mitochondria resulting in the regulation of mitochondrial functions. For example, a protein in the intermembrane space of mitochondria, called the VDAC modulator, can greatly increase the voltage dependence of VDAC channels, making VDAC channels close more easily. When added to isolated mitochondria, the modulator greatly decreases the permeability of mitochondrial outer membrane to ADP (Liu & Colombini, 1992). Many other agents that increase the probability of channel closure have also been shown to inhibit functions in isolated mitochondria by inhibiting the flux of metabolites through the mitochondrial outer membrane (König's polyanion: Colombini et al., 1987, Benz et al., 1988; osmotic pressure: Zimmerberg & Parsegian, 1986, Gellerich et al., 1993; cytosolic NADH: Zizi et al., 1994, Lee et al., 1994). More recently, it has been shown directly that ATP can move freely through VDAC channels in their high-conducting, "open," conformation but cannot permeate through the channels when they are in the low-conducting, "closed," conformation (Rostovtseva & Colombini, 1996, 1997). Similar results were found for metabolites such as citrate, succinate, and phosphate (Hodge & Colombini, 1997). The deletion of the major VDAC gene in yeast results in mitochondria with a 20-fold reduction in their outer membrane perme-

ability to NADH (Lee et al., 1998). All these suggest that the gating of VDAC channels plays an important role in controlling the rate of metabolite flux.

VDAC has also been found to be a component of the peripheral benzodiazepine receptor complex (McEnery, 1992), and has been found to bind to enzymes such as hexokinase and glycerol kinase. It has also been implicated in apoptosis (Zoratti & Szabò, 1995) and attachment of mitochondria to the cytoskeleton (Linden & Karlsson, 1996). It is therefore thought that VDAC may be involved in the regulation of a number of other metabolic processes.

It is reasonable to suspect that the different roles of VDAC might arise from the different isoforms expressed in one organism. The different isoforms in humans and wheat show different electrophysiological properties (Blachly-Dyson et al., 1993; Elkeles, Breiman & Zizi, 1997). For the yeast, *S. cerevisiae*, only one of its two VDAC isoforms has been shown to have channel-forming activity (Blachly-Dyson et al., 1997) and to play a significant role in the permeability of the mitochondrial outer membrane (Lee et al., 1998). In wheat, all three isoforms can form channels (Elkeles et al., 1997).

The existence of three mouse VDAC isoforms (named VDAC1, VDAC2 and VDAC3) has recently been demonstrated (Sampson, Lovell & Craigen, 1996*a*, *b*). All these three proteins are expressed at high levels in heart, kidney, brain and skeletal muscle and at lesser levels in all other tissues tested, with the exception that VDAC1 is not expressed in testes. VDAC2 and VDAC3 are highly expressed in testes (Sampson et al., 1996*a*, *b*), suggesting specialized functions for each isoform. VDAC3 was recently found to undergo tissue-specific alternative splicing that introduces a single amino acid into the amino terminal region of the protein (Sampson et al., 1998).

Clues to the specialized roles of the different isoforms may lie in the basic properties of each VDAC isoform. In this work, the cDNAs for each mouse protein was introduced into yeast cells lacking the major VDAC gene (Por1) so that each VDAC protein could be purified and studied in isolation. The presence of each of these proteins compensates for the inability of the VDAC-deficient yeast to grow at 37°C in medium containing a nonfermentable carbon source such as glycerol, although VDAC3 is less effective (Sampson et al., 1997). However, major differences were found in mitochondria isolated from the strains expressing the different isoforms and major differences were observed in the properties of the channels.

Materials and Methods

Expression of Mouse VDACs in Yeast

For each of the four VDAC isoforms, VDAC1, VDAC2, the two forms of VDAC3 lacking or containing the ATG codon, and the shortened form of VDAC3 (missing 38 amino acids from the N-terminus), oligonucleotide-directed mutagenesis was used to create *Nco*I sites at the start codon for both VDAC1 and VDAC2, or for VDAC3 (with and without the extra ATG and the shortened form) an *Afl*III site. The same strategy was used to generate *Nsi*I sites in the 3′-untranslated region of each gene. The individual oligonucleotide sequences, with the start codons underlined, are:

5′ VDAC1: CGCCCGAGACCATGGCCGTGCC
3′ VDAC1: CCACTTGTAGCCATGCATCTCTAC
5′ VDAC2: CCGCGACTTCGCCATGGCTGAG
3′ VDAC2: GGCAGCACTGATATGCATCCAAC
5′ VDAC3: CGGGTTGTAGACATGTGTAACAC
3′ VDAC3: GACAATTTCCATGCATGCCTTCC
5′ sVDAC3: GGCATGGTCAAGATAGATCTG

This allowed for the complete open reading frame of each VDAC cDNA to precisely replace the yeast VDAC1 (YVDAC1) gene previously cloned into a yeast single-copy shuttle vector (kindly provided by M. Forte, Oregon Health Sciences University; Blachly-Dyson et al., 1990). The oligonucleotides for each gene were used to amplify the cDNA inserts, the products were digested with the appropriate enzymes, and the fragments were subcloned to replace the yeast VDAC gene between the *Nco*I and *Nsi*I sites. From the starting ATG codon, the length of each cDNA insert was 1011 bp for VDAC1, 1079bp for VDAC2, and 1011, 1014, or 897 bp for VDAC3. The three constructs were then introduced into the YVDAC1-deficient yeast strain M22-2 by lithium acetate transformation (Gietz et al., 1992). The yeast were streaked onto media containing 2% glycerol as the sole carbon source, and incubated at 30 or 37°C for 6 days.

Preparation of Yeast Cells

The method has been described by Lee et al. (1998) and has been modified as below. A stock solution of cells was prepared. Yeast cells were first grown at 30°C in 0.67% (w/v) Bacto-yeast nitrogen base without amino acids, 0.1% (w/v) potassium phosphate, dropout mix (all amino acids minus uracil), and 2% lactic acid, pH 5.0 (neutralized with KOH). The cells were divided into test tubes when the OD reached 0.5 to 0.6 (at 600 nm) and stored at 4°C for later use. All the stock solutions were recultured once a month. For mitochondrial isolation, 6 ml of stock solution was inoculated into one liter of the same medium and grown with shaking at 30°C. Typically, four liters of cells were grown and harvested when an O.D. between 0.5 and 0.6 was reached.

The Isolation of Yeast Mitochondria

The isolation procedure is essentially that described by Daum et al. (1982) but modified as previously described (Lee et al., 1998) in order to obtain highly intact mitochondria. The final mitochondrial pellet was suspended in 0.6 M mannitol, 10 mM Tris.Cl, 0.6% PVP, 0.1 mM EGTA and 0.1% BSA, pH 7.2.

A portion of the above mitochondrial suspension was diluted 10-fold with R-medium (0.65 M sucrose, 10 mM Hepes, 10 mM potassium phosphate, 5 mM KCl and 5 mM $MgCl_2$, pH 7.2) in order to measure NADH oxidation. Another portion was hypotonically shocked, as follows, to break the mitochondrial outer membrane. It was incubated with 2 volumes of distilled water for 10 min on ice followed by the addition of 5 volumes of R-medium. Finally, 2 volumes of 2× R-medium was added to return the osmotic pressure to normal. This order of addition minimized damage to the inner mem-

brane, as determined by fumarase activity measurement (Lee et al., 1998).

