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Viroporins: discovery, methods of study, and mechanisms of host-membrane permeabilization

Antonio Alcaraz[1] and José L. Nieva[2,3]

[1]Laboratory of Molecular Biophysics, Department of Physics, University Jaume I, Castellón, Spain; [2]Instituto Biofisika (CSIC-UPV/EHU), University of the Basque Country (UPV/EHU), Bilbao, Spain and [3]Department of Biochemistry and Molecular Biology, University of the Basque Country (UPV/EHU), Bilbao, Spain

Review

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Corresponding author:
José L. Nieva;
Email: joseluis.nieva@ehu.eus

Abstract

The 'Viroporin' family comprises a number of mostly small-sized, integral membrane proteins encoded by animal and plant viruses. Despite their sequence and structural diversity, viroporins share a common functional trend: their capacity to assemble transmembrane channels during the replication cycle of the virus. Their selectivity spectrum ranges from low-pH-activated, unidirectional proton transporters, to size-limited permeating pores allowing passive diffusion of metabolites. Through mechanisms not fully understood, expression of viroporins facilitates virion assembly/release from infected cells, and subverts the cell physiology, contributing to cytopathogenicity. Compounds that interact with viroporins and interfere with their membrane-permeabilizing activity *in vitro*, are known to inhibit virus production. Moreover, viroporin-defective viruses comprise a source of live attenuated vaccines that prevent infection by notorious human and livestock pathogens. This review dives into the origin and evolution of the viroporin concept, summarizes some of the methodologies used to characterize the structure–function relationships of these important virulence factors, and attempts to classify them on biophysical grounds attending to their mechanisms of ion/solute transport across membranes.

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Introduction: discovery of viroporins and evolution of the field from a historical perspective

Cells become permeable to ions and small molecules after infection by cytopathic viruses (Kohn, 1979; Fernandez-Puentes and Carrasco, 1980). This 'membrane leakiness' phenomenon was thoroughly studied in the late 70s and early 80s of the past century by Carrasco and collaborators, mostly in the context of the picornavirus encephalomyocarditis (EMC) virus infection (Carrasco, 1977, 1981). Following their initial findings, these authors put forward a three-postulate hypothesis to explain the underlying mechanism and its cytopathophysiological implications: 1) Cell membrane depolarization and changes in the ion content of the internal milieu may favor intracellular viral replication by facilitating the translation initiation of viral RNA and by shutting off host-cell protein synthesis (Carrasco and Smith, 1976; Carrasco, 1977); 2) Given the lack of specificity for the permeating solute, holes through which small molecules could pass freely promote the cell membrane permeabilization to low molecular weight compounds; incorporation of viral proteins to the membrane could generate these holes (Carrasco, 1977, 1978);

and 3) Virus-induced permeabilization offers the opportunity to block the progression of infection by antibiotics capable of permeating selectively into infected *versus* non-infected cells (Carrasco, 1978, 1981; Contreras and Carrasco, 1979; Lacal and Carrasco, 1983).

In an earlier form, this hypothesis focused on the plasma membrane permeabilization phenomenon that occurs concurrent with the early stages of viral infection (Carrasco, 1977, 1981). Thus, the hypothesis advanced the implication in membrane leakiness of the viral coat proteins released in the plasma membrane after particle entry and decapsidation. Initial translation of viral RNAs would generate more of these membranolytic viral products, which would in turn incorporate into the plasma membrane, further contributing to enhance the cell permeability effect.

However, already in these early works, it was suggested that membrane permeabilization observed at the beginning of the replication cycle, and the cytopathic effects and cell killing phenomena observed during later stages of infection, could all be caused by a set of viral proteins involved in modifying the membrane (Carrasco, 1978). Indeed, parallel measurements revealed a correlation between host-cell protein synthesis inhibition and cell permeabilization occurring from the third hour after infection, that is, at the beginning of the late phase of infection by EMC virus (Lacal and Carrasco, 1982).

Therefore, the hypothesis was subsequently reformulated to account not only for the enhanced plasma membrane permeability observed during, or soon after, virus entry (early events), but also for similar processes occurring after mid-phases of cell infection (late events), ultimately leading to membrane disruption and cell lysis (Carrasco et al., 1989, 1993). Experimental evidence produced at the time revealed that modification of membrane permeability at the beginning of the late phase in infection was a widespread phenomenon observed in cells infected with a variety of both enveloped and naked viruses. In addition, triggering of the process required viral gene expression, suggesting that the synthesis of unidentified viral products was responsible for these changes. Furthermore, given the facts that before their transportation to the plasma membrane, these viral membrane proteins would translocate into the endoplasmic reticulum (ER), and that some viruses use the vesicular system to replicate their genomes, the first cell target of their action, once they are synthesized, was estimated to be the vesicular system (Carrasco et al., 1993).

Cloning and identification of several candidates in the beginning of the 90s of the past century led to the formal proposal of viroporins as a family of proteins, which, after individual expression in cells, reproduced certain aspects of the late membrane permeabilization phenomenon (Carrasco et al., 1993; Carrasco, 1995). The expression of cloned viroporin genes reproduced, in some instances, the changes in the ionic concentration within the cell and the disruption of ionic gradients and membrane potential observed upon infection. During the process, cell rounding and shrinkage takes place later, following a phenomenology overall similar to that observed upon treatment with membrane-active toxins. From the perspective of their microbial origin, viroporins were therefore considered as pathogen-encoded, pore-forming proteins with a capacity to cause an injury to the host cell (Carrasco et al., 1993). However, in contrast to bacterial toxins, viroporins do not need to travel from the producing cell to act on the target, and therefore are devoid of the elements that stabilize toxins in solution as non-constitutive membrane proteins (Cosentino et al., 2016; although see Madan et al., 2010a). Moreover, it appears that at the beginning of the replication cycle, viroporin-induced permeabilization of certain organelles could be involved in the prevention of cell killing, rather than in its promotion (see below).

Viroporins can conduct ions (H^+, Cl^-, Na^+, K^+, and Ca^{2+}) along electrochemical gradients and, therefore, also share some functional and structural features with ion channels (Fischer and Sansom, 2002; Becker et al., 2004; Wang et al., 2011; Hyser, 2015; Fischer et al., 2016). However, in comparison with the conventional ion channels existing in the host cells, most viroporins usually exhibit small size, low selectivity, and absence of ligand-regulated gating mechanisms (see section 'Mechanisms of viroporin-induced membrane permeabilization'). Notable exceptions to this rule are the K^+ channels encoded by chloroviruses, which share structural and functional features with those of bacterial and eukaryotic origin (Gazzarrini et al., 2003).

In many instances, composition-dependent physicochemical properties of the surrounding lipid bilayer such as surface charge, thickness, intrinsic curvature, or fluidity that modulate membrane polarization (Aguilella et al., 2014) and deformation (Phillips et al., 2009), can modify the ion-channel activity of viroporins (Fischer and Hsu, 2011; Whitfield et al., 2011; Verdia-Baguena et al., 2013; Aguilella et al., 2014; Largo et al., 2016; Largo et al., 2021). This in turn implies a degree of structural pliability that can confound not only the mechanisms of ion selectivity (Largo et al., 2021), but also, from a practical point of view, set hurdles to the resolution of *bona-fide* structures representing the functional versions of these integral membrane proteins (see, e.g., Ouyang et al., 2013; Oestringer et al., 2018).

Today, a consensus has been reached that the membrane permeabilization function of viroporins differs from the initial phenomenon of cell permeabilization induced by viral particles during entry (Nieva et al., 2012) (see, however, (Arroyo et al., 1995; Nieva et al., 2004; Zhang et al., 2023). Viroporins are actually regarded as virally encoded proteins that sustain virus assembly and egress, but that are also involved in the subversion of the host-cell physiology during the infection cycle by modulating a variety of processes, including apoptosis, autophagy, and inflammation (Nieva and Carrasco, 2015) (for comprehensive, excellent reviews on these issues, see Nieva et al., 2012; Giorda and Hebert, 2013; Hyser and Estes, 2015; Scott and Griffin, 2015; Nieto-Torres et al., 2015a; Fischer et al., 2016; Farag et al., 2020; Breitinger et al., 2022; Xia et al., 2022; Cedillo-Barron et al., 2024). Deletion of viroporin-encoding genes reduces the formation of virus progeny and mitigates pathogenicity. Hence, viroporins embody potential therapeutic targets for the development of antiviral drugs, a view firmly underpinned by observations indicating that some compounds can simultaneously interact with viroporins, inhibit their membrane-permeabilizing ability measured *in vitro*, and block virus production (Premkumar et al., 2004; Wilson et al., 2006a; Steinmann et al., 2007; Griffin et al., 2008; Khoury et al., 2010; Luscombe et al., 2010; Schwarz et al., 2011; Foster et al., 2014; Li et al., 2014; Takano et al., 2015; To et al., 2016; Wetherill et al., 2018; Jalily et al., 2020; Shaw et al., 2020; Luscombe et al., 2021; Park et al., 2021; Toft-Bertelsen et al., 2021; Ewart et al., 2023; Bekdash et al., 2024; Brown et al., 2024; Devantier et al., 2024). Moreover, viroporin-defective particles are being explored as live attenuated vaccines to protect against infection by diverse animal viruses affecting humans and livestock (Watanabe et al., 2009; Fett et al., 2013; Regla-Nava et al., 2015).

The increasing number of published works in the field reflects the wide distribution of viroporin function, not only among animal RNA viruses belonging to divergent families as Orthomyxoviridae (Pinto et al., 1992; Mould et al., 2003; Pinto and Lamb, 2006), Paramyxoviridae (Perez et al., 1997; Gan et al., 2008; Gan et al.,

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2012; Masante et al., 2014), Retroviridae (Ewart et al., 1996; Piller et al., 1996; Schubert et al., 1996; Gonzalez and Carrasco, 2001; Ma et al., 2002; Mehnert et al., 2008; Majeed et al., 2023), Coronaviridae (Madan et al., 2005; Zhang et al., 2015), Togaviridae (Melton et al., 2002; Sanz et al., 2005; Elmasri et al., 2022), Flaviviridae (Griffin et al., 2003; Wozniak et al., 2010; Gladue et al., 2012), Picornaviridae (Lama and Carrasco, 1992; Aldabe et al., 1996; Aldabe et al., 1997; van Kuppeveld et al., 1997; Ao et al., 2015; Shukla et al., 2015; Gladue et al., 2018a), Reoviridae (Han and Harty, 2004; Hyser et al., 2010; Hyser and Estes, 2015), or Caliciviridae (Wang et al., 2023, but also among DNA viruses (Wetherill et al., 2012; Royle et al., 2015; Luganini et al., 2018; Gladue et al., 2023; Gladue et al., 2024), and plant-infecting viruses (Nishikiori and Ahlquist, 2018; Chai et al., 2024; Gao et al., 2024). The purported small size and architectural simplicity led the proposal of an initial viroporin classification that took into account the number of transmembrane domains (TMDs) as the primary criterion (Nieva et al., 2012). Thus, two major groups were proposed, class I and class II, comprising viroporins containing one or two TMDs, respectively (Figure 1*a*). Class I viroporins include well-studied members as influenza A virus (IAV) M2, coronavirus (CoV) E, or human respiratory syncytial virus SH. Viroporins belonging to class II include hepatitis C virus (HCV) p7 or poliovirus (PV) 2B. These two major classes were further subdivided into subclasses A or B depending on their assumed ER membrane topology (N_{ter}-in or N_{ter}-out, respectively) (Figure 1*a*). Moreover, members of these two main groups can be generally classified within a larger family of virally encoded integral membrane miniproteins (DiMaio, 2014; Opella, 2015).

