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The mitochondrial channel VDAC has a cation-selective open state

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Abstract

The mitochondrial channel VDAC is known to have two major classes of functional states, a large conductance "open" state that is anion selective, and lower conductance substates that are cation selective. The channel can reversibly switch between open and half-open states, with the latter predominant at increasing membrane voltages of either polarity. We report the presence of a new functional state of VDAC, a cation-selective state with conductance approximately equal to that of the canonical open state. This newly described state of VDAC can be reached from either the half-open cation-selective state or from the open anion-selective state. The latter transition implies that a mechanism exists for selectivity gating in VDAC that is separate from partial closure, which may be relevant to the physiological regulation of this channel and mitochondrial outer membrane permeability.

Keywords: VDAC; Mitochondrial porin; Ion selectivity; Mitochondria; Patch clamp; Gating

1. Introduction

The permeability of the mitochondrial outer membrane to ions and metabolites is generally considered to be regulated by a 30-kDa protein that forms large-conductance ion channels in artificial membranes [1–5]. This protein is a major component of the mitochondrial outer membrane, constituting up to 50% of this membrane's protein in *Neurospora crassa* [6]. In planar phospholipid bilayers and liposomes, the ion channels formed by this protein display voltage-dependent transitions from a fully open (650 pS in 150 mM KCl) anion-selective state to a set of partially open substates that are generally described as cation selective. The most commonly observed partially open state has a conductance of about half that of the fully open state (300 pS). The electrophysiological characteristics of this channel, sometimes referred to as mitochondrial porin, are the basis for its acronym, VDAC (voltage-dependent, anion- selective channel) [5].

Structural models for VDAC generally invoke a porin-like beta-barrel motif, based on results from studies that include sequence analysis and CD spectroscopy [1,7–14]. The voltage-dependent changes in conductance state have been ascribed to major structural rearrangements in the beta-barrel that reduce the size of, and alter the distribution of fixed charges within, the lumen of the pore [1,15,16]. These studies include work with reagents like succinic anhydride and aluminum hydroxide as well as a variety of point mutations [17–22].

The cation-selective, lower-conductance substates of VDAC predominate at higher voltages and display greatly reduced permeability towards organic anions, such as respiratory substates and adenine nucleotides. This reduced permeability to large anions, attributed primarily to the reversal in charge selectivity [1], raises the possibility that large-scale transition of VDAC channels to such substates in vivo could down-regulate mitochondrial metabolism. Since the mitochondrial outer

Abbreviations: E_{rev}, equilibrium reversal potential; IMS, intermembrane space; TOM, translocase outer membrane; VDAC, voltage dependent anion-selective channel

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membrane, unlike the inner membrane, is not obviously electrogenic, it is unclear whether a transmembrane potential might be generated sufficient to induce the partially open state of VDAC observed in voltage clamping experiments. Several mechanisms have been proposed by which a significant Donnan potential might be generated across the outer membrane (e.g., [23]). Evidence supporting in situ VDAC closure includes a recent report that a substantial pH gradient may exist across the outer membrane, as measured by a pH-sensitive GFP protein bound to the outer surface of the inner membrane [24]. However, it is unclear how much of the signal is coming from GFP segregated in the intra-cristal space, which might be a sub-compartment of mitochondria distinct from the space between the inner and outer membrane [24,25]. Earlier experiments with isolated mitochondria showed that polyanions that increase VDAC's sensitivity to voltage induce changes in mitochondrial respiratory function consistent with reduced outer membrane permeability [2,26,27]. However, strictly speaking, these experiments are not proof that a membrane-potential-mediated mechanism for VDAC closure is in effect.

In this report, we describe a new permeability state of VDAC, a large-conductance, cation-selective substrate. Gating between this state and the more frequently observed anion-selective open state is not obviously voltage dependent. The implications of a voltage-*independent* selectivity gate in VDAC for regulation of mitochondrial outer membrane permeability are discussed.

2. Materials and methods

Mitochondria were isolated as previously described from wildtype yeast [28], rat liver [29], mouse FL5.12 cells [30] and *Neurospora crassa* [31]. Outer membranes were stripped from isolated mitochondria by the French press method [32] and further purified according to Mannella [31]. The purity of the membrane fractions was routinely assayed by Western blotting, and contamination of the outer membranes by inner membranes was typically less than 5% [33,34]. Outer membranes were reconstituted into proteoliposomes by dehydration–rehydration as previously described [28]. Purification of *Neurospora crassa* VDAC on a hydroxyapatite/celite column and reconstitution into liposomes were done according to Shao et al. [8].

