VDAC STRUCTURE AND FUNCTION: AN UP-TO-DATE VIEW

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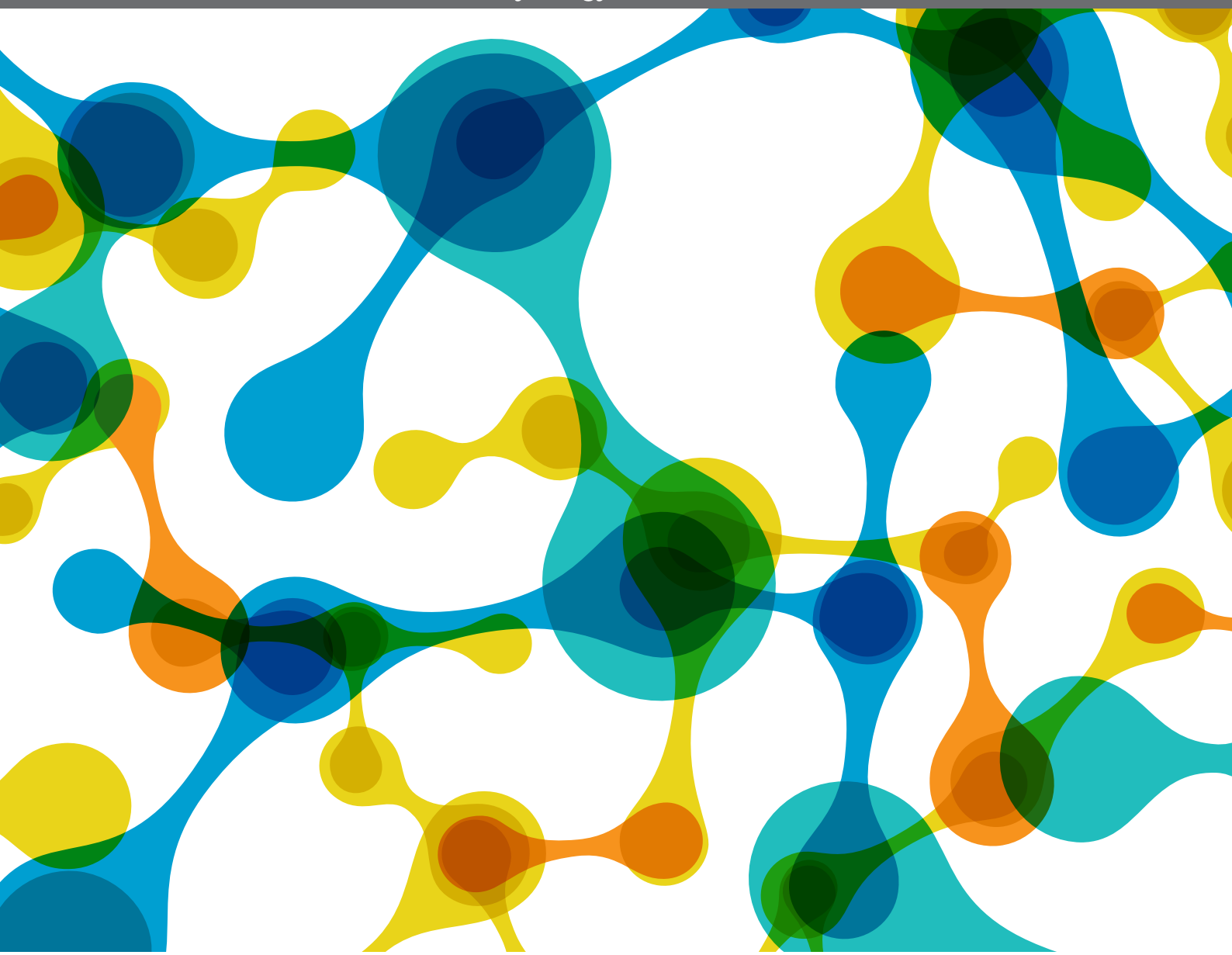
PUBLISHED IN: Frontiers in Physiology



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ISSN 1664-8714
ISBN 978-2-88976-066-4
DOI 10.3389/978-2-88976-066-4

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VDAC STRUCTURE AND FUNCTION: AN UP-TO-DATE VIEW

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Citation: De Pinto, V., Mahalakshmi, R., Messina, A., eds., (2022). VDAC Structure and Function: an Up-to-Date View. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-066-4

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EDITORIAL
published: 04 April 2022
doi: 10.3389/fphys.2022.871586

Editorial: VDAC Structure and Function: An Up-to-Date View

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Keywords: mitochondria, bioenergetics, outer membrane (OM), voltage dependence, biogenesis, oxidative stress, evolution, cancer biology

Editorial on the Research Topic

VDAC Structure and Function: An Up-to-Date View

The Special Issue "VDAC Structure and Function: an Up-to-Date View" investigated the latest findings obtained on VDAC (Voltage Dependent Anion-selective Channel), i.e., the channel protein allowing the permeability of the outer mitochondrial membrane. The discovery of VDAC in the outer mitochondrial membrane, about 50 years ago (Schein et al., 1976) was a breakthrough either in the evolution studies about the endosymbiotic theory and in the appreciation of organelle's bioenergetics. Two years ago, we launched this Special Issue about the latest updates in VDAC research in the knowledge that the understanding of the biochemistry of the protein and of the cell physiology linked to its function(s) has not yet reached the desired level. Another aim was to connect and focus the people working on VDAC: it is a fact, indeed, that many groups arrived serendipitously on VDAC and relatively few laboratories in the world are fully concentrated on it. Due to its relative abundance, VDAC was discovered in many diseases and in many cellular districts, but its involvement is not always known in full details. Just to mention an example of a recent high-throughput study that ended up on VDAC, in (Kim et al., 2021) it was found that VDAC3 inhibition has a primary role in TDP-43 pathways during inflammation.

As we wrote in the introduction to the Special Issue, "It is now time to build a unified description of the matter, by joining the contributions of the main laboratories in the world which devoted their efforts into this fascinating protein".

The excellent contributions have mainly achieved the goal. The introduction is in its historical perspective, which is now well described in the review by Benz, together with those which recently appeared in the literature (De Pinto, 2021; Mannella, 2021): the competition about VDAC discovery and functional elucidation was mainly a matter of few groups. In US three independent laboratories, led by Marco Colombini, Carmen Mannella and Mike Forte, and two in Europe based in Germany and Italy and led by Roland Benz and Vito De Pinto, competed in the discovery and elucidation of biochemical and biomolecular aspects of the VDAC family [see ref. (Colombini and Mannella, 2012) for the history of these achievements]. The knowledge of how research progressed in those years could be of great interest to newcomers to the VDAC field and to young researchers.

Next, as a theme of general interest, the import pathway of VDAC into mitochondria has been described by the group of Rapaport (Moitra and Rapaport) which clarified the role of a β-hairpin motif as an insertion signal, and put forward the model of the lateral gate to mechanistically explain the biogenesis and correct insertion of VDAC in the outer membrane. The work by Court group (Ferens et al.) about the deletion of the 19th strand of the *N. crassa* and its functional consequences upon cell transformation, agrees with those involving human VDAC2 expressed in *S. cerevisiae*

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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *08 February 2022*
Accepted: *08 March 2022*
Published: *04 April 2022*

Citation:
De Pinto V, Mahalakshmi R and Messina A (2022) Editorial: VDAC Structure and Function: An Up-to-Date View. Front. Physiol. 13:871586. doi: 10.3389/fphys.2022.871586

(Srivastava and Mahalakshmi, 2020) and does not challenge the results of biogenesis experiments but rather provides suggestive clues about the mechanisms of channel oligomerization.

Another general theme, not frequently dealt with is the genic regulation of VDAC expression. In her work, Zinghirino et al. proposed an overview of theirs and other's most recent results on the structure of VDAC promoters (Zinghirino et al.). In this paper they compared the three VDAC isoforms regulatory regions, detailing for the first time the presence of isoform-specific cis-acting elements for different transcription factors: they thus paved the way for a deeper understanding of the individual functions of each isoform. In the matter of VDAC isoforms characterization three more contributions described different aspects of this sought information. Magrì et al. reported about the VDAC family in *S. cerevisiae* with a definitive detail, defining the role of each isoform (Di Rosa et al.). Reina and Checchetto reviewed the VDAC3 mammalian isoform, the last to be discovered but target of many recent reports especially dealing with its putative role in the mitochondria redox balance. Also, the contribution by Karachitos et al. tries to explain the reason of the oxidative post-translational modifications of cysteines in VDAC3, in particular. Such redox PTM were subject of a cute Mass Spec study (Pittalà et al., 2021).

The determination of the 3D structure of the pore was a breakthrough in the field of VDAC because allowed to rationalize several hints and information (Hiller et al., 2010). This achievement laid the foundations for VDAC studies related to its involvement in diseases as biomarker or as the target for many new perspective drugs. The work by Shimizu et al. explains the function of a critical residue in VDAC (De Pinto et al., 1993) and connects this study with both the specific function in Ca++ movements in cardiac tissue and the different, antagonistic role of the VDAC isoforms in hearth physio-pathology. The physiological role of VDAC channel in the Ca++ intracellular traffic is an important issue since it is at a cross-road between the storages of the ER and mitochondria (Sander et al., 2021). Another joining point between the physiology and pathologies, where VDAC has a relevant role, was outlined (Heslop et al.; Rostovtseva et al., 2021; Shoshan-Barmatz et al.). The group of Maldonado reviewed the well-established connection between VDAC activity, as the Hexokinase binding receptor, and the Warburg effect, a hallmark of many cancers (Heslop et al.); in this respect the reports about the interaction of mainly disordered peptides, like the C-terminal of tubulin and α-synuclein, with VDAC, shed light on some potentially very important regulatory mechanisms of the pore-activity (Rostovtseva et al., 2021). The influence of a diffuse drug like Metformin on important aspects of mitochondrial dysfunction in energy metabolism and cell death is a clear example of how unexpected might be the VDAC activity (Shoshan-Barmatz et al.).

Last but not least, plant VDACs were described in the review by Pandey, illuminating the similarity/dissimilarities with animal VDACs (Ravi et al.); also, the specific interaction of plant phospholipids with the pore was highlighted and further details emerged from that (Saidani et al., 2021).

This Special Issue was aimed to build a collection of contributions well focused on the state-of-the-art of the structural and functional knowledge of VDAC: we believe it has achieved its goal. It goes without saying that so many answers open up so many new questions. A number of issues, indeed, that make the study of VDAC so topical and fascinating have not yet been resolved: 1) the implementation of voltage dependence in cells and the mechanism by which it occurs; 2) the mobility of the VDAC structure following stimuli or interactions with other proteins; 3) the reported presence of VDAC in other cell membranes and its purpose there. Most likely these topics will be the subject of other bright experiments and other focused Special Issues.

AUTHOR CONTRIBUTIONS

The text was elaborated by VDP and amended and enhanced by AM and RM.

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frontiers in Physiology

MINI REVIEW
published: 19 May 2021
doi: 10.3389/fphys.2021.675708

Voltage-Dependent Anion Selective Channel Isoforms in Yeast: Expression, Structure, and Functions

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Mitochondrial porins, also known as voltage-dependent anion selective channels (VDACs), are pore-forming molecules of the outer mitochondrial membranes, involved in the regulation of metabolic flux between cytosol and mitochondria. Playing such an essential role, VDAC proteins are evolutionary conserved and isoforms are present in numerous species. The quest for specific function(s) related to the raise of multiple isoforms is an intriguing theme. The yeast *Saccharomyces cerevisiae* genome is endowed with two different VDAC genes encoding for two distinct porin isoforms, definitely less characterized in comparison to mammalian counterpart. While yVDAC1 has been extensively studied, the second isoform, yVDAC2, is much less expressed, and has a still misunderstood function. This review will recapitulate the known and poorly known information in the literature, in the light of the growing interest about the features of VDAC isoforms in the cell.

Keywords: porin, VDAC, mitochondria, electrophyiology, yeast, outer mitochondrial membrane

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *03 March 2021*
Accepted: *20 April 2021*
Published: *19 May 2021*

Citation:
Di Rosa MC, Guarino F, Conti Nibali S, Magrì A and De Pinto V (2021) Voltage-Dependent Anion Selective Channel Isoforms in Yeast: Expression, Structure, and Functions. Front. Physiol. 12:675708. doi: 10.3389/fphys.2021.675708

INTRODUCTION

The passive diffusion of small hydrophilic molecules throughout outer membranes (OM) of Gram-negative bacteria, mitochondria and chloroplast is provided by the presence of integral membrane proteins commonly named *porins*. Characterized by a cylindrical shape, porins were firstly discovered in prokaryotes (Nakae, 1976) and subsequently in mitochondria (Schein et al., 1976; Colombini, 1979) and chloroplast (Smack and Colombini, 1985), supporting the endosymbiotic theory. Porins are generally arranged in a conserved β-barrel structure, with polar amino acids facing the hydrophilic compartments counterbalanced by non-polar residues in the hydrophobic membrane core (Benz, 1989; Rosenbusch, 1990; Zeth and Thein, 2010).

The first mitochondrial porin was identified in the unicellular ciliate *Paramecium tetraurelia* by Schein et al. (1976). In artificial membranes, the protein showed a maximal conductance at the transmembrane potential close to zero, which decreased as a function of both positive and negative voltage applied (Schein et al., 1976). Furthermore, the channel exhibited a slight preference for anions over cations in the high-conducting state (Schein et al., 1976; Benz et al., 1988). Given these electrophysiological features, mitochondrial porin was then named Voltage-Dependent Anion selective Channel (VDAC).

VDACs are ubiquitously expressed proteins of about 28–32 kDa, with an estimated pore dimension of ∼3–3.5 nm in diameter and ∼4–4.5 nm in height. The

number of VDAC isoforms varies significantly in many species, ranging from one or two in yeast, three in mammals and up to five in plants (Young et al., 2007). Anyway, they represent the most abundant protein family of the outer mitochondrial membrane (OMM), accounting for ∼50% of the total protein content (Mannella, 1998; Gonçalves et al., 2007). This confers the typical sieve-like aspect to the OMM, as revealed by atomic force microscopy experiments (Gonçalves et al., 2007, 2008).

While human and murine VDACs were extensively studied, the same was not for the *Saccharomyces cerevisiae* counterpart. *S. cerevisiae*, also known as the Baker's yeast, is a unicellular organism widely employed as a eukaryotic model. Its genome was completely sequenced in 1996 (Goffeau et al., 1996), making the genetic manipulation simpler through recombination techniques. Furthermore, most of the metabolic and cellular pathways, especially those involving mitochondria biogenesis and function, are conserved. This has led to define yeast "a smaller but not lower eukaryote" (Rine, 1989).

In the lights of these considerations, in this review we summarized all the literature information available so far about the structure, the electrophysiological features and the peculiar functions of the two VDAC isoforms expressed by the yeast *S. cerevisiae*.

THE STRUCTURE AND FUNCTIONS OF VDAC PROTEINS

From the time of their discovery, many hypotheses were formulated about VDAC three-dimensional structure. The alternation of hydrophobic and hydrophilic residues, as revealed by the sequence analysis, and a set of single-point mutagenesis experiments allowed the development of the first model, consisting in a transmembrane barrel made of 12 antiparallel β-strands and one amphipathic is α-helix (Blachly-Dyson et al., 1990; Thomas et al., 1993). In the early 2000s, by using computational approaches, *Neurospora crassa* and *S. cerevisiae* VDACs were modeled onto bacterial porins structures available at that time, predicting a 16 β-strands barrel structure with a globular α-helix corresponding to the first amino acid residues of the N-terminal domain (Casadio et al., 2002). The specific structure of N-terminus, already predicted by the first studies (Blachly-Dyson et al., 1990; Thomas et al., 1993), was experimentally confirmed by circular dichroism experiments performed by independent groups (Guo et al., 1995; Bay and Court, 2002; De Pinto et al., 2007). However, only several years later the three-dimensional structures of murine and human VDAC1 (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008) and zebrafish VDAC2 (Schredelseker et al., 2014) were determined by NMR spectroscopy, X-ray crystallography or a combination of these two techniques.

The β-barrel pore structure of VDAC proteins is built by 19 β-strands connected to each other by short turns and loops. This makes mitochondrial porins significantly different from bacterial general porins, which have an even, variable number of β-strands, commonly between 14 and 18 (Achouak et al., 2001; Nikaido, 2003). In VDAC, strands are anti-parallel except for the first and the last one, showing instead a parallel orientation. As predicted, in the first 26 N-terminal residues two short α-helix stretches were found. Although there are several differences in the specific location among the models, all authors agree that N-terminus does not take part in the barrel formation, as evinced also from the superposition of human and murine VDAC1 (Zeth and Zachariae, 2018). According to Bayrhuber et al. (2008) the sequence 7–17 (at the N-terminal end) is horizontally oriented inside the barrel and the sequence 3–7 contacts the pore wall. Similar findings have been found in the other models, where the presence of N-terminus within the channel lumen was justified by the presence of a specific hydrogen-bonding pattern between it and several specific residues located in different strands of the barrel (Ujwal et al., 2008) and/or by hydrophobic interactions (Hiller and Wagner, 2009).

As farther detailed, a putative role in the channel gating was assigned to the N-terminus (Shuvo et al., 2016). In fact, the domain is connected to the barrel by a glycine-rich motif, which confers flexibility. It is thus believed that N-terminal domain is capable to leave the lumen and to partially expose itself to the cytosolic environment, possibly mediating the interaction with the membrane or other proteins (Geula et al., 2012; Manzo et al., 2018). This hypothesis is supported by the transmission electron microscopy work by Guo et al. (1995) and, more recently, by the definition of VDAC topology within the OMM (Tomasello et al., 2013).

The pore allows the passive diffusion through the OMM of small ions (Na^+, Cl^-, and K^+) and metabolites up to ∼5,000 Da, including ATP/ADP and nucleotides, intermediates of Krebs' cycle (glutamate, pyruvate, succinate, malate) and NAD^+/NADH (Benz, 1994; Hodge and Colombini, 1997; Rostovtseva and Colombini, 1997; Gincel and Shoshan-Barmatz, 2004). Other small molecules are instead capable to modulate the pore activity and/or interaction of VDACs with cytosolic proteins and enzymes. By binding the channel, cholesterol preserves the structural integrity of VDAC and facilitates its insertion in lipid bilayers (De Pinto et al., 1989a; Popp et al., 1995; Hiller et al., 2008). Being a component of the OMM, cholesterol amount may vary according to the conditions, affecting in turn VDAC functionality (Baggetto et al., 1992; Pastorino and Hoek, 2008).

In this perspective, VDACs are widely considered essential for the maintenance of the mitochondrial bioenergetic and the communication between the organelle and the rest of the cell (as reviewed in Shoshan-Barmatz et al., 2010; De Pinto, 2021).

VOLTAGE-DEPENDENT ANION SELECTIVE CHANNELS HAVE ISOFORM-SPECIFIC FUNCTIONS

Both VDAC genes and proteins are evolutionary conserved. The three different mammalian VDAC isoforms are encoded by independent genes, each characterized by a similar intron-exon organization. VDAC2 gene has an additional pre-sequence placed upstream of the first exon that confers to the protein a 11-amino acids longer N-terminus (Messina et al., 2012). Furthermore, the proteins are characterized by high intra- and

inter-species sequence conservation. For instance, mammalian isoforms show up to ∼75% of sequence similarity, while yeast and human VDAC1 share about 70% of similar sequence (Young et al., 2007; Messina et al., 2012). This implies that all VDAC proteins should have similar structure/functions, as arises from computational simulations made for all the proteins for which the three-dimensional structure is not available yet (De Pinto et al., 2010; Guardiani et al., 2018). Accordingly, VDACs from mice, yeast, fruit fly, and human can substitute for each other in the regulation of metabolic fluxes if expressed in yeast mitochondria, as demonstrated by complementation assays performed in porin-less strain(s) on non-fermentable carbon sources (i.e., glycerol) at the restrictive temperature of 37°C (Sampson et al., 1997; Xu et al., 1999; Reina et al., 2010; Leggio et al., 2018). At the same time, the simultaneous presence of different isoforms has raised the question of distinct and non-redundant functions for each VDAC. While this issue is unexplained for yeast, several hypotheses have been put forward for high eukaryotes.

In mammals, VDACs show a tissue-specific distribution in which VDAC1 is the most ubiquitous (Cesar and Wilson, 2004; De Pinto et al., 2010). While controlling the overall permeability of OMM (Tomasello et al., 2009) and participating in Ca^{2+} homeostasis (De Stefani et al., 2012), VDAC1 interacts with proteins of Bcl-2 family and hexokinases, playing a crucial role for the activation of apoptosis (Shimizu et al., 2001; Abu-Hamad et al., 2008; Huang et al., 2013). Upon specific stimuli, VDAC1 undergoes oligomerization allowing MOM permeabilization, as well as the release of cytochrome c and/or mitochondrial DNA fragments (Keinan et al., 2010;. Kim et al., 2019).

VDAC2 was initially indicated as a pro-survival protein, being able to prevent the activation of the pro-apoptotic protein Bak (Cheng et al., 2003). In the last years, however, a mechanism in which VDAC2 is necessary for the activation of Bax, another pro-apoptotic member of Bcl-2 family, was proposed (Ma et al., 2014; Chin et al., 2018).

On the contrary, the specific role of VDAC3 remains not completely clarified yet. This isoform has specific and peculiar features: for example, in non-reducing conditions it forms small pores of about 90 pS in artificial membranes (Checchetto et al., 2014) while, in yeast devoid of endogenous porins, it complements the growth defect only partially (Sampson et al., 1997; Reina et al., 2010). In the last years, our group carried out site-direct mutagenesis experiments on human VDAC3 aimed at replacing cysteine with alanine residues. Cysteines, indeed, can undergo oxidation/reduction according to the environment. Mutations of single or multiple cysteines significantly increased the conductance of VDAC3 up to similar or identical values to those shown by the other isoforms (Reina et al., 2016a; Queralt-Martín et al., 2020), suggesting that the pattern of post-translational modifications (PTMs) modulates VDAC3 activity (Okazaki et al., 2015; Reina et al., 2016a). This hypotesis was later confirmed by mass spectrometry (Saletti et al., 2017; Pittalà et al., 2020). Given also the specific interaction of VDAC3 with stress sensor and redox-mediating enzymes (Messina et al., 2014), this isoform was indicated as a putative mitochondrial sensor of the oxidative stress (De Pinto et al., 2016; Reina et al., 2016b, 2020).

The pattern of cysteine oxidation was never studied in yeast. Isoform 1 has only two cysteines similar to mammal VDAC1. The same holds for yVDAC2, whose PTMs study by mass spectrometry is practically hindered by its paucity in the usual yeast strains.

THE VDAC GENES AND PROTEINS IN YEAST

Differently from mammals, *S. cerevisiae* genome is endowed with two different genes encoding for two distinct porin isoforms. As summarized in **Table 1**, the so-called *POR* genes are located in different chromosomes and they are very similar in length. A comparative genomic analysis has suggested that isoforms have been originated from genome duplication during the evolution. This phenomenon was postulated for *S. cerevisiae* and *Candida glabrata* but not for other Saccharomycetales fungi such as *Kluyveromyces lactis* or *S. pombe* in which VDAC paralogs were not detected (Kellis et al., 2004). At the same time, in *C. glabrata* the gene encoding for the second VDAC isoform is highly degenerate, raising the specific question about the maintenance of both *POR* copies in *S. cerevisiae* (Young et al., 2007).

Being encoded by the nuclear genome, all VDACs are synthesized by cytosolic ribosomes and subsequently imported into the OMM by specific evolutionary conserved protein complexes (Ulrich and Rapaport, 2015). This process was in-depth studied using the yeast as a cellular model. Briefly, the translocase of outer membrane (TOM) complex recognizes VDAC precursors onto OMM surface and drives the protein translocation through the main TOM complex subunit, Tom40 (Pfanner et al., 2004; Chacinska et al., 2009), which is itself a β-barrel protein (Araiso et al., 2019; Tucker and Park, 2019). The signal allowing the mitochondrial targeting is a hydrophobic β-harpin motif that interacts with Tom20, another subunit of TOM complex (Jores et al., 2016). The final assembly of VDAC into the OMM is achieved by the presence of a second complex, the sorting and assembly machinery (SAM) complex (also known with the acronym of TOB complex). Again, the main SAM complex subunit is the β-barrel protein Sam50/Tob55 (Kozjak et al., 2003; Takeda et al., 2021).

The expression levels of the two yeast VDAC isoforms appears profoundly different. A recent determination of the mitochondrial proteome at high-confidence identified an average copy number of 16,000 for yVDAC1 and 1–2 copy for yVDAC2 per single mitochondria (Morgenstern et al., 2017). This difference was attributed to the promoter strength, as hypothesized immediately after *POR* genes discovery (Blachly-Dyson et al., 1997). In fact, if *POR2* sequence is cloned downstream *POR1* promoter, yVDAC2 protein levels resemble those of yVDAC1 in physiological condition (Blachly-Dyson et al., 1997).

The primary sequences of yVDACs were determined after their identification. Multialigment analysis shown in **Figure 1A** revealed less than 50% of sequence identity between the two VDAC isoforms of *S. cerevisiae*. However, the computational analysis indicates high similarity in term

TABLE 1 | Main features of POR genes and proteins.

Gene	ORF	Location	Position	Size (bp)	Protein length (aa)
POR1	YNL055C	chrXIV	517.994–518.845	852	283
POR2	YIL114C	chrIX	149.143–149.988	846	281

Genetic information about POR genes and the coorrespondent proteins. Data were taken from Saccharomices Genome Database (https://www.yeastgenome.org).

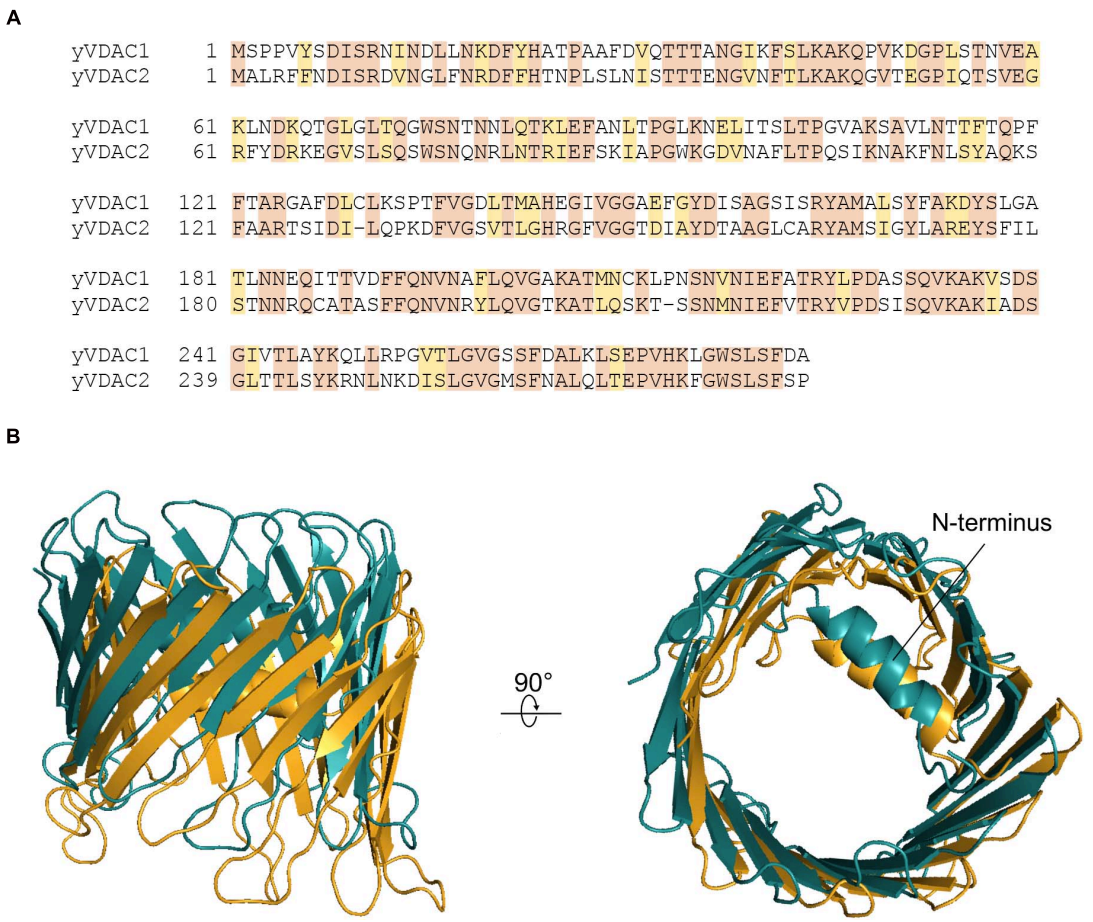


FIGURE 1 | Structural features of yeast voltage-dependent anion selective channel (VDAC) isoforms. **(A)** Multi-alignment analysis of the amino acid sequences of yVDAC1 and yVDAC2 (source: GeneBank). Colors highlight the conserved (orange) or similar (yellow) residues. **(B)** Lateral and top view of the superposition of yVDACs. The predicted structure of yVDAC1 (in brigthorange) and yVDAC2 (in teal) were obtained by homology modeling using MODELLER 9.24 software and the human VDAC1 structure (PDB ID: 2JK4) as template. Final figures were drawn using PyMol software.

of three-dimensional structures, as strengthened by homology modeling predictions (Guardiani et al., 2018) displayed in **Figure 1B**. Despite this, many substantial differences exist in the electrophysiological features, as fully described in the next paragraphs.

YEAST VDAC1: FROM THE DISCOVERY TO THE ELECTROPHYSIOLOGICAL CHARACTERIZATION

The first evidence of porin existence in yeast was observed by Mihara et al. (1982). They described a porin-like activity in isolated OMM fractions attributed to the presence of a single predominant ∼29 kDa protein comparable to that previously found in rat liver mitochondria (Mihara et al., 1982). This protein was generically called "porin" by analogy to the other similar proteins of Gram-negative bacteria. Only after the discovery of a second porin isoform it was formally named yVDAC1 or POR1.

The primary structure of yVDAC1 was deduced from the nucleotide sequence, revealing a 283 amino acid long molecule (Mihara and Sato, 1985). In comparison to the human homologous, it has similarity and identity values of 67 and 24%, respectively (Hiller et al., 2010).

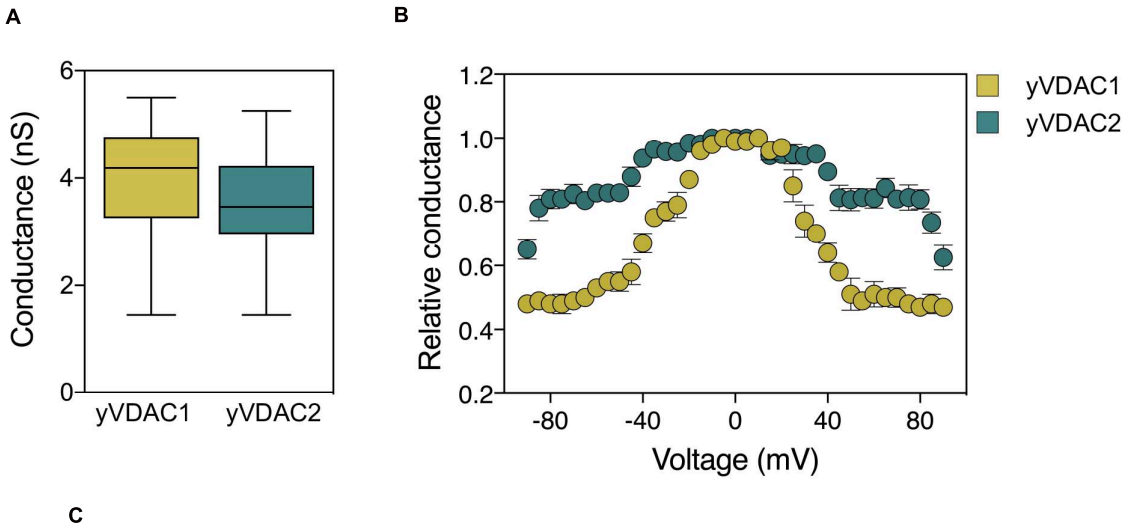
The electrophysiological properties of yVDAC1 were then investigated after protein isolation from mitochondria and

incorporation into planar lipid bilayer (PLB). The protein showed a high propensity to form pores in artificial membranes, characterized by an average conductance of ∼4 nS in 1 M KCl solution (Forte et al., 1987a). In a similar manner to what previously observed for VDACs extracted from *N. crassa* (Freitag et al., 1982), rat brain (Ludwig et al., 1986), and other mammalian tissues (De Pinto et al., 1987), the application of increasing positive and negative voltages, from 0 to ±60 mV, promoted a significative reduction of yVDAC1 conductance. In particular, a high-conducting or open state was observed at low voltages. Conversely, the application of potential, starting from ±10–20 mV, resulted in a symmetrical switch toward low-conducting or closed state(s) (Forte et al., 1987a). Notably, these data were recently confirmed by our group. In particular, we observed an average value of yVDAC1 conductance of ∼4.2 nS and a voltage-dependent behavior starting from ±20–30 mV (Guardiani et al., 2018).

The ion selectivity of yVDAC1 was also investigated. The protein prefers anion over cations in the open state, while in the closed state it becomes less anionic or more cation selective (Schein et al., 1976; Forte et al., 1987b; Colombini, 2016). These observations are in agreement with our recent report showing a ratio Cl^-:K^+ of 2:1 in the open state and 1:4 in the closed state (Guardiani et al., 2018). Similar electrophysiological features were detected for human VDAC1 (Reina et al., 2013) and for *Drosophila melanogaster* VDAC1 (De Pinto et al., 1989b).

A summary of the main electrophysiological features, as well as a comparison with those of yVDAC2, is shown in **Figure 2**.

Particularly interesting for the maintenance of electrophysiological features of VDACs is the N-terminal domain, as revealed by mutagenesis experiments. E.g., the mutations of Asp 15 to Lys or Lys 19 to Glu modified the sensitivity of yVDAC1 to the voltage applied, as well as the ion selectivity (Blachly-Dyson et al., 1990; Thomas et al., 1993). Remarkably, these residues are conserved in mammalian VDACs suggesting that they are essential for the proper functioning and gating of the channel. Also truncation or substitution of specific part of the N-terminus has similar effects. E.g., the truncated yVDAC1, missing the first 8 amino acids, showed an abnormal channel activity and a pronounced instability of the open state, which rapidly switched toward multiple low-conducting states (Koppel et al., 1998).



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	P_K/P_{Cl} ratio		
	Anionic state	Cationic state 1	Cationic state 2
yVDAC1	0.57 ± 0.09	3.9 ± 0.4	-
yVDAC2	0.30 ± 0.03	2.17 ± 0.11	16.24 ± 6

FIGURE 2 | Comparative analysis of the electrophysiological features of native yVDAC1 and yVDAC2 at the Planar Lipid Bilayer. **(A)** Analysis of conductance after yVDACs reconstitution into the artificial membrane. Experiments were performed with an applied constant voltage of +10 mV in 1 M KCl solution. **(B)** Analysis of the voltage-dependence of yVDACs isoforms. Experiments were performed by gradually increasing the voltage from 0 to ±100 mV. Data are expressed as mean of the relative conductance ± SEM. The relative conductance was calculated as the G/G_0, where G is the conductance at the given voltage while G_0 is the conductance values calculated at 0 mV applied. **(C)** Analysis of current-voltage performed in a 10-fold gradient 1/0.1 M cis/trans KCl in a voltage ramp with amplitude ± 60 mV. Values of reversal potential were used to estimate the permeability ratio of cations (P_K) over anions (P_{Cl}) by using the Goldman-Hodgkin-Katz equation in the three states.

The importance of the N-terminus for the channel function was demonstrated also for human VDAC isoforms. It is known that VDAC3 is the less active one in complementation assay of porin-less yeast performed on glycerol at 37°C or in the presence of acetic acid (a cell death inductor, Reina et al., 2010). Swapping experiments in which the first 20 N-terminal residues of VDAC3 were replaced with those from VDAC1 or VDAC2, showed increased life span and resistance to oxidative stress than porin-less yeast transformed with plasmids carrying wild-type VDAC3 sequence (Reina et al., 2010).

YEAST VDAC1 IS ESSENTIAL FOR THE PROPER MAINTENANCE OF MITOCHONDRIA

VDAC1 is by far the most abundant protein of yeast mitochondria, accounting for 7,000–19,000 copies per organelle, as for growth on glucose and glycerol, respectively (Morgenstern et al., 2017). Notably, this number overcomes of one and two orders of magnitude the copy number of the second and the third OMM most represented proteins, Tom40 and Sam50. From this study, we tried to estimate the overall OMM conductance, given by all the β-barrel proteins, and the specific contribution of yVDAC1. In this calculation, we included the putative pore-forming proteins recently discovered by Krüger et al. (2017), such as Mim1, Ary1, OMC7, and OMC8 as well as the β-barrel component of TOM and SAM complexes that can mediate small molecules exchanges across the OMM (Kmita and Budzińska, 2000; Antos et al., 2001). From this analysis emerged that the outer membrane of a single mitochondrion has an estimated permeability of ∼30 μS, 27 of which are provided by yVDAC1 (Magrì et al., 2020). It thus is clear that this isoform is mainly involved in the metabolic exchanges and in the maintenance of the communication between mitochondria and the rest of the cell.

Many information about yVDAC1 function arose from the study of Δ*por1* mutant, in which *POR1* was genetically inactivated. The strain was still viable, but it showed a marked growth impairment on media containing non-fermentable carbon sources (i.e., substrates which are mainly metabolized in mitochondria) at temperature of 37°C (Blachly-Dyson et al., 1990). More recently, our group performed an extensive characterization of Δ*por1* yeast in order to expand the knowledge of this model. Our results indicate that mitochondrial respiration is dramatically compromised in the absence of yVDAC1, since the expression of the respiratory chain subunits encoded by mtDNA, but not nuclear DNA, was completely abolished, as a consequence of the dramatic reduction of mtDNA (Magrì et al., 2020). In this context, the metabolites commonly addressed to the mitochondria, as pyruvate, are pushed toward a cytosolic utilization and the whole cell metabolism undergoes a complete rearrangement aimed to by-pass mitochondria. To survive in the absence of yVDAC1, the cells enhance the biosynthesis of phospholipids, which are then stored into lipid droplets (as an energy reserve) or in the plasma membrane, as schematized in **Figure 3** (Magrì et al., 2020). Overall, these results revealed once again the irreplaceable role of yVDAC1 for the OMM permeability and for mitochondrial metabolism.

An increasing body of evidence suggests an equally important function of yVDAC1 in mitochondrial biogenesis. It is known since many years that the inactivation of *POR1* affects the expression of specific subunits of TOM and SAM complexes (Galganska et al., 2008; Karachitos et al., 2009). However, only recently a direct involvement of yVDAC1 in this mechanism was demonstrated. The assembly of TOM complexes requires the presence of the constituent protein Tom40 and Tom22 (Model et al., 2001). Sakaue et al. (2019) demonstrated that yVDAC1 interacts with Tom22, preventing the transition from a dimeric to a trimeric form of the complex, essential for the import of specific proteins. Furthermore, yVDAC1 antagonizes Tom6, another regulator of TOM assembly (Sakaue et al., 2019). At the same time, yVDAC1 modulates also the Translocase of the inner membrane (TIM) complex activity, by its direct interaction with Tim22. In this contest, yVDAC1 was individuated as a coupling factor for protein translocation of carrier precursors into the inner mitochondrial membrane (IMM) (Ellenrieder et al., 2019). Notably, both these works supported this brand new role of yVDAC1 independent of its metabolic function (Edwards and Tokatlidis, 2019).

As a last point, the role of yVDAC1 in the regulation of yeast redox homeostasis is less characterized than in mammals. VDAC are intrinsically sensitive to oxidation (Saletti et al., 2017, 2018) and during the exponential and/or stationary growth phases they undergo oxidation/carbonylation (O'Brien et al., 2004). However, this effect is exacerbated in yeast strains devoid of the antioxidant enzymes superoxide dismutase (SOD), as expected. The cytosolic Cu/Zn SOD (SOD1), however, not only protects yVDAC1 from oxidation but it is required for the proper channel activity and expression of yVDAC1 (Karachitos et al., 2009). At the same time, the inactivation of *POR1* affects the expression of the mitochondrial Mn-SOD (SOD2) (Galganska et al., 2008, 2010), suggesting a mutual regulation between the two proteins.

THE CONTROVERSIAL STORY OF YVDAC2

Until 1996, yVDAC1 was believed the only porin isoform of *S. cerevisiae*. Two different events contributed to the discovery of the second VDAC: the availability of yeast genomic sequences and the increasing use of recombinant techniques aimed to inactivate specific genes. By the insertion of a functional *LEU2* gene in the *POR1* sequence, yVDAC1 was knocked-out and the Δ*por1* mutant was obtained (Dihanich et al., 1987). The mutant showed normal levels of other OMM proteins but reduced levels of cytochrome c_1 and cytochrome oxidase subunit IV (Dihanich et al., 1987). Surprisingly, Δ*por1* strain was still viable, even if it grew slower than the wild-type at 30°C (Dihanich et al., 1987). This result suggested the existence of some unknown alternative pathway through which small metabolites and ions could cross the OMM.

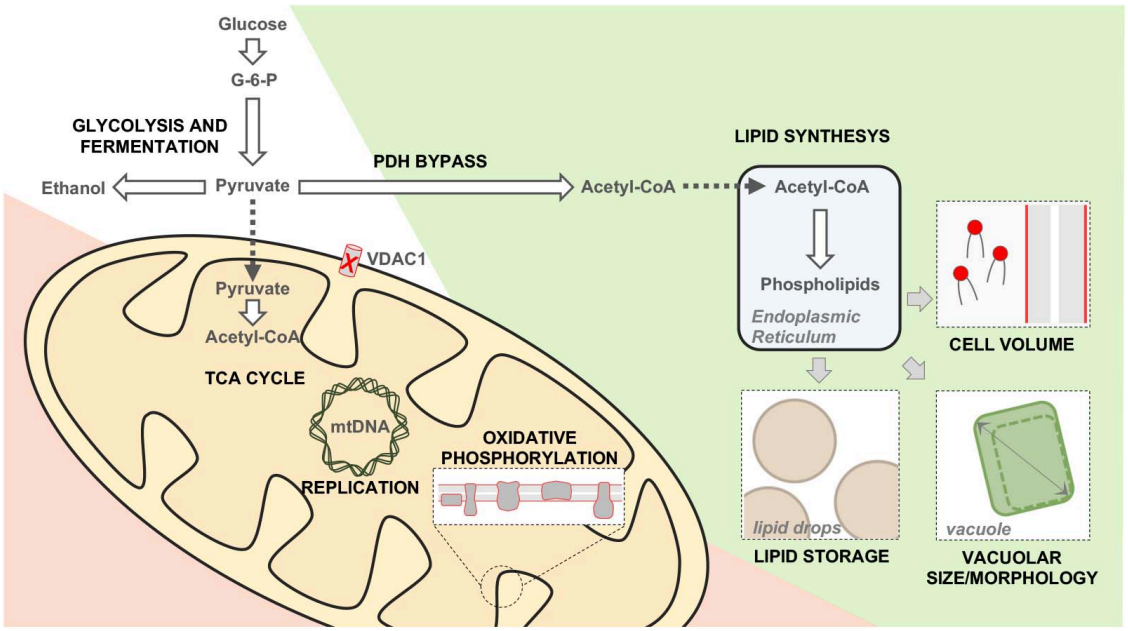

FIGURE 3 | Metabolic and phenotypic changes occurring in Δ*por1* cells. Schematic representation of the main changes occurring in yeast upon *POR1* gene inactivation. Metabolic down-regulated, upregulated or unchanged pathways are displayed in red, green and white, respectively. The absence of yVDAC1 reduces the nucleotides trafficking within mitochondria, affecting the replication/expression of the mtDNA. The mitochondrial utilization of pyruvate, TCA cycle and the functioning of the electron transport system are strongly compromised. On the other hand, the cytosolic conversion of pyruvate into acetyl-coA is enhanced (PDH by-pass). The latter is addressed toward the synthesis of phospholipids, which are then stored into lipid drops (an energy reservoir). Furthermore, the newly synthetized phospholipids contribute to build plasma membrane and to increase the vacuole dimension. As a consequence, the size of Δ*por1* cells is increased of about 30% in comparison to the wild-type.

The analysis of Δ*por1* growth on glycerol at the elevated temperature of 37 °C has revealed a specific defect (Blachly-Dyson et al., 1990). Glycerol, indeed, is a non-fermentable carbon source that forces the utilization of mitochondria (Gancedo et al., 1968). By screening a genomic *S. cerevisiae* library, Blachly-Dyson et al. (1997) identified a second VDAC isoform through its ability to correct this Δ*por1* growth defect. Then, a second porin gene, called *POR2* and encoding for yVDAC2, was individuated.

yVDAC2 was immediately indicated as a potential yVDAC1 substitute, even if some peculiarities emerged. For instance, the second yeast VDAC isoform was able to restore the growth defect of Δ*por1* only upon specific conditions. If *POR2* is present in low copy number (one or two copies per cell) it fails to substitute yVDAC1. Conversely, when *POR2* sequence is cloned downstream the *POR1* promoter it can successfully restore the yeast growth as in wild-type (Blachly-Dyson et al., 1997). Notably, Δ*por1* transformation with single copy plasmid, carrying the encoding sequences of mouse VDAC isoforms 1 and 2, completely recovers the yeast growth on glycerol at 37°C (Sampson et al., 1997). This suggested that yVDAC2 had pore-forming activity but such compensation was strictly depended on its concentration. However, all the efforts made by Blachly-Dyson et al. (1997) to isolate and incorporate yVDAC2 in artificial membrane failed, prompting the scientific community to question the pore-forming ability of yVDAC2 and its involvement in mitochondrial bioenergetics. This idea was supported by the low level of similarity between VDAC isoforms, consisting in only 49% (see **Figure 1A**). Also, not many information was obtained from deletion studies: differently from Δ*por1*, the genetic inactivation of *POR2* gene does not affect yeast growth in any condition, while the simultaneous absence of both endogenous porins, as in the double mutant Δ*por1*Δ*por2*, only exacerbates the yeast growth defect on glycerol typical of Δ*por1*.

YEAST VDAC2 HAS PORE FORMING ACTIVITY AND A PECULIAR ION SELECTIVITY

After its discovery, the interest in yVDAC2 has waned for almost two decades. However, in 2016, investigating the role of human SOD1 in yeast, we casually noticed a complete recovery of Δ*por1* growth defect on glycerol at 37°C in the presence of overexpressed hSOD1. In this condition, the expression level of *POR2*, as well as of other genes encoding for OMM β-barrel proteins (Tom40 and Sam50), was found significantly increased (Magrì et al., 2016b). Since the same results did not appear in Δ*por1*Δ*por2* yeast, we hypothesized that yVDAC2 might re-establish the OMM pore activity.

To definitely clarify this aspect, we established a collaboration with the group of prof. Hanna Kmita (Poznan, PL), aimed at purify with high yield yVDAC2. In the first attempt Δ*por1* strain was transfected with a plasmid carrying *POR2*

sequence and the protein was purified from Δ*por1* mitochondria, avoiding yVDAC1 contamination (Guardiani et al., 2018). It was called *native* yVDAC2. The second strategy applied the heterologous expression of a 6xHis-tagged yVDAC2 in *E. coli* (Magrì et al., 2019). Being a membrane protein, yVDAC2 localized in the inclusion bodies and needed high concentrations of denaturant to be purified, followed by a refolding step in presence of specific detergents (Engelhardt et al., 2007). Remarkably, this refolding method was successfully applied many times and by different groups, producing VDAC proteins with indistinguishable features from those native (Ujwal et al., 2008, Checchetto et al., 2014; Okazaki et al., 2015; Magrì et al., 2016a; Reina et al., 2016a; Queralt-Martín et al., 2020).

The electrophysiology at the PLB revealed that both native and recombinant proteins were able to form pores with the same, typical VDAC-like conductance of ∼3.6 nS in 1 M KCl, as displayed in **Figure 2A**) (Guardiani et al., 2018; Magrì et al., 2019). As for voltage-dependence, native yVDAC2 resembled yeast or human VDAC1, even if it began to close at ±40–50 mV. This suggests that yVDAC2 is slightly less sensitive to the applied voltage (**Figure 2B**). However, this specific aspect was amplified in the recombinant protein, which began to close only at ± 80–90 mV (Magrì et al., 2019). The difference between the native and recombinant forms of yVDAC2 raises the interesting question of whether the native yVDAC2 was subject to specific PTMs, not occurring in the heterologous expression in *E. coli*. The influence of PTMs in VDAC activity is indeed a rather unexplored subject.

Ion selectivity of yVDAC2 was particularly interesting. The computational analysis revealed a similar tridimensional structure for the two yeast VDAC isoforms but a net charge of +11 in the case of yVDAC2, in comparison to +1 of yVDAC1. Thus, anion selectivity was expected for yVDAC2 in the open state, as also predicted by bioinformatic analysis, with a chloride selectivity estimated 2–3 times higher than that displayed by isoform 1 (Guardiani et al., 2018). The analysis of native yVDAC2 at the PLB allowed the identification of up three states with different parameters of ionic selectivity: two of them appeared to be high-conductance states but with opposite selectivity (Guardiani et al., 2018; Magrì et al., 2019). In the open state, the ratio Cl^-: K^+ for yVDAC2 was 3:1, definitely more anionic than the corresponding state displayed by yVDAC1. The second high-conducting state showed a prominent cation selectivity (Cl^-: K^+ = 1:2) (Guardiani et al., 2018). This oddity is not entirely new with VDACs: a similar state was already observed for VDAC1 from mammals (Pavlov et al., 2005). The third state detected by studying yVDAC2 ion selectivity was a low-conducting and very cation-selective state (Cl^-: K^+ = 1:16, Guardiani et al., 2018). Such state was previously unseen in any studied VDAC. The ion selectivity of yVDAC isoforms is detailed in **Figure 2C**.

WHAT IS YVDAC2 FUNCTION?

Despite its demonstrated pore-forming activity, all evidences suggest that yVDAC2 plays only a marginal role in mitochondria bioenergetics. Indeed, the deletion of *POR2* does not significantly affect yeast growth on glycerol at 37°C although its simultaneous inactivation with *POR1* aggravates the growth defect (Blachly-Dyson et al., 1997). The involvement of yVDAC2 in mediating the OMM permeability to small molecules, such as NADH, was studied in Δ*por1* cells. Here, NADH permeability was found 20 times lower than in wild-type (Lee et al., 1998). However, similar results were obtained for the double mutant Δ*por1*Δ*por2*, excluding definitely the involvement of yVDAC2 in this pathway. Later, Tom40 was indicated as a valid substitute of yVDAC1 in Δ*por1* cells (Kmita and Budzińska, 2000; Antos et al., 2001).

A participation in the maintenance of energy homeostasis was also proposed for yVDAC2. SNF1 protein kinase is the orthologue of the mammalian AMP-activated protein kinase (AMPK), both important players in the regulation of cell growth and glucose metabolism in response to the energy limitation (Hedbacker and Carlson, 2008; Mihaylova and Shaw, 2011). It was shown that SNF1 co-precipitated with both yeast VDACs and SNF1 function was significantly affected only when both porin genes are simultaneously inactivated (Strogolova et al., 2012). For this reason, yVDAC2 was identified as a "co-sensor" of a stress signal upstream of SNF1, even if the precise mechanism was still unclear.

Anyway, given the pacucity of literature information, the residual hypothesis is that yVDAC2 acts as a rescue permeability mitochondrial pathway, expressed in presence of some undefined stimulus. In fact, the absence of yVDAC1 *per se* is not able to activate *POR2* gene expression (Magrì et al., 2020). On the contrary, the co-presence of an additional factor, such as the overexpressed hSOD1, induces *POR2* expression and restores the yeast growth defect of Δ*por1* cells (Magrì et al., 2016b).

Δ*POR1* YEAST, AN OPPORTUNITY TO STUDY VDAC ROLE IN HUMAN PATHOLOGIES

Despite the obvious absence of a nervous system in yeast, basic mechanisms and pathways underlying neurodegenerative diseases, such as mitochondrial dysfunction, transcriptional dysregulation, trafficking defects and proteasomal dysfunction, are extremely well conserved between humans and yeast, enabling detailed studies of the molecular events involved in those conditions.

Mitochondrial dysfunctions, along with defects in proteasomal activity and misfolded protein aggregations, are well-known molecular hallmarks of neurodegenerative disorders that can be easily recapitulated in a relatively simple system as the yeast. This is made possible by the presence of disease-associated human orthologues or by the introduction of a human protein directly linked to the disease of interested with easy manipulation techniques. For instance, yeast has been successfully used to investigate TDP43 and FUS dysfunction in amyotrophic lateral sclerosis (ALS), amyloid-β peptide and Tau in Alzheimer's disease, α-synuclein (αSyn) and Lrrk2 in Parkinson's disease, and Huntingtin in Huntington's disease (as reviewed in Miller-Fleming et al., 2008; Bharadwaj et al., 2010; Pereira et al., 2012; Rencus-Lazar et al., 2019). In this contest, VDAC proteins (and VDAC1 in particular) play a

crucial role in mediating mitochondrial dysfunction. In fact, most of the previously cited proteins are able to aggregate onto the cytosolic surface of mitochondria using VDAC as an anchor point (Magrì and Messina, 2017). Thus, the use of Δ*por1* mutant, transformed or not with plasmids carrying encoding sequences for human VDAC isoforms or mutants, represents an important opportunity to clarify the specific roles of porins in pathological contexts.

The involvement of human VDAC1 in mediating αSyn toxicity in Parkinson's disease was demonstrated for the first time in yeast. Rostovtseva et al. (2015) introduced αSyn expression in the Δ*por1* mutant, noticing a yeast growth defect on galactose only when the protein was expressed together with the human VDAC1. This finding supports the idea that mitochondrial dysfunction mediated by αSyn occurs through the modulation of VDAC1 permeability (Rostovtseva et al., 2015). Also, the specific ability of the three VDAC isoforms to counteract oxidative stress was investigated in yeast (Galganska et al., 2008), as well as the antibiotic minocycline specificity to interact with VDACs. These last studies revealed that minocycline interacts in a different manner with VDAC proteins and only isoform 3 is able to mediate the cytoprotective effect counteracting H_2O_2-mediated toxicity (Karachitos et al., 2012, 2016).

In the light of the emerging consideration of VDAC proteins as a pharmacological target in many diseases (Magrì et al., 2018; Shoshan-Barmatz et al., 2020), these few examples highlight the potential usage and the versatility of Δ*por1* cells for biotechnological and biomedical application.

CONCLUSION

Along with the increased interest of the scientific community in understanding VDACs role in apoptosis and mitochondrial dysfunctions, many studies have been carried out on mammalian or human porins, but significantly fewer for the yeast counterparts. Nevertheless, the complete understanding of *S. cerevisiae* VDACs functioning is equally important, especially considering its potential use as biomedical/biotechnological tool. The aim of this review was to collect all the information present in the literature about both yeast VDAC isoforms and to depict a framework as complete as possible. Despite this, several questions need to be addressed yet and deserved to be answered. One of above all: what is the physiological role of yVDAC2? Given the peculiar electrophysiological features here listed, it is indeed hard to believe that this protein is only a genetic heritage from a duplication event.

AUTHOR CONTRIBUTIONS

MCDR, FG, and SCN collected the information and prepared the reference list for the manuscript. AM drow the figures and wrote the manuscript. VDP supervised the work. All authors have read and approved the manuscript.

FUNDING

This research was conducted with the support of Università di Catania–linea PIACERI (Grants VDAC and ARVEST), Proof of Concept (Grant No. PEPSLA POC 01_00054) and AIM Linea 1 Salute (AIM1833071).

ACKNOWLEDGMENTS

Authors acknowledge Salvatore A. M. Cubisino for providing the help in the homology modeling figures preparation and Fondi di Ateneo 2020–2022, UniversitaÌ di Catania, linea Open Access.

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Conflict of Interest: FG, AM, and VDP are affiliated with we.MitoBiotech S.R.L, a spin-off company to the University of Catania.

All the authors declare that the research was conducted in the absence of any commercial of financial relationships that could be construed a potential conflict of interest.

frontiers in Physiology

MINI REVIEW
published: 05 August 2021
doi: 10.3389/fphys.2021.708695

VDAC Genes Expression and Regulation in Mammals

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VDACs are pore-forming proteins, coating the mitochondrial outer membrane, and playing the role of main regulators for metabolites exchange between cytosol and mitochondria. In mammals, three isoforms have evolutionary originated, VDAC1, VDAC2, and VDAC3. Despite similarity in sequence and structure, evidence suggests different biological roles in normal and pathological conditions for each isoform. We compared *Homo sapiens* and *Mus musculus* VDAC genes and their regulatory elements. RNA-seq transcriptome analysis shows that VDAC isoforms are expressed in human and mouse tissues at different levels with a predominance of VDAC1 and VDAC2 over VDAC3, with the exception of reproductive system. Numerous transcript variants for each isoform suggest specific context-dependent regulatory mechanisms. Analysis of VDAC core promoters has highlighted that, both in a human and a mouse, VDAC genes show features of TATA-less ones. The level of CG methylation of the human VDAC genes revealed that VDAC1 promoter is less methylated than other two isoforms. We found that expression of VDAC genes is mainly regulated by transcription factors involved in controlling cell growth, proliferation and differentiation, apoptosis, and bioenergetic metabolism. A non-canonical initiation site termed "the TCT/TOP motif," the target for translation regulation by the mTOR pathway, was identified in human VDAC2 and VDAC3 and in every murine VDACs promoter. In addition, specific TFBSs have been identified in each VDAC promoter, supporting the hypothesis that there is a partial functional divergence. These data corroborate our experimental results and reinforce the idea that gene regulation could be the key to understanding the evolutionary specialization of VDAC isoforms.

Keywords: VDAC mammalian genes, expression profile, gene structure, mitochondria, promoter methylation, core promoter elements, transcription factor binding sites

OPEN ACCESS

Edited by:
Tatiana Rostovtseva, National Institutes of Health (NIH), United States

Reviewed by:
Deborah Court, University of Manitoba, Canada
Arnon Henn, Technion Israel Institute of Technology, Israel

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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *12 May 2021*
Accepted: *02 July 2021*
Published: *05 August 2021*

Citation:
Zinghirino F, Pappalardo XG, Messina A, Nicosia G, De Pinto V and Guarino F (2021) VDAC Genes Expression and Regulation in Mammals. Front. Physiol. 12:708695. doi: 10.3389/fphys.2021.708695

INTRODUCTION

The presence of a family of β-barrel proteins in the mitochondrial outer membrane reflects that mitochondria originate from endosymbiotic bacteria. Indeed, in eukaryotic organisms, evolution has selected proteins that were able to confer ample permeability to the membrane while equipping them with structures that allow interaction with molecules bathing the membrane itself. With bacteria, where multiple porins are in place, it is also the case that there are more VDAC isoforms set up in eukaryotic mitochondria, likely to have derived from gene-duplication events.

VDAC isoforms vary in their number in many species, one or two in yeast, three in mammals, and up to 10 in plants, proposing a hypothesis that each has differentiated biological roles (Al Bitar et al., 2003; Wandrey et al., 2004; Young et al., 2007; Homblé et al., 2012; Messina et al., 2012). The multigenic family of VDAC evolved, following the divergence of animal and plant kingdoms, which explains the unrelated number of isoforms in the two kingdoms. In mammals, duplication events gave rise to a gene family composed of three different isoforms. Among them, VDAC3 is considered the ancestor while VDAC1 is the most recently evolved (Saccone et al., 2003; Young et al., 2007; Wojtkowska et al., 2012). The recent divergence of VDAC paralog genes has conserved a similar structure in the gene and protein organization and function (Young et al., 2007; Messina et al., 2012). The discovery of VDAC genes, in a human and a mouse, established the starting point for studying VDAC by various *in vivo* and *in vitro* approaches. Different statements have been reached regarding the expression and distribution of the different isoforms, the structure of the proteins, the channels functionality, and their involvement in many cellular mechanisms (Raghavan et al., 2012; Shoshan-Barmatz et al., 2020).

The three-dimensional structure of VDAC was determined as a transmembrane channel, consisting of 19 β-strands with an additional N-terminal region, containing elements of α-helix (De Pinto et al., 2007, 2008; Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). Interestingly, a conserved folding was demonstrated in the mouse and the human VDAC1 proteins, enough to overlap almost perfectly the two proteins. Also VDAC2 was determined from a crystal structure (Schredelseker et al., 2014; Eddy et al., 2019), while VDAC3 was only modeled on VDAC1 structure, using predictive tools. Nevertheless, the structural organizations of VDAC2 and VDAC3 proteins are undistinguishable with VDAC1 (De Pinto et al., 2010).

Functional experiments of VDAC channels reconstitution in artificial membranes demonstrated that VDAC isoforms are able to form pores (De Pinto et al., 2008; Messina et al., 2012; Raghavan et al., 2012). While VDAC1 and VDAC2 have a similar conductance, ion selectivity and voltage dependence, VDAC3 channel activity revealed different features (Reina et al., 2010, 2020; Checchetto et al., 2014; Saletti et al., 2018; Queralt-Martín et al., 2020).

Functional conservation of VDAC mouse and human orthologs was also demonstrated in different cellular contexts: by complementation assay in yeast, lacking the endogenous porin (Δpor1) with either mouse or human recombinant VDAC1 (Sampson et al., 1997; Reina et al., 2010), in mice by VDAC gene interruption, and in human cells by VDAC gene interference. In VDACs knock-out mice, physiological defects in tissues, requiring high energy support, are linked to an altered structure and functionality of the mitochondria (Anflous et al., 2001; Sampson et al., 2001). Silencing VDAC isoforms in cellular models confirmed the compromised mitochondrial functionality in the regulation of essential mechanisms, such as ATP production (Okada et al., 2004), Ca^{2+} flux through the OMM (De Stefani et al., 2012), balancing ROS impairment (De Stefani et al., 2012), apoptosis, and autophagy (Koren et al., 2010).

Although the structure and the function of each VDAC isoform are, frequently, the subjects of the study, the promoter elements, the transcriptional factors, and the potential epigenetic control mechanisms affecting the transcriptional activity of VDAC genes have never been comprehensively analyzed. We have recently started to study human VDAC genes and their transcriptional regulation (Guarino et al., 2020; Zinghirino et al., 2020). In this work, we provide additional information about murine VDAC genes counterparts as they are available in the public databases. We combined these data with the scarcely available related literature arising from expression and function of proteins. At the end, a detailed comparative overview of human and mouse VDAC genes promoter structure, expression, and regulation is reported. *Homo sapiens* and *Mus musculus* species were chosen as representative of the most evolved mammals, presenting a high degree of genome synteny and organization. The information acquired in the mouse will be invaluable to extend our knowledge about human physiology and pathology.

VDAC GENE STRUCTURE

The structural organization of mouse and human VDAC genes, of the coding sequence, and their chromosomal localization were defined by the late 1990s.

The VDAC1 protein sequence was determined by Edman degradation from human B cell hybridoma (Dermietzel et al., 1994). Later, VDAC1 and VDAC2 isoforms sequences were isolated, following a screening of human cDNA libraries (Blachly-Dyson et al., 1993). A few years later, two mouse VDAC coding genes were cloned from a brain cDNA library (Sampson et al.,

Abbreviations: AHRR, AHR-arnt heterodimers and AHR-related factors; AP1/AP1R, activator protein 1; AP-2, Activator protein-2; ATF7, activating transcription factor 7; BBCABW initiator, B = C/G/T, W = A/T; BCL2L1, Bcl-2-like 1; BCL6, B-cell lymphoma 6 protein; BECN1, Beclin 1; BEDF, BED subclass of zinc-finger proteins; BRAC, Brachyury gene, mesoderm developmental factor; BRE, B recognition element; CDXF, vertebrate caudal related homeodomain protein; CLOX, CLOX and CLOX homology (CDP) factors; CpG, 5′—C—phosphate—G-−3′; CREB, cAMP response element-binding protein; CREM, cAMP response element modulator; DB, database; DI, dual initiation; DPE, downstream promoter element; E2FF, E2F-myc activator/cell cycle; EBOX, E-box binding factors; EGRF, epidermal growth factor receptor; EPD, eukaryotic promoter database; ER, endoplasmic reticulum; ETSF, human and murine ETS1 factors; FANTOM, functional ANnotation of the mammalian genome; FOX, Forkhead (FKH)/Forkhead box (Fox); GATA1, GATA-binding factor 1; GTEX, genotype-tissue expression; GXD, gene expression database; HEAT, heat shock factors; HESF, vertebrate homologs of enhancer of split complex; HMG, high-mobility group family; IKZF, Ikaros zinc finger; Inr, initiator; KLFS, Krueppel-like transcription factors; LBXF, ladybird homeobox (lbx); lnc-RNA, long noncoding RNA; MOM, mitochondrial outer membrane; MYBL2, cellular and viral myb-like transcriptional regulators; MYOD, myogenic differentiation 1; NEUR, NeuroD, Beta2, HLH domain; NFE2L2, nuclear factor erythroid 2-related factor 2; NRF-1, nuclear respiratory factor 1; NRF-2, nuclear respiratory factor 2; Obs/Exp CpG, observed/expected CpG; OSRF, odd-skipped related factors; PBXC, Pre-B cell leukemia transcription factor; RACE, rapid amplification of cDNA ends; SOHLH, spermatogenesis and oogenesis basic helix–loop–helix; SP1, specificity protein 1; SRE, sterol repressor element; SRY, sex-determining region Y; TCT/TOP motif, polypyrimidine initiator; TEAD, TEA domain family member 1; TF, transcription factor; TFBS, tanscription factor binding site; TSS, transcription start site; VDAC, voltage-dependent anion channel; XCPE2, X core promoter element 2; ZFXY, Zfx and Zfy-transcription factors.

1996a), and, almost simultaneously, a third mouse isoform, later defined as VDAC3, was identified from a heart cDNA library (Sampson et al., 1996b). The mouse VDAC genes were localized in chr11 5q (VDAC1) and in chr14 10q (VDAC2), respectively (Sampson et al., 1996a,b). Correspondence with the chromosomal localization of human genes was identified and correctly defined by FISH experiments that localized VDAC1 to the chr5 q31 and VDAC2 to chr10 q22 positions (Messina et al., 1999). To understand the correlation between the gene and the structural and functional features of VDAC protein isoforms (Ludwig et al., 1986; De Pinto et al., 1987), mouse and human VDAC genes were also characterized. VDAC genes were assembled, using the genome-walking approach, the terminus of each gene defined by 5′ and 3′ RACE-PCR, the exon/intron junctions identified, the polyadenylation signal localized, and the transcription initiation sites predicted, using TSSG and TSSW software available at that time (Sampson et al., 1997; Messina et al., 2000).

The organization of the three genes, their exon size and composition, and exon-intron junctions turned out to be very similar, suggesting recent gene duplication events of the isoforms. VDAC1 and VDAC3 have the same number of exons, nine, with comparable size. VDAC2 contains additional exon coding for the extension of 11 amino acids of the N-terminal sequence. Alignment of the nucleotide sequences revealed 70% of identity among the three isoforms of the same species and 90% identity between the isoforms of a human and a mouse, in particular in the coding region (Messina et al., 2012; Raghavan et al., 2012).

To summarize, three VDAC genes of comparable size, structures, and with a high degree of sequence conservation are present in the mouse and the human, confirming the syntenic relationship between the two species (Young et al., 2007; Raghavan et al., 2012).

VDAC ALTERNATIVE SPLICE VARIANTS

The results of VDAC genes sequencing and structure reconstitution in the human and the mouse led to the identification of alternative splicing variants. Although the identification of two additional exons in the 5′-untraslated region (5′-UTR) of the human VDAC1 gene suggested the generation of two transcript variants, the proteins encoded did not change the amino acid sequences derived from the canonical transcript (Raghavan et al., 2012; https://www.ensembl.org/index.html). The human VDAC2 gene holds an additional exon2 in the transcript, responsible for its longer N-terminal sequence of additional 11 amino acids (Ha et al., 1993). This feature of the second isoform was maintained during mammalian evolution, but it is absent, for example, in fish. As for the human VDAC3 gene, an alternative transcript was found in various tissues, starting from an extra ATG located between exon 3 and exon 4 and identified as a short, alternative exon, consisting of a single ATG codon. This alternative transcript is translated in a shorter, truncated protein, with features comparable to the two other isoforms. The expression of this transcript variant was also identified in a rat and a mouse (Sampson et al., 1998). We have recently analyzed the human transcript sequences available in the main public resources (ENSEMBLE and GenBank) through the Genome Browser retrieval system of UCSC (Kent et al., 2002; http://genome.ucsc.edu). In this work, we compared human information with those found about murine VDAC isoforms. Several transcript variants are annotated for each isoform, falling into three different groups: coding proteins; processed transcripts; or RNA involved in nonsense mediated decay (Zinghirino et al., 2020). With the exception of the VDAC3 gene, holding two transcript variants in the mouse and six in the human, a comparable number of different protein-coding mRNA can be accounted for the VDAC1 and VDAC2 isoforms in both species (**Figure 1**). The alternative mRNAs in human VDAC1 have the same exons composition but differ in 5′ and/or 3′ UTR lengths. Instead, in the mouse, the mRNA translated into the functional protein VDAC1 might be subjected to alternative splicing at the C-terminal end of the coding region to generate transcript variants for longer or truncated polypeptides. A similar observation might be made with human and mouse VDAC2 transcript variants as is suggested by their structural organization.

