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The role of the SARS-CoV-2 envelope protein as a pH-dependent cation channel

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A novel human coronavirus known as severe acute respiratory syndrome (SARS)-coronavirus (CoV) 2 was isolated in Wuhan (China), and there followed an epidemic outbreak that started in December 2019. Coronaviruses include four main structural proteins: nucleocapsid protein (N), spike protein (S), membrane protein (M) and envelope protein (E) (McClenaghan *et al*. 2020).

SARS-CoV-2 E protein is a small integral membrane protein that has three main domains: the hydrophilic N-terminal domain, the hydrophobic transmembrane domain and the hydrophilic C-terminal region. Several studies have emphasized the importance of the hydrophobic transmembrane domain, which self-interacts to form a pentameric ion pore that has shown little or no ion selectivity. Thus it might act as a viroporin during virus infection and/or release (Sarkar & Saha, 2020). Topological analysis of SARS-CoV-2 E protein has shown that the N-terminus is localized within intracellular organelles, whereas the C-terminus is localized in the cytoplasm.

The E protein of SARS-CoV-1 has properties consistent with ion channel activity, and importantly, it shares 95% sequence identity with SARS-CoV-2 E. Together with functional studies of SARS-CoV-2 E in bacteria and in bilayer recordings, this information inspired Cabrera-Garcia *et al*. (2021), in a recent study published in *The Journal of Physiology*, to propose that SARS-CoV-2 E protein forms a cation channel.

In the study by Cabrera-Garcia *et al*. (2021), an innovative strategy was used to express SARS-CoV-2 E protein in mammalian NIH 3T3 and HEK 293S cells, which allowed the characterization of its ion channel activity. They synthesized and subcloned three constructs in a pcDNA3 vector: (i) a wild-type (WT) construct, which encoded E protein targeted to the intracellular site (i.e. SARS2-E-mKate2); (ii) a plasma membrane (PM) construct, which encoded E protein targeted to the PM (i.e. SARS2-E-Ala6-ΔPBM-PM-mKate2); and (iii) an untagged PM construct that lacked the fluorescent tag, mKate2 (i.e. SARS2-E-Ala6-ΔPBM-PM). The NIH 3T3 cells were transfected with the WT (SARS2-E-mKate2) construct, and then imaged with inclusion of the fluorescent pH-sensitive indicator DND-189, to determine whether intracellular expression of E protein affected the pH of the internal organelles. With increased E protein expression, they reported a time-dependent increase in the internal pH of intracellular organelles, including in particular lysosomes and the endoplasmic reticulum–Golgi intermediate compartment (ERGIC). Furthermore, membrane currents were measured for HEK 293S cells expressing the WT and PM constructs using whole-cell patch clamp to investigate E protein pore-forming activity. As expected, larger currents were obtained for the cells transfected with the PM construct, which confirmed that the membrane currents were associated with PM expression of E protein. The expression of the PM construct was also confirmed by measurements of membrane capacitance. HEK293S cells transfected with the PM construct had greater mean membrane capacitance and inward and outward current amplitudes. The normalized current density that was contributed by E protein was approximately ±1 pA/pF.

Electrophysiological measurements were also carried out with HEK 293S cells transfected with the PM construct to determine the pH-dependent activity of expression of E protein as an ion channel. Here, they looked at changes in extracellular pH, which confirmed the voltage dependence of the ion current through ion channels formed by E protein (i.e. the E current). The E current was approximately linear at pH 7.4, whereas at pH 6.0 it showed inward rectification. At pH 8.0 the E current was reduced over a range of holding potentials.

Changes in the intracellular and extracellular solutions facilitated the study of the selectivity of the ion channels formed by E protein. Na^+ and K^+ passed through these ion channels, while Cl^- showed little ion channel permeation, and the bulky *N*-methyl-D-glucamine$^+$ cation did not permeate through the channel. These observations led Cabrera-Garcia *et al*. (2021) to conclude that E protein forms an ion channel that is permeable to small monovalent cations, including Na^+ and K^+.

In addition, they performed two-electrode voltage clamp current recordings in *Xenopus laevis* oocytes injected with cRNA encoding WT or untagged PM E protein constructs. At pH 7.5, PM E protein produced linear currents over the entire voltage range examined (−100 to 70 mV). Importantly, the current amplitude increased with the amount of PM cRNA injected. Compared to PM E protein, WT E protein did not produce significant currents, as expected. After changing the pH of the bath solution, the mean peak current observed at pH 6.0 was greater than that observed at pH 9.0, whereas no significant currents were observed at pH 6.0 in non-injected oocytes.