Despite its wide generalization, this relatively simple, stereotype classification will need further revision as the number of the potential viroporin candidates increases. Thus, an updated classification should account for more complex folds adopted in membranes by larger individual members. In this regard, an additional Class III was proposed to account for two larger members of the family containing three hydrophobic TMDs, namely: CoV 3a protein (Lu et al., 2006; Kern et al., 2021) and rotavirus (RV) non-structural protein (NSP)-4 (Hyser et al., 2010; Pham et al., 2017) (Figure 1*b*, left). Some viroporins as the simian virus 40 (SV40) VP2 and VP3 proteins (Giorda et al., 2013), human cytomegalovirus (HCMV) US21 protein (Luganini et al., 2018), the Brome mosaic virus (BMV) protein 1a (Nishikiori and Ahlquist, 2018), or the norovirus (NoV) MLKL-like protein NS3 (Wang et al., 2023 (Figure 1*b*, right) probably fold in membranes adopting even more complex architectures. Interestingly, the possible coexistence of more than one pore–forming domain within a single sequence, as found in the foot-and-mouth disease virus 2B protein (Gladue et al., 2018a), could also contribute to increase the structural complexity of viroporins (Figure 1*c*).

Notwithstanding significant advances produced in the field (see, e.g., Nishikiori and Ahlquist, 2018; Wang et al., 2023), for the most cases, the molecular mechanisms that couple viroporin-induced cell membrane permeabilization to the stimulation of viral replication and egress are far from being elucidated. With the notable exception of the PV 2B, which has been described to hijack the cellular machinery to insert its TMDs and to facilitate glycosylation at canonical sites (Martinez-Gil et al., 2011; Bañó-Polo et al., 2013), detailed molecular descriptions on the biogenesis processes are also

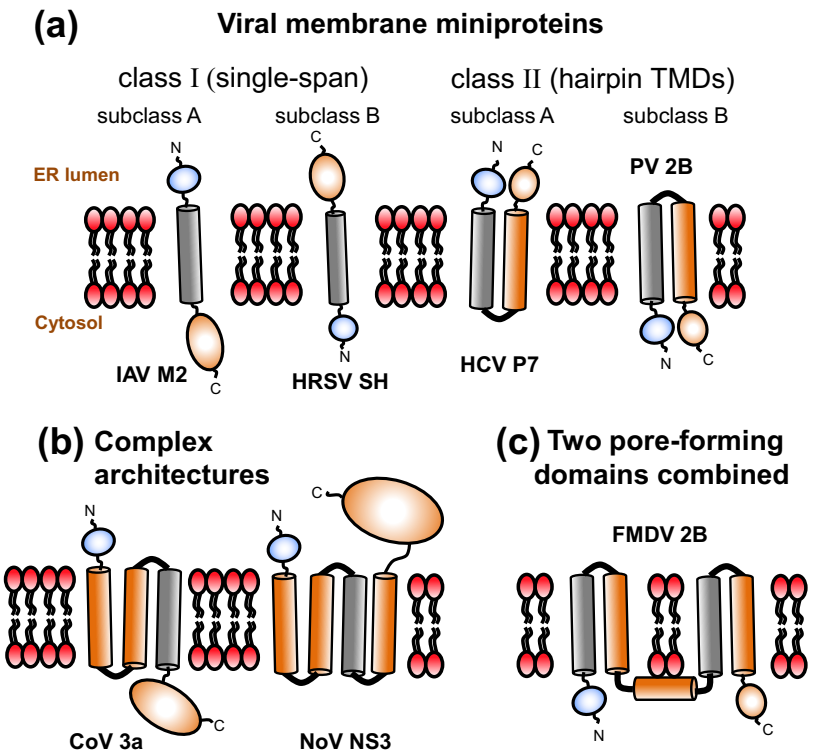

Figure 1. Viroporin classification. (*a*) Classes I and II comprise most known examples of conventional viroporins, and categorize them according to the number of transmembrane domains (TMDs). They are further divided into subclasses A and B considering the topology adopted in the endoplasmic reticulum (ER) membrane after synthesis (N-terminus facing the lumen or cytosolic side of the ER, respectively). These two classes may represent a subfamily within the larger group of integral membrane miniproteins of viral origin (DiMaio, 2014; Opella, 2015). (*b*) Examples of larger viroporins adopting more complex folds in membranes. Note that potential viroporins containing three TMDs were previously proposed to compose class III (Nieva et al., 2012). (*c*) At least a case of viroporin that includes two pore-forming domains has been reported in the literature (Gladue et al., 2018a). CoV, coronavirus; FMDV, foot-and-mouth disease virus; HRSV, human respiratory syncytial virus; IAV, influenza A virus; HCV, hepatitis C virus; NoV, norovirus; PV, poliovirus.

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missing for most viroporins. Even scarcer is our knowledge on the mechanisms relating ion-channel/pore assembly to the cytopathic effects observed in cells upon expression of these virally encoded products. In what follows, we summarize the methodological approaches used to characterize viroporin function and attempt, from a biophysical perspective, to systematize the mechanisms, both conventional (Wang et al., 2011) and unconventional (Hyser, 2015), that have been proposed to explain viroporin-induced ion–solute permeation and its inhibition. The fact that viral pathogens such as IAV or SARS-CoV-2, which are of concern due to their pandemic potential (Farheen et al., 2022; Zhang et al., 2022; Mostafa et al., 2024), encode archetypal viroporins supports the importance of this expanding field of research. Thus, given their relevance as therapeutic targets, when dealing with mechanisms, we will pay especial attention to the structure–function relationship of two prominent members of the family: IAV M2 and SARS-CoV E.

Permeability changes to ions and small solutes induced by viroporins in cell membranes

Permeability changes measured in cell plasma membranes

Assuming that viroporin-induced permeability changes reproduce a pattern akin to that observed in cultured mammalian cells upon viral infection, a series of expression-coupled cell permeability tests were devised to prove the viroporin activity of the cloned candidates. A common procedure involves the use of the antibiotic hygromycin B (HB), a protein translation inhibitor that cannot efficiently penetrate the cell membrane. Reduction of ^{35}S-labeled protein expression, followed by autoradiography after SDS-PAGE separation, can be observed in cells harvested from cultures previously induced to express the cloned viroporin gene. Inhibition of protein expression by HB has been reported in animal cells expressing among others PV 2B (Aldabe et al., 1996; Madan et al., 2010a), Paramyxovirus SH (Masante et al., 2014), E protein from murine hepatitis virus (Madan et al., 2005), NS3 protein of bluetongue virus (Han and Harty, 2004), human polyoma JC virus agnoprotein (Suzuki et al., 2010), or the Bovine ephemeral fever rhabdovirus α1 (Joubert et al., 2014). However, these measurements can neglect viroporin activity targeted at the cell endomembrane system and/or be limited by the cytotoxicity of the overexpressed protein. An alternative, more flexible method is to measure the increase in membrane permeability to small solutes induced in bacteria by the expression of the cloned genes. In the *Escherichia coli* BL21(DE3) system, the expression of potentially cytotoxic viroporins is tightly regulated by the inducible expression of the T7 RNA polymerase and the viroporin product, the latter cloned into a vector of the pET series after a T7lac promotor (Dubendorff and Studier, 1991; Lama and Carrasco, 1992). Induction of viroporin expression can arrest cell growth, or even promote lysis in the case of BL21(DE3) pLysS cells expressing T7 lysozime constitutively, a process that can be followed by the changes in the optical density of the culture (Lama and Carrasco, 1992; Sanz et al., 1994; Madan et al., 2005; Hyser et al., 2010; Strtak et al., 2019). Expression can also facilitate entry of HB (Madan et al., 2005) or induce the release to the medium of radioactivity from *E. coli* cells preloaded with [^{3}H]uridine (Perez et al., 1997; Madan et al., 2005) or [^{3}H]choline (Sanz et al., 1994).

These bacteria-based methods have become quite popular as a primary test for the presence of viroporin activity (Guo et al., 2013; Joubert et al., 2014; Ao et al., 2015). Used to discern among the distinct virally encoded products those bearing viroporin activity (Lama and Carrasco, 1992), or to map crucial residues for function (Perez et al., 1997), these assays are not, however, free in all instances from spurious effects arising from the massive incorporation of the expressed products to the bacterial membrane. To surpass this limitation, a more sophisticated procedure, called 'positive assay', has been developed (Taube et al., 2014). The assay takes advantage of the widespread, poor specificity of the viroporin ion-channel activity (i.e., its general implication in nonspecific homeostasis regulation; see section 'Mechanisms of viroporin-induced membrane permeabilization') and makes use of a K^+-uptake deficient bacteria strain requiring high concentration of this cation in the medium to grow. Upon expression of a channel-forming viroporin, the bacteria are able to thrive in low K^+ media. Thus, membrane permeability induced by viroporin activity impacts growth positively, avoiding potential toxicity issues. This approach has been recently applied to identification of new viroporins (Tomar et al., 2019) and to high-throughput screening of inhibitors (Lahiri and Arkin, 2022).

Despite the correlation existing between expression and permeabilization, it is still necessary to distinguish the intrinsic ion-channel activity of the virally encoded protein from that possibly performed by the host cell proteins. Thus, alternative, complementary approaches have made use of purified specimens, GST- or MBP- fusion proteins expressed in bacteria, or proteins and peptides produced through chemical synthesis, which allow for performing quantitative measurements. For instance, the addition to the culture medium of P3, a peptide representing the amphipathic TMD of the PV 2B protein, reproduced in BHK-21 cells the membrane permeabilization phenomenon induced by the single expression of 2B from an alphavirus replicon (Madan et al., 2007). Similarly, the external addition to COS cells of SV40 GST-VP2 and GST-VP3 induced the formation of pores with inner diameters in the 3–6 nm range (Giorda et al., 2013). Note that these pores are considerably larger than the canonical voltage-dependent channels of neurons that display radius quite below the nanometer (Sato et al., 1998; Moldenhauer et al., 2016).