Patch-clamp procedures and analysis used were as previously described [28,30]. Briefly, membrane patches were excised from proteoliposomes by moving the micropipette after formation of a giga-seal using microelectrodes with ~0.4-μm diameter tips and resistances of 10–30 MΩ. Unless otherwise stated, the solution in the microelectrodes and bath was 150 mM KCl, 5 mM HEPES, pH 7.4. Planar bilayers were formed across a 100-μm hole with a glass rod coated with phosphatidylcholine (Sigma, 2 mg /ml decane). Voltage-clamp conditions were established using a Dagan 3900 patch clamp amplifier in the inside-out mode. Voltages are reported as bath potentials, i.e., a net flow of cations from the bath to the pipette is considered to produce a positive current. Currents and voltages were visualized on two digital oscilloscopes; one showed the *I–V* curve while the other allowed monitoring current (and voltage) as a function of time. Unfiltered currents and voltages were recorded with time on VHS videotape using a Neurocorder digitizer and current traces were analyzed after passing the signal through a Frequency Devices low-pass filter at 2 kHz with 5 kHz sampling. Current traces were analyzed using the WinEDR program (Strathclyde Electrophysiological Software, courtesy of J. Dempster, University of Strathclyde, UK) and pClamp 8.0 (Axon Instruments Inc.). Origin 7.0 software (OriginLab Corp.) was used to convert the current and voltage verses time recordings into *I–V* plots. Ion gradients for selectivity measurements were established by perfusion of the ~0.5 mL bath with 5 mL of 30 mM KCl 184 mM Mannitol 56 mM Sucrose 5 mM HEPES pH 7.4 while the solution in the microelectrodes remained 150 mM KCl, 5 mM HEPES, pH 7.4. Permeability ratios were typically calculated from the reversal potential (E_{rev}) in the presence of a 150:30 mM KCl gradient as previously described [35], using the Goldman Hodgkin Katz equation [36] as described below. Agar bridges were routinely used to minimize junction potentials. Seals were typically 1–10 GΩ; the patch conductances were typically insignificant (<50 pS) and the reversal potentials were typically zero in the absence of channel activity and when the channels closed.

3. Results

The canonical electrophysiological behavior of VDAC is demonstrated in Fig. 1, for outer membranes from *Neurospora crassa* incorporated into a planar phospholipid bilayer and voltage-clamped by standard procedures. In this case, the response of the membrane, containing probably 2 VDAC channels, to a computer-generated triangular voltage ramp (lower curve, Fig. 1A) was monitored. At small membrane voltages, the current trace (upper curve, Fig. 1A) follows the voltage in a linear fashion. At higher voltages of either polarity (amplitude typically greater than 20 mV), step-like transitions

