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Characterization of Channel-Forming Activity in Muscle Biopsy from a Porin-Deficient Human Patient[1]

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A bioptic specimen from the muscles of a patient suffering from severe myopathy was inspected for the presence of human porin 31HL. Western blotting suggested that the specimen was free of the most abundant eukaryotic porin 31HL (HVDAC1). The specimen was treated with detergent and the soluble protein fraction was passed through a dry hydroxyapatite column. The passthrough of this column was inspected for channel formation in artificial lipid-bilayer membranes. The channel observed under these conditions had a single-channel conductance of about 2.5 nS in 1 M KCl, was cation selective, and was found to be virtually voltage independent. Experiments with a control specimen from a healthy human being, without any indication for muscle myopathy, revealed the presence of the voltage-dependent porin 31HL in the sample. It is discussed whether the patient's bioptic specimen contained another human porin, which has not been studied to date in its natural environment.

KEY WORDS: Porin deficiency; muscle biopsy; porin isoforms; VDAC; lipid-bilayer membrane; voltage dependence.

INTRODUCTION

Eukaryotic porins or VDACs (voltage-dependent anion-selective channels), are integral membrane proteins with an electrophoretic mobility corresponding to 35 kDa (SDS–PAGE) and a molecular mass 30–32 kDa that form transmembrane channels in biological and artificial membranes. These channels are permeable to solutes of molecular masses below 3 kDa. They were first discovered in the mitochondrial outer membrane of a variety of eukaryotic cells (Colombini, 1979; Freitag *et al.*, 1982; Lindèn *et al.*, 1982; Mannella *et al.*, 1989; Benz, 1994). The role of these membrane channels is the almost free access of small hydrophilic molecules to the mitochondrial intermembrane space, *i.e.*, they represent the major permeability pathway of the mitochondrial outer membrane. Besides this role in mitochondrial metabolism, there exists also emerging evidence that members of the same family of proteins are also present in the plasma membrane of eukaryotic cells (Thinnes *et al.*, 1989). Recently, we have purified a similar polypeptide from plasma membranes of cultured cells (Báthori *et al.*, 1999). Based on morphological and biochemical evidence (Lewis *et al.*, 1994; Junankar *et al.*, 1995; Reymann

[1] Key to abbreviations: HVDAC, human voltage-dependent anion channel; MVDAC, mouse voltage-dependent anion channel; n.v., normal value.

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et al., 1998), the multiple topological localization of eukaryotic porins now seems very likely (Báthori *et al.*, 1999), although there does not exist a good explanation for the function of eukaryotic porin in various cellular membranes.

Studies at the genetic level have demonstrated that in human (Messina *et al.*, 1998; Rahmani *et al.*, 1998) and mouse genome (Sampson *et al.*, 1997), several (at least three) genes exist that encode for eukaryotic porins. Their primary sequences are very similar (in the range of 70 to 90% amino acid identity) (Blachly-Dyson *et al.*, 1993; Sampson *et al.*, 1997). The presence of several porin genes in the genome may represent some "porin redundancy" or may be involved in some still unknown tissue-specific regulation. The isoforms of human (Blachly-Dyson *et al.*, 1993) and mouse porins (Xu *et al.*, 1999) have been expressed in yeast and studied in lipid-bilayer membranes. They all form partly voltage-dependent channels in the reconstituted system. However, the channel-forming unit contain, in this case, ergosterol, whereas the genuine sterol present in mammalian tissues is cholesterol, which may influence channel properties (De Pinto *et al.*, 1989b; Popp *et al.*, 1995). The discovery of the first human patient showing, in muscle biopsies, lack of porin 31HL (HVDAC1) allowed the possibility to obtain information in a naturally occurring defective porin system. In cultured fibroblasts from the same patient, porin 31HL (isoform 1) was expressed (Huizing *et al.*, 1994, 1996), showing a possible tissue-specific regulation of the gene. The lack of the porin isoform 1 (HVDAC1) in such muscle biopsies has been associated with a fatal mitochondrial encephalomyopathy. The biochemical characterization of mitochondria from the human patient pointed to a reduced functionality of mitochondria, with a most likely reduction of the exchange of mitochondrial metabolites between the inside and the outside of mitochondria, the typical function of porin (Huizing *et al.*, 1996).