Viroporins also display ion-channel activity in patch-clamped *X. laevis* oocytes (Pinto et al., 1992) and whole cells (Chizhmakov et al., 1996), provided that expressed recombinant forms are properly transported to the plasma membrane (Cabrera-Garcia et al., 2021; Harrison et al., 2022; Breitinger et al., 2023). Generalization of these electrophysiological approaches in the field followed the publication of the earliest works by Pinto et al. on the channel activity of IAV M2 protein (Pinto et al., 1992; Wang et al., 1994; Pinto and Lamb, 2006). Tetramers of this small protein make the virion envelope and the Golgi membrane permeable to protons upon its activation at low pH (Pinto and Lamb, 2006). During entry through the endocytic route, M2 activity allows the acidification of the virion core, facilitating the disassembly of the ribonucleoprotein complexes (Pinto and Lamb, 2006; Stauffer et al., 2014; Lamb, 2020). When located at the Golgi membrane, it prevents the acidification of this organelle, thereby protecting low-pH-activated spike hemagglutinin from premature conformational changes during transit to the plasma membrane (Pinto and Lamb, 2006; Lamb, 2020). Voltage clamp measurements of *X. laevis* oocytes and whole cells induced pH-dependent cation channel currents upon expression of M2 and homologous products BM2 and CM2 derived from influenza viruses B and C, respectively (Mould et al., 2003; Pinto and Lamb, 2006). Subsequently reported evidence supported specificity for proton conductance (Chizhmakov et al., 1996; Pinto et al., 1997), although the M2 channel showed capability to permeate NH_4^+ cations as well (Mould et al., 2000).

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Similar standard patch clamp approaches have been followed to test the activity of other potential viroporins including HCV p7 (Breitinger et al., 2016), Paramyxovirus SH (Gan et al., 2012), SARS-CoV E and 3a (Lu et al., 2006; Nieto-Torres et al., 2011; Toft-Bertelsen et al., 2021; Breitinger et al., 2023; Ewart et al., 2023), HIV-1 Vpu (Coady et al., 1998; Greiner et al., 2016), or Alphavirus 6K (Antoine et al., 2007), just to mention some examples. However, channel recordings in oocytes and whole cells requires performing a series of controls before interpreting these sole results as enough evidence of *bona fide* viroporin activity (Harrison et al., 2022; Breitinger et al., 2023; Ewart et al., 2023). Expression of HIV-1 Vpu in oocytes (Coady et al., 1998) or SARS-CoV E in whole cells (Nieto-Torres et al., 2011) can diminish membrane conductance, and in both instances this effect has been ascribed to interferences with plasma membrane expression of cell K^+ channels. Overexpression of not only viroporins (Antoine et al., 2007), but also other small integral membrane proteins (Shimbo et al., 1995), can evoke endogenous currents carried out by Ca^{2+}-activated Cl^{-1} channels. Even though pure specimens of SARS-CoV1 3a were previously shown to display ion-channel activity in planar bilayers (Castaño-Rodriguez et al., 2018), a recent series of published works support or oppose the viroporin-like role of SARS-CoV2 3a protein (Toft-Bertelsen et al., 2021; Harrison et al., 2022; Miller et al., 2023). The discrepancy in the reported electrophysiology data was suggested to arise from technical issues and/or the contribution of endogenous channels.

Permeability changes measured in the eukaryotic endomembrane system

Besides modifications of plasma membrane permeability, viroporins may alter permeability of cell organelles to ions and solutes upon synthesis. Most conspicuously, the activity of several viroporins can subvert interorganellar Ca^{2+} homeostasis, which plays important roles in pathophysiology (see Chami et al., 2006; Chen et al., 2019; Mehregan et al., 2022, for a discussion on this issue). Ca^{2+} stored at high concentrations in the ER and Golgi can be released into the cytosol on physiological stimuli that activate the InsP3R channels (Raffaello et al., 2016). This complex, multi-ionic process that also depends on the electric potential (Campbell et al., 2023) is followed by fast Ca^{2+} influx from the external medium through channels residing at the plasma membrane. The rapid and sustained increase in the cytosolic concentration of the free cation ($[Ca^{2+}]_{cyt}$) regulates a number of Ca^{2+}-dependent processes. Moreover, a local increase at ER contacts can also lead to Ca^{2+} overload of mitochondria through the uptake via VDAC-MCU channels (Singh and Mabalirajan, 2021). Ca^{2+} overload can result in turn in the long-term activation of the mPTP channel, which is accompanied by the release of pro-apoptotic factors and activation of cell death through the intrinsic pathway of apoptosis. Ca^{2+} signals directed to the mitochondria can also activate autophagy. Furthermore, if cells are properly primed by cytokines and PAMP ligands, these oscillations in the concentrations of mitochondrial and cytosolic Ca^{2+} can lead to inflammasome activation (Swanson et al., 2019).

Activation of cell death through apoptotic/necrotic pathways, cell content recycling through autophagy or activation of inflammatory responses can help the cell and tissues to cope with replicating viruses (Chami et al., 2006). Viroporin channels assembled at the ER membrane can prevent the sudden and sustained oscillation in intracellular calcium concentration and block activation of these cellular responses (Aldabe et al., 1997; Chami et al., 2003; de Jong et al., 2008; Hyser et al., 2010; Luganini et al., 2018). Thus, ER-targeting viroporins could suspend the infection-induced cell responses, providing the virus with sufficient time to replicate (Brisac et al., 2010).

Contrasting this assumption, other experimental results support that viroporin-induced increase in $[Ca^{2+}]_{cyt}$ can actually promote apoptosis or autophagy after virus infection (Berkova et al., 2003; Hyser et al., 2010; Crawford et al., 2012; Hyser and Estes, 2015). Interestingly, the increase in the cytosolic content of this cation may play a direct role in the assembly of new viral particles (Zhou et al., 2009; Amarasinghe and Dutch, 2014; Chen et al., 2019; Rahman et al., 2020). One well-studied example of a virus featuring calcium dependence for replication and assembly is RV (Hyser and Estes, 2015). In this case, the NSP4 viroporin activity appears to activate STIM1 in the ER, a process required to maintain the activation of calcium influx through channels in the plasma membrane (Hyser et al., 2013).

Estimating changes in ER and Golgi permeability coupled to expression of potential viroporins involves a distinct set of methods. One of those methods employs the sarco/endoplasmatic reticulum calcium ATPase (SERCA) inhibitor thapsigargin to measure the amount of calcium releasable from stores. Upon addition of this compound to cells, calcium uptake by the ER, but not its release into the cytosol, is blocked. Thereafter, sudden elevations of $[Ca^{2+}]_{cyt}$ occur due to the entry of extracellular Ca^{2+} via the Ca^{2+} channels in the plasma membrane, which are activated by the cation released from the organelle. Hence, the response to thapsigargin depends on the amount of calcium previously stored in the ER at high concentration, which might be altered upon expression and ER localization of several viroporins (Campanella et al., 2004; Luganini et al., 2018; Gladue et al., 2018a).

In addition, the fluorescent Ca^{2+} indicator Fura-2 has been used to measure changes of $[Ca^{2+}]_{cyt}$ that demonstrated an increase in individual cells expressing RV NSP4 protein (Berkova et al., 2003), but a reduction in the amount of releasable calcium in cells expressing the HCMV US21 protein (Luganini et al., 2018) or the Coxsackievirus 2B protein (Campanella et al., 2004). In the latter case, ER, Golgi, and cytosol were also selectively loaded with different versions of the Ca^{2+} indicator aequorin and its substrate coelenterazine to demonstrate that 2B decreases the cation content of both the ER and the Golgi (Campanella et al., 2004). Other Ca^{2+} indicators as Indo-1 (Hyser et al., 2010), GCaMP5G (Hyser et al., 2013; Strtak et al., 2019), and rhod-2/AM (Gladue et al., 2018a; Gladue et al., 2018b) have been described in the literature to measure $[Ca^{2+}]_{cyt}$ changes in cells expressing viroporins.

Dissipation of proton gradients at Golgi apparatus and ERGIC has been proposed as an additional viroporin function (Nieva et al., 2012), presumably to avoid premature low-pH activation of spike proteins in transit to the plasma membrane (Sakaguchi et al., 1996). Both HCV infection and expression of the p7 product increased the pH of acidic compartments, detected with LysoSensor Yellow/Blue DND-160 or LysoTracker Red DND-99, two pH fluorescent probes that exhibit a pH-dependent emission (Wozniak et al., 2010; Breitinger et al., 2016). Overexpression of infectious bronchitis CoV E also alters Golgi pH as evidenced from changes in the co-expressed pH-sensitive GFP mutant pHluorin-TGN38 (Westerbeck and Machamer, 2019). Similarly, SARS-CoV-2 E protein localized at the ERGIC and provoked an increase of pH in live NIH-3T3 cell internal organelles, as detected by changes in emission of Lysosensor Green DND-189 fluorescent probe (Cabrera-Garcia et al., 2021; Bekdash et al., 2024).

Notably, Wozniak et al. (2010) further isolated a light membrane vesicle fraction containing lysosomes and ER from cells

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expressing the HCV viroporin p7. These vesicles were loaded with the pH-dependent fluorophore 8-hydroxypyrene-1,3,6-trisulfonic acid and subsequently used to measure proton permeability. Following this *ex vivo* strategy, these authors demonstrated that H^+ efflux was promoted in vesicles bearing functional forms of p7, but not in those isolated from cells expressing non-functional mutants.

ER and Golgi compartments are also subject to profound remodeling by RNA viruses that replicate their genomes in the cytoplasm of the host cell. The involvement picornavirus 2B/2BC viroporin in the formation of new cytoplasmic vesicles or 'viroplasm', where genome replication may take place to protect double-stranded RNA intermediates from innate immune recognition, and affecting overall secretory pathways was well documented (Barco and Carrasco, 1995; Suhy et al., 2000; Gonzalez and Carrasco, 2003; de Jong et al., 2004; Hsu et al., 2010; Nieva et al., 2012). It has not been until recently, however, that a molecular mechanism coupling viroporin-induced permeability to the activation of functional vesicular compartments (spherules) at the ER has been put forward (Nishikiori and Ahlquist, 2018; Nishikiori and Ahlquist, 2021). Genome replication of the alphavirus BMV requires the activity of the membrane-associated RNA replication protein 1a. This protein also makes yeast ER membranes permeable to phenoxyl radicals that are generated by an engineered ascorbate peroxidase enzyme confined within the lumen of the organelle, and has therefore been defined as a viroporin (Nishikiori and Ahlquist, 2018). According to the proposed model (Nishikiori and Ahlquist, 2021), ER permeabilization through 1a viroporin activity releases oxidizing potential from the lumen of the organelle to the cytoplasm. Oxidizing radicals are in turn required for the generation of disulfide bridges and the stabilization of the 1a multimers that activate late RNA replication functions at the neck-like connection between the interior of the spherules and the cytosol.