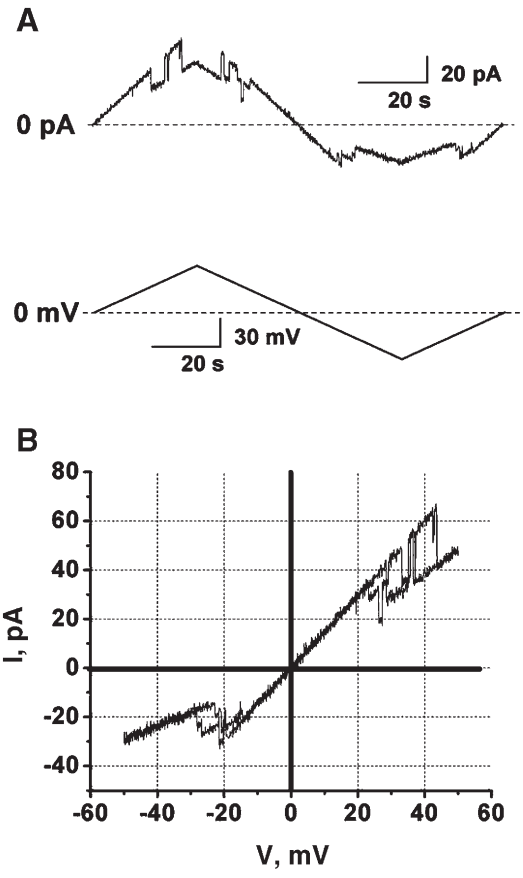

Fig. 1. Electrical characteristics of *Neurospora crassa* VDAC. (A) Current flow across a planar bilayer containing two VDAC from *Neurospora crassa* outer membranes (upper trace), in response to voltage ramping in the range ±50 mV (lower trace). Media in the baths on either side of the bilayer was symmetrical 0.15 M KCl 5 mM $CaCl_2$ 5 mM HEPES pH 7.4. (B) Current–voltage relationship (*I–V* curve) corresponding to the data in panel A.

are observed in the current trace that represent reversible transitions of VDAC to partially open substates. This same data can be represented as a plot of current vs. voltage (*I–V* plot), as in Fig. 1B. The plot is linear for the voltage range of −15 to 25 mV. Outside this range, the step-like current transitions in Fig. 1A are represented in the *I–V* curve as vertical shifts to short, linear regions of decreased slope, representing decreased conductance, $g = I/V$. At $V < -30$ mV, the slope of the *I–V* curve (0.55 nS) is about 40% of that at lower voltage amplitudes (1.47 nS), indicating that both VDAC channels have switched to the half-open substate.

In the experiment of Fig. 1, the buffers on both sides of the membrane have the same composition and, by necessity, the *I–V* curve intercepts both I and V axes at 0. With asymmetrical buffers, a non-zero intercept is possible if the channels in the membrane are "selective", i.e., have net differences in permeability towards the predominant anion and cation present. In this case, the intercept of the *I–V* curve with the I-axis is the net current at 0 mV, which will be positive for anion-selective channels and negative for cation-selective channels. Likewise, the intercept of the *I–V* curve with the *V* axis is the potential needed to be imposed across the membrane for zero net current, i.e., the so-called reversal potential that was derived from the Goldman Hodgkin Katz equation [36]:

$$E_{\mathrm{rev}} = \frac{\mathrm{RT}}{zF} \ln \frac{P_{\mathrm{K}}[K]_{\mathrm{pipette}} + P_{\mathrm{Cl}}[\mathrm{Cl}]_{\mathrm{bath}}}{P_{\mathrm{K}}[K]_{\mathrm{bath}} + P_{\mathrm{Cl}}[\mathrm{Cl}]_{\mathrm{pipette}}}$$

where *R* is the gas constant, *T* is temperature, *F* is Faraday constant and $[\,]_{\mathrm{pipette}}$ and $[\,]_{\mathrm{bath}}$ are the concentrations of the ions in the pipette and bath which were typically 150 mM and 30 mM KCl, respectively [36]. According to the above definitions, the reversal potential is negative for anion-selective channels and positive for cation-selective channels.

The response of a single VDAC channel to varying transmembrane potential in the presence of a five-fold KCl gradient is illustrated in Fig. 2. In this case, the channel was detected by patch-clamping a proteoliposome reconstituted with VDAC protein purified from *Neurospora crassa* mitochondria [8]. Voltage was ramped manually between −40 mV and +40 mV (lower curve, Fig. 2A). The current response of the membrane (upper curve, Fig. 2A) was complex, with at least 5 distinct phases evident, labeled a through e. The corresponding *I–V* curve (Fig. 2B) provides an interpretation of the current response of the membrane in terms of functional states of the VDAC channel it contained. In segments a and d, the channel occupied the canonical VDAC open state, with large conductance (slope corresponds to 597 pS) and anion selectivity (intercept on *V* axis = $E_{\mathrm{rev}} = -7$ mV; $P_{\mathrm{Cl}}/P_{\mathrm{K}}$ of 1.5). In segments b and e, corresponding to membrane potentials of large amplitude ($V < -20$ and $V > 25$), the slope is considerably smaller (216 pS) and the *V* axis intercepts range from 15 mV (for segment b; $P_{\mathrm{K}}/P_{\mathrm{Cl}}$ of 2.4) to 32 mV (for segment e; $P_{\mathrm{K}}/P_{\mathrm{Cl}}$ of 10). This behavior is consistent with normally observed lower conductance, cation-selective substates of VDAC. However, segment c is unusual, having a slope (630 pS) slightly greater than that of segments a and d, and an extrapolated *V* axis intercept (E_{rev}) of 27 mV ($P_{\mathrm{K}}/P_{\mathrm{Cl}}$ of 6). This corresponds to a fully open state of the VDAC channel with cation selectivity, a state not previously described. The VDAC channel switched to this cation-selective open state from the cation-selective partially open state at −20 mV (as voltage amplitude was decreasing towards the end of segment b), and the channel switched from this cation-selective open state to the anion-selective open state as the voltage amplitude decreased to −5 mV (at the end of segment c). (Note that manual control of the voltage allowed the instrument operator to lengthen the period over