In this study, we applied a purification procedure to the muscle bioptic specimen from another patient in whom we could observe lack of immunoreactivity to polyclonal antibodies raised against mammalian porin (HVDAC1). The electrophysiological analysis of a chromatographic HTP passthrough is described. These experiments were aimed at the characterization of "rescue" porin activities possibly present in mammalian tissues and suggest that the bioptic specimen of the patient contained another pore-forming activity.

MATERIALS AND METHODS

Clinical Case Report

At 18 months age, an isolated glycosuria was first noted in the patient, but no further biochemical investigations were carried out, in spite of recurrent infections presenting with fever, easy fatiguing, and muscle hypotonia since the age of 1 year. At 3 years, the child was suddenly hospitalized for continuous vomiting and unconsciousness. Metabolic acidosis was prominent (blood pH 7.27, HCO_3^- 2 mmol/L) with low plasma electrolytes (P, Mg, Cl, K, Na), ketonuria, electrolyturia, and massive bicarbonaturia. Weight and length were below the third percentile for his age. The level of urinary organic acids (mmol/mol creatinine detected) were: lactate 40 (n.v. 0–25,), 3-hydroxybutyrate 905 (n.v. <3), acetoacetate 52 (n.v. <2), 3-methylglutaconate 23 (n.v. <9), and 3-methylglutarate present in traces. Progressive tubular insufficiency with elevated transaminases, glycosuria, phosphaturia, generalized hyperaminoaciduria, and recurrent electrolyte derangements characterized the clinical course. Persistent lactic acidosis, with the plasma lactate ranging between 2 and 11 mmol/L, an increased lactate/pyruvate ratio, and a low plasma-free carnitine of 9.83 μmol/L v. n.v. 35–60 were also found.

Bone marrow examination showed general hypocellularity with marked erythroblastopenia and vacuolated erythroblasts. Pearl stain revealed the presence of sideroblasts. Eventually recurrent thrombocytopenia, anemia, and neutropenia with untreatable infections led to the death of this patient 1 month after the admission, despite all efforts: repeated fresh blood and plasma infusions, platelet-rich plasma concentrates, albumin, acetyl carnitine i.v., continuous electrolyte, and bicarbonate corrections.

Total DNA extracted from leukocytes was digested by the restriction enzyme PvuII (which linearizes the mtDNA) electrophoresed on agarose gel, blotted, and then hybridized with a ND1 probe. This revealed a normal band of 16.5 kb in control and an additional abnormal band (12 kb) corresponding to a shortened mtDNA molecule in the patient.

HTP Chromatography of Bioptic Material

The HTP [hydroxyapatite (Bio-gel HTP) Bio-Rad] chromatography of bioptic material from the above described patient was performed essentially as

described (De Pinto *et al.*, 1987). Because of the very small amount of bioptic specimen, the mitochondria purification was not attempted. After homogenization of the specimen, it was centrifuged at $600 \times g$ and the supernatant collected. This supernatant was spun down at $12{,}000 \times g$ and the resulting pellet (highly enriched in mitochondria) was resuspended in 3% Triton X-100, 10 mM Tris-HCl (pH 7.0), and 1 mM EDTA. After 30 min at 4°C, the solubilization mixture was centrifuged at $40{,}000 \times g$ for 15 min and the supernatant (0.2 ml) loaded onto a dry hydroxyapatite column (0.1 g). The elution was performed with the Triton X-100-containing buffer. The eluted first 0.3 ml were collected.

SDS Gel Electrophoresis and Immunoblotting Experiments

Polyacrylamide slab (12%) gel electrophoresis of aceton-precipitated samples was performed in the presence of 0.1% SDS.

Antiserum against bovine heart porin was raised in rabbit. A commercially available monoclonal antibody raised against the N-terminal end (first 18 amino acids) of the human porin (Calbiochem) was also used. The specimen's proteins separated on SDS–PAGE were transferred to nitrocellulose, incubated with the antiserum, and then with an anti-rabbit Ig horseradish peroxidase-linked antibody (Amersham). The peroxidase reaction was performed by 20 ml of a mixture of 0.05% 4-chloro-1-naphtol, 16% methanol, 0.5% BSA in 0.14 M NaCl, and 0.01 M phosphate (pH 7.0), with final addition of 12 μl of 30% H_2O_2. Alternatatively, the ECL (Amersham) protocol was used.