By comparison, the amount of evidence proving that viroporin activity can directly permeabilize mitochondrial membranes is very limited (Madan et al., 2008; Madan et al., 2010b; Nieva et al., 2012; Lee et al., 2018; You et al., 2019; Cedillo-Barron et al., 2024). However, the recent demonstration that mitochondrial membrane permeabilization by the NoV NS3 viroporin activates cell death processes for the induction of virus egress, constitutes a breakthrough in the field (Wang et al., 2023). It appears that permeabilization of mitochondria by the N-terminal domain of NS3 alters membrane potential and releases reactive oxygen species (ROS) and Cytochrome c to trigger programmed cell death and activation of cell plasma membrane lysis by Ninjurin-1, the latter process required for the release of the NoV particles to the medium. Depolarization of mitochondria can be followed by cytometry upon induction of viroporin expression in cells using the dye tetramethylrhodamine methyl ester, which is sequestered by active mitochondria (Lee et al., 2018; Wang et al., 2023). Mitochondrial ROS can be detected following a similar cytometry approach, after incubation of the viroporin-expressing cells with MitoSoX Red (Wang et al., 2023). In addition, mitochondria can be isolated from mice liver or cultured cells for incubation *in vitro* with purified forms of viroporins (You et al., 2019; Wang et al., 2023). Following this approach, it has been shown that, upon incubation of the isolated organelles with hepatitis B virus (HBV) X protein (You et al., 2019), membrane potential of mitochondria, which were stained with cationic dyes safranine O or JC-1, decreased significantly with respect to a carbonyl cyanide 3-chlorophenylhydrazone positive control. Using an alternative method, direct permeabilization of the mitochondrial membrane by NoV NS3 was assayed by determining Cytochrome c release into the medium (Wang et al., 2023.

Permeability changes measured in model membranes

Lipid vesicles: bulk measurements

A variety of minimal systems have been used to characterize viroporin function under more defined and controlled experimental conditions. These systems consist of model membranes of defined lipid composition incubated with pure protein/peptide specimens. Pore-forming activity can be assayed in liposomes through fluorescence-based methods both, in bulk and at the single-vesicle level. Bulk determinations of permeability changes should be preferably performed using large unilamellar vesicles (LUVs) with mean diameters ≥100 nm, which are devoid of the curvature stress and inter-layer surface asymmetry characteristic of small unilamellar vesicles produced by sonication. LUVs loaded with 5(6)-carboxyfluorescein at self-quenching concentrations, or high concentrations of fluorescent compounds together with quencher molecules (e.g., Tb^{3+}/DPA or ANTS/DPX pairs), can be readily prepared through extrusion of freeze-thawed, water-dispersed lipid multilayers followed by gel-filtration or repeated centrifugation to eliminate non-encapsulated probe (Agirre et al., 2002; StGelais et al., 2007; Foster et al., 2011; Gervais et al., 2011; Gladue et al., 2012; Largo et al., 2014; Largo et al., 2016; You et al., 2019). Release to the medium after viroporin-induced membrane permeabilization results in the dilution of the molecule pairs and concomitant increase in fluorescence intensity of the probe component, which can be monitored in a fluorimeter as a function of time. This procedure substantiated the capacity for permeabilizing membranes of a wide range of potential viroporins, including PV 2B (Agirre et al., 2002), HCV p7 (StGelais et al., 2007; Foster et al., 2011; Gervais et al., 2011), CSFV p7 (Gladue et al., 2012; Largo et al., 2014; Largo et al., 2016), HBV X protein (Lee et al., 2018; You et al., 2019), NoV NS3 (Wang et al., 2023), and alphavirus 6K (Dey et al., 2019), among others. In addition, more sophisticated experimental setups have been used to generate electrochemical and/or pH gradients across liposomal membranes and test the capacity for translocating different cations (Na^+, K^+, Ca^{2+}, and H^+) by viroporins as IAV M2 (Lin et al., 1997; Lin and Schroeder, 2001; Leiding et al., 2010; Peterson et al., 2011), HCV p7 (Wozniak et al., 2010; Gan et al., 2014), or SARS-CoV 3a (Kern et al., 2021; Miller et al., 2023).

Since vesicles are made of pure lipids, this type of experiments also allows straightforward determination of the dependence of pore-forming activity on membrane lipid composition, which in turn can provide clues about the organelle targeted by a given viroporin (van Meer et al., 2008). For instance, PV 2B activity appears to depend on the anionic nature of the phospholipid headgroup and the length and degree of unsaturation of its acyl chains (Agirre et al., 2002). Dependence of solute leakage on single lipids such as phosphatidylinositol or cardiolipin has been reported for viroporins that localize in the ER (Gladue et al., 2012; Largo et al., 2014; Largo et al., 2016) or mitochondria (You et al., 2019; Wang et al., 2023), respectively. Functional efficacy of M2 reconstituted in LUVs seems to depend on cholesterol (Leiding et al., 2010), a conspicuous component of Golgi and cell plasma membranes (van Meer et al., 2008).

LUVs can be loaded with compounds of different molecular weight sizes to prove solute-permeability through size-limited pores. Using this approach, molecules with a maximal size in the range of 1–10 kD have been described to leak out through pores established by potential viroporins (Agirre et al., 2002; Shukla et al., 2015; Wang et al., 2023). In the LUV system, efficient solute release occurring at protein-to-lipid mole ratios lower than 1:100 together

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with the existence of an MW cutoff for the permeating solute are usually good indicators of the measured permeability increase resulting from *bona fide* viroporin activity, and not from spurious processes as the bilayer disintegration that may occur coupled to the massive incorporation of hydrophobic protein moieties (detergent-like effect; Shai, 1999).

Fluorescence microscopy of single vesicles

In comparison, quantitative fluorescence microscopy performed in single vesicles provides more solid evidence on the capacity of viroporins to alter lipid bilayer permeability without compromising its overall integrity. For the purpose of lipid permeability testing, micrometer-sized giant unilamellar vesicles are produced by electroformation and labeled with a fluorescent lipid probe (e.g., N-(lissamine Rhodamine B sulfonyl) phosphatidylethanolamine) that allows their detection in a confocal microscope (see, e.g., Gladue et al., 2018a). Changes in permeability induced by potential viroporins are determined in the intact vesicles after addition to the external medium of a soluble fluorescent probe as Alexa Fluor 488 (MW: 0.6 kDa). Access of this fluorescent marker to the internal volume of vesicles after viroporin treatment denotes the occurrence of efficient membrane permeabilization. Application of this approach may offer useful information on the virpoporin-induced permeabilization mechanism. First, as mentioned before, it allows us confirming that the detected changes in permeability occur while preserving the integrity of the lipid bilayer architecture. Second, the degree of filling, which can be quantitatively determined from the ratio of fluorescence intensities measured in the lumen of the vesicle with respect to that measured in the external medium (Largo et al., 2018), may reveal if the process follows an all-or-none versus a graded mechanism of membrane permeabilization (Apellaniz et al., 2010; Gladue et al., 2018a). Stable pores assembled in lipid bilayers would tend to permeabilize membranes following the former mechanism, whereas transient, less defined structures are responsible for the latter. Third, the stability of the pores can be further tested after permeation to the Alexa Fluor 488 marker reaches equilibrium by incubating the vesicles with a second marker, for example, Alexa Fluor 647 (Gladue et al., 2018a). Incorporation of similar amounts of this second marker to the vesicle internal volume supports a pore-forming activity stable in time. Finally, the approximate size of the pore can be estimated using in the medium FITC-dextrans of different MW-s as permeant/impermeant solutes (Largo et al., 2018; You et al., 2019).

Electrophysiology in planar membranes

Ion-channel activity of viroporins can be also studied *in vitro* by means of electrophysiology in planar lipid bilayers (PLBs). This technique commonly employs a cell with two compartments separated by an inert film such as Teflon in which a small hole is made (typically 100–200 μm diameter orifice). Membranes can be formed at each side by different two main methods. The first one is the so-called solvent-containing membrane. Lipids dissolved in organic solvents are spread at both sides of the orifice. The formation of the bilayer can be controlled by with a microscope so that when monolayers formed in each side come into contact, a gray–black spot starts to spread over the film, explaining why bilayers formed following this procedure are called black lipid membranes (Mueller et al., 1963; Van Gelder et al., 2000; Winterhalter, 2000). Alternatively, solvent-free planar lipid membranes can be prepared by using the Montal–Mueller technique (Montal and Mueller, 1972) or slight modifications of it (Bezrukov and Vodyanoy, 1993). Aliquots of lipid in organic solvent are added onto the salt solution subphases at both compartments (so-called cis and trans) of a Teflon chamber. Since Teflon is lipophobic, the orifices must be pre-treated with some kind of solution to make it lipophilic. After evaporation of the organic solvents used, the level of solutions in each compartment is raised above the orifice, so the planar bilayer is formed by apposition (or opposition) of the two monolayers. Within this technique, capacitance measurements are used to monitor correct bilayer formation. The two methods described to form PLBs yield comparable results for ion-channel reconstitution in terms of measured conductance and selectivity. However, solvent-containing membranes appear to be slightly more flexible than the solvent free ones, which may have an impact in facilitating the insertion of extremely hydrophobic proteins (Winterhalter, 2000), as it is the case of the pore-forming domains of class I and II viroporins. Overall, PLBs provide the significant advantages of working in well-controlled artificial environment using only tiny amounts of material. Also, unlike liposomes, one has direct access to both sides of the membrane and therefore it is possible to form asymmetric membranes (Van Gelder et al., 2000).

Electrophysiological studies in PLBs with conventional ion channels usually show random transitions between open and closed states that allow to identify a canonical unitary conductance (Gutsmann et al., 2015). Quite in contrast, most studies with viroporins have encountered a large heterogeneity of results in terms of diversity of conductive levels and in their dynamic behavior (Mehnert et al., 2008; Montserret et al., 2010; Hyser, 2015; Largo et al., 2021). As shown in Figure 2a,b for CSFV p7 and SARS-CoV2-E, respectively, even when performing experiments with the same protocol and the same protein sample, it is possible to find traces with well-defined 'opening' and 'closing' rapid events, and others with larger current levels with longer lifetimes without almost any small flickering (Verdia-Baguena et al., 2013; Largo et al., 2016). Although such heterogeneity raised questions about reproducibility and/or the presence of detergent-like mechanisms (Hyser, 2015; Mehnert et al., 2008; Shai, 1999), electrophysiological characterizations of viroporins have proved to be solid when data collected are large enough to generate histograms that are statistically significant (see Figure 2a,b). Interestingly, pore size estimates arising from PLB can be satisfactorily compared to permeability assays with solutes of different molecular weight stablishing a certain cutoff (Largo et al., 2018). In the same line, data obtained in PLB become more trustworthy when regulatory factors of ion-channel activity are found in parallel in different techniques. For instance, pH or membrane composition that are critical for CSFV p7 permeabilization activity are found in PLB, leakage assay and even in atomic force microscopic imaging (see Figure 2c,d) (Largo et al., 2016; Largo et al., 2021). The relevance and interpretation of PLB experiments with viroporins will be discussed in detail below in section 'Mechanisms of viroporin-induced membrane permeabilization'.