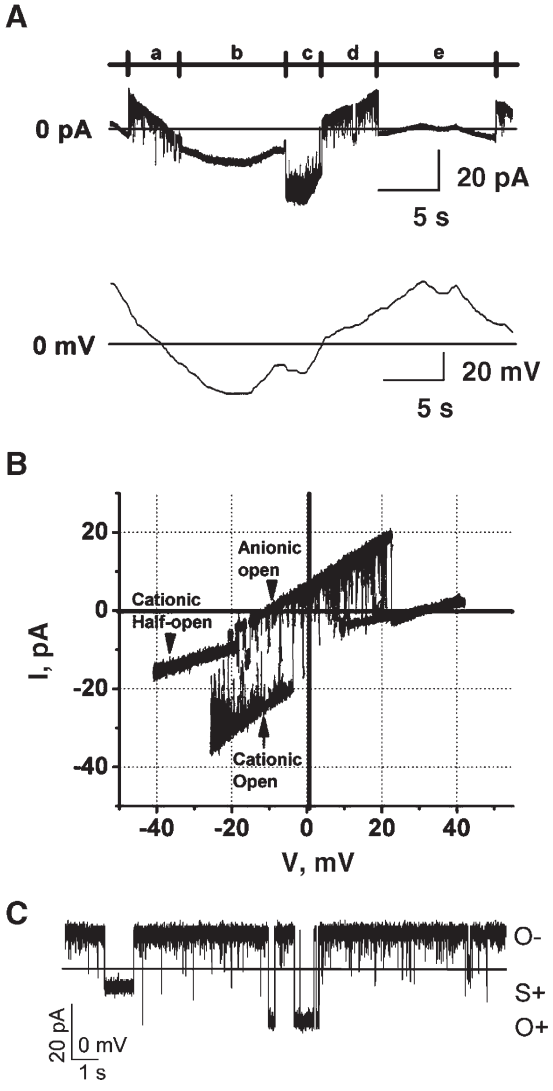

Fig. 2. Purified *Neurospora crassa* VDAC has a cation and an anion selective open state. (A) Current (upper trace) across a membrane patch excised from a liposome containing a single purified *Neurospora crassa* VDAC is shown and clamped at the indicated voltages (lower trace). Media in the pipette was 150 mM KCl, 5 mM HEPES (pH 7.4) and in the bath 30 mM KCl, 184 mM mannitol, 56 mM sucrose, 5 mM HEPES (pH 7.4). Segments in the current trace labeled a to e correspond to different functional states of the channel, as described in the text. (B) *I–V* curve corresponding to the data in panel A. Note the presence of two fully open states with different selectivity and a half-open state with cation selectivity. (C) A current trace at 0 mV is shown that illustrates transitions between the open anion-selective (O−), open cation-selective (O+), and half-open cation-selective (S+, substate) states. The line corresponds to 0 pA.

which the cation-selective open state was observed, e.g., by keeping the voltage below the amplitude that would induce a transition to the half-open state). Transitions between the cation- and anion-selective open states are also seen at 0 mV (Fig. 2C).