Lipid Bilayer Experiments

The methods used for the black-lipid bilayer experiments have been described previously (Benz *et al.*, 1978). The membranes were formed across a circular hole (surface area about 0.4 mm^2) in the thin Teflon wall separating two aqueous compartments from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, Alabama) in *n*-decane. Bilayer formation was indicated when the membrane turned optically black in reflected light. The current through the membranes was measured with two calomel electrodes switched in series with a voltage source and a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder. Zero-current membrane potentials were measured with a Keithley 617 electrometer 5–10 min after the application of a tenfold salt gradient across the membranes (Benz *et al.*, 1979).

RESULTS

Immunological Characterization of the Patients Muscle Biopsy Specimen for Eukaryotic Porin

In a preliminary study, we have better characterized the immunological activity of a polyclonal antiserum, which has been raised in rabbit against purified bovine heart mitochondrial porin and has been used in other previous reports (De Pinto *et al.*, 1989a, 1991). This characterization controlled whether other linear epitopes in the human porin 31HL (HVDAC1) were detectable by our pAb, in addition to the N-terminal end. These epitopes were chosen by computer predictions of the most exposed sequences (De Pinto *et al.*, 1991). Furthermore, we were interested to control whether our antibody (pAb) could recognize the N-terminal moiety in the second isoform of porin (HVDAC2). Figure 1 shows that our polyclonal Ab was able to cross react with human porin and human porin peptides. The most antigenic parts in the porin 31HL structure are thus the N- and the C-terminal ends of the protein (Fig. 1A). This means that our pAb is able to detect more linear epitopes of the protein and not just a single one. Synthetic peptides designed from the deduced N-terminus of HVDAC2, even when showing a clear sequence homology with the highly immunogenic N-terminal part of porin, did not show any affinity with our anti-porin Ab (Fig. 1B). Alternatively, the same dot blots were stained with a monoclonal Ab (mAb), raised against a synthetic peptide mimicking the N-terminal end of human porin 31HL, as a control (Fig. 1A and B).

This pAb was used for Western blot analysis of bioptic material obtained from patients affected by mitochondrial pathologies, mainly involving mtDNA deletions. Our attention was attracted by the result from a muscle bioptic specimen, which did not show any cross reactivity with our anti-porin pAb (Fig.2). This patient showed an overall picture of impaired metabolism, together with a large deletion in the mtDNA (see case report in the Material and Methods section). The patient died very soon and no further analysis could be made. We thus were interested to