Mechanisms of viroporin-induced membrane permeabilization

The characterization of the ion-channel activity of viral pore-forming proteins has been debated in recent years using a dichotomy expressed in various ways: conventional *versus* nonconventional channels (Delcour, 2015; Hyser, 2015, *bona fide* ion channels *versus* unregulated pores (Hyser and Estes, 2015), semi-transmembrane defects *versus* stable pores (Volovik et al., 2024),

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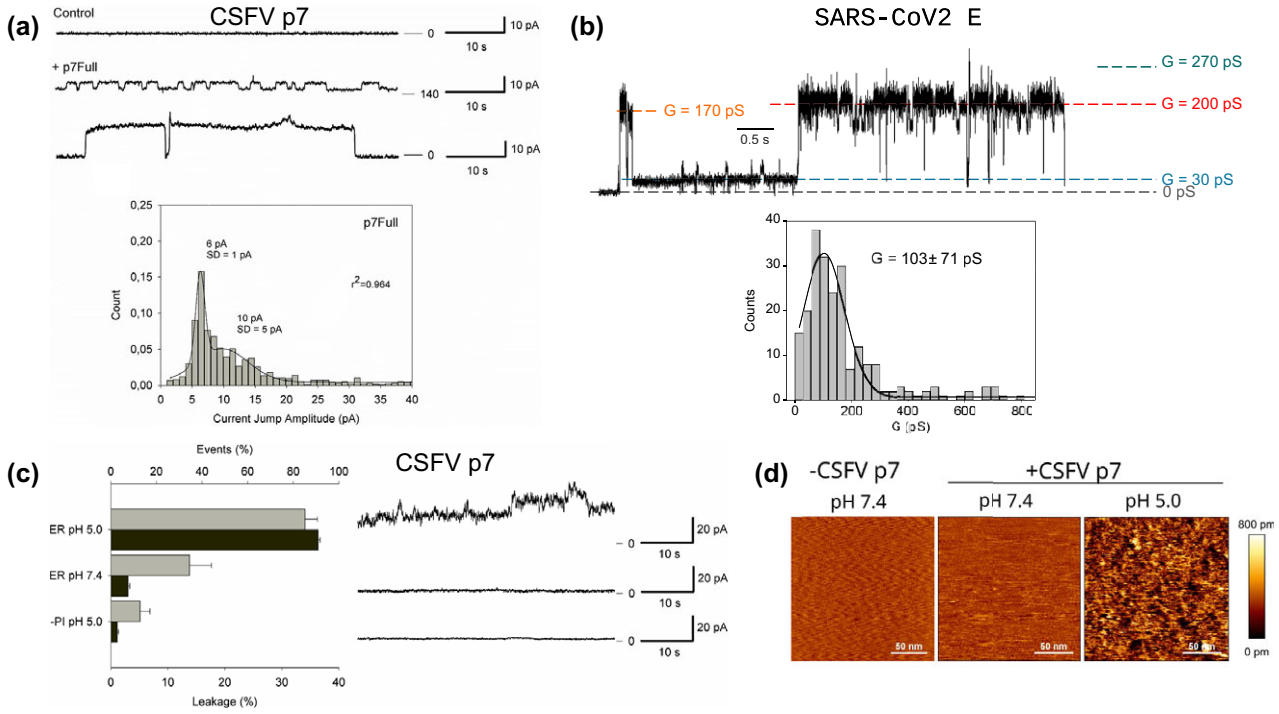

Figure 2. Electrophysiology and complementary approaches to study viroporin activity *in vitro*. (*a*) CSFV p7 ion channel activity in endoplasmic reticulum (ER)-like planar bilayers. Representative traces with current recordings without any protein addition (control) and after the addition of p7 protein show the different magnitudes of current jumps seen in experiments. Histogram of the current jump amplitude fitted to two Gaussian peaks. Current was recorded in 150 mM KCl, pH 5.0 at a potential of −50 mV. Reprinted with permission from Largo et al. (2016). (*b*) Representative current recordings of SARS-CoV2 E in ERGIC-mimetic lipid bilayers at 100 mM $CaCl_2$ show channel opening events with variable durations and conductance. Histogram of conductance jumps at +100 mV. Solid line indicates Gaussian fitting of the histogram. Reprinted with permission from Dregni et al. (2023). (*c*) Comparison between vesicle leakage and channel formation induced by CSFV p7. Left: Percentage of planar bilayers displaying IC activity (light gray) is compared to the leakage percentage induced by CSFV-p7 addition to LUV (protein-to-lipid ratio, 1:250) (dark gray). Right: Representative conductance recordings for the same conditions. Reprinted with permission from Largo et al. (2016). (*d*) Atomic force microscopic images in ER-like lipid bilayers at pH 7.4 in control conditions (left) and in the presence of CSFV p7 at pH 7.4 (center) and pH 5.0 (right). CFSV p7:lipid ratio was 1:800. The color bar indicates the height in the *z* dimension, being white the highest and black the lowest area. Reprinted with permission from Largo et al. (2021).

or the pore–channel dualism coined by Fisher in analogy to the particle–wave dualism of light (Mehnert et al., 2008). Interestingly, a recent publication directly raises the question whether how many and which SARS-CoV-2 viroporins are really ion channels (Harrison et al., 2022). The criteria that potential candidates must fulfill to be *bona fide* ion channels include the production of a robust conductance that can be associated with characteristic single-channel currents, display high ion selectivity and specific pharmacology (blocking), and finally, identify mutations that alter ion-channel function (Table 1). Negative or unclear answers to some of these questions may lead to uncharted territory where proteins form pores without obvious physiological relevance, or, in the event of the emergence of contradictory experimental evidence, a dual nature of viroporins may be assumed (Table 2). We would like to analyze here whether these conditions are actually useful by discussing them in the context of available structural and functional information about two representative viroporins, IAV M2 on one side, and SARS-CoV E protein (either SARS-CoV1 E or SARS-CoV2 E since they are almost identical (Surya et al., 2023) on the other side.

IAV M2 viroporin

IC activity of the *bona fide* IAV M2 channel and its blocking by amantadine/rimantadine are probably the viroporin activity and function inhibition processes that are best characterized on structural grounds (Schnell and Chou, 2008; Stouffer et al., 2008; Acharya et al., 2010; Sharma et al., 2010; Nieva et al., 2012; Scott and Griffin, 2015; Jalily et al., 2020; Lamb, 2020). A line of inquiry into the mechanism of proton conduction was followed by DeGrado et al. utilizing an optimized synthetic peptide that represented the tetramerization, pore-forming domain of M2 (Stouffer et al., 2008). An X-ray structure obtained at pH 6.5 with a 1.65 Å resolution provided insights into a potential M2 'intermediate' state (Acharya et al., 2010) (Figure 3*a*). In the lumen of this M2 pore structure, up to five side-chain layers can be defined, namely, the Val27 valve, Ser31, the His-box (the selectivity filter), the Trp-basket, and Asp44-Arg45; plus three clusters of immobilized water molecules H-bonded to the protein, designated as 'entry', 'bridging', and 'exit'. The comparison with structures previously solved at pHs close to 7.5 (neutral) (Schnell and Chou, 2008) and 5.0 (low pH) (Stouffer et al., 2008) suggested that tilting and bending of the helix N-terminus with respect to the pore axis can constrict the Val27 valve at low pH, whereas helix bundle opening, together with the flipping of Trp side chains in the basket, may result in water access at the C-terminus. The conformational oscillations between 'Open-out', 'Intermediate', and 'Open-in' linked to changes in the protonation state would facilitate H_3O^+ diffusion down the concentration gradient, the overall process being regulated by the rate of deprotonation of the His-box (Acharya et al., 2010).

Availability of crystal structures of M2 subsequently obtained under different experimental conditions enabled the refinement of this mechanism (Thomaston et al., 2015; Thomaston et al., 2017; Thomaston et al., 2019). 'Open-in' structures (also designated as 'Inward-open') were obtained at high (8.0) and low (5.0) pHs in lipid cubic phases with 1.10 Å resolution (Thomaston et al., 2015). These structures revealed that at the low pH, rather than organized in three

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Table 1. Viroporins purporting transport properties of conventional ion channels: relevant examples

	Characteristics	Examples of viroporins[a]
Selectivity	Highly selective and specific: current carried by a particular ionic specie while excluding all the others	IAV M2 (H^+) (Pinto et al., 1997; Lin and Schroeder, 2001); HCV p7 (predicted) (H^+) (Hyser, 2015); Kcv (K^+) (Gazzarrini et al., 2003); HPV16 E5 (H^+) (Wetherill et al., 2012)
Membrane-embedded structure	Protein homooligomer of α-helical bundles	IAV M2 (Pinto et al., 1992); IBV M2/ICV M2 (Hyser, 2015); RSV SH (Kochva et al., 2003); HPV16 E5 (Wetherill et al., 2012)
Conductance	Well-defined unitary conductance	IAV M2 (Tosteson et al., 1994)
Voltage dependence	Voltage dependent: Current rectification, asymmetric conduction	IAV M2 (Tosteson et al., 1994)
Membrane lipid dependence	Ion-channel function is (almost) independent of membrane composition	IAV M2 (Vijayvergiya et al., 2004)
pH gating	Yes	IAV M2 (Lin and Schroeder, 2001); HCV p7 (Hyser, 2015); HPV16 E5 (Wetherill et al., 2012); SARS-CoV1 E (predicted) (Antonides et al., 2022)
Calcium conduction	Specific Ca^{2+} conduction excluding any other ion	SARS-CoV1 E (predicted) (Antonides et al., 2022); coxsackievirus 2B (predicted) (de Jong et al., 2006); JCV Agnoprotein (predicted) (Suzuki et al., 2010)
Inhibition	Specific pharmacology: inhibitors	IAV M2 (Cady et al., 2009); hCoV–229 E (Wilson et al., 2006b)
Sequence dependence	Mutations abrogate function	IAV M2 (Balannik et al., 2010); HCV p7 (Wozniak et al., 2010); SARS-CoV1 E (Nieto-Torres et al., 2014); SARS-CoV2 E (Xia et al., 2022)

[a]References added to provide examples, not an exhaustive bibliography.