It is not known whether the identified alternative VDAC variants have any biological role. However, data collected by various collaborative projects available online report the expression of several mRNAs, including non-protein-coding transcripts. The variability of UTR sequences, as well as the presence of many non-coding RNAs, also supports the hypothesis that there are differentiated transcriptional regulatory mechanisms for each variant, depending on their cellular context.

VDAC GENE EXPRESSION IN DIFFERENT TISSUES

The very first information about the tissue-specific expression of VDAC isoforms in mammals was obtained by Northern blotting and RT-PCR experiments (Ha et al., 1993; Sampson et al., 1998). In general, a comparable level of VDAC transcripts in almost all human and mouse tissues was observed, with prevalence of VDAC1 isoform in every species. The highest amount of VDAC1 and VDAC2 transcripts was detected in brain, skeletal muscle, heart, digestive, and reproductive systems (Ha et al., 1993; Sampson et al., 1998). Interestingly, a peculiar expression of VDAC isoforms was observed in reproductive organs. Indeed, VDAC2 and VDAC3 are highly expressed in specific locations in sperm and oocyte, whereas VDAC1 was exclusively found in regions required to support gamete development, i.e., Sertoli cells (Hinsch et al., 2001; De Pinto et al., 2008). VDAC3 was also found to be essential for sperm motility; in the mice knockout of the VDAC3, the gene was associated with male infertility (Sampson et al., 2001). Furthermore, VDAC2 was detected in the acrosomal region where it finely regulates the balance between the life and death of these particular cells. In porcine oocytes, VDAC1 is localized in the plasma membrane and around the cortical area, whereas immunostaining of VDAC2 produced clusters of ring-like structures distributed over the cortical area in some stage oocytes (Cassará et al., 2010). Cryo-electron

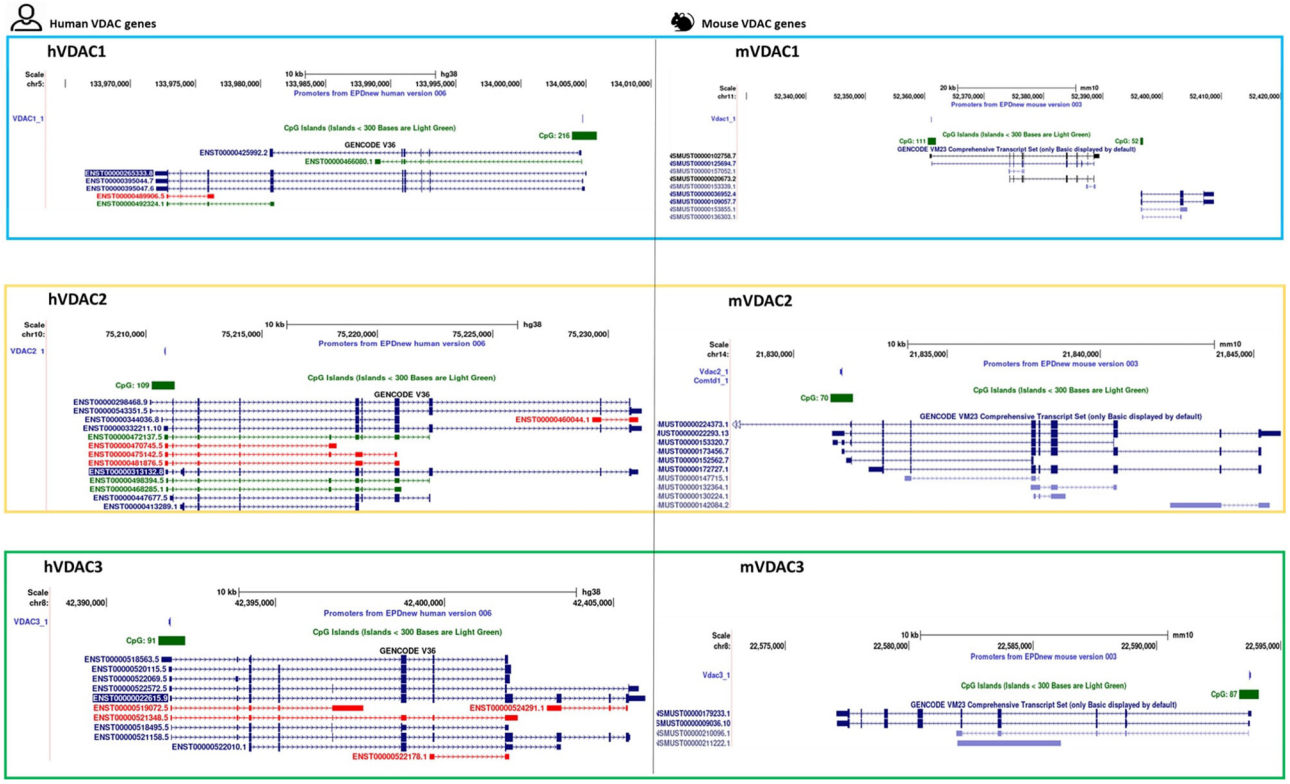


FIGURE 1 | VDAC isoforms in human and mouse. A UCSC schematic overview of transcript variants, showing the location of CpG island and the promoter sequence from EPD v.006 (human) and v.003 (mouse). In the left panel, the colors conventionally used in the GENCODE V36 display of human transcripts show the protein coding sequence in blue, the noncoding mRNA in green, and the retained one in red. In the right panel, the mouse protein gene transcripts are shown in black and dark blue, while other RefSeq transcripts are in light blue according to GENCODE VM23 track configuration.

tomography images of mammalian sperms resolved the structure of mitochondrial wrap around the flagellum cytoskeleton and revealed a network of proteins, including VDAC2 and VDAC3. Their role is essential for maintaining the interaction between mitochondria and cytoskeleton and to stabilize sperms during motility and activation (Leung et al., 2021).

To date, the information available on VDAC levels is primarily related to the investigation of changes between physiological and pathological conditions. These are corroborated by many results derived from large-scale genomic projects, high-throughput sequencing, and transcriptomics data. Recently, we have reported the expression profile of the human VDAC isoforms (Zinghirino et al., 2020) from the RNA-seqGTEx (Ardlie et al., 2015) and the RNA-seq CAGE RIKEN FANTOM 5 (FANTOM5) (Noguchi et al., 2017) Projects of Expression Atlas repository of EMBL-EBI (Papatheodorou et al., 2019). Here, we complement this information with the expression profile of VDAC mouse isoforms from the mouse gene expression database (GXD) (Baldarelli et al., 2021) and the FANTOM5 project repository (**Figure 2** and **Table 1**). The aim is to evaluate the expression profiles of VDAC isoforms between the two species.

Analyzing VDAC expression profiles in these datasets, we can confirm that human VDACs are ubiquitously expressed in all tissues but mainly enriched in skeletal muscle and heart. The level of VDAC2 is comparable or slightly lower than VDAC1 mRNA, while VDAC3 is less expressed than the other two isoforms (**Figure 2A**). The results extracted from GTEx were also confirmed by real-time PCR experiments in HeLa cells with quantification of VDAC2 and VDAC3 mRNA levels relatively to the VDAC1 transcript (Messina et al., 2012; Zinghirino et al., 2020). The comparison of human VDAC transcripts obtained from RNA-seq data from the GTEx project with those collected from GXD revealed that mice also show prevalence of VDAC1 expression, the highest in skeletal muscle (**Figure 2B**). We also found a predominant level of VDAC1 transcripts over the other isoforms in the heart, digestive system, and ovary. However, in contrast to human RNA-seq data, where VDAC3 is the most expressed isoform in testis, in mice, we observed that VDAC2 is the most expressed in this tissue.

Surprisingly, the data set from the FANTOM5 project on humans overturned information from previous repositories, showing a very poor presence of VDAC1 transcripts, while VDAC3 and VDAC2 were much more abundant (**Table 1**). These data, indeed, would highlight for the first time the prevalence of VDAC3 gene transcription on other isoforms, reflecting its higher promoter activity. We do not know whether this is an experimental effect of the dataset accumulation process, since the RNA-seq methodology—based on cap analysis of gene

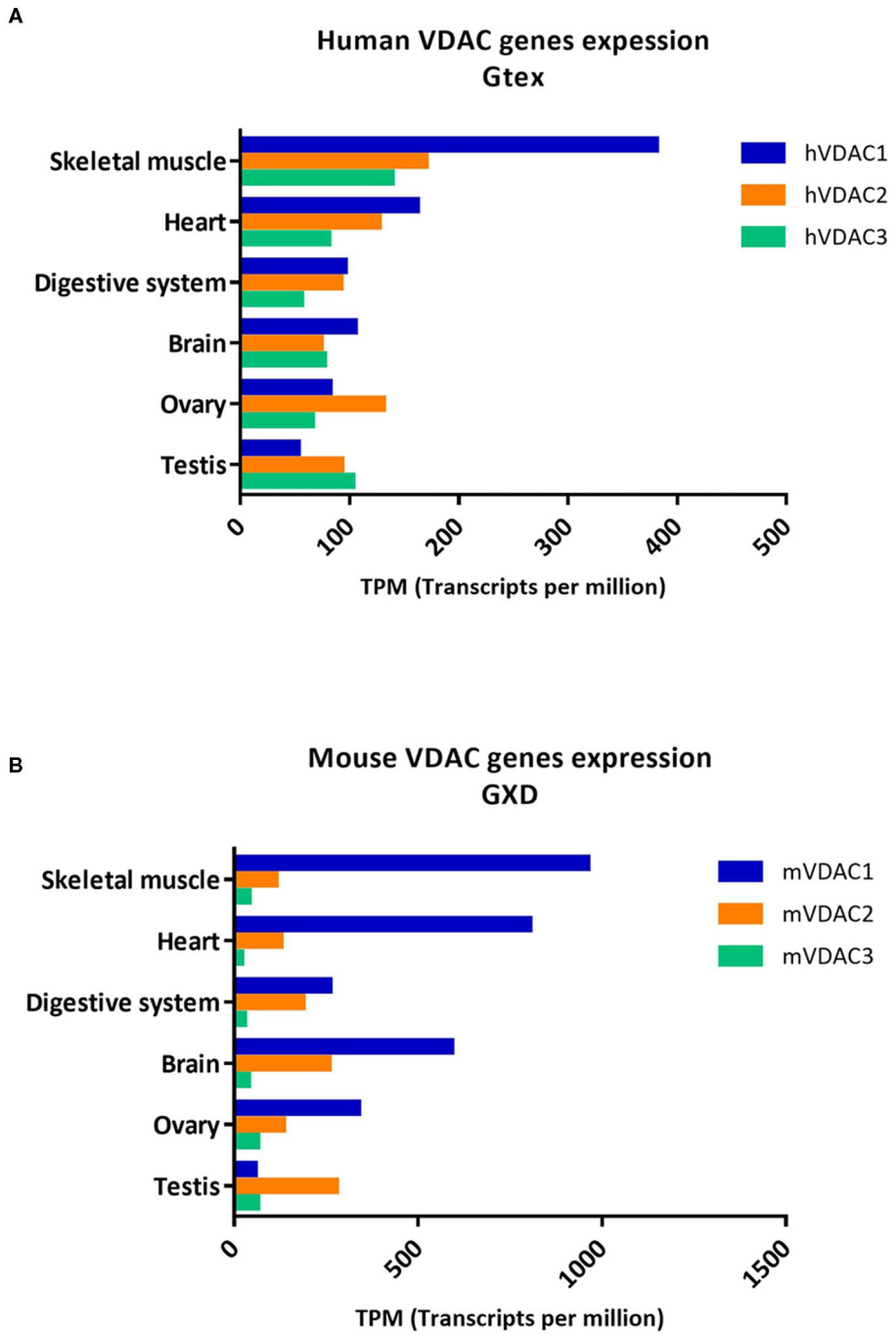


FIGURE 2 | Human and mouse VDAC isoforms genes expression in different tissues. The expression level of human and mouse VDAC genes in the most common tissues as obtained from **(A)** Genotype-tissue expression (GTEx) related to expression Atlas of EMBL-EBI open science resource (https://www.ebi.ac.uk/gxa/home); and from **(B)** MGI-Mouse gene expression database (GXD) (http://www.informatics.jax.org/gxd).

expression adopted by the FANTOM5 consortium—aims to identify exclusively active TSS located at the 5′-end of transcribed mRNA, which are not necessarily associated with the entire protein coding transcripts (Zinghirino et al., 2020).

The results reported for mouse VDAC isoforms expression in different tissues by the FANTOM5 project are more homogeneous in terms of tissue enrichment and confirm a comparable order of magnitude between VDAC1 and VDAC3

TABLE 1 | Human and mouse VDAC expression in different tissues by RNA-Seq CAGE FANTOM 5 project (TPM values).

Gene	Cerebellum	Diencephalon	Spinal cord	Heart	Lung	Pancreas	Colon	Ovary	Testis
VDAC genes expression by RNA-Seq CAGE FANTOM 5 (TPM values)									
hVDAC1	NR	0.5	NR	0.5	NR	NR	0.6	NR	0.7
hVDAC2	1	2	1	1	0.8	0.8	1	0,8	1
hVDAC3	89	82	59	107	30	35	38	62	107
mVDAC1	42	83	70	NR	6	4	10	3	0.6
mVDAC2	NR	0,6	0,6	NR	NR	4	6	1	6
mVDAC3	35	39	45	NR	18	11	25	21	152

RNA-seq cap analysis gene expression (CAGE) FANTOM5 data for the human and mouse VDAC gene were collected from the Expression Atlas repository (https://www.ebi.ac.uk/gxa/home). In the Table are the reported specific TPM values of each human VDAC isoform for representative tissues. NR: not reported in the dataset; TPM: transcripts per million.

isoforms expression, which are included in a range between 10 and 90 TPMI. VDAC2 is in a.5–6 TPMI range (**Table 1**).

One plausible explanation is that the level of each VDAC isoform expression is associated with the presence of the corresponding protein among tissues and, thus, to its putative specific biological role. For example, in mice, as well as in humans, VDAC3 is the most expressed isoform in testis and in tissues derived from different areas of the brain. In particular, in humans, VDAC3 expression is also reported to be high in other tissues as heart, cerebellum, diencephalon, spinal cord, and ovary. Among all the tissues tested in mice, VDAC2 is particularly highly expressed in the colon, testis, and pancreas, while, in humans, a prevalent level is registered in tissues or organs belonging to nervous system and circulatory apparatus. In these two last apparatuses and/or organs, VDAC1 expression is prevalent compared with the other tissues in both species.

VDAC GENE PROMOTER CORE ORGANIZATION AND TRANSCRIPTIONAL ACTIVITY

The earliest information about the regulatory regions of mouse and human VDAC genes was reported when the gene structure of these protein families was described (Sampson et al., 1997; Messina et al., 2000).

All mouse and human VDAC genes lack TATA-box elements, showing, on the contrary, enrichment of GC content. The transcription initiation sites (TSS) were predicted in murine VDAC, and the 5′-UTR located downstream was cloned and studied for its transcriptional activity by the CAT gene reporter assay. Interestingly, the putative VDAC1 promoter was the less active among the three isoforms and lacked any significant activity in antisense orientation, while VDAC2 predicted promoter showed the highest transcriptional activation level (Sampson et al., 1997). Only recently, human VDAC gene promoters have been investigated to highlight their structural and functional features (Zinghirino et al., 2020).

For each VDAC isoform, we selected the main promoter region found in the eukaryotic promoter database (EPD) and confirmed its role through the analysis of the best predictors of transcription available in the UCSC genome browser: CpG island location, the RNA polymerase II binding site, the chromatin-state model, and the enrichment levels of the H3K4me1 and H3K4me3 histone marks (**Figure 3**).

We also investigated the promoter core structure and motif elements distribution involved in VDAC gene transcription. A general overview of VDAC promoter regions reveals that, coherently, with the analysis of mammalian promoter properties (Bajic et al., 2006), the functional promoter elements are more enriched in the upstream rather than downstream TSS region. Moreover, the different promoter elements identified in the two species suggest a specific set of transcription initiation active domains in the control of TSS.

VDAC genes core promoter organization is similar to that of most TATA-less human promoters of ubiquitously expressed genes, where the presence of abundant GC regions, alternative binding sites Inr, DPE, and BRE assure basal transcription. In murine VDAC genes, several BRE elements are localized in VDAC2 and VDAC3 mouse genes, and, interestingly, BBCABW initiator elements are abundant, but only in murine VDAC gene promoters (**Figure 3**). The Inr-like sequence BBCABW (where B = C/G/T and W = A/T) is the most abundant sequence in the vicinity of the TSS and detected in focused promoters in humans (Vongoc et al., 2017). A noncanonical initiation site termed "the TCT/TOP motif" (polypyrimidine initiator), the target for translation regulation by the mTOR pathway, oxidative, and metabolic stress (Nepal et al., 2020), was also identified in human VDAC2 and VDAC3 promoter and in all murine VDACs. This element is an intriguing fingerprint for the VDAC genes family, at least in mammals, since it is conserved in mice and humans, suggesting that transcriptional activation or the translational control exerted through these elements might be linked to a specific biological context. One recent discovery is a set of genes termed dual-initiation (DI) promoter genes; these hold non-canonical YC initiation that are proximal to or intertwined with the canonical YR initiation in the same core promoter region. This promoter architecture reflects two regulatory functions, which can generate distinct sets of RNAs with different posttranscriptional fates correlated with developmental stages or different responses to environmental stimuli (Nepal et al., 2020; **Figure 3**).

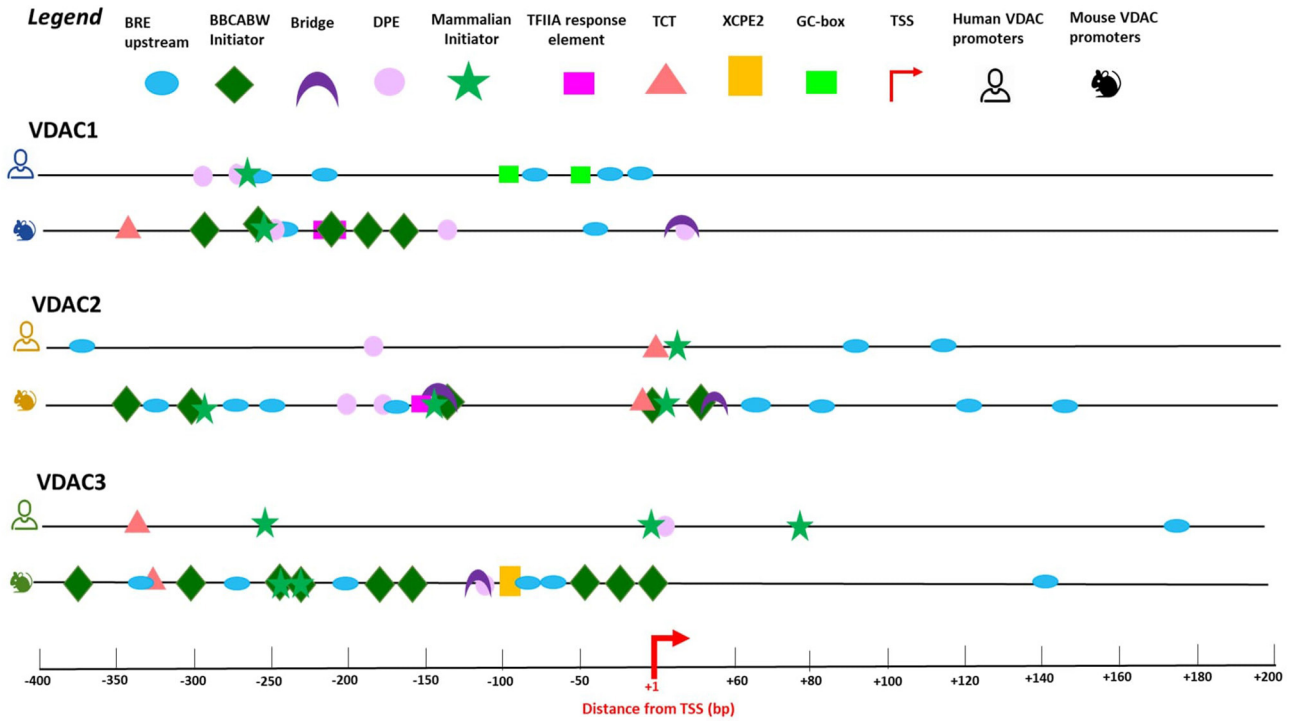


FIGURE 3 | Promoter binding motifs and their location in human and mouse VDAC genes. The promoter sequences of mVDAC1, mVDAC2, and mVDAC3 genes used in this work cover a wider region around the TSS compared with the annotated core promoter in EPD database (EPD new v.006), ranging from 400bp upstream the TSS to 200bp downstream the TSS. The promoter elements identified by the predictive tool ElemeNT v.2 (http://lifefaculty.biu.ac.il/gershon-tamar/index.php/resources) illustrated in the legend box are B recognition element (BRE); BBCABW, Mammalian initiator, Bridge motif, downstream core promoter element (DPE), TFIIA response element, polypyrimidine initiator (TCT), and X core promoter element 2 (XCPE2). The TSS site is indicated by a red arrow. The scheme displays the similarity (conserved motifs) and differences in the structure and distribution of mouse promoter elements with the results of promoter elements in human VDAC genes (Zinghirino et al., 2020). Each promoter binding motif was chosen with highest scoring consensus based on functionally recommended scores of ≥0.1 for BBCABW Initiator, Human TCT and TFIIA response element; ≥0.01 for Bridge, DPE, MTE, XCPE 1, and XCPE 2. The human output might be less informative than mouse representation due to a quality operating filtering. Few elements were found with a maximum score due to a redundancy of human promoter elements.

The promoter core analysis suggested that, with the exception of motifs predicted around the TSS of VDAC2 promoter, which is quite overlapping between the mouse and the human, the different promoter elements identified in the other isoforms suggest a specific set of transcription initiation active domains in the control of TSS.

We studied the transcriptional activity of the human VDAC promoter by gene reporter assays and, according to RNA-seq CAGEFANTOM5 results, we found that the VDAC3 promoter had the highest transcriptional activity, and VDAC1 promoter was, in contrast, the least active (Zinghirino et al., 2020). It could be hypothesized that these cells need to quantitatively regulate the level of VDAC3 mRNAs due to their different stability or to maintain a high level of transcripts in order to promptly respond to a particular stimulus.

VDAC GENE PROMOTER METHYLATION STATUS

An interesting aspect of the gene promoter is the methylation status of the gene in question, crucial for a complete understanding of gene regulation. To date, the available information on the methylation status of VDAC genes is very poor. Therefore, we have extrapolated an overview of the genomic GC enrichment by UCSC Genome Browser. We further reported the methylation profile extracted by MethBank database (Li et al., 2018; https://bigd.big.ac.cn/methbank/), which provided data on VDAC genes methylation status in different tissues, cell types, and/or developmental stages (**Table 2**).

In both species, the human and the mouse, VDAC1 gene owns the largest CpG island among isoforms, but both species are characterized by a comparable percentage of CpG as well as of C and G and of the observed/expected CpG ratio(Gardiner-Garden and Frommer, 1987). An extended CpG island in the promoter region is considered to be a feature of a more stable promoter activity compared with genes with a smaller CpG island, which needs to be specifically regulated (Elango and Yi, 2011). Although we suppose that VDAC1 is ubiquitously and stably expressed as a housekeeping gene, we also support the hypothesis that its transcriptional activity can be regulated in stress conditions when mitochondrial function needs to be assured in the cells (Guarino et al., 2020). For example, in tumor

TABLE 2 | Overview of CpG islands (CGIs) content in human and mouse promoters of VDAC genes.

Gene	CpG Position	CpG size (bp)	% CpG	% C or G	ObsCpG/ExpCpG ratio
VDAC genes expression by RNA-Seq CAGE FANTOM 5 (TPM values)					
hVDAC1	chr5:133339601-133341509	1,909	19.4	63.2	0.97
hVDAC2	chr10:76969993-76971002	1,010	21.6	71.6	0.84
hVDAC3	chr8:42249056-42249849	794	20.4	65.5	0.95
mVDAC1	chr11:52360505-52361866	1,362	16.3	62.6	0.83
mVDAC2	chr14:21831203-21831936	734	19.1	67	0.85
mVDAC3	chr8:22593328-22594117	790	22	68.2	0.95

Comparative analysis of CGIs in VDAC promoter genes was performed by the gene regulatory track of UCSC Genome Browser, showing position, size, GC content (values expressed in percentage) and an observed/expected CpG ratio (Gardiner-Garden and Frommer, 1987). Releases of the human and mouse reference genomes were GRCh37/hg19 and GRCm38/mm10, respectively.

cells and in placental trophoblasts from patients with recurrent miscarriages, the increase of EPB41L4A-AS1 lnc-RNA induced the enhancement of VDAC1 promoter activity by histone modification. Indeed, the chromatin region where the VDAC1 gene is located showed increase in H3K4me3, mediated by the histone lysine methyltransferase SET1A and reduced interaction with histone deacetylase HDAC2. In this situation, EPB41L4A-AS1 lnc-RNA is an important regulator of reprogramming tumor and trophoblast cells metabolism, since it can activate oxidative metabolism by enhanced mitochondrial function (Liao et al., 2019; Zhu et al., 2019).

Furthermore, we extracted the methylation profile of VDAC genes from MethBank Database for a panel of tissues and cell lines in humans and for a panel of cell types at different development stages in mice (**Table 3**). The level of CG methylation of the human VDAC genes promoter revealed that the VDAC1 promoter is less methylated than the other two isoforms. Interestingly, VDAC2 promoter shows the highest methylation level, especially in the brain, muscle, and heart. The low degree of VDAC1 promoter methylation in all the tissues considered may suggest the predominant expression of this isoform, allowing it to accomplish its main role of exchanging mitochondrial metabolites. The average methylation levels of mouse VDAC genes across different samples of a specific developmental stage were also investigated. As a general rule, DNA methylation pattern changes dynamically during development, showing a very low level in primordial germ line and after fertilization while a re-methylation process is found in the later stage of germ cell and embryo development. We found that comparable methylation percentage is associated with VDAC2 promoter, gene body, and the downstream gene region, while, in VDAC1 and VDAC3, the promoter methylation status can be absent (Zeng and Chen, 2019). The higher methylation status of mouse VDAC2 gene is also confirmed in two particular cell types, oocyte and sperm, where VDAC proteins play a specialized role, revealing that this gene might be subjected to fine regulation affected also by epigenetic mechanisms. In support of this hypothesis, in human males, abnormal methylation of CpG island of VDAC2 promoter determined a decrease of VDAC2 expression, leading to lack of sperm motility and male infertility (Xu et al., 2016).

VDAC GENE PROMOTER AND TRANSCRIPTION FACTOR BINDING SITES DISTRIBUTION

Having isolated mouse and human VDAC genes, we investigated their basic elements. The starting points of the transcription were set up and defined the predicted promoters as the upstream region, containing the regulatory elements of the expression of the genes.

Using the available bioinformatic approaches, the binding of some transcription factor was predicted and the conservation of their sequence into the promoter of VDAC genes of human and mouse observed (Sampson et al., 1997; Messina et al., 2000). A database search for transcription factors binding motifs revealed the presence of several SP1 and AP-2 sites, but the most significant identified TFBS were a sterol repressor element (SRE), an SRY, the testis-determining factor, and nuclear respiratory factor 2 (NRF-2) binding sites, which respectively led to hypothesize the involvement of VDAC1 in cholesterol traffic, sex determination, and mitochondrial biogenesis (Sampson et al., 1997; Messina et al., 2000). With the exception of these few notes, the regulative regions of VDAC genes and the mechanisms associated with their expression were not thoroughly investigated yet.

However, understanding the mechanisms triggering VDAC genes transcription may highlight the biological role of each isoform inside the cells and in different biological contexts. Thus, in our recent analysis of human VDAC promoter genes, we laid the foundation for studying the regulatory mechanisms of VDAC isoforms expression. In order to reveal the TFs that bind to the promoter of genes, JASPAR and UniBind were used for TFBS enrichment. We defined the distribution of TFBSs by scanning VDAC promoter sequences with three different genomic suites (Genomatix, JASPAR, UniBind) and crossed the results with the data experimentally validated by ChIP-Seq (ENCODE Project v3). With this approach, we found the most relevant families of TFs regulating VDAC genes expression (Zinghirino et al., 2020), confirming the central role of VDACs in regulating mitochondrial function in fundamental cell processes. Indeed, in all three VDAC promoters, the majority of identified TFs classes participate in many similar activities but are prevalently

TABLE 3 | Average methylation levels of VDAC genes across different normal human and murine samples from single-based resolution methylomes (SRMs) provided by MethBank (v.4.1).

Gene	Brain	Muscle	Heart	Digestive system	Placenta
Average methylation levels of VDAC promoters (β-value of 0–1)					
hVDAC1	0.26	0.23	0.24	0.22	0.31
hVDAC2	0.63	0.62	0.61	0.57	0.52
hVDAC3	0.42	0.42	0.40	0.35	0.32

Gene	2-cell	4-cell	E13	E6.5	E7.5	ICM	Oocyte	Sperm
mVDAC1	0.11	0.08	0.01	0.08	0.12	0.03	0.07	0.01
mVDAC2	0.48	0.41	0.02	0.19	0.25	0.17	0.69	0.2
mVDAC3	0.05	0.02	0.01	0.09	0.11	0.01	0.01	0.07

Methbank (http://bigd.big.ac.cn/methbank) contains the SRM profiles from a wide range of cell/tissue lines in human and from mouse embryonic development stages. According to MethBank, the promoter region is defined as the region 2000 bp upstream of transcription start site (TSS) for animals. Furthermore, the average methylation data are calculated as β-Value that reflects the methylation intensity at each CpG site. β-Values of 0–1 (represented from 0 to 1) indicate signifying percent methylation, from 0 to 100%, respectively, for each CpG site (Dhas et al., 2015). We specifically selected human and mouse SRMs of the methylation level in the VDAC gene promoter. On the left, the human SRM profiles of tissues associated with the specific expression pattern of VDAC genes as discussed in Zinghirino et al. (2020) are shown.

involved in cell life and death, differentiation and development, and metabolism regulation (Grandori et al., 2000; Thiel and Cibelli, 2002; Liu et al., 2004; Niederreither and Dollé, 2008; Qu et al., 2010; Woo et al., 2011; Kim et al., 2017).

Using the same approach, we integrated this analysis with the characterization of TFBS located on mouse VDACs promoters. The comparison between the human and the mouse revealed both functional similarities and divergence of regulatory regions.

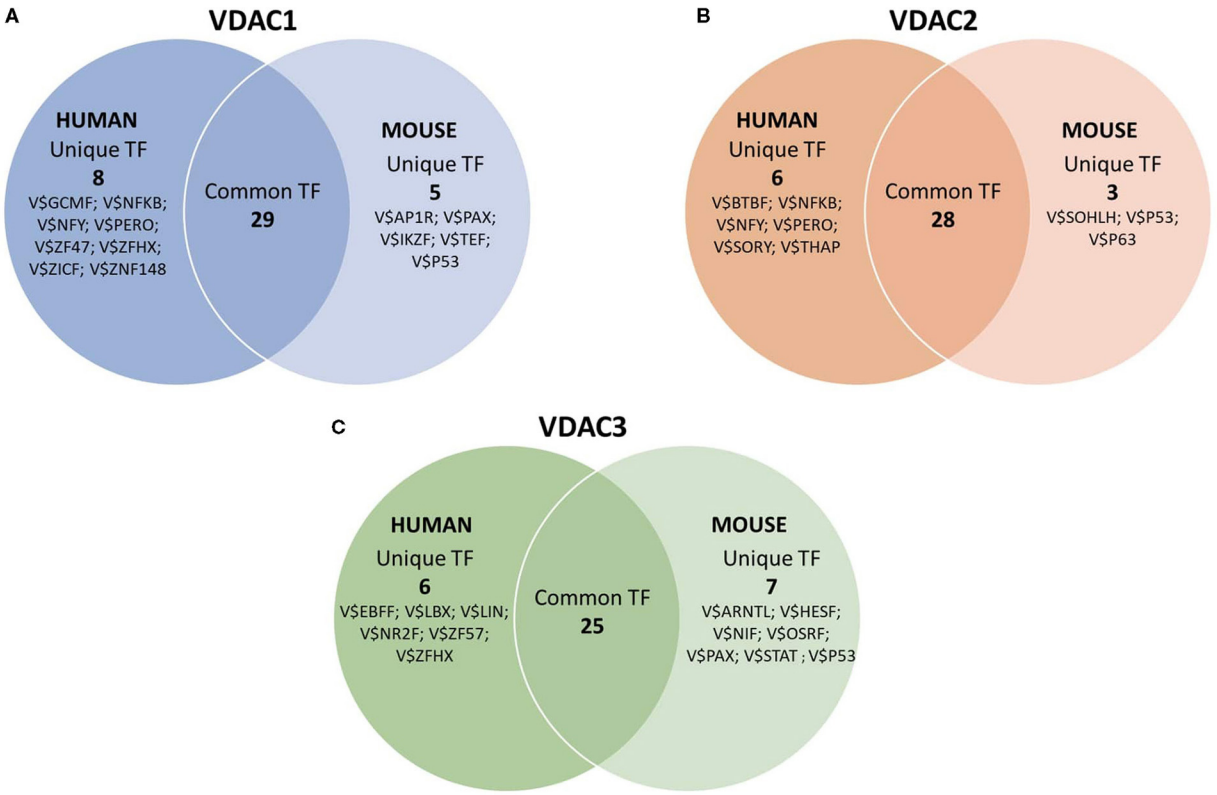
Comparing the VDACs genes of the two species, we brought to light a common fingerprint for main transcription factors; it comprises E2FF (E2F-myc cell cycle regulator), EBOX (E-box binding factors), KLFS (Krueppel-like transcription factors), NRF1 (nuclear respiratory factor 1), EGRF (EGR/nerve growth factor-induced protein C and related factors), MYOD (Myoblast determining factors) families, controlling cell survival, apoptosis, proliferation, differentiation, development, and metabolism regulation. The overlap of conserved transcription motifs reveals that there was a common evolutionary path of VDAC isoform regulatory regions in mammals. Meanwhile, other distinctive TF families exclusively characterize mouse or human VDAC promoters; however, in line with the previous observation, all of them fall back on those regulators involved in the main biological processes of growth, differentiation, and development in which cell, tissue, and organs need to be controlled in physiological and pathological conditions (**Figure 4**; **Supplementary Tables 1**–**6**).

Interestingly, we found that all mouse VDAC promoters bear specific motifs in their sequences recognized by the family of the p53 tumor suppressor transcription factors, including p63 in addition to p53. Their role in cell death, cell-cycle arrest, senescence, and metabolic regulation in response to cellular stress is well-known to affect cancer development (Chillemi et al., 2017; Fisher et al., 2020). Conservation of these TF families in mouse VDAC promoters allows us to hypothesize that the VDAC proteins are crucial in mitochondrial metabolism and in the balance of cell life and death. These functions were probably selected during the evolution of the VDAC gene family in mammals, and a more specialized role was acquired by each VDAC isoform in humans.

VDAC ISOFORMS SPECIFIC TRANSCRIPTION FACTORS

There are several examples of experimental evidence reported in the literature that confirm the crucial role that VDAC isoforms play in specific tissues or, in particular, biological contexts, but their transcriptional specificity and programming have not been fully elucidated until now.

The identification of specific transcriptional regulators in the human VDAC1 promoter has demonstrated a prevalent role for this isoform in mitochondrial function in conditions that force cells to keep the energy balance of mitochondria (Fang and Maldonado, 2018; Shoshan-Barmatz et al., 2020). In particular, we found families of regulators required for cell response to hypoxia (V$AHRR: AHR-arnt heterodimers and AHR-related factors; Labrecque et al., 2013), mitochondrial biogenesis and redox homeostasis (V$ETSF: human and murine ETS1 factors; V$PBXC: Pre-B cell leukemia transcription factor; Hayes and Dinkova-Kostova, 2014; Morgan and Pandha, 2020), and cell stress response (V$HEAT: heat shock factors; Morimoto, 2011). Similarly, after searching transcription factor binding site (TFBS) in mouse VDAC1 promoter, we confirmed the hypothesized role of VDAC1 and highlighted the presence of specific motifs recognized by transcription factors regulating growth (V$AP1R, activator protein 1; Hess et al., 2004) and developmental processes (Watkins et al., 2009) (V$TEAD: TEA domain family member 1; Zhou et al., 2016), which are mainly involved in tumorigenesis. Inspired by our bioinformatic prediction of TFBSs distribution, we wanted to investigate the regulation of human VDAC1 gene expression in metabolic



D

Common TF families Human and Mouse VDAC isoforms

Matrix family	Description
V$AP2F	Activator protein 2
V$CTCF	CTCF and BORIS gene family
V$EBOX	E-box binding factors
V$E2FF	E2F-myc activator/cell cycle regulator
V$EGRF	EGR/nerve growth factor induced protein C & related factors
V$ESR	Estrogen receptor binding
V$KLFS	Krueppel like transcription factors
V$MAZF	Myc associated zinc fingers
V$MYOD	Myoblast determining factors
V$NRF1	Nuclear respiratory factor 1
V$PLAG	Pleomorphic adenoma gene
V$ZF07	C2H2 zinc finger transcription factors 7
V$ZF02	C2H2 zinc finger transcription factors 2

FIGURE 4 | TFBS distribution on human and mouse VDAC promoters. The sequences of human and the promoter of mouse VDAC genes were analyzed by using JASPAR (http://jaspar.genereg.net/) and UniBind (https://unibind.uio.no/) databases, and the results were overlapped to find the most relevant TF families. A comparative analysis of common and unique TFBSs for each individual VDAC isoform of human and mouse was performed. Venn diagrams showed the number of common and unique TFBS clusters between human and mouse genes in **(A)** VDAC1, **(B)** VDAC2, and **(C)** VDAC3 promoters. **(D)** The results of the common TFBS families obtained from the comparative analysis of VDAC isoforms for each species were overlain to obtain the most evolutionarily conserved transcription factor families between a human and a mouse. In the Table, the Matrix family and the description of the main TFBS families shared among the three isoforms in both species are reported.

stress conditions (Guarino et al., 2020). The discovery of several binding sites for the NRF-1 and HIF-1α, transcriptional regulators involved in metabolic stress caused by nutrients (Scarpulla, 2006, 2012), and oxygen deficiency (Majmundar et al., 2010; Iommarini et al., 2017) in the VDAC1 promoter suggested a pivotal role for this isoform in the regulation of mitochondrial metabolism and energetic balance in cell adaptive response. We experimentally demonstrated that NRF-1 (nuclear respiratory factor 1) and HIF-1α (hypoxia-inducible factor 1-alpha) act as transcriptional activators of the VDAC1 promoter and can support the modulation of regulation of the VDAC1 promoter, following serum starvation and hypoxia (Guarino et al., 2020). Our results also suggested that the VDAC1 promoter activation in stress conditions is probably controlled by a more complex transcriptional apparatus, involving the cooperation of other transcriptional regulators. With the bioinformatics predictive tools and the ChIP-Seq Peaks data in ENCODE project validation, we predicted several transcription factors binding sites involved in mitochondrial biogenesis (CREB: cAMP response element-binding protein; SP1: specificity protein 1; ETS: human and murine ETS1 factors; CREM: cAMP response element modulator; ATF7: activating transcription factor 7; NFE2L2: nuclear factor erythroid 2-related factor 2; E2F), which might cooperate with NRF-1 or HIF-1α for the regulation of VDAC1 transcription. According to these data, it has been recently reported that the CREB effector of MAPK/ERK signaling pathway triggers the increase of VDAC1 transcription to activate apoptosis induced by H2O2 in cardiac microvascular endothelial cells. Such overexpression can be reestablished at a basal level when melatonin is used as an antioxidant to repair the injury (Xing et al., 2019). The experimental observation of VDAC1 transcriptional regulation in stress conditions confirms the importance of this protein in maintaining the correct balance in cell life and death by assuring the functionality of the mitochondria (Fang and Maldonado, 2018; Shoshan-Barmatz et al., 2020).

Analysis of the human VDAC2 promoter highlighted the presence of factors specifically involved in the development of specialized tissues and the organogenesis process mainly related to nervous system genesis (V$NEUR: NeuroD, Beta2, HLH domain; Kageyama et al., 2019) and growth [V$CLOX: CLOX and CLOX homology (CDP) factors; Li et al., 2010]. Corresponding findings arose from the analysis of the mouse VDAC2 promoter; however, different TFs are present, like V$SOHLH (spermatogenesis and oogenesis basic helix-loop-helix; Suzuki et al., 2012) and V$ZFXY (Zfx and Zfy-transcription factors; Fang et al., 2014), both associated with sex tissue development. The role of VDAC2 in this particular aspect is supported by the literature. GATA1 (GATA-binding factor 1) and MYBL2 (cellular and viral myb-like transcriptional regulators) were identified as regulators of the VDAC2 promoter in developing porcine ovary, determining its upregulation to inhibit autophagy through the interaction with BECN1 (Beclin 1) and BCL2L1 (Bcl-2-like 1). The region of the VDAC2 promoter involved in the regulation of ovary development identified in the *Sus scrofa* promoter is highly conserved in mice. This observation highlights the protective role of VDAC2 in ovary development in all mammals. An interesting finding is the identification of two different haplotypes associated with this promoter region, which determine a difference also in the transcriptional activity of the VDAC2 promoter (Yuan et al., 2015).

With regard to the human VDAC3 isoform, the identified TFBSs belong to various families, but those involved in organogenesis [V$FOX: Forkhead (FKH)/Forkhead box (Fox); V$LBXF: Ladybird homeobox (lbx)] (Friedman and Kaestner, 2006; Jennings et al., 2019) in the development of germinal tissues and sex determination (V$SOHLH; Suzuki et al., 2012) are the most abundant. The bioinformatic distribution of TFBS in the mouse VDAC3 promoter shows enrichment for some specific motifs required for mitochondrial stress response (V$HESF: Vertebrate homologs of enhancer of split complex; Ardlie et al., 2015), organs development (V$OSRF; Otani et al., 2014), tumor suppressor (V$P53; Sullivan et al., 2018).

The promoter analysis of motifs elements involved in gene transcriptional activity and the identification of the regulators by the TFBS distribution, mainly delineate, in the VDAC gene, clusters of evolutionarily conserved transcription motifs. However, we also simultaneously found some distinctive traits corresponding to TF families regulating mouse or human specific VDAC genes. From the biological point of view, the overall picture emerging from the analysis of the specific TFBS, characterizing VDAC promoters, reports a less undifferentiated role of VDAC isoforms in mice. All the isoforms in mouse seem to be involved in the mechanism of cell stress response, in particular in tumorigenesis, while in humans, this role has been assigned to VDAC1.

CONCLUSIONS

The activity of genes is a hallmark of the role of the proteins they encode. This aspect is even more relevant when analyzing a family of protein isoforms. In this work, we attempted to look for a nexus that could explain the evolution of three isoforms of the pore-forming protein VDAC and the need in the higher-evolved organisms to maintain such diversity. The comparison of promoter features of VDAC1-3 genes in close but different species as *H. sapiens* and *M. musculus* revealed that same functions, at least in terms of abundance/reduction of gene expression, are prevalently conserved among these two organisms, even though with some differences. The difference we found among human VDAC isoforms (Zinghirino et al., 2020) were mainly confirmed in mice, thus strengthening our feeling that the VDAC isoforms play different roles in the cell. The wealth of information stored in public databases appears a complex jungle. Our goal is to locate the classic needle in the haystack. With this work, we began to achieve this goal.

AUTHOR CONTRIBUTIONS

FG conceived the work. FZ, XP, AM, GN, VDP, and FG wrote the manuscript. FZ and XP equally performed the data collection and

analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This research was partly supported by Linea Piaceri 2020–2022 of the University of Catania, Italy to FG, AM, VDP, and by MIUR PNR Proof of Concept 2018 grant (codex: PEPSLA) to AM. The authors also acknowledge Fondi di Ateneo 2020-2022, Università di Catania, Linea Open Access and Linea CHANCE.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.708695/full#supplementary-material

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Conflict of Interest: AM, VDP, and FG have an intellectual collaboration with the company, we.MitoBiotech.srl.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Physiology

REVIEW
published: 05 August 2021
doi: 10.3389/fphys.2021.683920

VDACs: An Outlook on Biochemical Regulation and Function in Animal and Plant Systems

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The voltage-dependent anion channels (VDACs) are the most abundant proteins present on the outer mitochondrial membrane. They serve a myriad of functions ranging from energy and metabolite exchange to highly debatable roles in apoptosis. Their role in molecular transport puts them on the center stage as communicators between cytoplasmic and mitochondrial signaling events. Beyond their general role as interchangeable pores, members of this family may exhibit specific functions. Even after nearly five decades of their discovery, their role in plant systems is still a new and rapidly emerging field. The information on biochemical regulation of VDACs is limited. Various interacting proteins and post-translational modifications (PTMs) modulate VDAC functions, amongst these, phosphorylation is quite noticeable. In this review, we have tried to give a glimpse of the recent advancements in the biochemical/interactional regulation of plant VDACs. We also cover a critical analysis on the importance of PTMs in the functional regulation of VDACs. Besides, the review also encompasses numerous studies which can identify VDACs as a connecting link between Ca^{2+} and reactive oxygen species signaling in special reference to the plant systems.

Keywords: mitochondrial channel, biochemical regulation, phosphorylation, cell signaling, Ca^{2+}, reactive oxygen species, cellular homeostasis, CBL–CIPK

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *22 March 2021*
Accepted: *30 June 2021*
Published: *05 August 2021*

Citation:
Ravi B, Kanwar P, Sanyal SK, Bheri M and Pandey GK (2021) VDACs: An Outlook on Biochemical Regulation and Function in Animal and Plant Systems. Front. Physiol. 12:683920. doi: 10.3389/fphys.2021.683920

INTRODUCTION

Popularly known as the "powerhouse of the cell," mitochondria have risen from their classical biochemical functionality. The voltage-dependent anion channels (VDACs), first reported by Schein et al. (1976), are the most abundant, multi-functional family of pore-forming proteins present on the outer mitochondrial membrane (OMM; Benz, 1994; Colombini et al., 1996). The general structure of VDAC proteins can be described as a channel formed by 19 β-barrels and an N-terminal that folds in an α-helix, horizontally within the pore (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). The studies indicate that the permeability of the pore is majorly regulated by its voltage-dependent conformational state (open state shows slight preference for small anions while its closed state prefer cations) (Colombini, 1980; Benz et al., 1990; Hodge and Colombini, 1997), hence regulating organelle metabolism (Benz et al., 1988, 1990; Ludwig et al., 1988). The determination of VDACs as regulators of the OMM permeability, as well as in major molecular trafficking across the mitochondrial-cytoplasmic interface, revealed a new mitochondrial dimension on how they can regulate inputs according to external cues

(Colombini, 1979). Additional discoveries depicted their crucial role in cell signaling, cytoplasmic-mitochondrial communication, aging, cell life, as well as the controversial cell death mechanism (Crompton, 1999; Griffiths, 2000; Colombini, 2004; Baines et al., 2007; Ott et al., 2007). VDACs have been reported from all the eukaryotes studied so far (De Pinto et al., 2003). Three of its isoforms are present in mammals, including humans (Menzel et al., 2009; Raschle et al., 2009; De Pinto et al., 2010), wherein hVDAC3 is accepted as the oldest isoform, and hVDAC1 is the recently evolved isoform (Young et al., 2007). However, their numbers are variable in plants and are generally more as compared to mammals. The *Oryza sativa* and *Nicotiana tabacum* have three VDAC isoforms (Al Bitar et al., 2003; Tateda et al., 2009), *Arabidopsis thaliana* has four VDAC isoforms (Clausen et al., 2004; Tateda et al., 2011), and *Medicago trunculata*, *Lotus japonica*, *Phaseolus vulgaris*, and *Glycine max* contain at least five putative VDACs (Clausen et al., 2004; Wandrey et al., 2004; Saidani et al., 2016). Their variable number in plants indicates that they might have a more diverse role in plants. While protein purification, sequence analysis, and/or structure prediction have already been reported from plants like wheat, rice, pea, pearl millet, maize, and potato (Aljamal et al., 1993; Fischer et al., 1994; Heinss et al., 1994; Elkeles et al., 1995; Geiger et al., 1999; Al Bitar et al., 2003), plant VDACs still require high-resolution structure revelation.

Voltage-dependent anion channel, being one of the major membrane protein in the OMM, can be modulated by factors like chemical compounds and other proteins or cellular metabolites. In this review, we provide an update on the biochemical regulation of VDACs and their impact on the physiological function of VDACs, with a particular emphasis on the plant system. Post-translational modifications (PTMs) are essential mechanisms that diversify protein functions (Lodish, 1981). Several PTMs on VDACs are reported in animal systems and predicted in plant systems (Elkeles et al., 1995; Al Bitar et al., 2003; Martel et al., 2014) and might be responsible for the multi-functionality of these proteins. Therefore, it becomes imperative that we look at their PTMs via phosphorylation and other modes of modification. Lastly, we will tackle the potential role of VDAC in being the focal point through which calcium (Ca^{2+}) and reactive oxygen species (ROS) signaling may crosstalk in the cell. This information should help us understand the functional significance of VDACs and explore the possibility that VDACs might have a role in inter-organellar communication for major cellular processes in plants.

BIOCHEMICAL AND INTERACTIONAL REGULATIONS AFFECT VDAC FUNCTIONING IN PLANTS

In the following sections, we discuss the various factors that influence the biochemical, structural, and functional aspects of VDACs.

CHEMICAL COMPOUNDS: INTERACTIONAL INFLUENCES I

Natural compounds or extracts from plants have served humankind from ancient times owing to their medicinal properties and diversity. The modulation of VDACs is reported by plant-based compounds (depicted in **Figure 1**), usually through binding. Curcumin, a pigmented polyphenolic compound extracted from *Curcumin longa*, is known for its anti-cancer properties (Aggarwal et al., 2003). Curcumin modulates hVDAC1 when reconstituted in a lipid bilayer (Tewari D. et al., 2015). Molecular docking and mutational analysis have shown that curcumin interacts with hVDAC1 through N-terminus (Lys 15, Arg 18, and Asp 19) at one end and the amino acid Tyr 198 located in the inner wall of the channel. Closure of hVDAC1 by curcumin might contribute to its known pro-apoptotic property (Tewari D. et al., 2015). An essential oil constituent, precocene II, inhibits the production of tricothecene in *Fusarium graminearum*, the causal agent of fusarium head blight (Yaguchi et al., 2009). This fungal component has been shown to bind the single VDAC in *F. graminearum* through the affinity magnetic bead method. The precocene II is responsible for the increased level of superoxide in mitochondria (Furukawa et al., 2015) postulated due to gate closing. However, its role in trichothecene production needs further validation (Maeda and Ohsato, 2017). Hydrogen peroxide (H_2O_2) has been shown to modulate rat brain-VDAC1 through an electrophysiological approach. The conductance of VDAC was shown to increase upon H_2O_2 treatment, and its activity was restored by curcumin. The binding site of H_2O_2 on the channel identified through *in silico* molecular docking indicates a possibility of direct interaction between VDAC and H_2O_2 (Malik and Ghosh, 2020). Leaf extracts (aqueous-methanolic) from *Centella asiatica* (CA) quench ROS during ischemia-reperfusion injury by protecting N2a cells due to antioxidant property. However, leaf extracts from CA failed to prevent IR injury in VDAC1 knocked down-N2a cells, which indicates the involvement of VDACs in the protective effect of CA. This was followed by reconstitution of hVDAC1 in the lipid bilayer membrane, CA treatment resulted in its increased single-channel conductance and stabilized the open state of hVDAC1 (Tewari et al., 2016). This might indicate the modulation of VDACs by CA, aiding its protective effects. A phyto-cannabinoid CBD (cannabidiol) derived from *Cannabis* spp. demonstrates antileukemic activity. They directly binds to hVDAC1 and resulted in decreased channel conductance depicted using western blotting, and single channel conductance in the planar lipid bilayer thereby causing CBD-induced cell death (Rimmerman et al., 2013). *In-silico* hVDAC1-CBD interaction analysis led to the identification of three putative residues at the N-terminus (Thr9, Asp12, and Leu13) and five neighboring pore residues (Val146, Gln157, Gly175, Gln182, and His184). Steric interaction and hydrogen bonds stabilized the binding, with Thr9, Asp12, and His184 contributing most highest in the binding (Olivas-Aguirre et al., 2019). In the follow up report, the effect of CBD and curcumin, along with six other phenolic compounds, was tested for their anticancer activity.

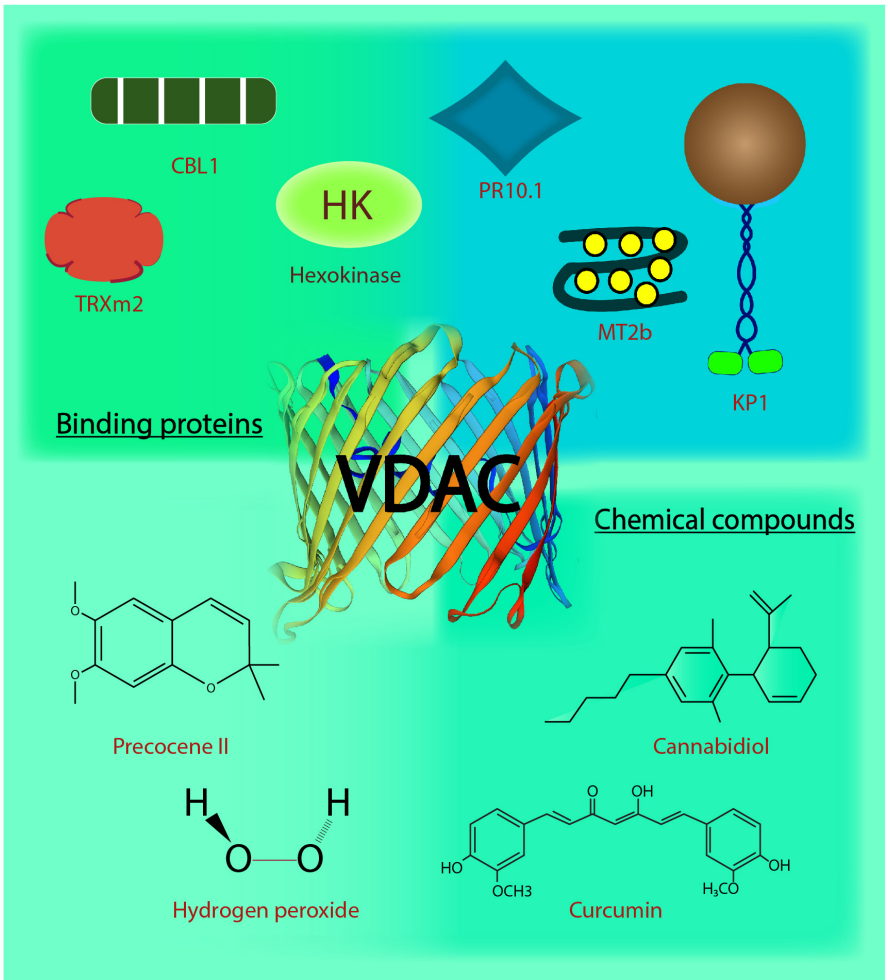

FIGURE 1 | Voltage-dependent anion channels in plants. The biochemical action and interaction of VDACs in plants affects their functional behavior. This image depicts the regulation and interaction of VDACs with binding proteins and chemical compounds. CBL1, Calcineurin B-like protein; TRXm2, chloroplast protein thioredoxin m2; MT2b, metallothionein 2b; H_2O_2, hydrogen peroxide; KP1, plant kinesin protein 1; PR10.1, pathogenesis-related 10.1; $C_{13}H_{16}O_3$, precocene II; $C_{21}H_{20}O_6$, curcumin; $C_{21}H_{30}O_2$, cannabidiol.

Out of these, CBD, curcumin and quercetin (another phenolic compound; Davis et al., 2009) preferred to interact on two specific residues (His184 and Thr9) predicted through *in silico* analysis and displayed cytotoxic effects in human T-ALL Jurket cell lines. CBD and curcumin were found to be the most conspicuous in causing cell death due to mitochondrial Ca^{2+} overload (Olivas-Aguirre et al., 2020). The regulation of human VDACs by plant extracts might indicate a new level of regulation operated by these plant-based metabolites, contributing to protective effects as witnessed in the case of apoptosis and cell death regulation. These plant-based natural compounds have potential to serve as anti-cancer drugs targeting mitochondria through VDACs. But, these prediction based on the above-mentioned studies lacks clinical potential viability and they need further exploration. Overall, the *in vitro* findings with reconstituted VDACs can help us infer better on the effect of plant extracts on VDACs and mitochondrial physiology in general. Moreover, their *in vivo* characterization will shed light on their degree of utilization. The identification of these candidate regulators from plants holds great therapeutic relevance.

PROTEINS: INTERACTIONAL INFLUENCES II

Voltage-dependent anion channel's interaction with many cytosolic proteins makes it a nexus for multiple cell signaling events. Interestingly, in plants, the reports on VDACs and their interacting partners (depicted in **Figure 1**) indicate that VDAC (and the particular partner) have a role in responding to abiotic and biotic stress, inter-organelle connection, metabolite flux at the cytosolic-mitochondrial interface, in addition to VDAC's probable role in programmed cell death. To begin with the role in stress signaling mechanism, a type 2 metallothioneins (MTs: small cysteine-rich, metal-binding proteins), have been shown to directly interact with AtVDAC3 in Arabidopsis

through yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays. The *AtMT2b* overexpression plants exhibited increased salt tolerance, and AtMT2b may act as a negative regulator of AtVDAC3 (Zhang et al., 2019). Further, plant-specific calcineurin B-like proteins (CBL) are one of the major Ca^{2+} sensors that integrate Ca^{2+} signaling to various abiotic stresses in plants (Pandey, 2008; Sanyal et al., 2015; Sanyal et al., 2016; Sanyal et al., 2020). AtVDAC1 in Arabidopsis physically interacts with AtCBL1 *in vitro*. Together, they regulate cold stress responses during plant development and seed germination (Li et al., 2013). This may also connect Ca^{2+} and ROS signaling pathways, extensively reviewed in the animal systems and discussed later in this review (Yan et al., 2006; Görlach et al., 2015; Feno et al., 2019). Recent discovery on mitochondrial regulation through interaction between chloroplast protein, thioredoxin m2 (AtTrx m2), and AtVDAC3, emphasizes an inter-organellar communication (Zhang et al., 2015). Y2H and pull-down assays have confirmed the physical interaction between the two, and BiFC assay has located the interaction on the mitochondria. This may act as another partner of VDAC3 for regulating ROS signaling and salt stress responses in plants (Zhang et al., 2015). VDAC3 from *Vitis piasezkii*' Liuba-8 was screened as an interacting partner of pathogenesis-related PR10.1 obtained from *Vitis pseudoreticulata*' Baihe-35-1 in Y2H library screening. The interaction was further confirmed through immunoprecipitation assays; together they function in imparting cell death mediated defense response to downy mildew disease caused by *Plasmopara viticola*, a biotrophic parasite in grapevine (Ma et al., 2018). The finding substantiates the functional role of plant VDACs in pathogen defense (Lee et al., 2009; Tateda et al., 2009). Several animal kinesins such as those from mouse cells, KIF1B as well as KIF5B and *Drosophila melanogaster*, KLP67A kinesin are involved in the movement of mitochondria (Nangaku et al., 1994; Pereira et al., 1997; Tanaka et al., 1998). A plant-specific kinesin, KP1, is also found to interact with plant mitochondrial AtVDAC3 in Arabidopsis. AtKP1 interacts with AtVDAC3 via its tail domain and regulates respiration during seed germination at low temperatures in plants (Yang et al., 2011). This interaction between microtubule motor protein and mitochondrial channel indicates possibilities of involvement of plant kinesins in controlling the movement of mitochondria, which requires further investigation.

Mitochondria are the hub of aerobic oxidation in eukaryotic cells. The glycolytic enzymes associate dynamically with mitochondria and support respiration. Cytoskeletal protein tubulin can induce a reduced respiration rate by reversible blocking of VDAC, as observed through patch-clamp and planar lipid bilayer technique in animal and fungal systems (Rostovtseva et al., 2008). The involvement of VDACs in the regulation of respiration is reported in plants as well. Glycolytic enzymes, such as aldolases, interact strongly with AtVDAC protein and the latter may anchor the enzyme at the mitochondrial surface, thus facilitating substrate channeling (Graham et al., 2007). These findings are important in the study of the regulation of metabolic flux and involvement of VDACs in the plant metabolic networks. Additionally, the interaction of VDACs by glycolytic enzyme, hexokinases through *in vitro* and *in vivo* studies is a well-studied concept in animals (Abu-Hamad et al., 2008; Galluzzi et al., 2008; Pastorino and Hoek, 2008) and is projected as a strategy to aid conventional chemotherapeutics (Pastorino et al., 2002; Abu-Hamad et al., 2008). Mitochondrial hexokinase modulates the function of endogenous VDAC in tobacco bright yellow cell-2 (BY2 cells) as well as heterologous expressed OsVDAC4 in *N. benthamiana* leaves. Their co-expression limits their toxicity (independently overexpressing both the proteins causes toxicity in plants and cell death in BY2 cell lines), resulting in healthy cells and leaves in plants. The expression ratio of VDAC–hexokinase and their interactions are essential in cell death pathways in plants (Kim et al., 2006; Godbole et al., 2013). The heterologous expression indeed provides a clue on the importance of VDAC–hexokinase interaction; however, the functional validation of this entire pathway in the endogenous rice system itself can conclude better about its actual functionality.

POST-TRANSLATIONAL MODIFICATIONS: REGULATION OF VDAC FUNCTION

Reversible PTMs affect VDACs and their interaction with other proteins. VDACs are modulated post-translationally by phosphorylation, acetylation, GlcNAcylation, oxidative post-translation modifications (Ox-PTMs), and ubiquitination. These modifications on VDACs will be discussed in the following sections.

REGULATION THROUGH PHOSPHORYLATION

Voltage-dependent anion channels undergo phosphorylation for modulation of their channel properties. To understand the biological significance of the phosphorylation event(s) in VDAC, it is necessary to identify the phosphorylation sites in VDACs, the protein mediating the phosphorylation, the physiological stimulus mediating the phosphorylation and the resultant change in VDAC (in particular) and physiology in general. *In vitro* and *in silico* analyses revealed many phosphorylation sites in isoforms of VDAC, which can play an important role in studying the function of VDAC in the near future. If we look into the studies performed till date to understand the phosphorylation events of VDAC, we can divide them into three main classes: (a) studies that proved VDAC can be phosphorylated which alters the channel properties, (b) studies where the whole proteome was examined to understand the PTM and (c) where the specific study on VDAC phosphorylation site was performed, followed by identification and validation.

The first group majorly consists of reports from the Ghosh laboratory (Bera et al., 1995; Bera and Ghosh, 2001; Banerjee and Ghosh, 2006; Gupta and Ghosh, 2017). They have used VDAC (not isoform-specific) from either rat liver (Bera et al., 1995; Bera and Ghosh, 2001) or rat brain (Banerjee and Ghosh, 2006; Gupta and Ghosh, 2017). This was followed by assessing the phosphorylation against protein kinase A (PKA)

(Bera et al., 1995; Bera and Ghosh, 2001), the effect of Bax and tBid binding on VDAC (Banerjee and Ghosh, 2006) and PKA mediated phosphorylation and c-Jun N-terminal kinase-3 (JNK3) (Gupta and Ghosh, 2017). They have identified PKA and JNK3 mediated phosphorylation of VDAC through *in vitro* kinase assays and followed it up with investigation of the changes in channel dynamics of VDAC due to phosphorylation in real time, using reconstitution in lipid membrane bilayer (Bera et al., 1995; Bera and Ghosh, 2001; Banerjee and Ghosh, 2006; Gupta and Ghosh, 2017). Their protocol, though, has given important insights into the phosphorylation of VDAC and the resultant change in the gating properties but lacks the information on the actual VDAC isoform being phosphorylated and also the phosphorylation site(s). Another similar study was reported by Baines and colleagues, where they analyzed the *in vitro* phosphorylation of mouse VDAC1 by protein kinase C (PKC). These studies gave some critical shreds of evidence on the PKC and VDAC interaction and probable regulation of mitochondrial permeability transition pore by PKC, but in terms of phosphorylation, the information did not yield more knowledge beyond just the event.

The second group of studies provided an improvement from the first group by indicating the exact site of phosphorylation in VDAC (and also the particular isoform) under a certain stimulus (or a disease). Tyr phosphorylation of pig VDAC1 and VDAC2 were reported using immunoblotting followed by mass spectrometry (MS) under hypoxia, with no specific site identified (Liberatori et al., 2004). Using anti-Phospho-Tyr antibodies, Schwertz et al. (2007) showed that in rabbits, VDAC1 underwent Tyr phosphorylation during myocardial ischemia by p38 MAPK. Ballif et al. (2008) used MS on peptides extracted from mouse brain and identified Tyr phosphorylation on VDAC1 (Tyr80 and Tyr208), VDAC2 (Tyr207), and VDAC3 (Tyr49). Another study performed on HeLa cells stimulated by epidermal growth factor (EGF) identified phosphor-sites [(Ser101, Ser102, and Ser 104) and Thr 107] in VDAC1 and (Ser115 and Thr118) VDAC2 by MS (Olsen et al., 2006). All three isoforms of rat VDACs were phosphorylated at one or more sites, presumably without any treatment. The study identified Ser12 and Ser136 in VDAC1, Tyr237 in VDAC2, and Thr33 and Ser241 in VDAC3. These results were confirmed by MS (Distler et al., 2006, 2007). Two different groups reported Ser117 as a possible phosphorylation site in mouse VDAC1 using different tissues, again presumably without treatment (Lee et al., 2007; Munton et al., 2007). Tewari S. G. et al. (2015) have used a phospho-mimetic VDACSer137Glu in their studies and have shown that it affects the channel properties of rat VDAC1 (the probable isoform). A quick check of the rat VDAC1 sequence in the UniProt database (Q9Z2L0) indicates that the Ser is at position 137 (at 136 position there is a Pro). Therefore, we believe that it is the same Ser reported by Distler and colleagues (and also claimed by Tewari and colleagues). These studies were advancements on the first group as they yielded exact information on the VDAC isoforms and phosphorylation sites, and the last report indicates that there is merit in examining them more carefully.

Addressing the third and final group, that looks into the VDAC phosphorylation sites followed by validation of experimental results. There are three main candidates here: (1) the phosphorylation of VDAC by glycogen synthase kinase-3 (GSK-3), (2) the phosphorylation of VDAC during endostatin treatment, and (3) phosphorylation of VDAC by NIMA-related protein kinase 1 (Nek1). VDAC can be phosphorylated by Akt and GSK-3 (proved from rat heart, mouse liver and humans VDAC) (Pastorino et al., 2005; Das et al., 2008; Sheldon et al., 2011; Martel et al., 2014). Pastorino et al. (2005) used VDAC precipitated from HeLa cells (different stimulus applied to perturb GSK-3 expression) using a mouse anti-VDAC antibody and then assessed the phosphorylation status using an anti-Phosphothreonine antibody. They further created a phosphor-mutant VDAC3Thr51Ala (from the consensus sequence for GSK-3 phosphorylation). An *in vitro kinase* assay showed that this particular variant was not phosphorylated by GSK-3 (Pastorino et al., 2005). By analyzing the mouse VDAC sequences provided in UniProt, we posit that the VDAC used in this study was either hVDAC1 (P21796) or hVDAC3 (Q9Y277) as they both have Thr at position 51. The phosphorylation of VDAC by GSK-3 at Thr residues were also validated in mice and human models by Martel et al. (2013) using both antibody (anti-Phosphothreonine) based approaches and *in vitro* kinase assay. This particular study used MS only to identify the VDAC isoform and not the phosphorylation site(s). But there are more Thr residues available (in humans, mice, and rats) that could be phosphorylated and need further validation. Nevertheless, Thr51 in human VDAC1 (or VDAC3) is a target of GSK-3 for modulating the channel properties. Yuan et al. (2008) showed that in endostatin-treated human cell line, VDAC1 could be immunoprecipitated by using a rabbit VDAC1 antibody, and its phosphorylation was detected on western blotting by probing with anti-Phosphoserine antibody. They created phosphor-mutant Ser12Ala and Ser103Ala, which showed reduced human VDAC1 accumulation after endostatin treatment, indicating that these sites are the probable phosphorylation sites. Hexokinase II, endostatin, PKC, and GSK-3 modulates VDAC function, however, it was hypothesized that either PKC or GSK-3 are the potential kinases regulating the phosphorylation of human VDAC1 in this case. Although there is no concrete proof of the identity of the kinases, this does question the earlier results, which had shown GSK-3 phosphorylating primarily Thr residues. Chen et al. (2009, 2010) reported that Nek1 interacts and phosphorylates human VDAC1 on Ser193 (by creating a phosphor mutant VDAC1Ser193Ala) residue using *in vitro* kinase assays. The Nek1 is regulated by TKL1 (Tousled-like kinase), which is also involved in chromatin assembly and DNA repair mechanism (Sunavala-Dossabhoy and De Benedetti, 2009). On investigating the entire TLK1-Nek1-VDAC1 module, VDAC1 was found to be a key link between mitochondria-mediated apoptosis and irreparable DNA damages (Singh et al., 2020).

We provide an interesting anecdote where it was shown that a kinase partner could modulate VDAC without phosphorylation. A Raf family Ser/Thr kinase, C-Raf, interacts and is targeted to OMM by Bcl2 protein in a kinase-substrate independent manner and is shown to suppress apoptosis in hemopoietic cell lines (Wang et al., 1994, 1996). However, a Bcl-2 independent mechanism controlled by C-Raf is also reported where C-Raf

targeted to OMM forms a complex with human VDAC1 observed in co-immunoprecipitation studies. No phosphorylation was reported for this interaction (in both *in vivo* and *in vitro* experiments) (Le Mellay et al., 2002).