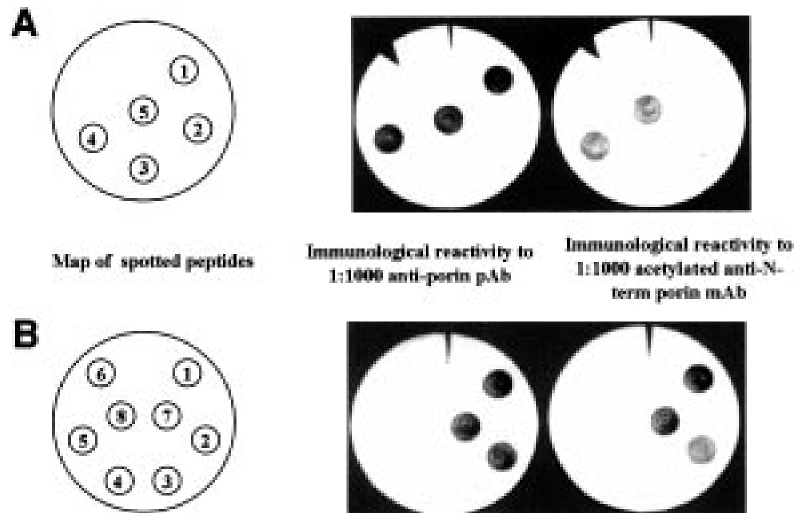

Fig. 1. Dot blots of synthetic porin peptides immunodecorated with anti-porin antibodies. (A) Peptides designed from the sequence of human VDAC1. In the scheme on the left side, the position of the spotted peptides is shown. 1, HVDAC1 C-ter (aa 271-282); 2, HVDAC1 predicted loop 1 (De Pinto *et al.*, 1991) (aa 104-123); 3, HVDAC1 loop 2 (aa 149-172); 4, HVDAC1 N-ter not acetylated (aa 1-19); 5, HVDAC1 (purified protein). Each peptide was (1 μg) spotted in the corresponding dot. The dark spots indicate their reactivity with the Ab shown in the figure. (B) Peptides designed from the sequence of human VDAC2. In the scheme on the left side, the position of the spotted peptides is shown. 1, HVDAC1 N-ter not acetylated (aa 1-19); 2, HVDAC1 N-ter not acetylated (aa 1-35); 3, HVDAC2 N-ter not acetylated (aa 1-19); 4, HVDAC2 N-ter acetylated (aa 1-19); 5, HVDAC2 N-ter not acetylated (aa 1-35); 6, HVDAC2 N-ter acetylated (aa 1-35); 7, HVDAC1 (purified protein); 8, control (non-porin peptide). Each peptide (1 μg) was spotted in the corresponding dot. The dark spots indicate their reactivity with the Ab shown in the figure.

investigate the channel-forming properties in this muscle biopsy specimen. It was already known, indeed, that mitochondria had to have a rescue-pore activity leading to some permeability of the outer membrane, otherwise the mitochondria just could not exchange any metabolite with the cytoplasm (Huizing *et al.*, 1994, 1996). Because of the very small amount of muscle specimen, it was not possible to obtain purified mitochondria. Instead the specimen was homogenized and a fraction enriched in mitochondria was obtained by centrifugation (the so called 600 × *g* supernatant). To this material, we applied a purification procedure, which employed a Triton X-100 solubilization followed by a chromatographic step on a dry HTP column. This procedure has been shown to be very selective for the isolation of mitochondrial proteins deeply embedded in the phospholipid bilayers, such as the mitochondrial inner membrane carriers and porin itself (De Pinto *et al.*, 1987). Half of the HTP eluate was run on a SDS–PAGE, but no protein band could be visualized by staining with Coomassie or with silver. This was most likely due to the very small amount of protein present in the sample. The rest of the HTP passthrough was used to exploit the presence of channel-forming activity.

Functional Study with the Muscle Biopsy Specimen

The functional study was possible because of the high sensitivity of BLM for the presence of mitochondrial porin, which in principle, allows the detection of single channels in a protein sample. Results of reconstitution experiments with HTP passthrough of the total 600 × *g* supernatant solubilized in Triton X-100 are shown in Figs. 3A and 4A. Most of the conductance steps had a single-channel conductance of about 2.5 nS in 1 M KCl, whereas the "normal" porin channels, which have under these conditions a conductance of 4 nS were very rarely observed (see the histograms in the Fig. 4). Single-channel experiments were also performed with other salts and other concentrations to investigate whether the channel detected in the bioptic material of the patient showed any peculiarity. In 1 M LiCl, the single-channel conductance was about 1.5 nS and a similar value was also calculated from single-channel records in 1 M potassium acetate. Experiments with 0.1 M KCl suggested that its single-channel conductance was a linear function of the aqueous salt concentration, which means that the channel present