Table 2. Viroporins displaying features of nonconventional ion channels and pores: relevant examples

	Characteristics	Examples of viroporins[a]
Selectivity	Mildly selective: multi-ionic character. Conduction of K^+, Na^+, Ca^{2+}, H^+, Cl^-, and OH^- without ion specificity	IAV M2 (Tosteson et al., 1994; Mould et al., 2000; Stauffer et al., 2014; Scott et al., 2020); HCV p7 (Premkumar et al., 2004; StGelais et al., 2007; Montserret et al., 2010); SARS-CoV E (Surya et al., 2023); SARS-CoV2 3a (Kern et al., 2021); RV NSP–4 (Pham et al., 2017)
Membrane-embedded structure	Homooligomer, heterooligomer, proteolipidic structures, unknown	HCV p7 (Hyser, 2015); FMDV 2B (Gladue et al., 2018a); NoV NS3 (Wang et al., 2023)
Conductance	Wide range of conductive levels	SARS-CoV1 E (Verdia-Baguena et al., 2021); SARS-CoV2 E (Surya et al., 2023); CSFV p7 (Largo et al., 2021)
Voltage dependence	Voltage-independent: ohmic conduction	HCV p7 (Whitfield et al., 2011); SARS-CoV1 E (Verdia-Baguena et al., 2012); SARS-CoV 3a (McClenaghan et al., 2020)
Membrane lipid dependence	Membrane composition strongly modulates ion-channel function	HCV p7 (Whitfield et al., 2011); SARS-CoV1 E (Verdia-Baguena et al., 2021); SARS-CoV2 E (Surya et al., 2023; Somberg et al., 2024; Volovik et al., 2024)
pH gating	pH modulates open channel conductance without gating	SARS-CoV1 E (Verdia-Baguena et al., 2021); SARS-CoV2 E (Surya et al., 2023); CSFV p7 (Largo et al., 2021)
Calcium conduction	Multi-ionic conduction including Ca^{2+} and multiple other cations and anions	SARS-CoV E; SARS-CoV2 3a (Kern et al., 2021); RV NSP–4 (Pham et al., 2017)
Inhibition	Broad-spectrum inhibitors, unspecific pharmacology	HCV p7 (StGelais et al., 2007); SARS-CoV2 E (Park et al., 2021)
Sequence dependence	Non-functional mutants not found	HCoV–229 E (Liu et al., 2021b)

[a]References added to provide examples, not exhaustive bibliography.

defined clusters, hydrogen-bonded water molecules formed a network along the pore from its entrance until the His-box selectivity filter. In combination with *in silico* simulations, these structures suggested that in the 'Inward-open' state H_3O^+ would channel protons through hydrogen bonds, which would orient in the water network as a function of pH to stabilize the protonation state of gating His37 residues. This mechanism of stabilization of the protonated His37 state received further support from diffraction studies performed at room temperature (Thomaston et al., 2017). In addition, M2 structures solved in the presence of bound adamantanes emphasized the role of interfering with this internal water network in the mechanism of action of these inhibitors (Thomaston et al., 2019).

Only few viroporins display an ionic specificity comparable to that of M2, and even in this paradigmatic case, there is some controversy about the actual nature of the ion conduction in the channel. Thus, some experiments of M2 have shown a large ionic specificity, since H^+ are transported in a ratio 10^5 to 10^6 with respect to of Na^+ and K^+ (Lin and Schroeder, 2001), while other investigations reported mild selectivity to alkali cations with no clear

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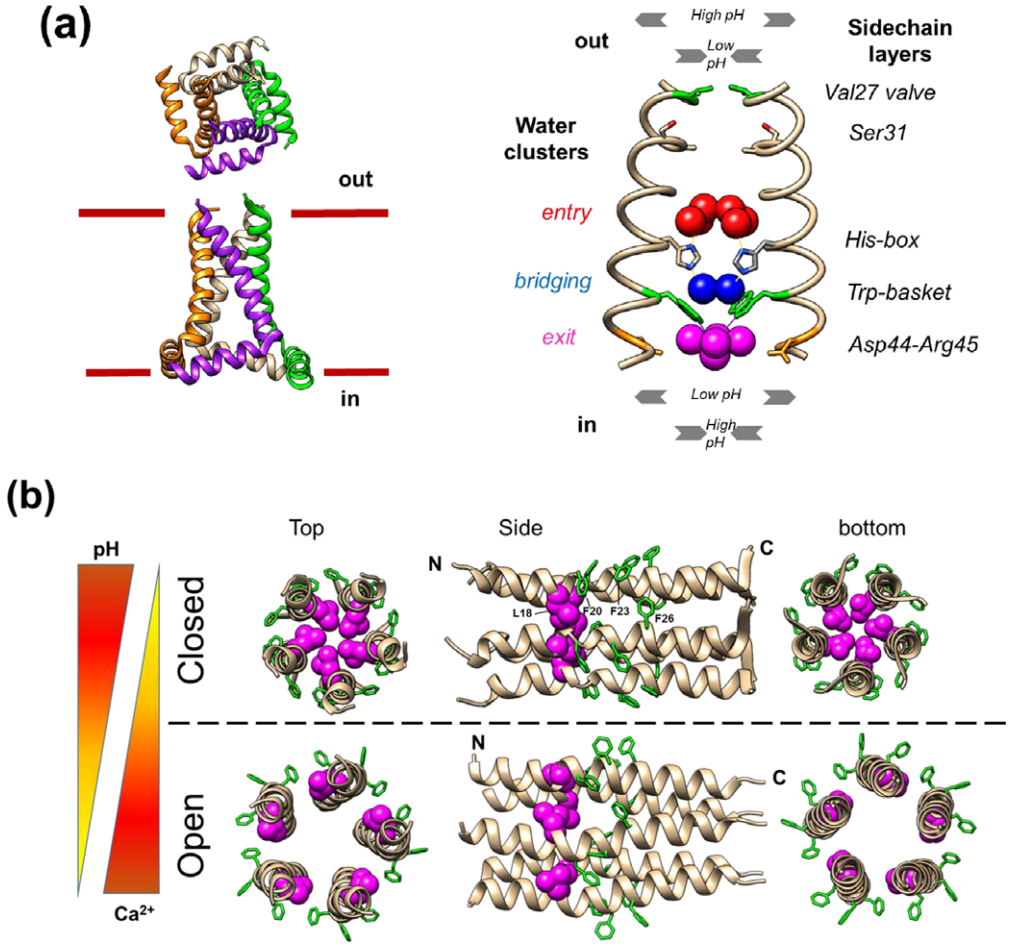

Figure 3. Structural features of IAV M2 and SARS-CoV2 E viroporins. (*a*) Structure of the proton-selective IAV M2. Left: tetrameric bundle structure solved by solid-state NMR spectroscopy in a lipid bilayer (PDB ID: 2L0J). Each monomer includes the transmembrane pore-forming domain (residues 22–46) and an interfacial amphipathic helix (residues 48–58); Right: internal anatomy of the pore-forming domain based on a crystal structure obtained with a resolution of 1.65 Å at the 'intermediate' pH 6.5 (PDB ID: 3LBW). Positions of the side-chain layers and water clusters are indicated. (*b*) Structures of the pore-forming transmembrane domain from SARS-CoV2 E solved by solid-state NMR spectroscopy in ERGIC-like bilayers. Top: a 'closed' state is favored at high pH and low Ca^{2+} concentration (PDB ID: 7K3G). Bottom: 'open' state adopted at low pH and high Ca^{2+} concentration (PDB ID: 8SUZ). Side chains of Leu18 are depicted to illustrate aperture of the pore. Side chains of Phe20, Phe23, and Phe26 undergo conformational changes coupled to the transition. Structure models rendered with Chimera (Pettersen et al., 2004).

specificity (Tosteson et al., 1994; Stauffer et al., 2014) or even permeability to small molecules such as anionic carboxyfluorescecin in liposomes or hygromicyin B in bacteria (Guinea and Carrasco, 1994; Scott et al., 2020; Devantier et al., 2024). Interestingly, such differences have been mechanistically interpreted in terms of a dual function of M2 in which the channel could mediate the efflux of alkali cations necessary to maintain a charge balance across the membrane once protons flow into the virion along their concentration gradient (Tosteson et al., 1994; Stauffer et al., 2014).

SARS-CoV E viroporin

Functional complexity has been associated with SARS-CoV E viroporin too (Surya et al., 2023). The preliminary evidence that the TMD of SARS-CoV1 E protein may form pentamers in gels (Parthasarathy et al., 2008) was subsequently supported by 3D structures derived from NMR spectroscopy of peptides solubilized in lipid micelles (Pervushin et al., 2009; Surya et al., 2018). The COVID-19 pandemic intensified notably research efforts on the structure and function of E as a potential target for antiviral drugs (To et al., 2016; Devantier et al., 2024). The result of this increased research activity was the publication of two high-resolution structures of the SARS-CoV-2 E TMD pore domain in ERGIC-like membranes determined by solid state NMR spectroscopy (Mandala et al., 2020; Medeiros-Silva et al., 2023) (Figure 3*b*). In the initially reported structure (Mandala et al., 2020), the SARS-CoV2 E TMD was modeled as a pentamer, whose structure featured a closed pore displaying Val and Leu interdigitation and an aromatic belt composed of Phe20, Phe23, and Phe26 (Figure 3*b*, top). Since SARS-CoV-1 E and SARS-CoV-2 E are very similar (they share 95% of the sequence and have comparable TMDs, only differing by three residue substitutions and one deletion localized at the C-terminus; Surya et al., 2023), the structural models for both proteins agree in showing quite rigid channels that include very narrow, almost impermeable, sections (approximately 2–3 Å) with no significant lipid involvement in the overall structure. Nonetheless, there are some differences such as minor changes in the N-terminal side and in the tilting of TMD helices and more importantly, the hydrophobic Phe26 is facing the pore lumen in SARS-CoV-1 E (Surya et al., 2018), whereas it is facing the lipid acyl chains in SARS-CoV-2 E (Mandala et al., 2020). For the sake of clarity, it should be mentioned that these structures of SARS-CoV-1 and SARS-CoV-2 E were

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obtained using truncated protein versions that differ in several charged residues outside the TM (Duart et al., 2021). These differences could slightly influence membrane topology and/or channel structure via electrostatic interactions. Anyway, detailed computational analysis confirmed that both structures represent a hydrophobically occluded pore in which the transition from the closed to the open states is not observed throughout the simulations (Yang et al., 2022).

Subsequent work suggested that in response to lower pH or higher Ca^{2+} concentration, the Phe20 and Phe26 residues could reorient their side chains causing the aperture of the pore (Medeiros-Silva et al., 2022; Medeiros-Silva et al., 2023) (see Figure 3*b*, bottom). The ubiquitous presence of both, protons and calcium cations, is a common theme underlying SARS-CoV E research for two reasons. On the one hand, expression of the E protein increases intracellular-Golgi pH (Cabrera-Garcia et al., 2021) and alters Ca^{2+} homeostasis triggering the activation of the NLRP3 inflammasome leading to IL-1β overproduction (Nieto-Torres et al., 2015a). On the other hand, E-induced pentameric structures delineate narrow aqueous pores with a precise architecture that could be potentially suited to yield H^+- and/or Ca^{2+}-activated channels that operate in resemblance to canonical ion channels in neurons (Hille, 2001; Delcour, 2015). Although both pieces of information seem the perfect match for each other, this line of reasoning could be an oversimplification of the actual situation if additional insights are considered.