The phosphorylation sites detailed in the above paragraphs (and summarized in **Table 1**) put forward a fundamental question regarding the identity of the "phosphorylation switches" that can be utilized for future studies to modulate the VDAC for *in vivo* research. Although VDACs have similar sequences, there are abundant proofs that phosphorylation sites can differ based on species and kinase involved. We believe that the best approach to deal with this problem is -after the identification of a kinase partner of VDAC, an *in vitro* kinase assay is to be followed to first answer the question of phosphorylation-based regulation. This should be then followed by identification of sites by MS following either perturbing the organism with the stimulus that is being investigated or alternatively, by MS after an *in vitro* kinase assay. The results obtained from the MS analysis should be used to create either phospho-mimic or phosphor-mutant, and then one can proceed to analyze the channel conductance of these variants. These approaches should give us better results for the identification of actual site(s). Distressfully, the data on plant VDAC phosphorylation is still lacking.

REGULATION THROUGH ALTERNATIVE ROUTES OF MODIFICATION

Other than phosphorylation, regulation of VDACs necessitates comprehension of its alternative modes of PTM. Its regulation has also been observed via nitrosylation/nitrosation, acetylation, oxidation, and interaction with ubiquitin proteins. *In vivo* PTMs by nitrosylation of VDACs have not been directly reported, however, VDAC activity is known to be affected by Nitrous Oxide (NO) (Cheng et al., 2011). Moreover, *in vitro* nitrosation using PAPA NON-Oate (PPN) at 25 μM showed that the conductance is reduced and the dwell time of rat VDAC is increased in the closed state. Also, at PPN100, the conductance was reported to be half of that is present in wild type (wt) (Tewari S. G. et al., 2015). The modification of VDACs induces significant changes in gating kinetics, which might affect mitochondrial (dys)function. The second type of PTM observed in VDACs is O-GlcNAcylation. O-GlcNAcylation affects protein-protein interactions, activity, stability, and expression (Dias and Hart, 2007). VDACs show an increase in O-GlcNAcylation in rats that exhibit low running capacity. This modification might increase mitochondrial stability (Darley-Usmar et al., 2012; Johnsen et al., 2013). Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3) is an interferon-stimulated through JAK/STAT signaling pathway during pancreatic ductal adenocarcinoma (PDAC) (Platanias, 2005; Neuzillet et al., 2015). It directly interacts and regulates VDAC2 through O-GlcNAcylation, which protects PDAC cells from chemotherapy-induced apoptosis (Wang Z. et al., 2020). The modification of VDACs by GlcNAcylation serves to increase mitochondrial stability. Protein modification through acetylation is also crucial in cellular function, though its biological significance is reported for limited substrates (Polevoda et al., 1999; Perrier et al., 2005; Caesar et al., 2006). It either occurs co-translationally at N-terminal residues or post-translationally on lysine (Lys) residue. Acetylation is reported at alanine, Ala 283 residue located at the N-terminal of VDAC1 from mature rat mitochondria, although its significance is not reported yet (Distler et al., 2007). However, they are suggested to function in protein-protein interaction, accumulation of mature protein in target organelles, and general protection of protein from degradation (Hershko et al., 1984; Manning and Manning, 2001; Pesaresi et al., 2003). Ox-PTMs, another type of PTMs reported in VDACs, is an emerging field, and it has broadened our understanding of redox regulation (Stipanuk et al., 2009; Ryan et al., 2014; Shakir et al., 2017). Cysteine (Cys) PTMs in VDAC isoforms have been recently determined using MS (Reina et al., 2020). There are two, nine, and six Cys residues in VDAC1, VDAC2, and VDAC3, respectively. Most of them are localized to facilitate their exposure to IMS (inter-membrane space). Detailed profiling of redox state of Cys and Met of rat VDAC3 has been reported. The evolutionary conservation of Cys modification in the three isoforms is observed in the rat and human VDACs (Saletti et al., 2017, 2018; Pittalà et al., 2020). A conserved oxidative status of Cys residues in rat and human VDAC1 is detected as trioxidized form (Cys127) and the reduced and carboxyamidomethylated form (Cys232) (Pittalà et al., 2020). However, in hVDAC1, there is no change in activity through the Ox-PTMs of the sulfhydryl groups. Cross-linking experiments deny the involvement of intermolecular S-S bridge leading to oligomerization (Aram et al., 2010; Teijido et al., 2012). In the case of VDAC2, the N-terminal Cys 8, 13, and 227 residues located on loop exposed to cytosol were reduced; Cys 47, 76, 103, and 210 residues, localized toward IMS were partially oxidized; and the Cys127 residue localized toward lipid environment was fully tri-oxidized (Pittalà et al., 2020). hVDAC3 contains six Cys2, 8, 36, 65, 122, and 229 residues. MS analyses showed that the Cys 2, 8, 122, and 229 residues were reduced, N-terminal Cys residue 2 was acetylated, residue 36 and 65 were reduced, and tri-oxidized to sulfonic acid (Saletti et al., 2018; Pittalà et al., 2020). Functional significance of the modifications in VDAC3 is reported for some of the residues. Electrophysiology and the ability to revert the growth phenotype of the yeast mutant, Δ*por1*, is strongly affected upon the deletion of Cys residues from engineered human VDAC3 (Okazaki et al., 2015; Reina et al., 2016). Cys 2, 8, and 122 residues seem to be more important for protein function; mutating them or a simultaneous mutation of any of these three Cys to Ala leads to restoration of large pores and the Δ*por1* phenotype (Reina et al., 2016; Queralt-Martín et al., 2020). Cys modifications vary with isoforms as well as their position on VDAC channel. They could function in ROS signaling in mitochondria and this link needs to be explored further. We do not know the significance of the Cys residues in plants. The Arabidopsis VDACs have lesser (or none) Cys residues, and this may indicate an evolutionary change in the way plant VDACs function (Sanyal et al., 2020). Overexpression of *VDAC3* is related to microglial I/R injury (Yao et al., 2018). VDACs exhibit temperature-sensitivity on activation and may contribute to hypothermic neuroprotection against oxygen-glucose deprivation/recovery (OGD/R) (Imada et al., 2010). It

TABLE 1 | The table shows the VDAC isoforms phosphorylated by candidate protein kinases detected by mass spectrometry (MS), site directed mutagenesis (SDM) and predicted sites with their functional consequences.

Isoform	Regulation	Phosphorylation site	Mode of site detection	Organism/organ interaction found in	Protein kinase	Functional relevance
VDAC1	Phosphorylation	S^{13}, S^{137}, S^{234}	Predicted (Wang C. et al., 2020)	Rat heart	PKCε	Cardio-protection (Baines et al., 2003)
VDAC1	Interaction	S^{12}, S^{103}	Experiment	Human microvascular endothelial cells	ES	Endostatin-induced endothelial cell apoptosis (Yuan et al., 2008)
VDAC1	Interaction	Not detected	None	Rat liver	C-Raf	Apoptotic suppression (Le Mellay et al., 2002)
VDAC1	Phosphorylation	S^{45}	Predicted (Wang C. et al., 2020)	Rat liver	PKA	Cytochrome mediated cell death (Banerjee and Ghosh, 2006)
VDAC1	Phosphorylation	S^{193}	Experiment	Human cell lines	Nek1	Mitochondrial Dysfunction and apoptosis (Chen et al., 2009)
VDAC1	Phosphorylation	S^{101*}, S^{102*}, S^{104*}, T^{107*}, S^{117}, T^{208}	Experiment	Rat liver, Human cell lines*	None	Not defined
VDAC1	Phosphorylation	S^{136}	Experiment	Rat liver	PKC	Not defined
VDAC1	Phosphorylation	S^{45}, S^{186}	Predicted (Wang C. et al., 2020)	Rabbit	p38 MAPK	Ischemia/reperfusion injury (Schwertz et al., 2007)
VDAC2	Phosphorylation	T^{51}	Experiment	Rat heart	GSK3	Ischemia/reperfusion injury (Pastorino et al., 2005; Das et al., 2008)
VDAC2	Phosphorylation	S^{115}, T^{118}	Experiment	Human cell lines	None	Not defined
VDAC2	Phosphorylation	T^{237}	Experiment	Rat liver	INSR (Distler et al., 2007)	Not defined
VDAC2	Phosphorylation	T^{207}	Experiment	Rat brain	None	Not defined
VDAC3	Phosphorylation	S^{241}	Experiment	Rat liver	None	Not defined
VDAC3	Phosphorylation	T^{33}	Experiment	Rat liver	None	Not defined
VDAC3	Phosphorylation	S^{137}	Experiment	Rat liver	None	Gating kinetics (Tewari S. G. et al., 2015)
VDAC3	Phosphorylation	T^{49}	Experiment	Rat brain	None	Not defined
VDAC	Phosphorylation	T^{6}, S^{104}, S^{137}	Predicted (Wang C. et al., 2020)	Rat brain	JNK3	Gating process
VDAC1	Phosphorylation	S^{44}, T^{45}, S^{103}, S^{233}, S^{264}	Predicted (Elkeles et al., 1995)	Wheat (*Triticum aestivum*)	PKC	Not defined
VDAC2	Phosphorylation	S^{41}, T^{42}, T^{223}, S^{262}, S^{270}	Predicted (Elkeles et al., 1995)	Wheat (*Triticum aestivum*)	PKC	Not defined
VDAC3	Phosphorylation	T^{44}, S^{102}, S^{225}, S^{264}	Predicted (Elkeles et al., 1995)	Wheat (*Triticum aestivum*)	PKC	Not defined
VDAC1	Phosphorylation	T^{81}, T^{156}, S^{206}	Predicted (Elkeles et al., 1995)	Wheat (*Triticum aestivum*)	Creatine kinase	Not defined
VDAC2	Phosphorylation	T^{78}, T^{164}, S^{204}, T^{214}, T^{252}	Predicted (Elkeles et al., 1995)	Wheat (*Triticum aestivum*)	Creatine kinase	Not defined
VDAC3	Phosphorylation	S^{71}, T^{80}, T^{164}	Predicted (Elkeles et al., 1995)	Wheat (*Triticum aestivum*)	Creatine kinase	Not defined
VDAC1, VDAC2, VDAC3	Phosphorylation	T^{51}, S^{109}, S^{269}	Predicted (Al Bitar et al., 2003)	Rice (*Oryza sativa*)	PKC	Not defined
VDAC1, VDAC2, VDAC3	Phosphorylation	T^{87}, T^{170}, S^{209}	Predicted (Al Bitar et al., 2003)	Rice (*Oryza sativa*)	Casein Kinase II	Not defined

*PKC, protein kinase C; ES, endostatin; C-Raf, Raf family Ser/Thr kinase; PKA, protein kinase A; Nek1, NIMA-related protein kinase 1; p38 MAPK, p38 MAP kinase; GSK3, glycogen synthase kinase; INSR, insulin receptor; JNK3, c-Jun N-terminal kinase-3. * Represents Human cell lines*

is facilitated by interaction between VDAC3 and ubiquitin. The extent of VDAC3 ubiquitination was found directly proportional to hypothermia duration, which may act as an endogenous protective pathway in hypothermia (Zhao et al., 2020). After many decades of research on VDACs, the molecular understanding of their PTM is still limited. The reports on the PTMs and regulation of VDACs are mostly limited to the identification of site and modes of regulation. Though several phosphorylation sites are present on VDACs, only a few are functionally characterized. More information on acetylation, GlcNAcylation, Ox-PTMs and ubiquitination is needed, particularly their functional characterization. Although phosphorylation has been studied more than other modifications, the mere identification of phosphorylation sites is not enough to relate to the functional attributes. Their biological significance needs to be examined critically to enable their implementation. Candidate interactors that regulate VDAC activity have immense therapeutic potential. In comparison, PTMs in the plant VDAC are still an emerging concept and need to be investigated substantially by the plant science community.

VDACs CONNECT Ca^{2+} AND ROS SIGNALING

Ca^{2+} is an important second messenger and plays a crucial role in signaling in all eukaryotes (Poovaiah et al., 1987; Clapham, 1995; Rudd and Franklin-Tong, 1999, 2001; Sanders et al., 1999; Trewavas, 1999; Harper, 2001; Pandey, 2008; Kudla et al., 2018; Tang et al., 2020). In a non-excitable cell, Ca^{2+} flux occurs between cytosol, plasma membrane (PM), and endoplasmic reticulum (ER). However, organelles such as mitochondria can also regulate Ca^{2+} signals (Patel and Muallem, 2011; Stael et al., 2012; Wagner et al., 2016; Costa et al., 2018). They are known to be critical regulators of cellular Ca^{2+} homeostasis. They can accumulate ions, including Ca^{2+} (up to several hundred times of initial Ca^{2+} concentration), from the suspended medium during electron transport (Rossi and Lehninger, 1963). The Ca^{2+} sequestration is an energy-dependent process (Gunter et al., 1994). Mitochondrial Ca^{2+} uptake and Ca^{2+} signals play a key role in cellular processes ranging from energy metabolism to cell death. Plant mitochondrial functions range beyond the passive storage/buffering of cytosolic Ca^{2+}; they generate unique Ca^{2+} signatures in response to the external stimuli. Cytosolic and mitochondrial Ca^{2+} signatures are generated on the perception of stimuli, particularly under stress. Also, similar to cytosolic Ca^{2+} signatures, mitochondrial Ca^{2+} signatures are differentially sensitive to stimuli. Exposure to cold, touch, and osmotic stress results in very similar temporal kinetics of cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_c$ and mitochondrial free Ca^{2+} concentration, $[Ca^{2+}]_m$. This reflects its role in buffering the $[Ca^{2+}]_c$, with the stimuli such as oxidative stress and touch showing an independent Ca^{2+} regulation of mitochondria in addition to cytosolic signatures (Logan and Knight, 2003). Ca^{2+} concentration in the cytosol and mitochondria is regulated by utilizing Ca^{2+} transporters across the IMM. However, to facilitate Ca^{2+} flux across the organelle, it needs to bypass the OMM. This regulation requires certain Ca^{2+} transport/regulating system on OMM. The regulating mechanism/molecular components responsible for this flux of Ca^{2+} from mitochondria may help understand the process and utilize it further. VDAC located on OMM shows permeability to Ca^{2+}, thus, acting as an important players in Ca^{2+} flux. VDAC is a candidate regulator that manages Ca^{2+} flow in and out of mitochondria (Wagner et al., 2016). It will be interesting to identify other regulatory components in such regulation and examine if it merges with cytosolic Ca^{2+} signaling for better stress adaptation in cells. However, the relationship between VDAC and Ca^{2+} is entrenched beyond their role in the Ca^{2+} movement. The role of VDACs regulating Ca^{2+} signaling in plants is beginning to emerge. Under unfavorable conditions, mitochondria connect and form a network with the rest of the cell to maintain cellular homeostasis (Zemirli et al., 2018). Ca^{2+} signaling is one of the modes of regulation. The physical interaction between AtVDAC1 and CBL1 is one such example, as described earlier. The expression profile of both *AtVDAC1* and *CBL1* indicates the role of this module in cold stress during seed germination (Li et al., 2013). Salt overly sensitive (SOS) pathway mediates Ca^{2+} based cellular signaling under salt stress (Ji et al., 2013). VDAC protein levels were elevated in response to short-term salinity exposure in maize roots (Zörb et al., 2010). Similarly, VDAC2 in plants may also be connected with Ca^{2+} signaling. AtVDAC2 might participate in stress response pathways, as the expression level of *SOS* genes changes with the expression level of *VDAC2* in qRT-PCR studies (Wen et al., 2011, 2014; Liu et al., 2015). Though a direct involvement of VDACs is not reported in plants, however evidence does show their involvement in Ca^{2+} signaling. This indirect Ca^{2+} involvement may be an additional mode of stringent regulation in cellular signaling. Since research based on plant VDAC-Ca^{2+} regulation is bound to flourish in future, it is important to optimize protocols for quantification of Ca^{2+} levels. The technical difficulties in measuring mitochondrial Ca^{2+} levels have been overcome through the development of several *in vivo* Ca^{2+} monitoring methods. The developments in the measurement of mitochondrial Ca^{2+} levels have been covered in several studies (Pozzan and Rudolf, 2009; Jean-Quartier et al., 2012; McKenzie et al., 2017; Fernandez-Sanz et al., 2019). Therefore, the *in vivo* dynamics of these Ca^{2+} signaling components and VDACs can shed more light on the real-time cellular Ca^{2+} status in unfavorable conditions.

VDAC mediated cyto-mitochondrial signaling as well as VDAC-mediated inter-organellar communication through Ca^{2+} in cellular metabolism and survival are vital to our understanding. Mitochondria can act as high capacity Ca^{2+} buffers that determine cytosolic $[Ca^{2+}]$ transients through regulation of the kinetic characteristics of Ca^{2+} channels or prevention of Ca^{2+} diffusion from the location of open channels. They may be critically involved in constraining Ca^{2+} signals in spatial terms to specific cellular domains (Rizzuto et al., 2012). The presence of Ca^{2+} hot spots on the mitochondrial surface in the regions closely apposed to Ca^{2+} channels localized on the PM or ER, contribute to the fast and high levels of Ca^{2+} uptake by mitochondria as observed in live cells (De Stefani et al., 2016). The proximity between the mitochondrial and

ER membranes favors Ca^{2+} exchanges between them. Their entwined endomembrane network may regulate intracellular Ca^{2+} signaling via VDACs. The transmission of Ca^{2+} signals between the two was imaged through Ca^{2+} sensitive GFPs and aequorin probes. The Ca^{2+} release from ER was coordinated with Ca^{2+} uptake from mitochondria via VDAC (Rapizzi et al., 2002). The coupling of the two organelles can be chaperone-mediated, which directly enhances the accumulation of Ca^{2+} in mitochondria. Glucose regulated protein 75, a cytosolic chaperone homologous to HSP70, physically connects VDAC1 (on OMM) to Inositol 1,4,5-triphosphate receptor (IP_3R: on ER) and is a determining factor for the interaction as its absence abolished the stimulatory effect. This direct enhancement of Ca^{2+} accumulation in mitochondria forms a molecular bridge (Szabadkai et al., 2006). Co-immunoprecipitation assays further indicate the complex formation of IP_3 receptor and VDAC1, regulated by apoptotic stimuli. VDAC1 silencing impaired the transfer of apoptotic Ca^{2+} signals selectively (De Stefani et al., 2012). Together, these reports demonstrate the regulation of inter-organellar communication through Ca^{2+} signaling, and VDACs constitute a significant part of this machinery. Ca^{2+} mediated inter-organellar connections in plants are beginning to emerge. This has been discussed in several reviews (Stael et al., 2012; Kmiecik et al., 2016; Himschoot et al., 2017; Costa et al., 2018; Liu and Li, 2019; Navazio et al., 2020).

Antioxidant systems in mitochondria regulate the maintenance of ROS at physiological levels through mitochondrial metabolism. ROS imbalances in mitochondria, either through overproduction or impaired antioxidant system, lead to mitochondrial dysfunction and induction of the apoptotic cascade. Such ROS imbalances in pathological conditions exhibit a Ca^{2+} overload in mitochondria (Feno et al., 2019). Since mitochondrial ROS can act as a signaling molecule and not just be a by-product of oxidation reaction during ATP production, it is interesting to look at its signaling aspect (Collins et al., 2012; Sena and Chandel, 2012; Sullivan and Chandel, 2014). Mitochondrial Ca^{2+} uptake impinges on enzymes of the TCA cycle and the activity of the electron transport chain (ETC), generating ROS signals under physiological conditions depending on tissue specificity. This signaling aspect depends on $[Ca^{2+}]$ threshold, which, when overcome, results in detrimental mROS levels, compromising mitochondrial bioenergetics and cellular functioning (Brookes et al., 2004; Görlach et al., 2015; Feno et al., 2019). The reciprocal interactions between the major signaling components, Ca^{2+} and ROS, regulating each other, form a "feed-forward, self-amplified loop." The resultant

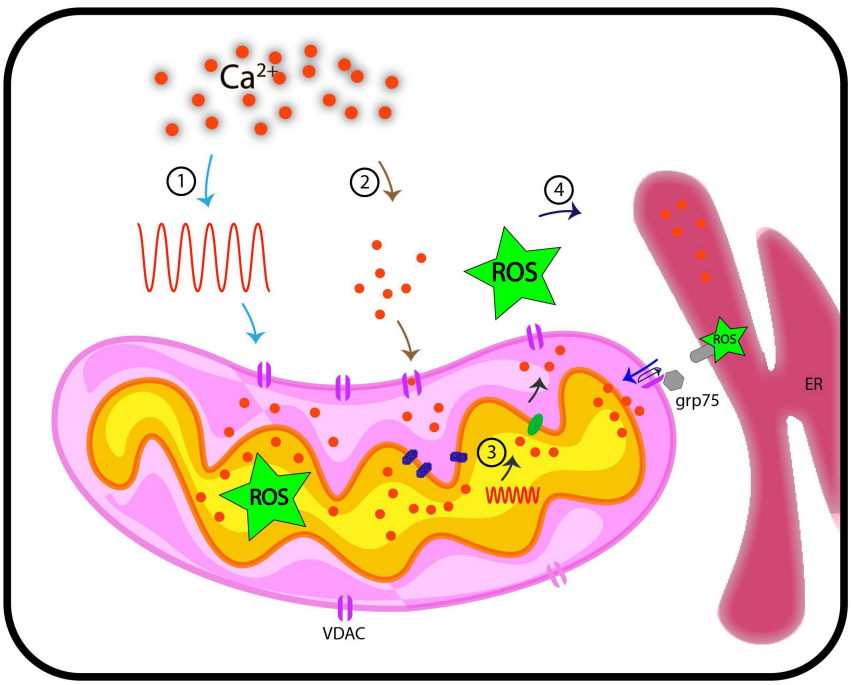

FIGURE 2 | Representation of functional implications of VDACs, Ca^{2+}, and ROS in cellular signaling. This hypothetical model traces pathways through which VDAC may regulate Ca^{2+} and ROS signaling in plants. We discuss here four possible routes that we currently posit are VDAC mediated connection between Ca^{2+} and ROS signaling. (1) When cytosolic Ca^{2+} level increases on perception of stimuli (generation of Ca^{2+} signature), these signals are transduced downstream, where they influence the activity of VDACs. This might results in increased level of ROS in mitochondria. (2) VDAC serves passively to regulate Ca^{2+} levels in mitochondria. (3) Stimuli such as oxidative stress (inclusive of increased ROS level) and touch response results in generation of Ca^{2+} signature in mitochondria, independent of cytosolic Ca^{2+} signature. (4) VDAC can be a candidate protein that leads to flux of Ca^{2+}/ROS from inter-membrane space to cytosol. Further, VDACs are known to connect ER based Ca^{2+} signaling and mitochondria through Ca^{2+} and ROS molecules and this may depict another possible mechanism of how VDAC connects Ca^{2+} and ROS. These hypothetical routes may (or may not) be connected. ER, endoplasmic reticulum; ROS, reactive oxygen species; grp75, glucose related protein 75; Ca^{2+}, calcium; VDAC, voltage-dependent anion channel.

oxidative damage can be more than that is directly caused by Ca^{2+} overload (Jou, 2008; Smaili et al., 2009; Peng and Jou, 2010). VDAC may serve as a junction for the inter-connection of the two signaling molecules, ROS and Ca^{2+}. The molecular link between Ca^{2+} and ROS through VDAC is evident, as already discussed. VDAC1 is linked to redox-sensitive ER Ca^{2+}-release channel, IP_3R by GRP-75 chaperone (Szabadkai et al., 2006), indicating that ROS and ER based Ca^{2+} signaling pathways are inter-linked. This inter-connection through VDAC is reported in yeast as well. ATP synthase along with the porin complex regulates Ca^{2+} homeostasis and permeability transition of mitochondria *in vivo* (Niedzwiecka et al., 2018). In lung cancer cells, a member of the Bcl-2 family, Mcl binds with VDAC1 and VDAC2. Disruption in this interaction limits Ca^{2+} uptake, which further inhibits ROS generation and shows VDAC-based Ca^{2+} dependent ROS production (Huang et al., 2014). The generation of oxidative damage in cardiac microvascular endothelial cell injury (CMEC injury) is based on IP_3R-VDAC-Ca^{2+}. Simulation of MAPK/ERK by melatonin inactivates cAMP response element-binding protein (CREB), thus, blocking oxidative stress damage responsible for cardiac dysfunction (Zhu et al., 2018). The closure of VDAC by phosphorothioate increased superoxide in mitochondria because its flux from the inner mitochondrial space to the cytosol is affected. This results in Ca^{2+}-induced mitochondrial permeability transition, causing the induced opening of permeability transition pores of high conductance in IMM due to excessive Ca^{2+} uptake (Tikunov et al., 2010). The notable roles of animal VDACs make the effort to pursue plant VDACs for their involvement in stress responses worthy. The research so far is pioneering in showing VDAC as a linking bridge between ROS and Ca^{2+} but we still lag in understanding the initiation of this loop. It is still emerging if mitochondrial Ca^{2+} signal generates a ROS signaling event or flux of ROS via mitochondrial stimulation of Ca^{2+} signaling in cytosol/mitochondria. It is still unclear whether the process is only facilitated by VDACs majorly and independently or does it need a complex to regulate the signaling. We expect studies in the future to depict the cytosolic and mitochondrial Ca^{2+} dynamics in the absence of VDAC in plants that can clarify the involvement of VDAC in the process. The simultaneous imaging of ROS and Ca^{2+} in reference to VDACs and their patterns in real-time also demand attention. Nevertheless, at present, it is an open question that needs to be investigated in plants. We also expect research in the future that can solve the mode of regulation in VDACs in these signaling events. It is a challenging opportunity to elucidate the entire signaling mechanism, including Ca^{2+}-ROS homeostasis, in the context of cell biology. The interconnection between pathways and the role of VDACs as the central node as shown in **Figure 2** is highly important and needs to be pursued further.

CONCLUSION AND FUTURE PERSPECTIVE

Significant knowledge on VDAC has accumulated in recent years. The functional range of VDACs as gating proteins has now expanded. We know the localization of VDACs, their modulation, biochemical regulation and functional implications in animals and, to some extent, in plants. Multi-localization of VDAC on different membranes, yet the difference in their functions, is an example of how nature increases diversity with existing tools in the cell. The multi-localization of VDAC can be predicted as a connecting route for better intra-cellular communication. However, its localization on PM is highly debatable. Different modes of regulation of VDACs make them versatile and hence, explains their ability to regulate and participate in multiple signaling events. The research on biochemical regulation is still limited and extensive intervention is the need of the hour. These channels are well known for molecular transport, and this function also puts them under the spotlight as communicators between cytoplasmic and mitochondrial signaling events. A mutual interplay between Ca^{2+} and ROS is also mediated by VDACs, and hence, they can be a Ca^{2+}-ROS connecting link. However, many interesting questions remain to be addressed. At present, the ongoing research on plant VDACs faces many challenges, with burning questions and controversies. Its structure, isoforms in plants, role in apoptosis, relation with PCD and the VDAC interactome are gray areas. The active components extracted from plants can modulate animal VDACs. Their functional significance on plant VDACs will be a great help to further our understanding. Fortunately, the once lagging plant VDACs have emerged as a new arena of research. Based on the studies so far, VDACs have immense potential in therapeutics and the improvement of crops. The ongoing research will further unmask its functional significance, particularly, in plants.

AUTHOR CONTRIBUTIONS

GP conceptualized the review. BR and GP wrote the manuscript. BR, PK, SS, MB, and GP reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

We are thankful to Delhi University (IoE/FRP grant), Department of Biotechnology (DBT), Science and Engineering Research Board (SERB), Council of Scientific and Industrial Research (CSIR), India for research funding in our lab. BR acknowledges CSIR for CSIR-SRF.

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frontiers in Physiology

MINI REVIEW
published: 12 August 2021
doi: 10.3389/fphys.2021.732742

The Biogenesis Process of VDAC – From Early Cytosolic Events to Its Final Membrane Integration

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Voltage dependent anion-selective channel (VDAC) is the most abundant protein in the mitochondrial outer membrane. It is a membrane embedded β-barrel protein composed of 19 mostly anti-parallel β-strands that form a hydrophilic pore. Similar to the vast majority of mitochondrial proteins, VDAC is encoded by nuclear DNA, and synthesized on cytosolic ribosomes. The protein is then targeted to the mitochondria while being maintained in an import competent conformation by specific cytosolic factors. Recent studies, using yeast cells as a model system, have unearthed the long searched for mitochondrial targeting signal for VDAC and the role of cytosolic chaperones and mitochondrial import machineries in its proper biogenesis. In this review, we summarize our current knowledge regarding the early cytosolic stages of the biogenesis of VDAC molecules, the specific targeting of VDAC to the mitochondrial surface, and the subsequent integration of VDAC into the mitochondrial outer membrane by the TOM and TOB/SAM complexes.

Keywords: beta-barrels, chaperones, mitochondria, outer membrane, TOM complex, VDAC

OPEN ACCESS

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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *29 June 2021*
Accepted: *26 July 2021*
Published: *12 August 2021*

Citation:
Moitra A and Rapaport D (2021) The Biogenesis Process of VDAC – From Early Cytosolic Events to Its Final Membrane Integration. Front. Physiol. 12:732742. doi: 10.3389/fphys.2021.732742

INTRODUCTION

Most of the outer membrane (OM) proteins in Gram-negative bacteria are membrane-embedded β-barrel proteins that are composed of anti-parallel β-strands forming a barrel shaped hydrophilic pore in the membrane. In eukaryotes, the presence of β-barrel proteins is restricted to the OM of mitochondria and chloroplasts that were derived from prokaryotic ancestors. The assembly of these proteins into their corresponding OM is in each case facilitated by a dedicated protein complex that contains a highly conserved central β-barrel protein termed BamA/YaeT/Omp85 in Gram-negative bacteria, Tob55/Sam50 in mitochondria, and probably OEP80 in plastids (Ulrich and Rapaport, 2015; Gross et al., 2021). These central components are related to each other and belong to the Omp85 superfamily (Gentle et al., 2005). Voltage Dependent Anion-selective Channels (VDACs) are abundant mitochondrial β-barrel proteins (Schein et al., 1976; Colombini, 1979). Their pore is composed of 19 anti-parallel β-strands whereas strands 1 and 19 are in parallel orientation to each other. VDAC, which was previously known as mitochondrial porin, functions as a channel for transport of metabolites, nucleotides, ions, and even small peptides (Benz, 1989). VDACs are found in mitochondria across the spectrum of life, from unicellular yeasts to plants and all higher eukaryotes. Bakers' yeast (*Saccharomyces cerevisiae*) has two genes encoding VDACs, *POR1* and *POR2*, while higher eukaryotes like humans have at least three isoforms, *VDAC1*, *VDAC2* and *VDAC3* and plants have up to five such genes (Young et al., 2007; Raghavan et al., 2012).

During the evolution of mitochondria from an ancient endosymbiont, most of the organellar genes, including those encoding predecessors of VDACs, were transferred to the nucleus, with the mitochondrial genome retaining the codes for only few key components of the respiratory chain complexes (Gray et al., 1999). VDACs are thus transcribed in the nucleus and translated on cytosolic ribosomes. Then, they need to be targeted to the correct sub-cellular organelle, namely the mitochondria, and ultimately integrated into the mitochondrial OM (MOM) with the help of dedicated import machineries. In this review, we will highlight recent studies that have discovered cytosolic factors associated with newly synthesized VDAC molecules, the elusive mitochondrial targeting information for VDAC, and finally the mechanisms of insertion and integration of VDAC into MOM.

EARLY CYTOSOLIC EVENTS OF NEWLY SYNTHESIZED VDAC MOLECULES

The first challenge of the biogenesis of VDAC is to keep the newly synthesized molecules in an import competent conformation (Freitag et al., 1982; Rapaport and Neupert, 1999). The rather hydrophobic β-strands that build the transmembrane segments are prone to aggregation in the cytosol. Thus, the newly synthesized VDAC precursors must be bound by cytosolic chaperones to shield these hydrophobic patches, preventing the emerging nascent chain from engaging in unfavorable intra- and inter-molecular interactions (Kim et al., 2013). This association with chaperones maintains them in an import-competent conformation. Recent studies, using yeast as a model system, demonstrate that newly synthesized VDAC molecules dynamically interact with Hsp70 chaperones (Ssa1/2) and their Hsp40 co-chaperones Ydj1 and Sis1 (**Figure 1**; Jores et al., 2018). Inhibiting the activity of the cytosolic Hsp70 chaperone, preventing its docking to the mitochondrial receptor Tom70, or co-depleting both co-chaperones Ydj1 and Sis1 resulted in a significant reduction in *in vivo* and *in vitro* import of VDAC into yeast mitochondria. Experiments utilizing Hsp70 inhibitors and pull-down assays demonstrated that the interactions between VDAC and Hsp70 chaperones and their physiological role are also conserved in mammalian cells. Moreover, a β-hairpin motif of VDAC, hypothesized to be the mitochondrial targeting signal (see below), was sufficient for the interaction with these (co-) chaperones. It should be emphasized that these (co-) chaperones support the import of not only β-barrel proteins but are also involved in the biogenesis of many additional proteins. Hence, so far, a targeting factor, which is dedicated solely to β-barrel proteins was not identified. The abovementioned chaperones, based on the mitochondrial targeting information, relay the nascent precursors to the receptors of the translocase of the outer membrane (TOM) of mitochondria. Other β-barrel proteins like Tom40 and Tob55/Sam50 appear to follow the same route as VDAC (Jores et al., 2018).

Currently, it is not clear whether the aforementioned cytosolic factors support biogenesis solely by preventing premature unfavorable aggregation or whether they also facilitate specific targeting. The contribution of the chaperone anchor Tom70, located at the mitochondrial surface, to the overall import process suggests that association with chaperones also increases the specificity of organellar targeting.

TARGETING OF VDAC TO THE MITOCHONDRIAL SURFACE

Most mitochondrial precursor proteins contain a cleavable N-terminal presequence that targets them to mitochondria. However, like the other mitochondrial β-barrel proteins, VDAC lacks a cleavable targeting signal. Hence, it remained unclear how the targeting information for VDAC was encoded. Various studies showed that bacterial and chloroplast β-barrel proteins could be targeted and assembled into yeast mitochondria (Walther et al., 2009; Ulrich et al., 2012, 2014). Conversely, VDAC could also be integrated into bacterial outer membranes and form pores there (Walther et al., 2010), suggesting that the targeting information for β-barrel proteins is conserved from bacteria to mitochondria and thus functional in both systems. Since none of the studies could identify a definitive linear amino acid sequence as the targeting signal, it was hypothesized that the targeting signal may be a structural feature of the β-barrel proteins.

Truncation studies showed that the last C-terminal β-strand of mitochondrial β-barrel proteins contains a stretch of amino acids that facilitate their interaction with the TOB/SAM complex. These residues were called the β-signal (Kutik et al., 2008). However, deletion or mutation of the β-signal did not interfere with the initial targeting of newly synthesized β-barrel proteins to mitochondria. Studies involving a bacterial trimeric autotransporter Yersinia adhesion A (YadA), where each subunit contributes four β-strands to a 12-mer β-barrel structure, demonstrated that such proteins can be targeted to mitochondria upon their expression in yeast cells (Müller et al., 2011). This finding implies that even a partial β-barrel structure (like four β-strands) is sufficient for specific mitochondrial targeting. Hence, it was further tested whether a β-hairpin structural motif, which is composed of two β-strands and a loop and represents the most basic repeating structural motif of β-barrel proteins, could be the elusive mitochondrial targeting signal. To support this possibility, it was shown that a peptide corresponding to the last β-hairpin of human VDAC1 could competitively inhibit the *in vitro* import of mitochondrial β-barrels (Jores et al., 2016). Moreover, hybrid proteins of this β-hairpin fused to soluble passenger domains like GFP or DHFR were targeted to mitochondria upon their expression in yeast cells. Such β-hairpin motif has an amphipathic characteristic as eventually, upon its incorporation into a membrane-embedded β-barrel, one phase of the motif will face the lipid core and hence is hydrophobic, whereas the opposite one will be exposed to the pore lumen and thus is rather hydrophilic. Importantly, it was discovered that optimal mitochondrial targeting depends on relative elevated hydrophobicity of those amino acid residues that face the lipid core of the membrane (Jores et al., 2016).

In most eukaryotic cells, mitochondria are the only organelles containing β-barrel proteins. The problem of specific targeting gets an interesting twist in plant cells where

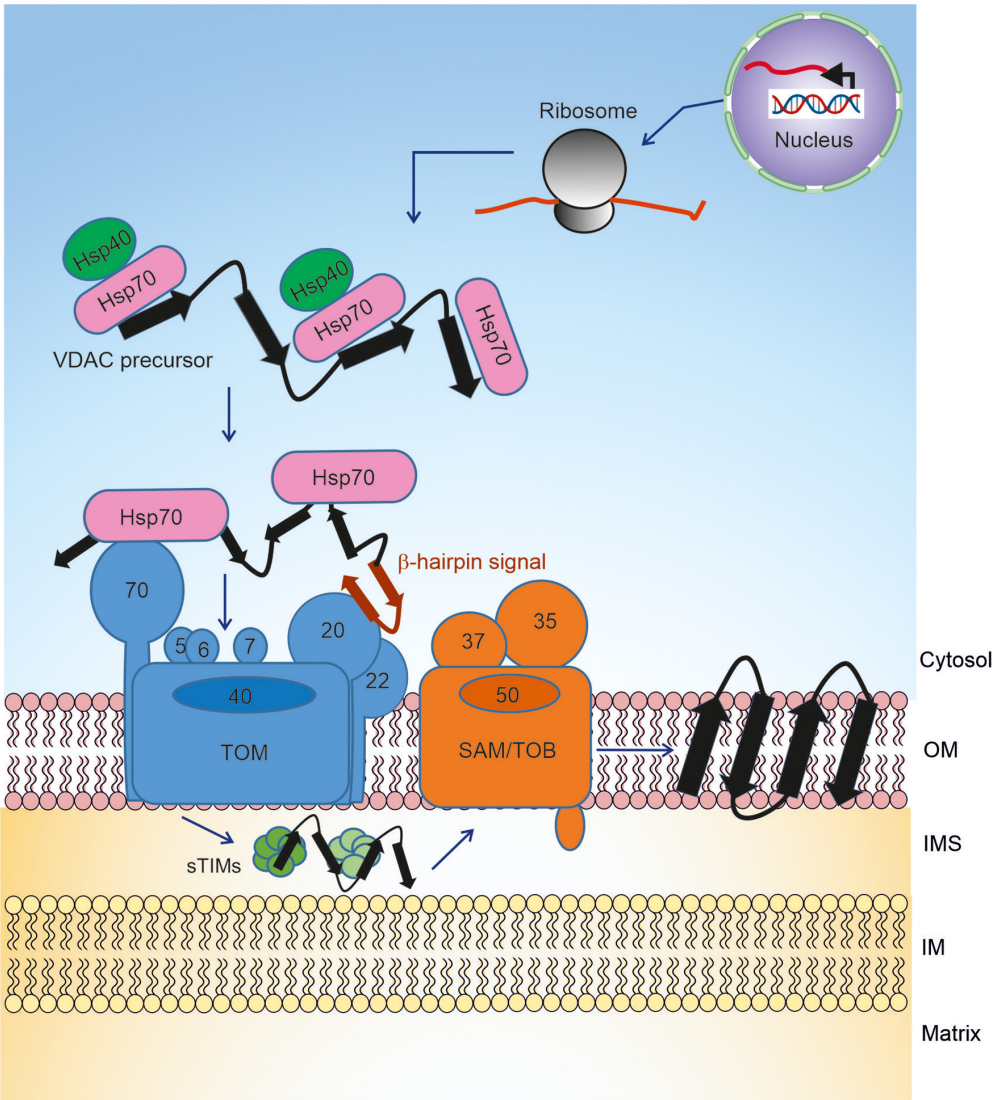

FIGURE 1 | Biogenesis pathway of VDAC. Precursors of VDAC are transcribed in the nucleus, translated on cytosolic ribosomes, and then transported to the mitochondrial surface with the help of chaperones. At the outer membrane, the precursors are initially recognized by receptors of the TOM complex and then translocated across the membrane via the pore formed by Tom40. In the IMS, the small TIM chaperones relay the newly synthesized VDAC molecule to the TOB/SAM complex, which facilitates the final steps of membrane integration.

plastids can be an alternative destination for such proteins. Klinger et al. (2019) addressed this issue and found that the hydrophobicity is not sufficient for the discrimination of targeting to chloroplasts or mitochondria. By domain swapping between mitochondrial (atVDAC1) and chloroplast (psOEP24) targeted β-barrel proteins, they could demonstrate that the presence of a hydrophilic amino acid at the C-terminus of the penultimate β-strand is also required for mitochondrial targeting. A variant of the chloroplast β-barrel protein psOEP24, which mimics such profile, was efficiently targeted to mitochondria (Klinger et al., 2019).

Collectively, it seems that the combined contribution of several β-hairpin motifs with a highly hydrophobic face assures proper mitochondrial targeting of VDAC.

MEMBRANE INTEGRATION OF VDAC BY THE TOM AND TOB/SAM COMPLEXES

Once the chaperone-associated VDAC precursors are targeted to mitochondria via the β-hairpin signal, they interact with the TOM complex at the mitochondrial surface to initiate organellar import (**Figure 1**). The TOM complex is comprised of the core complex and its peripheral import receptors. The core complex has a central translocon channel, formed by the integral β-barrel protein Tom40, along with several transmembrane accessory proteins namely Tom5, Tom6, Tom7, and Tom22 (Bausewein et al., 2017; Araiso et al., 2019; Tucker and Park, 2019). Tom20 and Tom70 are the receptors involved in the initial

recognition of multiple mitochondrial proteins (Neupert and Herrmann, 2007; **Figure 1**). Several studies hinted at the role of Tom20 in the recognition of β-barrel precursors (Rapaport and Neupert, 1999; Schleiff et al., 1999; Krimmer et al., 2001; Yamano et al., 2008). Using NMR, photo-crosslinking and fluorescence complementation assays, it was recently shown that the β-hairpin element of VDAC interacts with the mitochondrial import receptor Tom20 via the presequence binding region of the latter (Jores et al., 2016). Moreover, direct cross-linking of the β-hairpin motif to Tom70 and the observation that blocking this receptor interferes with the import of VDAC suggested that Tom70 also plays a role in the initial recognition of VDAC (Jores et al., 2016). The involvement of Tom70 can be either via direct recognition of the substrate protein or by serving as a docking site for the chaperone-substrate complex.

Following recognition by the import receptors, the VDAC precursors are translocated across the MOM via the Tom40 channel by interacting with a series of binding sites, probably with increasing affinities (Hill et al., 1998). Upon its emergence at the intermembrane space (IMS), the translocated VDAC molecule interacts with the small chaperones of the translocase of the inner membrane (small TIMs). The IMS chaperone system includes the small Tim proteins, Tim8, Tim9, Tim10, and Tim13 (Koehler et al., 1998). These small chaperones form alternating circular

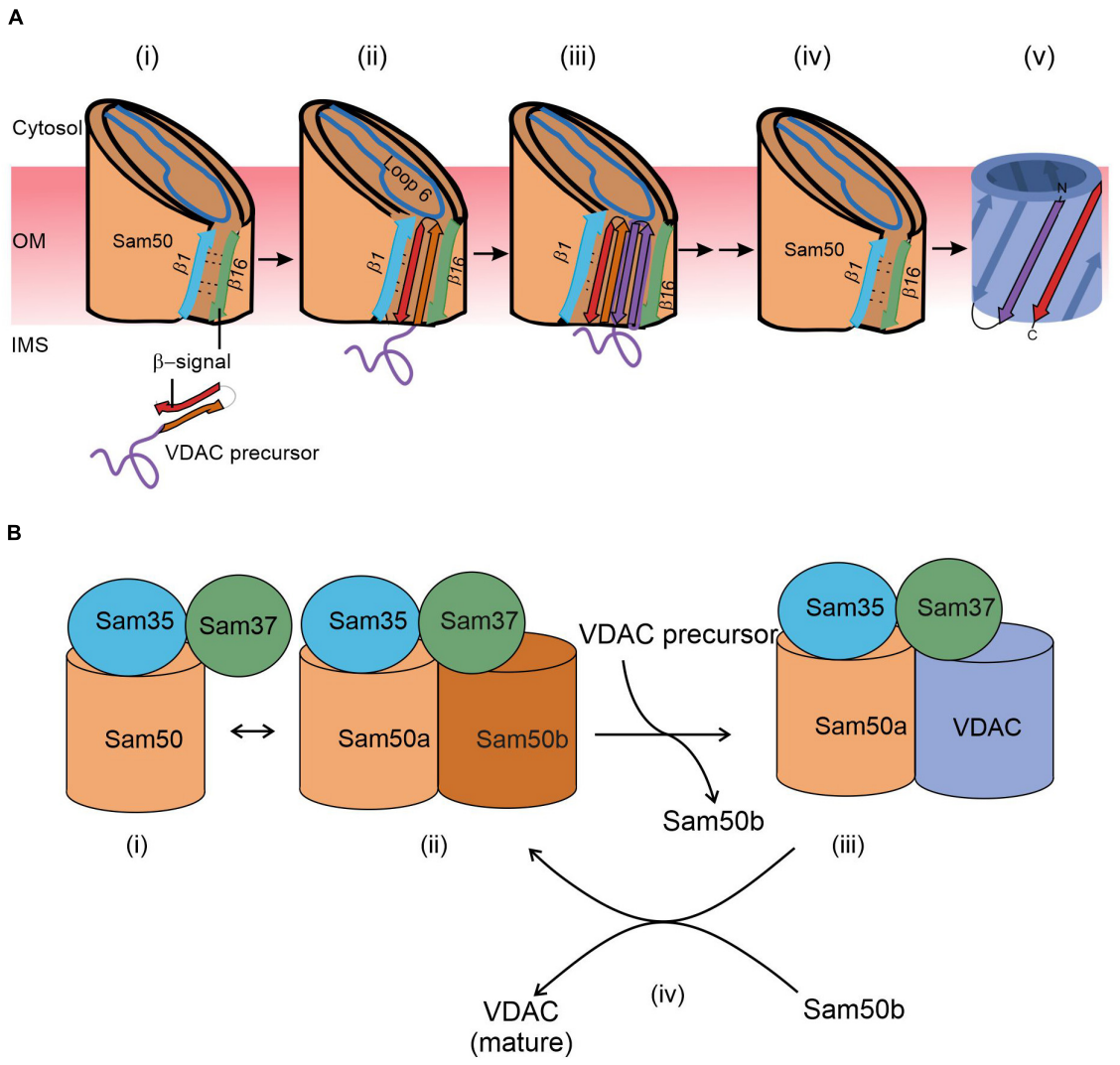

FIGURE 2 | A working model for the final steps of the membrane integration of VDAC. **(A)** Lateral insertion (adapted from Figure 8; Höhr et al., 2018). (i) VDAC precursors approach the outer membrane from the IMS. (ii) The C-terminal β-signal of VDAC precursor interferes with the Sam50 structure by binding to the β1 strand of Sam50 and disrupting the β1–β16 interactions within Sam50. This enforces opening of a lateral gate. (iii) The initial opening is followed by sequential insertion of additional precursor β-hairpins through the lateral gate of Sam50. (iv) The lateral gate of Sam50 re-closes to (v) release the fully formed β-barrel of VDAC into the MOM. **(B)** Barrel switching (based on Takeda et al., 2021). In its substrate-free state, the SAM complex is in equilibrium between the "monomeric" state consisting of Sam50a, Sam35, and Sam37 (i) and a "dimeric" species that contains a second (Sam50b) barrel (ii). The gradually formed VDAC barrel displaces Sam50b (iii). Finally, the fully folded VDAC molecule dissociates from the complex to be replaced by Sam50b (iv).

hexamers comprised of three subunits of Tim9 and Tim10, or three subunits of each Tim8 and Tim13 (Webb et al., 2006; Beverly et al., 2008). Site-specific cross-linking indicated that the small TIMs interact with the IMS-exposed part of the N-terminal extension of Tom40 (Shiota et al., 2015).

The small TIMs play an important role in the transfer of the β-barrel precursors of VDAC from the TOM complex to the sorting and assembly machinery (SAM) complex (Hoppins and Nargang, 2004; Wiedemann et al., 2004; **Figure 1**). The formation of a β-hairpin within the last two C-terminal β-strands of VDAC is crucial for the interaction of the precursors with the TIM chaperones. Structural and mechanistic studies revealed that TIM chaperones hold the VDAC protein precursors in a nascent chain-like extended conformation via multiple clamp-like binding sites (Weinhäupl et al., 2018). Such multiple weak and constantly reshuffling interactions ultimately allow for the efficient release of the precursor to the actual insertase, the SAM complex, which is also known as the topogenesis of outer-membrane β-barrel proteins (TOB) complex (**Figure 1**; Paschen et al., 2003; Wiedemann et al., 2003; Gentle et al., 2004). The Tim9/10 binding cleft for the β-barrel precursors has conserved hydrophobic residues for these interactions, and mutations in these residues are detrimental to the VDAC biogenesis and overall cell growth.

To facilitate a smooth transfer, the TOM and the TOB/SAM complex can form a super-complex bridged by the cytosolic domain of Tom22 and the peripheral TOB/SAM component, Mas37/Sam37 (Qiu et al., 2013). The core subunit of the TOB/SAM complex is the 16-stranded β-barrel protein Tob55/Sam50, that belongs to the Omp85 superfamily of proteins. Tob55/Sam50 has an N-terminal POTRA domain, which can bind the incoming substrate but is not essential for the β-barrel assembly process. In addition, the TOB/SAM complex harbors two cytosol-exposed peripheral subunits that are involved in formation of a TOM-TOB super-complex (Mas37/Sam37) and stabilization of the TOB/SAM bound form of the precursor (Tob38/Sam35).

Our understanding of the final steps in the biogenesis of the VDAC β-barrel precursors evolved dramatically in the last 5 years. Structural studies indicate the formation of a lateral gate between β-strands 1 and 16 of Sam50. Accordingly, and supported by intensive cross-linking assays, the lateral gate insertion model was put forward. This model suggests that the C-terminal β-signal of the precursor initiates opening of the gate by exchange with the endogenous Sam50 β-signal. In addition, loop 6 of Sam50 was found to be crucial for the VDAC precursor transfer to the lateral gate (Höhr et al., 2018). An increasing number of β hairpin–like loops of the precursor insert and fold sequentially and accumulate at the lateral gate (**Figure 2A**). Finally, hydrogen bonds are formed between the first and last β-strand to close the newly folded VDAC β-barrel. Upon folding at Sam50, the full-length newly formed β-barrel protein is laterally released into the outer membrane and the Sam50 lateral gate closes (**Figure 2A**). The opening of the putative lateral gate obtained further support from a recent report on the atomic structure of the SAM complex (Diederichs et al., 2020). Membrane thinning in the vicinity of the lateral gate can further facilitate insertion of the β-barrel protein into the lipid bilayer.

The membrane integration model recently obtained a new twist from structural studies. Based on detailed atomic structure of the SAM complex, the barrel swapping model envisions the SAM complex as formed by a SAM monomer (Sam50a along with Sam35 and Sam37) and a Sam50b second barrel (**Figure 2B**; Takeda et al., 2021). The precursor protein β-signal binds Sam50a as in the lateral gate insertion model. Then, the folded VDAC β-barrel slowly displaces Sam50b and takes its place. Sam37 that originally also interacts with Sam50b, gets gradually involved in interactions with the newly formed VDAC barrel (**Figure 2B**). Finally, this barrel dissociates from the SAM complex and is integrated into the MOM.

Of note, most of our current knowledge regarding the biogenesis of β-barrel proteins is based on biochemical and structural studies on fungal elements. While the atomic structure of the mammalian TOM complex appears to be rather similar to its fungal counterpart (Wang et al., 2020), not much is known about the SAM complex in higher eukaryotes. It is rather clear that the mammalian Sam50 is the central component of the complex. However, the precise functions of Metaxins1/2/3, which are homologous to yeast Sam35 and Sam37, is not clear yet.

PERSPECTIVES

Our understanding of the factors and machineries involved in the assembly of VDAC proteins into the MOM has made tremendous progress in the last 20 years. We now have detailed atomic structures of the membrane-embedded TOM and SAM complexes, and the hexamer of the small TIM chaperones that transfer the substrate from the former to the latter. Challenges for the future include the characterization of the mammalian SAM complex and to decipher how the various biogenesis steps of VDAC are regulated and adapted to the cellular physiological conditions. Moreover, it will be interesting to determine if after its insertion into the OM, oligomerization, additional folding, or post-translational modifications are necessary for VDAC to become fully functional.

AUTHOR CONTRIBUTIONS

AM and DR wrote the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (RA1028/8-2 to DR) and the IMPRS "From Molecules to Organisms" (AM).

ACKNOWLEDGMENTS

We thank Dr. Kai S. Dimmer for his comments on this manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 18 August 2021
doi: 10.3389/fphys.2021.724828

Glutamate 73 Promotes Anti-arrhythmic Effects of Voltage-Dependent Anion Channel Through Regulation of Mitochondrial Ca^{2+} Uptake

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Edited by:
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: 14 June 2021
Accepted: 22 July 2021
Published: 18 August 2021

Citation:
Shimizu H, Huber S, Langenbacher AD, Crisman L, Huang J, Wang K, Wilting F, Gudermann T, Schredelseker J and Chen J-N (2021) Glutamate 73 Promotes Anti-arrhythmic Effects of Voltage-Dependent Anion Channel Through Regulation of Mitochondrial Ca^{2+} Uptake. Front. Physiol. 12:724828. doi: 10.3389/fphys.2021.724828

Mitochondria critically regulate a range of cellular processes including bioenergetics, cellular metabolism, apoptosis, and cellular Ca^{2+} signaling. The voltage-dependent anion channel (VDAC) functions as a passageway for the exchange of ions, including Ca^{2+}, across the outer mitochondrial membrane. In cardiomyocytes, genetic or pharmacological activation of isoform 2 of VDAC (VDAC2) effectively potentiates mitochondrial Ca^{2+} uptake and suppresses Ca^{2+} overload-induced arrhythmogenic events. However, molecular mechanisms by which VDAC2 controls mitochondrial Ca^{2+} transport and thereby influences cardiac rhythmicity remain elusive. Vertebrates express three highly homologous VDAC isoforms. Here, we used the zebrafish *tremblor/ncx1h* mutant to dissect the isoform-specific roles of VDAC proteins in Ca^{2+} handling. We found that overexpression of VDAC1 or VDAC2, but not VDAC3, suppresses the fibrillation-like phenotype in zebrafish *tremblor/ncx1h* mutants. A chimeric approach showed that moieties in the N-terminal half of VDAC are responsible for their divergent functions in cardiac biology. Phylogenetic analysis further revealed that a glutamate at position 73, which was previously described to be an important regulator of VDAC function, is sevolutionarily conserved in VDAC1 and VDAC2, whereas a glutamine occupies position 73 (Q73) of VDAC3. To investigate whether E73/Q73 determines VDAC isoform-specific anti-arrhythmic effect, we mutated E73 to Q in VDAC2 ($VDAC2^{E73Q}$) and Q73 to E in VDAC3 ($VDAC3^{Q73E}$). Interestingly, $VDAC2^{E73Q}$ failed to restore rhythmic cardiac contractions in *ncx1* deficient hearts, while the Q73E conversion induced a gain of function in VDAC3. In HL-1 cardiomyocytes, VDAC2 knockdown diminished the transfer of Ca^{2+} from the SR into mitochondria and overexpression of VDAC2 or $VDAC3^{Q73E}$ restored SR-mitochondrial Ca^{2+} transfer in VDAC2 deficient HL-1 cells, whereas this rescue effect was absent for VDAC3 and drastically compromised for $VDAC2^{E73Q}$. Collectively, our findings demonstrate a critical

role for the evolutionary conserved E73 in determining the anti-arrhythmic effect of VDAC isoforms through modulating Ca^{2+} cross-talk between the SR and mitochondria in cardiomyocytes.

Keywords: mitochondria, voltage-dependent anion channel, calcium, cardiac rhythmicity, zebrafish

INTRODUCTION

Mitochondria govern a variety of cellular processes including bioenergetics, metabolism, reactive oxygen species production, cell survival, and Ca^{2+} homeostasis. In particular, a tight cross-talk between mitochondria and the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) was associated with important physiological and pathophysiological processes (Rizzuto et al., 1998; Gomez et al., 2016; Paillusson et al., 2016; Dia et al., 2020). In the heart, the close proximity of the SR and mitochondria facilitates Ca^{2+} cross-talk between these cellular organelles and provides a mechanism for the regulation of cardiac Ca^{2+} handling and adaptation to higher workload. This SR-mitochondria cross-talk depends on an array of inner and outer mitochondrial membrane channels, including the highly abundant voltage-dependent anion channels (VDACs) in the outer mitochondrial membrane, which critically regulate transport of ions, substrates, and metabolites across the outer mitochondrial membrane. VDAC dysfunction has previously been linked to pathophysiological outcomes (Feng et al., 2019; Santin et al., 2020), but the roles of VDAC proteins in cardiac function and disease are still poorly understood.

The vertebrate VDAC family consists of three paralogs, VDAC1, VDAC2, and VDAC3. While these proteins are highly similar in their sequences, structures, and biophysical properties, different biological functions have been noted among these isoforms (Naghdi and Hajnóczky, 2016; Caterino et al., 2017). In particular, there is a distinct requirement for VDAC2 during mammalian embryogenesis: Mice without functional VDAC1 or VDAC3 can survive to adulthood (Anflous et al., 2001; Sampson et al., 2001), but VDAC2 deficiency leads to embryonic lethality (Cheng et al., 2003). Furthermore, conditional ablation of VDAC2 specifically in cardiomyocytes causes cardiomyopathy suggesting an essential role for VDAC2 in maintaining the physiological function of the heart (Raghavan et al., 2012).

In cardiomyocytes, VDAC2 serves as a gatekeeper for transferring Ca^{2+} into mitochondria under both normal physiological and stressed conditions (Rosencrans et al., 2021; Sander et al., 2021). For example, knockdown of VDAC2 significantly extended Ca^{2+} sparks, supporting a physiological role for VDAC2 in Ca^{2+} cycling (Subedi et al., 2011; Min et al., 2012). Our laboratories previously showed that enhancing mitochondrial Ca^{2+} uptake with efsevin, a gating modifier of VDAC2 (Wilting et al., 2020), can suppress arrhythmogenesis in cardiomyocytes (Shimizu et al., 2015; Schweitzer et al., 2017). Efsevin efficiently restored rhythmic cardiac contractions in zebrafish *tremblor/ncx1h* mutant embryos, which are characterized by cardiomyocyte Ca^{2+} overload and chaotic cardiac contractions (Ebert et al., 2005; Langenbacher et al., 2005; Shimizu et al., 2015) and suppressed erratic, diastolic Ca^{2+} events in both a murine model for human catecholaminergic polymorphic ventricular tachycardia (CPVT) and in iPSC-derived cardiomyocytes from a CPVT patient (Schweitzer et al., 2017).

Overexpression of VDAC2 allows *tremblor/ncx1h* mutant zebrafish hearts to establish coordinated cardiac contractions and to preserve the integrity of myofibrils (Shimizu et al., 2015, 2017), indicating that *tremblor/ncx1h* can serve as an animal model for the study of cardiac Ca^{2+} regulation by VDAC. In this study, we investigate the potential of the three VDAC isoforms to regulate Ca^{2+} signaling in the heart, with the aim of identifying molecular moieties which confer isoform specificity. We show that overexpression of VDAC1, but not VDAC3, restored regular contractions in *tremblor/ncx1h* mutants comparable to VDAC2. We further show that VDAC1 and VDAC2 possess a glutamate at position 73 (E73), whereas VDAC3 contains a glutamine (Q73), a residue that was previously reported to be involved in VDAC regulation. Strikingly, substitution of VDAC3 Q73 for a glutamate confers a gain of function ability to restore rhythmic cardiac contractions in *tremblor/ncx1h* mutants while the converse exchange in VDAC2 abolishes its phenotype rescue ability. Finally, using cellular assays, we show that the glutamate at position 73 of VDAC proteins is essential for mediating mitochondrial Ca^{2+} uptake.

MATERIALS AND METHODS

Phylogenetic Analysis

Protein sequences used for phylogenetic analyses were downloaded from the NCBI database.[1] A full list of the NCBI accession IDs for each sequence used is found in **Supplementary File 1**. For analysis of vertebrate VDACs, full-length VDAC protein sequences were aligned using the MUSCLE algorithm in the MEGA7 application (Kumar et al., 2016), producing a 284 position alignment. For expanded analysis of vertebrate and non-vertebrate VDACs, full-length protein sequences were aligned using the ClustalW algorithm in MEGA7, producing a 397 position alignment. Phylogenetic analyses were performed with the software RAxML using a maximum likelihood method, the JTT substitution matrix, and empirical frequencies (Stamatakis, 2014). RAxML software was accessed using the CIPRES Science Gateway (Miller et al., 2010) and trees were visualized using the Interactive Tree of Life website (Letunic and Bork, 2007).

[1]https://www.ncbi.nlm.nih.gov/

Pairwise Comparison of VDAC Proteins

Human and zebrafish VDAC protein sequences were downloaded from the NCBI database and their percent identity was determined using a standard protein BLAST (blastp). Pairwise comparisons were visualized in R v3.6.3 using the ggcorrplot package.

Cloning

Plasmids containing the full-length zebrafish VDAC1, 2, and 3 cDNA were purchased from Open Biosystems and cloned into pCS2+ or pCS2+3xFLAG plasmid for mRNA synthesis. Point mutations VDAC2^{E73Q} and VDAC3^{Q73E} were inserted by SOE-PCR.

For HeLa cell transfections, coding regions of VDAC genes were cloned into the pCS2+ vector together with the NLS-EGFP fragments and the viral T2A sequence. The T2A sequence enables bicistronic expression of VDAC proteins and NLS-EGFP for the identification of VDAC-overexpressing cells.

Plasmids for the generation of transgenic lines were generated using the Tol2Kit (Kwan et al., 2007). Plasmids shLenti2.4G-mVDAC2 and shLenti2.4G-Ctrl for VDAC2 knockdown in HL-1 cells were generously provided by Dr. Yeon Soo Kim from Inje University, Gimhae, South Korea. Plasmids pCCLc-CMV, pCMVΔ8.91, and pCAGGS-VSV-G for lentivirus production were obtained as a gift from Dr. Donald Kohn, University of California Los Angeles, United States. For production of lentiviruses, the IRES-nlsEGFP element from p3E-IRES-nlsEGFPpA (Kwan et al., 2007) was fused to VDAC elements by subcloning into pCS2+ constructs, before the entire VDAC-eGFP element was fused into pCCLc-CMV using the In-Fusion HD Cloning Kit (TaKaRa; Wilting et al., 2020).

Zebrafish Husbandry, Generation of Transgenic Fish Lines, and Chemical Induction

Zebrafish were raised and maintained under standard laboratory conditions. The zebrafish *tremblor* (*tre*^{tc318d}) mutant line (ZDB-ALT-980203-1756) was bred and maintained as previously described (Langenbacher et al., 2005). To induce transgene expression in *Tg(myl7:Gal4EcR-EGFP-UAS-vdac1-FLAG)*, *Tg(myl7:Gal4EcR-EGFP-UAS-vdac2-FLAG)*, and *Tg(myl7:Gal4EcR-EGFP-UAS-vdac3-FLAG)*, 1 μM tebufenozide (TBF) was added to the embryo media at 1 day post-fertilization (dpf; Lu et al., 2017). Cardiac phenotypes were assessed at 2 dpf (Shimizu et al., 2015, 2017).

Zebrafish Injections

mRNA was synthesized from pCS2+-constructs using the SP6 mMESSAGE mMACHINE kit (Life Technologies). mRNA was injected into one-cell stage embryos collected from crosses of *tre*^{tc318} heterozygotes. Cardiac performance was analyzed by visual inspection of cardiac contractions on 1 dpf. and genotypes were confirmed at 2–3 dpf.

Zebrafish Expression Profiling

Total RNA of embryos was isolated using Trizol Reagent (Life Technologies) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Sequences for forward and reverse primers used for RT-PCR are provided in **Supplementary File 2**.

In-Situ Hybridization

Whole-mount *in situ* hybridization was performed as previously described (Chen and Fishman, 1996) using VDAC1, 2, and 3 probes spanning the entire coding sequences. For riboprobe synthesis, plasmids were linearized and *in vitro* transcription was performed using the DIG RNA labeling kit (Roche).

HeLa Ca^{2+} Uptake Assay

HeLa cells were transfected with expression plasmids using Lipofectamine2000 (Life Technologies). 24 h after transfection, cells were loaded with 5 μM Rhod2-AM (Life Technologies) in loading buffer (5 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and pH adjusted to 7.4 with NaOH) for 1 h at 15°C followed by an additional 30 min incubation in wash buffer (10 mM HEPES, 140 mM TEA-Cl, 1 mM $MgCl_2$, 2 mM Na-EGTA, 10 mM glucose, and pH adjusted to 7.4 with Trizma base) at 37°C to allow de-esterification of cytosolic AM esters. Prior to imaging, cells were permeabilized with 100 μM digitonin for 1 min at room temperature. After approximately 10 s of baseline recording, cells were exposed to an external Ca^{2+} pulse (final free Ca^{2+} concentration is calculated to be approximately 10 μM using WEBMAXC at https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcS.htm). Confocal images were recorded in internal buffer (5 mM K-EGTA, 20 mM HEPES, 100 mM K-aspartate, 40 mM KCl, 1 mM $MgCl_2$, 2 mM maleic acid, 2 mM glutamic acid, 5 mM pyruvic acid, 0.5 mM KH_2PO_4, 5 mM MgATP, and pH adjusted to 7.2 with Trizma base) every 0.6 s (Nikon Eclipse Ti microscope) at 545 nm excitation using 20x objective to monitor mitochondrial Ca^{2+} dynamics. Confocal images were analyzed and quantified using ImageJ (National Institutes of Health, Bethesda, MD).

Stable HL-1 Cell Lines

HL-1 cells (RRID:CVCL_0303) were a gift from William Claycomb (Louisiana State University) and were cultured as previously described (Claycomb et al., 1998). To knock down expression of the endogenous murine VDAC2 cells were lentivirally transduced with a construct expressing shRNA directed against murine VDAC2 (Min et al., 2012; Wilting et al., 2020) followed by selection using 3 μg/ml puromycin. This cell line was then again lentivirally transduced to overexpress zVDAC2, zVDAC2^{E73Q}, zVDAC3, and zVDAC3^{Q73E} using respective constructs. Stable cell lines were established by selecting for nlsGFP expression by FACS sorting.

SR-Mitochondria Ca^{2+} Transfer

Ca^{2+} transfer from the SR into mitochondria was measured as described previously (Schweitzer et al., 2017). In brief, HL-1 cardiomyocytes plated in a 96-well plate were loaded with 6 μM

Rhod-2 AM (Life technologies) and 0.12% (w/v) Pluronic® F-127 for 30 min at 37°C. After permeabilizing the cells with 100 mM digitonin in internal solution (in mM: 1 BAPTA, 20 HEPES, 100 L-Aspartic acid potassium salt, 40 KCl, 0.5 $MgCl_2$, 2 maleic acid, 2 glutamic acid, 5 pyruvic acid, 0.5 KH_2PO_4, 5 MgATP, and 0.46 $CaCl_2$; pH = 7.2 with KOH), cells were washed with internal solution before measurements. Rhod-2 fluorescence was monitored at excitation wavelength 540 ± 9 nm and emission wavelength 580 ± 20 nm with an Infinite® 200 PRO multimode reader (Tecan, Maennedorf, Switzerland). After recording 30 s of baseline fluorescence, 10 mM caffeine was added to release Ca^{2+} from the SR.

Statistical Analysis

Data are expressed as mean ± s.e.m. Statistical testing was carried out using student *t*-test unless otherwise specified. *Significance levels are expressed as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; and NS not significant.

RESULTS

Identification of Zebrafish VDAC Genes

In vertebrates, the VDAC gene family consists of three paralogs: VDAC1, VDAC2, and VDAC3 (Naghdi and Hajnóczky, 2016). We examined the zebrafish genome and found three genes encoding VDAC proteins with high sequence identity to human VDAC1, VDAC2, and VDAC3 (**Figure 1A**). To investigate whether these three zebrafish proteins represent homologs of the mammalian VDACs and examine the evolutionary relationships among VDAC homologs, we constructed phylogenetic trees of VDAC amino acid sequences using a maximum likelihood approach. Consistent with the findings of previous studies (Young et al., 2007; Wojtkowska et al., 2012), vertebrate VDACs clustered in three distinct clades representing homologs of VDAC1, VDAC2, and VDAC3 (**Figure 1B**). Within each VDAC clade, zebrafish VDACs clustered tightly with the VDACs of other fish species (medaka and pufferfish). Importantly, one zebrafish VDAC protein was a member in each VDAC clade based on strong bootstrap support, indicating that zebrafish VDAC1, VDAC2, and VDAC3 are the bona fide homologs of their mammalian equivalents (**Figure 1B**).

Interestingly, while some plants and fungi possess multiple VDAC paralogs, only a single VDAC protein is present in the majority of the invertebrate animal species surveyed (**Figure 1C**). This suggests that the three VDAC proteins present in zebrafish likely arose from duplication events occurring in a common ancestor of teleost and tetrapod animals (amphibians, reptiles, birds, and mammals). The retention of the three VDAC paralogs throughout vertebrate evolution may indicate that they have acquired differing and crucial functional roles in mitochondrial biology.