The Measurement of the Outer Membrane Intactness

The intactness of the mitochondrial outer membrane was quantitated by measuring the cytochrome c-dependent oxygen consumption (Douce et al., 1987). Forty μl of mitochondrial suspension was added to 3.0 ml of R-medium and preincubated for 3 min. To get hypotonically shocked mitochondria, 40 μl of mitochondrial suspension was added to 1.5 ml of distilled water and incubated for 3 min. Then 1.5 ml of 2× R-medium was added to restore the original osmolarity. The respiration rate was monitored, using a Clark oxygen electrode, after addition of 50 μl of 0.48 M ascorbate followed by 180 μg of cytochrome c and finally, KCN (0.2 mM final). The percent intactness = $(1 - V_{intact}/V_{shocked}) \times 100\%$, where V_{intact} and $V_{shocked}$ are the KCN-sensitive cytochrome c-dependent oxygen consumption of untreated and osmotically shocked mitochondria, respectively.

The Measurement of the Outer Membrane Permeability

We used the method previously developed by Lee et al. (1998) for yeast mitochondria. The rationale and the method itself are briefly outlined here.

When NADH is added externally to the isolated yeast mitochondria, it is oxidized primarily by an NADH dehydrogenase located on the outer surface of the mitochondrial inner membrane. The permeability of the mitochondrial outer membrane to NADH can be estimated by measuring the NADH oxidation rate by mitochondria. NADH oxidation was measured by monitoring the decrease in absorbance at 340 nm. Intact mitochondria and hypotonically shocked mitochondria were used as samples. About 36 μM NADH (final concentration) was added to start the reaction. Hypotonically shocked mitochondria consumed NADH faster than the intact mitochondria as indicated by a faster drop in NADH concentration with time (*see* Fig. 2 for examples). This increase in NADH oxidation rate was due to the removal of the barrier formed by the mitochondrial outer membrane. The lower the permeability of the outer membrane to NADH, the bigger the increase in NADH oxidation rate when the mitochondria are hypotonically shocked. Linear regression was used on each group of 13 adjacent points to obtain the oxidation rate (*see* Fig. 2 insets for examples). This produced minimum noise without significant filtration of the signal.

The permeability of the mitochondrial outer membrane was determined by taking the rate of NADH oxidation for intact mitochondria as the flux of NADH through the outer membrane and dividing it by the concentration difference of NADH across the membrane. The medium [NADH] was directly determined from the absorbance measurements. The intermembrane space [NADH] can be estimated from the oxidation rate of hypotonically shocked mitochondria. We assumed that (i) in the disrupted mitochondria the [NADH] in the intermembrane space is the same as in the medium, and (ii) the same local [NADH] around the NADH dehydrogenase should produce the same oxidation rate. Then we can determine the [NADH] in the intermembrane space of intact mitochondria by determining the [NADH] necessary to cause the same oxidation rate in the disrupted mitochondria. This [NADH] for the disrupted mitochondria was subtracted from the [NADH] for intact mitochondria with the same oxidation rate to obtain the concentration difference. The corresponding oxidation rate for the intact mitochondria divided by the concentration difference yields the permeability. At low NADH concentrations the calculated permeability values are not meaningful because the concentration differences were insignificant when compared to the noise.

The Assay of Protein Content

Mitochondrial protein was measured using the BCA method (Pierce, Rockford, IL) following addition of Triton X-100 to the mitochondrial suspension (1% w/v final). Bovine serum albumin was used as the standard.

Planar Phospholipid Membranes

The planar phospholipid membranes were generated by the monolayer method of Montal and Mueller (1972) as modified by Schein, Colombini & Finkelstein (1976) and Colombini (1987). The aqueous solutions were 1M KCl, 1mM $CaCl_2$, 1mM MES, pH5.8 (or as indicated) and the membranes were formed from a 5:1 asolectin:cholesterol (by weight) mixture. VDAC proteins were purified as previously described (Freitag, Benz & Neupert, 1983), as modified by Blachly-Dyson et al. (1990) and a 2–10 μl aliquot of each protein dissolved in 15% DMSO, 2.5% Triton X-100, 50 mM KCl, 10 mM Tris, 1 mM EDTA, pH 7.0 solution was added to one side of the chamber. VDAC inserted spontaneously and was studied under voltage-clamp conditions. Calomel electrodes with saturated KCl bridges were used to interface with the solution. For the ion selectivity experiments, the solution on one side contained 0.1 M KCl rather than 1 M KCl. In this paper the currents were converted to conductances for the convenience of the reader.

Current recordings in response to triangular voltage waves (4 mHz) were used to calculate the probability of the channel being open as a function of voltage and evaluate the two parameters that define the gating process: n, the gating valence, and V_0, the potential where the probabilities of channels being open or closed are equal. Error values reported are standard errors.

Liposome Permeability Measurement

The liposomes were made as follows: 20 mg of egg phosphatidylcholine (Sigma) and 1.75 mg of egg phosphatidylserine (Avanti Biochemicals), both dissolved in chloroform, were mixed together and dried down under N_2. One ml 1 mM KCl, 1 mM HEPES, pH 7.0 solution was added to the dry lipids and the material was sonicated (at 0 to 5°C). One ml of mitochondrial membranes suspended in 1 mM KCl, 1 mM HEPES, pH 7.0 containing 1mg protein was mixed into the lipid solution prepared above. The mixture was sonicated again and lyophilized overnight. Liposomes were produced by dispersing the dry material in 1 ml 20 mM KCl, 1 mM EDTA, pH 7.0. Note that whole mitochondrial membranes were used so as to obtain an indication of the overall channel content of the mitochondria by observing the rates of liposome reswelling. If purified VDAC proteins had been used one would wonder about the concentration of channels in the intact mitochondria and any effects of detergent solubilization, purification, etc.

Thirty microliters of this liposome suspension were added to 1.0 ml 20 mM KCl, 1 mM EDTA, pH 7.0 and the absorbance at 400 nm was monitored. The nonelectrolytes of different molecular weight were dissolved in 20 mM KCl, 1 mM EDTA, pH 7.0 and added to the liposomes when the liposomes had stabilized (no change in absorbance with time).

In the experiments with mouse VDAC1, the liposome suspension was centrifuged in a microfuge for 10 sec to reduce the light scattering. The observed slower nonelectrolyte flux is attributed to this added step.

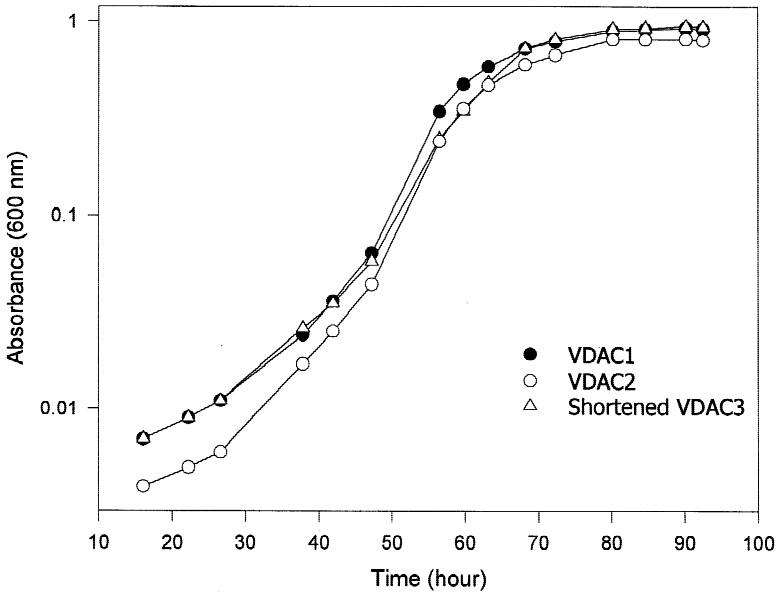


Fig. 1. The growth curves for yeast strains expressing mouse VDAC1, VDAC2, or the shortened VDAC3. The cell concentration was monitored by measuring the absorbance at 600 nm. The absorbance was log transformed and plotted as a function of time.