The cation-selective open state was observed with mammalian as well as fungal VDAC. In the experiment of Fig. 3, a liposome reconstituted with mitochondrial outer membranes from mouse cultured cells was patch-clamped as in the experiment of Fig. 2, with a 5-fold KCl gradient across the membrane. Typically, multiple channels are detected in patches from these preparations. Based on the slope (2.1 nS) of the *I–V* curve corresponding to the initial segment of the current trace (at low transmembrane potential), there are probably three VDAC channels in this excised patch, with a mean conductance of 700 pS. As the voltage (middle trace, Fig. 3A) was manually increased from 10 mV to approximately 45 mV, there was a shift in the *I–V* curve corresponding to a change in selectivity from anionic (E_{rev} = −2 mV, P_{Cl}/P_K of 1.1) to cationic (E_{rev}=24 mV, P_K/P_{Cl} of 4.6). This shift was accompanied by a small change in slope of the *I–V* curve, from 2.1 nS to 1.8 nS. If this conductance change were due to partial closure of one of the three VDAC channels (from 700 to 400 pS), the expected change in reversal potential would be small. (The net reversal potential is a weighted average of the reversal potentials of the individual channels, which for two anion-selective open channels and one cation-selective partially open channel would be around −9 mV). The much larger change in reversal potential indicates that all three channels have switched to a cation-selective state with E_{rev}=24 mV and mean conductance of 600 pS, only 100 pS smaller than the initial state. Following this transition, one or more of the channels flicker briefly between the cation-selective open and two lower conductance substates (Fig. 3B), before all three channels switch back to the usual anion-selective open state.

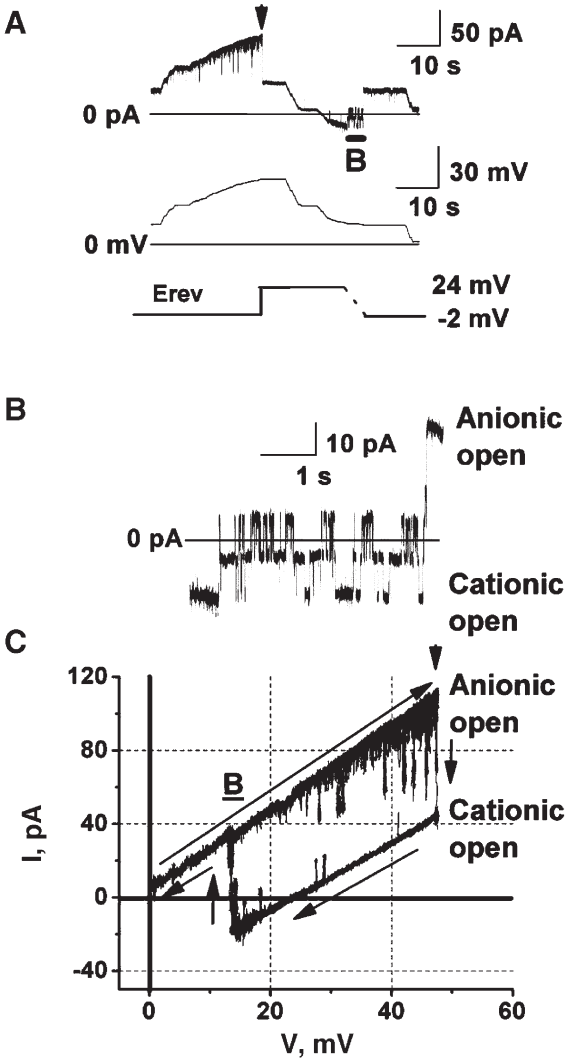

Fig. 3. Reversible transitions between open anion- and cation-selective states of mouse VDAC. (A) Current (upper trace) across a membrane patch excised from a liposome containing probably three VDAC from outer mitochondrial membranes from mouse FL5.12 cells is shown clamped at the indicated voltages (middle trace). E_{rev} indicates the reversal potential of the state occupied (lower trace). Media as in Fig. 2. Arrowhead indicates transitions from the anion-selective open state to a cation-selective open state for the channels that is also indicated in panel C. (B) An expanded region of the segment in the current trace labeled B in panels A and C, showing rapid spontaneous transitions between positive and negative currents. (C) *I–V* curve corresponding to the data in panel A, indicating the two long-lived states of these channels (anion- and cation-selective open states), along with short-lived cation-selective substates that are represented by numerous vertical deflections in this curve.