All Three VDAC Isoforms Are Expressed in the Hearts of Embryonic and Adult Zebrafish

We have previously shown that injecting wild-type VDAC2 mRNA in *tremblor/ncx1h* mutant embryos at the 1-cell stage allows *tremblor/ncx1h* mutant hearts to establish rhythmic Ca^{2+} transients and consequently preserves the integrity of myofibrils and maintains coordinated cardiac contractions (Shimizu et al., 2015, 2017). To investigate whether all three VDAC genes share this cardioprotective activity, we first examined if zebrafish VDAC1, 2, and 3 are expressed in the developing zebrafish heart. Whole-mount *in situ* hybridization revealed that all three VDACs are expressed in the embryonic heart (**Figure 2A**). Strong signals of VDAC transcripts are also detected in adult zebrafish hearts (both atrium and ventricle; **Figure 2B**), suggesting a role for VDAC proteins in the maintenance of cardiac physiology.

Diverged Cardioprotective Effects Among VDAC Genes

To evaluate the cardioprotective potential of VDAC1 and VDAC3, we injected FLAG-tagged VDAC1, 2, or 3 RNA into *tremblor/ncx1h* at the 1-cell stage (**Figure 3**). As expected, the majority of uninjected *tremblor/ncx1h* mutant hearts fibrillates where each individual cardiomyocytes contracts spontaneously but fails to coordinate with other cardiomyocytes within the same chamber to support a heartbeat (Langenbacher et al., 2005). However, a significantly higher number of FLAG-tagged VDAC2 RNA injected mutant embryos establish persistent and coordinated cardiac contractions as previous described (Shimizu et al., 2015, 2017), confirming a cardioprotective effect of VDAC2 against aberrant Ca^{2+} handling-induced arrhythmia. Interestingly, we observed that overexpression of VDAC1 and VDAC3 resulted in different effects on *tremblor/ncx1h* hearts. Similar to VDAC2, *tremblor/ncx1h* mutant embryos receiving VDAC1 mRNA established persistent cardiac contractions but the hearts of *tremblor/ncx1h* mutant embryos receiving VDAC3 RNA continued to fibrillate despite comparable expression levels as assessed by Western blot analysis against the flag epitope. These data demonstrate divergent cardioprotective effects among VDAC isoforms.

Cardiomyocyte-Specific Expression of VDAC1 and VDAC2 Is Sufficient to Restore Cardiac Contractions in ncx1 Deficient Mutants

RNA injection into newly fertilized eggs results in a global upregulation of VDAC proteins prior to the onset of Ca^{2+} handling defects in *tremblor/ncx1h* mutant embryos. This approach lacks cell type specificity and does not address the question of whether VDAC activation in cardiomyocytes alone is sufficient to suppress aberrant Ca^{2+} handling-induced cardiac dysfunction. To gain temporal- and cell type-specific resolution of the divergent effects of VDAC proteins on *ncx1* deficient hearts, we created transgenic lines *Tg:VDAC1, Tg:VDAC2, and Tg:VDAC3*, where the expression of FLAG-tagged VDAC isoforms as well as the GFP reporter is regulated by the cardiac-specific promoter *myl7* driven Gal4-ecdysone receptor fusion protein (Gal4-EcR; **Figure 4A**). We then crossed these transgenic fish into the *tremblor/ncx1h* mutant background, subjected the embryos to either vehicle (DMSO) or tebufenozide (TBF)

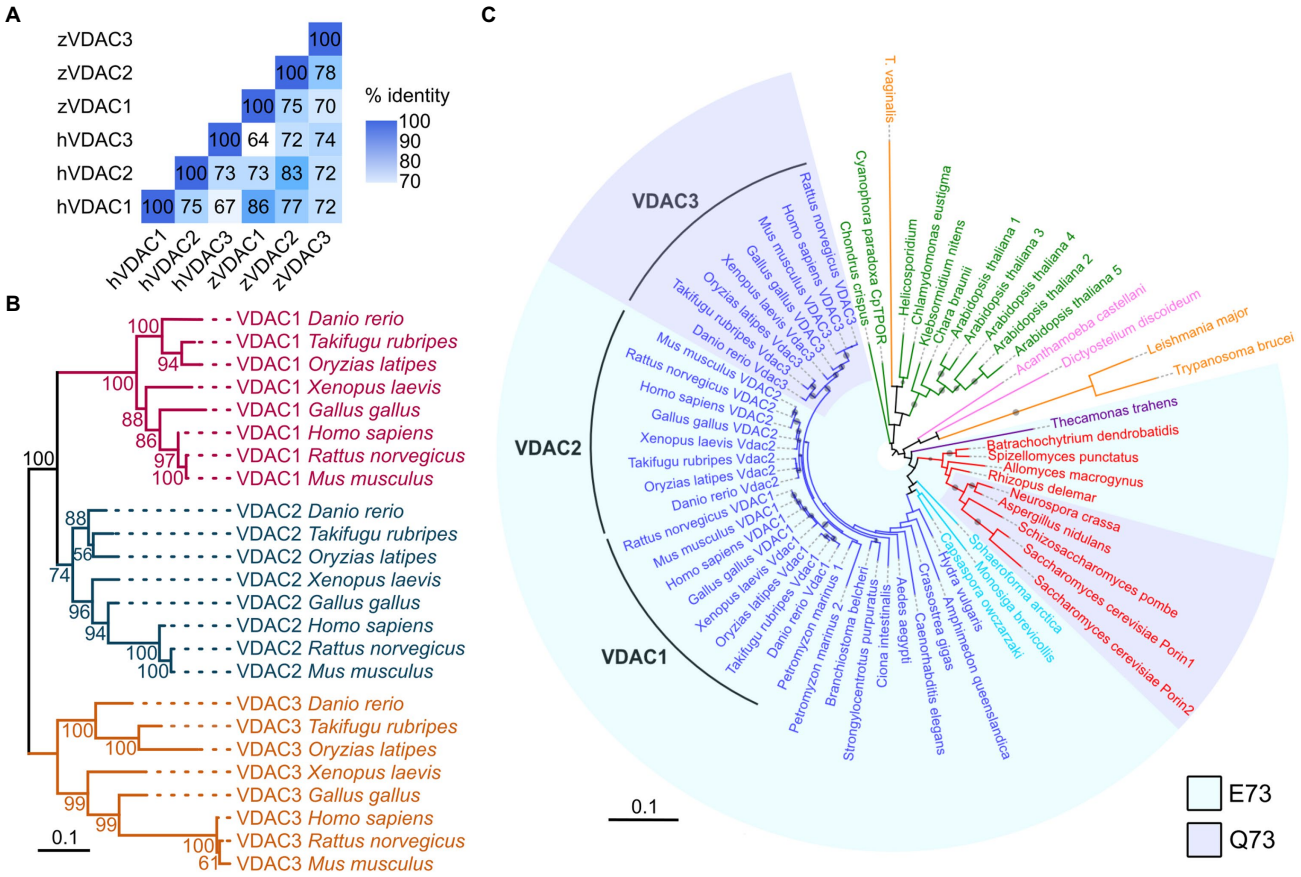

FIGURE 1 | VDACs are highly conserved proteins. **(A)** Pairwise comparison of sequence identity of human (h) and zebrafish (z) VDAC protein sequences. **(B)** Maximum likelihood phylogenetic analysis of 24 vertebrate VDAC proteins. Branches are colored based on VDAC gene families (VDAC1: magenta, VDAC2: blue, and VDAC3: gold). Nodes are labeled with bootstrap values in units of percentage. The scale bar for branch lengths indicates the mean number of inferred substitutions per site. **(C)** Maximum likelihood phylogenetic analysis of 63 VDAC homologs from 41 species. Branches and labels are colored based on phylogenetic groupings of species: blue – animals, light blue – Holozoa (excluding animals), red – fungi, purple – Apusomonadida, orange – Excavata, pink – Amoebozoa, and green – Archaeplastida. Clades are shaded to indicate if proteins contain glutamate (E, light blue) or glutamine (Q, light purple) at amino acid position 73 (with respect to human VDAC1) or are unshaded if this region of the protein lacks high homology with human VDAC1. The size of circles on branches represents bootstrap values and is only displayed for values of 50% and greater. The scale bar for branch lengths indicates the mean number of inferred substitutions per site.

treatment after 1 day of development, at which time the primitive heart has already formed and the *tremblor* mutant hearts are fibrillating (Langenbacher et al., 2005).

In the absence of TBF, Gal4-EcR is inactive and the transgenes are not expressed (**Figures 4B**,C; Lu et al., 2017). *In situ* hybridization analysis showed that TBF treatment effectively induced VDAC expression in the heart (**Figure 4B**). Western blotting further confirmed the induction of VDAC proteins by TBF treatment and detected comparable levels of induced VDAC proteins among three transgenic lines (**Figure 4C**). As expected, the majority of DMSO-treated transgenic *ncx1* deficient hearts exhibited fibrillation-like chaotic cardiac movement; coordinated and persistent cardiac contractions were observed in only approximately 14% of these embryos. Excitingly, about half of the hearts of *Tg:VDAC1;tremblor/ncx1h* and *Tg:VDAC2;* and *tremblor/ncx1h* embryos stopped fibrillating after TBF induction and began to contract. The hearts of *Tg:VDAC3;* *tremblor/ncx1h* embryos, on the other hand, continued to fibrillate after TBF treatment (**Figure 4D**). These findings parallel the divergent cardioprotective effects of VDAC1, 2, and 3 revealed by our global overexpression approach. In addition, because the induction of VDAC proteins in *tremblor/ncx1h* embryos occurs after the manifestation of cardiac fibrillation, our results demonstrate a cardiomyocyte autonomous suppressive effect for VDAC1 and VDAC2 on aberrant Ca^{2+} handling-induced cardiac dysfunction.

The N-Terminal Domain of VDAC Proteins Is Responsible for Their Divergent Cardioprotective Effect

Mechanisms by which VDAC proteins regulate cardiac contraction remain elusive at the molecular level. The differing abilities of VDAC isoforms to restore of cardiac contractions

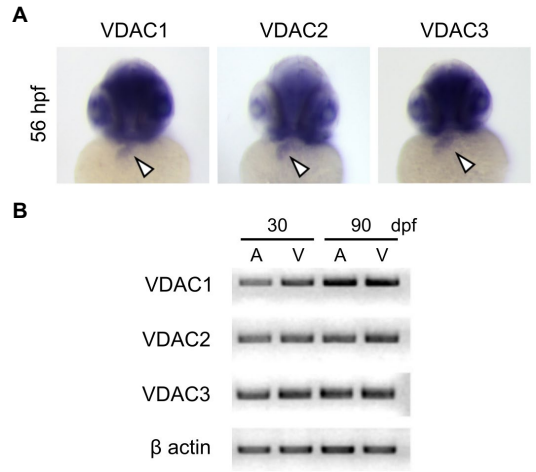

FIGURE 2 | Expression of VDAC isoforms during zebrafish embryonic development. **(A)** Whole-mount *in situ* hybridization analysis using full-length riboprobes reveals that all VDAC isoforms are expressed in the embryonic heart (arrowheads). **(B)** RT-PCR analysis of *VDAC* mRNA from zebrafish hearts at 30 and 90 days post-fertilization (dpf). Expression of all VDAC isoforms is maintained to adulthood in both the atrium **(A)** and the ventricle (V).

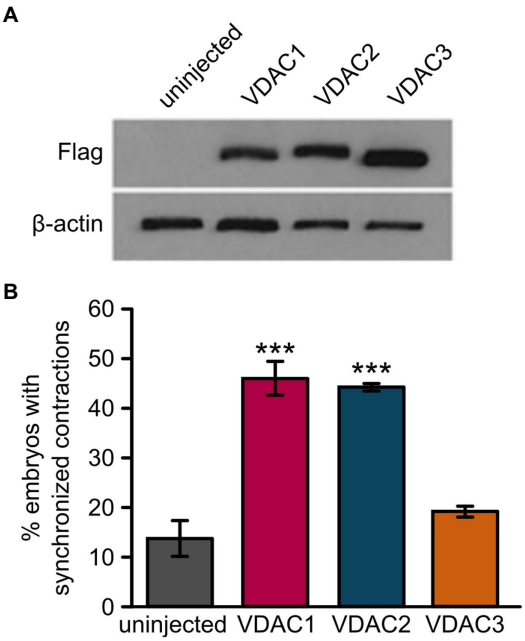

FIGURE 3 | Overexpression of VDAC1 or VDAC2 but not VDAC3 restores rhythmic cardiac contractions in *tremblor/ncx1h* mutants. **(A)** Western blot with anti-FLAG antibody after SDS-PAGE with lysate from 32 hpf uninjected embryos or embryos injected with 25 pg. of C-terminally FLAG-tagged VDAC mRNA demonstrates that all VDAC mRNAs are expressed at comparable levels in embryos. β-actin was used as a loading control. **(B)** While injection of 25 pg. VDAC1 or VDAC2 mRNA significantly restored rhythmic cardiac contractions in 1-day-old *tremblor/ncx1h* mutant embryos (46.5 ± 3.4%, N = 3, *n* = 120, and 44.8 ± 0.4%, N = 3, *n* = 134, respectively, as opposed to 14 ± 3.5%, N = 3, *n* = 175 in uninjected siblings), injection of 25 pg. VDAC3 failed to recapitulate this effect (19.4 ± 1.1%, N = 3, *n* = 114). Overall rescue percentages represent the mean rescue percentage ± s.e.m. from N independent experiments, using a total of n embryos.

in *tremblor/ncx1h* mutant hearts provide a reliable and robust platform to dissect critical domains for VDAC's ability to suppress aberrant Ca^{2+} handling-induced arrhythmogenic effects in cardiomyocytes. We created FLAG-tagged chimeric constructs consisting of the N-terminal half of VDAC2 and the C-terminal half of VDAC3 ($VDAC^{N2C3}$) and vice-versa ($VDAC^{N3C2}$; **Figure 5A**). We then injected synthetic RNAs made from these chimeric constructs into *tremblor/ncx1h* embryos. While Western blots showed that both $VDAC^{N2C3}$ and $VDAC^{N3C2}$ were expressed at comparable levels (**Figure 5B**), only those *tremblor/ncx1*h mutant embryos receiving $VDAC^{N2C3}$ mRNA manifested rhythmic cardiac contractions. The hearts of those mutant embryos receiving $VDAC^{N3C2}$ RNA continued to fibrillate (**Figure 5C**). These findings indicate that the anti-arrhythmic activity of VDAC proteins is determined by amino acids located in their N-terminal half.

E73 Is Evolutionary Conserved in VDAC1 and VDAC2

While the N-terminal half of zebrafish VDAC1, 2, and 3 are highly similar (67–74% similarity at the amino acid level), the amino acid at position 73 (with respect to zebrafish VDAC2) differs between VDAC1 and 2, which exhibit anti-arrhythmic activity, and VDAC3, which does not (**Figures 6A,B**). Residue 73 is located in β-sheet 4 of the channel facing the outside of the barrel (**Figure 6B**) and a glutamate is present at this position in zebrafish VDAC2 (E73), while a glutamine is present at the corresponding position in zebrafish VDAC3 (Q73). E73 is highly conserved among animals; it is present in all animal VDAC proteins we examined excluding the VDAC3 family (**Figure 1C**).

Interestingly, E73 also appears in some fungi and in several protists (including choanoflagellates) that are believed to be closely related to animals (**Figure 1C**), suggesting an early evolutionary origin and subsequent conservation of this residue. While E73 is present in early evolutionary branches of fungi (Blastocladiomycota, Chytridiomycota, Chytridiomycota, and Mucoromycota), Q73 is instead present in fungi from the subkingdom Dikarya. Therefore, Q73 appears to have evolved twice, once in animals (VDAC3) and once in a subset of fungi (Dikarya). Intriguingly, like E73, Q73 also appears to be highly conserved within the taxa that it evolved (**Figure 1C**). Together, the simultaneous conservation of E73 and Q73 suggests that these two residues may impart significant functional differences to the VDAC protein that in turn created an evolutionary pressure for their retention.

Given that upregulation of mitochondrial Ca^{2+} uptake restores cardiac contraction in *tremblor/ncx1h* mutant embryos (Shimizu et al., 2015) and that E73 was previously reported to have an essential role in Ca^{2+} handling (Israelson et al., 2007; Peng et al., 2020), we suspected that E73 may be a critical residue that determines the isoform-specific rescue effect of VDAC genes on *tremblor/ncx1h*'s arrhythmia phenotype. To investigate this possibility, we created two point mutants: $VDAC2^{E73Q}$ by replacing E73 in VDAC2 with a glutamine and $VDAC3^{Q73E}$ by substituting Q73 of VDAC3 with a glutamate. We then

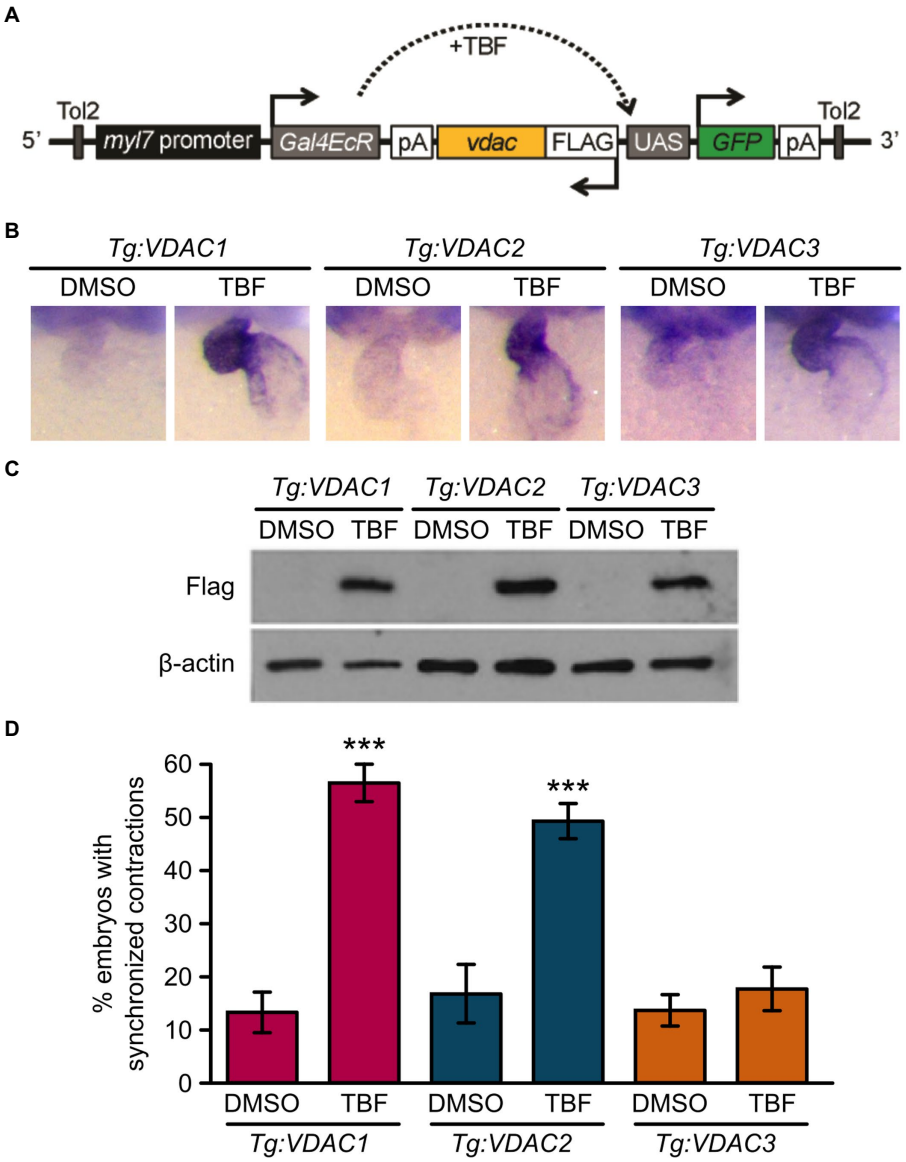

FIGURE 4 | Induction of VDAC1 and VDAC2 but not VDAC3 expression restores rhythmic cardiac contraction in transgenic *tremblor/ncx1h* lines. **(A)** Schematic diagram of VDAC transgenic construct. The cardiomyocytes-specific promoter *myl7* drives Gal4-ecdysone receptor fusion protein (Gal4EcR), which becomes transcriptionally activated in response to tebufenozide (TBF), an ecdysone receptor agonist and binds to the upstream activating sequence (UAS), resulting in the simultaneous expression of both FLAG-tagged VDAC and EGFP. Transgenic lines were bred in the *tremblor/ncx1h* background. **(B)** Whole-mount *in situ* hybridization analysis demonstrating that VDAC expression is induced specifically in the heart upon TBF treatment in transgenic zebrafish (*Tg:VDAC*). Embryos were treated with either DMSO or TBF from 24 hpf until they were fixed for *in situ* hybridization at 48 hpf. **(C)** Western blotting of 32 hpf transgenic embryo lysate with an anti-FLAG antibody showing that VDAC protein expression is induced after embryos are treated with TBF. β-actin was used as a loading control. **(D)** While only 13.5 ± 3.6% of DMSO-treated *Tg:VDAC1; tremblor/ncx1h* embryos exhibit rhythmic cardiac contraction (N = 3, *n* = 233), 56.72 ± 3.5% of TBF-treated *Tg:VDAC1; tremblor/ncx1h* embryos established rhythmic contraction (N = 3, *n* = 238). Similarly, as opposed to only 16.8 ± 5.7% of DMSO-treated *Tg:VDAC2; tremblor/ncx1h* embryos (N = 3, *n* = 161), 49.6 ± 3.1% of TBF-treated *Tg:VDAC2; tremblor/ncx1h* embryos showed cardiac contraction (N = 3, *n* = 227). In contrast, the effect of TBF-induced overexpression is minimal in *Tg:VDAC3; tremblor/ncx1h* (13.7 ± 2.9%, N = 3, *n* = 283 in DMSO-treated embryos compared to 17.9 ± 4.0%, N = 3, *n* = 373 in TBF-treated embryos). Overall rescue percentages represent the mean rescue percentage ± s.e.m. from N independent experiments, using a total of n embryos.

injected VDAC2^{E73Q} and VDAC3^{Q73E} RNA into *tremblor/ncx1h* mutant embryos. Western blots showed that both VDAC2^{E73Q} and VDAC3^{Q73E} are stably expressed in embryos (**Figure 6C**). Interestingly, opposite to wild-type VDAC2 and VDAC3 overexpression effects, the hearts that received VDAC2^{E73Q} RNA continued to fibrillate, whereas VDAC3^{Q73E} overexpression suppressed cardiac fibrillation and restored rhythmic cardiac contraction in *tremblor/ncx1h* mutant embryos (**Figures 6D,E**).

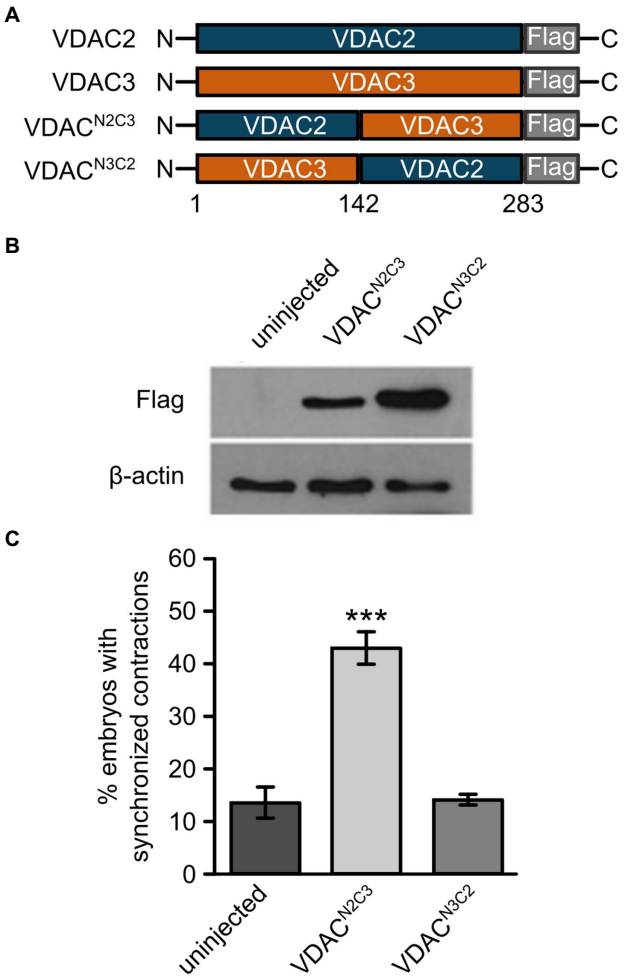

FIGURE 5 | The N-terminal domain of VDAC2 contains critical elements for its cardioprotective activity. **(A)** Schematic representation of VDAC2/3 chimeric constructs. Reciprocal chimeric constructs were generated by swapping the N- and C-terminal halves of VDAC2 and VDAC3 and tagging a FLAG epitope at the C-terminal end. VDAC^{N2C3}: The N-terminal half of VDAC2 (amino acids 1 to 142) was fused to the C-terminal half of VDAC3. VDAC^{N3C2}: The N-terminal half of VDAC3 was fused to the C-terminal half of VDAC2. **(B)** Western blot with anti-FLAG antibody after SDS-PAGE with lysates from 30 hpf uninjected embryos or embryos injected with 60 pg. FLAG-tagged chimeric VDAC mRNA. β-actin was used as a loading control. **(C)** Injection of VDAC^{N2C3} mRNA restored synchronized cardiac contractions in 1-day-old *tremblor/ncx1h* embryos (43.4 ± 2.8%, N = 3, *n* = 168 compared to 13.7 ± 3.1%, N = 3, *n* = 156 in uninjected siblings), whereas injection of VDAC^{N3C2} mRNA fails to produce this effect (14.2 ± 1.2%, N = 3, *n* = 162). Overall rescue percentages represent the mean rescue percentage ± s.e.m. from N independent experiments, using a total of n embryos.

E73 Controls VDAC-Dependent Mitochondrial Ca^{2+} Uptake

VDAC2 activation suppresses cardiac fibrillation in *tremblor/ncx1h* mutants by buffering excess Ca^{2+} into the mitochondria (Shimizu et al., 2015). To test whether VDAC's differential cardioprotective activities on *tremblor/nxc1h* mutant hearts are rooted in their differential Ca^{2+} transporting activity, we transfected HeLa cells with VDAC isoforms and measured mitochondrial Ca^{2+} uptake using Rhod-2 (**Figure 7A**). Cells transfected with VDAC2 showed increased mitochondrial Ca^{2+} levels compared to empty vector transfected control cells upon stimulation, whereas no significant differences in mitochondrial Ca^{2+} uptake between control and VDAC3 transfected cells were observed (**Figure 7B**). Given the previously observed correlation between position 73 and the cardioprotective activity of VDAC2 and VDAC3, we next examined whether E73 might determine the differential mitochondrial Ca^{2+} uptake activities between VDAC isoforms. Indeed, wild-type VDAC2 enhanced mitochondrial Ca^{2+} uptake, while VDAC2^{E73Q} showed a mitochondrial Ca^{2+} uptake profile comparable to control cells. In contrast, VDAC3 did not enhance mitochondrial Ca^{2+} uptake above control, while introduction of E73 into VDAC3 (VDAC3^{Q73E}) significantly enhanced mitochondrial Ca^{2+} uptake, demonstrating that E73 is a key determinant of VDAC2's mitochondrial Ca^{2+} uptake activity (**Figures 7B–D**).

E73 Critically Regulates VDAC2-Mediated Transfer of Ca^{2+} From the SR Into Mitochondria

Considerable differences in mitochondrial Ca^{2+} handling have been observed between excitable and non-excitable cells. In particular, in cardiomyocytes, VDAC2 was suggested to interact with RyR2, the major Ca^{2+} release channel on the SR, and thereby facilitate Ca^{2+} transfer from the SR to mitochondria (Subedi et al., 2011; Min et al., 2012). We therefore examined whether E73 regulates VDAC2's ability to mediate the transfer of Ca^{2+} from the SR into mitochondria in cultured HL-1 cardiomyocytes (Claycomb et al., 1998; Schweitzer et al., 2017; Wilting et al., 2020). Stable knockdown of the endogenously expressed murine VDAC2 by siRNA (Subedi et al., 2011; Min et al., 2012; Wilting et al., 2020) significantly reduced uptake of Ca^{2+} released from the SR by a caffeine pulse into mitochondria, while this uptake was comparable to native HL-1 cells when a scrambled control shRNA was used (**Figure 8**). We subsequently overexpressed our VDAC constructs, VDAC2, VDAC2^{E73Q}, VDAC3, and VDAC3^{Q73E} in mVDAC2 knockdown cells and evaluated their abilities to restore SR-mitochondria Ca^{2+} transfer. VDAC2 restored mitochondrial Ca^{2+} uptake to levels above baseline consistent with the idea of VDAC2 as the main mediator of SR-mitochondria Ca^{2+} transfer. Strikingly, replacing E73 of VDAC2 with a glutamine (VDAC2^{E73Q}) abrogated its ability to promote SR-mitochondria Ca^{2+} transfer (**Figure 8B**). On the other hand, while VDAC3 failed to restore SR-mitochondria Ca^{2+} transfer, introduction of E73 in VDAC3^{Q73E} restored SR-mitochondria Ca^{2+} transfer to control levels (**Figure 8C**). These data indicate that VDAC-mediated SR-mitochondria Ca^{2+} transfer is dependent on the presence of E73 in cardiomyocytes.

DISCUSSION

Despite structural and functional similarities, divergent biological functions have been noted among VDAC isoforms.

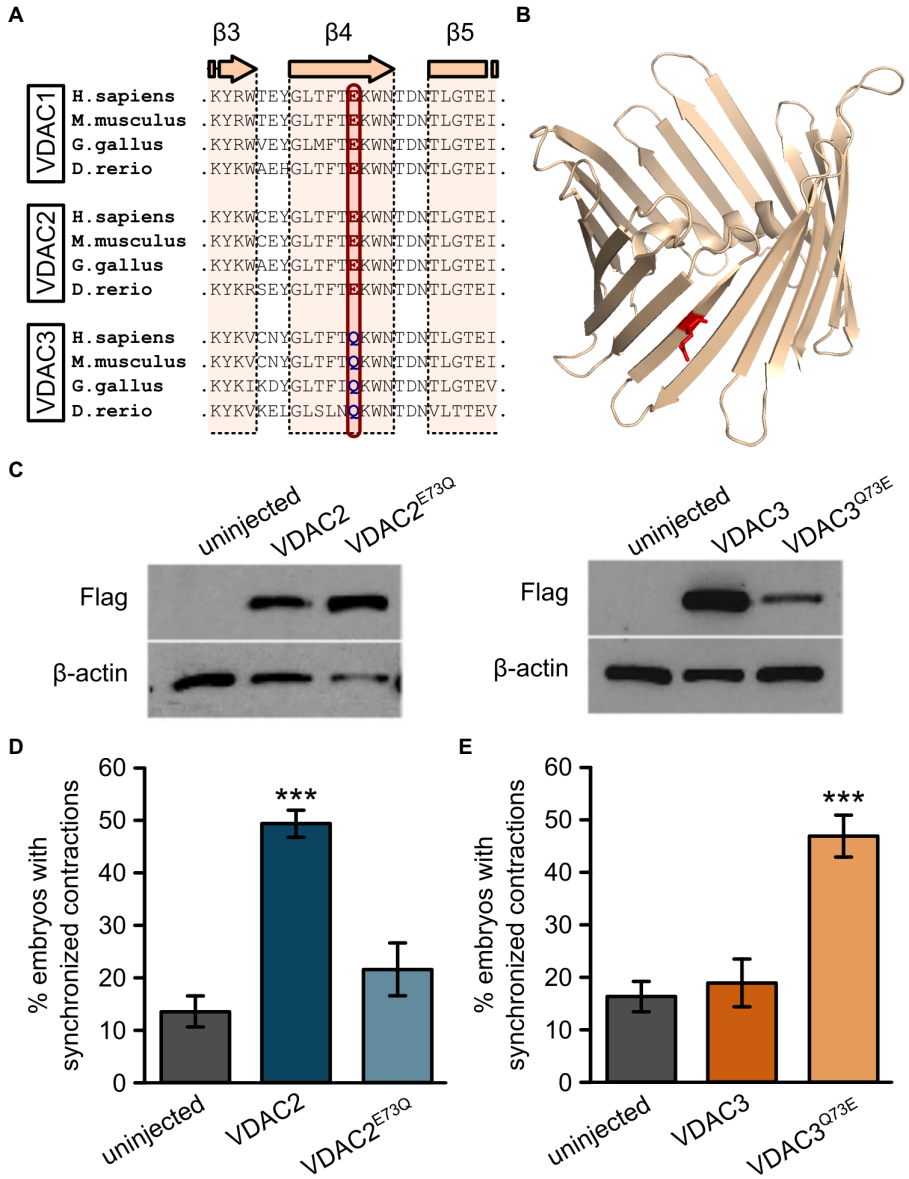

FIGURE 6 | E73 is the critical amino acid residue that determines the ability of VDAC2 to suppress cardiac fibrillation in the *tremblor/ncx1h* mutant. **(A)** Alignment of protein sequences of VDAC1, 2, and 3 from different species. In all the species examined the position corresponding to zebrafish residue 73 is consistently occupied by a glutamate **(E)** in VDAC1 and VDAC2, whereas this position is occupied by glutamine (Q) in VDAC3. **(B)** Three-dimensional model of the VDAC2 protein (pdb: 4bum) showing the location of amino acid 73 in β-sheet 4 in red. **(C)** Western blot analysis of lysates from 30 hpf uninjected embryos or embryos injected with 25 pg. FLAG-tagged wild type and point mutant VDAC mRNA. β-actin was used as a loading control. **(D)** Mutation of E73 to Q in VDAC2 abrogates its ability to suppress cardiac fibrillation in *tremblor/ncx1h* mutants (49.7 ± 2.8%, N = 3, *n* = 144 with VDAC2 in contrast to 21.7 ± 5.1%, N = 3, *n* = 155 with $VDAC2^{E73Q}$). **(E)** Vice-versa, by mutating Q73 to E, VDAC3 gained the ability to restore rhythmic cardiac contraction in *tre* mutants (19.0 ± 4.4%, N = 3, *n* = 182 with VDAC3 in contrast to 47.2 ± 4.3%, N = 3, *n* = 145 with $VDAC3^{Q73E}$). Overall rescue percentages represent the mean rescue percentage ± s.e.m. from N independent experiments, using a total of n embryos.

Accumulating evidence indicates that VDAC2 is a critical gatekeeper for mitochondrial Ca^{2+} entry in physiology and in disease within the heart (Min et al., 2012; Shimizu et al., 2015, 2017; Schweitzer et al., 2017; Wilting et al., 2020; Sander et al., 2021). In this study, we used zebrafish *tremblor/ncx1h* mutants as an *in vivo* platform to assess the ability of VDAC1 and VDAC3 to regulate cardiac rhythmicity through mitochondrial Ca^{2+} uptake. We demonstrate that like VDAC2, VDAC1 is capable of transporting Ca^{2+} across the outer mitochondrial membrane. When overexpressed, VDAC1 effectively restores rhythmic cardiac contractions in *ncx1* deficient hearts. VDAC3, on the contrary, cannot facilitate efficient Ca^{2+} transport across the mitochondrial membrane, demonstrating divergent roles for these isoforms

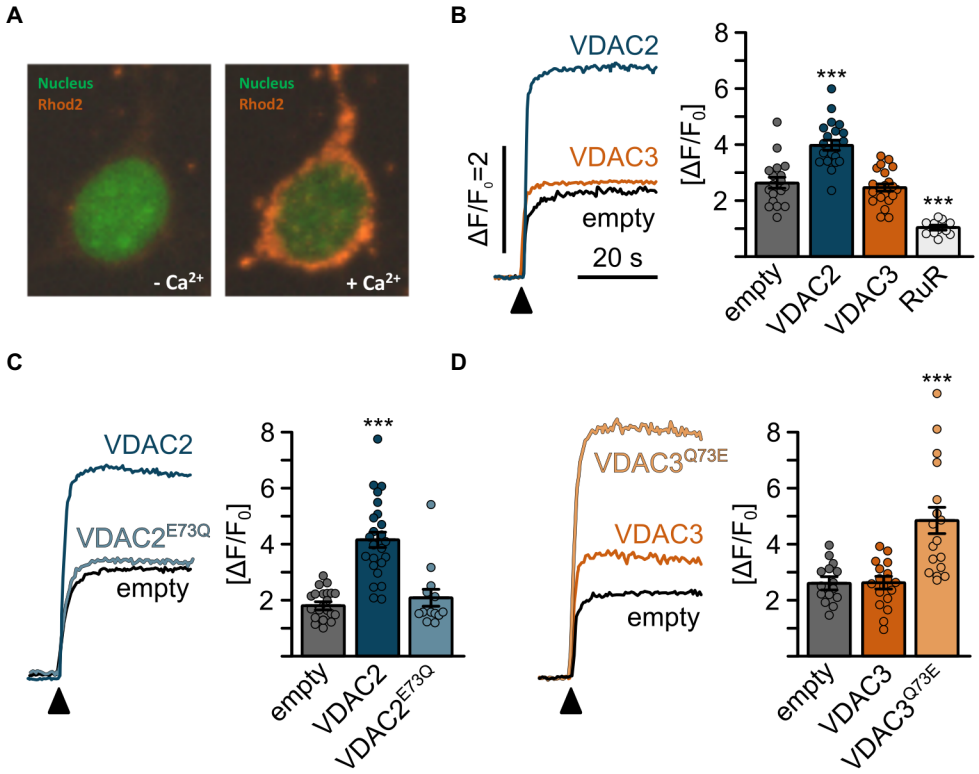

FIGURE 7 | Mitochondrial Ca^{2+} uptake in HeLa cells is promoted by E73. **(A)** Representative confocal images of permeabilized HeLa cells loaded with Rhod2 after transiently transfection with VDAC expression constructs. Mitochondrial Rhod2 fluorescence is minimal at the basal state (left). Upon addition of Ca^{2+}, the Rhod2 fluorescence rapidly concentrates in mitochondria (right). **(B-D)** Mitochondrial Ca^{2+} uptake assays of HeLa cells transiently transfected with VDAC constructs; **(B)** mitochondrial Ca^{2+} uptake was observed in empty vector transfected control cells (empty, $\Delta F/F_0 = 2.64 \pm 0.82$, $n = 18$), which was significantly enhanced by overexpression of VDAC2 ($\Delta F/F_0 = 3.99 \pm 0.80$, $n = 22$) but not VDAC3 ($\Delta F/F_0 = 2.47 \pm 0.61$, $n = 22$) and significantly inhibited by ruthenium red ($\Delta F/F_0 = 1.06 \pm 0.23$, $n = 12$). **(C)** While wild-type VDAC2 enhanced mitochondrial Ca^{2+} uptake ($\Delta F/F_0 = 1.83 \pm 0.52$ for empty cells vs. $\Delta F/F_0 = 4.18 \pm 1.4$ for VDAC2, $n = 22$ and 24, respectively), $VDAC2^{E73Q}$ failed to induce this effect ($\Delta F/F_0 = 2.09 \pm 1.15$, $n = 13$). **(D)** Conversely, wild-type VDAC3 did not induce a significant increase in mitochondrial Ca^{2+} uptake ($\Delta F/F_0 = 2.63 \pm 0.70$ for empty cells vs. $\Delta F/F_0 = 2.59 \pm 0.81$ for VDAC3, $n = 15$ and 18, respectively); however, $VDAC3^{Q73E}$ significantly increased mitochondrial Ca^{2+} uptake to $\Delta F/F_0 = 4.85 \pm 1.99$ ($n = 18$).

in Ca^{2+} trafficking. Structure-function analysis further revealed that the evolutionarily conserved E73/Q73 confers this divergent VDAC isoform-dependent calcium trafficking function.

Across all vertebrate species, a glutamate occupies position 73 in VDAC1 and VDAC2 (E73), while a glutamine is present at this position in VDAC3 (Q73). Using multiple model systems, we demonstrate that only isoforms with E73 are able to promote mitochondrial Ca^{2+} uptake and to regulate cardiac rhythmicity. While VDAC3 did not mediate mitochondrial Ca^{2+} uptake in its wild-type form, replacing Q73 with a glutamate allows VDAC3 to facilitate SR-mitochondrial Ca^{2+} transfer, enhance mitochondrial Ca^{2+} uptake, and suppress cardiac arrhythmia. These data are consistent with the established notion that E73 is an important regulator of VDAC function. However, the precise mechanism by which E73 influences Ca^{2+} trafficking remains elusive. Our data suggest that E73 confers cardiac protection by facilitating mitochondrial Ca^{2+} uptake. In line with these findings, E73 has been suggested to be involved in the Ca^{2+} transport activity of VDAC (Gincel et al., 2001; Ge et al., 2016; Peng et al., 2020) and is also the binding site for VDAC blockers like ruthenium red and its derivatives, which compete with Ca^{2+} for binding to this site (Israelson et al., 2007). Also supporting the notion that E73 can facilitate the inter-organellar transport of Ca^{2+} is the recent finding that it influences mitochondrial uptake of Ca^{2+} from lysosomes (Peng et al., 2020). Given the location of E73 on the outside of the fourth repeat of the VDAC barrel facing the lipid membrane (Ujwal et al., 2008; Schredelseker et al., 2014), it is unlikely that E73 serves as a direct binding site for Ca^{2+} ions, but instead may indirectly promote the uptake of Ca^{2+} by VDAC. Indeed, E73 alone was unable to modify voltage gating of purified VDAC1 channels (Queralt-Martín et al., 2019). An indirect effect is further supported by the findings that the positive charge of E73 can induce a thinning of the local plasma membrane (Villinger et al., 2010) and that E73 promotes binding of VDAC protein partners like hexokinase (Abu-Hamad et al., 2008) and mediates VDAC dimerization (Bergdoll et al., 2018). Though our data demonstrate an essential role for E73 in mitochondrial Ca^{2+} uptake, these reports clearly

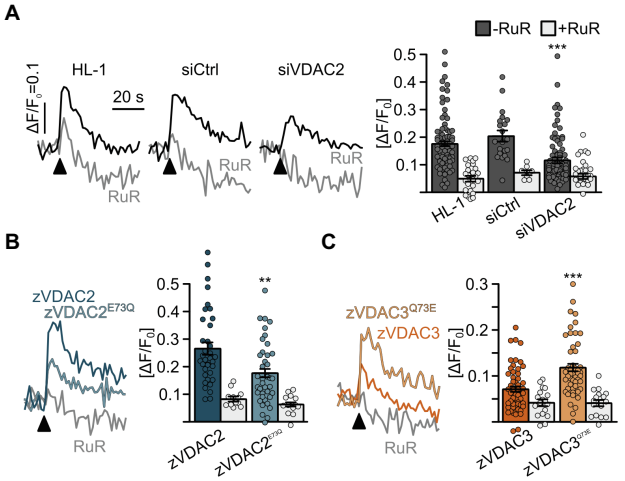

FIGURE 8 | Restoration of SR-mitochondria Ca^{2+} transfer in permeabilized HL-1 cardiomyocytes. **(A)** Knockdown of the endogenous mVDAC2 in HL-1 cells significantly reduced SR-mitochondria Ca^{2+} transfer after addition of caffeine (arrowhead) from $\Delta F/F_0 = 0.18 \pm 0.01$ ($n = 84$) to 0.12 ± 0.01 ($n = 83$) while a scrambled siRNA control did not affect Ca^{2+} transfer (0.20 ± 0.02, $n = 21$). **(B)** Wild-type zVDAC2 restored mitochondrial Ca^{2+} uptake to 0.27 ± 0.02 ($n = 36$), well above mVDAC2 knockdown cells. Overexpression of $zVDAC2^{E73Q}$ restored mitochondrial Ca^{2+} uptake to only 0.18 ± 0.02 ($n = 39$), which is significantly lower compared to wild-type zVDAC2. **(C)** Conversely, zVDAC3 did not restore mitochondrial Ca^{2+} uptake in mVDAC2 knockdown cells (0.12 ± 0.01, $n = 54$), while $VDAC3^{Q73E}$ restored mitochondrial Ca^{2+} uptake to $\Delta F/F_0 = 0.20 \pm 0.02$, ($n = 50$). Arrowheads indicate injection of 10 mM caffeine. Statistical analysis was performed using Kruskal-Wallis test with Dunn's *post-hoc* test.

highlight the need for further biochemical and structural studies to fully elucidate the molecular mechanisms of E73's involvement in VDAC-mediated Ca^{2+} transit.

In cardiomyocytes, VDAC2 interacts with the ryanodine receptor to shape intracellular Ca^{2+} signals (Subedi et al., 2011; Min et al., 2012). Knockout of VDAC2 alone is sufficient to attenuate SR-mitochondria Ca^{2+} transfer in cardiomyocytes and cardiac-specific elimination of VDAC2 was reported to result in early cardiac dysfunction (Raghavan et al., 2012). While our study suggests that both VDAC1 and VDAC2 are capable of mediating mitochondrial Ca^{2+} uptake in cardiomyocytes, VDAC2 appears to be more relevant to mammalian cardiac biology. VDAC1 is known to couple with the IP3-receptor to mediate mitochondrial Ca^{2+} uptake in non-excitable cells. However, inactivation of glycogen synthase kinase-3β was shown to reduce coupling of the IP3-receptor to VDAC1 in cardiomyocytes and to reduce mitochondrial Ca^{2+} uptake during cardiac ischemia-reperfusion (Gomez et al., 2016). It would thus be interesting to explore differential interaction of VDAC1 and VDAC2 with the ryanodine receptor or other Ca^{2+} release channels on various organelles to determine if VDAC2 plays a more acute and VDAC1 plays a more subtle role in healthy cardiomyocytes.

The presence of the highly conserved VDAC3 family, which lacks E73, in vertebrates suggests that this difference may impart crucial functional properties to VDAC3. Whether the VDAC proteins in vertebrates have evolved subtype-specific distinct functions, with each one assuming part of the role played by the single VDAC protein in basal metazoans, or if they have acquired novel functions are still an open question that could be examined to better understand the role of mitochondria in the evolution of the highly demanding vertebrate heart muscle. Whether the E73/Q73 residues identified in fungi by sequence homology are functionally and structurally homologous to zebrafish VDAC, E73/Q73 has not been studied. It would be interesting to examine whether E73 containing and/or Q73 containing fungal VDACs could substitute for the activity of VDAC2 in zebrafish embryos or mammalian cells, and whether these proteins exhibit differing calcium permeability properties. Most species of fungi examined have only a single VDAC protein, with the notable exception of *S. cerevisiae*. Q73 is present in Dikarya, a subkingdom of Fungi, while E73 is present in all other fungal species surveyed. It is intriguing to note that similar to the evolution of vertebrate VDACs, fungal E73 and Q73 are conserved within their respective clades, suggesting that differences at this residue may be functionally relevant. If indeed Q73 reduces the calcium permeability of fungal VDACs, this would suggest that a reduced requirement for mitochondrial calcium uptake or alternative routes of mitochondrial calcium uptake may have evolved in Dikarya. Alternatively, structural differences between animal and fungal VDACs may render fungal E73 less crucial for calcium uptake.

Together, our data demonstrate that the evolutionarily conserved E73 residue mediates the divergent anti-arrhythmic effects of VDAC isoforms by modulating mitochondrial Ca^{2+} uptake in cardiomyocytes. These findings underscore the important role of mitochondria in cellular Ca^{2+} dynamics and suggest that further exploring VDAC1- and VDAC2-mediated mitochondrial Ca^{2+} uptake may reveal novel regulatory mechanisms for normal cardiac physiology and heart disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by University of California Los Angeles Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

HS, SH, AL, LC, JH, KW, FW, and JS performed the experiments. JS and J-NC conceptualized the study. HS, JS, TG, and J-NC

acquired the funding. HS, AL, LC, JS, and J-NC wrote the manuscript. All authors commented on the manuscript.

FUNDING

This work was supported by the Heiwa Nakajima foundation (to HS), the Austrian Science Fund FWF (J3065-B11 to JS), the German research foundation (SCHR 1471/1-1 to JS, TRR 152 to TG), Deutsche Forschungsgemeinschaft, and the National Heart, Lung and Blood Institute (HL096980 and HL140472 to J-NC).

ACKNOWLEDGMENTS

The authors would like to acknowledge Brigitte Mayerhofer for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fphys.2021.724828/full#supplementary-material

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frontiers in Physiology

BRIEF RESEARCH REPORT
published: 17 September 2021
doi: 10.3389/fphys.2021.739001

A C-Terminally Truncated Variant of *Neurospora crassa* VDAC Assembles Into a Partially Functional Form in the Mitochondrial Outer Membrane and Forms Multimers *in vitro*

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The voltage-dependent anion-selective channel (VDAC) is a porin in the mitochondrial outer membrane (MOM). Unlike bacterial porins, several mitochondrial β-barrels comprise an odd number of β-strands, as is the case for the 19-β-stranded VDAC. Previously, a variant of a VDAC from *Neurospora crassa*, VDAC-ΔC, lacking the predicted 19th β-strand, was found to form gated, anion-selective channels in artificial membranes. *In vivo,* the two C-terminal β-strands (β18 and β19) in VDAC form a β-hairpin necessary for import from the cytoplasm into mitochondria and the β-signal required for assembly in the mitochondrial outer membrane resides in β19. The current study demonstrated that the putative 18-stranded β-barrel formed by VDAC-ΔC can be imported and assembled in the MOM *in vivo* and can also partially rescue the phenotype associated with the deletion of VDAC from a strain of *N. crassa.* Furthermore, when expressed and purified from *Escherichia coli*, VDAC-ΔC can be folded into a β-strand-rich form in decyl-maltoside. Size exclusion chromatography (SEC) alone or combined with multi-angle light scattering (SEC-MALS) and analytical ultracentrifugation revealed that, unlike full-length VDACs, VDAC-ΔC can self-organize into dimers and higher order oligomers in the absence of sterol.

Keywords: VDAC, mitochondrial porin, *Neurospora crassa*, dimers, decyl-maltoside

OPEN ACCESS

Edited by:
Angela Messina,
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Reviewed by:
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Andonis Karachitos,
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *09 July 2021*
Accepted: *11 August 2021*
Published: *17 September 2021*

Citation:
Ferens FG, Summers WAT, Bharaj A, Stetefeld J and Court DA (2021) A C-Terminally Truncated Variant of Neurospora crassa VDAC Assembles Into a Partially Functional Form in the Mitochondrial Outer Membrane and Forms Multimers in vitro. Front. Physiol. 12:739001. doi: 10.3389/fphys.2021.739001

INTRODUCTION

Voltage-dependent anion-selective channels, or mitochondrial porins, reside in the mitochondrial outer membrane and act as general channels that allow the bidirectional flow of metabolites across the membrane [MOM, reviewed in Young et al. (2007)]. Voltage-dependent anion-selective channel pores are modulated by interactions with multiple metabolites, including NADH (Zizi et al., 1994) and proteins such as tubulin (Rostovtseva et al., 2008), thereby contributing to the regulation of cellular metabolism (reviewed in Lemasters and Holmuhamedov, 2006; Rostovtseva and Bezrukov, 2008; Caterino et al., 2017; Shoshan-Barmatz et al., 2017; Magri et al., 2018; De Pinto, 2021). For example, voltage-dependent anion-selective channel (VDAC) interactions with hexokinases (Linden et al., 1982) can inhibit the induction of apoptosis (Azoulay-Zohar et al., 2004).

In mammalian systems, VDAC-protein interactions are also associated with a variety of cancers through the promotion of glycolysis and the reduction of apoptosis (reviewed by Mazure, 2017), with VDAC thus being a potential target of therapeutic agents (Shoshan-Barmatz et al., 2019). However, VDAC also participates in other processes (Endo and Sakaue, 2019), such as the import of some proteins into mitochondria (Ellenrieder et al., 2019) and the transport of cholesterol (Liu et al., 2006).

Unlike all characterized bacterial β-barrel proteins, which are composed of an even number of β-strands (Wimley, 2003), mammalian (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008) and zebra fish (Schredelseker et al., 2014) VDAC, expressed in *Escherichia coli* and folded in detergent, each formed a 19-stranded β-barrel with an N-terminal region that does not contribute to the barrel. Structural predictions for VDACs from multiple sources (Bay et al., 2012) support this model. A comparison of the VDAC isolated in decyl-maltoside (DM)-solubilized mitochondrial membranes of *Neurospora crassa* and the equivalent protein expressed in *E. coli* and folded in the same detergent support the equivalence of the native and recombinant forms (Ferens et al., 2019).

Targeting and assembly of β-barrel proteins into the MOM require two signals in the C-terminal β-strand. A C-terminal hydrophobic β-hairpin is recognized by the receptor Tom20 to initiate import through the TOM complex into the intermembrane space (Jores et al., 2016). Within the terminal β-strand is the β-signal (Kutik et al., 2008) that engages the topogenesis of β-Barrel proteins complex [TOB (Kozjak et al., 2003; Paschen et al., 2003)] or the sorting and assembly (SAM) complex (Wiedemann et al., 2003). This complex, related to the bacterial BAM complex (Gentle et al., 2004), assembles β-barrels in the MOM (reviewed in Hansen and Herrmann, 2019). Thus, the character of the amino acid side chains, rather than a precise sequence, defines the β-signal [Kutik et al., 2008; Imai et al., 2011 (**Figure 1B**)].

A C-terminal truncation variant of *N. crassa* VDAC, VDAC-ΔC, known as ΔC269-283Por (Popp et al., 1996), lacks the predicted terminal β-strand (β19, **Figure 1A**). In artificial membranes, it forms anion-selective gated pores similar to wild-type VDACs, but with 75% of the conductance (Popp et al., 1996). Similarly, an 18-β-stranded human hVDAC2 ($hV2^{18}$) lacking β19 forms a pore with about 90% of the conductance of hVDAC2 (Srivastava and Mahalakshmi, 2020). Both 18-stranded molecules form "noisier" channels, but only $hV2^{18}$ shows significantly reduced voltage-dependent gating. Whether this difference is due to an N-terminal His_6-tag on the *N. crassa* VDAC or the absence of a tag on $hV2^{18}$ remains to be elucidated.

In spite of this pore-forming ability, *N. crassa* VDAC-ΔC synthesized in rabbit reticulocyte lysate is not imported into isolated mitochondria, suggesting that targeting or assembly information is disrupted (Court et al., 1996). In contrast $hV2^{18}$ partially complements the lack of VDAC1 (Δ*POR1*) in *Saccharomyces cerevisiae,* allowing growth on non-fermentable carbon sources (Srivastava and Mahalakshmi, 2020), indicating that it can assemble in mitochondrial membranes.

In the MOM, VDAC exists in various oligomeric arrangements ranging from monomers, dimers, tetramers, and hexamers (Zalk et al., 2005; Geula et al., 2012), as reviewed by Shoshan-Barmatz et al. (2017), to large sheets (Goncalves et al., 2007; Hoogenboom et al., 2007). Hexagonal arrays were observed in negatively stained samples by electron microscopy (EM) upon the depletion of lipids from purified outer membranes (Mannella and Frank, 1984; Mannella et al., 1986) and detected by the atomic force microscopy of both purified outer membranes and spontaneously formed tubular, two-dimensional VDAC crystals (Hoogenboom et al., 2007). The self-association of detergent-solubilized VDAC has been observed in chemically cross-linked samples (Zalk et al., 2005; Malia and Wagner, 2007). In the absence of a cross-linker, *N. crassa* VDAC, expressed in *E. coli* and folded in DM, assembles into higher order structures only in the presence of sterol; models of dimers and of hexamers that resembled those observed by (Mannella et al., 1986) were obtained from a combination of SEC, alone or in combination with small-angle scattering (SEC-SAX) and analytical ultracentrifugation (AUC) (Ferens et al., 2019).

The oligomerization of VDAC regulates multiple processes. In mammalian cells, it is associated with the release of cytochrome *c* and apoptotic regulators (Shoshan-Barmatz et al., 2010), reviewed in (Shoshan-Barmatz et al., 2020). Recently, a role for oligomers of the mouse VDAC1 in the release of mitochondrial DNA during oxidative stress has also been shown (Kim et al., 2019). The N-terminal strand is critical for the oligomerization in human (Shoshan-Barmatz et al., 2010) and mouse (Kim et al., 2019) VDAC1.

In the current study, the functionality of VDAC-ΔC in *N. crassa*, the folded state of VDAC-ΔC, and the propensity of the latter to form oligomers were investigated using the protein expressed in *E. coli* and folded in DM.

MATERIALS AND METHODS

The *N. crassa* used in the study was grown as described (Davis and De Serres, 1970). Growth rates were measured in race tubes, containing Vogel's minimal medium at 22–23°C. An *N. crassa* strain in which the *POR1* gene is replaced by that for VDAC-ΔC was constructed by replacing the wild-type gene in FGSC 9718 (Colot et al., 2006), with the cDNA for the Δ269-283 Por truncation variant (Popp et al., 1996) downstream of a hygromycin-resistance gene (hygR) for selection (Colot et al., 2006) (**Supplementary Tables 1**, **2**). The "Por"/porin nomenclature for VDAC variants is from earlier studies on the *N. crassa* VDAC (Popp et al., 1996). Following selection on hygromycin, the strain was purified by repeated subculturing to remove untransformed nuclei; the absence of $por1^{+}$ nuclei was confirmed by PCR. The measurement of cytochrome spectra was carried out as described (Summers et al., 2012).

The expression, folding, and analysis of WT-VDAC and VDAC-ΔC were carried out as described (Ferens et al., 2019) (see **Supplementary File 1**). Circular dichroism (CD), size exclusion chromatography (SEC), and AUC experiments were carried out as performed in previous studies (Ferens et al., 2019).

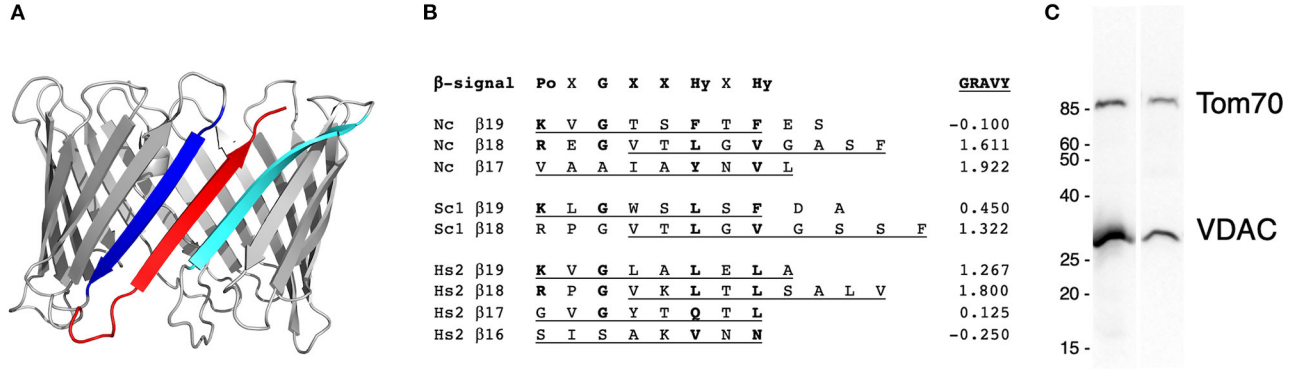

FIGURE 1 | The features and expression of VDAC-ΔC. **(A)** The structural model of *Neurospora crassa* VDAC. The model was generated using Phyre2 (Kelley et al., 2015) with human VDAC1 as the template for the entire sequence (PDB: 2K4T), with a 100% confidence score. β-strands β18 (blue), β19 (red), and β1 (cyan) are highlighted. **(B)** The alignment of the terminal β-strands of *N. crassa* VDAC (Nc, P07144), *Saccharomyces cerevisiae* Por1 (Sc, P04840), and human VDAC 2 (Hs2, P21796, and PDB 2K4T). β-strands predicted by the alignment with human VDAC1 (P21796, 2K4T) in Praline (Simossis and Heringa, 2005) are underlined. Non-X residues matching the β-signal motif (Kutik et al., 2008) are indicated in bold. The grand average of the hydropathicity [GRAVY (Gasteiger, 2006)] score, obtained from https://web.expasy.org/protscale/ is indicated in the right column. Po, polar; X, any residue; G, glycine; Hy, hydrophobic, after (Kutik et al., 2008). **(C)** The Western blot analysis of mitochondria isolated from the wild-type strain (FGSC 9718, left lane) and the strain expressing VDAC-ΔC (right lane). The blot was probed with antibodies against VDAC and Tom70, a component of the protein translocation machinery of the outer membrane (see **Supplementary File 1** for details). Size markers on the left are in kDa.

SEC-MALS (SEC with multi-angle light scattering, UV absorbance, and differential refractive index detectors) experiments were conducted under the same conditions as normal SEC runs. Data analysis was done using the software ASTRA by Wyatt Technology (Santa Barbara, CA, USA). After that, VDAC SEC-MALS data were analyzed using the protein-conjugate analysis method in ASTRA (Wyatt, 1993; Andersson et al., 2003; Slotboom et al., 2008). For the description of the method, the ratio of detergent/protein (g/g) in the complex is solved using the equation:

$$\frac{1}{1+\delta}\left(\frac{dn}{dc}\right)_{Protein} + \frac{\delta}{1+\delta}\left(\frac{dn}{dc}\right)_{Detergent} = \frac{\Delta RI}{\Delta A_{280}}\left(\left(\frac{1}{1+\delta}\right)\varepsilon_{0.1\%,\,Protein} + \left(\frac{\delta}{1+\delta}\right)\varepsilon_{0.1\%,\,Detergent}\right) \quad (1)$$

where δ is the ratio of protein/detergent (g/g), dn/dc is the refractive index increment (0.185 ml/g for protein and 0.146 ml/g for DM), ΔRI is the buffer-subtracted refractive index of the sample, ΔA_{280} is the buffer-subtracted absorbance of the sample at 280 nm, $\varepsilon_{0.1\%}$ is the extinction coefficient at 280 nm [0.79 ml/(mg*cm)] for VDAC, 0.82 ml/(mg*cm) for VDAC-ΔC, and 0 ml/(mg*cm) for DM]. With δ known, the dn/dc of the protein-detergent complex is determined:

$$\left(\frac{dn}{dc}\right)_{Complex} = \frac{1}{1+\delta}\left(\frac{dn}{dc}\right)_{Protein} + \frac{\delta}{1+\delta}\left(\frac{dn}{dc}\right)_{Detergent} \quad (2)$$

The concentration of the protein-detergent complex ($c_{Complex}$) can now be determined:

$$c_{Complex} = \left(\frac{dn}{dc}\right)_{complex}\Delta RI \quad (3)$$

The Rayleigh ratio (R_θ), is a measure of light-scattering intensity at angle θ from the incident light:

$$R_\theta = \left(\frac{I_\theta}{I_0}\right)_{Protein\ solution} - \left(\frac{I_\theta}{I_0}\right)_{Buffer} \quad (4)$$

where I_θ is the intensity of scattered light at angle θ from the incident light and I_0 is the intensity of the incident light. R_θ can be related to the molar mass of the protein-detergent complex ($M_{w,Complex}$) through the following equation (the Zimm method):

$$\frac{K^* c_{Complex}}{R_\theta} = \left(\frac{16\pi^2 n^2 < r_g^2 >}{3\lambda^2 M_{w,\,Complex}}\right)\sin^2\left(\frac{\theta}{2}\right) + \frac{1}{M_{w,\,Complex}} + 2A_2 c \quad (5)$$

where λ is the wavelength of the incident light, n is the refractive index of the solute, $< r_g^2 >$ is the root mean square radius, θ is the angle between the scattered light and incident light, A_2 is the second virial coefficient, and K* is an optical constant defined by the equation:

$$K^* = \frac{4\pi^2\left(\frac{dn}{dc}\right)^2_{Complex} n^2}{\lambda^4 N_A} \quad (6)$$

where N_A is Avogadro's number. With data points from multiple light-scattering detectors at different angles around the sample, a plot of $(K^*c)/R_\theta$ against $\sin^2(\theta/2)$ was fit with a linear function with the y-intercept equal to $(1/M_{w,Complex} + 2A_2c)$. For dilute SEC experiments, the second term of the y-intercept was assumed to be negligible, allowing for the determination of $M_{w,Complex}$ from the y-intercept:

$$\frac{1}{M_w} \approx \frac{1}{M_w} + 2A_2c \tag{7}$$

Finally, with $M_{w,Complex}$ and δ, the molar mass of the protein ($M_{w,Protein}$) can be determined:

$$M_{w,\,Complex} = (1 + \delta)\, M_{w,\,Protein} \tag{8}$$

RESULTS

VDAC-ΔC Partially Complements a ΔPor1 Strain

Although it forms pores in artificial membranes, *in vitro*-synthesized VDAC-ΔC cannot be imported into isolated mitochondria (Court et al., 1996). To determine whether VDAC-ΔC can function *in vivo*, a strain of *N. crassa* was generated, in which the coding sequence for VDAC was replaced by the cDNA-encoding VDAC-ΔC with the deletion of amino acid residues 269–283. The assembly of VDAC-ΔC in mitochondria was confirmed by the Western blotting of isolated mitochondrial proteins (**Figure 1C**).

To assess the functionality of the C-terminally truncated protein, the growth rate of the VDAC-ΔC strain was determined (**Table 1**). It grew at about 85% of the wild-type rate, which was faster than the VDAC-less strain, ΔPor1. Cytochrome spectra were used to assess the general functioning of the electron transport chain, as the absence of VDAC is associated with severe cytochrome defects in *N. crassa* (Summers et al., 2012). The levels of cytochromes *b* and aa_3, which function in complexes that include mitochondrially encoded subunits, were intermediate between those of the wild-type and the ΔPor1 strain. In VDAC-ΔC, an increase in cytochrome *c* was observed, which has previously been observed in *N. crassa* with defects in cytochromes *b* and aa_3 (Rifkin and Luck, 1971), including those lacking VDAC (Summers et al., 2012) or expressing an N-terminally truncated VDAC, ΔN-2-12Por (Shuvo et al., 2017), or the internally deleted Δ238-242Por (Ferens et al., 2017). Both of the latter molecules also partially complement the lack of VDAC.

Mitochondrial β-Barrel Motif Repeats in β18 and β19

The functioning of VDAC-ΔC indicates that the protein is targeted to and assembled in the MOM. Interestingly, the two motifs required for these processes are partially or fully within β19 (**Figure 1B**). The targeting of VDAC to the TOM complex requires the hydrophobic β-hairpin formed by β18 and β19 in the wild-type molecules. Given that the outer face of the VDAC pore interacts with the membrane, it is not unexpected that alternative pairs of terminal β-strands can form the requisite hydrophobic β-hairpins (**Figure 1B**). Similarly, the motif that captures the *N. crassa* β-signal can be found spanning part of the β18 from other organisms (**Figure 1B**, **Supplementary Table 3**). A survey of about 480 VDAC sequences (**Supplementary Table 3**) detected the β-signal in β19 in most sequences, with the exception of those from plants (87/162). A second copy of the β-signal was identified to overlap with β18 in 99% of the mammalian, vertebrate (>99%), yeast (100%), and fungal (98%) sequences sampled (**Supplementary Table 3**; see **Figure 1B**). Of the arthropod sequences examined, about 45% contained a putative β-signal in β18 and β19, and 52% of sequences contained three putative β-signals, two of which aligned with those in the other organisms and a third that shared three residues with those of β19 (**Supplementary Table 3**).

Recombinantly Expressed VDAC-ΔC Can Be Folded in DM

The full-length *N. crassa* VDAC oligomerizes in sterol-containing detergents (Ferens et al., 2019). To explore the ability of the VDAC-ΔC β-barrels to interact with each other, the protein was over-expressed in *E. coli*. It could be purified from inclusion bodies similarly to WT-VDAC and folded in DM using the same methods (Ferens et al., 2019). VDAC-ΔC folds into a predominantly β-strand conformation (**Supplementary Figure 1**), implying that the protein has formed a large β-sheet or β-barrel. SEC experiments (or Size exclusion chromatography experiments) (**Figure 2A**) revealed that the VDAC-ΔC in DM elutes in two major peaks, with one at an elution volume like monomeric WT-VDAC (∼14.2 ml). The additional peak elutes at about 13 ml, which is very similar to the elution volume of the WT-VDAC dimer that forms in the presence of the cholesterol analog cholesteryl-hemisuccinate (CHS) (Ferens et al., 2019). The deconvolution of the CD spectra of the two more prominent peaks in the elution profile estimated nearly identical secondary structure contributions, suggesting that both species are comprised of similarly folded VDAC molecules (**Supplementary Table 4** and **Supplementary Figure 1**). The VDAC-ΔC species had a ∼10% reduction in β-strand content relative to the previously examined WT-VDAC (**Supplementary Table 4** and **Supplementary Figure 1**). Due to the ambiguity in determining the oligomeric states of detergent-solubilized membrane proteins using SEC due to associated detergent molecules, we chose to investigate the two major peaks of DM-folded VDAC-ΔC using SEC-MALS to elucidate the oligomeric states of the two species.

VDAC-ΔC Folded in DM Is Primarily Comprised of a Mixture of Monomers, Dimers, and Tetramers

Both WT-VDAC and VDAC-ΔC were analyzed by SEC-MALS (**Figures 3A–D**). The elution was monitored by a UV detector, a MALS detector, and a differential refractometer in line with the column, allowing for the deconvolution of the protein molar masses of eluting protein-detergent complexes. The one major peak in the WT-VDAC sample was confirmed to be monomeric with a protein molar mass of 32 ± 1 kDa [theoretical

TABLE 1 | Genotypes and phenotypes of wild-types and strains expressing voltage-dependent anion-selective channel (VDAC) variants.

