



Published in final edited form as:

Virology. 2015 November ; 485: 330–339. doi:10.1016/j.virol.2015.08.010.

SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS E PROTEIN TRANSPORTS CALCIUM IONS AND ACTIVATES THE NLRP3 INFLAMMASOME

Jose L. Nieto-Torres^{1,*}, Carmina Verdiá-Báguena^{2,*}, Jose M. Jimenez-Guardeño¹, Jose A. Regla-Nava¹, Carlos Castaño-Rodríguez¹, Raul Fernandez-Delgado¹, Jaime Torres³, Vicente M. Aguilera^{2,§}, and Luis Enjuanes^{2,§}

¹Department of Molecular and Cell Biology, National Center of Biotechnology (CNB-CSIC), Campus Universidad Autónoma de Madrid, 28049 Madrid, Spain

²Department of Physics, Laboratory of Molecular Biophysics. Universitat Jaume I, 12071 Castellón, Spain

³School of Biological Sciences, Division of Structural and Computational Biology, Nanyang Technological University, Singapore 637551, Singapore

Abstract

Severe acute respiratory syndrome coronavirus (SARS-CoV) envelope (E) protein is a viroporin involved in virulence. E protein ion channel (IC) activity is specifically correlated with enhanced pulmonary damage, edema accumulation and death. IL-1 β driven proinflammation is associated with those pathological signatures, however its link to IC activity remains unknown. In this report, we demonstrate that SARS-CoV E protein forms protein-lipid channels in ERGIC/Golgi membranes that are permeable to calcium ions, a highly relevant feature never reported before. Calcium ions together with pH modulated E protein pore charge and selectivity. Interestingly, E protein IC activity boosted the activation of the NLRP3 inflammasome, leading to IL-1 β overproduction. Calcium transport through the E protein IC was the main trigger of this process. These findings strikingly link SARS-CoV E protein IC induced ionic disturbances at the cell level to immunopathological consequences and disease worsening in the infected organism.

Keywords

SARS-CoV; coronavirus; E protein; ion channel; viroporin; calcium; inflammasome; pathogenesis

[§]Corresponding authors, Luis Enjuanes. Department of Molecular and Cell Biology, National Center of Biotechnology (CNB-CSIC), Darwin 3, Campus Universidad Autónoma de Madrid, 28049 Madrid, Spain. Phone: +34-91-585 4555 L.Enjuanes@cnb.csic.es, Vicente M. Aguilera. Department of Physics, Laboratory of Molecular Biophysics, Universitat Jaume I, Av. Sos Baynat, s/n, 12071 Castellón, Spain. Phone: +34-96-472 8045 aguilell@uji.es.

*Both authors equally contributed to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Coronaviruses (CoVs) cause respiratory diseases in humans ranging from common colds to fatal pneumonias (Graham et al., 2013; Perlman and Netland, 2009). At the end of 2002, the etiological agent of severe acute respiratory syndrome (SARS-CoV) emerged in Guangdong province, Southeast China, initiating a global epidemic. Approximately 8000 people were infected by the virus, whose severe disease resulted in a 10% mortality rate (Drosten et al., 2003; Rota et al., 2003). The SARS-CoV epidemic was controlled by the summer of 2003, and the virus has not naturally reemerged since (<http://www.who.int>). However, in 2012 a closely related CoV appeared in Saudi Arabia causing the Middle East respiratory syndrome (MERS-CoV) (Zaki et al., 2012). MERS-CoV induces acute pneumonia similar to that caused by SARS-CoV, and is sometimes accompanied with renal failure (Danielsson and Catchpole, 2012; Zaki et al., 2012). MERS-CoV is now circulating and spreading throughout the human population, and to date has infected at least 1356 people leading to death in 484 cases. Initial cases were confined to countries of the Arabian Peninsula; however, new extensive infection clusters have been recently reported in other regions such as South Korea (<http://www.who.int>). Furthermore, CoVs similar to those causing SARS, MERS, and many other human and animal diseases have been detected in bat species circulating all over the globe (Annan et al., 2013; Chu et al., 2008; Drexler et al., 2010; Falcon et al., 2011; Muller et al., 2007; Quan et al., 2010). Bats are now considered the natural host for most CoVs, providing a perfect scenario for recombination events among different viral species leading to the emergence of new viruses, able to cross species barriers and cause devastating illness in other animals and humans (Graham et al., 2013). For this reason, development and implementation of broad spectrum treatments and general therapeutic strategies against CoVs are a high priority.

CoVs are enveloped viruses containing the largest positive-sense RNA genomes known, around 30 Kb, which encodes the viral replicase and a set of structural proteins: spike (S), envelope (E) and membrane (M), present in the viral envelope, and nucleocapsid (N) located inside the viral particle (Enjuanes et al., 2008). The S protein interacts with the cellular receptor to trigger viral entry into the host cell (Enjuanes et al., 2008; Wong et al., 2004). The E and M proteins actively participate in viral morphogenesis (de Haan et al., 1999; Lim and Liu, 2001; Nguyen and Hogue, 1997; Ruch and Machamer, 2012), and the N protein coats the viral genome to form a helicoidal nucleocapsid that remains protected within the viral envelope (Narayanan et al., 2000). Depending on the CoV species, there are other accessory proteins, some structural, that are also encoded by the genome. SARS-CoV encodes the highest number of accessory genes (3a, 6, 7a, 7b, 8a, 8b and 9b), which play diverse roles eventually related to pathogenesis (Enjuanes et al., 2008).

To analyze the mechanisms leading to the high virulence of SARS-CoV and MERS-CoV, several mutants affecting their different genes have been generated (Almazan et al., 2013; DeDiego et al., 2007; DeDiego et al., 2014a; Scobey et al., 2013; Yount et al., 2005). Remarkably, deletion of SARS-CoV E gene produced a virus that was attenuated in at least three different animal models and conferred protection against SARS-CoV challenge (DeDiego et al., 2007; DeDiego et al., 2011; DeDiego et al., 2008; Fett et al., 2013; Lamirande et al., 2008; Netland et al., 2010). Small deletions in different domains of the E

protein caused similarly attenuated viruses, and are promising vaccine candidates (Regla-Nava et al., 2015). In a similar approach, a MERS-CoV missing the E gene was generated, resulting in a replication-competent, propagation-defective virus which may also constitute the basis of a safe attenuated vaccine (Almazan et al., 2013).

The CoV E gene encodes a small transmembrane protein highly synthesized during infection (Maeda et al., 2001; Nieto-Torres et al., 2011; Raamsman et al., 2000) that mainly localizes to the Golgi apparatus and the endoplasmic reticulum Golgi apparatus intermediate compartment (ERGIC), where it facilitates virus production and morphogenesis (Cohen et al., 2011; Corse and Machamer, 2002; Maeda et al., 2001; Nal et al., 2005; Venkatagopalan et al., 2015). Notably, when the E protein is present, SARS-CoV overstimulates the NF- κ B inflammatory pathway (DeDiego et al., 2014b) and, through its PDZ-binding motif, interacts with the cellular protein syntenin, triggering p38 MARK activation (Jimenez-Guardeño et al., 2014). These signaling cascades result in exacerbated inflammation and immunopathology. The connection between the E protein and virulence has further encouraged the search for other E protein functions that may trigger disease worsening.