In the experiment of Fig. 4, a liposome reconstituted with outer membranes from rat liver mitochondria was patch-clamped with a 5-fold KCl gradient across the membrane, as in the experiment of Fig. 3. Only the *I–V* curve is shown, corresponding to the current response during a monotonic increase in voltage from −40 mV to 40 mV. The channel observed is VDAC based on the initial transition (at around −10 mV) from a lower conductance (slope=530 pS), cation selective (E_{rev}=8 mV P_K/P_{Cl} of 1.6) substate to a large conductance (slope=660 pS), anion-selective (E_{rev}=−3 mV, P_{Cl}/P_K of 1.2) state. As the voltage increased to 20 mV, a second transition occurred, to a large conductance (slope=680 pS), cation-selective (E_{rev}=6 mV; P_K/P_{Cl} of 1.4) state. Several transitions between the cation- and anion-selective "open" states also can be observed between 30 and 40 mV.

For obvious reasons, the novel, cation-selective open state of VDAC could only be detected when working with asymmetric ionic strength buffers and, even then, the frequency of observation was relatively low for all preparations of mitochondrial outer membranes or (in the case of fungal VDAC) isolated protein. Best data for frequency of observation of this novel open state for a single type of VDAC have been obtained with liposomes reconstituted with mouse cell mitochondrial outer membranes: the cation-selective open state was detected in 30% of the patches (N=18 patches). The length of time spent by VDAC in the cation-selective open state at constant voltage varied from fractions of a second to many seconds with transitions occurring from this state to either the anion-selective open state or to the cation-selective half-open state. The latter transitions tended to be favored at larger amplitude voltages,

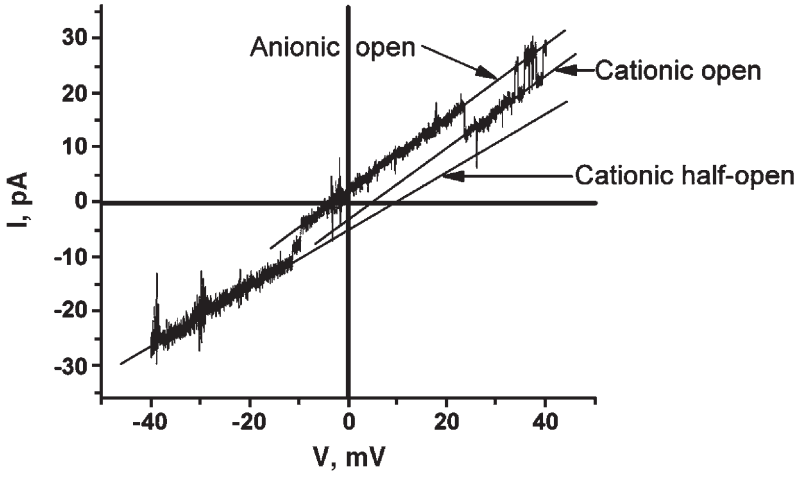

Fig. 4. Presence of open anion- and cation-selective states of VDAC from rat liver mitochondria. A current–voltage curve is shown for a single VDAC channel in a patch excised from a proteoliposome containing rat liver mitochondrial outer membranes. Note the reversible transitions between the open anion- and cation-selective states at 30–40 mV. Media as in Fig. 2.

as is the case with transitions from the anion-selective open state to the half-open state [1–3,37].

4. Discussion

The largest conductance (open) state of the mitochondrial channel VDAC from *Paramecium* was determined to be anion-selective and the lower conductance (half-open) substates cation-selective [1]. This relationship between channel size and charge selectivity was subsequently shown to hold for VDAC from many species, leading to the general belief that selectivity gating and partial closure in this channel are coupled. As reported above, VDAC from mammals and fungi incorporated into phospholipid liposomes also exhibits a cation-selective large-conductance state not previously described. The occurrence of a cation-selective "open" state in these varieties of VDAC is not itself surprising, since evidence for it can be found in previously published current traces [21] and a cation-selective form of VDAC has been described in *Drosophila* [7,38]. The significance of the cation-selective open state of mammalian and fungal VDAC is that transitions can occur to and from it and both the canonical anion-selective open state (Figs. 2, 3, 4) and cation-selective half-open state (Fig. 2). These findings are a clear indication that the processes involved in changing the charge selectivity and pore size of VDAC are distinct and separable events, and not necessarily coupled.

Since transitions involving the cation-selective open state are observed in experiments with VDAC from organisms that are evolutionarily divergent (fungi and mammals), with both purified VDAC protein and isolated mitochondrial membranes, this functional state is a conserved characteristic of the VDAC protein itself. Of course, interaction of VDAC with other proteins and co-factors may influence the frequency of observation and lifetime of any of the conductance states.