Strain	Genotype	Growth rate at 22 °C (cm/24 h)*	Cytochrome concentrations (nmol/mg protein)	Strain reference
FGSC† 9718 (wild-type)	Δ*mus51::bar*$^+$ (a)	7.7 ± 0.4‡	aa_3: 0.7 ± 0.1‡ *b:* 1.3 ± 0.1‡ *c:* 1.0 ± 0.2‡	Colot et al., 2006
ΔPor-1	Δ*por::hph*$^+$ Δ*mus51::bar*$^+$ *(a)*	1.6 ± 0.1‡	aa_3: 1.1 ± 0.0‡ *b*: 0.6 ± 0.1‡ *c*: 1.6 ± 0.1‡	Summers et al., 2012
VDAC-ΔC	*porΔC269-283* *hph*$^+$ Δ*mus51::bar*$^+$ *(a)*	6.3 ± 0.3	aa_3: 0.4 ± 0.0 *b:* 1.0 ± 0.1 *c:* 1.7 ± 0.2	This work

†*FGSC, fungal genetics stock center.*
‡*Summers et al. (2012).*
**n* ≥ 3*; average and standard deviation shown.*

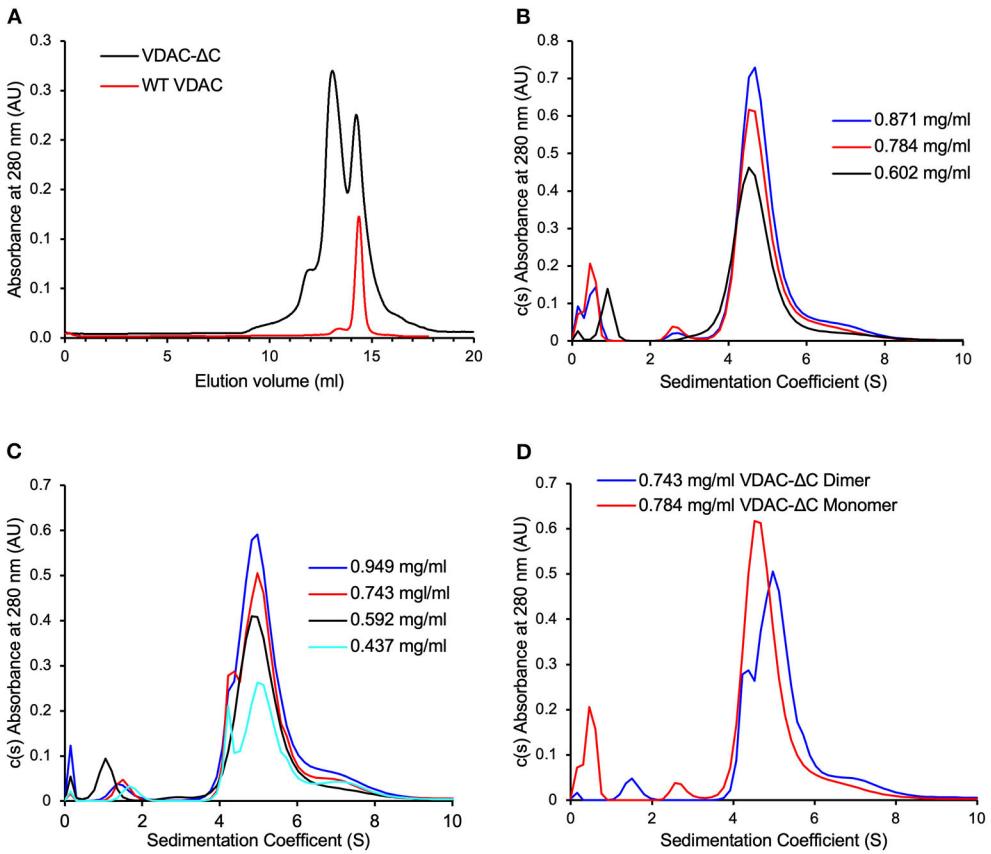

FIGURE 2 | VDAC-ΔC elutes as two major peaks, with the monomers and dimers not returning to equilibration after size exclusion chromatography (SEC) separation. **(A)** WT-VDAC, known to be monomeric, elutes as a single major peak with a similar elution volume to the VDAC-ΔC peak with larger elution volume **(B)** c(s) distribution of the VDAC-ΔC monomer SEC fractions at various VDAC concentrations. **(C)** c(s) distribution of the VDAC-ΔC dimer SEC fractions at various VDAC concentrations. **(D)** Comparison of the VDAC-ΔC monomer and dimer c(s) distributions at similar concentrations, showing differing populations in each sample.

M_w(tM_w) 31 kDa], which was in close agreement with previous AUC experiments that analyzed the WT-VDAC in DM (Ferens et al., 2019). VDAC-ΔC was eluted with a small shoulder peak, followed by two major protein-containing peaks. Of the two major peaks, the lower elution volume peak corresponded to the approximate M_w of a dimer at 69 ± 1 kDa (tM_w 60 kDa), while the higher elution volume peak corresponded to the approximate M_w of a monomer at 37 ± 2 kDa (tM_w 30

kDa). The small shoulder that preceded the two larger peaks had a protein molar mass of 112 ± 2 kDa and, likely, is a small population of VDAC-ΔC tetramers (tM_w120 kDa), although the overlapping dimer peak may be interfering with the accurate mass determination of this species. Furthermore, a downward sloping trend in protein mass across this peak was observed (**Figure 3D**). The deviation of the molar masses determined for the VDAC-ΔC species relative to theoretical values was likely due to the overlapping elution of the three species from the SEC column, which reduced the area of each peak that represented monodispersed species (**Figure 3D**) (See **Supplementary Table 5** for polydispersity values). Regardless, the values determined for the three VDAC-ΔC species were accurate enough to determine the oligomeric states, as the VDAC M_w of 30 kDa was far greater than the discrepancies between the theoretical and observed molar masses.

SEC-Separated VDAC-ΔC Monomers and Dimers Do Not Re-Equilibrate to Identical Populations

As presented above, the SEC elution profiles of VDAC-ΔC contained two major peaks, representing monomers and dimers, which were well defined in the elution profile. This suggested that there was either no equilibrium between the two species or that the rate of transition between monomeric and dimeric forms is very slow. To examine this further, we chose to analyze the SEC-separated monomer and dimer fractions through AUC sedimentation velocity experiments.

Due to the non-ideality caused by weak macromolecular interactions, the overall sedimentation coefficient (S) of noninteracting protein systems usually trends slightly down with increasing protein concentration (Schuck, 2013). In this study, the c(S) distributions of the VDAC-ΔC monomer (**Figure 2B**) and dimer fractions (**Figure 2C**) both showed a slight trend of increasing S with increasing VDAC concentrations, suggesting a concentration-dependent association, notably in the dimer sample (**Figure 2C**), where the resolution between monomer and dimer peaks was clearer. Furthermore, the proportion of monomers and dimers in the distribution seemed to be dependent on the overall VDAC concentration. The distributions of both the monomer and dimer samples contained the same range of species (**Figure 2D**), suggesting that the equilibration between the oligomeric states of VDAC was present; however, the mixture of species in each sample was clearly enriched in either monomer or dimer (**Figure 2D**), suggesting that the rate of exchange was very slow as there had not been a complete equilibration of the same population of species in the monomer and dimer samples at similar VDAC concentrations on the timescale of this experiment.

DISCUSSION

The current work revealed that *N. crassa* VDAC-ΔC can assemble into a functional form *in vivo*. In previous experiments, which used isolated mitochondria and *in vitro*-synthesized VDAC-ΔC, this variant was not imported into protease-resistant conformation mitochondria (Court et al., 1996). Several factors, alone or in combination, could account for this discrepancy. Newly synthesized β-barrel proteins are maintained in import-competent forms by association with Hsp70/Hsp40 family chaperones, which also contribute to their association with the import machinery (Jores et al., 2018). It is possible that either the heterologous (rabbit) chaperones are less effective with the *N. crassa* VDAC or that their concentration was insufficient. Furthermore, even though VDAC-ΔC is at least partially functional, its folded state in the MOM could be more protease-sensitive than that of the wild-type. Finally, the variant of VDAC that was tested in the *in vitro* experiments contained an N-terminal hexahistidinyl-tag (Court et al., 1996), while the variant in *N. crassa* was tag-less, as were the yeast truncation variants (Srivastava and Mahalakshmi, 2020). It is possible that the N-terminal tag interfered with import or generated a more protease-sensitive conformation, even though it did not impair pore function (Popp et al., 1996).

The *in vivo* results obtained with the *N. crassa* VDAC-ΔC agree with those involving $hV2^{18}$ expressed in *S. cerevisiae* (Srivastava and Mahalakshmi, 2020). The latter molecule and those lacking two ($hV2^{17}$) or three ($hV2^{16}$) C-terminal β-strands can partially complement a strain of yeast lacking the primary VDAC, Por1 (Srivastava and Mahalakshmi, 2020). Together, these two studies support the hypothesis that the redundant mitochondrial targeting and assembly information in fungal VDAC is functional. Conversely, the duplication of β19 or of β18 and β19 in $hV2^{20}$ and $hV2^{21}$ led to molecules that more effectively rescue the Δ*POR1* phenotype than do $hV2^{16}$ and $hV2^{17}$ (Srivastava and Mahalakshmi, 2020), presumably because they maintain the native signals in the C-terminal two β-strands.

Biophysical data support the hypothesis that the VDAC expressed in *E. coli* and folded in detergent (recombinant VDAC, $VDAC^R$) adopts a similar structure as that isolated from mitochondria (Ferens et al., 2019). The oligomeric state of $VDAC^R$ is influenced by the presence of sterol in the detergent micelles; a role for sterol in the biological function of VDAC has been predicted since early evidence of sterol copurifying with VDAC (De Pinto et al., 1989) was found and the more recent identification of sterol-binding regions in the detergent-folded mouse VDAC1 (Cheng et al., 2019). One of the five sterol-binding sites in mVDAC1 involves residue L279 in β19, and, if this binding site is present in *N. crassa* VDAC, it would be disrupted by deletion of β19.

Furthermore, *N. crassa* VDAC folded in DM is monomeric in the absence of sterol, and it is unclear whether the sterols promote a structure more amenable to multimerization or alter the character of the micelles such that they promote dimerization (Ferens et al., 2019). In particular, *N. crassa* VDAC is predicted to fold very similarly to vertebrate VDAC (Bay et al., 2012), with studies on human VDAC1 in detergent (Bayrhuber et al., 2008), rat VDAC1 in cultured cells (Geula et al., 2012), and zebrafish VDAC2 in LDAO (Schredelseker et al., 2014) suggesting a common model in which dimerization involves the interfaces produced by β1, β17, β18, and β19. Therefore, it was unexpected that VDAC-ΔC exited as a mixture of monomers and dimers in the absence of sterol (**Figures 2**

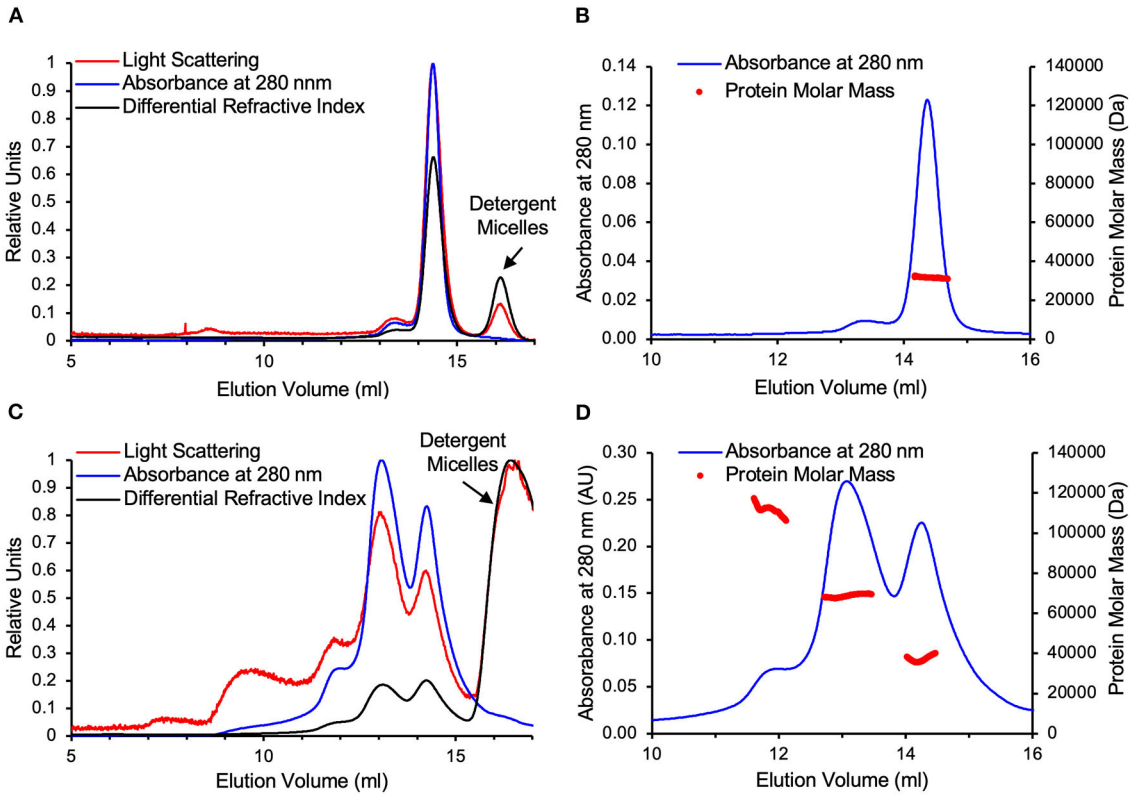

FIGURE 3 | WT-VDAC is monomeric and VDAC-ΔC is composed of monomers and dimers. **(A)** WT-VDAC. SEC with multi-angle light scattering (SEC-MALS) raw data on A relative scale. **(B)** WT-VDAC protein masses across the VDAC peak eluted from the SEC column. **(C)** VDAC-ΔC raw data on a relative scale. **(D)** VDAC-ΔC protein masses across the two major peaks eluted from the SEC column. WT-VDAC and VDAC-ΔC elutions are followed by the elution of excess detergent micelles from the injected samples, which are easily distinguished from VDAC species as they scatter visible light and alter the refractive index but do not absorb UV light. Detergent micelle peaks are indicated in **(A)** and **(C)**.

and **3**). This change in the propensity to form oligomers in DM micelles relative to WT-VDAC (**Figure 3B**) could be due to the creation of a new interaction interface from the assumed interaction of β18 and β1 in antiparallel orientations. This could create a VDAC with a more "bacterial-like" porin architecture. Bacterial porins have been found to form dimers, trimers, and larger oligomers in detergent micelles (Wimley, 2003). Alternatively, the abovementioned sterol-binding site may be interrupted by the VDAC-ΔC deletion. Sterol regulates the WT-VDAC oligomeric state, and the deletion of part of a sterol-binding site may decouple the regulation of the oligomeric state from the binding of sterol molecules. Our AUC analysis suggested that there was some equilibration between VDAC-ΔC species when the SEC-separated monomer and dimer fractions at various concentrations were examined. However, the rate of equilibration was likely to be very slow, which could be due to experimental factors such as the use of detergent micelles as a membrane mimetic system. An obvious complication is the three-dimensional environment of the interaction of the protein-detergent complexes in the solution as compared with the two-dimensional interaction environment of a lipid bilayer. Alternatively, a subset of the VDAC-ΔC molecules in the samples may have been misfolded and, thus, unavailable to form larger species or disassociate into smaller ones. However, the CD spectra of VDAC-ΔC monomer and dimer (**Supplementary Figure 1**) samples suggest that the VDAC-ΔC molecules in these samples are comprised of a predominantly β-strand fold, which is expected of correctly folded VDAC pores.

Both VDAC and its phylogenetic relatives, Tom40 and Mdm10, exist as 19-stranded β-barrels, which are unlike all known bacterial porins [(Pusnik et al., 2009; Flinner et al., 2013), reviewed in (Bay et al., 2012)]. It has been proposed that, rather than being derived from an ancestral bacterial porin, the progenitor of VDAC and Tom40 was assembled in the last common eukaryotic ancestor from a series of ββ-hairpin units. The resulting 20-stranded barrel was involved in the protein import activity that evolved as genes migrated from early mitochondria to the nucleus (Pereira and Lupas, 2018). The N-terminal helical segment has also been proposed to be derived from the first strand of an ancestral 20-stranded β-barrel as the need for a pore regulatory component arose.

The biological advantage of the 19-β-strand form of VDAC was assessed by generating 16–21 stranded versions of hVDAC2 (Srivastava and Mahalakshmi, 2020). As revealed by enthalpy

determinations (ΔH), the 19-β-stranded wild-type form was less stable in bicelles than 18- and 20-stranded variants. Chemical unfolding experiments revealed that the stability of wild-type hVDAC2 was the most sensitive to the environment provided by the lipid head groups. Thus, it was hypothesized that the metastable, 19-stranded molecule allows superior responses to changes in membrane composition, subsequently allowing hVDAC2 to participate in events such as cytochrome *c* release that, in turn, triggers apoptosis. It is also the most compatible with the conductance and gating properties of the molecule (Srivastava and Mahalakshmi, 2020). However, it is unclear whether gating was a selective criterion prior to the divergence of VDAC, Tom40, and Mdm10.

Pore formation and channel gating are among the many functions exhibited by VDAC-ΔC that also are observed in WT-VDAC (Popp et al., 1996; Srivastava and Mahalakshmi, 2020). Herein, it was shown that VDAC-ΔC retains the ability to be imported into mitochondria and inserted into the MOM due to the presence of a second β-signal in β18, which appears to be widely conserved in VDACs from fungi and animals. Furthermore, VDAC-ΔC retains the ability to form dimers and tetramers in detergent micelles, although the conditions under which oligomers form are altered relative to WT-VDAC, which requires the presence of sterol. Thus, VDAC β19 is not essential for pore formation in the MOM; however, it may be necessary for the appropriate self-association of VDAC in response to the surrounding lipid environment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**. Other data is available upon request to the corresponding author.

AUTHOR CONTRIBUTIONS

WS and AB generated the VDAC-ΔC strain and carried out the biological analysis. FF developed the biophysics pipeline, performed the experimental analyses, and analyzed the data. FF, DC, and JS designed the experiments and prepared the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Natural Sciences and Engineering Council of Canada [RGPIN-05930-2016 (DC) and RGPIN-004970-2018 (JS)]. FF was partially supported by the Faculty of Science and the GETS Program, Faculty of Graduate Studies, University of Manitoba (UM). JS is a Canada Research Chair in Structural Biology and Biophysics. We acknowledge the assistance of Richard Adegboyega (UMURA), Emily Ardebol (Faculty of Science URSA), and Nancy Ngyuen (BSc Coop) in collecting and analyzing the VDAC sequences and Max Qually (BSc Honors) for analyzing VDAC-ΔC strains.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.739001/full#supplementary-material

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frontiers in Physiology

REVIEW
published: 26 October 2021
doi: 10.3389/fphys.2021.734226

Historical Perspective of Pore-Forming Activity Studies of Voltage-Dependent Anion Channel (Eukaryotic or Mitochondrial Porin) Since Its Discovery in the 70th of the Last Century

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *30 June 2021*
Accepted: *24 September 2021*
Published: *26 October 2021*

Citation:
Benz R (2021) Historical Perspective of Pore-Forming Activity Studies of Voltage-Dependent Anion Channel (Eukaryotic or Mitochondrial Porin) Since Its Discovery in the 70th of the Last Century. Front. Physiol. 12:734226. doi: 10.3389/fphys.2021.734226

Eukaryotic porin, also known as Voltage-Dependent Anion Channel (VDAC), is the most frequent protein in the outer membrane of mitochondria that are responsible for cellular respiration. Mitochondria are most likely descendants of strictly aerobic Gram-negative bacteria from the α-proteobacterial lineage. In accordance with the presumed ancestor, mitochondria are surrounded by two membranes. The mitochondrial outer membrane contains besides the eukaryotic porins responsible for its major permeability properties a variety of other not fully identified channels. It encloses also the TOM apparatus together with the sorting mechanism SAM, responsible for the uptake and assembly of many mitochondrial proteins that are encoded in the nucleus and synthesized in the cytoplasm at free ribosomes. The recognition and the study of electrophysiological properties of eukaryotic porin or VDAC started in the late seventies of the last century by a study of Schein et al., who reconstituted the pore from crude extracts of *Paramecium* mitochondria into planar lipid bilayer membranes. Whereas the literature about structure and function of eukaryotic porins was comparatively rare during the first 10 years after the first study, the number of publications started to explode with the first sequencing of human Porin 31HL and the recognition of the important function of eukaryotic porins in mitochondrial metabolism. Many genomes contain more than one gene coding for homologs of eukaryotic porins. More than 100 sequences of eukaryotic porins are known to date. Although the sequence identity between them is relatively low, the polypeptide length and in particular, the electrophysiological characteristics are highly preserved. This means that all eukaryotic porins studied to date are anion selective in the open state. They are voltage-dependent and switch into cation-selective substates at voltages in the physiological relevant range. A major breakthrough was also the elucidation of the 3D structure of the eukaryotic pore, which is formed by 19 β-strands similar to those of bacterial porin channels. The function of the presumed gate an α-helical stretch of 20 amino acids allowed

further studies with respect to voltage dependence and function, but its exact role in channel gating is still not fully understood.

Keywords: mitochondria, eukaryotic pore, VDAC, single channel, porin, electrophysiology, evolution

ORIGIN OF BACTERIA AND CELL ORGANELLES

The age of earth is around 4.5 billion years. Stromatolites represent presumably the oldest fossils that date back to something like 3.7 billion years (Garwood, 2012; Nutman et al., 2016). How life on earth started is still a matter of debate. One hypothesis suggests that molecules serving as the basis of life formed from atoms under the input of energy from different sources as proposed by the Miller-Urey experiment (Miller, 1953; Osinski et al., 2020; Takeuchi et al., 2020). Cell-like particles may have formed in the Hadean eon within such a primordial broth from a variety of small molecules. They represent the origin of life, which began about 4 billion years ago (Garwood, 2012; Takeuchi et al., 2020). The first organisms on earth are not known and they did not leave any evidence of their live. What we know are the oldest fossils in the form of sedimentary rocks, known as stromatolites (Margulis et al., 1986; Nutman et al., 2016). They were formed by microbial communities containing photosynthetic bacteria and cyanobacteria and represent the oldest sign for the existence of photosynthesis (Awramik, 1992; Gérard et al., 2009). It is possible that these bacteria were Gram-negative, which means that the cytoplasm was surrounded by two membranes. Their outer membranes may be considered as permeability barriers similar to those of modern Gram-negative bacteria and the mitochondrial outer membrane.

The Last Universal Common Ancestor (LUCA) is a bacteria-like organism, which existed before the bacterial cell lineage divided into the different kingdoms (Theobald, 2010; McInerney, 2016; Weiss et al., 2016). The LUCA is joint ancestor of bacteria and archaea and existed presumably within the time of begin of earth and the oldest fossils, which means that the division of the cell lineage occurred about 4 billion of years ago (Di Giulio, 2011). No fossils of this organism are known but the comparison of the genomes of all modern organisms allowed the identification of a set of about 355 genes, which could have been present in the LUCA (Weiss et al., 2016). The genes code for proteins involved in energy metabolism, synthesis of amino acids, and the equipment for transcription and translation of protein synthesis (Chioccioli et al., 2020; Koonin et al., 2020). It seems to be possible that the life of LUCA depended on hydrogen and metals favoring a deep sea vent environment for its existence (Weiss et al., 2016). Nevertheless, also other environments for the existence of LUCA are possible, for example, in ponds of different salinity and pH and in an N_2-CO_2 atmosphere together with dry-wet cycles and UV light (Sasselov et al., 2020). The LUCA divided subsequently into the different kingdoms of life: bacteria, eukaryote, and archaea.

THE ENDOSYMBIOTIC THEORY AND THE DEVELOPMENT OF MITOCHONDRIA FROM STRICTLY AEROBIC BACTERIA

A similar concept as described for the LUCA exists also for the LECA, the Last Eukaryotic Common Ancestor (Hedges, 2002; Koonin, 2015; O'Malley et al., 2019). The first eukaryotic cell represents presumably a genomic hybrid of bacteria and archaea (Katz, 2012; Grau-Bové et al., 2015). Special for the LECA is its endosymbiotic capacity because between one and 2 billion years ago specialized Gram-negative bacteria were taken up. This provided a considerable advantage for the eukaryotic host because its energy metabolism was characterized before endosymbiosis by anaerobic fermentation and is now complemented with cellular respiration (Margulis, 1981). The endosymbionts were presumably α-proteobacteria and cyanobacteria (Gray, 2012; Degli, 2014; Nowack and Weber, 2018). This can be concluded from the homology of aerobic respiration between mitochondria and α-proteobacteria and of photosynthesis between chloroplasts and cyanobacteria (Martijn et al., 2018; Nowack and Weber, 2018).

Besides the genes coding for the respiration chain, there exist also other indications for the close relation of mitochondria with Gram-negative bacteria. All proteins residing in the outer mitochondrial membrane and in the intermembrane space are encoded in the nucleus and synthesized on cytoplasmic ribosomes of the host cell and are imported post-translationally into mitochondria with modifications of amino acids in particular of cysteines (Saletti et al., 1859; Grevel et al., 2019). This suggests that since the event of endosymbiosis about 1–2 billion years ago many genes of the protomitochondrion came under control of the eukaryotic host. The mitochondrial proteins produced in the cytoplasm of the host cells are imported *via* the mitochondrial outer membrane import TOM complex into the intermembrane space using a not yet fully identified import signal, which is presumably related to the β-strands of the imported proteins (Bausewein et al., 2020; Drwesh and Rapaport, 2020; Moitra and Rapaport, 2021). This is based on the observation that the β-hairpin element of eukaryotic porins interacts with the mitochondrial import receptor Tom20 (Jores et al., 2016). From there, the mitochondrial outer membrane proteins are assembled by the multi-subunit TOB/SAM system (sorting and assembly machinery) in the mitochondrial outer membrane with the help of the hexamer of the small TIM chaperones (Moitra and Rapaport, 2021). The SAM system has a high homology to the barrel-assembly machinery BAM of Gram-negative bacteria, which provides also evidence that Gram-negative bacteria were ancestors of mitochondria (Pereira and Lupas,

2018; Diederichs et al., 2020, 2021; Takeda et al., 2021). Accordingly, the bacterial outer membrane represents the ancestor of the mitochondrial outer membrane. However, whereas the porins of the bacterial outer membranes have only passive sieving properties in bacterial metabolism, it seems that the mitochondrial outer membrane including the eukaryotic porin or Voltage-Dependent Anion Channel (VDAC) plays also an active and important role in mitochondrial and cellular metabolism (Benz, 1994a,b; Shoshan-Barmatz et al., 2006, 2010; Gatliff and Campanella, 2012; Grevel and Becker, 2020). It binds different kinases (Fiek et al., 1982; Brdiczka, 1990; Adams et al., 1991; De Pinto et al., 2003) and evidence has been provided that eukaryotic porins play also an important role in mitochondria-mediated apoptosis, protein translocation, and are also involved in response to drugs (Shoshan-Barmatz et al., 2010; Grevel et al., 2019; Grevel and Becker, 2020). This applies also to its interaction with the 18 kDa translocator protein, also known as peripheral benzodiazepine receptor, which mediates cholesterol transport between mitochondrial membranes, cytochrome C release, and apoptosis (Gatliff and Campanella, 2012; Bader et al., 2019; Betlazar et al., 2020).

A VOLTAGE-DEPENDENT PORE WAS DISCOVERED IN CRUDE EXTRACTS OF PARAMECIUM MITOCHONDRIA

The most interesting property of eukaryotic porins or VDACs is their voltage dependence, which is clearly no reconstitution artifact. The voltage dependence of eukaryotic porins was first discovered in extracts of *Paramecium aurelia* mitochondria by Schein et al. (1976). They dissolved mitochondrial-rich fractions from *Paramecium* supplemented with asolectin in hexane and used this lipid-protein mixture for membrane formation. This means that they spread the crude protein-lipid mixture on the surface of electrolyte solutions in a Teflon cell. Membranes were then formed from the protein-lipid layers across a small hole in a thin Teflon by the Montal-Mueller method (Montal and Mueller, 1972). Schein et al. (1976) observed frequently high conductance channels/pores in these solvent-depleted membranes. The number of reconstituted channels in the folded membranes was somewhat dependent how much from the mitochondria-rich fractions were present in the lipid-protein mixtures (Schein et al., 1976). Highest pore-forming activity was observed in fractions 50–55 of the sucrose gradient. The open channels had a conductance of 200 pS in an asymmetric solution of 20 mM $MgCl_2$ versus 20 mM $CaCl_2$, or 750 pS in an asymmetric solution of 1 M KCl versus 0.1 M KCl (Schein et al., 1976). The current-voltage behavior of the channel was ohmic, resulting in a linear current-voltage curve (Schein et al., 1976).

At voltages smaller than 10 mV, the channels were frequently in the "open" configuration. However, they switched a higher voltages in closed configurations, which could be observed in experiments when the voltage was switched to ±20 mV. The maximum voltage dependence was reached at about ±40 to ±50 mV transmembrane potential and appeared to be symmetric with respect $V_m = 0$ mV (Schein et al., 1976). **Figure 1** shows an example of the voltage dependence of human eukaryotic porin 1 (hVDAC1) reconstituted into solvent-containing membranes made of diphytanoyl phosphatidylcholine/n-decane membranes. The experiment started by an application of +10 mV to about 50 reconstituted hVDAC1 pores, followed by application of −10 mV. At ±20 mV applied to the membrane, the current through the pores started to decrease, higher positive, and negative voltages resulted in a stronger decrease of the current and a faster exponential decay from the initial current to the final current level at longer times (see **Figure 1**). The closing of the eukaryotic porin is a relatively slow process as **Figure 1** clearly demonstrates. The invers process, i.e., the reopening of the pores, when the voltage is switched off is much faster, which means that it is difficult to measure it precisely (Schein et al., 1976). It is sometimes so fast that it cannot be resolved properly (Colombini, 1979; Benz, 2004).

The decrease of the current at higher positive and negative voltages than ±10 mV could be analyzed using a similar approach as proposed by Schein et al. (1976) assuming a Boltzmann distribution of the open and closed states of the pore:

$$N_o / N_c = \exp\left(\frac{nF(V_m - V_0)}{RT}\right) \quad (1)$$

Where F, R, and T are Faraday's constant, gas constant, and absolute temperature, respectively; n is the number of gating charges moving through the entire membrane potential. V_0 is the midpoint potential, where one half of the pores are open and the other half are closed, i.e., $N_o / N_c = 1$. The open to closed ratio of the pores is given by the analysis of the experimental results of experiments similar to **Figure 1** according to:

$$N_o / N_c = (G - G_{\min}) / (G_0 - G) \quad (2)$$

G is the membrane conductance at a given membrane potential V_m. G_0 and G_{min} are the conductance at zero voltage, when all pores are in the open configuration and when all pores are in the closed one at very high voltage, respectively. The use of the Boltzmann distribution allows also the derivation of the activation energy for the voltage-dependent gating process of eukaryotic porins (Schein et al., 1976). The activation energy is given by $W(V_m)$, which is equivalent to the energy of one mole pores between the open and the closed configuration, i.e., it has the form (Schein et al., 1976):

$$N_o / N_c = \exp\left(-\frac{W(V_m)}{RT}\right) \quad (3)$$

Comparison of Eqs. (1) and (3) shows that $W(V_m)$ is given by $nF(V_m\text{-}V_0)$, which means that the activation energy nFV_0 is about 5.8 kJ/mol (1.38 kcal/mol), which is a very low energy

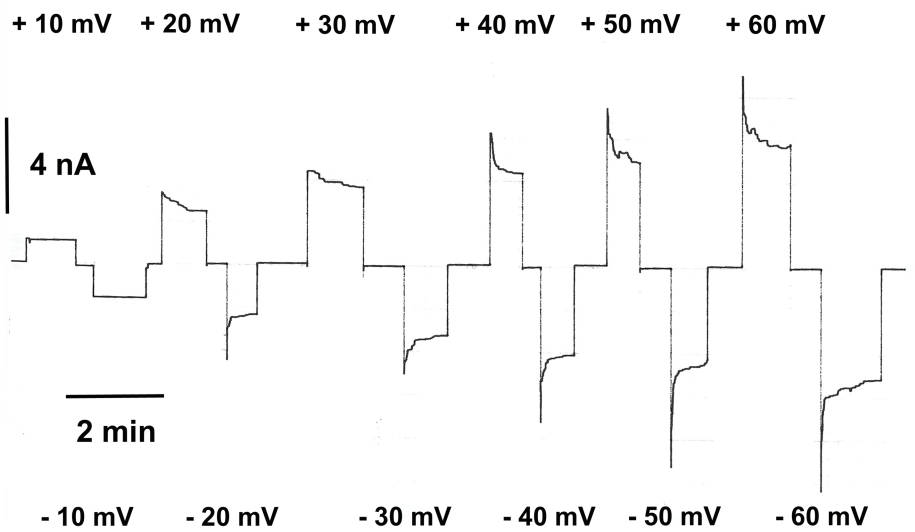

FIGURE 1 | Relaxation of the membrane current in the presence of eukaryotic porin 1 from humans (hVDAC1, Porin 31HL; Benz et al., 1992). The membrane potential was first switched to +10 mV and then to −10 mV applied to the cis-side of the membrane containing about 50 hVDAC1 pores. Note that the membrane current did not decrease at these voltages. Then, higher positive and negative voltages were applied which resulted in a substantial exponential decrease of the membrane current. The membrane was formed of diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 0.5 M KCl (pH 7.2); T = 20°C.

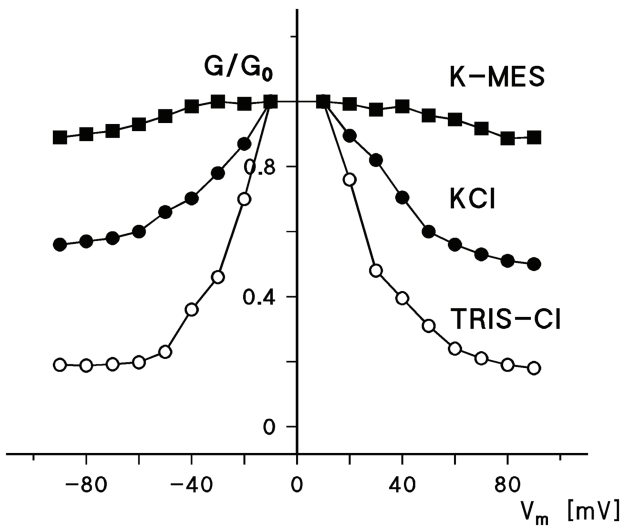

FIGURE 2 | Ratio of the conductance, G, at a given voltage, V_m, divided by the conductance, G_0, at 10 mV as a function of the voltage. The aqueous phase contained either 0.5 M KCl, 0.5 M K-MES, or 0.5 M TRIS-HCl (pH in all cases 7.2). The cis-side contained about 10 ng/ml hVDAC1 [Porin 31 HL (Benz et al., 1992)]. The sign of the voltage is given with respect to the cis-side, the side of the addition of Porin 31HL.

that is needed to shift the eukaryotic pores from the open into the closed configuration.

Figure 2 shows the ratio of the conductance, *G*, at voltages between ±10 and ± 90 mV divided by the initial conductance G_0 as a function of the applied voltage for experiments similar to that shown in **Figure 1** for 0.5 M KCl, 0.5 M K-MES, and 0.5 M TRIS-Cl (mean values of three experiments taken under the same conditions). The combination of the cations and anions was chosen to show the voltage dependence of human eukaryotic porin 1 (hVDAC1, Porin 31HL) in dependence of cations and anions of different mobility because this provides not only some information on voltage dependence but also on ion selectivity of the open and closed states of the pore. It is obvious that the voltage dependence was in all cases similar. However, G/G_0 was found to be dependent on the type of the cation and anion present in the aqueous solution, indicating a selectivity change when the pores switched in the closed configuration at higher voltages. The exact value for the permeability ratio of potassium and chloride was difficult to obtain because the mobility of TRIS and MES inside the pore is not known. However, because of the comparably small mobility of TRIS and MES in the aqueous phase, it is possible that P_{cation}/P_{anion} of the closed hVDAC1 pore is very high (around 10) in contrast to the selectivity of the open pore where P_{cation}/P_{anion} is about 0.5 (Benz et al., 1992). The selectivity of the closed state may be even higher if it is impermeable for anions. On the other hand, it is also evident from **Figure 2** that potassium is almost equally mobile through the open and the closed state, because of the low mobility of MES in the aqueous phase. This represents another proof that the closed state has completely different properties for the permeation of charged solutes than the open state.

The voltage dependence of the pores formed by Porin 31HL (hVDAC1) can be analyzed using Eq. (1) and semilogarithmic plots of the ratio N_0/N_c as a function of the transmembrane potential, V_m, calculated from the experimental results according to Eq. (2) as shown in **Figure 3**. The slope of the straight line for the application of negative voltages for an e-fold change in N_0/N_c was about 12.5 mV, which suggested that the number of charges involved in the gating process was approximately

2.0. A similar analysis for positive voltages resulted in a slope for an e-fold change of N_0/N_c of 11.9 mV, which suggested that the gating charge of the right branch of the voltage dependence of hVDAC1 is about 2.1. The midpoint potentials of the two N_0/N_c distributions for negative and positive voltages with respect to the addition of porin 31HL (human eukaryotic porin 1) were − 27.4 mV and + 35 mV, respectively. This indicated a slight asymmetry in the midpoint potential, V_0, where the number of open and closed channels was balanced, i.e., $N_0/N_c = 1$. It is noteworthy that the voltage dependence of eukaryotic porin (VDAC) in the first study of *Paramecium* mitochondria exhibited a much higher voltage dependence because n was about 4.5 and the midpoint potentials of N_0/N_c were around ±20 mV (Schein et al., 1976). In a more recent study of eukaryotic porin of *Paramecium tetraulia*, where the eukaryotic porin was purified to homogeneity, the voltage dependence of the reconstituted pore was lower with about 2 gating charges and a midpoint potential for N_0/N_c of 32 mV (Ludwig et al., 1989).

The voltage dependence of eukaryotic porins from a variety of eukaryotic organisms was investigated in detail in many studies: *Paramecium* (Schein et al., 1976; Doring and Colombini, 1984; Ludwig et al., 1989); Mammals: rat (Roos et al., 1982; Colombini, 1983; Ludwig et al., 1986), rabbit (De Pinto et al., 1987a), bovine (De Pinto et al., 1987a), pig (De Pinto et al., 1987a), and human brain (Bureau et al., 1992); Fish: *Anguilla anguilla* (De Pinto et al., 1991c); Plants: potato (Heins et al., 1994; Lopes-Rodrigues et al., 2020), pea (Fischer et al., 1994), corn (Smack and Colombini, 1985; Aljamal et al., 1993; Fischer et al., 1994), wheat (Blumenthal et al., 1993), and pea root plastid porin (Fischer et al., 1994; Popp et al., 1997); Other organisms: *Neurospora crassa* (Freitag et al., 1982c), yeast (Ludwig et al., 1988), and *Dictyostelium* (Troll et al., 1992); and Flies: *Protophormia* (Wiesner et al., 1996) and *Drosophila* (De Pinto et al., 1989a; Aiello et al., 2004; Komarov et al., 2004). Common to all of these studies is that the eukaryotic porins of all these eukaryotes formed high-conducting channels in reconstituted systems. They were all in their open configuration at small transmembrane voltages smaller or equal to 10 mV (Benz, 1994b). At higher voltages, they switched into substates. The analysis of the voltage dependence in terms of the above used formalism showed that the number of gating charges for almost all pores formed by these eukaryotic porins was around two, which means that an e-fold change in N_0/N_c occurred, when the voltage across the membrane was changed by about 12 mV (De Pinto et al., 1987a; Benz, 1994b, 2004). The midpoint potential for the distribution of the open and closed pores (i.e., $N_0 = N_c$) was in many cases either symmetrical or slightly asymmetrical with values around ±30 mV to ±40 mV (De Pinto et al., 1987a; Benz, 1994b, 2004).

ISOLATION AND PURIFICATION OF EUKARYOTIC PORINS

Schein et al. (1976) had already the idea that voltage-dependent pore was present in the mitochondrial outer membrane of the *Paramecium* mitochondria, because of its high permeability. This was revealed in a study by Colombini (1979), where he could explicitly show that the pore-forming activity came from the outer membrane of rat liver mitochondria, but not from fractions containing inner membranes. The pore had a conductance of 0.45 nS and 4.5 nS in 0.1 and 1 M KCl, respectively. The first biochemical evidence for the identity of the pores in the mitochondrial outer membranes of rat liver and mung beans was provided by Hiroshi Nikaido and coworkers (Zalman et al., 1980). In analogy to their work with bacterial porins, they were able to reconstitute fragments of the mitochondrial outer membranes into vesicles from soybean lipids and demonstrated that the vesicles became permeable for low molecular mass carbohydrates but not for high molecular mass dextrans. Following different biochemical procedures, Zalman et al. (1980) were able to identify a protein in the mitochondrial outer membranes of mung bean mitochondria with a molecular mass of about 30 kDa, which obviously was responsible for the permeability properties of the reconstituted vesicles. It is quite difficult to establish a potential across vesicles membranes or to study the permeability of charged solutes in the liposome system, which means that the putative voltage dependence of the pore could not be studied. Nevertheless, the diffusion of carbohydrates with a molecular mass up to 8 kDa suggested indeed that Zalman et al. (1980) identified the eukaryotic porin of mung beans as a general diffusion pore.

A similar study was performed by Mihara et al. (1982) to identify yeast porin. They isolated yeast porin as a 29 kDa polypeptide from the mitochondrial outer membranes of yeast mitochondria. To verify their results, they demonstrated that yeast porin was not accessible for protease treatment as long the protein was localized in the mitochondrial outer membrane (Mihara et al., 1982). When the protease digestion was performed in the presence of detergent yeast porin was no longer protected. They were also the first to notice that *in vitro*-synthesized yeast porin using yeast total RNA had the same molecular mass as the native protein and did not exhibit any additional leader sequence. It was incorporated directly into intact mitochondria and not into rough endoplasmic reticulum (Mihara et al., 1982). A membrane potential across the inner mitochondrial membrane was not important for this process (Mihara et al., 1982). A similar conclusion was obtained from the import of porin from *N. crassa* synthesized in homologous or heterologous cell-free systems into mitochondria (Freitag et al., 1982b).

The identification of other eukaryotic porins also proceeded at the same time. Roos et al. (1982) were the first to identify a mammalian eukaryotic porin from rat liver. Rat liver mitochondria were sub fractionated. When the OM fraction obtained by centrifugation steps was treated with detergent it showed pore-forming activity in artificial lipid bilayer membranes (Roos et al., 1982). Rat liver porin was identified as a 32 kDa protein using different biochemical methods and the reconstitution of the protein into artificial lipid bilayers. Rat liver porin formed voltage-dependent pores in lipid bilayers with a single-channel conductance of about 4 nS in 1 M KCl, which is typical for eukaryotic porins (Colombini, 1979, 1980, 1983). The molecular mass of rat liver porin was confirmed

by other groups (Lindén et al., 1982a; Colombini, 1983). However, in contrast to a putative purification of rat liver porin by a Concanavalin A-containing column, eukaryotic porins are pure polypeptides (Benz, 1994b), which means that the 300-fold purification of rat liver porin (rVDAC) by chromatography across this column has presumably nothing to do with eukaryotic porins as glycoproteins Colombini, 1980, 1983).

The purification of eukaryotic porins until 1982 was always dependent on the fractionation of the mitochondrial membranes using different methods, such swelling and shrinking of mitochondria followed by density gradient centrifugation (Roos et al., 1982; Lindén et al., 1982a). This procedure and related methods were always accompanied by a substantial loss of outer membrane material because it is in part tightly associated with the mitochondrial inner membrane (van der Laan et al., 2016). This means that it was a considerable breakthrough when eukaryotic porins could be isolated from detergent-solubilized whole mitochondrial membranes of *N. crassa* by using the method of Freitag et al. (1982a). In a first step, mitochondria were lysed by an osmotic shock and the total mitochondrial membranes were obtained by centrifugation. Next, the detergent-solubilized mitochondrial membrane proteins were applied to a dry hydroxyapatite (HTP) column and the eluate was passed in a second step through a dry HTP/celite column in a ratio of 1:1 (w/w). Using this method, *N. crassa* porin was almost pure (Freitag et al., 1982a). Following the isolation and purification of different eukaryotic porins, the method was refined (De Pinto et al., 1987b). Finally, the mitochondrial membrane proteins were dissolved in 3% Triton X-100 using a low protein/detergent ratio and then passed only once through a dry HTP/celite column in a ratio of 2:1 (w/w) (De Pinto et al., 1987b). This procedure resulted in eukaryotic porins in particular from mammals of high purity and was successfully applied many times to the purification of eukaryotic porins by the Bari/Catania group of research into mitochondria (Ludwig et al., 1986, 1988; De Pinto et al., 1987a,b; Carbonara et al., 1996). This easy purification method allowed also further investigations of structure and function of eukaryotic porins and their interaction with different detergents (De Pinto et al., 1989b, 1991a,b; De Pinto and Palmieri, 1992).

PRIMARY SEQUENCES OF EUKARYOTIC PORINS

The first two primary sequences of eukaryotic porins that became known were those of yeast and *N. crassa* (Mihara and Sato, 1985; Kleene et al., 1987). Mammalian porins could not be sequenced from their cDNA at that time because their sequence is only distantly related to the porins of the microorganisms despite a similar length and a relative large fraction of hydrophilic amino acids. That was possible, when human porin (hVDAC1, porin 31HL) was sequenced by direct amino acid sequencing (Kayser et al., 1989). Shortly after, eukaryotic porins from higher eukaryotic cells could be cloned in different organisms, such as mouse and humans (Blachly-Dyson et al., 1993, 1994; Ha et al., 1993; Sampson et al., 1996). Mammalian genomes contain the genes coding for three VDAC species. The genes have the same exon-intron structure (Young et al., 2007). The proteins have the same length but they exhibit some differences, in particular in the number of cysteines. The differences in the primary sequence of the three VDAC isoforms in mammals did not alter the structure of the splicing sequences and the organization of the three genes (Pinto and Messina, 2004; Young et al., 2007; De Pinto, 2021). The most abandoned version of the VDAC isoforms is VDAC1, which was extensively studied *in vivo* and *in vitro* (Benz, 1994b, 2004; Báthori et al., 2006; Shoshan-Barmatz et al., 2020). However, also the other two human isoforms were studied in recent years (Checchetto et al., 2014; Gattin et al., 2015; Queralt-Martín et al., 2020). The results of these studies were to some extent controversial, because hVDAC3 was a small channel-forming component in one of the studies (Checchetto et al., 2014), whereas it has in a more recent study quite normal electrophysiological properties (Queralt-Martín et al., 2020). This means that the three human eukaryotic porins have a similar single-channel conductance (see **Table 1**) and the pores formed by the three human isoforms are all voltage-dependent with some modifications (Benz, 2004; Gattin et al., 2015; Queralt-Martín et al., 2020). The expression of the isoforms may be tissue-specific but their function in mitochondrial outer membrane permeability and in other cellular important functions, such as apoptosis, is still a matter of debate (De Pinto et al., 2016; Shoshan-Barmatz et al., 2020; Shimizu et al., 2021). Three genes coding for analogs of VDAC1 have not only be found in mammals but also in the genome of the fruit fly *Drosophila melanogaster* (Aiello et al., 2004; Komarov et al., 2004). All of them with one exception code for pore-forming proteins with properties similar to most eukaryotic porins with some modification of the voltage dependence (Aiello et al., 2004; Komarov et al., 2004). Careful analysis of the genes and their comparison with those of other eukaryotic porins from insects suggested that the genes evolved by duplication from an ancestral gene (Komarov et al., 2004).

The genetic organization of eukaryotic porins in plants appears to be even more complicated (Kusano et al., 2009; Homblé et al., 2012). The genome of the popular model organism in plant biology and genetics, *Arabidopsis thaliana,* contains at least four or five genes coding for eukaryotic porin-like proteins (Lee et al., 2009; Tateda et al., 2011; Berrier et al., 2015). A similar number of eukaryotic porin genes has been found in *Lotus japonicus* and soybean, where also up to five genes were found (Wandrey et al., 2004). Localization analysis of the different isoforms of eukaryotic porins in *Arabidopsis* indicated specific functions of the porins including DNA and RNA transport (Tarasenko et al., 2021). Some of the plant porins have similar subcellular localizations (Tateda et al., 2011, 2012). Knockout mutants of AtVDAC2 and AtVDAC4 show despite similar subcellular localizations severe defects in growth indicating that these eukaryotic porins have an important function in *Arabidopsis* (Tateda et al., 2012). Many eukaryotic porins from plants have been studied in lipid bilayer membranes (see **Table 1**). Their single-channel conductance seems to be a little smaller than those of porins from other organisms, but plant porins show similar voltage dependences as most eukaryotic porins with some modifications. From the many porins of

TABLE 1 | Single-channel conductance of eukaryotic (mitochondrial) porins (VDACs) from different eukaryotic organisms in 1 M KCl, pH 6, if not indicated otherwise.

Eukaryotic porin (VDAC)	G (nS)	References
Human VDAC1 (Porin 31HL)	4.3	Benz et al., 1992
	4.1	Blachly-Dyson et al., 1993
Human VDAC2	4.0	Blachly-Dyson et al., 1993
	2.0 and 4.0	Gattin et al., 2015
Human VDAC3	3.9	Queralt-Martín et al., 2020
Rat liver	4.3	Roos et al., 1982
Beef heart	4.0	Benz et al., 1985
Rabbit liver	4.0	Benz et al., 1985
Rat brain	4.0	De Pinto et al., 1987a
Rat kidney	4.0	De Pinto et al., 1987a
Pig heart	3.5	De Pinto et al., 1987a
Anguilla anguilla	4.0	De Pinto et al., 1991c
Drosophila melanogaster VDAC	4.5	De Pinto et al., 1989a
CG17140	3.4/1 M NaCl	Komarov et al., 2004
Protophormia	4.5	Wiesner et al., 1996
Neurospora crassa	4.5	Freitag et al., 1982c
Yeast	4.5	Forte et al., 1987a
	4.2	Ludwig et al., 1988
	4.2	Blachly-Dyson et al., 1993
Paramecium	4.5	Colombini, 1979
	2.4	Ludwig et al., 1989
Pea mitochondria	1.5 and 3.7	Schmid et al., 1992
Pea root plastids	1.5 and 3.7	Fischer et al., 1994
Maize root plastids	1.5 and 3.7	Fischer et al., 1994
Solanum tuberosum POM 34	2.0 and 3.5	Heins et al., 1994
Maize mitochondria	1.5 and 3.7	Carbonara et al., 1996
Phaseolus coccineus	3.7	Krammer et al., 2014
Arabidopsis	0.5/300 mM KCl	Berrier et al., 2015

The pores were measured at low transmembrane potentials where almost all pores should be in their open configuration. All pores formed by these proteins were anion selective in their open state (i.e., at small transmembrane voltage). If not indicated otherwise, the single-channel conductance of the eukaryotic porins refers to VDAC1.

Arabidopsis, only AtVDAC3 has been studied in lipid bilayers in some detail (Berrier et al., 2015). Again, its properties were quite similar as found for most eukaryotic porins,

IDENTIFICATION OF EUKARYOTIC PORIN AS DICYCLOHEXYLCARBODIIMIDE (DCCD)-BINDING PROTEIN IN THE MITOCHONDRIAL OUTER MEMBRANE

When pig heart mitochondria are treated with low doses (1.5 nmol/mg of mitochondrial protein) of C14-labeled dicyclohexylcarbodiimide (DCCD), three mitochondrial polypeptides of approximately 9, 16, and 33 kDa bound DCCD (Houstěk et al., 1981; De Pinto et al., 1985). The two smaller DCCD-binding proteins are parts of the F_0F_1 ATPase localized in the mitochondrial inner membrane (Houstěk et al., 1981). The 33 kDa DCCD-binding protein present in the mitochondrial outer membrane of pig heart was identified as the eukaryotic porin based on biochemical evidence and electrophysiological experiments although DCCD-binding did not change the characteristics of the pore (De Pinto et al., 1985). However, labeling of porin with DCCD resulted in the loss of hexokinase binding to porin (Nakashima et al., 1986; Nakashima, 1989), because porin is the hexokinase-binding protein (Fiek et al., 1982; Lindén et al., 1982b). Fifty percent inhibition of hexokinase binding occurred at very low levels of DCCD by less than 2 nmol of DCCD/mg of mitochondrial protein (Nakashima, 1989). Water-soluble carbodiimides had no effect on hexokinase binding on porin, indicating that the binding place was in a hydrophobic environment. DCCD-binding to proteins suggested that a negatively charged amino acids exist in a hydrophobic environment (De Pinto et al., 1985; Nakashima, 1989). This amino acid was identified as glutamate 72 in the sequence of bovine heart eukaryotic porin (De Pinto et al., 1993). The role of this negative charge in mitochondrial metabolism of the three VDAC isoforms in Zebrafish was studied in detail recently because the homologous glutamate 73 is present in VDAC1 and VDAC2 but not in VDAC3 and plays an important role in regulation of Ca2+ uptake (Shimizu et al., 2021). Diafenthiuron is an acaricide and insecticide developed by Ciba-Geigy that cannot longer be used as a pesticide because of its toxicity (Kayser and Ellinger, 2001). Its active form is the carbodiimide CGA 140'408, which labels also components of the mitochondrial ATPase together with the eukaryotic porin of the fly *Protophormia* (Wiesner et al., 1996). Reconstitution experiments with the CGA 140'408-modified porin of *Protophormia* showed also no significant effects on the characteristics of channel formation by *Protophormia* porin similar as described above for the binding of DCCD to pig heart porin (De Pinto et al., 1985; Wiesner et al., 1996).

RECONSTITUTION OF EUKARYOTIC PORINS IN LIPID BILAYER MEMBRANES

The first reconstitution of eukaryotic porin (VDAC) occurred *via* the enrichment of mitochondrial particles from *Paramecium* mitochondria with endogenous asolectin followed by the formation of solvent-depleted membranes according to the Montal-Mueller method (Montal and Mueller, 1972). For this, the protein-lipid mixtures were spread with hexane on the aqueous surface of the membrane cell for membrane formation (Schein et al., 1976). The pores were present in the membranes immediately after its formation. The number of pores incorporated into the membranes by this method depended on the concentration of porin in the protein-lipid mixtures (Schein et al., 1976). The reconstitution of the eukaryotic porins occurred late on similarly as the incorporation of bacterial porins into lipid bilayers (Benz et al., 1978). After isolation and purification of eukaryotic porins, they were added to the aqueous electrolyte solution bathing preexisting membranes (Colombini, 1979; Roos et al., 1982; Freitag et al., 1982c) or to preexisting lipid vesicles (Zalman et al., 1980; Mihara et al., 1982). The characteristics of the pores formed by eukaryotic porins were not dependent on the method of bilayer formation, either painted of folded,

although this was occasionally claimed (Colombini, 1983). It seems that these pores, similar as bacterial porins form their own sphere and their properties, are only little dependent on the lipid environment in the membranes.

Figure 4 shows the stepwise increase of membrane current when a eukaryotic porin (Porin 31HL) was added to the aqueous 1 M KCl solution bathing a painted black lipid membrane from diphytanoyl phosphatidylcholine/n-decane. The steps were mostly directed upward at low transmembrane potential, as it was already outlined above, when the voltage dependence of the same eukaryotic pore was discussed. The single-channel conductance of Porin 31HL was under these conditions about 4 nS (see the histogram of the current fluctuations obtained with Porin 31HL).

The histogram of all current fluctuations observed with Porin 31HL showed two maxima for pore distribution (Benz et al., 1992). One is centered on 4 nS and the other one around 2 nS (**Figure 5**). The higher single-channel conductance refers presumably to the first opening of a Porin 31HL pore by reconstitution of one pore protein into the membrane. The lower conductance represents pores that adopted a sublevel of conductance and reopened again, i.e., these pores reflect the closed state of the Porin 31HL pore, which seems to be around 2 nS.

Similar experiments were performed with many eukaryotic porins in different research groups. Typical for the pores formed by the eukaryotic porins is an open state between 4 and 4.5 nS at small voltages. However, histograms of all current fluctuation show also pores around 2 nS, which could refer to substates of the open pore. Plant porins have a somewhat smaller conductance just a little below 4 nS at low voltage. In the case of plant porins, also a second maximum was observed at 1.5 nS. The hypothesis is that this maximum also referred to the closed state or voltage-gated substates of the mitochondrial pore.

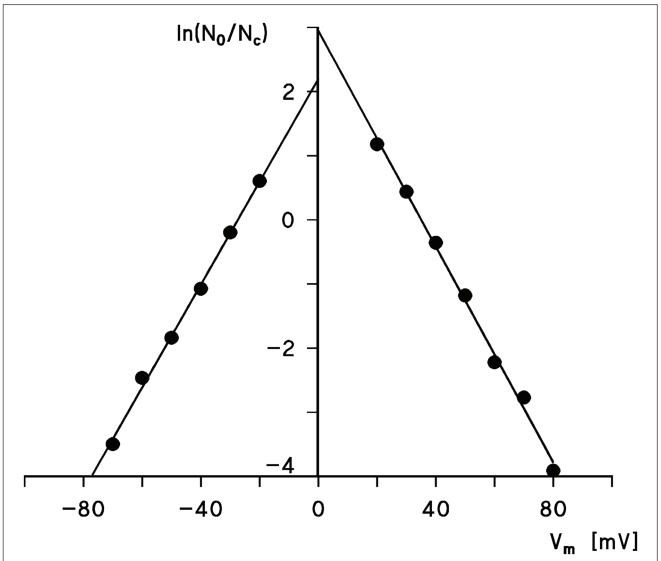

FIGURE 3 | Semilogarithmic plot of the ratio, *N0/Nc*, as a function of the transmembrane potential V_m. The data were taken from **Figure 2**. The slope of the straight lines is such that an e-fold change of *N0/Nc* is produced by a change in V_m of 12.5 mV (left side) and 11.9 mV (right side), corresponding to gating charges, n = 2.0 and 2.1, respectively. The midpoint potential of the *N0/Nc* distribution (i.e., $N_0 = N_c$) was at 27.4 mV (left side) and 35 mV (right side).

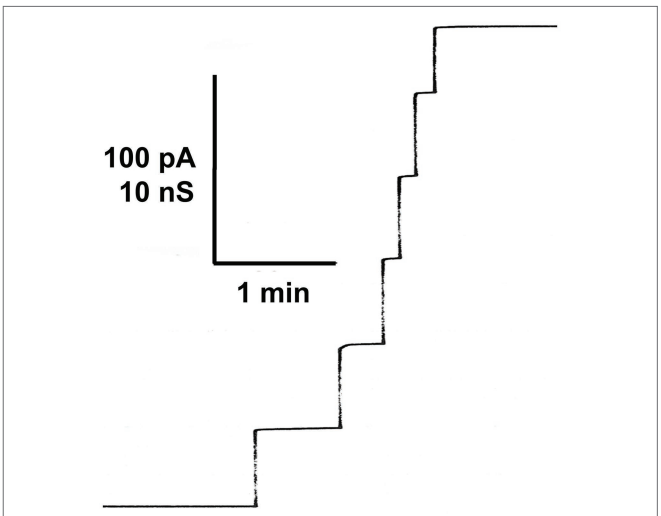

FIGURE 4 | Stepwise increase of the membrane current (given in pA) after the addition of porin 31HL (hVDAC1) to a black lipid bilayer membrane given as a function of time. The aqueous phase contained 5 ng/ml Porin 31HL and 1 M KCl (Benz et al., 1992). The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The voltage applied was 10 mV; T = 20°C.

IONIC SELECTIVITY OF THE OPEN STATE OF PORES FORMED BY EUKARYOTIC PORINS

The single-channel conductance of pores formed by eukaryotic porins followed in salts composed of different cations and anions approximately the mobility of the different ions in the aqueous phase (Colombini, 1979; Roos et al., 1982). This result suggested together with the high single-channel conductance (see **Table 1**) that eukaryotic porin pores are wide and water filled. Nevertheless, the pores showed some preference for anions. The ionic selectivity of channels/pores reconstituted into artificial lipid bilayers can be measured by the zero-current membrane potential as the result of a salt gradient applied across the membranes. This could be performed by using a high impedance electrometer connected to electrodes with salt bridges when the gradient is established across the membranes (Benz et al., 1979). It is also possible to measure current-voltage curves and extrapolating the current to zero. The corresponding voltage provides the same information (Schein et al., 1976). **Table 2** shows examples of zero-current membrane potentials obtained for several eukaryotic porins using salts composed of anions and cations of different mobility in the aqueous phase. It is evident from the data in **Table 2** that the mobility of the ions in the aqueous phase has a substantial

influence on the zero-current membrane potential of the pores and the permeability ratio P_{anion}/P_{cation}. For KCl that is composed of equally mobile potassium ions and chloride, the pores are slightly anion selective. This changes remarkably, when potassium ions or chloride are replaced by lithium ions and acetate, respectively. For LiCl, the potential is more negative, whereas it gets positive for K-acetate. This result is typical for general diffusion pores similar to general diffusion pores of the bacterial outer membranes (Benz, 1994a). The voltage dependence of the eukaryotic porins changes this picture, because all eukaryotic porins are cation-selective in the closed state (Benz, 1994b, 2004). The switch from open-anion selectivity to closed-cation selectivity of eukaryotic porins adopts presumably an important role in regulation of mitochondrial energy metabolism.

CONDUCTANCE OF THE CLOSED STATES OF EUKARYOTIC PORINS

The conductance of the closed state of eukaryotic porins could be evaluated from single-channel recordings extended over longer times. At voltages between 20 mV and 30 mV, the pores close more frequently but not too often, which means that the residual conductance associated with the closing pores could be estimated from the current recordings. This procedure allows a meaningful analysis of the conductance of the closed eukaryotic pores as it is shown in **Table 3** for KCl. Included into **Table 3** are also the results of this type of experiment when salts composed of different anions and cations were used. The results indicate again (similar to **Figure 2**) that open and closed states of the eukaryotic pores have different selectivity.

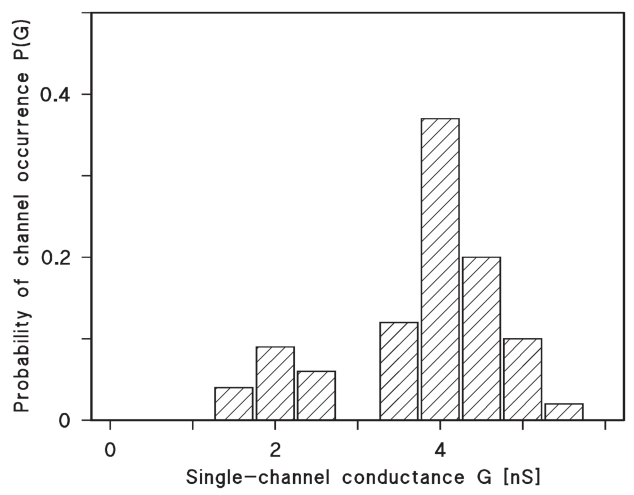

FIGURE 5 | Histogram of conductance fluctuations observed with membranes of diphytanoyl phosphatidylcholine/n-decane in the presence of Porin 31HL (Benz et al., 1992). P(G) is the probability for the occurrence of a conductance step with a certain single-channel conductance (given in nS). The aqueous phase contained 1 M KCl. The voltage applied was 10 mV. The mean value of all upward directed steps was 4.3 nS for the right-side maximum and 2.4 nS for the left-side maximum (in total 288 single events); T = 20°C.

This could be concluded from the observation that the single-channel conductance of the closed state of the pore was considerably smaller for Tris-HCl than for K-MES, despite a similar aqueous mobility of K^+ and Cl^-. This result suggested again that the closed state(s) of mitochondrial porins is cation-selective, otherwise, the relatively small conductance difference

TABLE 2 | Zero-current membrane potentials, V_m, of lipid bilayer membranes the presence of rat liver, yeast, human eukaryotic porin1, human eukaryotic porin2, and *Paramecium* porins measured for 10-fold gradients of different salts. V_m is defined as the potential of the dilute side (10 mM/100 mM) relative to that of the concentrated side (100 mM/1 M); P_{anion}/P_{cation} was calculated from the Goldman-Hodgkin-Katz equation (Benz et al., 1979).

Salt	V_m [mV] 10-fold gradient	P_{anion}/P_{cation}	References
Rat liver			
KCl (pH 6)	−11	1.7	Roos et al., 1982
LiCl (pH 6)	−24	3.4	Roos et al., 1982
Potassium acetate(pH 7)	+14	0.50	Roos et al., 1982
Yeast			
KCl (pH 6)	- 7	1.4	Ludwig et al., 1988
KCL	- 11	1.8	Forte et al., 1987a
KCl	- 11	1.8	Blachly-Dyson et al., 1993
LiCl (pH 6)	−20	2.6	Ludwig et al., 1988
Potassium acetate (pH 7)	+14	0.5	Ludwig et al., 1988
Paramecium			
KCl (pH 6)	−11	1.7	Ludwig et al., 1989
LiCl (pH 6)	−24	3.4	Ludwig et al., 1989
Potassium acetate(pH 7)	+14	0.50	Ludwig et al., 1989
hVADAC1			
KCl	−11.1	1.8	Blachly-Dyson et al., 1993
hVDAC2			
KCl	−10.9	1.8	Blachly-Dyson et al., 1993
hVDAC3			
KCl	−5.4	1.3	Queralt-Martín et al., 2020

TABLE 3 | Average single-channel conductance of the open and closed states of human (Porin 31HL; Benz et al., 1992) and yeast (Ludwig et al., 1988) porins in different 0.5 M salt solutions.

Salt	Open state [nS]	Closed state [nS]
Human porin (Porin 31 HL)		
KCl	2.4	1.4
K-MES	0.70	0.65
Tris-HCl	1.5	0.30
Yeast porin		
KCl	2.3	1.3
K-MES	0.95	0.65
Tris-HCl	1.5	0.30

The pH of the aqueous salt solutions was adjusted to 7.2. The protein concentration was between 5 and 10 ng/ml; Vm = 30 mV, T = 25°C. The single-channel conductance of the closed state was calculated by subtracting the conductance of the closing events from the conductance of the initial opening of the pores.

TABLE 4 | Average single-channel conductance of the open and the polyanion-induced closed state of rat liver porin in different 0.5 M salt solutions (Benz et al., 1990).

Salt	Open state [nS]	Closed state [nS]
KCl	2.2	1.2
LiCl	1.8	0.40
K-acetate	1.1	0.85
K-MES	0.88	0.74
Tris-Cl	1.5	0.25

The pH of the aqueous salt solutions was adjusted to 7.2. The membrane voltage was 10 mV at the cis-side. The aqueous phase contained in the measurements of the closed state 0.1 μg/ml polyanion added to the cis-side.

for K-MES between open and closed state and the big difference for Tris-HCl cannot be understood.

EUKARYOTIC PORES ARE CLOSED *IN VITRO* AND *IN VIVO* BY A SYNTHETIC POLYANION

König et al. (1977, 1982) described effects of a 10 kDa synthetic polyanion (a copolymer of methacrylate, maleate, and styrene in a 1:2:3 proportion) on mitochondrial metabolism. Dependent on its concentration, the polyanion was able to inhibit anion transport, respiration, ATPase activity, and ADP/ATP exchange activity of rat liver mitochondria (König et al., 1977, 1982). The polyanion is by far too big to enter the intermembrane space of mitochondria through the outer membrane pore and to act with inner membrane components, which means that its action on mitochondrial metabolism was something like a mystery. However, reconstitution experiments with eukaryotic porin demonstrated that the polyanion bound to porin and changed its voltage dependence (Colombini et al., 1987; Benz et al., 1988). Application of small voltages of −5 mV or less negative to the cis-side of the membranes, where porin and polyanion were added, resulted already in pore closure (Benz et al., 1988; De Pinto et al., 1989a). The mitochondrial pore was always in the open configuration when positive potentials were applied to the cis-side (Benz et al., 1988).

Careful analysis of the polyanion-induced closed state of rat liver porin demonstrated that it showed an interesting analogy to the voltage-mediated closed state (see **Table 4**). This means that the polyanion, although it is not able to enter the pore, interacts with the gate. It pulls the gate (presumably the α-helical N-terminus) to the side of the polyanion and changes thus the voltage dependence of the gate (Benz et al., 1988). The effect of the polyanion on mitochondrial metabolism was also studied in intact mitochondria, because it allowed the evaluation of the role of the outer membrane pore on different features of mitochondrial metabolism (Benz et al., 1990; Benz and Brdiczka, 1992; Brdiczka, 1993). The addition of 30 μg polyanion per mg mitochondria completely blocked adenylate and creatine kinases. Similarly, peripheral kinases, such as hexokinase and glycerolkinase, were also completely inhibited, when mitochondrial, but not external ATP, was used (Benz and Brdiczka, 1992). Disruption of the mitochondrial outer membrane by detergent completely restored the activity of all peripheral kinases, which clearly indicated that compartment formation exists in the intermembrane space of intact mitochondria (Benz and Brdiczka, 1992; Brdiczka, 1993; Gellerich et al., 1993; Ahmadzadeh et al., 1996; Brdiczka et al., 1998). These results suggest that the mitochondrial outer membrane pore could be involved in the control of mitochondrial metabolism *via* its voltage dependence (Benz and Brdiczka, 1992; Liu and Colombini, 1992). Important for this could be the close apposition of mitochondrial inner and outer membrane that a voltage across the outer membrane is induced *via* capacitive coupling of inner and outer membranes, in which also the folding of the inner membrane may be involved (Benz, 1985; Mannella et al., 2013).