One of the most striking functions displayed by the CoV E protein is ion channel (IC) activity. The CoV E protein self assembles in membranes forming pentameric protein-lipid pores that allow ion transport (Pervushin et al., 2009; Torres et al., 2007; Verdia-Baguena et al., 2012; Wilson et al., 2006; Wilson et al., 2004). Interestingly, lipid head-groups are integral components of the pore and regulate ion conductance and selectivity (Verdia-Baguena et al., 2013; Verdia-Baguena et al., 2012). The SARS-CoV E protein showed a mild preference for cations (Na^+ , K^+) over anions (Cl^-) when reconstituted in membranes mimicking the charge and composition of those of the ERGIC/Golgi. In this scenario, the E protein IC showed no selectivity for any particular cation (Verdia-Baguena et al., 2012), though the relevance and consequences of these IC properties in a cellular context remains unknown.

Alteration of ion, and specially, Ca^{2+} homeostasis in favor of infection has been already demonstrated in several viral systems. To that end a wide range of viruses encode ion conductive proteins similar to E protein, named viroporins (Nieva et al., 2012). Highly pathogenic RNA viruses such as human immunodeficiency virus-1 (HIV-1), hepatitis C virus (HCV), influenza A virus (IAV), picornaviruses and CoVs, encode one or more of these proteins (Nieva et al., 2012). Viroporins participate in several steps of the life cycle and are usually linked with pathogenesis. Whether IC properties could promote pathways leading to disease worsening has been unknown for a long time. Recently, it was described that point mutations that specifically inhibited SARS-CoV E protein IC activity caused attenuation (Nieto-Torres et al., 2014). Mice infected with E protein IC proficient viruses presented extensive disruption of the pulmonary epithelia and edema accumulation (Nieto-Torres et al., 2014). Edema is the major determinant of acute respiratory distress syndrome (ARDS), the pathology induced by SARS-CoV, leading to death (Graham et al., 2013; Hollenhorst et al., 2011; Matthay and Zemans, 2011). Edema and an IL-1 β mediated proinflammatory response was increased in the lung parenchyma when E protein IC activity was present (Nieto-Torres et al., 2014).

IL-1 β is a potent proinflammatory cytokine crucial in resolving infectious processes; however, its overproduction has been correlated with diverse severe inflammatory diseases such as asthma, ARDS, gout, atherosclerosis and Parkinson's (Dinarello, 2009; dos Santos et al., 2012; Martinon et al., 2006; Pugin et al., 1996; Strowig et al., 2012). Organisms tightly control IL-1 β production through macromolecular complexes called inflammasomes, which are mainly expressed in macrophages and dendritic cells although other cell types, such as those of the bronchiolar epithelium, synthesize their components (Ichinohe et al., 2010; Triantafilou and Triantafilou, 2014). One highly studied inflammasome is the nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome, relevant in the pulmonary tissue. This complex is composed of the sensing protein NLRP3, the adaptor component apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and the catalytically inactive procaspase-1 (Elliott and Sutterwala, 2015; Latz et al., 2013). The inflammasome components are synthesized under precise danger stimuli, such as molecular patterns associated to infections. However, a second signal is sequentially needed to induce their assembly, which triggers the inflammasome. This leads to the processing of procaspase-1 into active caspase-1, which cleaves inactive pro-IL-1 β into its mature form IL-1 β that is released to the extracellular media to stimulate proinflammation (Elliott and Sutterwala, 2015; Latz et al., 2013). The presence of ionic imbalances within cells is a main trigger of the NLRP3 inflammasome assembly and activation (Latz et al., 2013). Interestingly, several viroporins stimulate this pathway through alteration of cell ion homeostasis, frequently involving Ca²⁺ imbalances (Ichinohe et al., 2010; Ito et al., 2012; Triantafilou et al., 2013a; Triantafilou and Triantafilou, 2014).

There is clear evidence correlating NLRP3 inflammasomes and IL-1 β driven proinflammatory cascades with worsening of several respiratory diseases, including those caused by viruses (dos Santos et al., 2012; McAuley et al., 2013; Pugin et al., 1996; Triantafilou and Triantafilou, 2014). However, the specific role that viral proteins with IC activity may play in this process has not been fully determined. Overproduction of IL-1 β in the airways of the lungs of mice infected with E protein IC proficient SARS-CoVs, strongly suggested that ion conductivity may be stimulating the inflammasome (Nieto-Torres et al., 2014). Here we demonstrate that SARS-CoV E protein forms a Ca²⁺ permeable channel in ERGIC/Golgi membranes. E protein IC activity alters Ca²⁺ homeostasis within cells boosting the activation of the NLRP3 inflammasome, which leads to the overproduction of IL-1 β . This data supports that SARS-CoV E protein Ca²⁺ channel activity may play a role in disease, through overstimulation of inflammasomes leading to immunopathology. Pharmacological inhibition of this pathway may constitute the basis for combined therapeutics applicable for SARS-CoV and other viruses.

Results

The SARS-CoV E protein forms Ca²⁺ permeable ion channels

Previously, we reported that the SARS-CoV E protein forms channels that are moderately selective for cations in membranes mimicking the ERGIC/Golgi (Verdia-Baguena et al., 2013; Verdia-Baguena et al., 2012). Within cells, the movement of different cations through

the E protein pore should be dictated by their respective gradients. Ca^{2+} possesses the highest asymmetrical distribution between the ER-Golgi lumen (hundred μM) and the cytoplasm (around 100 nM) (Zhou et al., 2009), which should allow the flow of this cation through the E protein IC channel if it is permeable. To test whether this is the case, E protein ICs were first reconstituted in artificial neutral lipid membranes in order to exclude any effect coming from the lipid charge, using 100 mM CaCl_2 solutions. Current jumps corresponding to the assembly of channels in the lipid membrane were detected (Figure 1A). The most frequent current jump, representing the insertion of a single E protein IC in the lipid membrane, displayed an intensity of 7.2 ± 2.4 pA.

Interestingly, values multiple of 7.2 pA were also detected, corresponding to the insertion of two (14.4 pA) or three channels (21.6 pA), reinforcing the previous observation (Figure 1A and 1B). Because lipid charge can affect channel conductance (Verdia-Baguena et al., 2013; Verdia-Baguena et al., 2012), single channel conductance was analyzed in ERGIC/Golgi-like membranes that contained approximately 20% negatively charged lipids. Interestingly, the E protein IC showed a slightly higher unitary current jump of 11 ± 4 pA under these conditions, and multiples of this value, evidencing the insertion of additional channels, were also detected (Figure 1C and D). The presence of the negatively charged lipid head-groups in the channel pore may facilitate Ca^{2+} flow through the channel enhancing current intensity under these conditions. This effect was already reported for E protein and monovalent cations (Verdia-Baguena et al., 2012).