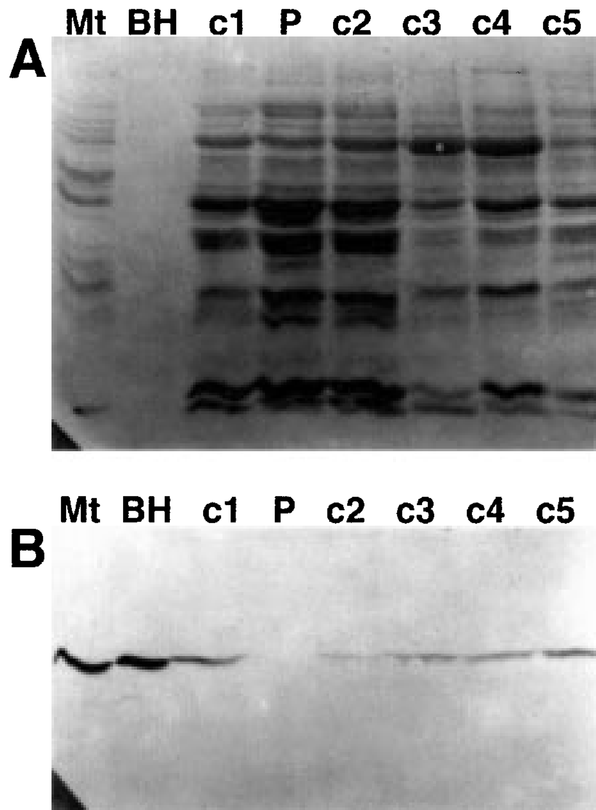

Fig. 2. Immunoblots of patient's muscle bioptic tissues with anti-porin antiserum. Samples of 600 × *g* SNT from patient's and from controls' muscle bioptic specimens were electrophoresed on SDS–PAGE and blotted to nitrocellulose. The membrane was stained with Ponceau red (A) to control the protein amount in each lane. Immunostaining (at a serum dilution of 1:1000) was performed with the polyclonal antiserum raised against bovine heart mitochondrial porin, characterized in Fig. 1. Staining was with secondary anti-rabbit Ab (Amersham) and 4-chloro-1-naphthol (B). Lane Mt, bovine heart mitochondria; BH, purified bovine heart porin; P, mitochondrial fraction from the patient reported in this work; c1–c5, mitochondrial fractions obtained from other patients affected by mitochondrial pathologies.

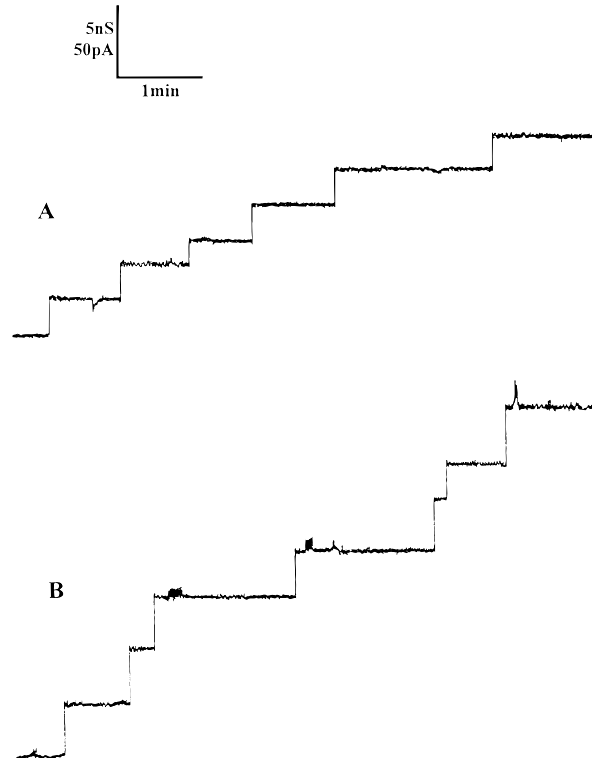

Fig. 3. (A) Single-channel record of a diphytanoyl phosphatidylcholine/*n*-decane membrane in the presence of 50 μl of the passthrough of the HTP column of the patient's bioptic specimen. The aqueous phase contained 1 M KCl (pH 6). The applied membrane potential was 20 mV; $T = 20°C$. (B) Single-channel record of a diphytanoyl phosphatidylcholine/*n*-decane membrane in the presence of 50 μl of the passthrough of the HTP column of human bioptic specimen without any indication of the existence of a myopathy. The aqueous phase contained 1 M KCl (pH 6). The applied membrane potential was 20 mV; $T = 20°C$.

in the HTP passthrough of the bioptic specimen did not exhibit a binding site or point net charges.

In further experiments, we studied the ionic selectivity of the channel obtained from the bioptic specimen. Zero-current membrane potential measurements allow the calculation of the permeability ratio P_C divided by P_A in multichannel experiments. Diphytanoyl phosphatidylcholine/*n*-decane membranes were formed either in 10 or in 50 mM KCl solution and small amounts of the HTP passthrough were added to the aqueous phase on one side of the membrane (the *cis* side) when the membranes were in the black state. After incorporation of about 100 channels into a membrane, a tenfold KCl gradient was established by addition of small amounts of 3 M KCl salt solution to one side of the membrane. In all experiments performed with the specimen, the more diluted side of the membrane became positive, which indicated preferential movement of potassium ions through the channel. The zero-current membrane potentials for a tenfold gradient of KCl was about 28 mV, on average (four experiments). Analysis of the zero-current membrane potential using the Goldman–Hodgkin–Katz equation suggested that anions also could have a certain permeability through the channel because the ratio of the permeability coefficients P_C divided by P_A was about 4. It is noteworthy that normal mitochondrial or eukaryotic porins are slightly anion selective in the open state [P_C divided by $P_A = 0.5$ (Roos *et al.*, 1982; Benz *et al.*, 1992); see also below]