RENATURATION AND RECONSTITUTION OF EUKARYOTIC PORINS OBTAINED BY HETEROLOGOUS EXPRESSION IN BACTERIA

During the first time of research into the characteristics of eukaryotic porins, these proteins were always isolated from mitochondria. However, modern research into the properties of channel-forming proteins needs very often mass production and site-directed mutagenesis of the proteins. This is possible in the case of eukaryotic porins but it is very complicated and time consuming to bring the mutated proteins back into mitochondria. Thus, it was of interest to express eukaryotic porins in bacteria and to renature eukaryotic porins for research purposes. This was possible although translation of eukaryotic porins *in vivo* and *in vitro* differs considerably. Nevertheless, two early studies describe the renaturation processes of eukaryotic porins in some detail. Pfaller et al. (1985) described for the first time the possibility to make eukaryotic porin from *N. crassa* water soluble. In this form, the protein binds to the surface of mitochondria and blocks the import of the porin precursors. The water-soluble porin may also be renatured by treatment with low doses of detergents and needs the presence of sterols in the lipid bilayer membranes (Pfaller et al., 1985). In fact, the presence of cholesterol in a ratio of five cholesterol per one polypeptide has been detected in purified eukaryotic porin using different detergents (De Pinto et al., 1989b). Sterols were also necessary when the properties of mutated *N. crassa* porin were studied in lipid bilayer membranes. Following the renaturation process of different eukaryotic porins, it seems that sterols seem to be necessary, although they may modulate the properties of the pores, in particular of plant porins (Popp et al., 1995; Carbonara et al., 1996; Mlayeh et al., 2010; Lopes-Rodrigues et al., 2020; Saidani et al., 2021). However, in mass production and functional renaturation of two human isoforms of human porin (hVDAC1 and hVDAC2) and of potato VDAC36, no indication for the need of cholesterol/sterol for porin structure and function was observed (Engelhardt et al., 2007; Lopes-Rodrigues et al., 2020; Najbauer et al., 2021). On the other hand, ergosterol interacts with eukaryotic porin of *N. crassa*

(Bay and Court, 2009) and stigmasterol seems to be important for proper function of bean seed VDAC (Saidani et al., 2021) and sterols were found to be important for renaturation of VDAC from pea root plastids (Popp et al., 1995). This means presumably that it is an open question whether sterols are important for porin function and/or could only accelerate the renaturation process but are essentially not needed for the formation of some functional pores.

STRUCTURE OF THE MITOCHONDRIAL OUTER MEMBRANE PORE

The presence of a voltage-dependent outer membrane pore in mitochondria was an interesting feature in research into mitochondria and attempts were made to visualize the pores. X-ray diffraction of oriented outer mitochondrial membranes from plants suggested a special location of the proteins in the membrane plane (Mannella, 1981). Further electron microscopic analysis of *Neurospora* mitochondria showed crystalline arrays of putative pores in the outer membranes if the membranes were dialyzed against low salt buffers (Mannella and Frank, 1984; Mannella, 1987). Single repeating units contained three pores, which was revealed by lipid bilayer experiments (Mannella et al., 1983). Analysis of the pores using uranyl acetate suggested that the outer membrane pore formed cylinders with an outer diameter of 5 nm and an inner core of about 1.8 to 2 nm (Mannella and Frank, 1984). Many attempts were made besides the electron microscopic analyses to resolve the 3D structure of the mitochondrial pore. However, all these attempts were unsuccessful for a very long time presumably because the pore was deeply buried in the outer membrane and did not show intermolecular interactions (De Pinto et al., 1987b).

Starting with eukaryotic porins from yeast (Mihara and Sato, 1985) and *N. crassa* (Kleene et al., 1987), the primary structure of many eukaryotic porins became known during the last 25 years as it is discussed in part 4 of this review. Common to all of them is the length of about 280 amino acids without an obvious leader sequence and the balanced distribution of hydrophobic and hydrophilic amino acids. The genomes of many eukaryotic organisms, in particular of mammals and plants, code very often for several porins with yet not fully understood functions (Anflous and Craigen, 2004; Pinto and Messina, 2004; Graham and Craigen, 2005; Tateda et al., 2011). Secondary structure predictions suggested the existence of many β-strands within the primary sequence of eukaryotic porins similar to the situation in bacterial ones (Benz, 1994a,b). However, all mitochondrial porins known to date contain at the N-terminal end a stretch of about 20 to 25 amino acids that forms an α-helical structure, probably involved in voltage-dependent gating of the pore because its deletion leads to the loss of voltage dependence (Popp et al., 1996; Runke et al., 2006; De Pinto et al., 2007; Zachariae et al., 2012). A comparison of the many primary sequences of eukaryotic porins shows that the sequences have all a similar length, but otherwise, the homology is not very obvious because only a few amino

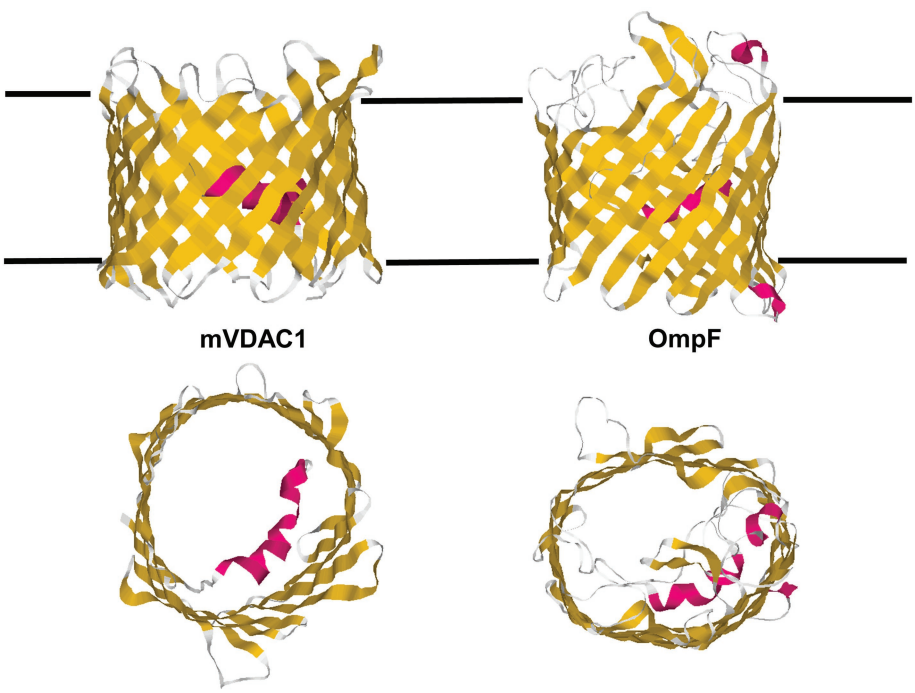

FIGURE 6 | Structure of the mitochondrial outer membrane pore (mVDAC1) and an OmpF monomer of *E. coli*. β-strands within the protein structures are shown in yellow and α-helical stretches in red. The 3D structures of the proteins are shown from the side in direction to the surface of the mitochondrion and the cell (upper structures) and from the surface of the mitochondrion and the bacterial cell (structures down). mVDAC1 (PDB code: 2JK4) is the 3D structure of mouse mitochondrial porin (Ujwal et al., 2008). OmpF (PDB code: 2OMF) represents the structure of the major outer membrane protein of *E. coli* (Cowan et al., 1992).

acids are conserved (Benz, 2004). Only near amino acid 90 of most porins a triplet of the form GLK can be found, which is highly conserved but its function is unknown (Runke et al., 2000). The phylogenetic relationship of the β-barrel mitochondrial outer membrane proteins TOM40 (involved in protein transport) and eukaryotic porin was studied in detail by Young et al. (2007) and Bay et al. (2012). The authors suggested from their analysis that these proteins share a common evolutionary origin, meaning that the lineage of both protein families was co-evolutionary and formed later paralogues.

The 3D structure of the mitochondrial pore was for a longer time a matter of debate. Based on secondary structure predictions, electrophysiology and mutagenesis several models were developed (Forte et al., 1987b; Blachly-Dyson et al., 1990, 1993; Peng et al., 1992; Benz, 1994b; Casadio et al., 2002; Anflous and Craigen, 2004). One model assumed that eukaryotic porin is exclusively formed by 16 or 18 β-strands. The other model suggested also that the pore contains β-strands, but only 12–13 strands in combination with the α-helix as part of the channel wall. At the end, three groups were successful to derive simultaneously the 3D structure of eukaryotic porins using different techniques (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). Hiller et al. (2008) used the technique of solution NMR to study recombinant hVDAC1 reconstituted in detergent micelles. In this case, the location of the N-terminus was not resolved. Bayrhuber et al. (2008) derived the 3D structure of hVDAC1 from a combination of NMR-spectroscopy and X-ray crystallography. Ujwal et al. (2008) succeeded to crystallize murine VDAC1 (mVDAC1). The three studies agreed in the basic structure of the mitochondrial pore that forms a β-barrel with 19 β-strands. Two structures show the location of the N-terminal α-helix horizontally midway in the pore, restricting its size (Bayrhuber et al., 2008; Ujwal et al., 2008). This means that the α-helix has a strategic position to control the passage of metabolites and ions through the mitochondrial pore. This structure was criticized and Colombini (2012) insisted in the structure of VDAC with one α-helix and 13 β-strands tilted at a 46° angle toward the surface of the mitochondrial outer membrane. However, the structure of VDAC shown in **Figure 6** has many times been realized that we can consider it as the real structure of the eukaryotic pore.

Figure 6 shows the schematic structure of mouse VDAC as it was obtained by X-ray crystallography (Ujwal et al., 2008) in comparison with the 3D structure of the most abandoned bacterial porin OmpF of *E. coli* (Cowan et al., 1992). The mitochondrial pore is formed by 19 β-strands (18 are antiparallel and β-strands one and 19 are parallel) in contrast to 16 antiparallel β-strands of OmpF. It is clear from a comparison of the two 3D structures that the architecture of the two outer membrane pores is quite similar. This has presumably to do with the history of bacterial and mitochondrial outer membrane pores. It has presumably also to do with translation and assembly of both pores. The β-strands of both β-barrel cylinders are tilted by 30–40° toward the surface of the membranes. The dimensions of the eukaryotic porin are 35 Ă for the height and 40 Ă for the width. The N-terminal α-helix (amino acids 1–21) is located inside the β-barrel cylinder and acts there as a gate, but also as a stabilizing element for the mitochondrial pore similar to the external loop 3 of OmpF that is folded inside the bacterial pore (Cowan et al., 1992; Ujwal et al., 2008). Despite the location of the N-terminus inside the eukaryotic pore, it has a high ion permeability. It is approximately the same as OmpF trimers (Benz, 1994a,b). It is noteworthy, that Tom40, the major component of the mitochondrial outer membrane import machinery is also a member of the VDAC-family and shows the same structure of 18 antiparallel β-strands and one pair of parallel β-strands (Zeth and Zachariae, 2018). The most interesting point of the comparison of bacterial and mitochondrial porins is the fact that bacterial outer membrane pores have only passive properties, whereas mitochondrial porins adopted during evolution an active role in mitochondrial metabolism.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

I would like to thank Dieter Brdiczka, Vito De Pinto, Walter Neupert, Ferdinando Palmieri, and Friedrich P. Thinnes for the fruitful and excellent collaboration during many years and my collaborators Otto Ludwig, Elke Maier, Birgit Popp, and Angela Schmid for their excellent work with porins from mitochondria on which this review is largely based. My own research was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 29 September 2021
doi: 10.3389/fphys.2021.742839

VDAC Modulation of Cancer Metabolism: Advances and Therapeutic Challenges

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Most anionic metabolites including respiratory substrates, glycolytic adenosine triphosphate (ATP), and small cations that enter mitochondria, and mitochondrial ATP moving to the cytosol, cross the outer mitochondrial membrane (OMM) through voltage dependent anion channels (VDAC). The closed states of VDAC block the passage of anionic metabolites, and increase the flux of small cations, including calcium. Consequently, physiological or pharmacological regulation of VDAC opening, by conditioning the magnitude of both anion and cation fluxes, is a major contributor to mitochondrial metabolism. Tumor cells display a pro-proliferative Warburg phenotype characterized by enhanced aerobic glycolysis in the presence of partial suppression of mitochondrial metabolism. The heterogeneous and flexible metabolic traits of most human tumors render cells able to adapt to the constantly changing energetic and biosynthetic demands by switching between predominantly glycolytic or oxidative phenotypes. Here, we describe the biological consequences of changes in the conformational state of VDAC for cancer metabolism, the mechanisms by which VDAC-openers promote cancer cell death, and the advantages of VDAC opening as a valuable pharmacological target. Particular emphasis is given to the endogenous regulation of VDAC by free tubulin and the effects of VDAC-tubulin antagonists in cancer cells. Because of its function and location, VDAC operates as a switch to turn-off mitochondrial metabolism (closed state) and increase aerobic glycolysis (pro-Warburg), or to turn-on mitochondrial metabolism (open state) and decrease glycolysis (anti-Warburg). A better understanding of the role of VDAC regulation in tumor progression is relevant both for cancer biology and for developing novel cancer chemotherapies.

Keywords: cancer, glycolysis, metabolic flexibility, metabolic reprogramming, metabolism, mitochondria, voltage dependent anion channels, Warburg

OPEN ACCESS

Edited by:
Vito De Pinto, University of Catania, Italy

Reviewed by:
Tuuli Käämbre, National Institute of Chemical Physics and Biophysics, Estonia
Catherine Brenner, Délégation Paris-Villejuif-01, Center for the National Scientific Research (CNRS), France

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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *16 July 2021*
Accepted: *25 August 2021*
Published: *29 September 2021*

Citation:
Heslop KA, Milesi V and Maldonado EN (2021) VDAC Modulation of Cancer Metabolism: Advances and Therapeutic Challenges. Front. Physiol. 12:742839. doi: 10.3389/fphys.2021.742839

INTRODUCTION

The relative contribution of aerobic glycolysis and oxidative phosphorylation (Oxphos) to overall ATP generation, determine cancer bioenergetics. Cancer metabolism, however, involves not only chemical reactions to cope with energy demands, but also those necessary to maintain anabolism and catabolism. The study of cancer metabolism preceded the discovery of oncogenes

and tumor suppressors by approximately 50 years, becoming one of the oldest areas of research in cancer biology. A different metabolism in tumor cells, compared to non-proliferating cells, is regarded essential to develop and maintain malignant characteristics. A "reprogrammed metabolism," considered by many a hallmark of cancer and observed quite generally across many types of tumor cells, is a major driver of tumor metabolism (Cerezo and Rocchi, 2020; Faubert et al., 2020). Metabolic reprogramming, induced by oncogenic mutations among other factors, refers to the enhancement or suppression of specific metabolic pathways in tumor cells that improve the cellular fitness required for rapid cell division. Despite its relevance for long-term cell survival, reprogramming is not sufficient to explain metabolic adaptations to fast changing demands. It is very likely that rapid metabolic changes be driven by fast acting mechanisms that modulate specific pathways or proteins not depending on reprogramming. In particular, regulation of the conductance of voltage dependent anion channels (VDAC), located in the outer mitochondrial membrane (OMM) will be described here as a mechanism potentially involved in fast metabolic responses.

Regardless of the relatively long history of research on cancer metabolism, the interest on the role of mitochondrial metabolism in tumors was limited until the end of the 20th century. A 2021 updated PubMed search using the words mitochondrial metabolism, or mitochondrial metabolism and cancer, showed only ~33 and 12% of the total publications during the period 1921–2000. The number of indexed publications for both topics exponentially increased in the last 2 decades. Even more strikingly, another search using the words VDAC and cancer, showed only ~3% of the total publications, reported from 1986 to 2000.

The relevance of mitochondria for cellular metabolism started to be unveiled in the early 20th century, when oxidations in the mitochondrial matrix and ATP synthesis, were identified as main functions of the organelle. However, a mechanistic explanation reconciling observation with a theory to explain the link between oxidation and ATP synthesis was lacking. The chemiosmotic hypothesis of Mitchell (1966) solved the problem by postulating a proton electrochemical gradient across the inner mitochondrial membrane (IMM) as the energy-rich intermediate of Oxphos (Mitchell and Moyle, 1967). Mitchell (1966) proposed that the flow of electrons through complexes of the electron transport chain (ETC) was coupled to the outward translocation of H^+ across the IMM creating a proton motive force (Δp) used by the ATP synthase to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). The hypothesis of Mitchell (1966) was later confirmed by experimentation in mitochondria, chloroplasts, and bacteria.

A major trigger to research in cancer metabolism were the seminal findings made in the early 20th century by the German biochemist Otto Warburg, who showed in tumor slices and ascites cancer cells, that tumors produce more lactic acid than non-tumor cells even at physiological partial pressures of oxygen (Warburg et al., 1927; Warburg, 1930). This particular phenotype, called Warburg effect, is characterized by enhanced aerobic glycolysis. In tumor cells, relatively low cytosolic ATP/ADP ratios caused by a partial suppression of mitochondrial metabolism prevents glycolysis inhibition mediated by high ATP. Warburg (1956) even proposed that damaged mitochondria were the origin of cancer. According to his hypothesis, only those cells with irreversible but incomplete damage to respiration capable of increasing the conversion of glucose to lactic acid (fermentation) become cancerous. The provocative hypothesis of failing mitochondria as the origin of cancer was quickly challenged by Weinhouse (1956) and others, who demonstrated both high glycolysis and oxidative metabolism in cancer tissues. In fact, all tumors display a certain level of enhanced glycolysis that always coexists with functional mitochondria. Measurements of oxygen consumption, mitochondrial membrane potential ($\Delta\Psi$), 1,4-dihydronicotinamide adenine dinucleotide (NADH) production, and ATP generation, among other parameters, have confirmed that tumor mitochondria are metabolically active (Nakashima et al., 1984; Maldonado et al., 2010; Mathupala et al., 2010; Lim et al., 2011; Moreno-Sanchez et al., 2014; Singleterry et al., 2014). Variations in the relative contribution of mitochondrial ATP to cellular bioenergetics among different tumor types and even within the same tumor, suggest a dynamic regulation of oxidative metabolism.

In this review, we will describe the biological effects induced by changes in the conformational states of VDAC; how an increase of VDAC conductance reverses the Warburg phenotype and promotes cell death; and finally, the relevance of VDAC as a pharmacological target to develop novel cancer chemotherapies.

VDAC STRUCTURE AND REGULATION OF CONDUCTANCE

The discovery of VDAC in mitochondrial extracts from *Paramecium tetraurelia*, followed by the identification in mammalian cells, opened a new avenue in the research and understanding of mitochondrial metabolism (Schein et al., 1976; Colombini, 1979). VDAC, comprising three isoforms, is a polypeptide of ~30 kDa (VDAC 1 and 3: 280 amino acids; VDAC 2: 291 amino acids). In most mammalian cells, including cancer cells, VDAC1 and 2 are the most abundant isoforms, whereas VDAC3 is the least expressed, except for testis and spermatozoa (Sampson et al., 1997; De Pinto et al., 2010). VDAC β-barrels enclose an aqueous channel of ~3 nm internal diameter in the open state, that allows the passage of molecules up to ~5 kDa (Colombini, 1980, 2012; Mannella, 2021). The current consensus about VDAC structure shows differences with the originally proposed model by Colombini that was based on biochemical and functional data (Colombini, 2009, 2012). Structural studies using NMR and X-ray crystallography have shown VDAC1 and VDAC2 as a transmembrane β-barrel protein with 19 β-strands, mostly anti-parallel, except for strands 1 and 19. Both isoforms also have an N-terminal, α-helical region located within the pore (Ujwal et al., 2008; Hiller et al., 2010).

Voltage dependent anion channels inserted in non-polarized or weakly polarized membranes (close to 0 mV), stays mostly in the high conductance open state. By contrast, positive or negative membrane potentials induce conformational changes to several lower conductance closed states (maximal at −45 or +45 mV; Bowen et al., 1985; Colombini, 1989). Although, it is currently not possible to determine membrane potentials across the OMM in live cells, plausible theoretical approaches suggest the existence of polarization of the OMM in intact cells (Lemeshko, 2021). Moreover, the ~0.6 pH difference reported between the cytosol and the mitochondrial intermembrane space (IMS) (Porcelli et al., 2005), corresponds to a −15–20 mV potential. Since this value of membrane potential falls in the range in which reconstituted VDAC display a mild decrease in conductance (Zizi et al., 1998), it is theoretically possible that voltage actually contributes to the regulation of VDAC opening.

Voltage dependent anion channels are selective for anionic metabolites and small cations, as showed initially by Colombini, and later confirmed by other groups (Choudhary et al., 2010; Villinger et al., 2014; Colombini, 2016). The open state of VDAC allows the flux of anions, including most respiratory substrates, ATP^{4-}, ADP^{3-}, $HPO4^{2-}$, phosphocreatine^{2-}, and AMP, among others. In the closed state, VDAC favors a non-selective flux of cations including Na^+, K^+, and Ca^{2+} (Tan and Colombini, 2007; Colombini, 2012; Sander et al., 2021). VDAC closure, induced by voltage, increases the flux of Ca^{2+} up to 10-fold (Tan and Colombini, 2007). Moreover, the magnitude of Ca^{2+} flux through VDAC is influenced by the type and amount of each isoform present (only VDAC1 and 2 seem involved in Ca^{2+} signaling); post-translational modifications (phosphorylation and monoubiquitinylation); and possibly interactions with partner proteins including Bcl-xL and translocator protein (TSPO), among others (Sander et al., 2021). However, a higher flux of calcium through VDAC in mitochondria of intact cells does not necessarily correlates with an increase in the Ca^{2+} content in the matrix. After entering the IMS, Ca^{2+} still needs to be transported through the IMM by the mitochondrial calcium uniporter holocomplex (Fan et al., 2020). Thus, mitochondrial uptake of Ca^{2+} is subjected to multiple levels of regulation both at the OMM and the IMM. It remains to be determined experimentally if VDAC-mediated increase in the flux of Ca^{2+}, actually influences Ca^{2+} content in the mitochondrial matrix modifying mitochondrial metabolism.

Overall, every physiological or pharmacological regulation of VDAC to induce a change from the open state to the closed states, reduces or increases the flux of negatively charged metabolic substrates and cations, respectively. Although gating and selectivity for VDAC1 and VDAC2 are very similar in different cell types, the detailed molecular determinants of voltage gating are still incompletely understood. A structural model proposes that the N-terminus of VDAC1 lying inside the pore, parallel to the wall, moves to the lumen blocking the passage of metabolites (Shuvo et al., 2016). Regardless of the mechanism controlling gating, the flux of polar metabolites through VDAC is determined mostly by their charge and size (Colombini, 1980, 2004).

Even though VDAC was initially considered constitutively open, like an "all-time open gateway," subsequent research both *in vitro* and in intact cells, showed regulation of VDAC conductance by several endogenous molecules. VDAC conductance has been shown to be modulated by α/β tubulin heterodimers (Rostovtseva et al., 2008; Timohhina et al., 2009; Maldonado et al., 2013); hexokinase (Pastorino et al., 2002; Al Jamal, 2005); bcl2 family members (Tsujimoto and Shimizu, 2000); glutamate (Gincel et al., 2001); and NADH (Zizi et al., 1994). It has also been demonstrated that post-translational modifications, mainly phosphorylation by protein kinases, GSK3β, PKA, and protein kinase C epsilon (PKCε), blocks or inhibits association of VDAC with other proteins, such as Bax and tBid, and regulates VDAC opening (Heiden et al., 2001; Baines et al., 2007; Das et al., 2008). Moreover, PKA-dependent VDAC phosphorylation and GSK3β-mediated VDAC2 phosphorylation increase VDAC conductance and also the sensitivity to tubulin inhibition (Bera et al., 1995; Das et al., 2008; Sheldon et al., 2011). VDAC opening is also modulated by protein-protein interactions with actin, p53, mitochondrial creatine kinase, and alpha-synuclein, among others (Rovini, 2019; Kanwar et al., 2020).

Overall, the movement of metabolites through VDAC dynamically depends on the concentration gradient of each permeant molecule reaching the OMM, the electric field, the number of functional VDAC channels, the selectivity-permeability to a particular metabolite, and the open probability of the channel.

VDAC OPENING, MITOCHONDRIAL METABOLISM, AND WARBURG EFFECT

VDAC1, VDAC2, and VDAC3, at the interphase between mitochondria and cytosol, are strategically located to control the flux of metabolites and ATP entering or leaving mitochondria. At present, it is unclear if the flux of metabolites and nucleotides through VDAC is different among tumor cell types, and if it is isoform specific. To access the mitochondrial matrix, most anionic substrates that cross the OMM only through VDAC, are further transported through the IMM by several finely tuned specific carriers (Palmieri and Pierri, 2010). Pyruvate, fatty acids, and the amino acids glutamine (quantitatively the most important amino acid utilized by several tumors), glycine, serine, leucine, isoleucine, valine, and tryptophan, generate acetyl-coenzyme A (AcCoA) that fuels the Krebs (tricarboxylic acid) cycle. A cycle of oxidation generates NADH, and dihydroflavine-adenine nucleotide ($FADH_2$), electron donors to the ETC. Electrons flowing through the ETC or respiratory chain, formed by complexes I-IV, reduce the final acceptor molecular O_2 to H_2O, while simultaneously generating single electron leaks from complexes I, II, and III to form the superoxide anion ($O2^{\bullet-}$), that is further converted into other reactive oxygen species (ROS) (Chance et al., 1979; Mailloux, 2020). The metabolic fitness of mitochondria in any cell type, also depends on the generation of $\Delta\Psi$ formed when complexes I, III, and IV drive H^+ translocation from the matrix to the

IMS, where it generates a negative transmembrane $\Delta\Psi$ and a ΔpH, both components of the proton motive force (Δp). Ultimately, Δp drives ATP synthesis from ADP and Pi by complex V (F_1F_O-ATP synthase): (Hoek et al., 1980; Nicholls and Ferguson, 2013). Newly synthesized ATP is transported to the cytosol through the adenine nucleotide translocator, located in the IMM, and exchanged for ADP in a 1:1 molar ratio to be finally released to the cytosol through VDAC (Klingenberg, 2008; Allouche et al., 2012). AMP, another adenine nucleotide, also cross the OMM through VDAC. Therefore, regulation of VDAC opening influences the ATP/AMP ratio, which in turn, modulates the activation of AMP-activated protein kinase (AMPK); (Colombini, 2016; Shevade et al., 2018). Thus, the magnitude of metabolite fluxes to support Oxphos and ATP synthesis, ultimately depends on VDAC conductance (**Figure 1**).

In general, approximately 95% of ATP in quiescent cells is produced by Oxphos, with the remaining 5% generated through the pay-off phase of glycolysis in the cytosol, and the succinyl-CoA ligase reaction of the Krebs cycle. Full mitochondrial oxidation of glucose generates ~32 moles of ATP, as estimated by different methods, compared to the 2 moles of ATP/mole of glucose during glycolysis (Brand, 2005). Although the total amount of mitochondrial ATP calculated considers the currently accepted proton stoichiometry for ATP synthesis, ATP/ADP-Pi exchange, respiration, and the malate/aspartate shuttle, the actual ATP yield could be less due to proton leak into the mitochondrial matrix (Brand, 2005; Rich and Marechal, 2010; Walker, 2013; Wikstrom et al., 2015). The relatively low ATP demand for cell division compared to the energy requirements for maintenance of cellular functions, mainly the activity of the Na^+-K^+ ATPase, suggests that ATP generation is not a limiting factor to sustain rapid cell proliferation (Kilburn et al., 1969; Schwenke et al., 1981; Veech et al., 2019; Seyfried et al., 2020). In non-proliferating cells, high VDAC conductance promotes an oxidative metabolism, generating cytosolic ATP/ADP ratios 50–100 times higher compared to mitochondria. High ATP-ADP in the cytosol inhibits phosphofructokinase-1 (PFK-1), a rate limiting step in the glycolytic pathway, among other possible mechanisms blocking glycolysis (Schwenke et al., 1981; Jenkins et al., 2011). The ATP-dependent inhibitory mechanisms, together with regulation of other pathways, may be key to explain the reciprocal dependence between mitochondrial metabolism and glycolysis in several tumor types. By contrast, a partial suppression of mitochondrial metabolism in cancer cells contributes to a low cytosolic ATP/ADP ratio; releasing the brake on glycolysis and favoring the Warburg phenotype. Compared to non-proliferating cells, cancer cells generate ~10–90% of total ATP by glycolysis (Nakashima et al., 1984; Griguer et al., 2005). Tumor cells also display "glucose avidity," an increased uptake of glucose compared to non-proliferating cells. In clinical settings, the preferential incorporation of the radioactive glucose analog fluorodeoxyglucose by tumor cells is used in positron emission tomography (PET) to diagnose cancer (Zhu et al., 2011). A downside of PET scan that leads to false positives is the inability to distinguish between tumor cells and non-tumor cells with a high rate of glucose uptake, including tumor infiltrating lymphocytes.

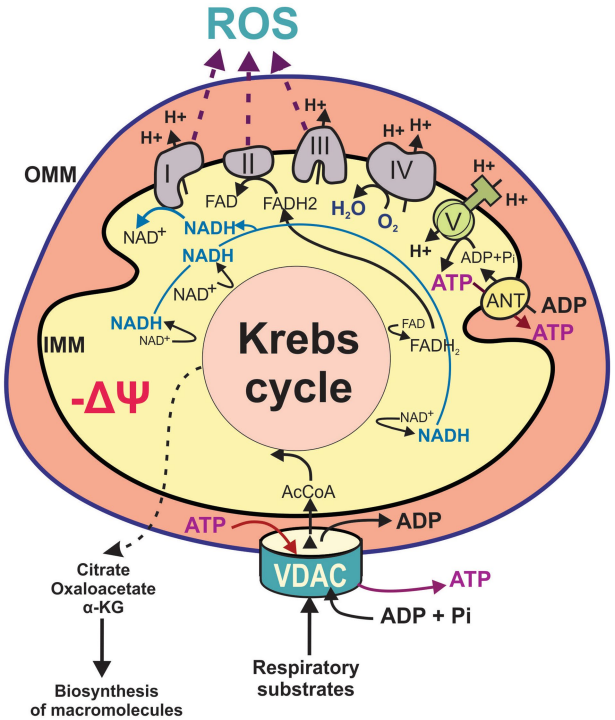

FIGURE 1 | Main features of mitochondrial metabolism. Flux of metabolites including the respiratory substrates pyruvate, adenosine diphosphate (ADP), and inorganic phosphate (Pi) and certain amino acids, cross the outer mitochondrial membrane occurs through voltage dependent anion channels (VDAC). Catabolism of respiratory substrates in the Krebs cycle generates 1,4-dihydronicotinamide adenine dinucleotide (NADH) and dihydroflavine-adenine nucleotide ($FADH_2$), which fuel the electron transport chain (ETC; complexes I–IV) to support oxidative phosphorylation. The Krebs cycle also produces metabolic intermediaries released to the cytosol for the biosynthesis of proteins and lipids. Proton pumping by the respiratory chain across inner mitochondrial membrane (IMM) generates mitochondrial $\Delta\Psi$. Protons moving back across MIM into the matrix drive ATP synthesis from ADP and Pi by the F_1F_0-ATP synthase (complex V). Mitochondrial ATP is exported from the matrix by the adenine nucleotide transporter (ANT) and released to the cytosol through VDAC. Flow of electrons through complexes I, II, and III also generates reactive oxygen species. AcCoA, acetyl CoA; α-KG, alpha ketoglutarate; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; and ROS, reactive oxygen species.

The conformational open or closed state of VDAC, by regulating the flux of respiratory substrates, is a major determinant of cytosolic ATP/ADP ratios to favor or oppose the pro-proliferative Warburg phenotype. The predominance of open or closed conformational states of VDAC, not only determines the bioenergetics efficiency of mitochondria, but also the ability to contribute metabolic intermediaries to the synthesis of amino acids, fatty acids, nucleotides, cholesterol, glucose, and heme (Spinelli and Haigis, 2018). Rapidly dividing cells face a constant challenge to produce new macromolecules to approximately double the biomass before mitosis. In the Warburg metabolism, the major sources of carbon backbones are glucose, glutamine, and fatty acids. Similar to glycolytic intermediates, Krebs cycle intermediates are also used as precursors for biosynthesis of macromolecules. A continuous

supply of precursors, sustained by anaplerotic pathways, "refills" the cycle with intermediates to replace those that have been already used for biosynthesis. Glutaminolysis, which produces α-ketoglutarate from glutamine, and pyruvate carboxylation, which produces oxaloacetate from glucose/pyruvate are major contributors to anaplerotic fluxes in cancer cells (Bott et al., 2019; Martins et al., 2020; Kiesel et al., 2021). Oxidation of the branched-chain amino acids (BCAAs) isoleucine and valine also provides an anaplerotic flux in some tumors. If glucose supply is sufficient for energy generation, glutamine-derived α-ketoglutarate and oxaloacetate are utilized for the synthesis of nonessential amino acids, whereas citrate exported to the cytosol, is converted into AcCoA and utilized for the synthesis of fatty acids, cholesterol, and amino acids. Glutamine is also a nitrogen donor in purine and pyrimidine synthesis, and a precursor for the synthesis of the antioxidant glutathione (Owen et al., 2002; Still and Yuneva, 2017; Li and Le, 2018; DeBerardinis and Chandel, 2020). Recently, one-carbon metabolism, a set of reactions that transfer one-carbon units (methyl groups) from serine and glycine, has been shown to be important for *de novo* synthesis of purines and thymidylate synthase in highly proliferative tumors (Meiser and Vazquez, 2016).

Overall, VDAC operates as a biological switch that, in the on-phase (open state), maximizes the flux of metabolites for optimal mitochondrial function, whereas during the off-phase (closed state), minimizes mitochondrial metabolism (**Figure 2**). Thus, regulation of only this channel has an amplifying effect on several intra and extra-mitochondrial pathways modulating cancer metabolism and bioenergetics. The dynamic changes in ATP and biosynthesis demands, ranging from seconds to minutes or even hours, imply the coexistence of both metabolic reprogramming and fast acting regulatory mechanisms. VDAC is, very likely, one of the rapidly adapting mechanisms that are responsive to interactions with other OMM and cytosolic proteins and soluble factors, along with transient or permanent posttranslational modifications. At present, it is unknown if metabolic reprogramming, that affects several mitochondria-related pathways, modulates VDAC conductance.

Beyond the role in mitochondrial metabolism, VDAC is also a prognostic biomarker for certain types of human cancer (Jozwiak et al., 2020; Wersall et al., 2021). High expression of VDAC1 has been associated with unfavorable outcomes in cancers from lung, head and neck, breast, and liver (Grills et al., 2011; Yang et al., 2019; Jozwiak et al., 2020). High transcript levels of VDAC2 in multiple tumors, including melanoma, epithelial thyroid tumors, and breast cancer have been reported in the cancer databases cBioportal,[1] and the Human Protein Atlas.[2] In head and neck cancer and in liver cancer, high expression of VDAC2 is also associated with a poor outcome. Interestingly, VDAC3 expression seems not to be of prognostic value for human cancer suggesting isoform specific effects on cell proliferation.

[1]http://www.cbioportal.org
[2]http://www.proteinatlas.org

MITOCHONDRIA, TUMOR METABOLIC FLEXIBILITY, AND TUMOR HETEROGENEITY

The term metabolic inflexibility, coined in the late 90's, refers to inadequate responses of skeletal muscle to fuel changes in insulin-resistant obese patients (Kelley and Simoneau, 1994; Kelley et al., 1999). By contrast, metabolic flexibility alludes to the ability of muscle cells to switch from fatty acid to glucose oxidation. A "mitocentric" concept of nutrient metabolism describes the storage, utilization, and conversion of nutrients into other metabolites, as a critical process to monitor energy homeostasis (Trepanowski et al., 2011; Gambardella et al., 2020; Motori et al., 2020). Research to identify the molecular origins of metabolic flexibility has focused mostly on the interplay between glucose and fatty acids, and/or the aberrant production of the fatty acid precursor, malonyl-CoA. Mitochondria, as an integral metabolic hub, are major contributors to metabolic flexibility. Similar to muscle cells, most cancer cells are metabolically flexible. The different quantitative contributions of mitochondria to cellular bioenergetics, together with genomic instability and differences in the microenvironment, are important determinants of tumor heterogeneity (Kuipers et al., 2017; Barcena-Varela and Lujambio, 2021; Wei et al., 2021). Tumor metabolic heterogeneity is increasingly recognized as a factor causing failures in cancer treatment (Gentric et al., 2017; Kim and DeBerardinis, 2019). Whether a tumor displays a predominantly glycolytic or oxidative metabolism depends on gene expression as well as temporary and long-term epigenetic stimuli. In addition, the dynamic relative contribution of glycolysis and Oxphos is influenced by the type of primary or metastatic tumor, intra-tumor regional differences, temporal variations in the energetic demands and availability of glucose, fatty acids, ketone bodies, and certain amino acids (Jose et al., 2011; Alam et al., 2016; Duraj et al., 2021; **Figure 2**).

A reciprocal dependence of mitochondrial metabolism and enhanced glycolysis has been shown in several cancer cell types under different experimental conditions, including hypoxia and limitations in the availability of nutrients. The magnitude of Oxphos inhibition during hypoxia is influenced by the cell type and duration of the hypoxic exposure. Prolonged hypoxia increases glycolysis in MCF-7 cells but not in HeLa cells, although, Oxphos is the predominant source of ATP in both cell types (Rodriguez-Enriquez et al., 2010). Interestingly, in solid tumors, the respiratory chain is still fully functional at oxygen levels as low as 0.5%, indicating that cancer cells exposed to <2% oxygen in rapidly growing and heterogeneously perfused tumors still produce ATP by Oxphos (Vaupel et al., 2001; McKeown, 2014). In some human cell models, hypoxia induced the synthesis of a C-terminal truncated form of VDAC1, with similar channel activity and voltage dependency as the full-length channel. Truncated VDAC1 was linked to an upregulation of both Oxphos and glycolysis, as well as to resistance to apoptosis (Brahimi-Horn et al., 2012; Brahimi-Horn and Mazure, 2014; Mazure, 2016; Cunha-de Padua et al., 2020). By contrast, knockout of VDAC1 in mouse embryonic fibroblasts (MEF)

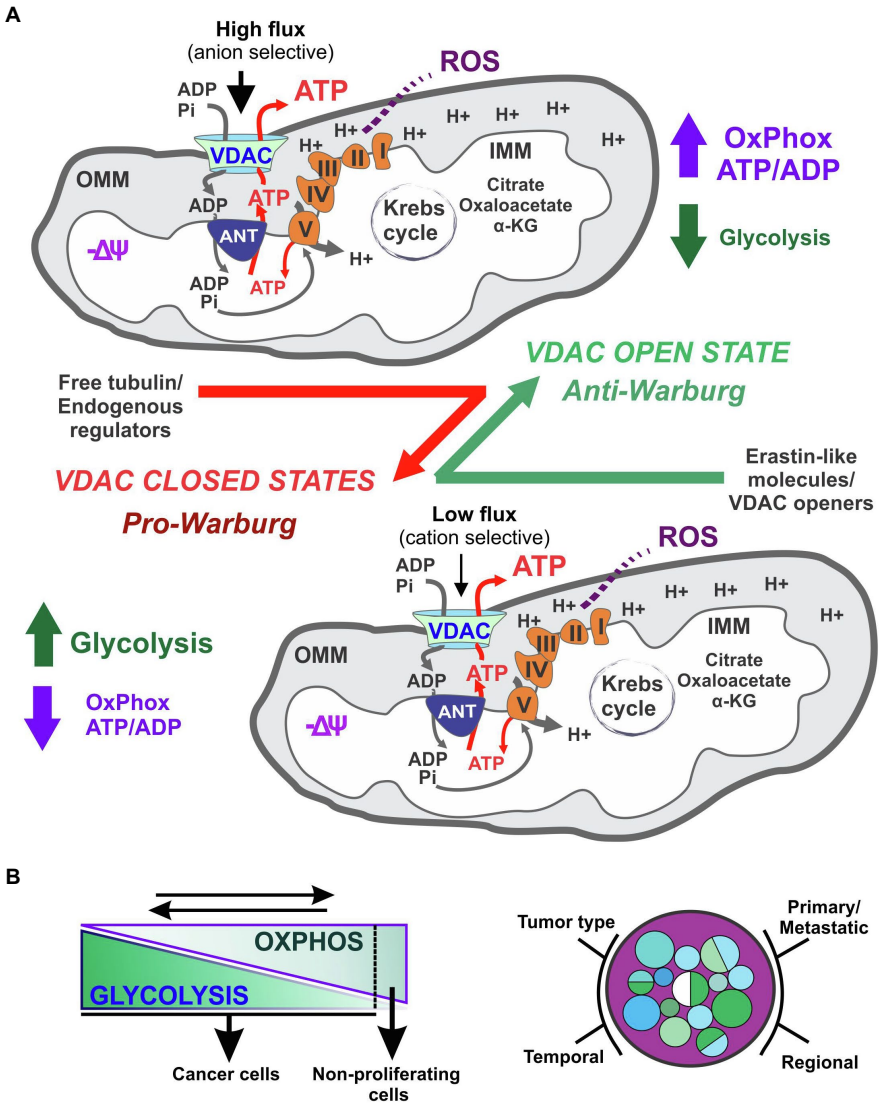

FIGURE 2 | Voltage dependent anion channels regulation of cancer bioenergetics in metabolically flexible tumors. VDAC opening in cancer cells promotes oxidative metabolism and reverses the Warburg phenotype **(A)**. The contribution of glycolysis to cancer cell bioenergetics is influenced by the type of tumor, and regional differences, among other variables. A dynamic reversal of the Warburg phenotype influences cell proliferation **(B)**.

expressing oncogenic RAS, favors tumor development in mice by promoting metabolic reprogramming (Brahimi-Horn et al., 2015). Beyond hypoxia, under conditions that decrease pyruvate oxidation in the Krebs cycle, mitochondria from tumor cells adapt to oxidize more glutamine as an energy source sustaining tumor growth both through aerobic glycolysis and Oxphos (Mullen et al., 2012). Nutrient availability not only influences tumor growth but also induces a switch from aerobic glycolysis to Oxphos in lymphoma and breast cancer cell lines cultured in glucose-free media (Smolkova et al., 2010; Robinson et al., 2012). If access to glucose and glutamine is limited, tumor cells adapt to utilize instead, lactate, methionine, arginine, cysteine, asparagine, leucine, acetate, and even lipids and proteins from the microenvironment to cope with the energy demands (Kreis et al., 1980; Clavell et al., 1986; Scott et al., 2000; Commisso et al., 2013; Kennedy et al., 2013; Comerford et al., 2014; Keenan and Chi, 2015). Overall, tumors display several metabolic alternatives to support continuous cell division regardless of unfavorable environmental conditions.

VDAC AS A PHARMACOLOGICAL TARGET

Conventional chemotherapeutic agents promote cell death or arrest cell proliferation by blocking DNA synthesis and replication, inhibiting specific enzymes or receptors, or by destabilizing or stabilizing microtubules. By contrast, "metabolic" approaches for cancer treatment have mostly focused on the inhibition of glycolysis. Only more recently, mitochondria and mitochondrial

metabolism emerged as a source of targets to prevent or slow tumor progression (Adachi et al., 2004; Doherty and Cleveland, 2013; Bhat et al., 2015; Weinberg and Chandel, 2015; Fang and Maldonado, 2018; Curcio et al., 2021). In general, a restriction of mitochondrial oxidation of substrates decreases the amount of each intermediary released to the cytosol for the biosynthesis of macromolecules. By contrast, enhancement of mitochondrial metabolism leads to increased oxidation of substrates and ATP generation, increased ROS formation with subsequent oxidative stress, and reversal of the Warburg phenotype. These two ways of modulating mitochondrial metabolism decrease cell proliferation and promote cell death (**Figure 3**).

A remarkable and unique feature of VDAC is the ability to globally control mitochondrial metabolism by simply increasing or decreasing the conductance. In a broad sense, VDAC is a "first-step" in mitochondrial metabolism. Although, mitochondrial bioenergetics is regulated at multiple levels, VDAC opening in cancer cells leads to three major biological effects: maximization of full oxidation because of augmented entry of substrates into mitochondria; subsequent decrease in glycolysis due to a high cytosolic ATP/ADP ratio promoted by maximum generation of mitochondrial ATP; and increased formation of ROS following the enhanced activity of the *ETC*. Particularly relevant for cancer metabolism, VDAC has been shown to also serve as a docking site for a group of cytosolic proteins including hexokinase-II and the Bcl-2 family of proteins. While VDAC1 is considered a proapoptotic protein and VDAC2 seems to be anti-apoptotic, some controversy still remains about the role of VDAC for apoptotic cell death (Camara et al., 2017; Shoshan-Barmatz et al., 2017; Chin et al., 2018). Regardless, because of the obvious relevance of apoptosis for tumor growth, VDAC1 has emerged as a candidate to develop VDAC1-targeting molecules. Several peptides (mastoparan, mitoparan, and TEAM-VP); an oligonucleotide (G3139); molecules of unrelated structures (dicyclohexylcarbodiimide, estradiol, among several others); and more recently a miRNA (miR-7), have been reported to target VDAC and be pro or anti-apoptotic. These different molecules either regulate VDAC1 activity, expression, oligomerization, and interaction with HK-II or posttranslational modifications. An excellent review providing a brief description of compounds interacting mostly with VDAC1, have been published by De Pinto group (Magri et al., 2018).

Regardless of older and recent developments including a resolved NMR structure of VDAC1 with NADH bound (Bohm et al., 2020), and the identification of a cholesterol binding site (Budelier et al., 2017), drug discovery of VDAC modulators still faces major challenges. Despite the growing number of compounds that have an effect on VDAC, potential isoform specificities and identification of ligand binding sites for each of the reported molecules, are still unknown. An analysis of the structures of anti-cancer drugs acting on VDAC shows a lack of common structural motifs. In addition, a well-defined druggable binding site has not been yet identified. To add more complexity, and even when electrophysiology of VDAC inserted into lipid membranes is likely the best method currently available to study the effect of a molecule on VDAC conductance, the results may not be definitive. A different lipid composition of the artificial membranes compared to the OMM, lack of cytosolic soluble factors and protein interactions, limited knowledge about the effect of posttranslational modifications, potential artifacts introduced during the isolation and insertion of VDAC, as well as isoform specific responses, may lead to inconclusive results. Moreover, a compound of interest could eventually affect VDAC gating inserted into bilayers not only by interacting directly or indirectly with the channel, but also by altering the lipid bilayer surrounding the channel (Rostovtseva et al., 2020).

In the last 10 years, we showed that VDAC regulates mitochondrial metabolism in live cancer cells using a combination of knockdown strategies and confocal microscopy of $\Delta\Psi$ and NADH, among other techniques. We also provided evidence that increased cytosolic free tubulin dynamically correlates with changes in $\Delta\Psi$, suggesting a direct effect of free tubulin on VDAC opening (Maldonado et al., 2013). Our initial findings about the effect of VDAC regulation by free tubulin on mitochondrial metabolism in intact cells were published shortly after dimeric α/β tubulin was shown to block VDAC conductance in lipid bilayers and in isolated mitochondria (Rostovtseva et al., 2008). We also showed that VDAC1 and 2 isolated from VDAC double-knockdown HepG2 cells in all combinations, inserted into lipid bilayers, were equally sensitive to tubulin inhibition. By contrast, VDAC3 was insensitive at tubulin concentrations even 5-fold higher than those used to inhibit VDAC1 and 2 (Maldonado et al., 2013). In parallel with those experiments, we showed that the blockage of the inhibitory effect of tubulin on VDAC by erastin, increased mitochondrial metabolism (Maldonado et al., 2013). Erastin is a VDAC 1 and 2-binding molecule identified in a synthetic lethal chemical screening shown to induce non-apoptotic cell death (Dolma et al., 2003). Interestingly, cell death induced by erastin was blocked by antioxidants but not prevented by pan-caspase inhibitors, suggesting that erastin-induced cell death was ROS-dependent (Yagoda et al., 2007). The effect of erastin on mitochondrial metabolism was independent of the inhibition of the cystine/glutamine antiporter system x_c^-, another known target of erastin (unpublished). More recently, using a cell-based high throughput screen, we identified a series of erastin-like compounds that enhance mitochondrial metabolism, promote oxidative stress leading to mitochondrial dysfunction, and decrease glycolysis as measured by lactate release (anti-Warburg; Wright et al., 2001; DeHart et al., 2018a,b). Lately, we showed that mitochondrial dysfunction promoted by erastin/erastin-like molecules was mediated by a ROS-dependent translocation of activated JNK to mitochondria (Heslop et al., 2019). In summary, the VDAC–tubulin interaction represents a new pharmacological target to turn a pro-proliferative phenotype into a cytotoxic, mitochondrial-dependent pro-oxidant metabolism. Based on our studies on VDAC regulation in cancer, we have proposed that VDAC opening is a pro-oxidant anti-Warburg switch that promote cancer cell death (Maldonado, 2017; Fang and Maldonado, 2018; Heslop et al., 2019).

Overall, pharmacologically-induced VDAC opening, as achieved by reversal of the inhibitory effect of tubulin on VDAC, triggers two distinct and nearly simultaneous effects:

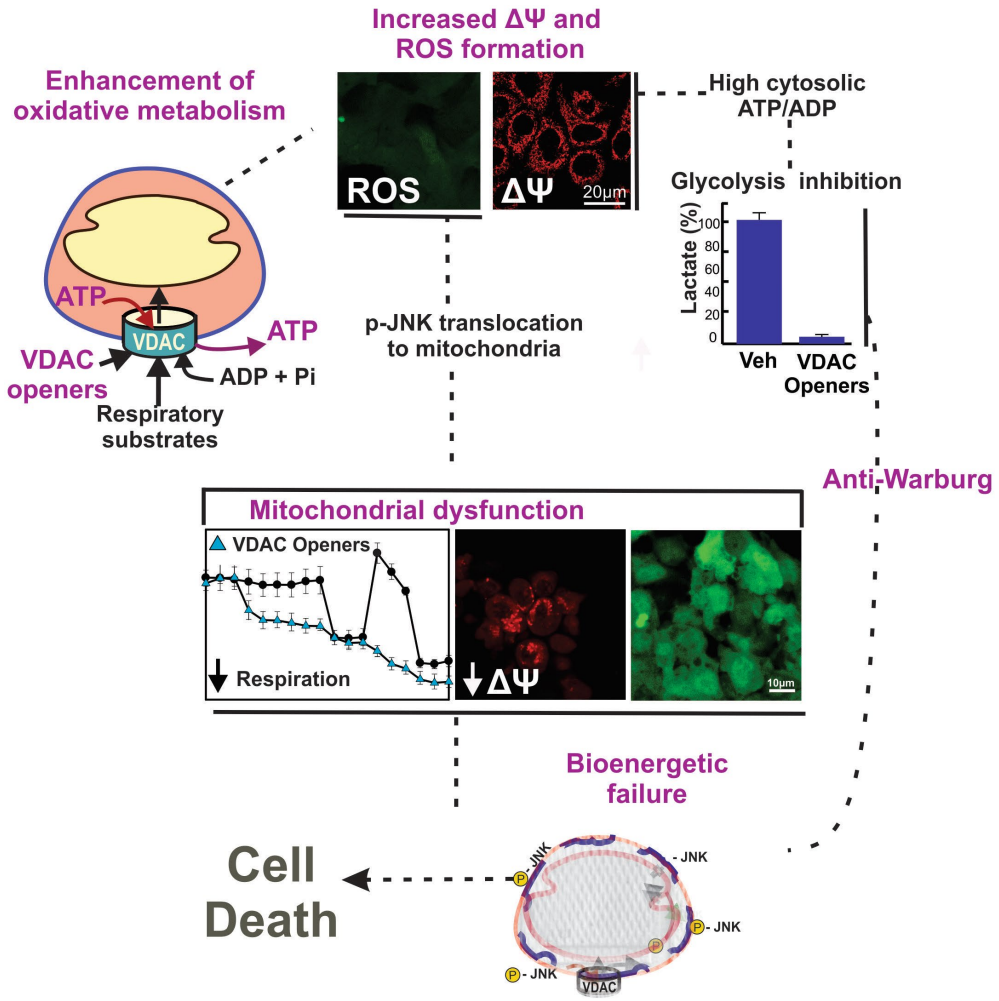

FIGURE 3 | Working model of a two-hit mechanism of cell death induced by VDAC opening. VDAC-tubulin antagonists and potentially other VDAC openers increase the flux of metabolites into and out of mitochondria. VDAC opening increases mitochondrial metabolism, promotes high cytosolic ATP/ADP ratios, increases oxygen consumption, and decreases glycolysis (anti-Warburg). VDAC opening also increases mitochondrial ROS formation causing oxidative stress and mitochondrial dysfunction. Images are from confocal fluorescence microscopy of SNU-449 hepatocarcinoma cells loaded with chloromethyl-20,70–dichlorodihydrofluorescein diacetate (ROS), and tetramethyl rhodamine methyl ester (**ΔΨ**).

the increase of mitochondrial metabolism and Oxphos with subsequent decrease of glycolysis (anti-Warburg), and the increase in ROS formation causing oxidative stress (**Figure 3**). Because of the metabolic heterogeneity of tumors, it is possible that the adverse effects of ROS accumulation and glycolysis inhibition on cell survival and proliferation be different among cells. A VDAC-dependent increase in ROS production would be likely more detrimental for highly glycolytic cells, constitutively not exposed to high levels of mitochondrial ROS. Conversely, the reversal of the Warburg effect would damage more those highly glycolytic cells that survive oxidative stress and continue proliferating, or low glycolytic cells with a presumably constitutively higher basal level of ROS. The combination of reversal of Warburg metabolism and oxidative stress by erastin-like compounds caused cell death to human hepatocarcinoma cell lines in culture and slowed tumor growth in a xenograft model of Huh7 hepatocarcinoma cells (DeHart et al., 2018a,b).

An intriguing possibility is that VDAC be implicated also in controlling metabolic fluxes in cancer stem cells (CSC), also called stem-like cancer cells, tumor-initiating cells, or cancer-initiating cells. CSC comprising 1–2% of total cells in most types of cancer, have been found in breast, lung, colon, brain, head and neck, prostate and liver tumors, among others (Batlle and Clevers, 2017). Because of the capabilities for self-renewal, tumorigenesis, invasion, and migration, CSC are associated with high risk of metastasis and relapses after chemotherapy. CSC display either a more oxidative or a more glycolytic profile, are plastic, undergo phenotypic transitions, and depending on the tumor of origin, differentiate into non-stem tumor cells to sustain tumor growth (Fonseca et al., 2017; Gupta et al., 2019). An interesting study showed that the interaction between VDAC2 and a subunit of the phosphofructokinase 1 tetramer regulates glucose metabolism and modulates the phenotypic reprogramming of glioma stem

cells (Zhou et al., 2018). This first work on the potential role of VDAC as a glycolytic regulator of the phenotype transition between CSC and non-stem cancer cells is opening new avenues to study VDAC as a potential therapeutic target. Although, a perspective about the role of mitochondria-tubulin interactions in the regulation of mitochondrial structure and function in CSCs has recently been reviewed, there is no actual evidence that the VDAC-tubulin interaction or VDAC opening modulates CSC plasticity and proliferation (Kim and Cheong, 2020).

CONCLUDING REMARKS

Research on different aspects of cancer bioenergetics and cancer metabolism clearly showed that tumors are metabolically heterogeneous, and that many tumors, if not all, are metabolically flexible. Moreover, the pro-proliferative Warburg phenotype can be reversed by increasing oxidative metabolism. In that regard, VDAC opening is an attractive mechanism to target pharmacologically, because it serves the dual purpose of increasing mitochondrial metabolism and ROS formation, while at the same time decreasing glycolysis. The dual action of VDAC openers as anti-Warburg and promoters of oxidative stress, represent a two-hit mechanism to induce cell death. An advantage of VDAC as a target relies on the fact that regulation of only one protein has an amplifying effect, both on mitochondrial function and subsequently on biosynthetic reactions occurring in the cytosol, which depend on mitochondrial intermediaries. As it was developed here, the understanding of endogenous mechanisms regulating VDAC conformational states is relevant for cancer biology and for potential pharmacological applications. The search for drugs promoting VDAC opening directly, or indirectly by blocking endogenous regulators like is the case for VDAC-tubulin, open a new era in the development of novel metabolism-oriented cancer chemotherapy.

AUTHOR CONTRIBUTIONS

KH contributed with the search for updated literature, compilation of sources, writing and figures preparation. VM contributed with writing and preparation of **Figure 2**. EM organized the structure of the review, intervened in all the steps of the review preparation, contributed to the writing, and made final adjustments to the text and figures. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NIH grant NCI R01CA184456 and SCTR Pilot Project UL1 TR001450-SCTR to EM; and the Abney Foundation Fellowship from MUSC Hollings Cancer Center to KH.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 04 October 2021
doi: 10.3389/fphys.2021.730048

Adverse Effects of Metformin From Diabetes to COVID-19, Cancer, Neurodegenerative Diseases, and Aging: Is VDAC1 a Common Target?

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Metformin has been used for treating diabetes mellitus since the late 1950s. In addition to its antihyperglycemic activity, it was shown to be a potential drug candidate for treating a range of other diseases that include various cancers, cardiovascular diseases, diabetic kidney disease, neurodegenerative diseases, renal diseases, obesity, inflammation, COVID-19 in diabetic patients, and aging. In this review, we focus on the important aspects of mitochondrial dysfunction in energy metabolism and cell death with their gatekeeper VDAC1 (voltage-dependent anion channel 1) as a possible metformin target, and summarize metformin's effects in several diseases and gut microbiota. We question how the same drug can act on diseases with opposite characteristics, such as increasing apoptotic cell death in cancer, while inhibiting it in neurodegenerative diseases. Interestingly, metformin's adverse effects in many diseases all show VDAC1 involvement, suggesting that it is a common factor in metformin-affecting diseases. The findings that metformin has an opposite effect on various diseases are consistent with the fact that VDAC1 controls cell life and death, supporting the idea that it is a target for metformin.

Keywords: apoptosis, cancer, metabolism, metformin, hexokinase, COVID-19, mitochondria, VDAC

OPEN ACCESS

Edited by:
Radhakrishnan Mahalakshmi, Indian Institute of Science Education and Research, Bhopal, India

Reviewed by:
Francesca Guarino, University of Catania, Italy
Amandine Rovini, Centre Hospitalier Universitaire de Limoges, France

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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *24 June 2021*
Accepted: *24 August 2021*
Published: *04 October 2021*

Citation:
Shoshan-Barmatz V, Anand U, Nahon-Crystal E, Di Carlo M and Shteinfer-Kuzmine A (2021) Adverse Effects of Metformin From Diabetes to COVID-19, Cancer, Neurodegenerative Diseases, and Aging: Is VDAC1 a Common Target? Front. Physiol. 12:730048. doi: 10.3389/fphys.2021.730048

OVERVIEW: METFORMIN'S MOLECULAR AND CELLULAR ASPECTS, PROPOSED TARGETS, AND THERAPEUTIC MODE OF ACTION

Metformin is a biguanide derivative [3-(diaminomethylidene)-1,1-dimethylguanidine] that was first extracted from the flowers of goat's rue, the French lilac (Galega officinalis) (Bailey, 2017). Here, we present its reported effects, in addition to type 2 diabetes mellitus (T2DM) (Viollet et al., 2012), on several diseases such as cancer and cardiovascular and neurodegenerative diseases (Ghatak et al., 2011; Mazza et al., 2012; Rizos and Elisaf, 2013; Foretz et al., 2014; Kasznicki et al., 2014; Scheen et al., 2015; Hitchings et al., 2016; Novelle et al., 2016; Lv and Guo, 2020; Dardano and Del Prato, 2021). The complex and heterogeneous molecular basis of these diseases suggests that many biological signaling pathways are influenced by metformin; therefore, it is

very difficult to pin down its underlying mechanism(s) of action. It is proposed that it acts on metabolism (Da Silva et al., 2010), which is tightly linked to the cell signaling pathways involved in proliferation and survival, with their dysregulation associated with various diseases. Metformin acts via multiple mechanisms/signaling pathways including AMP-activated kinase (AMPK) signaling, the mammalian target of rapamycin (mTOR) (Pernicova and Korbonits, 2014; Howell et al., 2017), and inflammatory, mitochondrial (Owen et al., 2000; Foretz et al., 2014; Luengo et al., 2014), and insulin signaling, as well as cell death signaling whose dysregulation is associated with some diseases (Viollet et al., 2012). Metformin increased the ratio of AMP/ATP and suppressed mitochondrial respiratory chain complex I, resulting in increased AMPK signaling, and reduced glucagon signaling (Pernicova and Korbonits, 2014). Metformin downregulates oxidative phosphorylation genes, AKT and p38, and type I interferon response pathways (interleukin 1β and interferon γ) (Titov et al., 2019), inhibits mTOR (Kalender et al., 2010; Howell et al., 2017), stimulates the blood cellular landscape, and increases reactive oxygen species (ROS) production (Mogavero et al., 2017). Moreover, metformin treatment has been associated with various classifications of age-related cognitive decline, showing mixed results with both positive and negative findings (Campbell et al., 2018).

A special issue devoted to "Metformin: Beyond Diabetes" has recently been published (Bost et al., 2019). Here, we focused on the functions of the mitochondria and their governor protein VDAC1 in the effects of metformin. Mitochondria are responsible for produce energy and perform other functions associated with essential metabolism and cell signaling. Mitochondrial dysfunction is present in many diseases from T2DM to cancer, cardiovascular diseases, obesity, renal diseases, and all neurodegenerative diseases (Sorrentino et al., 2018). Moreover, we have introduced VDAC1 as a protein that possibly mediates the multiple effects of metformin. It is overexpressed in several diseases, and its overexpression is induced by apoptosis inducers. Accumulated data showed that VDAC1 overexpression is common in many diseases (T2DM, cancer, Alzheimer's' disease, Parkinson's disease, cardiovascular diseases, and more) that are affected by metformin-affecting diseases (**Table 1**). The relationship between VDAC1 and the reported diverse effects of metformin and the major proposed metformin mechanisms of action are presented here.

VOLTAGE-DEPENDENT ANION CHANNEL: ISOFORMS, MITOCHONDRIA FUNCTION, AND OVEREXPRESSION

Mitochondria play a fundamental role in metabolism, not only by producing the main energy for cellular functions, but they also play a crucial role in almost all aspects of cell biology and regulate cellular homeostasis, metabolism, innate immunity, cell death (apoptosis, necroptosis, pyroptosis ferroptosis, autophagy, necrosis), epigenetics, and more (Wallace, 2005; Mcbride et al., 2006; Murphy and Hartley, 2018). Because mitochondria metabolism dysregulation is associated with several severe diseases, mitochondria are a potential target for therapeutic intervention (Schirrmacher, 2020).

Mitochondria contain about 1,000 different proteins with different functions that depend on the exchange of metabolites and ions between the cytosol and mitochondria. Therefore, metabolites must be transported across both the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The voltage-dependent anion channel 1 (VDAC1) allows the transfer of metabolites across the OMM, while the IMM is equipped with many transporters, the carrier proteins, each of which is responsible for transporting specific metabolites across the IMM (Shoshan-Barmatz et al., 2010, 2015; Colombini, 2016; Shoshan-Barmatz et al., 2017a,b; De Pinto, 2021).

Thus, VDAC1, as a multi-functional protein, is a key regulator of mitochondrial function serving as a mitochondrial gatekeeper. It controls the metabolic and energetic crosstalk between the mitochondria and the rest of the cell, and it is also one of the key proteins in mitochondria-mediated apoptosis (Shoshan-Barmatz et al., 2010, 2015; Shoshan-Barmatz and Ben-Hail, 2012; Magri et al., 2018; De Pinto, 2021; **Figure 1**).

In mammals, three isoforms of VDAC (VDAC1, VDAC2, and VDAC3) have been identified and shown to share many structural and functional properties (De Pinto et al., 2010; Raghavan et al., 2012; Zeth and Zachariae, 2018; Messina et al., 2012). The three isoforms are expressed in most tissue types, with VDAC1 expression being higher in most, but not all tissues than that of VDAC2 and VDAC3. VDAC1 is also the most abundant and best studied isoform (De Pinto et al., 2010; Messina et al., 2012; Raghavan et al., 2012), and VDAC2 was reported as a pro-apoptotic protein, interacting with Bax (Roy et al., 2009), yet its effect in apoptosis is controversial (Maurya and Mahalakshmi, 2016), expressed mainly in cancer, but not in the brain.

Voltage-dependent anion channel 1 is composed of 19 transmembrane β-strands connected by flexible loops, forming a β-barrel, and a 26-residue-long N-terminal region that lies inside the pore (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). However, the N-terminus domain can be translocated from the internal pore to the channel surface (Geula et al., 2012a), and it can interact with hexokinase (HK) (Azoulay-Zohar et al., 2004; Zaid et al., 2005; Abu-Hamad et al., 2008; Shoshan-Barmatz et al., 2008a, 2010; Neumann et al., 2010), Aβ (Thinnes, 2011; Smilansky et al., 2015), and other proteins such as Bcl-2 and Bcl-xL (Shimizu et al., 1999, 2000; Malia and Wagner, 2007; Abu-Hamad et al., 2009; Arbel and Shoshan-Barmatz, 2010; Shoshan-Barmatz et al., 2010; Arbel et al., 2012).

Purified and membrane-embedded VDAC1 can assemble into dimers, trimers, tetramers, hexamers, and higher-order complexes (Zalk et al., 2005; Shoshan-Barmatz et al., 2008b, 2010, 2013, 2015, 2017a,b; Zeth et al., 2008; Keinan et al., 2010; Betaneli et al., 2012; Geula et al., 2012a; Shoshan-Barmatz and Golan, 2012; Shoshan-Barmatz and Mizrachi, 2012; Boulbrima et al., 2016). Contact sites between VDAC1 molecules in dimers and higher oligomers have also been identified (Geula et al., 2012b).

The positioning of VDAC1 at the OMM also allows its interaction with proteins involved in the integration of

TABLE 1 | Voltage-dependent anion channel 1 overexpression is a common factor in metformin-affecting diseases.