Additional measurements were performed in aqueous solutions with decreasing CaCl_2 concentrations, to approach Ca^{2+} amounts found in the ERGIC/Golgi lumen. IC conductance (G), which is the ratio between the current intensity and the applied voltage, was calculated. Single channel conductance scaled almost linearly with CaCl_2 concentration in neutral membranes (Figure 1E). This reflects that ion conduction inside the E protein IC is similar to the conduction of the solution, which increases as the electrolyte concentration rises. These data indicate that the interaction between the channel and the permeating ions is weak, and are in accordance with E protein forming a neutral pore in these conditions, as previously reported for monovalent cation salts (Verdia-Baguena et al., 2013). In ERGIC/Golgi membranes two different regimes depending on the range of CaCl_2 concentrations were observed. A linear relation between conductance and CaCl_2 concentration was again detected in high concentrated solutions. However, IC conductance in ERGIC/Golgi membranes was independent of CaCl_2 concentration in low concentration solutions (below 0.2 M), and higher than in neutral membranes (Figure 1E). This result suggests that E protein acts as a charged protein-lipid pore in ERGIC/Golgi membranes. In this scenario, the conductance is regulated by the balance between the ions that flow into the channel to neutralize the excess of negative charges and the bulk electrolyte concentration. Collectively, these data indicate that the E protein forms channels that are highly conductive in CaCl_2 . Furthermore, E protein ICs worked very efficiently when reconstituted in ERGIC/Golgi membranes under Ca^{2+} concentrations approaching those found in the lumen of the organelles (hundred μM).

To specifically test E protein selectivity for Ca^{2+} , i.e. the ability of the IC to select Ca^{2+} either by its charge or by its intrinsic properties, reversal potential (Erev) experiments were

performed. The rationale of these experiments is provided next. ICs were reconstituted in lipid membranes that separated two solutions, one with high and the other with low CaCl_2 concentration. This concentration gradient induces the movement of ions through the channel to equilibrate their asymmetric distribution. The potential applied across the channel, which is required to counteract this ion movement leading to zero electric current is the Erev. The Erev can be transformed into channel permeability for Ca^{2+} (PCa^{2+}) and Cl^- (PCI^-) by using the Goldman-Hodgkin-Katz equation (Hodgkin and Katz, 1949). To test whether lipid charge may have an effect on E protein, selectivity measurements were performed in neutral, ERGIC/Golgi similar charged membranes, and fully negative-charged membranes. The E protein IC showed $\text{PCa}^{2+}/\text{PCI}^-$ values of 0.2 (1/5) in neutral membranes. The value of this permeability ratio is barely the same expected for a non-selective neutral pore just reflecting the different diffusivities of Ca^{2+} and Cl^- . Therefore, E protein channel is equally selective for Cl^- and Ca^{2+} in neutral membranes. Interestingly, the ratio $\text{PCa}^{2+}/\text{PCI}^-$ increased when the E protein IC was assembled in membranes containing negatively charged lipids, being 0.5 in ERGIC/Golgi membranes and 1.8 in negative membranes (Figure 2). These data indicated that lipid charge largely influenced the channel preference for Ca^{2+} showing that under conditions mimicking the ERGIC/Golgi environment, E protein displayed a mild selectivity for Ca^{2+} .

Calcium cations and pH modulate E protein channel selectivity

Multivalent ions, such as Ca^{2+} , can modulate ion transport across ICs by interacting with the internal charges of their pores (Alcaraz et al., 2009; Garcia-Gimenez et al., 2012; Queralt-Martin et al., 2011). Therefore we tested whether E protein ion selectivity could be modified by the presence of a range of small CaCl_2 concentrations. Ion selectivity was measured using a ten-fold concentration gradient of KCl at pH 6 upon symmetrical addition of very small CaCl_2 concentrations (mM range), at both sides of the membrane. By applying the Erev, the flow of K^+ and Cl^- ions down their electrochemical potential gradient is prevented. Therefore, Erev measures the relative preference of the channel for K^+ cations over Cl^- anions, but not for Ca^{2+} , because calcium concentrations are the same at both sides of the pore. To analyze the relevance of Ca^{2+} on IC selectivity, this type of experiments were performed in neutral and in negatively-charged membranes (Figure 3A). In charged membranes the addition of small amounts of Ca^{2+} induced significant changes in the channel Erev, whereas in neutral membranes the effect was very small, although still measurable. These results indicate that Ca^{2+} interaction with E protein channel, modifying the effective pore charge, mainly occur with the lipid charges that line the pore, rather than with acidic residues of E protein TM domain.

Previously we reported that pH also modulates E protein channel selectivity by protonation and deprotonation of the titratable residues present in the pore (Verdia-Baguena et al., 2013). The effect of Ca^{2+} and pH on E protein selectivity was simultaneously tested. Erev titration was studied in the presence or absence of 15 mM CaCl_2 under different pH conditions (Figure 3B). In negatively-charged lipid membranes, rising pH induced sequential deprotonation of lipid head-groups and E protein glutamic acid residues as their respective pK_a (1.73 for lipid head-groups and 4.5 for glutamic acid) were overtaken, conferring an excess of negative charges to the channel and making it cation selective. The

addition of Ca^{2+} when E protein is reconstituted in charged membranes reduces the cationic selectivity or increases the anionic selectivity (i.e., it shifts the reversal potential towards less negative values), and reversal potential values become closer to those obtained in neutral membranes. This indicates that Ca^{2+} ions interact with the acidic protein residues and the negative lipid head-groups and decrease the effective negative charge of the E protein pore. This effect was especially patent at physiological values of pH (pH 6-7.5). In addition, this interaction of Ca^{2+} ions with the protein acidic residues shifts their effective pK_a towards lower values. These results indicate that both Ca^{2+} and H^+ ions change E channel transport properties by modifying the charge of the pore, and further support that Ca^{2+} enters within E protein IC.

Identification of SARS-CoV E protein ion channel mutants lacking Ca^{2+} transport

In order to assess the biological impact of SARS-CoV E protein with and without Ca^{2+} channel activity, we evaluated previously constructed mutants containing amino acids substitutions known to affect the E protein IC. Previously we demonstrated that mutations N15A and V25F in the transmembrane domain of SARS-CoV E protein abolished ion conductance in KCl and NaCl solutions (Verdia-Baguena et al., 2012). New conductance measurements were performed in ERGIC/Golgi membranes in 100 mM CaCl_2 solutions (Figure 4). Wildtype E protein transmembrane peptides showed conductance values in the range of 110 pS, whereas no conductance was observed for N15A or V25F mutants, indicating that these mutations also inhibited Ca^{2+} transport, and probably prevent all ion passage as it was previously demonstrated that they also failed to transport K^+ , Na^+ and Cl^- (Verdia-Baguena et al., 2012).

