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# The mitochondrial VDAC of bean seeds recruits phosphatidylethanolamine lipids for its proper functioning

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ABSTRACT

The voltage-dependent anion-selective channel (VDAC) is the main pathway for inorganic ions and metabolites through the mitochondrial outer membrane. Studies recently demonstrated that membrane lipids regulate its function. It remains, however, unclear how this regulation takes place. In this study, we show that phospholipids are key regulators of *Phaseolus* VDAC function and, furthermore, that the salt concentration modulates this regulation. Both selectivity and voltage dependence of *Phaseolus* VDAC are very sensitive to a change in the lipid polar head from PC to PE. Interestingly enough, this dependence is observed only at low salt concentration. Furthermore, significant changes in VDAC functional properties also occur with the gradual methylation of the PE group pointing to the role of subtle chemical variations in the lipid head group. The dependence of PcVDAC gating upon the introduction of a small mole fraction of PE in a PC bilayer has prompted us to propose the existence of a specific interaction site for PE on the outer surface of PcVDAC.

Eventually, comparative modeling and molecular dynamics simulations suggest a potential mechanism to get insight into the anion selectivity enhancement of PcVDAC observed in PE relative to PC.

## 1. Introduction

The exchange of ions and metabolites between the cytoplasm and the mitochondrial matrix requires their translocation through two membranes: the mitochondrial outer (MOM) and inner (MIM) membrane. In plants, their transport through the MIM is achieved by highly specific transporters that belong to the mitochondrial carrier family (MCF) [1], four types of potassium channels: ATP-sensitive, ATP-insensitive, Ca-sensitive and Ca-insensitive [2] and ABC transporters [3]. The MIM also hosts the oxidative phosphorylation (OXPHOS) system that sets up a proton electrochemical potential difference, which drives ATP synthesis by ATP-synthases. In contrast to the diversity of carriers in MIM, the voltage-dependent anion-selective channel (VDAC) is the major pathway for metabolite and ion transport through the MOM. A typical feature of VDAC is its voltage dependence: at low voltage ($|V| < 20$ mV), the channels are in their open state featuring a high conductance. They switch to subconductance states upon increasing the voltage amplitude. In *Neurospora crassa*, both open and subconductance states are permeable to small inorganic ions but unlike the open state, the subconductance states are impermeable to metabolites suggesting that gating of VDAC is able to control their flux through the MOM [4–6]. The open state of the channel is believed to be described by the 3D structures of mammalian VDAC isoform 1 and zebrafish VDAC isoform 2 that consist in a β-barrel of 19 β-strands and an N-terminal α-helix leaning onto the inner wall about half way along the pore [7–10].

Studies on MIM proteins from yeasts, mammals and plants have provided a wealth of data showing that membrane lipids act as structural elements and functional regulators of these proteins. For instance, the presence of cardiolipin is essential for the activity of the ADP/ATP carrier, a MCF carrier [11], and of several OXPHOS proteins such as cytochrome (Cyt) C oxidase [12–15], NADH dehydrogenase, Cyt bc1, and ATP synthase [16,17]. In addition, phospholipids such as cardiolipin, phosphatidylethanolamine (PE), phosphatidyl glycerol, phosphatidyl inositol and phosphatidylcholine (PC) occupy specific binding sites in high-resolution crystal structures of the OXPHOS proteins Cyt C oxidase [18,19], Cyt bc1 complex [20], and of the ADP/ATP carrier [21]. In these structures, the tightly bound lipids interact with the protein through ionic interactions and/or hydrogen bonds mediated by their polar head or through van der Waals interactions with their acyl chains. In contrast, little is known about the effect of lipids on the function and regulation of MOM proteins. There are some clues indicating that lipids affect VDAC function. For instance, the gating of

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*Neurospora crassa* VDAC (NcVDAC) is altered by the presence of non-lamellar lipids such as cardiolipin and PE [22]. This finding was explained by the non-specific collective physicochemical properties of these lipids. Other studies on mammalian VDAC however suggested the occurrence of direct interactions between the channel and lipids. In particular, structural and biochemical studies led to the conclusion of the existence of a shell of tightly bound phospholipids around the protein [23,24] and of specific cholesterol binding sites [9,25,26].

The plant VDAC isolated from *Phaseolus coccineus* (PcVDAC) belongs to the canonical isoform of VDAC found in all eukaryotic kingdoms that features similar secondary structure content, electrophysiological properties (conductance, ion selectivity, voltage-dependence) [27–31] and ATP transport [32]. We previously showed that both voltage dependence and selectivity of PcVDAC are sensitive to the sterol content in the membrane and that these effects might arise from specific protein-sterol interactions [33]. In addition a change from high to low KCl salt concentration inhibited the typical voltage-dependent gating of PcVDAC reconstituted in soy phospholipids and increased the VDAC selectivity towards anions [33]. Such a decrease in voltage gating with KCl concentration has never been observed as far for VDACs from other organisms, as this was reported for instance in *Paramecium aurelia* [34], mammalian [35] or in the fungus *Neurospora crassa* [22,36,37]. This suggests that the voltage gating concentration dependence is specific for plant VDAC.