Diseases	VDAC1 state	Function	Ref.	Metformin association	Ref.
Type 2 diabetes (T2DM)	Overexpressed	Impairs generation of cellular ATP and induced apoptosis	Ahmed et al., 2010; Gong et al., 2012; Sasaki et al., 2012; Zhang E. et al., 2019	Improves glucose tolerance	Maruthur et al., 2016; Palmer et al., 2016; Sanchez-Rangel and Inzucchi, 2017
Cancer	Overexpressed	Increases cancer cell metabolic activity	Abu-Hamad et al., 2006; Koren et al., 2010; Arif et al., 2014; Shoshan-Barmatz et al., 2015; Arif et al., 2017; Shoshan-Barmatz et al., 2017a; Pittala et al., 2018	Anti-cancer activity	Chen et al., 2017; Andrzejewski et al., 2018; Biondani and Peyron, 2018; Xie et al., 2020
Alzheimer's disease (AD)	Overexpressed	Neuronal cell death	Perez-Gracia et al., 2008; Cuadrado-Tejedor et al., 2011; Manczak and Reddy, 2012	Neuroprotective	Qiu and Folstein, 2006; Hsu et al., 2011; Rotermund et al., 2018
Parkinson's disease (PD)	Interaction with alpha-synuclein	Regulates VDAC1 conductance and VDAC1-mediated Ca^{2+} transport	Rostovtseva et al., 2015; Rosencrans et al., 2021	Reverses certain PD phenotypes	Bayliss et al., 2016; Lu et al., 2016; Ryu et al., 2018
Epilepsy	Increased expression	Apoptosis, alerts energy charge	Jiang et al., 2007	Decreases seizure frequency and duration, stops seizures	Zhao et al., 2014; Yang et al., 2017; Nandini et al., 2019
Depression/Bipolar disease	Upregulation of VDAC and TSPO	TSPO-VDAC complex down-regulates mitophagy proteins and NLRP3 inflammasome activation	Nahon et al., 2005; Scaini et al., 2019	Anti-depressant	Guo et al., 2014
Cardiovascular diseases (CVDs)	Overexpressed	Cardiomyocyte cell death	Lim et al., 2001; Schwertz et al., 2007; Liao et al., 2015; Tong et al., 2017; Jiang et al., 2018; Tian et al., 2019; Yang et al., 2019; Klapper-Goldstein et al., 2020	Reduces risk of CVDs among patients with T2DM	Norwood et al., 2013; Griffin et al., 2017; Rena and Lang, 2018; Mohan et al., 2019
Non-alcoholic fatty liver disease (NAFLD)	Overexpressed	Mediates transport of fatty acids across the OMM	Lee et al., 2011; Tonazzi et al., 2015; Pittala et al., 2019	Attenuates the onset of NAFLD	Koren et al., 2010; Brandt et al., 2019
Inflammatory bowel disease (IBD) and gut microbiota composition	Overexpressed	Mediates apoptosis, and inflammation	Verma et al., 2021	Affects IBD and intestinal microbiota and is a barrier in small intestine	Brandt et al., 2019; Ouyang et al., 2020; Tseng, 2021
Rheumatoid arthritis (RA)	Increased VDAC1 oligomerization	Induces cardiac cell death and functional impairment in RA	Zeng et al., 2018	Improves the pathogenesis of RA	Matsuoka et al., 2020
COVID-19	Overexpressed	Induction of apoptosis	Thompson et al., 2020	Decreases risk of death in T2DM affected by COVID-19	Chen X. et al., 2020; Luo et al., 2020; Scheen, 2020; Bramante et al., 2021

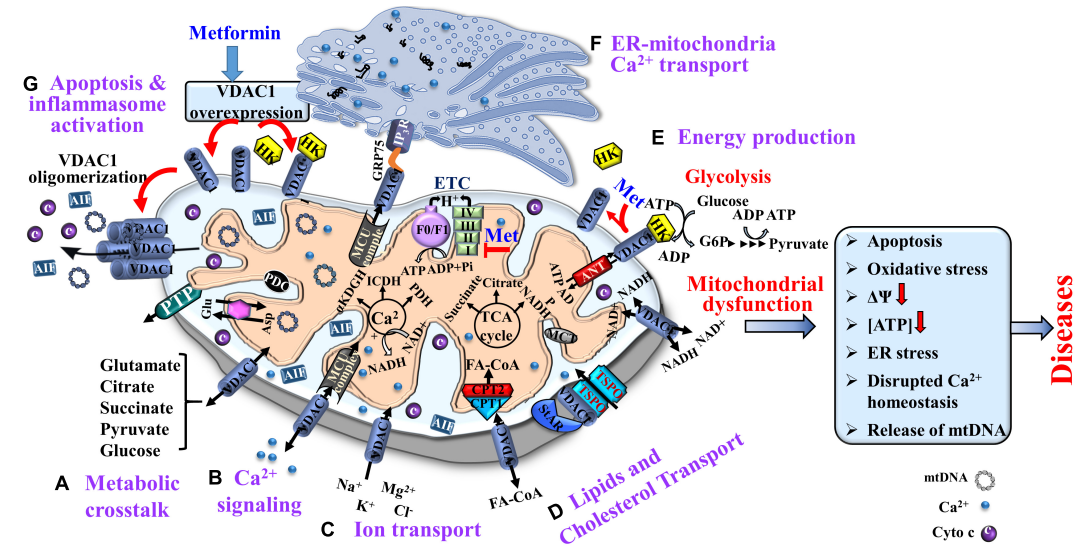

FIGURE 1 | Voltage-dependent anion channel 1, a multi-functional protein, controlling cell and mitochondria functions. VDAC1 functions include: **(A)** Controlling metabolic crosstalk between mitochondria and the rest of the cell; **(B)** Acting as a Ca^{2+} transporter in and out of the intermembrane space (IMS); **(C)** Mediating ion transport including Ca^{2+} that was transported by the MUC complex into the matrix, where Ca^{2+} regulates energy production via activation of the TCA cycle enzymes: pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), and α-ketoglutarate dehydrogenase (α-KGDH); **(D)** Mediating lipid transport such as of acyl-CoAs across the OMM to the IMS, to be converted into acylcarnitine by CPT1a for further processing by β-oxidation and the transported cholesterol; **(E)** Mediating cellular energy production by transporting ATP/ADP and NAD^+/NADH from the cytosol to the IMS, and regulating glycolysis via association with HK. Metformin induces HK detachment; **(F)**. Involvement in structural and functional association of the mitochondrion with the ER; and **(G)** Participation in apoptosis via its oligomerization, allowing cytochrome *c* release, and apoptotic cell death and mtDNA release that triggers inflammosome activation. Metformin induced VDAC1 overexpression, VDAC1 oligomerization, and apoptosis. As HK is acting as an anti-apoptotic protein, its detachment by metformin further enhances apoptosis. The TCA cycle, electron transport chain (ETC), ATP synthase (F_oF_1), and key ER-mitochondria association proteins are indicated. It is proposed that metformin may affect pathological conations that are associated with dysfunction of mitochondria activities.

mitochondrial functions with other cellular activities. Indeed, VDAC1 is considered a hub protein, as it interacts with over 100 proteins (Rostovtseva and Bezrukov, 2008; Shoshan-Barmatz et al., 2017a,b; Kanwar et al., 2020).

It functions as a docking site for diverse mitochondrial, cytosolic, nuclear and ER proteins that together mediate and/or regulate metabolic, apoptotic, and other processes in normal and diseased cells. The VDAC1 interactome includes proteins that are involved in signal transduction anti-oxidation, metabolism, apoptosis, DNA- and RNA-linked proteins, and more (Caterino et al., 2017; Shoshan-Barmatz et al., 2017a,b).

VDAC1 interacts with proteins involved in energy homeostasis such as adenine nucleotide translocase (ANT), tubulin, glycogen synthase kinase (GSK3), creatine kinase, and hexokinase (HK), and it interacts with proteins that regulate apoptosis such as Bax, Bcl-2, and Bcl-xL, and in HK functions as an anti-apoptotic protein (Shoshan-Barmatz et al., 2010; Shoshan-Barmatz and Mizrachi, 2012; Shoshan-Barmatz et al., 2017a,b). Thus, VDAC1 appears to be a convergence point for a variety of cell survival and death signals, mediated through its association with various ligands and proteins that link energy, redox. Thus VDAC1 signaling pathways in mitochondria and other cell compartments (**Figure 1**).

VDAC1 functions as a hub protein that regulates ATP production, Ca^{2+} homeostasis, and apoptosis —all crucial for proper mitochondrial function and, consequently, for normal cell physiology. Thus, alterations in VDAC1 functions are associated with mitochondrial dysfunction.

This is well demonstrated by silencing VDAC1 expression in cell lines and different cancer mouse models using specific siRNAs. We demonstrated that silencing this expression resulted in metabolic reprogramming that altered the expression of over 2,000 genes, many of which belong to mitochondria, glycolysis, and other pathways associated with metabolism. Moreover, VDAC1 silencing inhibited tumor growth, modulated the tumor microenvironment, eliminated tumor oncogenic properties (e.g., angiogenesis, stemness), and induced differentiation into normal-like cells (Arif et al., 2014, 2017, 2018, 2019a,b; Amsalem et al., 2020).

The association of VDAC1 with various diseases (Shoshan-Barmatz et al., 2020; Varughese et al., 2021) is reflected in its overexpression. VDAC1 is overexpressed in cancer (Arif et al., 2014, 2017; Shoshan-Barmatz et al., 2015; Shoshan-Barmatz et al., 2017a,b; Pittala et al., 2018), Alzheimer's disease (AD) (Perez-Gracia et al., 2008; Cuadrado-Tejedor et al., 2011; Manczak and Reddy, 2012), T2DM (Ahmed et al., 2010; Sasaki et al., 2012; Zhang E. et al., 2019), autoimmune diseases such as lupus (Kim et al., 2019b), cardiovascular diseases (CVDs) (Klapper-Goldstein et al., 2020), inflammatory bowel diseases (IBDs) (Verma et al., 2021), non-alcoholic fatty liver disease (NAFLD) (Pittala et al., 2019), COVID-19 (Luo et al., 2020; Scheen, 2020; Bramante et al., 2021),

and others (**Table 1**). As VDAC1 overexpression induces apoptotic cell death (Godbole et al., 2003; Zaid et al., 2005; Abu-Hamad et al., 2006; Ghosh et al., 2007; Weisthal et al., 2014), its overexpression in these diseases may be a common mechanism in their pathologies. It is not clear whether VDAC1 overexpression leads to the disease or if the disease state results in VDAC1 overexpression.

In post-mortem brain of patients with Down Syndrome (DS) and Alzheimer's disease (AD), the levels of VDAC1 and VDAC2 were altered (Yoo et al., 2001). In the DS cerebellum, total VDAC1 protein was elevated, whereas VDAC2 showed no significant alterations.

In AD brains, VDAC1 was significantly decreased in the frontal cortex and thalamus. VDAC2 was significantly elevated only in the temporal cortex. However, other studies showed that, in AD, VDAC1 is overexpressed early in the disease (Fernandez-Echevarria et al., 2014).

Finally, in cancer, VDAC1 (Abu-Hamad et al., 2006; Koren et al., 2010; Arif et al., 2014, 2017; Shoshan-Barmatz et al., 2015, Shoshan-Barmatz et al., 2017a,b; Pittala et al., 2018) and VDAC3 (Jozwiak et al., 2020) are overexpressed, and shown to be essential for cancer development (siRNA). VDAC2 was found to be required, for BAX-mediate apoptosis (Chin et al., 2018).

This review focuses on the relationship between VDAC1 and the reported diverse effects of metformin. For other VDAC isoforms, no published data are available, except for a report demonstrating, by using a proteomic approach, that in metformin-treated MCF-7, VDAC2, was found to be upregulated along with the proapoptotic proteins p53, Bax, and Bad (Al-Zaidan et al., 2017). With respect to T2DM, pancreatic β-cells express both VDAC1 and VDAC2 (Ahmed et al., 2010; Zhang E. et al., 2019). Under glucotoxic conditions (20 mM glucose), INS-1E cells significantly overexpressed VDAC1, whereas VDAC2 levels were reduced (Ahmed et al., 2010). However, islets from T2D pancreas donors show upregulated *VDAC1* mRNA, while *VDAC2* mRNA is suppressed, compared with islets in healthy donors (Zhang E. et al., 2019). In addition, in T2D β cells, VDAC1 levels were decreased in endoplasmic reticulum–mitochondria contact sites (Thivolet et al., 2017). Thus, not only altered *VDAC* gene expression, but also its sub-cellular localization could lead to mitochondrial dysfunction. Thus, the involvement of VDAC2 in metformin effects can not be ruled out due to its cellular functions in apoptosis (Naghdi and Hajnoczky, 2016).

Metformin also has been reported to increase VDAC1 expression levels in NCaP cells along with increased the levels of $IP3R_1$, $IP3R_2$, $IP3R_3$, and MCU mRNA, as well as VDAC1 protein (Loubiere et al., 2017), and in polycystic ovary syndrome (PCOS)-like rats treated with metformin. Zhang et al. (2017) revealed that treatment with metformin increased VDAC expression and decreased superoxide dismutase 1 (SOD1) in PCOS-like rats compared to control rats (Zhang et al., 2017). In addition, metformin in the presence of citral, but not in its absence, increased VDAC expression (Duan et al., 2021). Moreover, VDAC1 is overexpressed in diseases that were found to be modulated by metformin (**Table 1**).

METFORMIN MODE OF ACTION

The major proposed metformin mechanisms of action include modulating cell metabolism, inducing apoptosis, mitochondrial dysfunction, ER stress, inflammation, and more (Vial et al., 2019; **Figure 2**). These proposed mechanisms point to the complexity of metformin action at the molecular and cellular levels, as presented below.

Metformin Modulation of AMPK and mTOR Signaling, and Mitochondrial Functions

Metformin acts on the central cell metabolism and on several major signaling pathways including glucose metabolism and energy-sensing that involve the cellular energy sensor AMPK and mTOR signaling (Howell et al., 2017). It has been shown that metformin activates the AMPK pathway via ATM (ataxia telangiectasia mutated), LKB1 (liver kinase B1) activation, and inhibition of the mTOR pathway, leading to a reduction in protein synthesis and cell growth (Howell et al., 2017). Metformin can activate p53 by activating AMPK, thereby, inhibiting the cell cycle (Saraei et al., 2019).

AMP-activated kinase activation is required for gluconeogenesis suppression and stimulation of glucose uptake by peripheral tissues (Musi et al., 2002). However, it was recently shown that metformin inhibits hepatic gluconeogenesis in transgenic mice without AMPK or its upstream activator LKB1 (Foretz et al., 2014).

It is widely documented that metformin is one of the most potent drugs that activates AMPK (Foretz et al., 2014). Stimulating AMPK activity, affects age-related disorders including cancer, CVDs, diabetes, neurocognitive decline, and more (Wang et al., 2011; Coughlan et al., 2014). Activation of AMPK initiates the phosphorylation of tuberin and raptor (mTOR cascade proteins), leading to the rapid inhibition of mTOR pathway activity (Shaw, 2009).

At the same time, there are several cellular targets that can drive the metformin effect independently from AMPK (Viollet et al., 2012; Foretz et al., 2014). These include the electron chain complexes (ETCs) I (Owen et al., 2000), II, and IV (Drahota et al., 2014), serine-threonine liver kinase B1/AMP-activated protein kinase complex (LKB1/AMPK) (Shaw et al., 2005), adenylate cyclase (Miller et al., 2013), AMP deaminase (Ouyang et al., 2011), NADPH oxidase (Piwkowska et al., 2010), and mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014). However, other targets are also proposed such as HK and VDAC1 (see section "Hexokinase-VDAC1 Interaction as a Metformin Target").

Metformin affects glucose consumption, lactate production, oxidative metabolism, and ATP levels similarly to those promoted by insulin alone, suggesting that metformin modulates the key enzymes involved in glycolysis regulation such as HK and phosphofructokinase (PFK) (Da Silva et al., 2010).

Accumulated evidence suggests the involvement of mitochondria in metformin activities (Vial et al., 2019), and raises the question of exactly how metformin enters the

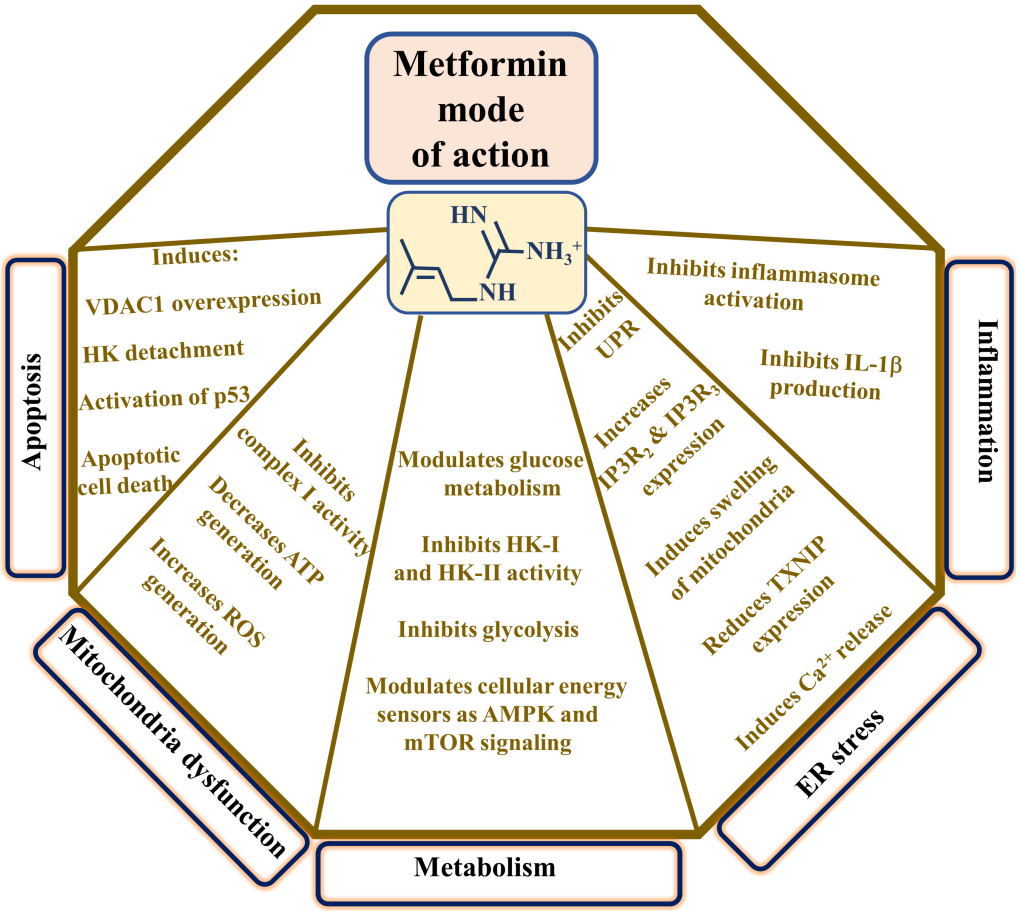

FIGURE 2 | Proposed metformin mode of action. The major proposed metformin mechanisms of action such as inducing apoptosis, mitochondrial dysfunction, modulating cell metabolism, and affecting ER stress and inflammation are presented. These are reflected in the indicated metformin effects. TXNIP indicates thioredoxin-interacting protein and UPR, the unfolded protein response.

mitochondria. Metformin distribution and cell penetration are mediated by tissue-specific transporters counting plasma membrane monoamine transporter (PMAT) in the intestine, organic cation transporter 1 (OCT1) in the liver, and both organic cation transporter 2 (OCT2) and multidrug and toxin extruder (MATE)1/2 in the kidneys (Gormsen et al., 2016). It should be noted that exactly how metformin enters the mitochondria is unclear (Fontaine, 2014). The intra-mitochondrial accumulation of phenformin, another biguanide, has been shown to involve the mitochondrial organic cation/carnitine transporter 1 (OCTN1) (Shitara et al., 2013). Metformin affecting mitochondrial function via modulation of the multifunctional OMM protein VDAC1 modulation requires no metformin transport into the mitochondria.

One of the proposed metformin targets is the mitochondrial respiratory chain protein complex-I (Owen et al., 2000; Foretz et al., 2014; Luengo et al., 2014), but it is not clear if metformin inhibits complex-I by direct interaction (Fontaine, 2014). In isolated mitochondria, very high concentrations of metformin (20–100 mM) inhibit complex-I activity, while micromolar concentrations are required for its inhibition in various cell types (El-Mir et al., 2000) or *in vivo* in skeletal muscle from healthy and diabetic rats (Wessels et al., 2014). It should be noted that clinically relevant metformin concentrations are $<100\ \mu$ M.

Several explanations have been proposed for this discrepancy between the metformin concentration required for complex-I inhibition in the *in vitro* and *ex vivo* experiments and the clinically relevant concentrations (He and Wondisford, 2015), including that the positive charge of metformin slows it accumulation within the matrix due to the transmembrane electrochemical potential ($\Delta\Psi$) (Bridges et al., 2014).

Metformin inhibiting complex-I activity reduces ATP production and elevates the levels of AMP and ADP. The increase in the AMP/ATP ratio with increased AMP leads to inhibition of gluconeogenesis and activation of AMPK (Miller et al., 2013; Foretz et al., 2014; Pernicova and Korbonits, 2014).

Hexokinase-VDAC1 Interaction as a Metformin Target

The first step of glycolysis is catalyzed by HK, with the isoforms HK-I or HK-II known to bind to the OMM through VDAC1

(Azoulay-Zohar et al., 2004; Zaid et al., 2005; Abu-Hamad et al., 2008; Shoshan-Barmatz et al., 2008a, 2010; Neumann et al., 2010; Shoshan-Barmatz et al., 2017a,b). This has a metabolic benefit as phosphorylation of glucose by VDAC1-bound HK is coupled to the mitochondrial-produced ATP with ATP channeling enhancing glycolysis. The binding of HK to VDAC1 has another important aspect in inhibiting cytochrome *c* (Cyto c) release and, subsequently, apoptosis occurring in cells expressing native, but not E-72Q-mutated VDAC1 (Zaid et al., 2005; Abu-Hamad et al., 2008; Arzoine et al., 2009). Hence, HK by binding to VDAC1, provides the cell with both a metabolic benefit and apoptosis suppression.

Metformin has been shown to directly inhibit the enzymatic activity of HK-I and HK-II through an allosteric modification of HK structure, leading to the inhibition of glucose-6-phosphate (G-6-P) production, thereby, inhibiting glycolysis (Marini et al., 2013; Salani et al., 2013; Picone et al., 2016). Also, metformin induces the detachment of HK-II from its binding site in the OMM (Salani et al., 2013, 2014). HK-I or HK-II detachment from the mitochondria has been shown to activate apoptosis (Azoulay-Zohar et al., 2004; Zaid et al., 2005; Abu-Hamad et al., 2008; Shoshan-Barmatz et al., 2008a, 2010). Thus, metformin detachment of HK-I/HK-II is expected to result in apoptotic cell death. Indeed, metformin acting through HK and VDAC1 not only impairs metabolism, but also induces mitochondrial dysfunction and cell death (Marini et al., 2013; Salani et al., 2013). This may explain the pro-apoptotic effect of metformin on cancer cells that overexpress HK-I/HK-II (Smith, 2000).

Detaching HK from VDAC1 has also been shown to impair glutamate transporter-mediated glutamate uptake (Jackson et al., 2015). Thus, it is expected to impair the uptake of excitatory neurotransmitter glutamate, affecting synaptic activity.

Collectively, the above strongly suggests that metformin's mode of action involves the mitochondria, as inhibition of complex I, glycerophosphate dehydrogenase, and HK can affect the NAD/NADH ratio and ATP production. Also, as emphasized above, the HK–VDAC1 complex is critical in metabolism and apoptosis, and in detaching HK from VDAC1, leading to impairment of mitochondrial activity and apoptosis induction. Moreover, metformin increases VDAC1 expression levels, shifting the equilibrium from monomeric to oligomeric VDAC1, thereby, leading to apoptotic cell death. Detachment of HK from VDAC1 and induction of VDAC1-associated cell death can explain metformin's anti-cancer effect via the induction of apoptosis.

The mechanisms underlying metformin's protective effects in several diseases, and the link between metformin, HK and VDAC1 are presented below (section "Cancer, Metformin, VDAC1, and HK"). Among the proposed metformin neuroprotection activity is its inhibition of the lipid phosphatase Src homology 2 domain, containing inositol-5-phosphatase 2 (SHIP2), which when elevated, reduces Akt (protein kinase B) activity (Hori et al., 2002). Metformin, by inhibiting SHIP2 activity, stimulates Akt activity, and thus, the phosphorylation of HK by Akt, which was shown to increase its binding to VDAC (Roberts et al., 2013), thereby, protecting against apoptosis.

Metformin Modulating Apoptosis: Mitochondria, VDAC1, and HK as Key Factors

Along with regulating cellular energy and metabolism, VDAC1 is involved in mitochondria-mediated apoptosis, participating in the release of apoptotic proteins, and interacting with the anti-apoptotic proteins, Bcl_2 and Bcl-xL, and HK, overexpressed in cancers (**Figure 1G**).

Apoptotic signals change the mitochondrial membrane permeability, allowing the release of apoptogenic proteins such as Cyto *c*, apoptosis-inducing factor (AIF), and SMAC/Diablo from the intermembrane space (IMS) into the cytosol (Kroemer et al., 2007; Shoshan-Barmatz et al., 2015). These proteins participate in complex processes, leading to the activation of proteases and nucleases, thereby to degradation of proteins and DNA, and cell death. Several hypotheses regarding the mechanism of mitochondria-mediated apoptosis have been proposed (Garrido et al., 2006). Our and others' studies demonstrated that upon apoptosis induction by various reagents such as chemotherapy drugs, arbutin, prednisolone, cisplatin, viral proteins, elevated cytosolic Ca^{2+}, or UV irradiation, VDAC1 expression levels were increased (Shoshan-Barmatz et al., 2020). The overexpressed VDAC1 leads to its oligomerization to form a large pore, allowing the release of mitochondrial pro-apoptotic proteins (Zalk et al., 2005; Shoshan-Barmatz et al., 2008b, 2013; Ujwal et al., 2009; Keinan et al., 2010; Huang et al., 2015; Ben-Hail et al., 2016). We further demonstrated that VDAC1 oligomerization is a dynamic process, and that it is a general mechanism common to numerous apoptotic stimuli, acting via different initiating cascades (Zalk et al., 2005; Shoshan-Barmatz et al., 2008b, 2013; Keinan et al., 2010, 2013; Weisthal et al., 2014; Huang et al., 2015; Ben-Hail and Shoshan-Barmatz, 2016; Ben-Hail et al., 2016). Moreover, recently, we identified VDAC1-interacting molecules such as diphenylamine-2-carboxylate (DPC) (Ben-Hail and Shoshan-Barmatz, 2016) and new molecules developed in our lab such as VBIT-4 and VBIT-12 that were found to prevent VDAC1 oligomerization and subsequent apoptosis. Furthermore, cyathin-R, a cyathane-type diterpenoid, was found to induce apoptosis in Bax/Bak-depleted cells, but not when VDAC1 that was inhibited by DPC was depleted (Huang et al., 2015).

Based on these results we proposed a novel model in which VDAC1 exists in a dynamic equilibrium between monomeric and oligomeric states, with apoptosis inducers shifting the equilibrium toward oligomers, forming a large channel that enables Cyto c release, leading to cell death (Zalk et al., 2005; Shoshan-Barmatz et al., 2008b, 2013; Keinan et al., 2010; Kasznicki et al., 2014; Huang et al., 2015; Ben-Hail et al., 2016). Furthermore, a correlation between drug effectiveness in apoptosis induction and VDAC1 expression levels has been reported (Castagna et al., 2004; Lai et al., 2006; Tajeddine et al., 2008). Moreover, not only apoptosis inducers, but also stress and pathological conditions can induce VDAC1 overexpression and,

thus, trigger apoptosis (Keinan et al., 2013; Weisthal et al., 2014; Shoshan-Barmatz et al., 2020).

Metformin-induced apoptosis (Ben Sahra et al., 2010a; Malki and Youssef, 2011; Sancho et al., 2015) can be mediated via inducing VDAC1 overexpression and its oligomerization. Treatment of mice for 3 months with metformin increased the expression of VDAC1 in the cortex, but not in the hippocampus (Wijesekara et al., 2017). Moreover, in the cortical region, plasmalemmal VDAC1 (pl-VDAC1) was found as oligomers in areas where metformin induced Aβ-aggregate accumulation, and apoptotic neurons were observed (Wijesekara et al., 2017). In addition, metformin increased VDAC1 expression levels in NCaP cells (Loubiere et al., 2017) and in polycystic ovary syndrome-like rats (Zhang et al., 2017), and in the presence of citral (Duan et al., 2021).

Considering the pro-apoptotic effects of VDAC1 overexpression leading to its oligomerization, and subsequently to apoptotic cell death, we suggest that metformin, as do other apoptosis inducers and stress conditions, induces apoptosis (Ben Sahra et al., 2010a; Malki and Youssef, 2011; Sancho et al., 2015) via triggering VDAC1 overexpression and increasing the expression levels of p53, Bax, and Bad, while reducing the expression levels of Akt, Bcl-2, and Mdm2 (Malki and Youssef, 2011).

Previously, it was demonstrated that apoptosis induced by various reagents disrupted intracellular Ca^{2+} ($[Ca^{2+}]i$) homeostasis (Keinan et al., 2013; Weisthal et al., 2014). Moreover, it has been shown that pro-apoptotic agents inducing upregulation of VDAC1 expression levels are Ca^{2+}-dependent (Keinan et al., 2013; Weisthal et al., 2014; Shoshan-Barmatz et al., 2015). Metformin has been shown to induce ER stress and Ca^{2+} released from the ER and, subsequently, its uptake by the mitochondria, leading to mitochondrial swelling (Loubiere et al., 2017). Interestingly, metformin significantly increased the levels of mRNA encoding for IP_3R_2 and IP_3R_3 (Loubiere et al., 2017). Thus, the increase in cytosolic Ca^{2+} may be responsible for VDAC1 overexpression, as found for other inducers of this overexpression (Keinan et al., 2013; Weisthal et al., 2014; Shoshan-Barmatz et al., 2015).

Recently, we showed that metformin interacted with purified VDAC1, and inhibited the channel conductance of bilayer-reconstituted VDAC1 (Zhang E. et al., 2019). The direct interaction of metformin with VDAC1 may modulate VDAC1 activity, thereby, mitochondrial functions. This metformin-VDAC1 interaction is currently subjected to further studies. This together with metformin inducing VDAC1 overexpression and apoptotic cell death, may suggest that metformin-apoptosis induction involves VDAC1.

METFORMIN'S MULTIPLE EFFECTS ON VARIOUS DISEASES

Metformin, besides being the first-line medication used to treat T2DM, was shown to be a potential drug candidate to treat several other diseases including various cancers, cardiovascular diseases, diabetic kidney disease, neurodegenerative diseases, renal diseases, obesity, inflammation, COVID-19 in diabetic patients, and aging (**Figure 3**; Reina and De Pinto, 2017; Magri et al., 2018; Shoshan-Barmatz et al., 2020).

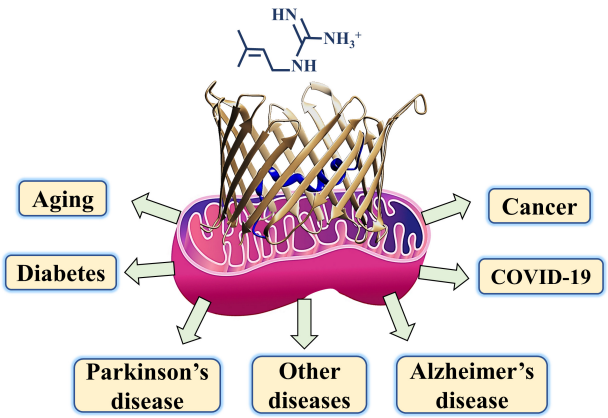

FIGURE 3 | A schematic presentation of metformin targeting diseases such as diabetes, COVID-19, cancer, neurodegenerative diseases, and aging with mitochondria and their gatekeeper VDAC1 proposed as a common target.

Diabetes, Mitochondria, VDAC1, and Metformin

Diabetes mellitus (DM) is a group of chronic metabolic disorders characterized by hyperglycemia that ultimately leads to damage of different body systems (American Diabetes, 2014). It is the ninth major cause of mortality worldwide (Zheng et al., 2018), exerting a global public health threat. Patients with untreated DM or prolonged hyperglycemia may suffer from polyuria, polydipsia, polyphagia, weight loss, and blurred vision (Galtier, 2010). DM is growing at epidemic proportions, becoming increasingly prevalent in all countries. It is estimated to increase to 700 million cases by the year 2045 (English and Lenters-Westra, 2018; IDF, 2019). It was also reported that in 2014, there were 422 million people who had diabetes (NCD Risk Factor Collaboration., 2016), and 5.1 million deaths among people between 20 and 79 years old were attributed to it in 2013 (Zimmet et al., 2016). Type 2 diabetes mellitus (T2DM) is more prevalent than type 1 (T1DM) and in adults, 90–95% of patients with diabetes have T2DM.

Type 2 diabetes mellitus management consists mainly of drugs that reduce insulin resistance and glucose uptake in the intestine, as well as reduce gluconeogenesis in the liver, and drugs that increase glucose excretion through the urine. These drugs include metformin, sulfonylureas, and SGLT2 (sodium-glucose transporter protein 2) inhibitors (Tan et al., 2019). The use of metformin as a therapeutic agent began in France in 1957, and was approved for use in Canada in 1972, and in the United States by the Food and Drug Administration (FDA) in 1994 for use by those with non-insulin-dependent T2DM. Today, metformin is the first-line, leading oral antidiabetic drug prescribed for the treatment of T2DM (Pernicova and Korbonits, 2014; Hotta, 2019) either alone or in combination with

thiazolidinediones, sulfonylureas, or other hypoglycemic agents (Maruthur et al., 2016).

Metformin, as an anti-hyperglycemic agent, improves glucose tolerance in patients with T2DM by lowering both basal and postprandial plasma glucose (Maruthur et al., 2016; Palmer et al., 2016; Sanchez-Rangel and Inzucchi, 2017). It increases glucose uptake and utilization by intestinal cells and lactic acid formation in these and liver cells (Mccreight et al., 2016). It reduces liver glucose production, slows glucose transfer to the blood, increases glucose utilization by muscle cells (in anaerobic glycolysis, due to the suppression of mitochondrial function and aerobic respiration in these cells), lowers insulin resistance, and increases incretin activity, and especially glucagon-like peptide 1 (GLP-1), which contributes to raising insulin levels and lowering blood glucagon levels. In addition to the effects on glucose level, metformin also contributes to the suppression of fatty acid synthesis and gluconeogenesis, and the removal of insulin sensitivity reduces blood levels of LDL cholesterol and triglycerides (Viollet et al., 2012).

The proposed metformin mode of action in regulating blood glucose level is not completely understood, and multiple potential mechanisms have been proposed. It enters liver cells mainly through OCT1, and suppresses the mitochondrial respiratory chain (complex-I). It also reduces ATP production and increases AMP levels activating AMPK; inhibits glucagon-induced elevation of cAMP with reduced activation of protein kinase A (PKA); and decreases gluconeogenesis (liver glucose production) (Foretz et al., 2014; Madiraju et al., 2014). As a result, glucose depletion in the cell increases, and the cell reduces glucose formation and increases the amount of glucose transferred from the blood.

Mitochondria have been connected to the pathophysiology of diabetes with changes in their quality, quantity, and function reported to occur in diabetics (Sivitz and Yorek, 2010).

Recently, we found that VDAC1 expression levels were increased in islets from T2DM and non-diabetic organ donors under glucotoxic conditions (Zhang E. et al., 2019). The overexpressed VDAC1 is mistargeted to the plasma membrane of the insulin-secreting β cells, resulting in a loss of ATP, and thereby no insulin secretion occurs. Moreover, VDAC1 antibodies, as well as metformin, and specific VDAC1-interacting molecule VBIT-4, restore the impaired generation of ATP and glucose-stimulated insulin secretion in T2DM islets (Zhang E. et al., 2019). Furthermore, treatment of db/db mice with VBIT-4 prevents hyperglycemia, and maintains normal glucose tolerance and physiological regulation of insulin secretion (Zhang E. et al., 2019). These metformin effects are not mediated via activation of AMP kinase (Foretz et al., 2014) or through antioxidant effects such as an AMPK inhibitor (MRT199665), nor do the antioxidants N-Acetyl cysteine influence metformin's effects.

These findings suggest that VDAC1 is a diabetes executer protein that can be targeted by its interacting molecules, as indicated above (Zhang E. et al., 2019). Moreover, high glucose enhances VDAC1 expression levels by elevating the expression of SREBP1 and SREBP2, the transcription factors of VDAC1 (Zhang E. et al., 2019).

Several recent studies have identified bacterial effectors of metformin therapy (Pryor et al., 2019), Metformin signatures in the human gut microbiome of T2DM were demonstrated using 784 available human gut metagenomes, and proposed mechanisms contributing to the beneficial effects of the drug on the host's metabolism (Forslund et al., 2015). Metformin-induced changes in T2DM patients are expressed by the significant decrease in *Verrucomicrobia* and *Firmicutes* and an increase in *Actinobacteria* and *Bacteroidetes* (Zhang et al., 2015; De La Cuesta-Zuluaga et al., 2017; Nakajima et al., 2020). Furthermore, *Escherichia*, *Streptococcus*, *Subdoligranulum*, *Clostridium*, *Bacteroides*, and *Collinsella* were the genus-level bacteria that increased, whereas *Ruminococcus* and *Faecalibacterium* bacteria decreased (Rosario et al., 2018; Nakajima et al., 2020). For example, when metformin was given to healthy young men, their *Bilophila wadsworthia* and *Escherichia/Shigella spp.* increased, whereas their *Clostridium spp.* and *Intestinibacter spp.* decreased (Bryrup et al., 2019).

In addition, metformin was found to alter upper small intestinal microbiota that impact the sodium-dependent glucose cotransporter (SGLT1) sensing glucoregulatory pathway (Baur and Birnbaum, 2014).

The link between metformin effects on gut microbiota, and VDAC1 is not clear, yet it is likely related to VDAC1 function as transporter of verity of metabolites that their levels can be affected by the microbiota and its modulation by metformin.

Cancer, Metformin, VDAC1, and HK

Generally, diabetic patients are more expected to develop a variety of cancers (Shikata et al., 2013; Collins, 2014; Cignarelli et al., 2018; Kang et al., 2018; Scully et al., 2020). These patients are at increased risk of developing cancers such as breast, prostate, pancreatic, and non-small cell lung (NSCLC) cancer compared to non-diabetic patients (Richardson and Pollack, 2005; Pierotti et al., 2013).

In the past decades, several epidemiologic studies have linked numerous *in vitro* and *in vivo* studies, along with epidemiological, clinical, and preclinical evidence supporting the anti-cancer activity of metformin (Ben Sahra et al., 2010b; Evans et al., 2010; Jalving et al., 2010; Dowling et al., 2011; Zhang et al., 2011; Rizos and Elisaf, 2013; Kasznicki et al., 2014; Higurashi et al., 2016; Mohamed Suhaimi et al., 2017).

The molecular mechanisms associated with the anti-cancer activity of metformin are complex and include several targets and pathways (Saini and Yang, 2018). Several potential mechanisms proposed for its ability to suppress cancer *in vitro* and *in vivo* include: (a) activation of the LKB1/AMPK pathway, (b) induction of cell cycle arrest, and/or apoptosis, (c) inhibition of protein synthesis, (d) reduction in circulating insulin levels, (e) inhibition of the unfolded protein response (UPR), (f) activation of the immune system, and (g) eradication of cancer stem cells (Kourelis and Siegel, 2012).

Metformin anticancer effects include AMP-activated protein kinase activation, mTOR inactivation, mitogen-activated protein kinase 1 (MEK)/extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway inhibition (Pernicova and Korbonits, 2014). It is also suggested

that metformin has an anti-tumor effect by lowering insulin levels and disabling the mTOR in the cell (Del Barco et al., 2011; Foretz et al., 2014; Zhang et al., 2018).

In vitro, metformin exhibits a strong anti-proliferative action on cancer cell lines derived from the breast, colon, ovaries, pancreas, lung, and prostate (Andrzejewski et al., 2018), as well as in leukemia, and pancreatic and colorectal cancers (Chen et al., 2017; Biondani and Peyron, 2018; Xie et al., 2020). Metformin in combination with 5-FU strongly inhibited colorectal cancer (Wang S. Q. et al., 2019), and it was shown to suppress cancer initiation and progression in genetic mouse models (Chen et al., 2017). It was shown to selectively inhibit metastatic colorectal cancer with the KRAS (Kirsten rat sarcoma viral oncogene homolog) mutation, inhibiting cell proliferation by inactivating both RAS/ERK and AKT/mTOR signaling (Xie et al., 2020). Metformin suppresses cancer stem cells (CSCs) in the tumor, and enhances the responsiveness of glioma cells to temozolomide (Zhang et al., 2010). It has also been demonstrated that metformin can be used as a co-adjuvant, reverting the resistant-like pattern of a human glioma cell line both *in vitro* and *in vivo* (Rattan et al., 2012).

Metformin has been shown to facilitate DNA repair, which is critical for cancer prevention (Lee et al., 2016). It was proposed that it targets pancreatic CSCs, but not their differentiated non-CSCs (Sancho et al., 2015). It is further proposed that mitochondrial inhibition by metformin creates an energy crisis and induces CSC apoptosis (Sancho et al., 2015).

In prostate cancer cells, the combination of metformin and 2-deoxyglucose (2-DG) (that binds to HK) drastically reduced intracellular ATP levels through the inhibition of the mitochondrial complex 1 and glycolysis (Ben Sahra et al., 2010a). Metformin was also shown to affect the glycolytic rate by directly inhibiting HK-II activity and its interaction with the mitochondria (Salani et al., 2014). *In silico* models suggest that metformin mimics G6P (glucose 6-phosphate) features and binds to its binding site in HK (Salani et al., 2013). The HK–VDAC1 complex formation is regulated by Akt (protein kinase B) (Majewski et al., 2004) and glycogen synthase kinase 3 beta (GSK3β), while the HK–VDAC complex is disrupted by VDAC phosphorylation (Pastorino et al., 2005). Cancer cells express high levels of mitochondria-bound HK that not only enhances glycolysis, but also protects against mitochondria-mediated apoptosis via direct interaction with VDAC1 (Bryson et al., 2002; Azoulay-Zohar et al., 2004; Zaid et al., 2005; Abu-Hamad et al., 2008, 2009; Arzoine et al., 2009). Thus, metformin inhibits HK activity and induces HK detachment from the VDAC1, resulting in both inhibiting cancer cell metabolism and inducing apoptosis. It should be noted, however, that some clinical trials have failed to show a protective association between metformin and survival in colorectal cancer (CRC) patients with T2DM (Cossor et al., 2012; Mc Menamin et al., 2016). VDAC1 has been shown as a critical protein in cancer development and survival (Mazure, 2017) and many anti-cancer compounds were shown to mediates their activity via targeting VDAC1 (Reina and De Pinto, 2017; Magri et al., 2018). Metformin has been consistently shown to reduce the risk of various types of cancer including the breast, colon, ovaries, pancreas, lung, prostate, leukemia, and colorectal cancers (Chen et al., 2017; Andrzejewski et al., 2018; Biondani and Peyron, 2018; Xie et al., 2020) (see section "Cancer, Metformin, VDAC1, and HK"). Thus, it is possible that the anticancer effects of metformin may involve some common pathophysiological mechanisms (Del Barco et al., 2011). Among these the common hallmarks of cancer are reprogramming of energy metabolism and resisting cell death (Hanahan and Weinberg, 2011).

Cancer cells need excess energy and metabolites are required for cell proliferation and migration to distant organs for metastasis. Metabolic reprogramming in cancer cells is a significant pathogenic mechanism in cancer involving flexibility of the metabolic machinery. VDAC1, by regulating the metabolic and energetic functions of mitochondria, controls the fate of cancer cells. The overexpression of VDAC1 in various tumors obtained from patients, and in tumors established in mouse models, as well as in cancer cell lines (Arif et al., 2014, 2017; Shoshan-Barmatz et al., 2015, Shoshan-Barmatz et al., 2017a,b; Pittala et al., 2018), points to its significance in high energy-demanding cancer cells. Indeed, the pivotal role of VDAC1 in regulating cancer cellular energy, metabolism, and viability is reflected in the findings that downregulation of VDAC1 expression reduced cellular ATP levels, metabolite exchange between the mitochondria and cytosol cell proliferation, and tumor growth (Abu-Hamad et al., 2006; Koren et al., 2010; Shoshan-Barmatz and Golan, 2012; Arif et al., 2014, 2017).

Metformin via interacting with VDAC1 and modulating its conductance it can affect cancer cell metabolism. Metformin also blocks the Warburg effect in energy metabolism of cancer cells (Del Barco et al., 2011). Metformin is well recognized for its effects on the activation of AMPK, followed by the inhibition of mTOR (Viollet et al., 2012) and its activation is commonly observed in many types of cancer cells (Hanahan and Weinberg, 2000).

Another hallmark of cancer cells is their ability to suppress pro-apoptotic pathways and/or to activate anti-apoptotic mechanisms (Fulda, 2009; Hanahan and Weinberg, 2011) associated with drug resistance (Johnstone et al., 2002). Cancer cells overexpress anti-apoptotic proteins, such as the Bcl-2 family of proteins and HK, preventing the release of Cyto c from the mitochondria. VDAC1, by interacting with the anti-apoptotic proteins and HK, protects tumor cells from cell death (Shimizu et al., 1999, 2000; Pastorino et al., 2002, 2005; Pedersen et al., 2002; Shi et al., 2003a,b; Azoulay-Zohar et al., 2004; Zaid et al., 2005; Mathupala et al., 2006; Malia and Wagner, 2007; Abu-Hamad et al., 2008, 2009; Arzoine et al., 2009; Arbel and Shoshan-Barmatz, 2010; Shoshan-Barmatz et al., 2010; Arbel et al., 2012; Geula et al., 2012a).

In addition, overexpression of VDAC1 is induced by various apoptosis-inducing conditions such as chemotherapy drugs, UV irradiation, and viral proteins that increase the level of VDAC1 expression, with apoptosis being correlated with VDAC1 expression levels (Shoshan-Barmatz et al., 2010, 2013, 2015, 2020; Keinan et al., 2013; Shoshan-Barmatz et al., 2017a,b). Metformin's anti-cancer effects can be mediated through induction of VDAC1 overexpression, as shown in NCaP cells (Loubiere et al., 2017) in polycystic ovary syndrome-like rats (Zhang et al., 2017) and

the presence of citral in RD cells (Duan et al., 2021). VDAC1 overexpression leads to apoptosis induction, as presented in Section "Metformin Modulating Apoptosis: Mitochondria, VDAC1, and HK as Key Factors."

Finally, apoptosis and VDAC1 overexpression, as induced by pro-apoptotic agents, are Ca^{2+}-dependent (Keinan et al., 2013; Weisthal et al., 2014; Shoshan-Barmatz et al., 2015). As VDAC1 controls intracellular Ca^{2+} homeostasis, metformin disrupting calcium fluxes involving ER and mitochondria (Loubiere et al., 2017) may involve VDAC1.

Thus, the anti-cancer effects of metformin can be mediated through induction of VDAC1 overexpression and thereby apoptosis, affecting cell metabolism and/or Ca^{2+} homeostasis.

Metformin also affects cancer cells' resistance to various drugs via modulating the activity or levels of ATP-binding cassette (ABC) transporters. The ABC family of transporters mediate the transport of a variety of compounds at the cost of ATP hydrolysis. Among the ANC transporters are the multiple drug resistant (MDR) proteins MDR1-P-glycoprotein (Pgp), MRP1 (the multidrug resistance protein 1, ABCC1), and others, which in cancer cells can cause resistance to various drugs (Borst and Elferink, 2002). Pgp, and MRP1 confer treatment resistance via the exclusion of drugs such as etoposide, daunorubicin, vinblastine, doxorubicin, and others (El-Awady et al., 2016; Joshi et al., 2016; Wijdeven et al., 2016). For example, P-gp has been shown to be overexpressed in various cancers such as in 52% of acute lymphocytic leukemia (ALL) patients, and this is correlated with reduced survival and treatment resistance (Olarte Carrillo et al., 2017).

Finally, metformin has been shown to affect cancer cells resistance to several drugs, such as by reducing the expression of MDR protein (**Table 2**).

The molecular relationship between T2DM and tumorigenesis has not yet been fully elucidated. Previous studies have suggested that there are several factors associated with patients with T2DM, that make them more likely to develop tumors. These include insulin resistance that leads to increased levels of insulin and insulin-like growth factor (I/IGF), which could bind to receptors and activate the downstream phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling pathways, leading to cell proliferation (Gallagher and Leroith, 2010; Djiogue et al., 2013). An additional factor is inflammation, suggesting that the insulin resistance characterizing T2DM may produce a large number of cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL)-6, and IL-1β (Donath and Shoelson, 2011). These cytokines activate nuclear factor-κB and Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT-3) pathways, which are important signaling pathways in tumorigenesis (Neurath and Finotto, 2011).

Type 2 diabetes mellitus patients treated with metformin showed a decreased risk of developing cancer. A study encompassing 27 clinical trials (∼24,000 patients) showed that in people at early stages of colon and rectum cancer, metformin improved recurrence-free survival by 37%, and cancer-specific survival by 42%, and in early stage prostate cancer, it increased recurrence-free survival by 17% and cancer-free survival by 42%, compared with non-metformin users (Coyle et al., 2016). In head and neck cancer, diabetic patients treated with metformin had a 46% reduction in the risk of developing this cancer type compared to non-diabetic patients (Figueiredo et al., 2016). Similarly, the risk of gastric cancers in metformin users decreased by 55% compared with non-users (Tseng, 2016). Most of the studies showed that metformin inhibited cancer development, and showed no evidence of cancer stimulation (Anisimov, 2015).

Neurodegenerative Disorders, Mitochondria, HK, VDAC1, and Metformin

Neurodegenerative disorders (NDs) include multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), epilepsy, amyotrophic lateral sclerosis (ALS), depression, and others. In 2015, about 46 million people globally were diagnosed with dementia, and > 6 million suffered from PD (Feigin et al., 2017). Currently, there is a growing need to search for a potential medication to treat neurodegenerative disorders worldwide.

A meta-analysis of 28 longitudinal studies demonstrated that people with diabetes had a 73% increased risk of developing dementia and a 56% increased risk of developing AD compared to the general population (Campbell et al., 2018). The mechanism linking diabetes and dementia is multifactorial, with evidence supporting the involvement of chronic low-grade inflammation, oxidative stress, vascular effects, increased cerebral amyloid-β peptides, hyperinsulinemia, and brain insulin resistance, among others (Craft, 2007; Ahtiluoto et al., 2010).

Metformin crosses the blood–brain barrier (BBB) rapidly and induces various therapeutic benefits in the brain such as enhanced learning capacity, and neuroprotective effects. It also boosts memory function and anti-inflammatory activities (Labuzek et al., 2010; Pintana et al., 2012; Guo et al., 2014; Shivavedi et al., 2017).

Metformin showed pharmacological neuroprotective efficacy in neurological diseases (Ryu et al., 2018; Demare et al., 2021), including AD (Ou et al., 2018), PD (Curry et al., 2018), and HD (Sanchis et al., 2019), with its potential use enhancing neuroprotection against apoptotic cell death (El-Mir et al., 2008), stimulating neurogenesis, improving spatial memory (Wang et al., 2012; Fatt et al., 2015), and prolonging the lifespan of mice (Martin-Montalvo et al., 2013). It was found that metformin and sulfonylureas treatment decreased the occurrence of T2DM dementia, and lowered the risk of PD in T2DM patients (Hsu et al., 2011; Wahlqvist et al., 2012a,b). Furthermore, metformin was suggested as a possible therapy of choice for diabetic patients with cognitive dysfunction, acting as an indication of changes in thinking and memory function (Pintana et al., 2012).

The positive effects of metformin were linked to a decrease in the opening of the mitochondrial permeability transition pore (PTP) that prevents the release of Cyto *c* and causes cell death (Guigas et al., 2004; Detaille et al., 2005; Lablanche et al., 2011).

In addition, the lipid phosphatase Src homology 2 domain, containing inositol-5-phosphatase 2 (SHIP2), is elevated in

TABLE 2 | Metformin decreases cancer cell resistance to chemotherapy.

Disease	Metformin effect	References
ALL	In patients with higher ABCB1 gene expression levels, the combined use of metformin with chemotherapy is beneficial.	Ramos-Penafiel et al., 2018
Breast cancer	Metformin reduces the expression of MDR protein markers, prevents the growth of treatment-resistant breast cancer, and fosters re-sensitization.	Davies et al., 2017
Breast cancer	Metformin re-sensitized multidrug-resistant breast cancer cells (MCF7/5-FU and MDA-MB-231) to 5-fluorouracil (5-FU), Adriamycin, and paclitaxel reduced their invasive potential and reversed the epithelial-mesenchymal transition (EMT) phenotype.	Qu et al., 2014
Nasopharyngeal carcinoma (NPC)	Metformin reduced the expression of PECAM-1, which controls the expression of the multi-drug expression of resistance-associated proteins (MRPs) that contribute to cisplatin resistance of irradiated CNE-1 cells.	Sun et al., 2020
Breast cancer	In breast cancer and MCF7/DOX cells, metformin lowers Pgp activity.	Shafiei-Irannejad et al., 2018
Triple negative breast cancer (TNBC)	Metformin increases cisplatin's anti-proliferative, anti-migratory, and anti-invasion effects in TNBC cells. Metformin also reduces the upregulation of RAD51 expression by triggering RAD51 proteasomal degradation.	Lee et al., 2019

the brain of diabetic db/db mice (Hori et al., 2002). SHIP2 overexpression reduces Akt activity and enhances apoptosis (Polianskyte-Prause et al., 2019). Metformin directly binds to SHIP2 phosphatase, and in the skeletal muscles and kidneys of db/db mice, it reduces catalytic activity and restores Akt activity, preventing apoptosis (Polianskyte-Prause et al., 2019). These findings can be connected to HK, as phosphorylation of HK by Akt increases its binding to mitochondria (Roberts et al., 2013), thereby, protecting against apoptosis. These studies suggest a link between metformin and VDAC1 in the prevention of neuronal apoptosis in these diseases.

However, metformin was reported to affect the progression and severity of AD and other forms of dementia (Campbell et al., 2018), and lower cognitive function in patients with diabetes (Moore et al., 2013). Metformin-induced cortex mitochondrial dysfunction is associated with an overall increase of the risk of AD onset (Picone et al., 2016), and mitochondria-mediated cell death was linked to neuronal death witnessed in neurological disorders and associated with caspase-mediated apoptosis (Gervais et al., 1999; Li et al., 2000; Friedlander, 2003; Petrozzi et al., 2007; Mattson et al., 2008; Radi et al., 2014).

It has been shown that brains from AD patients contain high levels of nitrated VDAC1, pointing to oxidative damage from VDAC1 (Sultana et al., 2006), and feasibly affecting cell energy and metabolite homeostasis (Ferrer, 2009). Moreover high-levels of VDAC1 were demonstrated in the dystrophic neurites of Aβ deposits in the brains of post-mortem AD patients (Yoo et al., 2001; Perez-Gracia et al., 2008; Cuadrado-Tejedor et al., 2011; Manczak and Reddy, 2012), and in the thalamuses of mice with neurodegeneration in the Batten disease model (Kielar et al., 2009), and changes in thalamic VDAC protein levels were found to be related to spatial cognitive deficits in an animal model of Wernicke–Korsakoff syndrome (Bueno et al., 2015). Interestingly, it is also reported that in AD, VDAC1 levels are decreased in the frontal cortex, and VDAC2 is elevated in the temporal cortex (Rosa and Cesar, 2016).

Overexpression of VDAC1 is associated with apoptosis (Shoshan-Barmatz et al., 2015). This overexpression in various neuronal diseases is proposed to be associated with neuronal cell destruction and HK detachment, resulting in both inhibiting cell metabolism and inducing apoptosis. Thus, HK detachment from VDAC1 by metformin can explain the reported negative effects of metformin on neurodegenerative disorders.

These findings indicate that metformin possesses both pro-survival and pro-apoptotic activities in neurodegenerative diseases, but the factors mediating these opposite effects are not clear. Here, we suggest that these metformin effects are mediated via metformin interaction with HK and VDAC1, proteins that regulate cellular energetics and cell death.

Alzheimer's Disease and Metformin

Alzheimer's disease is characterized by progressive memory loss and a decline in cognitive function. The pathological hallmarks of the AD brain include neurofibrillary tangles (NFTs; composed of abnormal hyperphosphorylated tau protein) and amyloid plaques (Aβs) (Brion et al., 1985). Tau is involved in microtubule stabilization (Johnson and Stoothoff, 2004), associated with synaptic loss, and has been correlated with cognitive impairments in AD patients (Arriagada et al., 1992).

The underlying biological mechanisms leading to sporadic forms of AD have still not been defined, but these are proposed to involve mitochondrial dysfunction, cholinergic dysfunction, Aβ plaque formation, tau accumulation, inflammation, DNA damage, inflammatory response, hormone regulation, and lysosomal dysfunction (Dorszewska et al., 2016).

Obesity, metabolic syndrome, and T2DM were proposed to contribute to impaired cognitive function, increasing the risk for the development of dementia including AD (Ott et al., 1999; Arvanitakis et al., 2004). A recent meta-analysis of longitudinal studies suggests that the relative risk for AD is approximately 1.5-fold higher among persons with T2DM (Cheng et al., 2012).

It has been shown that T2DM is correlated with twice the risk of dementia (Hsu et al., 2011). Therefore, metformin has been proposed as a potential neuroprotective agent in T2DM patients as it is able to reduce the chances of AD onset (Hsu et al., 2011). The proposed mechanism for metformin inhibiting the development of dementia in patients with diabetes is by preventing hyperinsulinemia, which contributes to amyloid-β plaque formation in the brain and the onset of AD (Qiu and Folstein, 2006). Another study showed that metformin reduced tau phosphorylation in primary neuron cultures from a tau transgenic mouse (Kickstein et al., 2010).

Overexpression of VDAC1 in affected regions of AD brains (Perez-Gracia et al., 2008; Cuadrado-Tejedor et al., 2011; Manczak and Reddy, 2012) and in β-cells of T2D (Ahmed et al., 2010; Gong et al., 2012; Sasaki et al., 2012; Zhang E. et al., 2019) has been reported. As neuron loss, mainly due to apoptosis, occurs in AD brains (Colurso et al., 2003; Lezi and Swerdlow, 2012; Sabirov and Merzlyak, 2012; Silva et al., 2012; Smilansky et al., 2015) and VDAC1 overexpression induces apoptotic cell death (Godbole et al., 2003; Zaid et al., 2005; Abu-Hamad et al., 2006; Ghosh et al., 2007; Lu et al., 2007; Weisthal et al., 2014), its overexpression in AD and in T2DM may be a common mechanism in these pathologies.

Aβ triggered HK-I detachment from mitochondria, decreasing HK-I activity in cortical neurons (Saraiva et al., 2010). In addition, in the postmortem brain tissue of AD mice and patients, HK levels were decreased, while VDAC1 levels were elevated (Cuadrado-Tejedor et al., 2011). In addition, HK-I detachment from mitochondria was observed in AD models (Rossi et al., 2020). It is well demonstrated that HK binds to VDAC1 and that its dynamic association with VDAC1 (Zaid et al., 2005; Abu-Hamad et al., 2008; Pastorino and Hoek, 2008; Arzoine et al., 2009) is known to modulate the metabolic coupling between cytosol and mitochondria by regulating both glycolysis and oxidative phosphorylation.

Parkinson's Disease and Metformin

Parkinson's disease is a progressive neurodegenerative disease characterized by both motor and non-motor features, and is the second most common neurodegenerative disorder (Rizek et al., 2016). Metformin reversed certain PD phenotypes in PD mouse models through AMPK-dependent and independent pathways (Bayliss et al., 2016; Lu et al., 2016; Ryu et al., 2018). It lowered α-synuclein phosphorylation and upregulated neurotrophic factors in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Katila et al., 2017), and prevented the loss of dopamine-producing brain cells in a model of PD (Lu et al., 2016).

Metformin mitigated neuronal damage, strengthened antioxidant activity, and increased muscle and locomotive functions in an MPTP-triggered PD mouse model (Patil et al., 2014). Similar findings were found where metformin-ameliorated MPTP induced dysfunction of dopaminergic neurons, elevated striatal dopamine output, and improved motor injuries in a mice model via microglia-overactivation-induced neuroinflammation inhibition, and enhanced AMPK-mediated autophagy (Lu et al., 2016). Metformin also suppressed AMPK-independent development of L-DOPA-induced dyskinesia and impaired glycogen synthase kinase 3β (GSK3β) activity, without affecting elevated mTOR or ERK signaling observed in a mouse model of PD (Ryu et al., 2018). Similar results showed neuroprotective effects of metformin and inhibition of degeneration of nigrostriatal dopamine in a PD mouse model (AMPK knockout) (Bayliss et al., 2016). This raises the feasibility that metformin could be a potential therapeutic agent in suppressing complications of L-DOPA-induced motor complications in PD (Freitas et al., 2017; Sportelli et al., 2020).

α-synuclein, a presynaptic neuronal protein, interacts with VDAC1 and regulates VDAC1 conductance and VDAC1-mediated Ca^{2+} transport (Rostovtseva et al., 2015; Rosencrans et al., 2021). Metformin-induced ER stress resulted in Ca^{2+} release from the ER and its uptake by the mitochondria, leading to mitochondrial alterations (Loubiere et al., 2017). Thus, α-synuclein, by inhibiting VDAC-mediated Ca^{2+} transport, can prevent metformin-mediated mitochondrial Ca^{2+} overload and the associated mitochondria dysfunction.

COVID-19, Diabetes, Mitochondria, VDAC1, and Metformin

The COVID-19 pandemic has been the focus of global concern since its outbreak in December 2019 when a new coronavirus (SARS-CoV-2), was first discovered in Wuhan, China. This virus, that rapidly spread around the world, is characterized by a severe acute respiratory syndrome (Perlman, 2020).

It is identified by the presence of a "crown" structure observed by electron microscope. The whole genome has been sequenced and is composed of a single-stranded RNA about 30Kb in length (GenBank no. MN908947), encoding 9,860 amino acids (Chen L. et al., 2020).

The surface of the COVID-19 virus is covered by a large number of spike glycoproteins that are responsible for binding to the host receptor and membrane fusion (Huang et al., 2020; Letko et al., 2020). To date, most evidence points toward angiotensin-converting enzyme 2 (ACE2) as the primary receptor for virus entry into host cells (Zhao et al., 2020). Genetic polymorphisms of ACE2 are associated with hypertension, cardiovascular disease, stroke, and diabetes (Ranadheera et al., 2018).

Several reports indicate that people with diabetes who become infected by COVID-19 have more severe consequences and a higher risk of mortality compared to non-diabetic individuals (Apicella et al., 2020). Retrospective studies in patients with T2DM hospitalized for COVID-19 suggest that anti-hyperglycemic agent metformin treatment is associated with a threefold decreased risk of death.

A study involving more than 2,500 people with COVID-19 and with T2DM from 16 hospitals in China found an increased incidence of acidosis, although this incidence was not associated with greater mortality in people treated with metformin during hospitalization (Cheng et al., 2020). In addition, metformin was significantly associated with reduced mortality in women with obesity or T2DM who were admitted to hospitals with the virus (Bramante et al., 2021).

Immunomodulatory and antiviral activity of metformin and its potential implications in treating COVID-19 and lung injury has been reported (Chen L. et al., 2020).

It has been hypothesized that ACE2 causes acute lung injury by triggering autophagy through the AMPK/mTOR pathway (Zhang X. et al., 2019). AMPK increases the expression of ACE2 and its stability by phosphorylating ACE2 Ser^{680} (Liu et al., 2019). Thus, it was proposed that metformin could prevent the entry of SARS-CoV-2, as well as activation of ACE2 through AMPK-signaling (Sharma et al., 2020).

In addition, the SARS-CoV-2 life cycle depends on modulating the mTOR protein and pathway. mTOR signaling is necessary for viral translation, and its interruption inhibits viral growth and replication (Ranadheera et al., 2018). Metformin, an FDA-approved mTOR inhibitor, when administered as an anti-hyperglycemic drug in diabetes patients, was found to simultaneously act as an anti-hyperglycemic and antiviral agent (Lim et al., 2021), offering benefits in patients with COVID-19. This correlation could justify the reduced risk of mortality in metformin-treated compared with non-treated diabetic patients. However, additional studies are necessary to further elucidate the exact role of mTOR inhibitors and modulators in the treatment of COVID-19.

Many viruses modulate mitochondria (Monlun et al., 2017; Tiku et al., 2020). The connection of mitochondria and VDAC1 to the metformin effects on cell function presented above has also been demonstrated for COVID-19 (Thompson et al., 2020). Recently, it was shown that SARS-CoV-2 RNA and proteins are localized to the mitochondria, hijacking the host cell's mitochondrial function, and manipulating metabolic pathways to their own advantage (Singh et al., 2020; Ajaz et al., 2021).

It was demonstrated that metabolic programs define dysfunctional immune responses in severe COVID-19 patients (Thompson et al., 2020). Moreover, VDAC1 expression level was highly increased in T-cells from these patients, leading to mitochondrial dysfunction and apoptosis (Thompson et al., 2020). In addition, COVID-19 patients' T-cells underwent apoptosis that was inhibited by VBIT-4 (Thompson et al., 2020), a compound that targets VDAC1 oligomerization and prevents apoptosis (Ben-Hail et al., 2016). Further, VBIT-4 restored insulin secretion in T2DM islets and maintained normal glucose levels and insulin secretion in db/db mice (Zhang E. et al., 2019). Moreover, HK-II was found to be highly expressed in T-cells in acutely ill COVID-19 patients, but not in other viral diseases (Thompson et al., 2020).

These findings point to the likelihood that the mitochondria, VDAC1, and HK are involved in metformin-reduced mortality of T2DM induced by COVID-19.

It should be mentioned that metformin can be considered to be either a friend or foe of SARS-CoV-2-infected patients with diabetes (Ursini et al., 2020).

METFORMIN AS AN ANTI-AGING AGENT

The nine biological hallmarks of aging include mitochondrial dysfunction, altered intercellular communication, loss of proteostasis, telomere attrition, deregulated nutrient sensing, genomic instability, cellular senescence, stem cell exhaustion, and epigenetic alterations. All have been associated with various neurodegenerative diseases (Hou et al., 2019). Several studies using preclinical models suggest that metformin is improving health span and lifespan (Martin-Montalvo et al., 2013; De Haes et al., 2014; Alfaras et al., 2017; Piskovatska et al., 2019).

In a rat model and human neuronal cell cultures, metformin has been reported to significantly stimulate the formation of new neurons, i.e., neurogenesis, but there has been no sufficient evidence of clinical trials to confirm these findings to date (Potts and Lim, 2012). Mice treated with metformin have been found to live nearly 6% longer than controls, and diabetic patients treated with metformin live 15% longer than healthy individuals without diabetes (Bannister et al., 2014).

Metformin is considered an anti-aging medication as it has been shown to affect many factors that accelerate aging., such as protecting against DNA damage, mitochondrial dysfunction, and chronic inflammation (Formoso et al., 2008; Martin-Montalvo et al., 2013; Cameron et al., 2016; Garg et al., 2017; Valencia et al., 2017). Metformin increases the levels of mTOR and AMPK, which are considered to be longevity-promoting signaling molecules in cells (Martin-Montalvo et al., 2013; De Kreutzenberg et al., 2015). Finally, it has been reported that the AMPK activity declines with age (Salminen et al., 2011). Thus, the finding that metformin activates AMPK may support the suggestion that it is an agent that prevents age-related disorders including cancer, cardiovascular disease, obesity, and neurocognitive decline (Wang et al., 2011; Coughlan et al., 2014; Wang B. Z. et al., 2019).

A decline in mitochondria quality and activity is associated with normal aging and correlated with the development of a wide range of age-related disorders, particularly neurodegenerative diseases. Impaired mitochondrial function includes decreased oxidative phosphorylation (OxPhos), ATP production, mitochondrial dynamics, and mitochondrial quality control, as well as a significant increase in ROS generation, diminished antioxidant defense, and enhanced mitochondria-mediated apoptosis (Chistiakov et al., 2014; Sun et al., 2016). In addition, an accumulation of mutations in mitochondrial DNA (mtDNA) causes adverse effects including altered expression of OxPhos complexes, thereby, decreasing energy production and enhancing ROS generation (Wallace, 2010).

Several mechanisms underlying the anti-aging effect of metformin have been proposed including metformin reducing the production of mitochondrial ROS through the inhibition of complex I (Owen et al., 2000); upregulating ER glutathione peroxidase (Fang et al., 2018); regulating mitochondrial biogenesis and senescence through AMPK-mediated H3K79 methylation (Karnewar et al., 2018); decreasing the opening of the mitochondrial permeability transition pore (mPTP) (Guigas et al., 2004); and inducing autophagy by AMPK activation, regarded as health span-promoting and pro-longevity properties (Piskovatska et al., 2019), and with beneficial effects on chronic inflammation (Saisho, 2015), a state known to contribute to the development and progression of all age-related disorders.

Finally, metformin via binding to SHIP2 prevented Akt inhibition (Polianskyte-Prause et al., 2019) allowing Akt to phosphorylate HK, thereby, increasing its binding to mitochondria (Roberts et al., 2013) and preventing apoptosis.

Several studies demonstrated age-related changes in VDAC isoform expression levels and posttranslational modifications (Groebe et al., 2010). Moreover, an age-dependent increase in VDAC1 in the cerebral cortex of mice has been demonstrated (Manczak and Reddy, 2012).

The increase in VDAC1 expression levels by metformin (Loubiere et al., 2017; Zhang et al., 2017; Duan et al., 2021) can explain its pro-apoptotic effect relevant to cancer therapy. However, no clear mechanism is presented to link between metformin 's anti-aging activity and VDAC1. A possible link is metformin inhibiting mPTP opening, and activating mitophagy which removes damaged mitochondria, and is inhibited in aging cells (Rottenberg and Hoek, 2021). VDAC1 has been proposed as one of the components of mPTP (Vianello et al., 2012) and metformin, by blocking VDAC1 conductance (Zhang E. et al., 2019), may inhibit mPTP opening. Thus, metformin's anti-aging effects may be associated with its effects on mitochondria, HK, and VDAC1 functions. It should be noted that most of metformin's anti-aging effects were observed at doses that substantially exceed the recommended therapeutic doses in humans (Novelle et al., 2016). Clearly, better understanding of the mechanisms underlying metformin's effects on health-span and life extension in non-diabetics requires further studies.

METFORMIN: CONTROVERSIAL RESULTS AND SIDE EFFECTS

Although metformin used in treating various diseases including diabetes, cancer, obesity, and neurodegenerative and cardiovascular diseases, there are some precautions necessary with its use. Studies on its association with various classifications of age-related cognitive decline have shown diverse results with both positive and negative effects.

The proposed "anti-aging" activity of metformin is a controversial subject in general. The suggestion that it decreases the risk, progression, and severity of AD and other forms of dementia in individuals without diabetes is not supported by the available evidence (Campbell et al., 2018).

Clinical studies have reported that long-term metformin use increased the risk of AD among patients over 65 years old (Imfeld et al., 2012), and T2DM patients treated with it had over two to three times more impaired cognitive function than non-treated patients (Moore et al., 2013). Yet, another cohort study reported that patients with diabetes co-treated with sulfonylureas and metformin alleviated the risk of dementia by up to 35% over an 8-year period (Hsu et al., 2011).

Metformin inhibition of mitochondrial respiration (El-Mir et al., 2000; Wessels et al., 2014) has been shown to contribute to the development of PD. In a cell culture model, it was found to increase Aβ formation (Chen et al., 2009; Picone et al., 2016), and in a population-based study, it increased the rate of AD (Imfeld et al., 2012) and lowered cognitive function in patients with diabetes (Moore et al., 2013).

A recent study demonstrates that metformin increased the generation of Aβ by promoting β- and γ-secretase-mediated cleavage of APP in SH-SY5Y cells. Also, it caused autophagosome accumulation in Tg6799 AD model mice, and it was concluded that it may aggravate AD pathogenesis by promoting amyloidogenic APP processing in autophagosomes (Son et al., 2016). It is proposed that metformin induces Aβ generation by activating AMPK, inhibiting the mTOR pathway, which results in upregulated autophagy and abnormal accumulation of autophagosomes enriched in APP, BACE1, and-secretase, facilitating amyloidogenic Aβ production and AD progression (Son et al., 2016).