An important question is whether the newly described cation-selective open state is an intermediate in the pathway of closure and/or re-opening of VDAC, or whether it is a distinct functional state. In the experiments of Figs. 2–4, transitions involving the cation-selective open state do not occur in any particular order with respect to the other two states. In Fig. 2A and B, the cation-selective open state switches between the canonical half-open and open states, consistent with it being an intermediate to channel re-opening. However, in Figs. 2C, 3 and 4, the cation-selective open state predominantly switches with the anion-selective open state, with no noticeable long-lived transitions occurring to the cation-selective half-open state, even at voltages above 40 mV (Fig. 4). This is not the behavior expected if, for example, the cation-selective open state were a precursor to partial closure.

The reversal potentials associated with the large-conductance cation-selective state fell in a wider range, 6 mV (Fig. 4) and 24–27 mV (Figs. 2, 3), than those of the anion selective open state (−2 to −7 mV in the same experiments). The values of the single channel conductances were more consistent, falling in a range (600–680 pS) very similar to that of the anion-selective open state (600–700 pS). Systematic studies of the cation-selective large-conductance state of VDAC are needed to definitively characterize its electrophysiological properties, as well as its permeability to organic anions such as respiratory substates and adenine nucleotides. Colombini [1,39] has argued convincingly that the reduced permeability displayed towards succinate^{2-}, citrate^{3-}, and inorganic phosphate by the conductance substates of VDAC is probably due more to the change in charge selectivity (from anionic to cationic) than to reduced pore size, since the inferred pore diameter in the half-open state (0.9 nm) is still considerably larger than the dimensions of these solutes (~0.5 nm). Similarly, the newly described cation-selective open state might display reduced permeability to some or all anionic metabolites despite its large conductance. To undertake the necessary experiments, conditions will need to be found that increase the frequency of occurrence and lifetime of the cation-selective open state. It will be interesting to determine whether known modulators of VDAC (such as anionic polymers and NADH) affect the frequency of occurrence or stability of this state.

There is reason to believe that cation-selective states of VDAC might predominate in the native mitochondrial outer membrane. The overall charge selectivity of the mitochondrial outer membrane has been examined by directly patch-clamping mitochondria isolated from mouse liver [40] and mouse cultured cells [30]. Single-channel recordings could not be obtained in these studies, presumably because of the high density of pores in the native membrane. When the outer membranes are clamped at potentials (±5–15 mV) well below those required for partial closure of reconstituted VDAC (20–40 mV), the selectivity is consistently cationic, with a P_{K+}/P_{Cl-} of ~2 [30]. The outer-membrane protein import channel TOM is cation-selective (P_{K+}/P_{Cl-} of 3.6) [30] and will likely contribute to the overall charge selectivity of the outer membrane. However, since VDAC is the predominant protein in the mitochondrial outer membrane [6], the cation-selectivity of the membrane implies that a large fraction of the VDAC channels are in cation-selective states. Since transitions to the partially open substates generally occur at larger transmembrane potentials than those employed in these experiments, while transitions to the cation-selective open state may occur at low voltages and show no obvious voltage dependence, the possibility is raised that the latter state of VDAC is prevalent in native mitochondrial outer membranes. The low frequency of occurrence of the cation-selective open state with reconstituted VDAC argues against this possibility, but occupancy of this state could be influenced by any number of factors involved in reconstitution of the channel protein.

In studies of the effects of point mutations on the reversal potential of yeast VDAC, Blachly-Dyson et al. inferred that a change in the net fixed charge inside VDAC's pore as small as −2 is sufficient to reverse the channel's selectivity from anionic to cationic [12]. This suggests that small-scale conformational changes (such as movement of flexible domains in or near the pore lumen) [14] could account for changes in charge selectivity, without invoking the kind of large-scale re-arrangements associated with voltage-dependent partial closure, involving removal of multiple transmembrane strands from the wall of the pore [12]. Since the mitochondrial outer membrane may not have a significant transmembrane potential, it is tempting to speculate that physiological regulation of outer membrane permeability might be achieved by charge selectivity gating alone, involving only minor structural alterations in VDAC. The physical basis of this gating and how it might be regulated remains to be determined.

Acknowledgments

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