Quantitation of VDAC Expression

HA tagged constructs were generated by PCR amplification using oligonucleotides designed to add a 3′ HA epitope. PCR products were T-A cloned into pBluescript. The inserts were subject to automated DNA sequencing to confirm that no changes were erroneously introduced. Inserts excised from pBluescript with either *Nco*I and *Nsi*I (VDAC1 and VDAC2) or *Afl*III and *Nsi*I (VDAC3 derivatives) and were ligated into pN2 (pTZ19U backbone), a plasmid that contains the native POR1 promotor and terminator sequences. These inserts containing the transcription regulatory regions and the protein coding regions were then excised with either PstI and BamHI (VDAC1), PstI and SmaI (VDAC2), or HindIII and BamHI (VDAC3 derivatives) and cloned into pSEYC58, a single-copy yeast shuttle vector. Transfections into *S. cerevisiae* were carried out by the lithium acetate method (Gietz et al.,1991) and transformants were selected by growth at 30°C on ura-YPD medium. Mitochondrial extracts were made by the method of Daum, Böhni & Schatz (1982). Protein content was measured by BCA protein assay (Pierce), and 230 micrograms of each extract was electrophoresed on a 12% polyacrylamide gel at 150 volts, electrotransferred to PVDF membrane (Boehringer Mannheim) for 1 hr at 300 milliamps, and Western blotted with an anti-HA/horseradish peroxidase conjugate (Boehringer Mannheim) according to the methods of Sambrook, Fritsch & Maniatis (1989). Protein loads were normalized by Western blots to a subunit of the yeast outer membrane polypeptide translocator, TOM22 (antibody kindly provided by Dr. K. Tokatlidis, University of Basel, Switzerland).

Results

In presenting the experimental results we will be referring to 5 different gene products expressed in yeast cells missing the chromosomal copy of the yeast VDAC1 gene. The expressed genes from mice are referred to as VDAC1, VDAC2, and VDAC3. Since VDAC3 has a 1-codon exon that is alternatively spliced, 2 possible gene products could be produced and so both of the possible coding sequences were expressed. The extra codon is a possible start site. Thus, if the translation machinery were to start at that site it would produce a protein that is shorter by 38 amino acids. This shorter form of VDAC3 will be referred to as the shorter version. The other 2 forms are referred to as either having or lacking the extra ATG codon or the extra methionine.

The Growth of Yeast Cells Expressing Different VDAC Proteins

The different VDAC genes were expressed in yeast lacking the yeast major VDAC gene (YVDAC1). The growth rate of each strain was measured. An experiment on a group of three strains is illustrated in Fig. 1. It shows that the presence of VDAC1, VDAC2, or the shortened version of VDAC3 does not cause significant differences in the growth of yeast cells in lactate medium. Other experiments showed that yeasts harboring VDAC3 with or without the extra methionine have the same rate of growth (*data not shown here*).

The Permeability of the Outer Membrane of Mitochondria Containing Different VDAC Proteins

VDAC forms the major pathway by which metabolites cross the mitochondrial outer membrane. Thus the permeability of the outer membrane of mitochondria isolated from cells expressing each of the different mouse VDAC isoforms should yield information on the ability of that isoform to allow metabolites to cross the outer membrane. The only method available to quantitatively

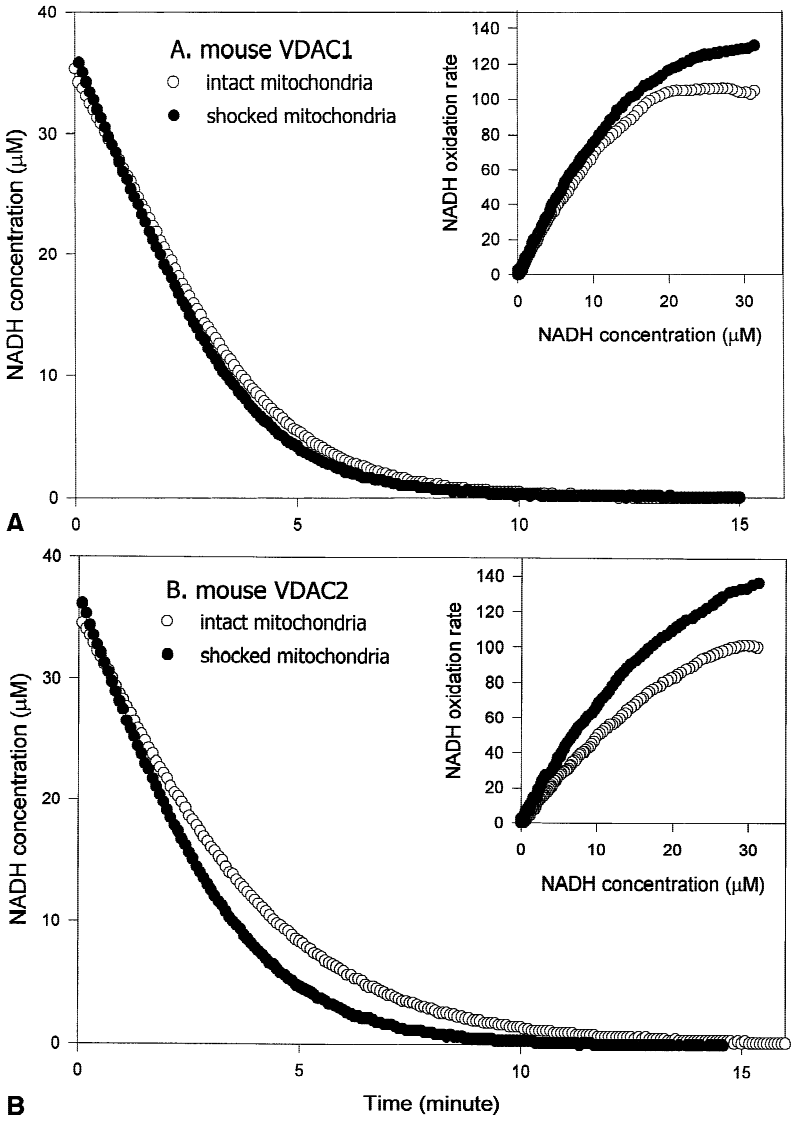
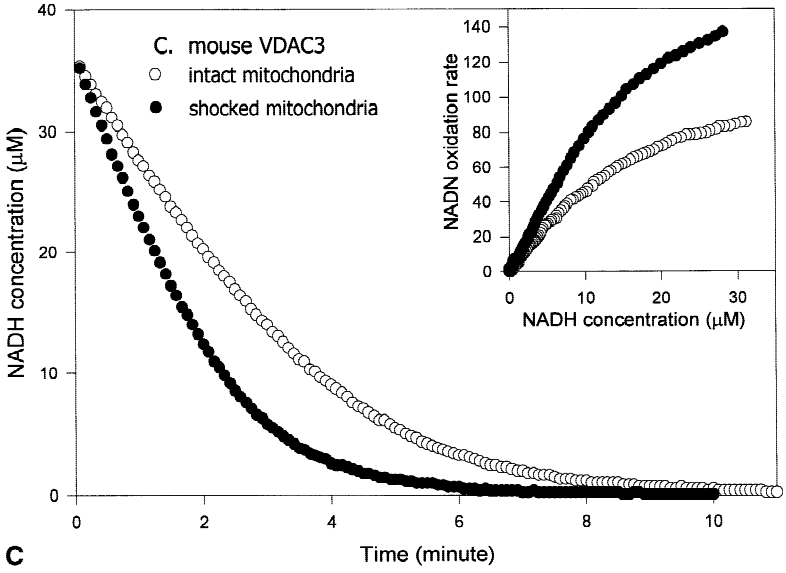