In addition, potential side effects of metformin were reported. Typically, gastrointestinal side effects, including diarrhea, nausea, flatulence, indigestion, vomiting, and abdominal discomfort, dominate in individuals taking it (Nasri and Rafieian-Kopaei, 2014). Long-term metformin use resulted in vitamin B12 deficiency (Liu et al., 2014; Niafar et al., 2015), interfering with the absorption of B12 in the terminal ileum (Bauman et al., 2000). Low B12 levels contribute to higher concentrations of artery-clogging homocysteine, an independent risk factor for cardiovascular disease (Ganguly and Alam, 2015). The association between metformin and impaired cognitive function has been linked at least in part to metformin-induced B12 deficiency (Moore et al., 2012; Kim et al., 2019a).

Another side effect is that metformin increases the levels of lactate in mice and humans. Although it is extremely rare, lactic acidosis may cause dizziness, muscle pain, tiredness, difficulty breathing, irregular heartbeat, and stomach pain with diarrhea (Scheen and Paquot, 2013).

SUMMARY

The interest in metformin has been significantly revitalized during the last years due to its potential repositioning for treatment of many diseases. Metformin has been proposed as a treatment for cancer, and neurodegenerative and other diseases. However, it is not clear what factors mediate its pro-survival or pro-apoptotic activities. Several mechanisms were proposed including activation of the LKB1/AMPK pathway, causing cell cycle arrest, inducing apoptosis, inhibiting protein synthesis and unfolded protein response (UPR), reducing circulating insulin levels, modulating PTP opening, inhibiting mitochondrial complex I, inducing ER stress and increased Ca^{2+} cellular levels, activating the immune system, and more. Here, we propose that metformin interacts with HK, and alters its binding toVDAC1. Together with VDAC1, it regulates cellular energetics and cell death by these proteins. This suggests that metformin's multiple effects also involve HK and VDAC1, which are both shown to be associated with cancer and neurodegenerative diseases. In cancer, metformin detaches HK from VDAC1, allowing apoptosis, and in neurodegenerative diseases, it interferes with

HK phosphorylation and, thereby, allows its bind to VDAC1, protecting against cell death.

AUTHOR CONTRIBUTIONS

UA and EN-C contributed to literature search and helped in writing. AS-K contributed in preparing the summary models and the references. MDC helped in the final version of the manuscript. VS-B wrote the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.730048/full#supplementary-material

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frontiers in Physiology

ORIGINAL RESEARCH
published: 13 December 2021
doi: 10.3389/fphys.2021.750627

Redox-Sensitive VDAC: A Possible Function as an Environmental Stress Sensor Revealed by Bioinformatic Analysis

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Voltage-dependent anion-selective channel (VDAC) allows the exchange of small metabolites and inorganic ions across the mitochondrial outer membrane. It is involved in complex interactions that regulate mitochondrial and cellular functioning. Many organisms have several VDAC paralogs that play distinct but poorly understood roles in the life and death of cells. It is assumed that such a large diversity of VDAC-encoding genes might cause physiological plasticity to cope with abiotic and biotic stresses known to impact mitochondrial function. Moreover, cysteine residues in mammalian VDAC paralogs may contribute to the reduction–oxidation (redox) sensor function based on disulfide bond formation and elimination, resulting in redox-sensitive VDAC (rsVDAC). Therefore, we analyzed whether rsVDAC is possible when only one VDAC variant is present in mitochondria and whether all VDAC paralogs present in mitochondria could be rsVDAC, using representatives of currently available VDAC amino acid sequences. The obtained results indicate that rsVDAC can occur when only one VDAC variant is present in mitochondria; however, the possibility of all VDAC paralogs in mitochondria being rsVDAC is very low. Moreover, the presence of rsVDAC may correlate with habitat conditions as rsVDAC appears to be prevalent in parasites. Thus, the channel may mediate detection and adaptation to environmental conditions.

Keywords: VDAC, parasite, cysteine oxidation, redox sensor, environmental stress, spermatozoa

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *30 July 2021*
Accepted: *12 November 2021*
Published: *13 December 2021*

Citation:
Karachitos A, Grabiński W, Baranek M and Kmita H (2021) Redox-Sensitive VDAC: A Possible Function as an Environmental Stress Sensor Revealed by Bioinformatic Analysis. Front. Physiol. 12:750627. doi: 10.3389/fphys.2021.750627

INTRODUCTION

Environmental stress of varying severity always exists in any organism's habitat. Given the diversity of organisms and their habitats, the diversity may be affected by different environmental conditions. The three main types of environments can be broadly distinguished as terrestrial, aquatic, and semi-terrestrial (semi-aquatic). The habitats available within these environments can be characterized by a set of parameters, including light, temperature, pH, atmospheric or hydrostatic pressure, salinity, and oxygen pressure, as well as individual combinations of these parameters (Edery, 2000; Rothschild and Mancinelli, 2001). An environmental factor is determined as stressful based on the organism's tolerance against it. Abiotic factors such as temperature, radiation, oxygen pressure, and

changes in water availability can exert stress *via* disturbances in gas exchange, water management, and nutrient production (Lesser, 2006; Lushchak, 2011; Sokolova et al., 2012; Wang and Komatsu, 2018). Biotic factors, including predators, competitors, and parasites (e.g., Forsman and Martin, 2009) can also be stressful to organisms. In the case of parasites, both the internal and external environments of a parasitic host dictate the outcome of their infection, resistance, susceptibility, and transmission (Prado et al., 2021). Moreover, the internal conditions of parasitic hosts may constitute a greater constraint upon survival than external conditions (Tinsley, 1999).

Unicellular organisms are exposed to stress conditions through their whole surface. Multicellular organisms regulate their response to stress in a more complex manner, but with cell response as the basis of all response types. Many different organisms are known for their resistance strategies to environmental stress, which indicate efficient cellular anti-stress mechanisms. These mechanisms may protect against intracellular oxidative stress imposed by environmental stress conditions, including increased temperature, not optimal oxygen pressure or high salinity (Laksanalamai and Robb, 2004; Wang et al., 2006; Bagnyukova et al., 2007; Sinha et al., 2013). The state of oxidative stress threatens the functioning of whole cells, especially that of the mitochondria. Reactive oxygen species (ROS), formed mainly during cellular respiration performed by mitochondria, are important signaling molecules but also markers of oxidative stress. Their excess is dangerous due to the direct impact of ROS or ROS-mediated regulation on cell structure and function (e.g., Chung, 2017).

The most common anti-oxidative stress cellular strategy involves maintaining ROS homeostasis. Available data indicate that the homeostasis may be provided by voltage-dependent anion-selective channel (VDAC) (e.g., Shoshan-Barmatz et al., 2010, 2020; De Pinto, 2021; Rostovtseva et al., 2021). This relatively simple, monomeric β-barrel channel at the interface between mitochondria and the cytosol is described as a highly conserved protein of the mitochondrial outer membrane, present in nearly all eukaryotic species examined to date (Colombini, 2012). VDAC performs and regulates inorganic ion and metabolite transport between mitochondria and the cytoplasm under both physiological and pathological conditions (Kroemer et al., 2007; Li et al., 2013; Rostovtseva et al., 2021). The contribution of VDAC in ROS homeostasis (Reina et al., 2010; Sanyal et al., 2020) is based on its transport of superoxide anion (Han et al., 2003), its important role in ROS production (Fang and Maldonado, 2018; Heslop et al., 2020), and its role in regulating the amount and activity of anti-oxidative enzymes (Gałgańska et al., 2008).

Voltage-dependent anion-selective channel is the most abundant protein in the mitochondrial outer membrane and has been relatively well studied since its discovery in 1976 (Schein et al., 1976). As summarized by Rostovtseva et al. (2021) in their comprehensive review, besides being a strictly regulated transport pathway between the mitochondrion and cytosol, VDAC also interacts with a numerous of mitochondrial and cytosolic proteins, which makes the channel a key element in and regulator of communications between mitochondria and cytosol. Moreover, VDAC forms homo- and hetero-complexes with additional functional subunits (e.g., Shoshan-Barmatz et al., 2010, 2018). However, the identification of VDAC paralogs indicates the presence of VDAC variants with slight amino acid differences that undoubtedly perform specified but not yet fully explained functions (De Pinto, 2021).

Three VDAC paralogs were identified in vertebrate mitochondria (Sampson et al., 1997; Messina et al., 2012). The presence of VDAC paralogs was also reported in other multicellular organisms such as invertebrates (Sardiello et al., 2003) and plants (Elkeles et al., 1997; Al Bitar et al., 2003; Wandrey et al., 2004; Lee et al., 2009; Wojtkowska et al., 2012; Hemono et al., 2020; Sanyal et al., 2020), as well as in unicellular organisms such as yeasts *Saccharomyces cerevisiae* (Blachly-Dyson et al., 1997; Di Rosa et al., 2021) and *Candida glabrata* (Wojtkowska et al., 2012) and protists *Trypanosoma brucei* (Flinner et al., 2012) and *Cyanophora paradoxa* (Wojtkowska et al., 2012). Thus, it is assumed that VDAC-encoding genes were duplicated independently in different lineages of eukaryotic organisms, several times during their evolution (Sampson et al., 1996; Saccone et al., 2003; Young et al., 2007). The resulting VDAC-encoding gene redundancy might indicate a need to innovate their existing function and a tendency to duplicate genetic material, as observed in invertebrates, plants, and vertebrates (Saccone et al., 2003). Thus, the following question arises: could the trigger factor be oxidative stress imposed by habitat conditions?

Identifying the function of individual VDAC paralogs is currently one of the main topics concerning VDAC research (De Pinto, 2021). One of the most important aspects of the research is the study of post-translational modification of VDAC paralogs by focusing mainly on cysteine residues. The significance of the number of cysteine residues, as well as their localization and oxidation state in individual mammalian VDAC paralogs, have been indicated by the De Pinto research group (Messina et al., 2012; De Pinto et al., 2016; Reina et al., 2020) and verified by other researchers (Okazaki et al., 2015; Karachitos et al., 2016; Queralt-Martín et al., 2020). The presence of ROS could result in the variable oxidation of cysteine residues exposed to the VDAC interior (including the flexible N-terminal region) or present in the connection loops between 19 β-strands forming the channel (Reina et al., 2010; Okazaki et al., 2015). As oxidative modifications of cysteine residues in VDAC proteins are not detected in other proteins of the mitochondrial outer membrane (Reina et al., 2020), it has been speculated that such modifications could have a regulatory function (including channel gating and conductance) as well as mitochondrial ROS buffering capacity (Okazaki et al., 2015; De Pinto, 2021).

Among the modifications occurring in cysteine residues, disulfide bond formation was shown to affect gating properties and conductance of VDAC (Okazaki et al., 2015; De Pinto et al., 2016; Reina et al., 2020). Accordingly, available data on human VDAC paralogs indicate that cysteine residues in the flexible N-terminal region are crucial for this bond formation. The same probably applies to *Drosophila melanogaster* VDAC paralogs (Komarov et al., 2004). Therefore, the presence of cysteine residues in the N-terminus could be a prerequisite

for VDAC to serve as a sensor of the reduction–oxidation (redox) state (Queralt-Martín et al., 2020). Thus, the following questions arise: (1) is the sensor function possible when only one VDAC variant is present in mitochondria, and (2) is the sensor function possible for all VDAC paralogs present in mitochondria? To answer these questions, we used currently available VDAC amino acid sequences to analyze the number of cysteine residues and their location, with particular emphasis on the N-terminus. Next, we examined the relationship between the sequences and the studied species' ecology. The obtained results suggest that the presence of redox-sensitive VDAC (rsVDAC) proteins may be important for adaptation to environmental conditions.

MATERIALS AND METHODS

Construction of the Database

UniProt (The UniProt Consortium, 2021) was used to compile a list of VDAC paralogs of non-vertebrate and non-plant organisms (**Supplementary File 1**). Next, the database was enriched with records of paralogs from previously obtained organisms (using

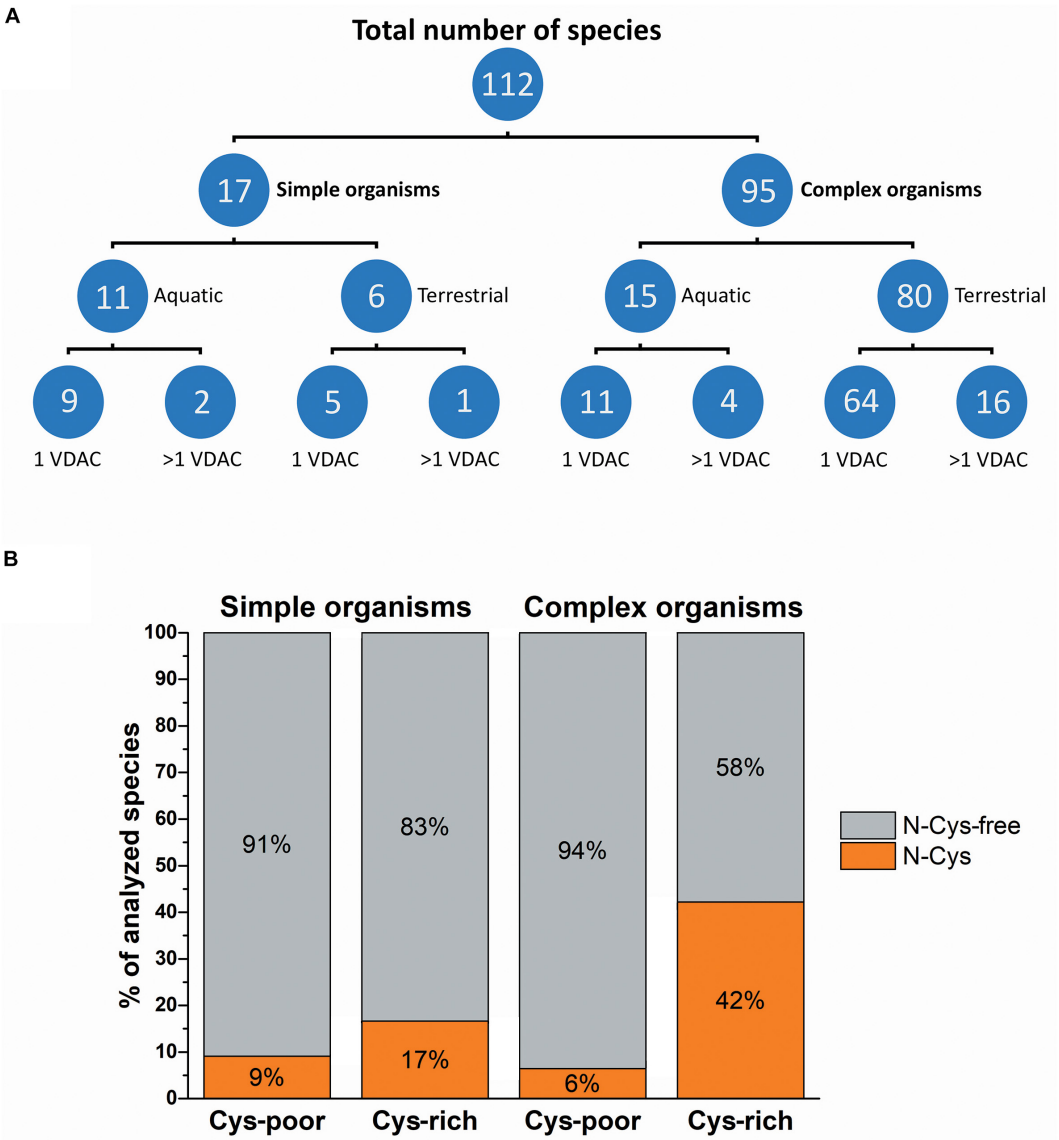

FIGURE 1 | The complexity of organisms and their inhabited environment in relation to the number of VDAC variants and their cysteine residue content. **(A)** The number of organisms classified in terms of complexity, inhabited environment, and the number of genes encoding VDAC proteins. **(B)** Classification of organisms based on the number and localization of cysteine residues in VDAC proteins. Cys-poor: a set of organisms in which only one VDAC variant or at least one VDAC paralog (if present) contains fewer than two cysteine residues in the primary structure; Cys-rich: a set of organisms in which only one VDAC variant or all VDAC paralogs (if present) have more than one cysteine residue in the primary structure; N-Cys: a set of organisms in which only one VDAC variant or all VDAC paralogs (if present) have at least one N-terminal cysteine residue; N-Cys-free: a set of organisms that have at least one VDAC variant with no N-terminal cysteine residue.

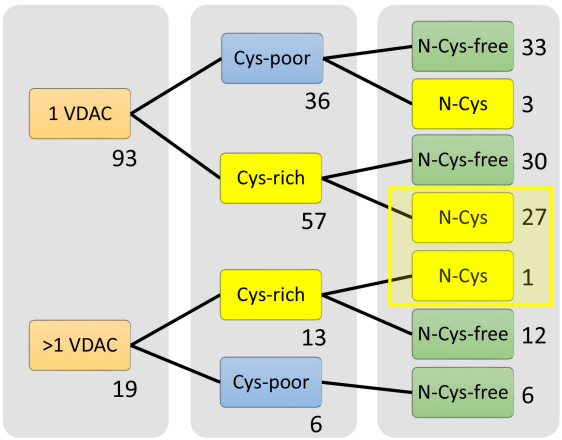

FIGURE 2 | Selection of organisms that possess the only redox-sensitive VDAC (rsVDAC) variant in mitochondria. The analysis indicates the number of cysteine residues and their location in the VDAC secondary structure, as well as the absence or presence of VDAC paralogs, that is, the presence of only one VDAC variant (1 VDAC) or VDAC paralogs (>1 VDAC). Cys-poor: a set of organisms in which only one VDAC variant or at least one VDAC paralog (if present) has fewer than two cysteine residues in the primary structure; Cys-rich: a set of organisms in which only one VDAC variant or all VDAC paralogs (if present) have more than one cysteine residue in the primary structure; N- Cys-free: a set of organisms that have at least one VDAC variant with no N-terminal cysteine residue; rsVDAC, redox-sensitive VDAC.

sequences of 250–380 amino acids) and with organisms from systematic groups missing in the list. Sequences containing "Fragment" annotations (except of *Hydra vulgaris*) and those whose amino acid sequences did not start with methionine were removed. The list was supplemented with the VDAC sequence predicted for the tardigrade *Milnesium tardigradum*, based on data kindly provided by Felix Bemm (Max Planck Institute for Developmental Biology, Tübingen, Germany). Finally, sequences were blasted using Blastp to determine whether different records for a given organism were paralogs or products of the same genes (Altschul et al., 1997).

Prediction of the Voltage-Dependent Anion-Selective Channel Structure

The SSPRo (Pollastri et al., 2002; Cheng et al., 2005) and DIpro (Baldi et al., 2004; Cheng et al., 2006) servers available at the Scratch Protein Predictor[1] were used to estimate the secondary structure of the available VDAC sequences and predict the presence of disulfide bonds, respectively. 3D structures were predicted by applying the Iterative Threading ASSEmbly Refinement (I-TASSER) method (Roy et al., 2010; Yang et al., 2015; Yang and Zhang, 2015). The predicted solutions were visualized using YASARA.[2]

RESULTS AND DISCUSSION

To perform all analysis, a database (**Supplementary File 1**) was built using a collection of species categorized by various parameters, such as a type of environment (terrestrial or aquatic), organism complexity (simple or complex), and the number of VDAC paralogs, including the number (poor or rich) and location (N-terminus free) of cysteine residues (**Figure 1**). We assumed that a complex organism contained multiple organ

[1]http://scratch.proteomics.ics.uci.edu/

[2]www.yasara.org

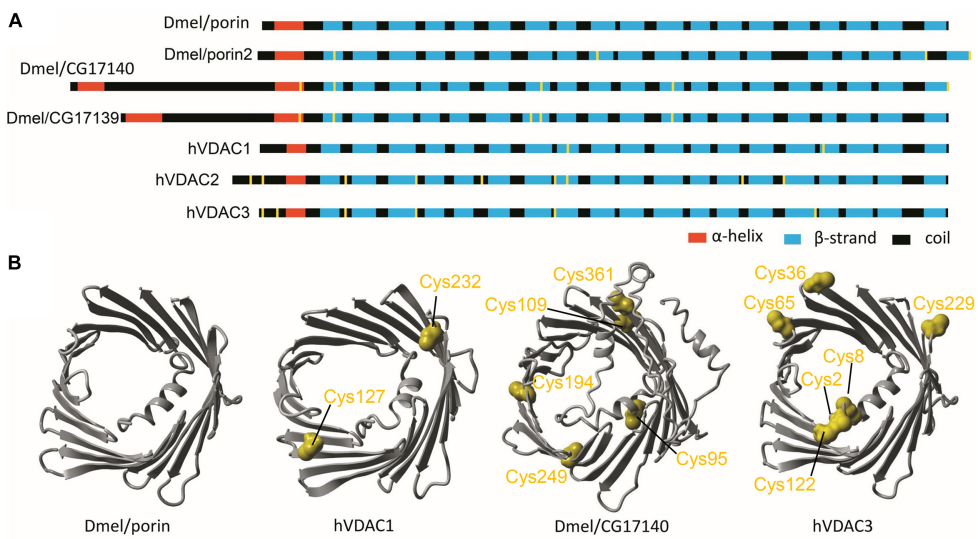

FIGURE 3 | *Drosophila melanogaster* and human VDAC paralogs as models in studies of redox-sensitive VDAC. **(A)** The secondary structure of *D. melanogaster* and human VDAC paralogs with cysteine residues marked in yellow. **(B)** The tertiary structure predicted for ubiquitously expressed *D. melanogaster* and human paralogs (Dmel/porin and hVDAC1, respectively) as well as for paralogs with limited expression, mainly in *D. melanogaster* male reproductive tracts and human testes (Dmel/CG17140 and hVDAC3, respectively). Cysteine residues are marked in yellow.

systems with different functions (Novikoff, 1945). As shown in **Figure 1A**, most of the studied organisms categorized by complexity and inhabited environment possess only one VDAC gene. This group mostly includes terrestrial and complex organisms. Due to the lack of VDAC paralogs, various aspects of mitochondrial functioning in these organisms depend on the properties of only one VDAC variant. Therefore, we checked the number of cysteine residues and their location in the secondary structure of VDAC proteins of simple and complex organisms (**Figure 1B**). We applied the following parameters to perform the operations: one VDAC or at least one VDAC paralog (if present) contained fewer than two cysteine residues in the primary structure (Cys-poor) and one VDAC or all VDAC paralogs (if present) had more than one cysteine residue in the primary structure (Cys-rich). Moreover, the presence of cysteine residue(s) in the N-terminal region upstream of the β1 strand was applied as an additional parameter, as the residue is obligatory for the possibility of disulfide bond formation in human VDAC paralogs (Okazaki et al., 2015; De Pinto et al., 2016; Reina et al., 2020). The results allowed us to distinguish organisms with VDAC(s), in which disulfide bonds could potentially form. Moreover, the possibility of this type of VDAC occurring was more frequent in complex organisms (42%) than in simple organisms (17%).

Next, we analyzed the interactions between the number of cysteine residues, their location in the secondary structure, and the number of VDAC paralogs present in the mitochondria of studied organisms (**Figure 2**). We found organisms that exclusively contained VDAC characterized by a few cysteine residues (including those within the N-terminus). We assumed that the number and distribution of cysteine residues allowed for their over-oxidation and, consequently, modulation of the channel under oxidative conditions. Therefore, we described this type of VDAC as a rsVDAC. This term refers to data on widely studied VDAC paralogs of humans and *D. melanogaster*. The latter has four VDAC paralogs: Dmel/porin, Dmel/porin2, Dmel/CG17140, and Dmel/CG17139. Dmel/porin is ubiquitous, while the remaining three paralogs are expressed exclusively in the male reproductive organ (Graham and Craigen, 2005). The knockout of *D. melanogaster* VDAC-encoding genes results in partial lethality, mitochondrial respiration defects, abnormal muscle mitochondrial morphology, synaptic dysfunction, and male infertility (Graham et al., 2010). All four paralogs were expressed in yeast *Saccharomyces cerevisiae* cells lacking yVDAC1 (Komarov et al., 2004), and only Dmel/porin and Dmel/porin2 complemented the absence of yVDAC1. Interestingly, the other two paralogs (Dmel/CG17140 and Dmel/CG17139) are characterized by the presence of several cysteine residues, one of which is located within their N-terminus (**Figure 3A**). Moreover, electrophysiological analysis showed that Dmel/CG17139 does not form a channel. Conversely, Dmel/CG17140 forms a channel in lipid membranes, but is far less voltage-dependent, unlike the canonical Dmel/porin or Dmel/porin2 (Komarov et al., 2004). Thus, Dmel/CG17140 only starts to close slightly at very high potential values (above 110 mV), whereas Dmel/porin2 closes at a potential of approximately 30 mV. Three paralogs, namely VDAC1, VDAC2, and VDAC3, have been detected in humans and other vertebrates. Human VDAC1 (hVDAC1) is ubiquitous and show the highest expression level, whereas hVDAC2 and hVDAC3 are highly abundant in the testes (Yamamoto et al., 2006). Accordingly, VDAC3 knockout in mice causes male infertility (Sampson et al., 2001). Although hVDAC1 and hVDAC2 can rescue the conditional lethal phenotype of yeast cells deficient for yVDAC1, hVDAC3 is almost unable to restore the wild-type phenotype (De Pinto et al., 2010) when the disulfide bond between cysteine residue 2 or 8 (Cys2/Cys8), located at the N-terminus region, and Cys122 is formed (Okazaki et al., 2015). The permanently reduced state of a cluster of close cysteine residues in hVDAC2 and hVDAC3 has been shown to sustain disulfide bond formation in the protein (Pittalà et al., 2020). Such a modification alters the electrophysiological properties of the formed channel, resulting in a lack of voltage dependence of the channel and consequently, the channel remains open. Interestingly, swapping the N-terminus of hVDAC1 with hVDAC3 (which eliminates the N-terminal cysteine residues in hVDAC3) restores the canonical activity of the formed channel and the ability to complement the lack of yVDAC1, as well as confers resistance to yeast against oxidative stress conditions (Reina et al., 2010).

Based on these correlating data, we assume that hVDAC3 and Dmel/CG17140 are orthologs due to their electrophysiological properties and tissue specificity. Both proteins were also used as our model of the assumed rsVDAC (**Figure 3B**), that is, VDAC containing multiple cysteine residues, with at least one within the N-terminus. The N-terminus is described as the most flexible segment of VDAC, which, in turn, facilitates interactions with other cysteine residues under oxidative conditions (Okazaki et al., 2015). However, it remains unclear if Dmel/CG17139 could be described as an rsVDAC. Based on the present analysis, the secondary structure and distribution of cysteine residues are very similar to Dmel/CG17140; however, limited available experimental data exclude the paralog channel activity.

Numerous parasitic species were found in the group of organisms that contained the assumed rsVDAC. As shown in **Figure 4**, 28 out of 112 studied species were

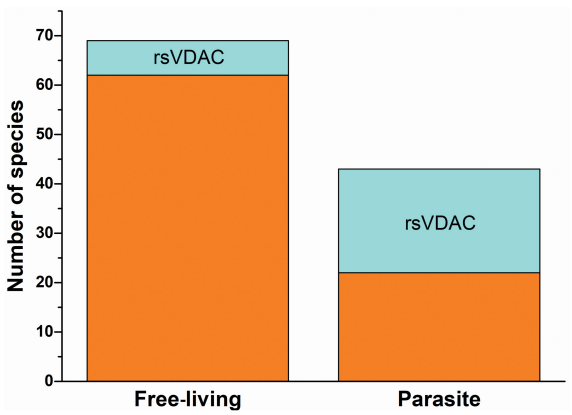

FIGURE 4 | The occurrence of redox-sensitive VDAC in free-living organisms and parasites.

assigned to the rsVDAC group, of which 21 were parasitic species, including obligatory ones (both internal and external) (**Table 1**). This finding suggests that metabolic and environmental conditions typical for parasitic organisms may support the presence of only one VDAC variant, which may be a redox sensor. In the case of free-living organisms, we noted the presence of at least one VDAC variant that displayed exceptionally low or no probability of cysteine residue oxidation, excluding the function of the redox sensor. Based on the human and *D. melanogaster* models, we believe this type of VDAC to be the most abundant and ubiquitous.

TABLE 1 | List of organisms with redox-sensitive VDAC being the only VDAC variant, classified by their lifestyle and the presence or absence of cysteine residues in selected regions of the VDAC.

Species	Lifestyle	β1Cys	(β7–β8)Cys	β15Cys	C-Cys
Steinernema glaseri	Parasitic (internal)	No	No	No	No
Wuchereria bancrofti	Parasitic (internal)	Yes	Yes	Yes	Yes
Brugia pahangi	Parasitic (internal)	Yes	No	Yes	Yes
Loa	Parasitic (internal)	Yes	Yes	Yes	Yes
Onchocerca flexuosa	Parasitic (internal)	Yes	Yes	Yes	Yes
Litomosoides sigmodontis	Parasitic (internal)	Yes	No	No	Yes
Angiostrongylus costaricensis	Parasitic (internal)	No	No	Yes	No
Brugia malayi	Parasitic (internal)	Yes	Yes	Yes	Yes
Intoshia linei	Parasitic (internal)	No	No	No	No
Onchocerca volvulus	Parasitic (internal)	Yes	Yes	Yes	Yes
Steinernema carpocapsae	Parasitic (internal)	Yes	Yes	No	No
Brugia timori	Parasitic (internal)	Yes	Yes	Yes	Yes
Enterobius vermicularis	Parasitic (internal)	No	No	Yes	No
Ixodes scapularis	Parasitic (external)	Yes	No	Yes	No
Frankliniella occidentalis	Parasitic (external)	No	No	Yes	No
Amblyomma aureolatum	Parasitic (external)	Yes	No	Yes	No
Rhipicephalus pulchellus	Parasitic (external)	Yes	No	Yes	No
Haemaphysalis longicornis	Parasitic (external)	Yes	No	Yes	No
Rhipicephalus appendiculatus	Parasitic (external)	Yes	No	Yes	No
Ornithodoros turicata	Parasitic (external)	Yes	No	Yes	No
Ixodes ricinus	Parasitic (external)	Yes	No	Yes	No
Chilo suppressalis	Free-living	No	No	No	No
Papilio machaon	Free-living	No	No	No	No
Dinothrombium tinctorium	Free-living	No	No	Yes	No
Hadrurus spadix	Free-living	Yes	Yes	Yes	No
Salpingoeca rosetta	Free-living	No	No	No	No
Leptidea sinapis	Free-living	No	No	No	No
Operophtera brumata	Free-living	No	No	No	No

Marine eukaryotic organisms use redox-based mechanisms that mediate sensing and adaptation to environmental stress (Van Creveld et al., 2015). However, little is known about the role of ROS in the signaling of environmental stress conditions. ROS are toxic molecules that can cause severe damage to cells and, therefore, are strictly regulated by a wide range of antioxidant systems (Antonucci et al., 2021; Čapek and Roušar, 2021). However, it is crucial that a moderate amount of ROS act as second messenger molecules in a very complex network of signals in the cell (Tauffenberger and Magistretti, 2021). VDAC is involved in changes in the redox states of the cytosol and mitochondria (Gałgańska et al., 2008) and may act as a mitochondrial oxidative marker, participating in ROS signaling (Reina et al., 2016). Therefore, it is also possible that VDAC participates in the sensing of environmental stress conditions. If that is the case, the evolution of the VDAC structure would depend significantly on the inhabited environment.

Cyanidioschyzon merolae is a unicellular extremophilic eukaryotic organism adapted to high-sulfur acidic hot spring habitats. This organism has only one gene encoding VDAC, which contains four cysteine residues, all of which are located outside the N-terminus. By contrast, the tardigrade *Hypsibius dujardini*, which can survive under extreme conditions, also has one VDAC, with the protein containing only one cysteine residue located at the N-terminus. Both the VDAC proteins did not qualify as rsVDAC in our analysis. Instead, the group of organisms with the assumed rsVDAC was dominated by parasites. Using hVDAC3 as the rsVDAC model, we verified the presence and location of cysteine residues in assumed rsVDAC being the only VDAC variant in mitochondria. The regions containing cysteine residues included the N-terminus, the β1 strand, the segment containing β7 and β8 strands and the β15 strand (**Figure 5A**). Cysteine residues in the β1 and β15 strands turned out to be quite common in parasites, whereas those in the β7 and β8 segment were characteristic of internal parasites (**Figure 5B** and **Table 1**). In hVDAC3, the β7 and β8 segment is the location for Cys122, which is responsible for forming disulfide bonds with the N-terminal cysteine residues (Okazaki et al., 2015). A cysteine residue is also present in this region of Dmel/CG17140 (**Figures 3**, **5A**), suggesting its potential role in redox sensitivity. Accordingly, the presence of two cysteine residues in modified mouse VDAC1, one at the N-terminus (Val3Cys) and the second one in β7 segment (Lys119Cys) resulted in the formation of disulfide bond and strong deviation from the typical native channel gating under oxidative condition (Mertins et al., 2012). In further studies, it was demonstrated that the N-terminal dynamics were essential for voltage gating (Zachariae et al., 2012; Zeth and Zachariae, 2018). We also indicated the presence of cysteine residue(s) at the C-terminus only in internal parasites (**Table 1**). It should be noted that cysteine residue(s) in the location is (or are) less common in VDAC of free-living organisms that have only one VDAC variant meeting our criteria of rsVDAC.

Thus, what are the features that distinguish parasites from free-living organisms? It is suggested that hosts of parasites can

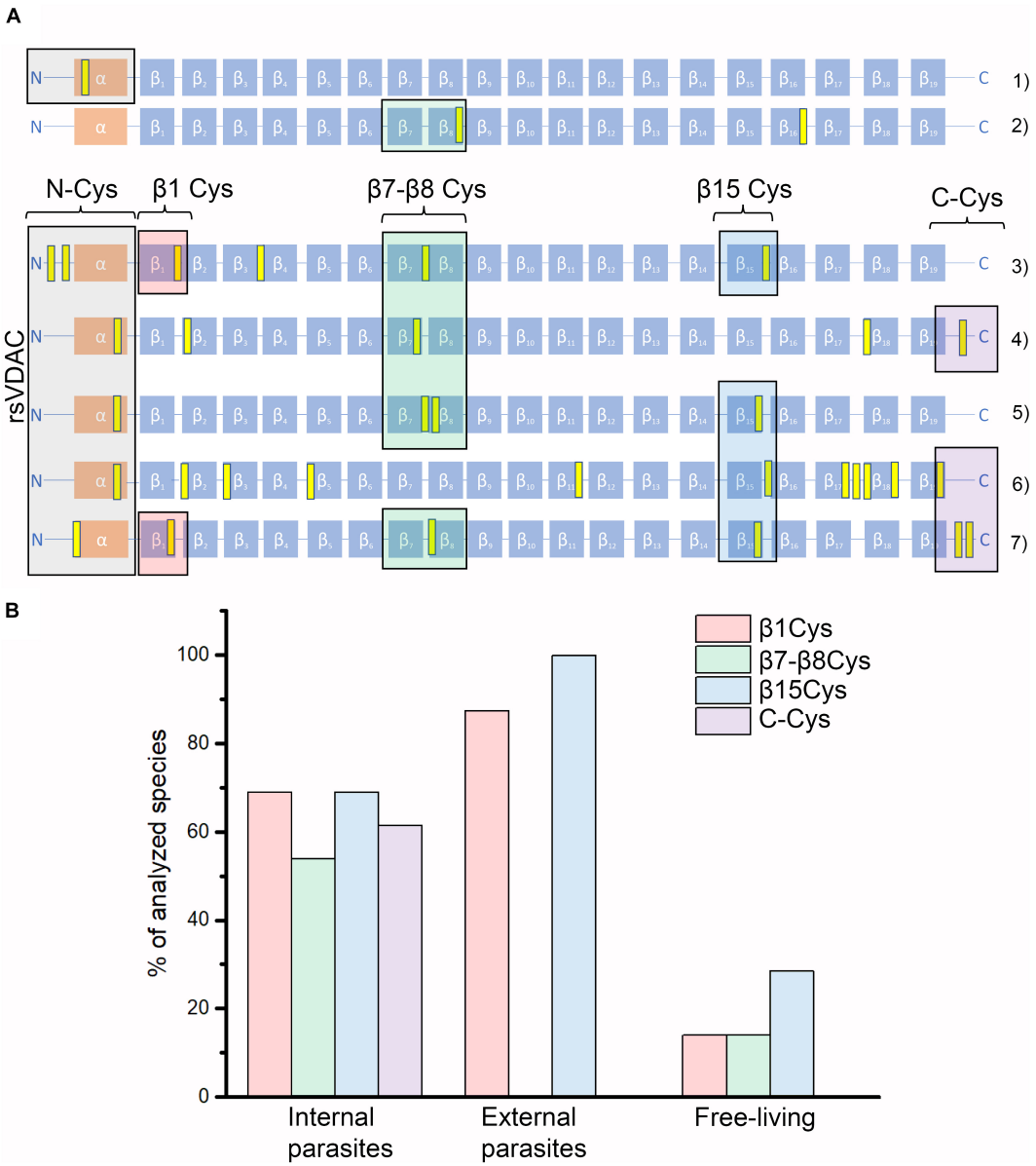

FIGURE 5 | VDAC variants that differ in their number and location of cysteine residues. **(A)** Graphical representations of the studied organisms' VDAC secondary structure with indicated locations of cysteine residues (marked in yellow). They represent "redox-insensitive" VDAC (1–2) and redox-sensitive VDAC (3–7). (1) *Hypsibius dujardini* (free-living); (2) human VDAC1; (3) human VDAC3; (4) Dmel\CG17140; (5) *Aceria tosichella* (free-living); (6) *Folsomia candida* (free-living); and (7) *Onchocerca flexuosa* (parasitic, internal). **(B)** The percentage of studied organisms with their assumed rsVDAC, being the only VDAC variant in mitochondria, including their lifestyle and the presence of cysteine residues in the selected regions of VDAC proteins (see also **Table 1**). Besides rsVDAC, *A. tosichella* and *F. candida* possess also "redox-insensitive" VDAC.

be considered a safe environment, and the external environment to which parasites are exposed, for example, during transmission, as hostile ones (Tinsley, 2007). Conversely, many years of coexistence with the host body requires suitable adaptation, such as a strong antioxidant system, which may serve as a defense strategy against host-generated ROS (Chiumiento and Bruschi, 2009). Perhaps the reduced imbalance between ROS generated by the host and the antioxidant system requires the presence of stronger redox sensors in some parasites. The same idea may apply to mature spermatozoa enriched in rsVDAC (Reina et al., 2016). Spermatozoa are foreign to both the male who produces them and the female who receives them (Clark and Schust, 2013). The organs of the female reproductive tract are subject to being colonized by pathogens and, therefore, have developed multiple adaptations to impede the invasion and proliferation of such pathogens. In addition to physical (production of a cleansing outward flow of fluid and secretion of a viscoelastic mucus) and chemical (acidification of the vaginal fluid) impediments in the

female tract, immunological barriers could include components of the innate immune system, including inflammatory responses, ROS, and antimicrobial peptides, that could potentially damage spermatozoa (Ford, 2004; Wigby et al., 2019).

CONCLUSION

The imbalance between ROS production and antioxidant capacity – which causes oxidative stress – is a common feature of cells exposed to environmental stress conditions. Our hypothesis was based on the assumption that, in some organisms, VDAC amino acid sequences form proteins sensitive to redox changes. Specifically, cysteine residues can potentially be oxidized and form disulfide bonds that alter the properties of the formed channel. The available data indicate that the bond formation requires the presence of cysteine residue in the flexible N-terminus. A large group of organisms, most often possessing only one VDAC variant, do not contain this type of VDAC that we termed "redox-sensitive." Redox-sensitive VDACs may form adjacent to "redox-insensitive" ones in organisms with VDAC paralogs, such as *D. melanogaster* or humans. The redox-sensitive paralogs are often expressed in specific tissues and are not ubiquitous. Finally, our observations indicate that certain organisms – mainly parasites – have only one, but potentially redox-sensitive, VDAC variant. Thus, there is a possibility that VDAC evolution may depend on environmental conditions and that the channel may mediate detection and adaptation to environmental stress.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AK conceived, designed, and made the analyses. HK supervised the performed analyses. AK and HK wrote the final version of the manuscript. MB contributed to the database and wrote sections of the manuscript. WG created the database, helped in the analysis, and wrote sections of the manuscript. All authors have read and approved the final manuscript.

FUNDING

This work was supported by the research grants of National Science Centre, Poland, Grant Numbers 2017/26/D/NZ1/00075 and 2016/21/B/NZ4/00131.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.750627/full#supplementary-material

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frontiers in Physiology

MINI REVIEW
published: 10 January 2022
doi: 10.3389/fphys.2021.784867

Voltage-Dependent Anion Selective Channel 3: Unraveling Structural and Functional Features of the Least Known Porin Isoform

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Voltage-dependent anion-selective channels (VDAC) are pore-forming proteins located in the outer mitochondrial membrane. Three isoforms are encoded by separate genes in mammals (VDAC1-3). These proteins play a crucial role in the cell, forming the primary interface between mitochondrial and cellular metabolisms. Research on the role of VDACs in the cell is a rapidly growing field, but the function of VDAC3 remains elusive. The high-sequence similarity between isoforms suggests a similar pore-forming structure. Electrophysiological analyzes revealed that VDAC3 works as a channel; however, its gating and regulation remain debated. A comparison between VDAC3 and VDAC1-2 underlines the presence of a higher number of cysteines in both isoforms 2 and 3. Recent mass spectrometry data demonstrated that the redox state of VDAC3 cysteines is evolutionarily conserved. Accordingly, these residues were always detected as totally reduced or partially oxidized, thus susceptible to disulfide exchange. The deletion of selected cysteines significantly influences the function of the channel. Some cysteine mutants of VDAC3 exhibited distinct kinetic behavior, conductance values and voltage dependence, suggesting that channel activity can be modulated by cysteine reduction/oxidation. These properties point to VDAC3 as a possible marker of redox signaling in the mitochondrial intermembrane space. Here, we summarize our current knowledge about VDAC3 predicted structure, physiological role and regulation, and possible future directions in this research field.

Keywords: VDAC3, electrophysiology, planar lipid bilayer, redox signaling, human pathologies

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Mitochondrial Research, a section of the journal Frontiers in Physiology

Received: *28 September 2021*
Accepted: *10 November 2021*
Published: *10 January 2022*

Citation:
Reina S and Checchetto V (2022) Voltage-Dependent Anion Selective Channel 3: Unraveling Structural and Functional Features of the Least Known Porin Isoform. Front. Physiol. 12:784867. doi: 10.3389/fphys.2021.784867

Abbreviations: ADP, Adenosine DiPhosphate; ALS, Amyotrophic Lateral Sclerosis; ATP, Adenosine TriPhosphate; Bcl2, B-cell lymphoma 2; BRAF, B-Raf and V-Raf murine sarcoma viral oncogene homolog; Cys, Cysteines; FTLD, FrontoTemporal Lobar Degeneration; HBV, hepatitis B virus; HK, hexokinase; HRAS, Harvey rat sarcoma virus; hVDAC, human Voltage-Dependent Anion-selective Channels; KCl, potassium chloride; KRAS, Kirsten rat sarcoma viral oncogene homolog; LDAO, lauryl dimethylamine oxide; MAMs, mitochondrial-associated membrane proteins; MDVs, mitochondrial-derived vesicles; MOM, mitochondrial outer membrane; mVDAC, mouse Voltage-Dependent Anion-selective Channels; PLM, Planar Lipid Membranes; PPIs, Protein-Protein Interactions; pS, pico Siemens; ROS, Reactive Oxygen Species; rVDAC, rat Voltage-Dependent Anion-selective Channels; SO3H, sulfonic acid; TAP-Tag, Tandem Affinity Purification Tag; Tm, melting temperature; VCP, Valosin-Containing Protein; VDAC, Voltage-dependent anion-selective channels; zfVDAC2, zebrafish Voltage-Dependent Anion-selective Channels; α-syn, α-synuclein; ES, embryonic stem.

INTRODUCTION

The Voltage-dependent anion-selective channels (VDACs) are pore-forming proteins, also known as porins, localized in the mitochondrial outer membrane (MOM). These small proteins (30–35 kDa) are the main pathway for the flux of ions and metabolites between mitochondria and the cytoplasm. VDACs are involved in many cellular functions, including Adenosine DiPhosphate and Adenosine TriPhosphate transfer, Reactive Oxygen Species (ROS) signaling, hexokinase anchoring, and apoptosis (Vander Heiden et al., 2000). In mammals and most chordates, three VDAC isoforms have been characterized: VDAC1, VDAC2, and VDAC3 (Ha et al., 1993; Sampson et al., 1996, 1998; Messina et al., 2012).

The data emerging in the last decades denote that VDAC isoforms in mammals show differences in (i) the mitochondrial localization: VDAC1 and VDAC2 are colocalized within the same restricted area in the MOM, while VDAC3 is widely distributed on the MOM (Neumann et al., 2010; Okazaki et al., 2015); (ii) the channel activity and voltage dependence: both VDAC1 and VDAC2 are maximally open at 0 mV and that they enter a lower-conductance state. They work mainly as anion channels in the −40 a +40 mV voltage range, while outside of this range, they function as cation channels. On the contrary, VDAC3 did not exhibit typical voltage gating and electrophysiological properties (Checchetto et al., 2014; Okazaki et al., 2015); and (iii) the N-terminal sequence and its contribution to cell viability and survival: the N-terminal end of VDACs contains amphipathic α-helix elements with functionally relevant properties (Ujwal et al., 2008). A remarkable difference in the number of cysteines in the VDAC N-terminal sequences, VDAC3 has two cysteines at positions 2 and 8, VDAC2 also has two cysteines, but only one of them corresponding to the VDAC3 In addition, VDAC1 and 2 N-termini have additional residues, the target of carbonylation reactions, while VDAC3 does not have them; and (iv) finally the specific Protein-Protein Interactions (PPIs). These data lead to hypothesize a more specialized role for each isoform in different biological contexts. The PPIs of VDAC1 have been described more in-depth than those of VDAC2 and VDAC3 (Caterino et al., 2017).

This review will report progress in understanding the VDAC3 function, focusing on its structure, and discussing various models proposed for voltage gating, its modulation, and its overall role as a channel (see **Figure 1**).

OVERVIEW OF VOLTAGE-DEPENDENT ANION SELECTIVE CHANNEL 3: FROM GENE TO PROTEIN STRUCTURE

In higher eukaryotes, the structure of VDAC genes is very similar. The genes have the same coding-exon organization, the same size, with the VDAC2 gene containing an additional first exon encoding for the short presequence of 11 amino acids, a feature of this isoform. The size of the VDACs introns varies, but the exon-intron organization is conserved among the whole group (Young et al., 2007). Evolutionary analysis indicates that VDAC3 is the oldest of the vertebrate VDAC genes, suggesting that multiple isoforms arose from gene duplication and VDAC3 diverged from the primordial VDAC first, with VDAC1 and VDAC2 arising more recently. VDAC3 is placed on a separate branch of a phylogenetic tree, suggesting that this isoform has a distinctive physiological function from VDAC1 and VDAC2 (Sampson et al., 1996). The observation indirectly supports this prediction that VDAC3 does not rescue the porin-less yeast temperature-sensitive phenotype completely but generates a lower level of growth under restrictive conditions (Sampson et al., 1997).

Transcription of the VDAC isoform genes was detected with many techniques and indicated that the three mammalian VDAC isoforms are ubiquitously expressed. To date, very little is known about the mechanisms of VDAC3 gene regulation. Although its transcript is less abundant within the cell, the *VDAC3* promoter exhibits the highest transcriptional activity compared to VDAC2 and, particularly, VDAC1. In this regard, it has been hypothesized that VDAC3 transcripts could be less stable than VDAC1 ones or that their levels are kept constitutively high to readily increase VDAC3 expression in response to specific stimuli (Zinghirino et al., 2020). Consequently, the VDAC3 promoter contains a polypyrimidine stretch that has been featured as a specific target of oxidative stress (Nepal et al., 2020).

Former studies based on structure prediction suggested that VDAC isoforms folded similarly to bacterial porins (Mihara and Sato, 1985; Kleene et al., 1987; Young et al., 2007). About 20 years after the primary structure elucidation, the VDAC1 3D structure was solved combining NMR spectroscopy and X-ray crystallography approaches in three different laboratories (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008), while the zfVDAC2 structure was solved by Schredelseker et al. (2014) and Gattin et al. (2015). Both VDAC1 from mice or humans and VDAC2 from zebrafish fold into a novel structure comprised of 19 β-strands and an N-terminal α-helix that adopted several conformations. A similar topology with 19 β-strands and a helix was recently resolved in TOM40, also located in the MOM (Araiso et al., 2019; Tucker and Park, 2019). VDAC3 shares about 60–70% sequence identity with VDAC1 and VDAC2. The sample preparation and spectroscopic methods described by Eddy et al. for VDAC2 will likely apply to this isoform as well (Eddy et al., 2019), but unfortunately, the 3D structure of VDAC3 has not yet been obtained, and only a few pieces of information are available so far. However, modeling of the hVDAC3 sequence on the structure of hVDAC1 or mVDAC1 showed a remarkable similarity, indicating that this isoform is possibly folded like the other two known VDACs.

Several investigations have focused on the N-terminal segment, which resides in the lumen and is not part of the VDACs barrel. The N-terminal region constitutes a mobile component of the protein that exhibits motion during voltage gating (Mannella et al., 1992; Mannella, 1997; Najbauer et al., 2021) and may modulate the interaction of the antiapoptotic proteins, HK and Bcl-2, to their binding sites (Shi et al., 2003; Abu-Hamad et al., 2009; Arzoine et al., 2009). The N-terminal sequence of VDACs is composed of 25 residues, except for VDAC2, where there is an N-terminal extension of 11 residues that do not change the channel activity. The VDAC isoforms also differ in their cysteine

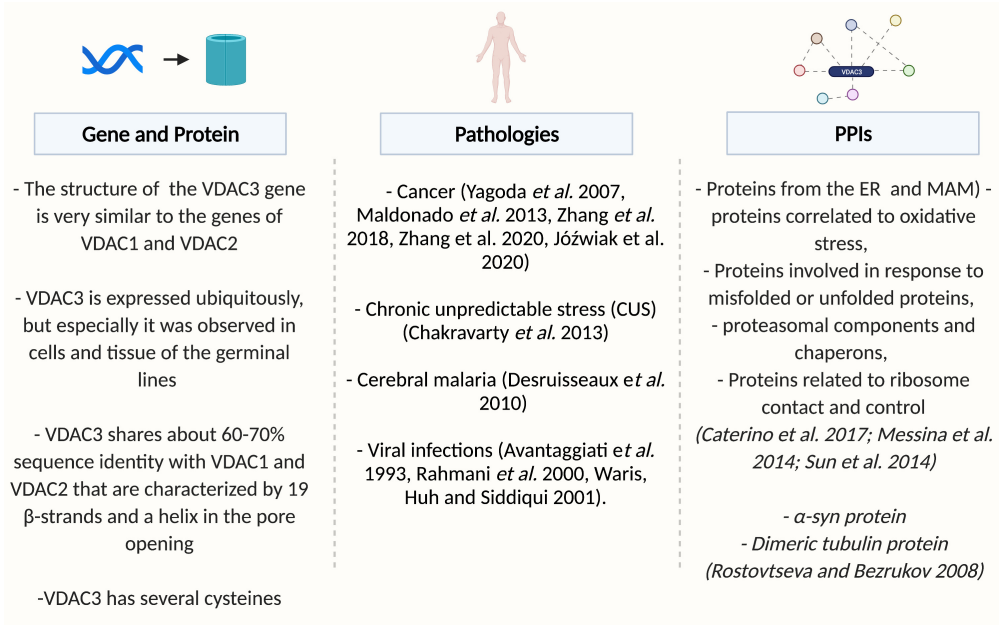

FIGURE 1 | Overview of VDAC3. Created by BioRender.com.

content. Human VDAC3 has six cysteines: four of these residues are predicted to be in the connection loops between β-strands, protruding toward the intermembrane space (IMS; Cys36, Cys65, Cys122, and Cys229), and two are in the N-terminal domain (Cys2 and Cys8). VDAC1 lacks cysteines at the N-terminus and has only two residues located on the β-strands (i.e., Cys 127 and Cys232 in humans). VDAC2 also has many cysteines (nine in humans and six in mice), but only one of them is conserved in the N-terminus in a position corresponding to VDAC3. The predicted location of hVDAC3 cysteines suggests that they are highly accessible to soluble oxidative molecules and related to some specific biological function.

To explore the role of the N-terminal domain of VDAC3, a set of chimerical proteins was created by swapping the first 20 amino acids of VDAC3 N-terminal with homologous sequences of the other isoforms and then expressing them in the yeast strain of *Saccharomyces cerevisiae* Δpor1 strain. Such replacement is sufficient to change the features of the protein radically. Insertion of the N-terminus of VDAC1 confers activity to VDAC3 and increases life span, indicating more efficient bioenergetic metabolism and/or better protection against ROS. However, also substitution with the N-terminus VDAC2 improves the ability of VDAC3 to complement the absence of endogenous porin1 in yeast, although to a lesser extent (Reina et al., 2010).

CHANNEL ACTIVITY OF VOLTAGE-DEPENDENT ANION SELECTIVE CHANNEL 3

The voltage-dependent characteristics of VDAC1 and VDAC2 have been extensively demonstrated, while those of VDAC3 has only recently been examined in detailed biophysical and electrophysiological studies (**Table 1**).

Initially, Xu et al. (1999) demonstrated that mVDAC3 exhibited electrophysiological properties strikingly different from the other isoforms. mVDAC3 rarely insert into artificial membranes and did not gate well even at high membrane potentials (do not exhibit voltage-gating up to 80 mV; Xu et al., 1999). Subsequently, differences in the human isoform were also observed. As reported in Checchetto et al. (2014), the LDAO-solubilized hVDAC3 showed channel activity with very small conductance (90 pS in 1M KCl) compared to hVDAC1 conductance (>3,500 pS in 1M KCl), allowing passage of both chloride and gluconate anions. Unlike VDAC1, the VDAC3 channel was open even at transmembrane voltages higher than ±40 mV and showed a relatively high probability of opening even at ±80 mV. In addition, the pores were only slightly voltage-dependent and tended to adopt low conductance states preferentially at negative voltages lower than positive voltages (Checchetto et al., 2014). The small conductance matches the cellular performance of hVDAC3 expressed in yeast strain *S. cerevisiae* Δpor1 strain, where only partial growth recovery under non-permissive conditions (i.e., 2% glycerol at 37°C) was obtained (Xu et al., 1999; Reina et al., 2010; Checchetto et al., 2014; Okazaki et al., 2015). Careful analyzes showed that, after treatment with reducing agents, VDAC3 occasionally reaches the characteristic conductance level of a fully open VDAC (Okazaki et al., 2015; Reina et al., 2016). Very recently, the group of De Pinto further confirmed what was previously reported in Checchetto et al. (2014) using nanodisc-stabilized human VDACs: accordingly, in the absence of any reductants, VDAC3 inserted into artificial membranes as small, non-gated channels

TABLE 1 | Summary of information on VDAC3 channel activity.

Protein	mVDAC3 (Xu et al., 1999)	hVDAC3 (Checchetto et al., 2014)	hVDAC3 (Okazaki et al., 2015)	hVDAC3 (Queralt-Martín et al., 2020)	hVDAC3 (Conti Nibali et al., 2021)
Refolding detergent	5% DMSO, 2.5% Triton X-100	1% (v/v) LDAO	0,4% (v/v) LDAO	0,1% (v/v) LDAO	1% (v/v) LDAO
Reducing agent	The presence of DTT resulted in a modest increase in insertion of VDAC3 channels.	None	None	DTT	Without and with 5 mM DTT
Bilayer composition	Asolectin:cholesterol (5:1)	Asolectin (2 mg/ml)	POPE:POPC (8:2)	DOPC:DOPE:DOPG (1:1:2)	1% DiPhPC
Conductance	It does not show a clear preferred state for this channel in a phospholipid membrane.	∼90 pS in 1M KCl	∼500 pS in 250 mM KCl	∼3.9 nS in 1M KCl	In the absence of DTT ∼0.7 nS; in the presence of DTT ∼3 nS in 1M KCl
Voltage dependence	No	No	No	Yes	No A perfect overlap of the voltage dependence between all three isoforms was obtained only when the cysteines were removed from the hVDAC3 sequence
Ion selectivity	Similar to mVDAC1 and mVDAC2, mVDAC3 resulted in the same molecular weight cutoff, indicating that this protein could also form channels that allow the flux of large nonelectrolytes across the mem- brane.	N.D.	N.D.	Similar to hVDAC1	In the presence of DTT similar to hVDAC1 and hVDAC2 The results of these experiments further corroborate the importance of cysteine redox state in pore function, and they foster the hypothesis that the selectivity of the channel is correlated to the size of the unitary conductance.

(Conti Nibali et al., 2021). When cysteines are found to be reduced in mass spectrometry analysis (following DTT and iodoacetamide treatment) it means that those cysteines were probably involved in disulfide bridges (otherwise DTT could not have reduced them back to SH). The main hypothesis for this discrepancy in hVDAC3 conductance was proposed to arise from the lipids used in planar lipid bilayer experiments and the difficulty in obtaining stable and homogeneous VDAC3 proteins. As reported in Queralt-Martín et al. (2020), VDAC3 forms canonical pores responsive to membrane voltage, even though with a much lower insertion rate than isoforms 1 and 2, and exclusively following highly-reducing refolding procedures. The authors correlated these differences with the lower stability of hVDAC3 in LDAO detergent. Using a highly reactive thiol-specific fluorochrome, they performed a thermal stability assay on mVDAC1 and hVDAC3, confirming a dramatic change in melting temperatures between the two isoforms (hVDAC3 T_m = 29°C, mVDAC1 T_m = 56°C). The outcome revealed that hVDAC3 has lower protein stability than hVDAC1 when solubilized in LDAO. The lower T_m value of hVDAC3 may explain the formation of noisy channels in the PLM due to the insertion of the unproperly folded hVDAC3. In this paper, the authors suggested that the best insertion yield is achieved using lipid bicelles made from 2-dimyristoyl-sn-glycero-3-phosphocholine (Queralt-Martín et al., 2020).

More often, the protein appears to form aggregates during protein purification. Another possible reason for such strange channel behavior of VDAC3 was attributed to the number and endomitochondrial location of exposed cysteine residues, which predominantly protrude toward IMS. According to mass spectrometry analysis, these amino acids follow an oxidative pattern that is conserved throughout evolution and does not include irreversible oxidations, as it was instead found in VDAC1 and VDAC2 cysteines (Saletti et al., 2018; Pittalà et al., 2020). In hVDAC3, Cys2, Cys8, Cys122, and Cys229 were identified as completely reduced, while Cys36 and Cys65 were detected in both the reduced and trioxidized form. The consequence is that the reduced Cys, even though it can be oxidized, is always reduced back to -SH and avoids being irreversibly oxidized. Then, they are candidates to be protected in a disulfide bridge, or their function is linked to their reduction, indicating that they are structurally and functionally crucial for the protein itself. The swapping experiments mentioned above have already suggested the importance of N-terminal cysteine residues in the pore activity of VDAC3 (Reina et al., 2010).

Later, electrophysiological data reported in Okazaki et al. (2015) and Reina et al. (2016) confirmed the essential role of such sulfur-containing amino acids in channel gating, proposing that the N-terminal residues Cys2 and Cys8 could form transient disulfide bonds capable of modulating the pore diameter or changing the charges exposed on the protein surface (Amodeo et al., 2014; Guardiani et al., 2016) and therefore its conductance. The current flow through VDAC3 is dramatically reduced, compared to VDAC1 and VDAC2, under non-reducing conditions (i.e., ∼90 pS vs. ∼3.5 nS in 1M KCl, respectively).

A pivotal role for VDAC3 cysteines in modulating mitochondrial ROS has also been proposed (Reina et al., 2016). To date, however, this point is still speculation since no empirical evidence is available. In this regard, the latest data in the literature seem to support this hypothesis, at least indirectly: for instance, Zou et al. (2018) described the correlation between VDAC3 knockout and mitochondrial ROS overload in renal tubules in mice subjected to high salt intake. However, it is not clear how this "ROS buffering" activity should take place: one possible explanation is that under mitochondrial stress, conformational changes induced by –SO3H formation could function as signals for incorporation of VDAC3 into MDVs, responsible for the removal of oxidized proteins and closely involved in mitochondrial quality control (Soubannier et al., 2012; Reina et al., 2020). MDVs contain numerous oxidized proteins derived mainly from the MOM: VDAC1 has been listed among these proteins (Soubannier et al., 2012), whereas information on the presence of VDAC3 is not yet available.

To address their physiological role (Queralt-Martín et al., 2020) analyzed the activity of the hVDAC3 cysteine-less mutant (in which all six cysteine residues were replaced with alanines) and compared it with the WT form. The PLM results suggested that cysteine residues do not significantly affect the stability or functionality; however, they affect the ability of hVDAC3 to interact with other proteins (e.g., α-synuclein).

VOLTAGE-DEPENDENT ANION SELECTIVE CHANNEL 3-PROTEIN INTERACTIONS

Protein interaction networks are crucial to understanding cell functions and pathways and developing successful therapies to treat human diseases.

In 2014, the VDAC3 interactome was defined *in vivo* by a TAP-Tag immunoprecipitation strategy and mass spectrometry identification (Messina et al., 2014). The crucial interactions correlate VDAC3 with: (i) proteins from the endoplasmic reticulum and MAM, (ii) proteins correlated with the response to oxidative stress, (iii) proteins involved in the response to misfolded or unfolded proteins, (iv) proteasomal components and chaperons, and (v) proteins related to ribosome contact and control (Messina et al., 2014).

In the context of PPIs, it has recently been reported that the main difference between VDAC3 and the other VDAC isoforms concerns associations with cytosolic proteins involved in mitochondrial metabolism, especially α-syn and the dimeric tubulin (Rostovtseva and Bezrukov, 2008). Several studies establish the involvement of α-syn in mitochondrial dysfunction. A detailed examination of the blockage kinetics of rVDAC1 reconstituted into planar lipid membranes defines that at nanomolar concentrations, α-syn reversibly causes time-resolved reversible blockages of the channel conductance. α-Syn induces two distinct blocked states, depending on its concentration and the applied voltage. Two steps characterize the blocked state in terms of conductance: a blocked state with a conductance of ∼40% that of the open state and a second deeper state with a conductance of ∼17% that of the open state (at potentials $V \geq 30$ mV). α-Syn blocks rVDAC1 from both sides of the channel, but only when a negative potential is applied from the side of the α-syn addition, suggesting that the negatively charged C-terminal region of α-syn is responsible for the blockage of rVDAC1 (Rostovtseva et al., 2015). These recent data show that similarly to rVDAC1, α-syn interacts with VDAC3 but 10–100 times less effectively (Queralt-Martín et al., 2020). An important role is attributed to the VDAC3 cysteines. Queralt-Martin and colleagues, using a cysteine-less hVDAC3 mutant, showed that the cysteine residues do not significantly affect hVDAC3 stability or functionality, as previously indicated (De Pinto et al., 2016; Reina et al., 2016), but they are responsible for the voltage asymmetry in the on-rate of α-syn-hVDAC3 interaction (Queralt-Martín et al., 2020). Likewise, the authors reported that VDAC3 is blocked by tubulin 10 times less effective than isoform 1, supporting the hypothesis that VDAC3 is primarily open when VDAC1 is closed via tubulin interaction (Queralt-Martín et al., 2020).

Voltage-dependent anion selective channel 3 is involved in the recruitment of PINK1/Parkin, cytosolic proteins involved in a pathway regulating mitochondrial quality control and promoting the selective autophagy of depolarized mitochondria (mitophagy; Narendra et al., 2008, 2010; Geisler et al., 2010; Truban et al., 2017; Ge et al., 2020). Loss of its function causes profound morphological and functional alterations in mitochondria, associated with the pathogenesis of Parkinson's disease. Sun et al. (2012) proposed that VDACs are part of the machinery that recruits Parkin to the organelle. Thus, they assumed that VDACs act as mitochondrial docking sites to recruit Parkin from the cytosol to mitochondria. The authors observed that in the absence of all three VDACs, the recruitment of Parkin to defective mitochondria and consequent mitophagy was compromised (Sun et al., 2012).

Another important VDAC-interactor is the VCP, a central and important element of the ubiquitin system. VCP is implicated in numerous neurodegenerative disorders. For example, its gene mutations cause frontotemporal dementia associated with inclusion body myopathy, early-onset Paget disease, familial ALS, and FTLD. Furthermore, VCP seems to act on VDAC3, addressing it toward microtubules through the traffic of cytoplasmic granules and enriching near the centrosome (Messina et al., 2014).

All these PPIs interactions are of great interest. They can have significant implications for mitochondrial bioenergetics and open

the way to discover new possible specific *in vivo* functions of the VDAC3 isoform hitherto unexplored.

ROLE OF VOLTAGE-DEPENDENT ANION SELECTIVE CHANNEL 3 IN PATHOLOGIES

Due to its crucial role in cellular metabolism and apoptosis, VDAC proteins are implicated in a wide range of diseases (Caterino et al., 2017; Magrì et al., 2018), including cancer (Maldonado et al., 2010; Reina and De Pinto, 2017; Magrì et al., 2018), neurodegenerative disorders, including Parkinson's disease (Rostovtseva et al., 2015), Amyotrophic Lateral Sclerosis (Magrì et al., 2016; Magri and Messina, 2017), and Alzheimer's disease (Manczak and Reddy, 2012). The knowledge about VDAC3 involvement in pathologies is very restricted. Studies with erastin, a small molecule compound that selectively kills human tumor cells carrying the oncogenes HRAS, KRAS, or BRAF, raised the possible connection between VDAC3 and cancer (Yagoda et al., 2007; Maldonado et al., 2013). In particular, a role of isoform 3 in the hepatocarcinogenesis induced by HBV infection was proposed by Zhang et al. (2018, 2020): a specific miRNA (miR-3928v) was shown to directly target and down-regulate VDAC3 expression and to promote hepatocarcinoma malignancy, by a still unclarified molecular mechanism. Recently, Józwiak et al. (2020) reported a significant increase in VDAC3 expression in non-metastatic endometrial cancers compared to normal tissue[1]. However, alterations in VDAC isoform 3 mRNA levels have also been registered in pathologies different from cancer, such as chronic unpredictable stress (Chakravarty et al., 2013), cerebral malaria (Desruisseaux et al., 2010), and viral infections (Avantaggiati et al., 1993; Rahmani et al., 2000; Waris et al., 2001).

A peculiar expression of VDAC3 isoform was observed in cells and tissue in the germinal lines of different organisms. Although VDAC1 is predominantly located in cells of reproductive organs required for the development of gametes (Hinsch et al., 2001; Specchia et al., 2008), VDAC2 and VDAC3 are expressed in a specific portion of sperm and oocyte, and genetic variants or aberrant regulation of these genes are correlated with infertility (Sampson et al., 1997; Pan et al., 2017). VDAC3-deficient mice are healthy, but males are infertile with a disassembled sperm tail, the flagellum essential for sperm motility. In VDAC3-deficient mice, the normal structures found in spermatids within the testes suggest that the defect develops with the maturation of the sperm in the transition from the testes to the epididymis. Each microtubule doublet has a corresponding outer dense fiber, all of which are morphologically distinguishable. Two of the outer dense fibers that are associated with microtubules 3 and 8 terminate within the principal piece and form the longitudinal columns of the fibrous sheath that partition the axoneme into two unequal compartments (Sampson et al., 2001). The VDAC3 gene might affect the energy supply for spermatogenesis and Leydig cell steroidogenesis and, finally, affect spermatogenesis (Hinsch et al., 2004).

Although VDACs are highly conserved between species, the specific function of each isoform remains unknown. To understand the specialized biological role of VDACs isoforms, recently, using the main available public resource reporting high-throughput data of international collaborative projects (Zinghirino et al., 2020) was performed a systematic analysis of human VDAC gene promoters was performed to highlight their structural and functional features. In particular, the authors underlined that the most active promoter controls VDAC3, enriching in GC repetitions, suggesting an epigenetic control mechanism capable of reducing transcript expression. Factors binding sites found in the VDAC3 promoter belong to various families, but those involved in the development of germinal tissues, organogenesis, and sex determination are the most abundant, confirming the experimental evidence of its crucial role in fertility (Sampson et al., 2001).

FUTURE PERSPECTIVES AND CONCLUSION

In general, the data available nowadays confirm that the VDAC3 function is still not fully discernible. In the beginning, VDAC isoforms were considered rescue vessels to make up for deficiencies in other abundant isoforms. Whether or not VDAC3 knockout or overexpression could alter the expression of other isoforms has been addressed by Craigen's group (Wu et al., 1999): accordingly, mouse ES cells are the first mammalian VDAC to knock out a model in which a compensatory increase in VDAC1 expression was registered for VDAC2−/− and VDAC3−/−.

Several hints make the study of this protein a very intriguing and promising field for acquiring deeper basic knowledge and for the development of diagnostic and therapeutic approaches to a wealth of pathologies such as cancer, respiratory or reproductive system diseases, renal or dermatological diseases, some myopathies, frontotemporal dementia, and neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

Both authors contributed to the writing of the manuscript.

FUNDING

The authors are grateful to the Italian Ministry (PRIN2010CSJX4F to VDP and IS), BIRD PRID SEED 2021 (CHEC_BIRD21_01) and Dotazione Ordinaria della Ricerca Dipartimentale (DOR) to VC funding for financial support.

ACKNOWLEDGMENTS

The authors thank Ildikò Szabò (IS) and Vito De Pinto (VDP) for their critical reading of the manuscript and useful discussion.

[1]https://www.proteinatlas.org/ENSG00000078668-VDAC3/tissue

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