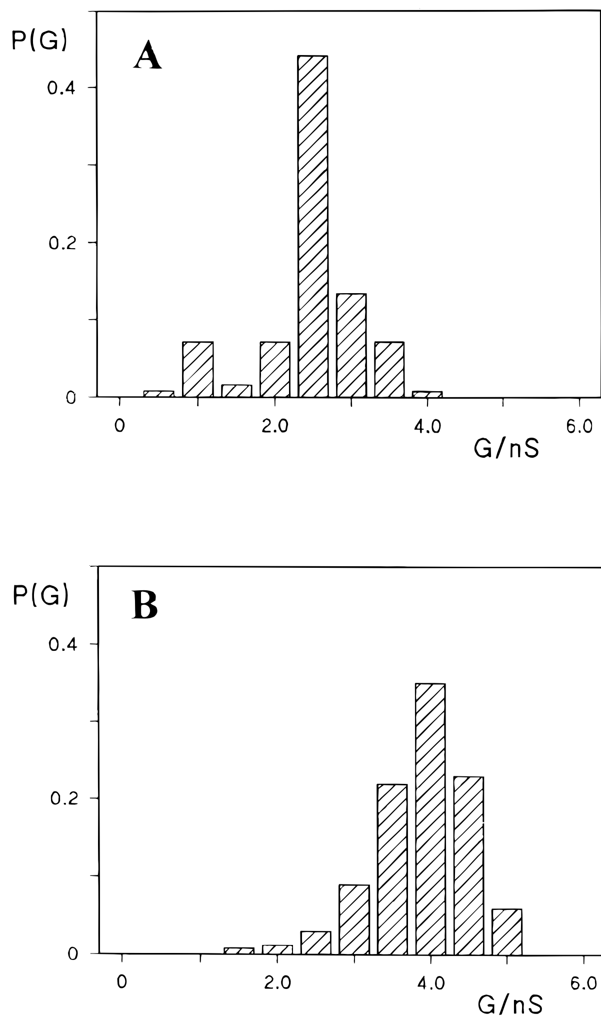

Fig. 4. (A) Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit observed for 186 single-channel fluctuations obtained from experiments with HTP passthrough of the patient's bioptic specimen. Same conditions as described in Fig. 3A. The mean of the single-channel fluctuations was 2.7 nS. Note that the 4 nS channel of porin 31HL was almost completely absent. (B) Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit observed for 206 single-channel fluctuations obtained from experiments with HTP passthrough of a normal human bioptic specimen as a control. Membrane experiments performed at the same conditions as in Fig. 3B. The mean of the single-channel fluctuations was 3.8 nS. Note that the 4 nS channel of porin 31HL was the prominent channel observed under these conditions.

A characteristic feature of all eukaryotic porins studied to date is their voltage dependence. Starting with about 20 to 30 mV, they close in an approximately symmetric fashion. To check whether the channel observed in the bioptic specimen was also voltage dependent, we used multichannel experiments. Small amounts of the HTP passthrough were added to the aqueous 0.1 M KCl solution on one side of a black diphytanoyl phosphatidylcholine/*n*-decane membrane (the *cis* side). After 30 min, about 20 channels were reconstituted into the membrane. Then, different potentials were applied to *cis* side of the membrane: first 10 and then −10 mV. These experiments were repeated with voltages between 20 and 100 mV. Interestingly, we found almost no voltage dependence even when the membrane potential was as high as 120 mV (data not shown). The experiments were analyzed in the following way: the membrane conductance (G) as a function of voltage, V_m, was measured when the voltage was applied for some time (up to 1 min) following the voltage step V_m. G was divided by the initial value of the conductance (G_o, which was a linear function of the voltage) obtained immediately after the onset of the voltage. In other cases, G was divided by the conductance, G_o at 5 mV, which was virtually voltage independent. The closed squares of Fig. 5 correspond to the voltage dependence of the channel from the bioptic specimen (mean of four membranes) when the sample was added to the *cis* side of the membrane.