Fig. 2. NADH oxidation by mitochondria isolated from yeast (lacking the yeast VDAC1 gene) expressing mouse VDAC1, mouse VDAC2, mouse VDAC3 without the extra methionine, or no mouse VDAC. The NADH oxidation was monitored by the decrease in absorbance at 340 nm in a cuvette with a 1-cm path length. The absorbance was converted to [NADH] by dividing it by the molar extinction coefficient (6.22×10^3). The oxidation rate (inset) was generated as described in methods and divided by the amount of mitochondrial protein added (69 μg for VDAC1, 70 μg for VDAC2, 96 μg for VDAC3). The units for the inset are nmole NADH/min.mg protein. (*Fig. 2 continued on next page.)*

measure the permeability of the outer membrane in yeast mitochondria involves measuring indirectly the permeability to NADH by measuring the NADH oxidation rate (Lee et al., 1998). Yeast mitochondria have an NADH dehydrogenase on the outer surface of the inner membrane that consumes NADH in the intermembrane space (Ohnishi et al., 1966). The rate at which this dehydrogenase oxidizes exogenously added NADH can be used to calculate the permeability of the outer membrane to NADH.

Figure 2*A* shows the raw data on the NADH oxidation obtained from mitochondria containing the mouse VDAC1 protein. NADH was added at time 0. As shown in the figure, the shocked mitochondria consume NADH slightly faster than the intact mitochondria. This faster rate is due to the removal of the barrier formed by the outer membrane. The inset in Fig. 2*A* shows the same data expressed as oxidation rates.

The corresponding data for mitochondria containing VDAC2 and VDAC3 are shown in Fig. 2*B* and *C*, respectively. Note the larger difference in NADH oxidation rates between the intact and shocked mitochondria in these figures. This indicates a reduced outer membrane permeability to NADH, especially in the case of mitochondria containing mouse VDAC3. While Fig. 2*C* shows data collected from mitochondria isolated from cells expressing the VDAC3 gene lacking the ATG exon, almost identical results were obtained when the ATG exon was present or with the shortened version of VDAC3. However, this difference between the intact and disrupted mitochondria is smaller than that observed with mitochondria lacking the mouse VDAC genes (Lee



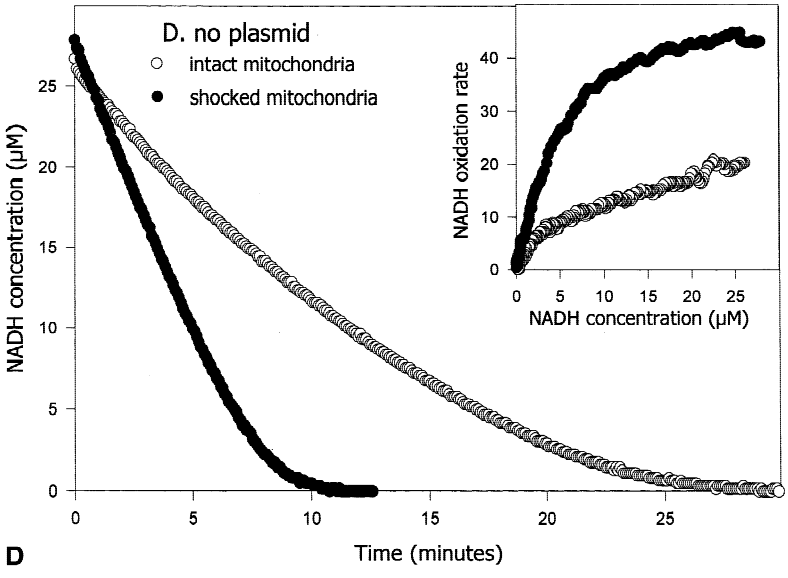


Fig. 2 *(continued).*

et al., 1998), indicating the outer membrane permeability of mitochondria containing any of the mouse VDAC proteins is elevated by the presence of any of these proteins.

It is important to note from the insets in Fig. 2 that the NADH oxidation rates of the mitochondria with the damaged outer membrane were essentially the same regardless of the mouse VDAC present. This indicates that the outer membrane permeability is the important factor limiting the oxidation rate in intact mitochondria.

The permeability of the mitochondrial outer membrane was quantitated as previously described (Lee et al., 1998). This can be done for each point on the respiration curve until the [NADH] is too low to yield reliable values. All the permeability values are summarized in Fig. 3. The permeability of the outer membrane of mitochondria depends on the medium [NADH] and this is consistent with previous reports of the ability of NADH to close VDAC channels (Zizi et al., 1994). The permeability of the outer membrane of mitochondria containing mouse VDAC1 ranges from 20 to 61 cm^3/min.mg protein (depending on the medium [NADH]). This is 2 to 3 times that of mitochondria containing mouse VDAC2 and 4 times that of mitochondria containing any of the forms of mouse VDAC3. It is comparable to the permeability of the outer membrane of the wild type yeast mitochondria (20 to 120 cm^3/min.mg protein) (Lee et al., 1998).

Expression Levels of Different VDAC Genes

All the VDAC genes are carried by the same single copy plasmid and use the same promoter for expression.

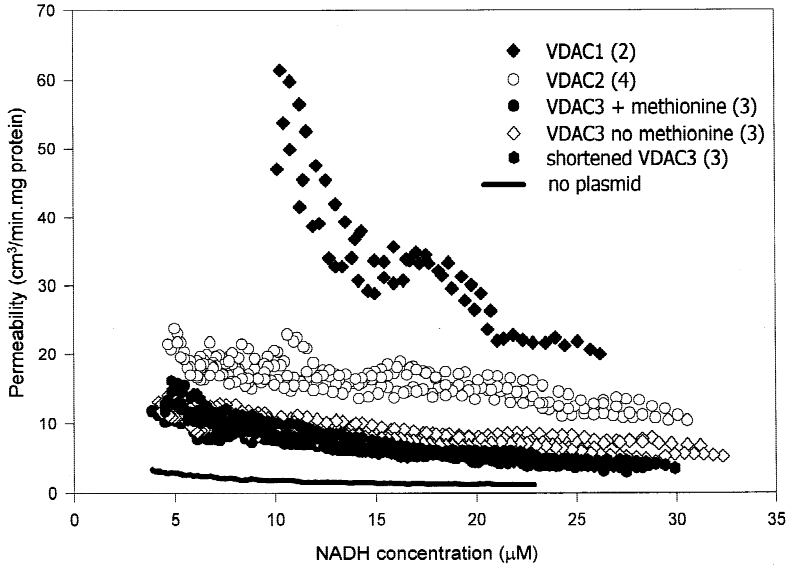


Fig. 3. A comparison of the permeability of the outer membrane of mitochondria isolated from yeast expressing mouse VDAC1, mouse VDAC2, mouse VDAC3 with and without the inserted methionine, or the shortened VDAC3. The solid line at the bottom shows the permeability measured without the expression of any mouse VDAC (from Lee et al., 1998). The numbers of experiments performed on different batches of mitochondria are indicated in parentheses. All data collected are illustrated.