Thus, experimental evidence obtained for both SARS-CoV-1 E and SARS-CoV-2 E supports that fully functional open channels can be obtained without acidification of the medium or the presence of calcium (Wilson et al., 2004; Parthasarathy et al., 2008; Verdia-Baguena et al., 2021; Surya et al., 2023). Furthermore, although experiments performed with SARS-CoV2 E in (Xia et al. 2021) were consistent with an amplitude and open probability that gradually increased when pH decreased, larger permeabilities were also found for monovalent cations than for divalent ones. In contrast, systematic experiments reported in Verdia-Baguena et al. (2013, 2021)) indicate that both low pH and addition of calcium over salts of monovalent cations substantially decreased the channel conductance of SARS-CoV1 E. Thus, exhaustive electrophysiological characterization of SARS-CoV1 E and SARS-CoV2 E seems to indicate that these channels can transport different types of ions (Na^+, K^+, Ca^{2+}, H^+, and Cl^-) with only mild selectivity and no ion specificity (Wilson et al., 2004; Verdia-Baguena et al., 2013; Verdia-Baguena et al., 2021). In this scenario, we may speculate that E-induced channels would allow multi-ionic transport so that their ion conduction depended on the overall composition of the cellular compartments that they connect (Appenzeller-Herzog and Hauri, 2006) and not on proton and calcium concentration gradients alone. Interestingly, recent studies suggest that SARS-CoV2 E could perturb calcium homeostasis not directly connecting diffusively compartments, but indirectly acting as an exoregulin affecting the SERCA (Berta et al., 2024).

The existence of a complex behavior regarding the mechanism of ion permeation and the role of Ca^{2+} in the SARS-CoV2 E-induced channels is also evident in the computational field. Thus, in contrast to recent models based on NMR spectroscopy data (Medeiros-Silva et al., 2022; Medeiros-Silva et al., 2023), some computational studies identify Leu10 and Phe19 as the hydrophobic gates of the SARS-CoV2 E channel (Cao et al., 2020), showing also that the channel should be impermeable to divalent cations and relatively low permeable to monovalent ones. Interestingly, the collapse of the pentameric NMR structure into a closed configuration, expelling water from interior, has been reproduced by subsequent computational approaches using different membrane compositions, enhanced sampling methods, or extended equilibration with constraints (Mehregan et al., 2022; Cubisino et al., 2024). However, recent molecular dynamics simulations suggest a new arrangement for the SARS-CoV2 E TMD monomers in the pore to effectively conduct ions (Cubisino et al., 2024). Following a similar route to previous studies in which collapsed pore structures in α-helical barrels are turned into conductive ones (Scott et al., 2021), each E TMD monomer was rotated 180° so that the short amphipathic helices faced the interior of the pore and the long hydrophobic helix stretches oriented toward the exterior, in direct contact with the membrane (Cubisino et al., 2024). Despite this new arrangement involved a U-turn in monomers forming the pore, calcium was decisive again acting as an amplifier of the ionic current, being the calculated *in silico* conductance almost two orders of magnitude higher when calcium was added over 50 mM KCl than in pure 150 mM KCl (Cubisino et al., 2024). Again, these computational predictions are at odds with experimental results showing that Ca^{2+} added over KCl acts as a partial blocker and not as an enhancer (Verdia-Baguena et al., 2021), mimicking the anomalous mole fraction effect observed in calcium selective channels and other nonspecific pores (Gillespie et al., 2008).

Up to this point, the discussion of the physiological role of the E protein usually has mainly included pentameric structures. However, a growing number of studies involving different techniques suggest that E-induced oligomerization may be heterogenous and dynamic, including pentamers but also other structures (from monomers or dimers to even decamers; Surya et al., 2023) that are crucially regulated by membrane composition in terms of membrane charge, cholesterol and other constituents (Somberg et al., 2024; Volovik et al., 2024). In particular, electrophysiological recordings of both SARS-CoV1 E with SARS-CoV2 E show a large variability of conducting states (average conductance values are usually obtained via histograms of currents involving large error bars; Verdia-Baguena et al., 2012; Verdia-Baguena et al., 2013; Verdia-Baguena et al., 2021; Surya et al., 2023) whose properties are strongly lipid-dependent (Aguilella et al., 2014). Interestingly, SARS-CoV1 E channels are more conductive in neutral lipids than in charged ones in concentrated solutions (Aguilella et al., 2014), what clashes with purely electrostatic arguments suggesting that charged lipids should induce ion accumulation in the channel mouths and hence increase channel conductance (Queralt-Martin et al., 2018). This emphasizes the importance of protein–lipid interactions and particularly the role of the hydrophobic mismatch in pore formation (Grau-Campistany et al., 2015; Grau-Campistany et al., 2016).

Although the critical influence of lipids on the conductive properties of SARS-CoV1 E with SARS-CoV2 E channels is beyond doubt, and it has been suggested that these pores have proteolipidic structure (Aguilella et al., 2014; Verdia-Baguena et al., 2012), it is unclear what this term implies structurally. In the literature of pore-forming proteins, the term 'proteolipidic' usually refers to toroidal pores where the walls of the channel are formed by both protein monomers and lipid molecules in some sort of intercalated fashion (Gilbert et al., 2014; Cosentino et al., 2016; Vandenabeele et al., 2023). Since none of the resolved pentameric structures of the E protein show significant lipid presence, we may speculate that proteolipidic pores correspond to arrangements involving a low number of monomers (Surya et al., 2023; Volovik et al., 2024), or alternatively, lipid molecules interact strongly with some specific residues (e.g., Phe19, Phe20, or Phe 26) that act as hydrophobic gates of the pore (Cao et al., 2020).

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Overall, it is unknown why high-resolution structural studies mainly report very tight pentameric E channel structures in closed configurations that are allegedly activated by particular stimuli (Ca^{2+} and H^+), whereas most available functional data suggest a variety of E-triggered mildly selective pores in which lipid molecules may exert a tight modulation. We hypothesize that the existence of contrasting evidence could be a manifestation of the protein versatility and its capacity to play different functional roles (the so-called dualism). Such flexibility seems to be obvious in the case of SARS-CoV E protein, but, as discussed previously, appears subtly even in the case of IAV M2.

Indeed, in the general context of viroporin-mediated pathophysiological effects, the existence of dual mechanisms can be understood considering that the successive stages of the virus cycle may involve permeabilization mechanisms of entirely different complexity. Thus, hijacking of the host cell biosynthetic machinery and hindering immune responses during the early stages of viral infection may probably involve highly selective pores (usually narrow and poorly conductive (Aguilella et al., 2011) with specificity for certain ions (H^+ and Ca^{2+}) (Nieva et al., 2012), whereas more conductive and nonselective pores may be more suitable for later steps where indiscriminate membrane disruption and cell death occur (Nieto-Torres et al., 2014; Largo et al., 2021). However, the dualism may also express the general concern in the field of pore-forming proteins about the physiological relevance of some results obtained either in cell culture experiments (where the protein under scrutiny may inadvertently coexist with other native pore-forming proteins) or after applying *in vitro* techniques that may not reproduce the actual membrane environment favoring the pore assembly (Cosentino et al., 2016). It has been also argued that the use of unrealistic high mole fractions of proteins in experiments may lead to artifactual membrane defects that allow polar molecules to cross the membrane nonspecifically (Delcour, 2015; Perera et al., 2016).

Functional classification of viroporins: conventional versus unconventional ion channels

In an effort to overcome the confusion created when a given viroporin shows both traits corresponding to canonical *bona fide* ion channels and other characteristics suggesting unclear regulation, we have attempted a classification of viroporins taking into account the functional data available in the literature (Figure 4 and Tables 1 and 2). We adopted the terminology conventional/unconventional ion channels suggested by A. Delcour, remarking that the notion of ion channel is too restrictive when refers only to the conventional voltage-dependent channels of neurons (and similar ones), showing well-defined single channel conductance, a highly selectivity for particular ions, tightly gated by certain stimuli (ligands and/or voltage) and typically constructed as oligomers of α-helical segments ('barrel staves') forming a narrow aqueous pore (Delcour, 2015) (notably, these characteristics match the requirements commented above in Harrison et al., 2022, discussing whether certain viroporins are really ion channels). As we will discuss later on this section, many well-known pore-forming entities (i.e., bacterial and mitochondrial porins, toxins, and connexins) that share common traits with most viroporins do not meet all these criteria, so that all them can be grouped under the notion of unconventional ion channels (Delcour, 2015; Hyser and Estes, 2015; Syrjanen et al., 2021) rather than being in a gray zone of non-reproducible pores like those created by detergent-like mechanisms (Shai, 1999).

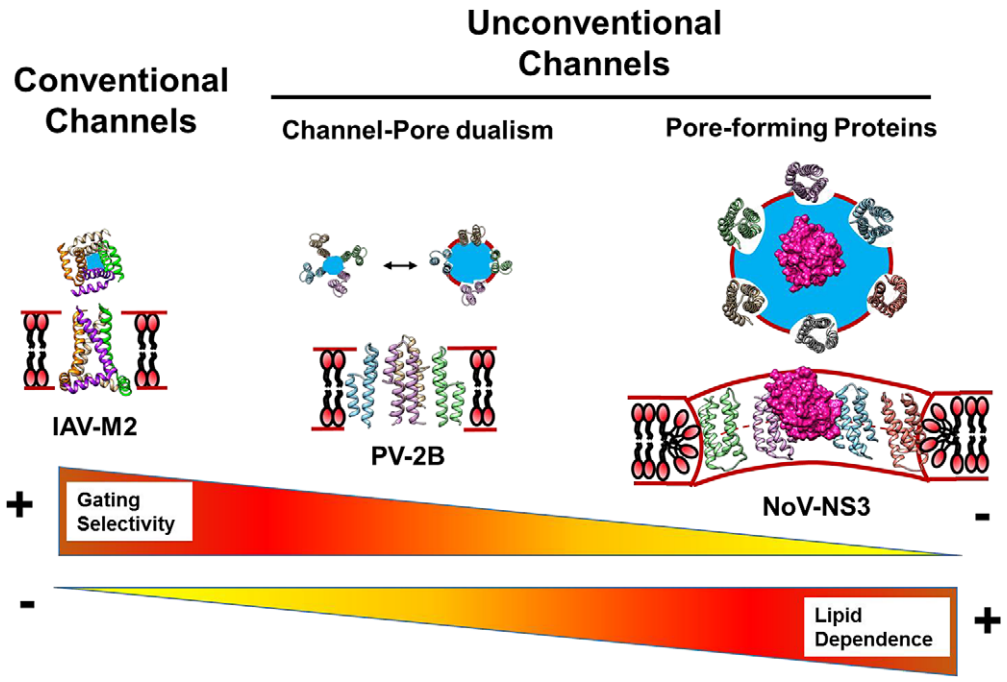

Figure 4. Mechanisms of membrane permeabilization by viroporins. Class IA IAV M2 exemplifies the case of a conventional channel, operated by the pH gradient and selective for protons. The structure of the conducting pore is stabilized through interactions of the helix bundle with the surrounding membrane lipids (model based on structure with PDB ID: 2L0J). Class IIB PV 2B, or more generally the 2B protein of enteroviruses, portraits features of a conventional channel that conducts Ca^{2+}, but also behaves as a pore allowing free diffusion of solutes below approximately 1,000 Da, whose aperture seems to depend on anionic phospholipids bearing long, unsaturated acyl chains. 3D structure of PV 2B hairpin transmembrane domain derived from Alpha-Fold (Senior et al., 2020). The more complex pore-forming domain of NoV NS3 assemble pores in the outer mitochondrial membrane that allow leakage of Cytochrome c to the cytosol (monomers based on the PDB ID: 4BTF structure). In analogy with the mitochondrial apoptosis-induced channel, the model proposes the release of the protein (depicted in magenta) through toroidal proteolipidic megapores that depend on cardiolipin (Vandenabeele et al., 2023).