Ca^{2+} transport through SARS-CoV E protein channel activates the NLRP3 inflammasome

Alteration of cellular ion homeostasis by SARS-CoV E protein IC could have several implications. Previously, we linked E protein IC with IL-1 β triggered proinflammation in the lungs of mice, leading to epithelial cell damage and death (Nieto-Torres et al., 2014). Higher levels of mature IL-1 β were detected in the airways of infected animals when E protein IC activity was present, suggesting that ion conductance could stimulate the inflammasome. To determine whether this was the case, the inflammasome complex was reconstituted in Vero E6 cells by transient transfection of its components (NLRP3, ASC and procaspase-1) and the inactive pro-IL-1 β (Lo et al., 2013), in the absence or presence of E protein, with or without IC activity. All the components of the inflammasome, besides the different versions of E protein were efficiently expressed within cells (Figure 5A). When pro-IL-1 β was transfected as a control, no significant level of active IL-1 β was detected in the supernatant. However, when all the components were supplied to the cells, the inflammasome was stimulated, and active IL-1 β was detected in the media in the range of 600 pg/ml (Figure 5B). Interestingly, the production of IL-1 β was significantly enhanced in the presence of E protein with IC activity (IC+) (>800 pg/ml). This stimulation was IC activity dependent, as the E protein mutants lacking ion conductance (N15A and V25F), represented in the figure as EIC⁻¹ and EIC⁻², respectively, did not boost IL-1 β levels (Figure 5B). Stimulation levels found with wildtype E protein were in the range of those previously reported for other inflammasome activating proteins, using this system (Lo et al., 2013).

Imbalances in Ca^{2+} within cells have been described as an inflammasome inducer (Ito et al., 2012; Murakami et al., 2012; Triantafilou et al., 2013b). To test the specific contribution of SARS-CoV E protein Ca^{2+} transport to inflammasome activation, the complex was reconstituted in cells in the presence or absence of E protein. Cells were subsequently treated with the cell permeant calcium chelator BAPTA-AM (Figure 6A). This compound enters cells and binds Ca^{2+} preventing inflammasome activation (Ito et al., 2012). Increasing amounts of BAPTA-AM markedly decreased the levels of secreted IL-1 β in the presence of E protein (Figure 6A, INFL EIC⁺), reaching levels close to those obtained when the inflammasome was assembled alone (Figure 6A, INFL). The Ca^{2+} ionophore ionomycin stimulated active IL-1 β accumulation in the cell supernatant at levels similar to those seen with E protein, further confirming that Ca^{2+} imbalances boost IL-1 β production (Figure 6B). No significant differences in cell viability were found for any of the treatments (Figure 6C). Collectively, these results indicate that SARS-CoV E protein activates the NLRP3 inflammasome through its Ca^{2+} transport ability.

Discussion

Ion conductive proteins are widely distributed among viruses, being especially common in RNA viruses (Nieva et al., 2012). Conductance of ions facilitate diverse processes of the viral life cycle such as entry, takeover of organelles to serve as platforms for viral replication, protection of viral proteins from acidic cell compartments, and trafficking of nascent virions (Nieva et al., 2012). In addition, viroporins are often linked to pathogenesis, and in general, mutant viruses lacking them are attenuated, in many cases serving as effective vaccines (DeDiego et al., 2007; Netland et al., 2010; Watanabe et al., 2009; Whitehead et al., 1999). Viroporin removal is frequently accompanied by a defect in viral production, which by itself can explain virulence attenuation. However, recent studies have indicated that IC activity may specifically trigger pathways leading to pathology. Previously we demonstrated that SARS-CoVs proficient in E protein IC activity caused increased damage of the pulmonary epithelia and edema accumulation (Nieto-Torres et al., 2014). These disease symptoms correlated with an immunopathological response mediated by proinflammatory cytokines such as IL-6, TNF and IL-1 β , the latter being a crucial mediator of this cascade. IL-1 β overproduction is linked to a wide range of inflammatory pathologies including those caused by respiratory viruses (dos Santos et al., 2012; McAuley et al., 2013; Pugin et al., 1996; Triantafilou and Triantafilou, 2014). Here, we report for the first time that CoV E protein formed an IC that transported Ca^{2+} in ERGIC/Golgi membranes, where this protein locates, which may have important consequences on cell physiology. In fact, Ca^{2+} leakage through E protein IC induced the activation of the NLRP3 inflammasome resulting in overproduction of IL-1 β . This finding together with previous *in vivo* observations indicates that IC activity correlates with proinflammation and pathology.

Generally, viroporins form poorly selective ion channels (Nieva et al., 2012). Therefore, the subcellular location where viroporins assemble and the conditions of that particular environment are crucial determinants of their impact on cellular ionic homeostasis. Previously, we reported that SARS-CoV E protein showed mild selectivity for cations (Na^+ and K^+) when reconstituted in ERGIC/Golgi membranes, mostly conferred by the negative charges of the lipids (Verdia-Baguena et al., 2013; Verdia-Baguena et al., 2012). High

concentration gradients are found for Na⁺ and K⁺ between the cell interior and the extracellular media (Dubyak, 2004). However, there is no known asymmetric distribution for either of these ion species between the lumen of ERGIC/Golgi and the cell cytoplasm (Chandra et al., 1991; Schapiro and Grinstein, 2000). Accordingly, minimal net transport of Na⁺ and K⁺ through E protein pore should be expected, and therefore the biological relevance of these processes may be also limited. In contrast, the ER and Golgi apparatus store high amounts of calcium ions by the action of pumps such as the sarcoendoplasmic reticulum Ca²⁺ ATPase (SERCA) and the secretory pathway Ca²⁺ ATPase (SPCA) (Wuytack et al., 2002). This creates an enormous gradient of around 1000-fold between the lumen of these organelles and the cytoplasm (Zhou et al., 2009). The gradient allows controlled eventual and temporal leakages of Ca²⁺ into the cytoplasm that trigger several processes relevant to cell physiology. Interestingly, we have shown above that SARS-CoV E protein IC was also permeable to Ca²⁺ in ERGIC/Golgi membranes. Furthermore, Ca²⁺ interacted with the negative charges of the protein-lipid pore, modulating its properties. Several other viroporins are known to transport Ca²⁺, resulting in leakage of this cation from its intracellular stores. Rotavirus NSP4 protein as well as Coxsackievirus, encephalomyocarditis virus, and poliovirus 2B proteins deplete ER and/or Golgi Ca²⁺ concentrations in favor of viral proliferation (Campanella et al., 2004; Crawford et al., 2012; de Jong et al., 2008). Alteration of protein trafficking, manipulation of apoptosis, and control of autophagy are some of the processes controlled by these Ca²⁺ effluxes. Whether some of these aspects are influenced in a similar manner during SARS-CoV infection will be explored in future experiments.

Protons (H⁺) are also actively confined to the lumen of the Golgi apparatus and those of the organelles of the secretory pathway in a process that acidifies their interior and creates a gradient with the cytoplasm (Paroutis et al., 2004).