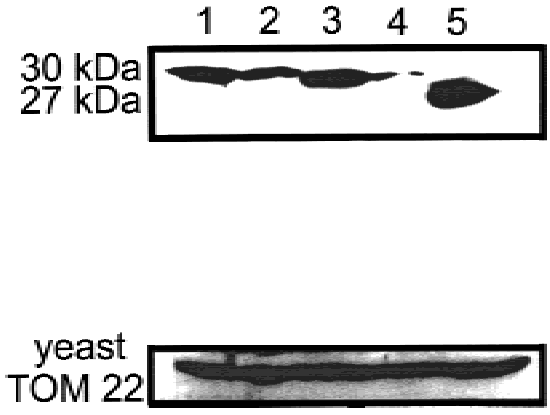


Fig. 4. Quantitation of each VDAC isoform in mitochondrial extracts. The yeast strain lacking the VDAC1 gene, was transformed with each mouse VDAC tagged with the HA epitope and a Western blot carried with anti-HA antibody (upper panel) followed by anti-yeast TOM22 antibody (lower panel) as a control for mitochondrial content. Lanes 1-5: mVDAC1-HA, mVDAC2-HA, mVDAC3-HA, mVDAC3+ATG-HA, mVDAC3-delta N38-HA (shortened VDAC3). Three blots were performed and representative results are shown.

However, the actual amount of VDAC found in the mitochondria, per mg of mitochondrial protein was quite different. For these experiments, HA epitopes were placed on each protein. In this way, differences in antibody avidity could be circumvented. Westerns are shown in Fig. 4. The results were corrected by the expression level of TOM22. This correction did not significantly affect the results.

VDAC1 was present at 3 times the level of VDAC2 (Table 1). The amount of VDAC3 present depended on the version that was expressed. The truncated version of VDAC3 was present in roughly equal amounts to VDAC1 but others were expressed at lower levels.

Table 1. Relationship between expression level and outer membrane permeability

Isoform	Normalized[a] outer membrane permeability	Normalized[a] expression level	Ratio
VDAC1	1	1	1
VDAC2	0.4	0.33	1
VDAC3 + ATG	0.25	0.2	1
VDAC3 − ATG	0.25	0.5	0.5
VDAC3 truncated	0.2	1	0.2

[a] Both the outer membrane permeabilities and the expression levels were normalized to that observed for VDAC1.

When compared to the permeability levels (Table 1) the expression levels correlated with the permeability levels for VDAC1, VDAC2 and the VDAC3-containing the extra methionine. The other versions of VDAC 3 yielded lower permeability levels in relationship to the expression levels. The fact that the permeability of the outer membrane of mitochondria expressing these different VDAC proteins all have higher values than that of mitochondria without these genes (whose permeability ranges from 1 to 5 cm^3/min.mg protein; Lee et al., 1998) indicates that these different versions of VDAC can all form some kind of pathway for NADH, presumably channels, in the mitochondrial outer membrane. However, the pathways formed favor the flux of NADH across the outer membrane to different extents.

The Electrophysiological Properties of Different VDAC Isoforms

To characterize the channel forming properties of these different VDAC proteins, all the proteins were purified

and added to planar phospholipid membranes and studied by electrophysiological methods.

VDAC1 and VDAC2 can insert easily into a phospholipid membrane and form channels in the membrane. Their stepwise insertion can be clearly seen in Fig. 5, top two panels. While most of the VDAC1 channels inserted in the normal-conductance state, many of VDAC2 channels inserted in a lower-conductance state, as shown in Fig. 6, the histograms of insertion events. Rather than merely being a broad distribution, there is strong evidence for 2 populations of VDAC2 channels. This became evident when experiments were performed in the presence of a salt gradient when both conductance and selectivity were measured. Both VDAC1 and VDAC2 have the normal ion selectivity in the typical high-conductance, "open," state. However, VDAC2 channels that inserted in a lower conductance state also had a lower anion selectivity. Thus the results were grouped into two populations (*see* Table 2) with one outlier. This grouping shows the excellent correlation between conductance and selectivity and is consistent with the existence of at least 2 different VDAC2 channels. Extended recordings of single channels of VDAC 2 (e.g. Fig. 7) show that a VDAC2 channel with a lower conductance could not be converted into one with a higher conductance by varying the applied voltage. Both types of channels underwent voltage-dependent closing in an indistinguishable way but upon reopening they returned to their original open-state conductance. Thus, we found no way of converting the lower conducting VDAC2 channels into the higher conducting ones.

In contrast to VDAC1 and VDAC2, mouse VDAC3 did not insert easily into planar phospholipid membranes. In each experiment a maximum of only one or two channels inserted, and often no channels inserted at all. Similar results were obtained whether the negatively charged soybean phospholipids or the neutral diphytanoyl phosphatidylcholine phospholipids were used to make the membranes. The presence of DTT resulted in a modest increase in insertion of VDAC3 channels. As many as 5 or 6 channels inserted, as shown in Fig. 5, bottom panel, our best result. (The channel insertion records illustrated for VDAC1 and VDAC2 are quite typical.) Figure 6 shows the distribution of the conductances of the insertions for VDAC3 (without the additional methionine) observed over more than 20 experiments. It does not show a clear preferred state for this channel in a phospholipid membrane. Similar results were obtained with mouse VDAC3 containing the extra methionine.

It was even more difficult to get VDAC3 to insert into a membrane in the presence of an ion gradient. The two successful experiments we did showed the right value for the ion selectivity (about 12 mV for a 10-fold KCl gradient, anion preference), although the corresponding conductance is substantially lower (0.68 nS and 0.71 nS) than that for the typical VDAC channel, including mouse VDAC1 and VDAC2 under the same conditions.

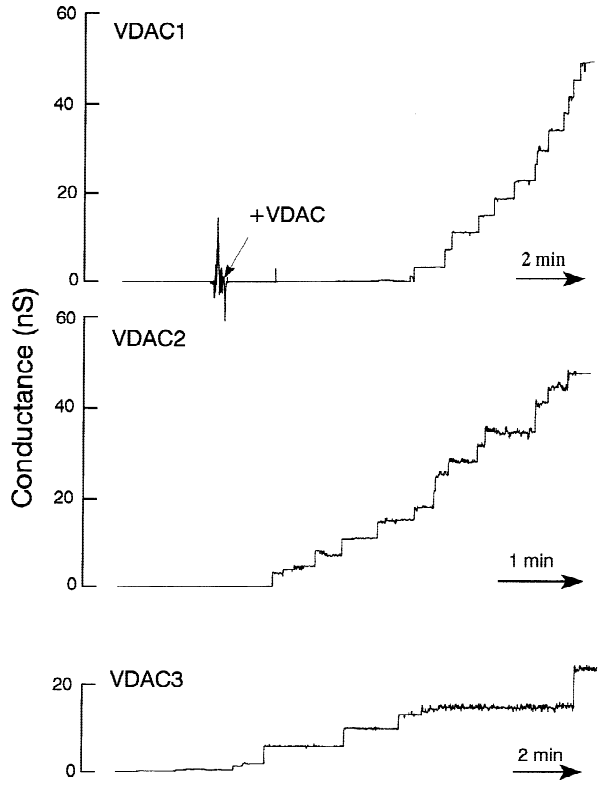


Fig. 5. Illustrations of the insertion process of different mouse VDAC channels. A membrane was made as described in Methods. A small aliquot of VDAC sample (2 μl to 10 μl) was added to one side of the membrane and stirred for about 20 sec. VDAC channels then inserted spontaneously into the membrane. The mouse VDAC3 illustrated is the one without the inserted methionine. For mouse VDAC3, 1 mM of DTT was present in the buffered solutions bathing the membrane.