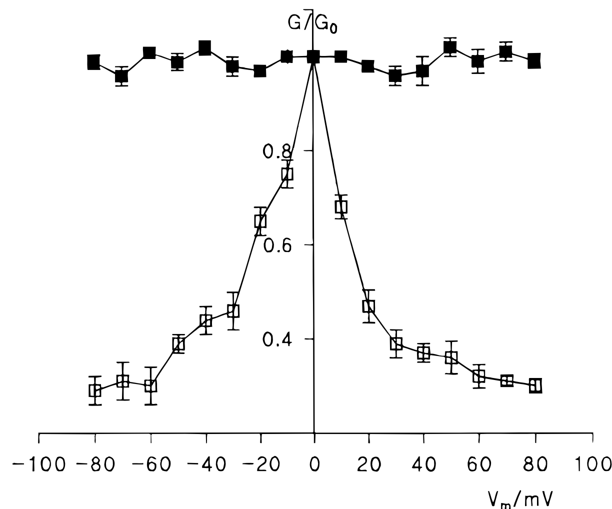

Fig. 5. Ratio of membrane conductance G at a given membrane potential (V_m) divided by the conductance G_o at 5 mV as a function of the membrane potential V_m. The open squares indicate the measurements, in which 50 μl of the HTP passthrough of the biopsy specimen of the patient were added to the *cis* side of lipid-bilayer membranes. The closed squares show measurements, in which 50 μl of the passthrough of the control specimen were added to the *cis* side of membranes. (The membrane potential refers always to the *cis* side of the membrane.) The aqueous phase contained 0.1 M KCl. The membranes were formed from diphytanoyl phosphatidylcholine dissolved in *n*-decane. $T = 20°C$. Means (±SD) of four membranes are shown.

The results suggested that the channel had only minor voltage dependence, if any.

Control Experiments with a Human Biopsy Specimen

Because of the small amount of the bioptic specimen and the lack of protein bands in the HTP passthrough, control experiments were required. For this, human bioptic specimens were taken without any indication for the lack of mitochondrial porin or the presence of any other mitochondrial pathology. These samples were also homogenized and the $600 \times g$ supernatant was also passed through a HTP column. The passthrough was analyzed in a similar way in experiments with lipid-bilayer membranes. Single-channel conductance measurements revealed the presence of the "normal" porin channels with a conductance of 4 nS (see Fig. 3B and 4B). Similarly, we also performed with the passthrough of the control specimen, zero-current membrane potential measurements and found the channel essentially non specific or slightly anion selective (mean of four experiments) as it is typical for eukaryotic porins of different sources (Benz, 1994). Finally, we also studied the voltage dependence of the control specimen. In this case, we observed strong voltage dependence, as illustrated in Fig 5 (open squares). At about 10 mV, the channels started to close in a more or less symmetric manner, although the protein was only added to one side of the membrane (*cis* side)(see Fig. 5). Analysis of the data of Fig. 5, using a previously postulated formalism, revealed that the number of gating charges moving for channel gating through the entire membrane was about two, which was in good agreement to the voltage dependence of other eukaryotic porins (Benz *et al.*, 1992; Benz, 1994; Popp *et al.*, 1996). Taken together, it was clear that the porin in the control specimen behaved similarly if not identically to most eukaryotic porins studied to date and, in particular, the same as porin 31HL in similar experiments (Benz *et al.*, 1992).

DISCUSSION

In this work, we describe the characterization of a bioptic muscle specimen obtained from a patient who was suffering from a severe muscle myopathy and who died at the age of 3 because of this disease. The muscle specimen lacked porin 31HL, as judged from Western blots, with the total protein using a previously well-characterized antiserum. At a molecular level, the patient showed the presence of a large deletion in his mtDNA. At variance with another reported porin-deficiency case, where porin deficiency was the only molecular indication of a defect in the mitochondrion (Huizing *et al.*, 1996), the case reported here showed multifactorial damaging of mitochondria. It may thus be possible that the primary defect could be in some still unknown mechanism of concerted genetic activity necessary for the correct biogenesis of mitochondria, including the correct assembly of porin in the mitochondrial outer membrane.