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Figure 4 depicts a gradient that goes from conventional channels to unconventional ones as regards gating, selectivity, and lipid implication in the pore structure of viroporins. Our figure suggests that because of their dual character, most viroporins occupy a continuous portion of the spectrum, rather than a well-defined position in it that would allow a more precise classification. At one end of the spectrum, we have placed IAV M2, the paradigm of *bona fide* ion channel regarding the specificity of the transported ion, the operation of a gating mechanism, and the adoption of robust, functional structures irrespective of the lipid matrix. At the other end, we propose NoV NS3 as an example of viroporin displaying the capacity to generate macropores (toroidal pores), in this case within the outer membrane of mitochondria, which, following a mechanism akin to that displayed by proteins of the BAX/BAK/BOK family (Vandenabeele et al., 2023), includes lipids of high curvature. Somewhere in the middle, we have placed PV 2B or, more generally, non-structural 2B proteins from picornaviruses, which are simultaneously described as ion channels displaying specificity for the transported ion (i.e., Ca^{2+}), or pores with a size cutoff of approximately 1,000 Da for the permeant solutes.

Tables 1 and 2 show the characteristic features of conventional and unconventional channels, respectively, with some viroporins that match one or the other category. The characterization of these tables aims to provide relevant examples to understand the concepts rather than attempting an exhaustive classification, in this sense some excellent reviews are available (Hyser and Estes, 2015; To et al., 2016; Devantier et al., 2024). From the point of view of transport phenomena, differences between highly selective and specific channels like M2 and other weakly selective viroporins such as SARS-CoV2 E lie on the compromise between two complementary properties, permeability (how many molecules flow through the system and how fast they do it) and selectivity (how a desired molecule is separated from the rest) (Hille, 2001; Aguilella et al., 2011). However, this balance cannot be understood only in terms of pore size: the ability to display specific selectivity for a particular specie requires highly sophisticated structure-based physicochemical mechanisms. For example, aquaporins that allow flux of water molecules in cells while being impermeable to protons (Agre et al., 1993) operate by a combination of hydrophobic effects and precise steric restraints (Hub and De Groot, 2008). Also, in potassium channels, coordination of the protein channel carbonyl group with unsolvated permeant ions is essential to achieve 1,000-fold K^+/Na^+ selectivity (Noskov et al., 2004). Since the majority of viroporins are relatively small proteins with almost no conserved motifs characteristic of specific canonical channels (with the exception of the chlorovirus Kcv potassium channel; Gazzarrini et al., 2003), one could expect a very limited amount of specific viroporin ion channels. As seen in Table 1, only few examples are available apart from Kcv, all of them specific to protons such as IAV M2 and HPV16 E5, configuring pH-gated channels. These sophisticated structures that allow specificity are well-defined oligomeric protein structures where lipid molecules have no significant presence (Devantier et al., 2024; Hyser, 2015). Accordingly, membrane composition has limited impact on viroporin performance as ion channel (see Table 1). Also, the existence of critical conserved motifs in the amino acid sequence allows to identify both mutations inhibiting the channel function and a specific pharmacology in terms of channel blocker (see Table 1). However, even in these conventional channels, contradictory information appears, such as IAV M2 conducting alkali cations (Tosteson et al., 1994; Stauffer et al., 2014), or the alleged behavior of HCV p7 as a proton channel (given the presence of a highly conserved motif similar to the M2 selectivity filter; Hyser, 2015), whereas conduction of monovalent and divalent cations is observed (Premkumar et al., 2004; Montserret et al., 2010) (see Table 2).

Accordingly, having a narrow pore is a necessary but not sufficient condition to observe specific ion selectivity, extremely precise structural arrangements are necessary. This idea is crucial to understand why tireless structure-based and computational studies trying to turn viroporins into calcium-specific ion channels find extreme difficulties: viroporins described up to date can conduct calcium together with other ions but not while excluding any other ions as canonical calcium channels do (Wu et al., 2016; see Table 2) (see also Hyser and Estes, 2015; Verdia-Baguena et al., 2021). As mentioned above, a relevant example in this sense are molecular dynamic simulations trying to turn closed structures of SARS-CoV E into calcium-activated channels (Cubisino et al., 2024), where it is acknowledged that calcium conducting mechanisms do not present any regularity compared to the Ca^{2+} specific selectivity of calcium channels (Liu et al., 2021a).

In contrast to specialized channels such as IAV M2, chlorovirus Kcv, and HPV16 E5, other viroporins can display high conductive levels, weak ionic selectivity, permeability to small solutes and ohmic (not rectifying) conduction (see Table 2). Such traits usually involve pore diameters that can be significantly larger than for conventional channels so that they have been named 'large-pore channels' (Syrjanen et al., 2021) in a dramatic twist of the channel/pore dichotomy. Their function can be described embracing the notion of unconventional ion channels, meaning that their regulatory mechanisms can be unusually complex compared to the conventional channels, involving multiple bioelectrochemical stimuli (diverse salt ions, membrane constituents, small solutes, solution pH, metabolites, drugs, etc.) that operate together in a concerted manner coupled by electrochemical gradients and electroneutrality requirements. Within this line of reasoning, the large heterogeneity in the conductive levels and weak selectivity found in most studies of viroporins (see Table 2) may not arise from failed attempts to achieve a 'canonical' homo-oligomeric conformation, but are probably linked to the capacity of viroporins to form a variety of hetero-oligomeric structures with flexible architectures in which even membrane lipids may be involved (Verdia-Baguena et al., 2013; Cosentino et al., 2016). Well-characterized examples of viroporins support this structural heterogeneity, for example, HCV p7, which has been observed to form hexamers and heptamers (Clarke et al., 2006; Ouyang et al., 2013).

Likewise, the absence of a marked ion discrimination capacity in relatively highly conductive structures could be reasonably associated with the physiological role of facilitating the exchange of small solutes, including metabolites, between different cellular compartments. Relevant examples of such unconventional behavior can be found in maltoporin (LamB), a maltodextrin transport-channel in which the static (different levels) and dynamic (variations with time) disorder found in ion conductance values does not affect sugar binding specificity (Bezrukov et al., 2000; Kullman et al., 2006). Also, bacterial porins, mitochondrial channels, and connexin gap junctions that show weak ionic selectivity may display sophisticated specificity for antibiotics and small metabolites like ATP (Rostovtseva et al., 2002; Danelon et al., 2006; Alcaraz et al., 2009; Syrjanen et al., 2021).

One of the factors that blurs the distinction between conventional and unconventional channels is understanding how selected mutations abrogate channel function. In canonical viroporins, nonfunctional mutants arise from the existence of a well-defined structure and a detailed knowledge of the mechanism of pore function.

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For instance, in IAV M2, the identification of H37xxxW41 motif as responsible for its gating and proton selectivity is used to engineer non-functional mutants (Balannik et al., 2010). Non-functional mutants have also been found in the case of SARS-CoV1 E and SARS-CoV2 E (Surya et al., 2023; Verdia-Baguena et al., 2012). However, quite in contrast to IAV M2, it is intriguing how profound is the impact that single mutations such as N15A and V25F exert on the activity of these viroporins, particularly when having in mind the versatility of the CoV E protein (Verdia-Baguena et al., 2021; Surya et al., 2023) and the ability of lipid molecules to stabilize different proteolipidic conformations in the bilayer (Grau-Campistany et al., 2016; Largo et al., 2016; Perini et al., 2022). Of note, it must be pointed out that problems in the rationalization of functional experiments with mutants involving amino acid replacements may have an origin in effects induced by collateral impacts on protein expression and/or localization and membrane insertion. Last but not least, Tables 1 and 2 also reflect the sharp contrast between the well-defined specific pharmacology (see Devantier et al., 2024, for extensive details) of conventional channels like IAV M2 (Cady et al., 2009) and the use of broad-spectrum channel inhibitors like in unconventional ones SARS-CoV1 E and SARS-CoV 3a (Nieto-Torres et al., 2015b). To date, the pharmaceutical approach to viroporins has targeted the direct blocking of channel activity or the disruption of protein oligomerization. In most cases, this has been achieved with generic inhibitors that show low potency and a variety of unwanted (or unknown) adverse effects. In this sense, remarkable efforts to develop viroporin-targeting drugs, are described in a recent review (Devantier et al., 2024).

Concluding remarks

From the widespread use of the term 'viroporin' in the literature, we may conclude that this field of research is already firmly established among virologists, molecular biologists, and biophysicists studying the pathophysiological effects of the cell membrane permeability phenomena that occur during viral infections. We anticipate the establishment of two big categories of viral proteins implicated in those processes: integral membrane mini-proteins, which are grouped so far as viroporins belonging to class I or II, and larger products that adopt more complex folds in membranes still awaiting a systematic classification. As the approaches to study the structure and function of viroporins increase in number and complexity, concerns regarding the reliability of the experimental results still persist in the area. At any rate, a combination of systems (e.g., testing effects of expressed proteins in cells and of pure specimens in model membranes) and techniques (e.g., monitoring liposome permeability changes and ion-channel activity measured in PLBs) appears to be advisable to circumvent experimental uncertainties arising in this research field.

In relation to the permeation mechanisms, we intended to emphasize that most viroporins display the ability to form multiple ion-conducting structures that are strongly regulated by the environment as regards channel gating, selectivity, and lipid implication in the pore structure. A good part of the existing literature categorizes these findings in two extremes: *bona fide* ion channels with well-defined function (proton or calcium channels) versus unregulated pores that may be either artifactual or anecdotical. Our approach suggests that most viroporins are versatile enough to act in a dual channel/pore fashion occupying an intermediate position between these two extremes. Therefore, transport properties of viroporins can be understood in the context of unconventional ion channels such as bacterial or mitochondrial porins, toxins, or gap junctions that are probably designed, not only to regulate ionic transport, but also to respond to diverse cellular stimuli acting as second messengers in the permeation of larger molecules such small solutes or metabolites.

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