E protein has been implicated in various stages of the coronavirus life cycle, including virus assembly, budding, envelope formation and release. It interacts with other viral and cellular proteins, and has several molecular functions, including its putative role as an ion channel (McClenaghan *et al*. 2020; Singh Tomar & Arkin, 2020). Initially, studies provided structural insights into the putative mechanism of action of E protein as a conformation-dependent ion channel that is permeable to H^+ ions. Now these WT and PM constructs designed by Cabrera-Garcia *et al*. (2021) have enabled functional investigation of E protein ion channel activity, and its influence on intracellular proton homeostasis.

Recent studies have shown that only a small proportion of E protein that is expressed during SARS-CoV-2 infection is incorporated into the virion envelope. The rest is localized in the ER–Golgi complex region, and in particular, in the

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ERGIC of human cells (McClenaghan *et al.* 2020; Sarkar & Saha, 2020). Here, the transmembrane domain of E protein oligomerizes and forms a pentameric ion-conducting hydrophilic pore, or viroporin, similar to E protein of some other coronaviruses, such as those that cause SARS and Middle East respiratory syndrome (Sarkar & Saha, 2020). While also confirming the results of previous studies, Cabrera-Garcia *et al.* (2021) demonstrated: (i) perinuclear (ERGIC) localization of WT SARS-CoV-2 E protein; (ii) regulation of the luminal pH of intracellular organelles by SARS-CoV-2 E protein; and (iii) regulation of SARS-CoV-2 E protein ion channel activity through changes in proton concentrations. E protein forms cation channels that are permeable to Na^+ and K^+ (Cabrera-Garcia *et al.* 2021). In coronaviruses, channel formation by E protein has been shown to be critical for infectivity and pathogenicity (Singh Tomar & Arkin, 2020).

The importance of E protein in infectivity and pathogenicity is also shown by the proposal that attenuated viruses lacking E protein can be used as vaccine candidates. According to *in silico* studies and pH imaging, E protein ion channels are permeable to H^+, although this could not be confirmed directly by the electrophysiological measurements of Cabrera-Garcia *et al.* (2021) due to the resolution limits of the recording technique. For this reason, there is also the possibility of using improved techniques to allow detection of such small currents. However, all of these findings taken together suggest that SARS-CoV-2 E protein has a similar effect on intracellular proton homeostasis and ion gradients across organelle membranes to some of the other coronavirus viroporins. The consequence of these changes is facilitated viral assembly and propagation, through increased S protein stability in infected cells, with more severe pathogenesis also being seen due to disruption of cellular homeostasis.

Data from several studies have suggested that the viroporin channel activity of SARS-CoV-1 E protein that occurs in a pentameric form has an essential role in viral pathogenesis and in the development of the characteristic signs of acute respiratory distress syndrome. Further, mutation of the hydrophobic valine 25 residue to phenylalanine in the transmembrane domain of SARS-CoV-1 E protein can completely abolish the formation of an oligomeric structure, while mutation of asparagine 15 to alanine can reduce the probability of pentamer formation (Das *et al.* 2021).

Studies have shown that the transmembrane domain of SARS-CoV-2 E protein, which is responsible for its oligomerization, has a completely conserved amino-acid sequence (residues 15–37 of E protein) compared to that of SARS-CoV-1 E protein. Targeted inhibition of the transmembrane domain of SARS-CoV-2 E protein, and in particular, block of the amino acids involved in its oligomerization, might inhibit pentamer formation, and thence the pathogenic effects of SARS-CoV-2. Indeed, drug screening using various computational techniques (e.g. artificial-intelligence-based deep learning, pattern recognition) have revealed the potential for the beta-lactam antibiotic nafcilin to bind to the transmembrane domain of SARS-CoV-2 E protein, which will prevent E protein oligomerization (Das *et al.* 2021). Moreover, the pathogenic mechanism of SARS-CoV-2 E protein can be inhibited by block of its ion channel pore, as described for hexamethylene amiloride, amantandine, memantine and gliclazide. According to recent *in silico* studies, this effect has also been described for tretinoin and some other compounds. The techniques described by Cabrera-Garcia *et al.* (2021) can now serve as a good model for E protein expression and *in vitro* drug screening of potential inhibitors and modulators of E protein ion channel activity: high-throughput, automated patch clamp and imaging with fluorescent indicators.

Overall, the study of Cabrera-Garcia and colleagues (2021) has demonstrated that SARS-CoV-2 E protein is an important therapeutic target. Their study provides an innovative and useful model for detailed electrophysiological characterization of this viroporin that also allows screening for compounds with inhibitory activity towards the E protein ion channel. For the future: (i) further insights into the function of the E protein ion channel might be gained by studies of channel gating in combination with mutagenesis; (ii) potential inhibitors or modulators of E protein can be screened *in vitro* and subsequently evaluated for antiviral activity against SARS-CoV-2; and (iii) interactions between SARS-CoV-2 E and S proteins remain to be investigated.

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Additional information

Competing interests

None.

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Sole author.

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Keywords

electrophysiology, envelope protein, ion channel, pathophysiology, SARS-CoV-2

Supporting information

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