So far, all efforts to observe channel-forming activity by the shortened VDAC3 have been unsuccessful. Re-isolation of mitochondria in the presence of protease inhibitors did not change the outcome, unlike reports with wheat VDAC (Elkeles et al., 1997). This reduces the likelihood that the shortened VDAC3 was being degraded by proteases during the isolation process. Purification of the shortened VDAC3 from larger batches of mitochondria and the use of samples directly from mitochondrial membranes, all failed to insert channels into planar membranes. Immunobinding assays on the purified VDAC samples show that the protein amounts of these different isoforms (including the shortened VDAC3) in the samples after purification were similar, excluding the possibility that the lack of channel activity in the shortened VDAC3 was due to the loss of this protein during the purification process (*data not shown*).

VDAC1 and VDAC2 have the normal voltage-dependent gating activity. They demonstrated the nor-

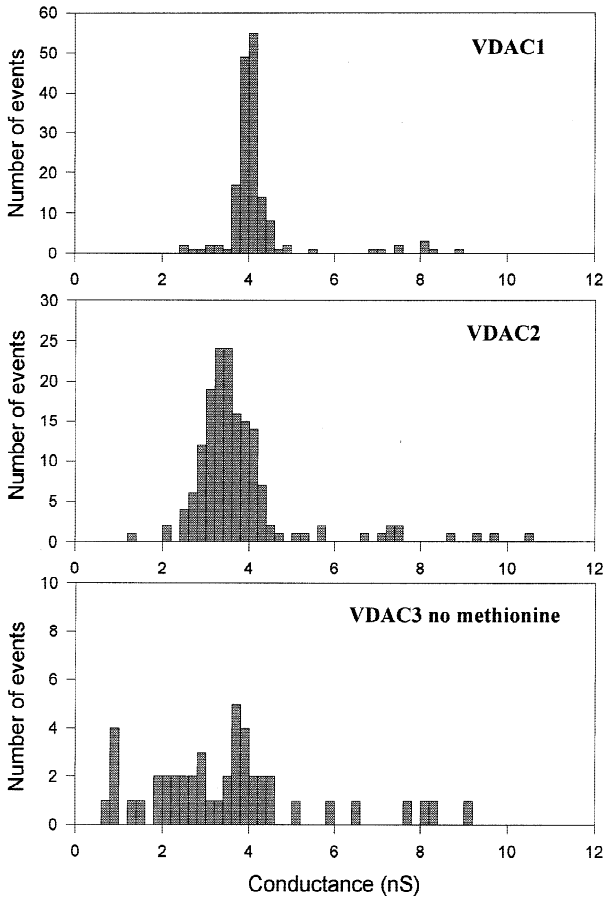


Fig. 6. Histograms of insertion events from experiments such as those illustrated in Fig. 5.

mal symmetrical response to voltages of opposite signs. Figure 7 shows the gating process of a single VDAC1 and VDAC2 channel in response to a linearly changing membrane potential. Channel closure is evident at both positive and negative potentials. The values of the parameters that characterize the voltage dependence of channels, n and V_0, were measured in multichannel membranes and are summarized in Table 2. Note that despite the normal voltage gating, the VDAC2 channel illustrated never achieved the high-conducting state seen with VDAC1. This indicates that the lower conducting state is not a partially closed state but a lower-conductance open state.

Mouse VDAC3 channels did not respond normally to membrane potential changes. Generally, a voltage as high as 80 mV did not close the channels as expected. The recording of the only single channel we found to gate is shown in Fig. 7. It fluctuated very quickly between the closed and open states even at high voltages. For all the other experiments, the channels did not gate and therefore we could not measure the values of the voltage dependence parameters, n and V_0. Addition of pronase to the buffered solutions bathing the membrane did not improve the voltage dependence, indicating that it is unlikely that some bound proteins caused the VDAC3 channels to stay in the open state even at high membrane potentials.

Measurement of Nonelectrolyte Permeability

To get more information on whether the shortened VDAC3 can form channels, the different VDAC proteins were incorporated into liposomes and the permeabilities of the liposomes were measured. Hyperosmotic solutions containing nonelectrolytes of different sizes were used to induce liposome shrinkage (monitored as an increase in absorbance at 400 nm, Fig. 8). If the nonelectrolytes were permeable, reswelling would be observed as the nonelectrolytes entered the aqueous compartments within the liposome. This was detected as a decrease in absorbance following the initial increase. The rate of reswelling is a measure of the degree of permeability of the liposome membrane. Lack of reswelling indicates impermeability of the nonelectrolyte and allows an estimation of the molecular weight cutoff. Control experiments are illustrated when there was a slow reduction in absorbance with time due to a slow sedimentation of the liposomes. In controls, the liposome suspension was merely shaken and allowed to stand in the spectrophotometer. Thus any reswelling would require that the rate of decrease in absorbance be greater than that observed in the control.

As shown in Fig. 8, in all cases the liposomes showed the same molecular weight cutoff between 3,400 and 6,800, indicating that all forms of mouse VDAC proteins can form channels of similar size. In all cases reswelling was clear when PEG 3400 was added but no significant reswelling was detected when PEG 6800 was added. VDAC3 without (illustrated in the figure) or with the inserted methionine produced the same liposome permeability. Similarly, liposomes containing the shortened VDAC3 resulted in the same molecular weight cutoff, indicating that this protein could also form channels that allow the flux of large nonelectrolytes across the membrane.

The amount of mitochondrial protein incorporated into the phospholipid mixture used to form the liposomes was the same for all isoforms. The rates of reswelling were comparable, indicating similar permeabilities to nonelectrolytes. A previous report on identical experiments using mitochondria isolated from the identical yeast strain but lacking the mouse-VDAC containing plasmid showed a much lower molecular weight cut off (between 500 to 700 Daltons; Blachly-Dyson et al., 1997).

Table 2. Electrophysiological properties of different VDAC isoforms

VDAC isoform	Average conductance (nS)	Conductance in ion gradient (nS)	Reversal potential (mV)	Voltage dependence	
				n	V_0 (mV)
VDAC1	4.25 ± 0.09(166)	1.77 ± 0.03 (8)	11.3 ± 0.1 (8)	2.51 ± 0.02 (8)	29.7 ± 1.1 (8)
VDAC2	3.79 ± 0.10(163)	1.71 ± 0.01 (3)	11.9 ± 0.9 (3)	2.55 ± 0.06 (7)	29.8 ± 0.7 (7)
		1.55 ± 0.01 (5)	5.2 ± 0.1 (5)		
		1.52 (1)	0.9 (1)		