To gain insight into how the phospholipid environment affects the function of PcVDAC we investigated in this work its electrophysiological properties in planar lipid bilayers composed of either PC or PE in high and low KCl concentration. These two lipids are the most abundant constituents of the MOM lipid bilayer [38] and make up to ca. 80–90% of the total amount of MOM phospholipids in plants [39–42]. We show that at low KCl concentration PcVDAC loses its voltage-dependence in PC but not PE. In addition, the anion selectivity of PcVDAC open state also features a significant decrease in PC compared to PE. Furthermore, the recovery of both VDAC voltage-dependence and ion selectivity depends on the degree of methylation of the PE polar head. Remarkably, a very low amount of PE added to PC (2% PE, mol/mol) is enough to restore PcVDAC voltage-dependence suggesting that PE forms specific interactions with PcVDAC. Using molecular modeling and molecular dynamics (MD) simulations we propose that ionic interactions formed by acidic residues with the PE head groups may be liable for altering VDAC ion selectivity.

## 2. Material and methods

### 2.1. VDAC purification

Seeds from *Phaseolus coccineus* were soaked in tap water for 18 h and mitochondrial membranes were isolated from the cotyledons by differential centrifugation steps and further purified on a 28% Percoll gradient as described previously [27]. Purification of the most abundant PcVDAC isoform (32 kDa, UniProt/Swiss-Prot accession number: Q4PKP6) was achieved using the chromatofocusing technique [28].

### 2.2. VDAC reconstitution and electrophysiology

The purified PcVDAC was reconstituted in planar lipid bilayers as described previously [27,43]. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE) and methylated dioleoylphosphatidylethanolamine ($PE(CH_3)_1$ and $PE(CH_3)_2$) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipids were dissolved in hexane to a final concentration of 2% (*w/v*). Planar lipid bilayers were formed by folding two lipid monolayers over a hole (110–150 μm in diameter) made in a 25 μm thick Teflon partition that separated two Teflon experimental chambers. Before each experiment, the partition was treated with a solution of hexadecane/hexane (2.5%, *v/v*). Ag/AgCl electrodes connected in series with a salt bridge (1 M KCl in 1% agar) were used to connect the experimental chambers to the electronic equipment. The *trans* compartment is defined as the one connected to the ground and the voltage was applied to the *cis* compartment. For channel reconstitution into a planar lipid bilayer, proteins were added to the *cis* compartment. KCl solutions were buffered with 10 mM HEPES at pH 7.5.

Current recordings were performed as described previously [33] using a BLM120 amplifier (BioLogic, France). Data were filtered at 300 Hz (5-poles linearized Tchebichev filter), digitized at 44.4 kHz with a DRA200 interface (BioLogic, France) and stored on CD for further processing using a homemade program written in the MATLAB environment (The MathWorks, The Netherlands). The conductance mentioned in the text refers to the chord conductance calculated from the steady state current recorded when a voltage step is applied across the lipid bilayer. The voltage-dependence of PcVDAC was assessed from multichannel experiments using a symmetric 5 mHz triangular wave with an amplitude of ± 70 mV. The probability of finding VDAC in its open state was calculated following the standard procedure [44] using part of the wave corresponding to the channel reopening [22,33]. The probability of finding PcVDAC in the fully open state can be described by a Boltzmann distribution as follows: $\ln(P_o(V) / (1 - P_o(V)) = \pm n e (V - V_h) / kT$, where $P_o(V)$ is the probability of occurrence of the open state, $V_h$ is the voltage at which half of the channels are in their open state, n is a measure of the steepness of the voltage dependence, V is the voltage applied across the membrane, *e* is the elementary electronic charge, k is the Boltzmann constant and T is the absolute temperature. The '+' is used for the negative voltages and the '−' sign for positive voltages. $P_o(V)$ was calculated from the relative change in membrane conductance: $P_o(V) = (G(V) - G_{min}) / (G_{max} - G_{min})$, where $G_{max}$ and $G_{min}$ are the maximal and the minimal conductance. $G_{max}$ is obtained at low applied voltages ($|V| < 20$ mV, where $|V|$ is the modulus of V) when the channels are in the open state and $G_{min}$ is calculated at high applied voltages ($|V| > 50$ mV) when the channels have switched to a subconductance state.

We used single channel experiments for ion selectivity experiments. The reversal potential (zero-current potential) was set to zero in the presence of identical KCl molality on both sides of the membrane. The *cis* compartment was afterwards perfused three times its volume with a solution of different KCl molality and then we recorded the change of reversal potential ($E^{rev}$). Salt solutions were prepared in molal concentrations to give an integer value for the salt activity ratio on both sides of the membrane (0.81 and 0.133 *m* correspond to 0.5 and 0.1 activity, respectively).

### 2.3. Statistics

Experimental data are shown as the mean ± standard error of the mean (N = number of experiments). The statistical significance between different means was estimated using either a *t*-test or an analysis of variance (one-way ANOVA).

### 2.4. Molecular dynamics simulations

A 100-ns long MD simulation was performed on the mVDAC1 structure (PDB ID: 3EMN) [8] embedded in either a palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) or in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid bilayer. Another 100-ns long MD simulation in which all POPE molecules were changed into POPC was also carried out. All simulations were made using the program NAMD2.9 [45] with the CHARMM27 force field [46,47]. For more details about the simulation and their analysis, see the Supplementary material. Figures depicting the protein were prepared with VMD [48].