Considering that the E protein IC weakly interacts with circulating ions and that pH can modulate its net charge, it is highly likely that protons will also flow through the IC within cells. This is not an isolated case, as several viroporins such as HCV p7 and IAV M2 are known to transport protons, and others such as the 2B protein of the *picornaviridae* family transport both H⁺ and Ca²⁺ (de Jong et al., 2006; Wang et al., 1994; Wozniak et al., 2010). Alkalinization of the Golgi lumen is crucial to protect acid-sensitive viral progeny and prevent premature activation of viral proteins involved in entry processes (Sakaguchi et al., 1996; Wozniak et al., 2010). SARS-CoV E protein IC mutants did not show profound growth defects, although they were outgrown in competition assays by IC proficient viruses, which suggests better proliferation when E protein ion conductance was present (Nieto-Torres et al., 2014). Whether alkalinization of intracellular compartments by E protein IC may assist in SARS-CoV production remains to be explored. Besides these considerations, it cannot be excluded that IC activity may have a greater impact in SARS-CoV production. Inhibition of E protein IC activity may be compensated by the action of two other viroporins encoded by SARS-CoV, the 3a and 8a (Chen et al., 2011; Lu et al., 2006); further experiments are being performed to answer this question.

Disruption of ion homeostasis can have profound deleterious effects for the cells; consequently, they have evolved mechanisms to sense and control these disturbances. Ion

Author Manuscript

Author Manuscript

Author Manuscript

imbalances associated with infecting pathogens are recognized by inflammasomes, which are components of the innate immune system. Inflammasomes orchestrate proinflammatory responses to fight viral infections, IL-1 β being one of the major players (Strowig et al., 2012). However, increasing evidence indicates that overstimulation of this pathway can lead to undesirable effects for the organism. In fact, immunopathology rather than direct viral destruction of infected cells is the main cause of severe disease in many viral illnesses (McAuley et al., 2013; Meduri et al., 1995; Triantafilou and Triantafilou, 2014). SARS-CoVs that lack E protein IC activity induced less proinflammation and active IL-1 β , suggesting that IC activity could be a trigger of this pathway (Nieto-Torres et al., 2014). Indeed, wildtype E protein but not its IC⁻ mutants boosted the production of mature IL-1 β through the mediation of a reconstituted inflammasome. Ca²⁺ was the main trigger of this process, as chelation of this ion abolished enhanced IL-1 β production, and a Ca²⁺ ionophore produced a similar simulation. E proteins from other human respiratory CoVs, such as MERS-CoV and HCoV-229E, also display ion channel properties (Surya et al., 2015; Verdia-Baguena et al., 2012; Wilson et al., 2006). Investigating whether these proteins may favor Ca²⁺ transport and activation of the inflammasome represents a relevant issue. Furthermore, the identification of a possible correlation between the extent of inflammasome activation and the disease symptoms caused by these viruses, highly deleterious in the case of SARS-CoV and MERS-CoV, and mild in the case of HCoV-229E, could provide key information on the impact of this pathway on the pathological outcome induced by different CoVs, and will be analyzed in future experiments.

This study provides new insights into the molecular mechanisms governing CoV viroporin activity and the consequences of this function in viral pathogenesis. What is more, IL-1 β overproduction has been related with the pathology induced by SARS-CoV and other respiratory viruses (Triantafilou and Triantafilou, 2014). Our results suggest that development of specific IC inhibitors and implementation of novel compounds decreasing inflammasome driven immunopathology (Coll et al., 2015) may be a valuable complement to other antiviral approaches for the treatment of these infectious diseases.

Materials and Methods

Cells

The African green monkey kidney-derived Vero E6 cells were kindly provided by Eric Snijder (Medical Center, University of Leiden, The Netherlands). Cells were grown at 37 °C with an atmosphere of 98% humidity and 5% CO₂, in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 25 mM HEPES, 2 mM L-glutamine (SIGMA), 1% non-essential amino acids (SIGMA) and 10% fetal bovine serum (FBS, BioWhittaker).

Peptide synthesis and ion channel measurements in artificial lipid membranes

Synthetic peptides representing the full-length SARS-CoV E protein, or its transmembrane domain (amino acids 7 to 38) containing point mutations that inhibited ion channel activity (N15A and V25F), were generated by standard phase synthesis and purified by HPLC, as previously described (Verdia-Baguena et al., 2012). Ion channels were reconstituted in lipid membranes composed of diphytanoyl phosphatidylcholine (DPhPC), diphytanoyl

phosphatidylserine (DPhPS), or a mixture of 59% dioleoyl phosphatidylcholine (DOPC), 24% dioleoyl phosphatidylethanolamine (DOPE), and 17% dioleoyl phosphatidylserine (DOPS) (Avanti polar lipids, Inc., Alabaster, AL). The membranes separated two chambers filled with electrolytic solutions of CaCl_2 or KCl. Ion channel insertion was achieved by adding 0.5-1 μl of a 300 $\mu\text{g/ml}$ solution of synthetic protein or peptides in a acetonitrile:isopropanol (40:60) buffer to one of the chambers (cis). All measurements were performed at room temperature (23 ± 1 °C).

The single-channel conductance was obtained from current measurements under an applied potential of +100 mV in symmetrical salt solutions of 1 M CaCl_2 buffered with 5 mM HEPES at pH 6. The conductance values were evaluated using the Gaussian fit tool of Sigma Plot 10.0 (Systat Software, Inc).

For the selectivity measurements the reversal potential (E_{rev}) was obtained under a ten-fold salt concentration gradient (500 mM / 50 mM). One or several channels were inserted into the bilayer causing a net ionic current due to the concentration gradient. Then the ionic current was manually set to zero by adjusting the applied potential. The potential needed to achieve zero current represents the E_{rev} ; conversion of E_{rev} into the channel permeability ($P_{\text{+}}/P_{\text{-}}$) was by done using the Goldman-Hodgkin-Katz (GHK) equation (Hodgkin and Katz, 1949). The effect of Ca^{2+} on E protein ion selectivity was defined by measuring E_{rev} in a ten-fold KCl concentration gradient (500 mM / 50 mM) buffered with 5 mM HEPES at pH 6, in the presence of increasing concentrations of CaCl_2 . The functional interaction of Ca^{2+} with E protein-lipid pore was evaluated measuring E_{rev} in DPhPC and DPhPS membranes under different pH values, with or without 15 mM CaCl_2 .

Inflammasome reconstitution in Vero E6 cells

Sub-confluent monolayers of Vero E6 cells seeded onto 24-well plates were transfected using Lipofectamine 2000 (Invitrogen) and a set of plasmids encoding the components of the NLRP3 inflammasome (Lo et al., 2013; Wu et al., 2014): 200 ng of pcDNA4-proIL-1 β , 25 ng of pcDNA4-NLRP3-HA, 20 ng pcDNA4-ASC-mCherry, and 10 ng of pcDNA4-procaspase-1-Myc, with 100 ng of empty pcDNA3, as a control, or 100 ng of either pcDNA3-E, pcDNA3-EN15A or pcDNA3-EV25F. Cell lysates and culture supernatants were collected 24 h after transfection. The secreted active IL-1 β was measured by ELISA according to the manufacturer's specifications (eBioscience).