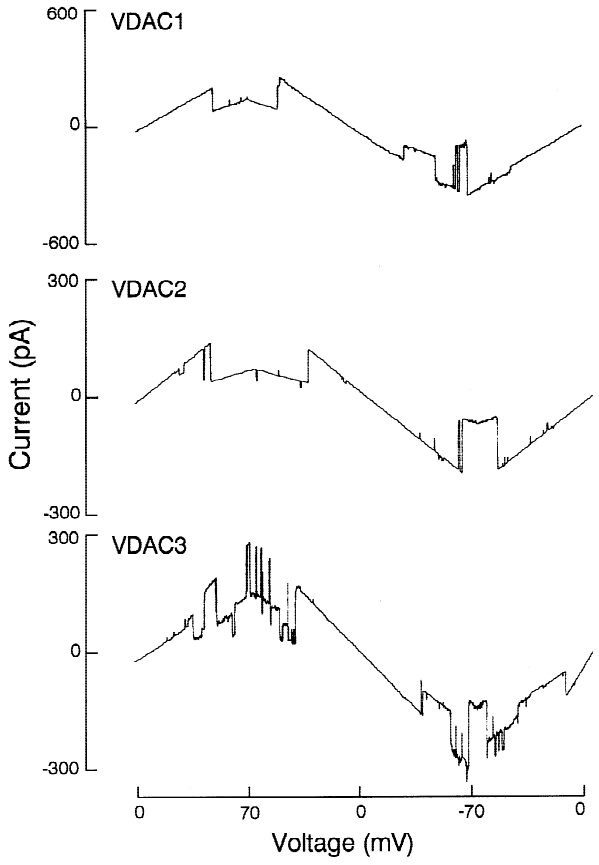


Fig. 7. The voltage gating processes of different reconstituted mouse VDAC channels. Current records of single-channel experiments are illustrated. Voltage was applied in the form of a slow (4 mHz) triangular wave traveling between −70 and +70 mV. A steep slope indicates that the channel is in the highest conducting, open, state while shallower slopes indicate the channel is in a low-conducting, closed, state.

Discussion

When VDAC channels are isolated from a wide variety of organisms (fungi, plants, animals) and reconstituted into planar phospholipid membranes, the observed characteristic properties: single-channel conductance, ion selectivity, and voltage dependence, are highly conserved. However, this method may favor the insertion of one isoform over another. The mouse VDAC isoform that corresponds to this canonical VDAC is mouse VDAC1. VDAC3 has very different properties and it inserts with great difficulty into planar membranes. Thus, in a typical VDAC extraction and reconstitution experiment, this isoform would contribute only a very small minority of channels. A small fraction of aberrant channels would tend to be either not noticed or considered to be damaged or denatured. The current results force one to reconsider such cavalier conclusions.

The properties of VDAC2 are quite similar to those of VDAC1. However, there is clear evidence of 2 populations of channels. The broadness of the peak in the histogram of insertion events (Fig. 6) only hints at this. One shoulder overlaps with the conductance values for VDAC1. However, the main portion of the peak occurs at conductance values that are 15% lower. When individual channels were examined over long periods of time and subjected to triangular voltage waves (Fig. 7), the open-channel conductance remained unchanged. Those VDAC2 channels with a lower open-state conductance did not switch to the higher conductance level but they did switch, in a voltage-dependent manner, from a from the open to the low-conducting "closed" states. Thus the breadth of the peak in the histogram was not the result of the varying conductance of individual channels but due to a population of channels that varied in conductance. The VDAC2 channels that insert with reduced conductance are different in some unknown way. Selectivity measurements bolster the 2-populations hypothesis. As shown in Table 2, when the conductance and selectivity of individual channels were measured simultaneously (in the presence of a salt gradient) most of the observations naturally grouped into 2 populations of distinct conductance and selectivity. Note the very low variance. The one outlier is included for completeness. The presence of two populations of VDAC2 channels might arise from some cellular regulatory process that results in long-lasting changes in the properties of the protein. The lowered conductance and lowered selectivity could combine to make these channels less permeable to metabolic anions. Selectivity has been shown to be very important in

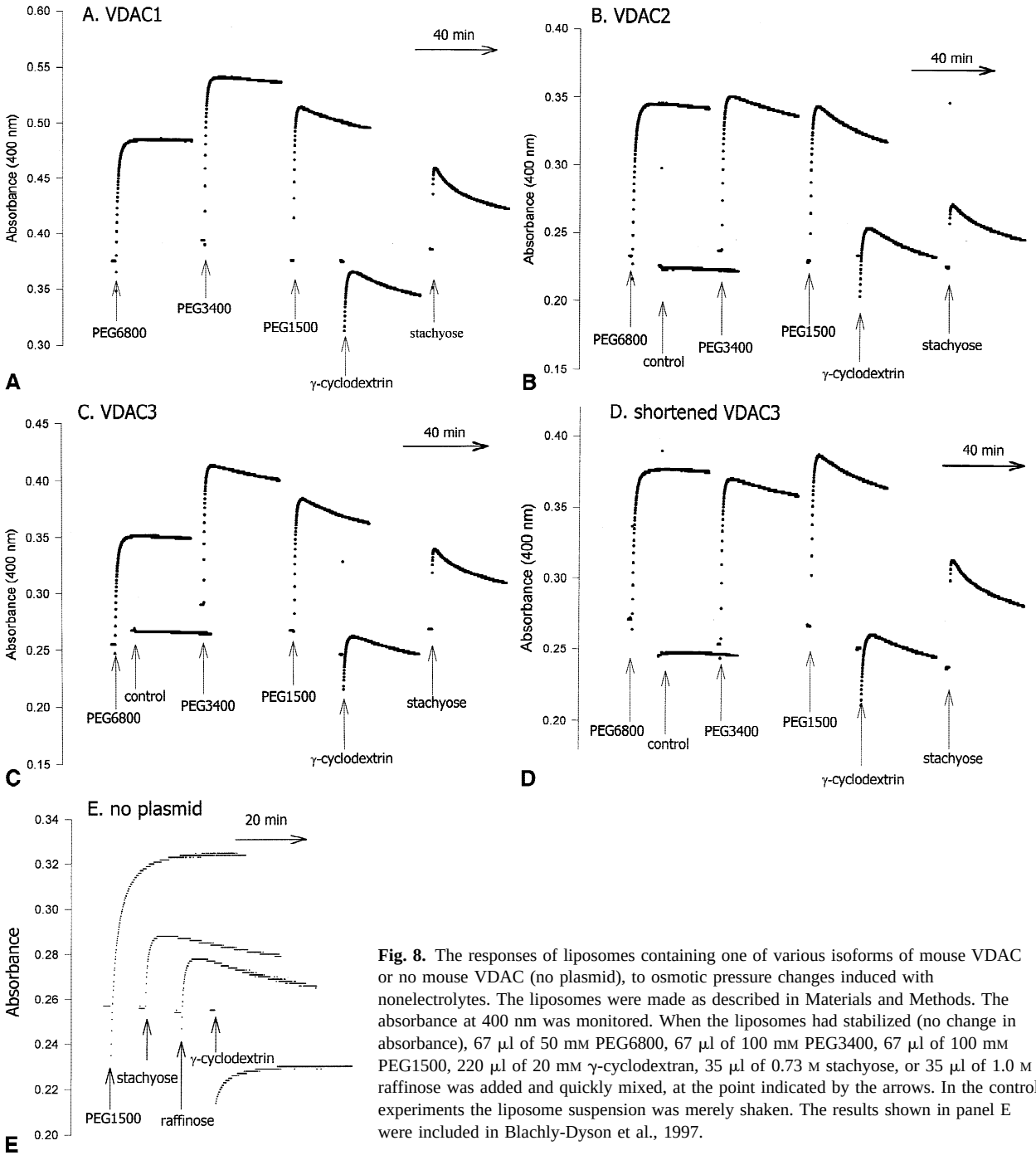


Fig. 8. The responses of liposomes containing one of various isoforms of mouse VDAC or no mouse VDAC (no plasmid), to osmotic pressure changes induced with nonelectrolytes. The liposomes were made as described in Materials and Methods. The absorbance at 400 nm was monitored. When the liposomes had stabilized (no change in absorbance), 67 μl of 50 mM PEG6800, 67 μl of 100 mM PEG3400, 67 μl of 100 mM PEG1500, 220 μl of 20 mM γ-cyclodextran, 35 μl of 0.73 M stachyose, or 35 μl of 1.0 M raffinose was added and quickly mixed, at the point indicated by the arrows. In the control experiments the liposome suspension was merely shaken. The results shown in panel E were included in Blachly-Dyson et al., 1997.

controlling the flux of metabolic anions such as ATP, citrate, succinate, and phosphate (Rostovtseva & Colombini, 1996, 1997; Hodge & Colombini, 1997).