We inspected the bioptic specimen of the patient for channel-forming activity. Using a previously developed method (De Pinto *et al.*, 1987), we passed the detergent extract of a mitochondrially enriched fraction of the specimen through a dry HTP column. The passthrough of the column, which normally contains the porin fraction, was used for lipid-bilayer membrane experiments. Although proteins could not be detected in the passthrough on SDS–PAGE, probably because of the low protein content, it was able to form channels with a single-channel conductance of about 2.5 nS in 1 M KCl in the membranes. The normal eukaryotic porin channel, which has a conductance of 4 nS under these conditions, was not observed in these reconstitution experiments. Interestingly, the channel was cation selective, which also differs from the properties of the normal human porin 31HL (HVDAC1) (Benz *et al.*, 1992). Another important feature of the channel reconstituted from the patient's bioptic specimen was the absence of any voltage dependence of the channel. This was surprising because all eukaryotic porins purified from tissues or cells where they were naturally occurring and studied to date are voltage dependent and switch to cation-selective closed states when the membrane voltage exceeds 20 to 30 mV. Thus, the channel observed in the bioptic specimen of the patient had properties which differed from those of human porin 31HL (Benz *et al.*, 1992).

The low protein concentration of the bioptic muscle specimen of the patient made it necessary to investigate whether a similar human specimen without any indication for myopathy behaved similarly in lipid-bilayer experiments. The HTP passthrough from a control specimen was inspected for channel formation in lipid-bilayer membranes. Again we observed channels in the reconstitution experiments. However, this time the properties of the reconstituted channels were found to be indistinguishable from those that have been

observed previously in reconstitution experiments with porin 31HL (Benz *et al.*, 1992). This means that the channels were slightly anion selective, voltage dependent, and had a single-channel conductance of 4 nS in 1 M KCl.

The Channel Observed in the Patient's Bioptic Specimen Could Be Another Porin Isoform

When we consider that the channel observed in the patient's specimen is not porin 31 HL, the question arises of whether it could be another mitochondrial porin. A possible candidate for this is HVDAC2, because it has been demonstrated that its voltage dependence decays after reconstitution in lipid-bilayer membranes of the protein expressed in yeast (Blachly-Dyson *et al.*, 1993). It is now clear that several porin genes are present in the genome of eukaryotes (Blachly-Dyson *et al.*, 1993; Sampson *et al.*, 1997) and at least three genes encoding for very similar proteins (in the range 70– 90% homology) have been found in human and mouse. At least one of these genes can produce alternatively spliced isoforms (Sampson *et al.*, 1998). The genetic system underlying the porin expression in higher mammals and the possible functional roles of this group of proteins are not yet understood. Despite the presence of several genes in the mammals' genome, the only porin isoform purified so far as a protein from human tissues or cells has been the so-called porin 31HL, also termed "HVDACl" or "porin isoform 1" by the various authors (Thinnes *et al.*, 1989; Blachly-Dyson *et al.*, 1993; Messina *et al.*, 1998). Information about the functional features of a second porin isoform (HVDAC2) has been obtained by expressing the corresponding cDNA in yeast strains where the normal porin gene has been deleted (Blachly-Dyson *et al.*, 1993). Under these conditions, HVDAC2 is localized in the mitochondrial membrane (Yu *et al.*, 1995) and shows a functional pattern very similar to that known for HVDAC1, with the only exception of the voltage-dependence decay (Blachly-Dyson *et al.*, 1993). We have shown here that the channel-forming protein in the patient's specimen lacks voltage dependence. It thus seems to be possible that it was HVDAC2. On the other hand, we cannot exclude the possibility that the channel observed in the patient's specimen could be identical to mouse VDAC3 (MVDAC3), because several forms of this protein, including a truncated one, have recently been expressed in yeast (Xu *et al.*, 1999) and also show no voltage dependence in reconstituted system, together with a reduced permeability in intact mitochondria as compared with MVDAC1 and MVDAC2. However, this is still tentative because the only eukaryotic porin isolated from mammalian tissue is HVDAC1 (porin 31HL). The others have been isolated from yeast, which could influence their channel properties. Eukaryotic porins contain either ergosterol (Freitag *et al.*, 1982) or cholesterol (De Pinto *et al.*, 1989b). The sterols are essential for channel activity, but may also influence the channel properties, including voltage dependence (Popp *et al.*, 1995).

The existence of tissues, which lack the most studied porin 31HL on a genetic basis, is thus an interesting tool to isolate and characterize alternative isoforms of porin. This is especially important with porin isoforms alternative to isoform 1, since they were never isolated or studied in their physiological situation. In this paper we show for the first time the electrophysiological features of a pore-forming protein present in a tissue where the most abundant porin 31HL was absent and which could be HVDAC2 or HVDAC3. Further efforts to purify it in larger amounts are still necessary for more detailed studies, with the final aim to discriminate the physiological role of the various porin isoforms in mammalian cells.

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