The electrophysiological properties of VDAC3 were highly variable for unknown reasons.

The permeability measurements of the outer membrane of intact and functional mitochondria show large differences in NADH permeability depending on which mouse gene was expressed. Each VDAC gene was expressed in yeast using the identical plasmid and promoter. However, the use of HA-tagged isoforms to measure of the expression level showed that the steady-state level of these proteins in the outer membrane varied significantly from one isoform to another. The lower outer membrane permeability of mitochondria containing VDAC2 or VDAC3 with the extra methionine, can

be readily accounted for by a reduced amount of VDAC protein. However, the reduced permeability of mitochondrial outer membranes containing the other isoforms, cannot be accounted for in this way. In addition, since there is quite a bit of evidence that not all VDAC channels are open in the outer membrane, in fact the majority may be closed (Lee et al., 1994), it is reasonable to conclude that the probability of the channels being open, in the isolated mitochondrion, varies with the particular isoform. There may be a differential regulation of mouse VDAC channels in yeast mitochondria.

After reconstitution into liposomes, all the isoforms induced very similar permeabilities to nonelectrolytes indicating that each isoform can form channels with a similar permeability. In addition, they display a similar effective size with a molecular weight cutoff between 3400 and 6800. This is the normal cutoff for VDAC channels (Colombini, 1980). This does not mean that all these isoforms have the same permeability to NADH because charge selectivity might come into play. From the planar membrane experiments, the single-channel conductances properties are not different enough to have resulted in different fluxes in the liposome experiments. Thus the differences observed in the outer membrane permeability experiments were most likely not due to intrinsic permeability differences in the VDAC3 isoforms but rather due to a combination of different levels of expression (for VDAC2 and VDAC3 with the extra methionine) or regulation of channel opening probability (for the other VDAC3 isoforms).

The differences in the magnitude of the observed permeability between the liposome and planar membrane experiments are understandable on the basis of channel insertion into membranes. In the liposome experiments, the VDAC channels have no choice but to incorporate in some form into the liposome membranes. The rehydration at room temperature of the lyophilized proteolipid contains no detergent to keep intrinsic membrane proteins out of the newly formed membranes. By contrast, insertion into planar membranes requires that the protein overcome some energy barrier to go from the detergent-solubilized state into the phospholipid matrix. It has been shown that VDAC channels from some sources can catalyze this process and accelerate it by 10 orders of magnitude (Xu & Colombini, 1996, 1997). From our observations, this may also be true for mouse VDAC1 and 2 but perhaps not for VDAC3. In any case, the poor insertion of the full length VDAC3 and the lack of insertion of the shortened VDAC3 could be attributed to the lack of auto-directed insertion.

Different isoforms of the VDAC channel have also been found in other species. Humans have two known VDAC proteins, HVDAC1 and HVDAC2, while a human VDAC3 homologue can be found in human EST databases. Their amino acid sequences are 75% identical (Blachly-Dyson et al., 1993). Comparisons of the sequences among the different mouse VDAC proteins and the sequences between mouse VDAC and human VDAC show that the three mouse VDACs are 70% identical to one another, and mouse VDAC1 is 90% identical to HVDAC1 while mouse VDAC2 is more than 90% identical to HVDAC2. What is more, mouse VDAC2 is predicted to have an extension of 12 amino acids at the amino terminus compared to an 11 amino acid extension found in HVDAC2 (relative to VDAC1 from yeast and humans). This indicates that mouse VDAC1 is evolutionarily more related to HVDAC1, while mouse VDAC2 is closer to HVDAC2 (Sampson et al., 1996*a*, *b*). A comparison of their electrophysiological properties supports this hypothesis. Both HVDAC1 and mouse VDAC1 have the typical channel conductance, ion selectivity, and voltage dependence indicating that they are the prototypic version. Mouse VDAC2 is very similar to HVDAC2: both have normal voltage gating characteristics, and both tend to insert in a lower conductance state. The major difference is that HVDAC2 loses its voltage dependence shortly after reconstitution into planar membranes (Blachly-Dyson et al., 1993) while mouse VDAC2 does not. The mouse VDAC3 protein sequence is the most divergent being only 66% identical to HVDAC1 and 70% identical to HVDAC2. This is more evident in its very different properties suggesting a different physiological role.

Sampson et al. (1998) discovered the existence of a 1-codon ATG exon in mouse VDAC3 gene. The presence of this ATG exon raises the possibility that it could be an alternative start site. In yeast, only the full length was detected and the extra methionine did not significantly alter the growth rate (*data not shown*). However, the levels of the HA-tagged VDAC3 containing the extra methionine was present at about half the level found when the methionine was absent. Yet, the outer membrane permeability to NADH of mitochondria containing either form of VDAC was unchanged indicating that VDAC3 channels lacking the extra methionine were more likely to be closed in intact mitochondria. Unfortunately, the high variability in the electrophysiological properties of these two channels did not allow us to detect differences in the open probability of these channels.

The shorter VDAC3 used in these experiments was the form that would be produced if translation began at the second start site. From the Ha-tagged experiments, this protein is incorporated at the same level as VDAC1 and yet the NADH permeability is very low but similar to that found with the other versions of VDAC3. Among the possible interpretations are: (i) the protein is maintained at a higher level than the other VDAC3 forms because of a lower NADH permeability; (ii) the shortened VDAC3 is more likely to be in a closed state under

these conditions. The liposome experiments demonstrate that this shortened version still forms large channels but does not address the ability of these channels to facilitate NADH flux. Since we were unable to reconstitute these into planar membranes we cannot say anything about the open probability.

VDAC3 may serve other functions in cells that were not examined in these experiments. Its major physiological role may be in regulating the functions of other proteins rather than forming a pathway for metabolites. There are indications that the shortened VDAC3 can more effectively compensate for the loss of yeast VDAC1 by allowing better growth at 37°C on glycerol medium (Sampson et al., 1998). Thus further work is required to clarify the situation.

The physiological roles of these different mouse VDAC isoforms need further study, but from our experiments it could be concluded that VDAC1 and VDAC2 have a more important role in determining the permeability of mitochondrial outer membrane than VDAC3. In mouse testes tissue VDAC1 is not expressed, leaving VDAC2 as the candidate most likely responsible for metabolite flux through the mitochondrial outer membrane. It is unclear why both VDAC1 and VDAC2 are expressed in the other tissues. Perhaps each is regulated differently. Gating for one might be coarsely tuned and the other finely tuned. Permeability induced by VDAC1 seems to be much more sensitive to NADH than that resulting from VDAC2. There may also be different regulatory proteins acting on the different isoforms.

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