Cell protein extract preparation and western blot assays

Cells were lysed in a buffer containing Tris/HCl 10 mM, EDTA 1mM, NaCl 150 mM, IGEPAL 1%, and complete protease inhibitor (Roche) at pH 8. Inflammasome proteins were detected by western blot using a mouse anti-HA antibody (Sigma) to detect NLRP3-HA, mouse anti-mCherry (Abcam) to bind ASC-mCherry, rabbit anti-Myc (Abcam) to label procaspase-1-Myc, and rabbit anti-human IL-1 β (Cell signaling). SARS-CoV E protein was detected using a polyclonal antiserum produced in rabbits (Nieto-Torres et al., 2011). As a loading control, beta-actin was labeled using a mouse monoclonal antibody (Abcam). Bound antibodies were detected using HRP-conjugated goat anti-rabbit IgG and rabbit anti-mouse

IgG and the Immobilon Western chemiluminescence substrate (Millipore), following manufacturer's specifications.

Effect of BAPTA-AM and ionomycin treatments on inflammasome activation

Fresh supplemented DMEM containing the indicated concentration of the cell permeant calcium chelator BAPTA-AM (Life technologies) was added to cells 4 h after transfection of the inflammasome components and incubated 30 min at 37 °C. Then, media was removed and a second treatment with fresh media containing BAPTA-AM was performed. Supernatants were collected 20 h post BAPTA-AM treatment. Ionomycin (Life technologies) was added at 1 μM 4 h after transfection and incubated for 20 h.

Cell viability assay

The MTT colorimetric method was used to measure cell metabolic activity. Cell media of transfected cells was replaced by fresh DMEM supplemented with 10% FCS and 500 μg/ml of MTT. Cells were incubated 2 h at 37 °C. After that, media was removed and a solution containing 0.04 M HCl 0.1% Nonidet P-40 in isopropanol was added. Plates were gently mixed to obtain a homogeneous solution, and optic density was measured at 570 nm.

Acknowledgments

The work done by the authors was supported by grants from the government of Spain (BIO2013-42869-R, FIS2013-40473-P), Generalitat Valenciana (Prometeo 2012/069), Fundació Caixa Castelló-Bancaixa (P1-1B2012-03) and a U.S. National Institutes of Health (NIH) project (5P01AI060699). JLN, JMJ and JAR received contracts from NIH. CCR received a contract from Fundacion La Caixa. We thank professor Ming-Zong Lai (Institute of Molecular Biology, Taipei) for kindly providing us plasmids encoding the inflammasome components. We thank Marga Gonzalez for her technical assistance.

References

- Alcaraz A, Nestorovich EM, Lopez ML, Garcia-Gimenez E, Bezrukov SM, Aguilera VM. Diffusion, exclusion, and specific binding in a large channel: a study of OmpF selectivity inversion. *Biophys J*. 2009; 96:56–66. [PubMed: 19134471]
- Almazan F, DeDiego ML, Sola I, Zuñiga S, Nieto-Torres JL, Marquez-Jurado S, Andres G, Enjuanes L. Engineering a replication-competent, propagation-defective Middle East respiratory syndrome coronavirus as a vaccine candidate. *mBio*. 2013; 4:e00650–00613. [PubMed: 24023385]
- Annan A, Baldwin HJ, Corman VM, Klose SM, Owusu M, Nkrumah EE, Badu EK, Anti P, Agbenyega O, Meyer B, Oppong S, Sarkodie YA, Kalko EK, Lina PH, Godlevska EV, Reusken C, Seebens A, Gloza-Rausch F, Vallo P, Tschapka M, Drosten C, Drexler JF. Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. *Emerg Infect Dis*. 2013; 19:456–459. [PubMed: 23622767]
- Campanella M, de Jong AS, Lanke KW, Melchers WJ, Willems PH, Pinton P, Rizzuto R, van Kuppeveld FJ. The coxsackievirus 2B protein suppresses apoptotic host cell responses by manipulating intracellular Ca²⁺ homeostasis. *J Biol Chem*. 2004; 279:18440–18450. [PubMed: 14976205]
- Chandra S, Kable EP, Morrison GH, Webb WW. Calcium sequestration in the Golgi apparatus of cultured mammalian cells revealed by laser scanning confocal microscopy and ion microscopy. *J Cell Sci*. 1991; 100:747–752. [PubMed: 1814929]
- Chen CC, Kruger J, Sramala I, Hsu HJ, Henklein P, Chen YM, Fischer WB. ORF8a of SARS-CoV forms an ion channel: experiments and molecular dynamics simulations. *Biochim Biophys Acta*. 2011; 1808:572–579. [PubMed: 20708597]

- Chu DK, Peiris JS, Chen H, Guan Y, Poon LL. Genomic characterizations of bat coronaviruses (1A, 1B and HKU8) and evidence for co-infections in *Miniopterus* bats. *J Gen Virol*. 2008; 89:1282–1287. [PubMed: 18420807]
- Cohen JR, Lin LD, Machamer CE. Identification of a Golgi complex-targeting signal in the cytoplasmic tail of the severe acute respiratory syndrome coronavirus envelope protein. *J Virol*. 2011; 85:5794–5803. [PubMed: 21450821]
- Coll RC, Robertson AA, Chae JJ, Higgins SC, Munoz-Planillo R, Inserra MC, Vetter I, Dungan LS, Monks BG, Stutz A, Croker DE, Butler MS, Haneklaus M, Sutton CE, Nunez G, Latz E, Kastner DL, Mills KH, Masters SL, Schroder K, Cooper MA, O'Neill LA. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med*. 2015; 21:248–255. [PubMed: 25686105]
- Corse E, Machamer CE. The cytoplasmic tail of infectious bronchitis virus E protein directs Golgi targeting. *J Virol*. 2002; 76:1273–1284. [PubMed: 11773403]
- Crawford SE, Hyser JM, Utama B, Estes MK. Autophagy hijacked through viroporin-activated calcium/calmodulin-dependent kinase kinase-beta signaling is required for rotavirus replication. *Proc Natl Acad Sci USA*. 2012; 109:E3405–E3413. [PubMed: 23184977]
- Danielsson N, Catchpole M. Novel coronavirus associated with severe respiratory disease: case definition and public health measures. *Euro Surveill*. 2012; 17:20282. [PubMed: 23041021]
- de Haan CAM, Smeets M, Vernooij F, Vennema H, Pottier PJM. Mapping of the coronavirus membrane protein domains involved in interaction with the spike protein. *J Virol*. 1999; 73:7441–7452. [PubMed: 10438834]
- de Jong AS, de Mattia F, Van Dommelen MM, Lanke K, Melchers WJ, Willems PH, van Kuppeveld FJ. Functional analysis of picornavirus 2B proteins: effects on calcium homeostasis and intracellular protein trafficking. *J Virol*. 2008; 82:3782–3790. [PubMed: 18216106]
- de Jong AS, Visch HJ, de Mattia F, van Dommelen MM, Swarts HG, Luyten T, Callewaert G, Melchers WJ, Willems PH, van Kuppeveld FJ. The coxsackievirus 2B protein increases efflux of ions from the endoplasmic reticulum and Golgi, thereby inhibiting protein trafficking through the Golgi. *J Biol Chem*. 2006; 281:14144–14150. [PubMed: 16540472]
- DeDiego ML, Alvarez E, Almazan F, Rejas MT, Lamirande E, Roberts A, Shieh WJ, Zaki SR, Subbarao K, Enjuanes L. A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo. *J Virol*. 2007; 81:1701–1713. [PubMed: 17108030]
- DeDiego ML, Nieto-Torres JL, Jimenez-Guardeño JM, Regla-Nava JA, Alvarez E, Oliveros JC, Zhao J, Fett C, Perlman S, Enjuanes L. Severe acute respiratory syndrome coronavirus envelope protein regulates cell stress response and apoptosis. *PLoS pathogens*. 2011; 7:e1002315. [PubMed: 22028656]
- DeDiego ML, Nieto-Torres JL, Jimenez-Guardeño JM, Regla-Nava JA, Castaño-Rodríguez C, Fernandez-Delgado R, Usera F, Enjuanes L. Coronavirus virulence genes with main focus on SARS-CoV envelope gene. *Virus Res*. 2014a; 194:124–137. [PubMed: 25093995]
- DeDiego ML, Nieto-Torres JL, Regla-Nava JA, Jimenez-Guardeño JM, Fernandez-Delgado R, Fett C, Castaño-Rodríguez C, Perlman S, Enjuanes L. Inhibition of NF-kappaB mediated inflammation in severe acute respiratory syndrome coronavirus-infected mice increases survival. *J Virol*. 2014b; 88:913–924. [PubMed: 24198408]
- DeDiego ML, Pewe L, Alvarez E, Rejas MT, Perlman S, Enjuanes L. Pathogenicity of severe acute respiratory coronavirus deletion mutants in hACE-2 transgenic mice. *Virology*. 2008; 376:379–389. [PubMed: 18452964]
- Dinarello CA. Interleukin-1beta and the autoinflammatory diseases. *N Engl J Med*. 2009; 360:2467–2470. [PubMed: 19494224]
- dos Santos G, Kutuzov MA, Ridge KM. The inflammasome in lung diseases. *Am J Physiol Lung Cell Mol Physiol*. 2012; 303:L627–633. [PubMed: 22904168]
- Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, Goettsche M, Seebens A, Niedrig M, Pfefferle S, Yordanov S, Zhelyazkov L, Hermanns U, Vallo P, Lukashev A, Muller MA, Deng H, Herrler G, Drosten C. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. *J Virol*. 2010; 84:11336–11349. [PubMed: 20686038]

- Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med*. 2003; 348:1967–1976. [PubMed: 12690091]
- Dubyak GR. Ion homeostasis, channels, and transporters: an update on cellular mechanisms. *Adv Physiol Educ*. 2004; 28:143–154. [PubMed: 15545343]
- Elliott EI, Sutterwala FS. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. *Immunol Rev*. 2015; 265:35–52. [PubMed: 25879282]
- Enjuanes L, Gorbalenya AE, de Groot RJ, Cowley JA, Ziebuhr J, Snijder EJ. The Nidovirales. In: Mahy BWJ, Van Regenmortel M, Walker P, Majumder-Russell D, editors. *Encyclopedia of Virology*. Third Edition. Elsevier Ltd.; Oxford: 2008. p. 419-430.
- Falcon A, Vazquez-Moron S, Casas I, Aznar C, Ruiz G, Pozo F, Perez-Brena P, Juste J, Ibanez C, Garin I, Aihartza J, Echevarria JE. Detection of alpha and betacoronaviruses in multiple Iberian bat species. *Arch Virol*. 2011; 156:1883–1890. [PubMed: 21766197]
- Fett C, DeDiego ML, Regla-Nava JA, Enjuanes L, Perlman S. Complete protection against severe acute respiratory syndrome coronavirus-mediated lethal respiratory disease in aged mice by immunization with a mouse-adapted virus lacking E protein. *J Virol*. 2013; 87:6551–6559. [PubMed: 23576515]
- Garcia-Gimenez E, Alcaraz A, Aguilera VM. Divalent Metal Ion Transport across Large Biological Ion Channels and Their Effect on Conductance and Selectivity. *Biochem Res Int*. 2012; 2012:245786.
- Graham RL, Donaldson EF, Baric RS. A decade after SARS: strategies for controlling emerging coronaviruses. *Nat Rev Microbiol*. 2013; 11:836–848. [PubMed: 24217413]
- Hodgkin AL, Katz B. The effect of sodium ions on the electrical activity of giant axon of the squid. *J Physiol*. 1949; 108:37–77. [PubMed: 18128147]
- Hollenhorst MI, Richter K, Fronius M. Ion transport by pulmonary epithelia. *J Biomed Biotechnol*. 2011; 2011:174306.
- Ichinohe T, Pang IK, Iwasaki A. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol*. 2010; 11:404–410. [PubMed: 20383149]
- Ito M, Yanagi Y, Ichinohe T. Encephalomyocarditis virus viroporin 2B activates NLRP3 inflammasome. *PLoS pathogens*. 2012; 8:e1002857. [PubMed: 22916014]
- Jimenez-Guardeño JM, Nieto-Torres JL, DeDiego ML, Regla-Nava JA, Fernandez-Delgado R, Castaño-Rodríguez C, Enjuanes L. The PDZ-binding motif of severe acute respiratory syndrome coronavirus envelope protein is a determinant of viral pathogenesis. *PLoS pathogens*. 2014; 10:e1004320. [PubMed: 25122212]
- Lamirande EW, DeDiego ML, Roberts A, Jackson JP, Alvarez E, Sheahan T, Shieh WJ, Zaki SR, Baric R, Enjuanes L, Subbarao K. A live attenuated SARS coronavirus is immunogenic and efficacious in golden Syrian hamsters. *J Virol*. 2008; 82:7721–7724. [PubMed: 18463152]
- Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol*. 2013; 13:397–411. [PubMed: 23702978]
- Lim KP, Liu DX. The missing link in coronavirus assembly. Retention of the avian coronavirus infectious bronchitis virus envelope protein in the pre-Golgi compartments and physical interaction between the envelope and membrane proteins. *J Biol Chem*. 2001; 276:17515–17523. [PubMed: 11278557]
- Lo YH, Huang YW, Wu YH, Tsai CS, Lin YC, Mo ST, Kuo WC, Chuang YT, Jiang ST, Shih HM, Lai MZ. Selective inhibition of the NLRP3 inflammasome by targeting to promyelocytic leukemia protein in mouse and human. *Blood*. 2013; 121:3185–3194. [PubMed: 23430110]
- Lu W, Zheng BJ, Xu K, Schwarz W, Du L, Wong CK, Chen J, Duan S, Deubel V, Sun B. Severe acute respiratory syndrome-associated coronavirus 3a protein forms an ion channel and modulates virus release. *Proc Natl Acad Sci USA*. 2006; 103:12540–12545. [PubMed: 16894145]
- Maeda J, Repass JF, Maeda A, Makino S. Membrane topology of coronavirus E protein. *Virology*. 2001; 281:163–169. [PubMed: 11277690]

- Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*. 2006; 440:237–241. [PubMed: 16407889]
- Matthay MA, Zemans RL. The acute respiratory distress syndrome: pathogenesis and treatment. *Annu Rev Pathol*. 2011; 6:147–163. [PubMed: 20936936]
- McAuley JL, Tate MD, MacKenzie-Kludas CJ, Pinar A, Zeng W, Stutz A, Latz E, Brown LE, Mansell A. Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. *PLoS pathogens*. 2013; 9:e1003392. [PubMed: 23737748]
- Meduri GU, Headley S, Kohler G, Stentz F, Tolley E, Umberger R, Leeper K. Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome overtime. *Chest*. 1995; 107:1062–1073. [PubMed: 7705118]
- Muller MA, Paweska JT, Leman PA, Drosten C, Grywna K, Kemp A, Braack L, Sonnenberg K, Niedrig M, Swanepoel R. Coronavirus antibodies in African bat species. *Emerg Infect Dis*. 2007; 13:1367–1370. [PubMed: 18252111]
- Murakami T, Ockinger J, Yu J, Byles V, McColl A, Hofer AM, Horng T. Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc Natl Acad Sci USA*. 2012; 109:11282–11287. [PubMed: 22733741]
- Nal B, Chan C, Kien F, Siu L, Tse J, Chu K, Kam J, Staropoli I, Crescenzo-Chaigne B, Escriou N, van der Werf S, Yuen KY, Altmeyer R. Differential maturation and subcellular localization of severe acute respiratory syndrome coronavirus surface proteins S, M and E. *J Gen Virol*. 2005; 86:1423–1434. [PubMed: 15831954]
- Narayanan, K.; Maeda, J.; Maeda, A.; Makino, S. Mechanism of specific coronavirus RNA packaging into virus particles, 19th Annual Meeting; Colorado: American Society for Virology; 2000. p. 84
- Netland J, DeDiego ML, Zhao J, Fett C, Alvarez E, Nieto-Torres JL, Enjuanes L, Perlman S. Immunization with an attenuated severe acute respiratory syndrome coronavirus deleted in E protein protects against lethal respiratory disease. *Virology*. 2010; 399:120–128. [PubMed: 20110095]
- Nguyen VP, Hogue BG. Protein interactions during coronavirus assembly. *J Virol*. 1997; 71:9278–9284. [PubMed: 9371586]
- Nieto-Torres JL, DeDiego ML, Alvarez E, Jimenez-Guardeño JM, Regla-Nava JA, Llorente M, Kremer L, Shuo S, Enjuanes L. Subcellular location and topology of severe acute respiratory syndrome coronavirus envelope protein. *Virology*. 2011; 415:69–82. [PubMed: 21524776]
- Nieto-Torres JL, DeDiego ML, Verdía-Baguena C, Jimenez-Guardeño JM, Regla-Nava JA, Fernandez-Delgado R, Castaño-Rodríguez C, Alcaraz A, Torres J, Aguilera VM, Enjuanes L. Severe acute respiratory syndrome coronavirus envelope protein ion channel activity promotes virus fitness and pathogenesis. *PLoS pathogens*. 2014; 10:e1004077. [PubMed: 24788150]
- Nieva JL, Madan V, Carrasco L. Viroporins: structure and biological functions. *Nat Rev Microbiol*. 2012; 10:563–574. [PubMed: 22751485]
- Paroutis P, Touret N, Grinstein S. The pH of the secretory pathway: measurement, determinants, and regulation. *Physiology (Bethesda)*. 2004; 19:207–215. [PubMed: 15304635]
- Perlman S, Netland J. Coronaviruses post-SARS: update on replication and pathogenesis. *Nat Rev Microbiol*. 2009; 7:439–450. [PubMed: 19430490]
- Pervushin K, Tan E, Parthasarathy K, Lin X, Jiang FL, Yu D, Vararattanavech A, Soong TW, Liu DX, Torres J. Structure and inhibition of the SARS coronavirus envelope protein ion channel. *PLoS pathogens*. 2009; 5:e1000511. [PubMed: 19593379]
- Pugin J, Ricou B, Steinberg KP, Suter PM, Martin TR. Proinflammatory activity in bronchoalveolar lavage fluids from patients with ARDS, a prominent role for interleukin-1. *Am J Respir Crit Care Med*. 1996; 153:1850–1856.
- Quan PL, Firth C, Street C, Henriquez JA, Petrosov A, Tashmukhamedova A, Hutchison SK, Egholm M, Osinubi MO, Niezgodá M, Ogunkoya AB, Briese T, Rupprecht CE, Lipkin WI. Identification of a severe acute respiratory syndrome coronavirus-like virus in a leaf-nosed bat in Nigeria. *mBio*. 2010; 1:e00208–00210. [PubMed: 21063474]

- Queralto-Martin M, Garcia-Gimenez E, Mafe S, Alcaraz A. Divalent cations reduce the pH sensitivity of OmpF channel inducing the pK(a) shift of key acidic residues. *Phys Chem Chem Phys*. 2011; 13:563–569. [PubMed: 21046046]
- Raamsman MJB, Locker JK, de Hooge A, de Vries AAF, Griffiths G, Vennema H, Rottier PJM. Characterization of the coronavirus mouse hepatitis virus strain A59 small membrane protein E. *J Virol*. 2000; 74:2333–2342. [PubMed: 10666264]
- Regla-Nava JA, Nieto-Torres JL, Jimenez-Guardeño JM, Fernandez-Delgado R, Fett C, Castaño-Rodríguez C, Perlman S, Enjuanes L, DeDiego ML. SARS coronaviruses with mutations in E protein are attenuated and promising vaccine candidates. *J Virol*. 2015; 89:3870–3887. [PubMed: 25609816]
- Rota PA, Oberste MS, Monroe SS, Nix WA, Campganioli R, Icenogle JP, Peñaranda S, Bankamp B, Maher K, Chen M-H, Tong S, Tamin A, Lowe L, Frace M, DeRisi JL, Chen Q, Wang D, Erdman Dd, Peret TCT, Burns C, Ksiazek TG, Rollin PE, Sanchez A, Liffick S, Holloway B, Limor J, McCaustland K, Olsen-Rassmussen M, Fouchier R, Gunther S, Osterhaus ADME, Drosten C, Pallansch MA, Anderson LJ, Bellini WJ. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science*. 2003; 300:1394–1399. [PubMed: 12730500]
- Ruch TR, Machamer CE. The Coronavirus E Protein: Assembly and Beyond. *Viruses*. 2012; 4:363–382. [PubMed: 22590676]
- Sakaguchi T, Leser GP, Lamb RA. The ion channel activity of the influenza virus M2 protein affects transport through the Golgi apparatus. *J Cell Biol*. 1996; 133:733–747. [PubMed: 8666660]
- Schapiro FB, Grinstein S. Determinants of the pH of the Golgi complex. *J Biol Chem*. 2000; 275:21025–21032. [PubMed: 10748071]
- Scobey T, Yount BL, Sims AC, Donaldson EF, Agnihothram SS, Menachery VD, Graham RL, Swanstrom J, Bove PF, Kim JD, Grego S, Randell SH, Baric RS. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proc Natl Acad Sci USA*. 2013; 110:16157–16162. [PubMed: 24043791]
- Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. *Nature*. 2012; 481:278–286. [PubMed: 22258606]
- Surya W, Li Y, Verdia-Baguena C, Aguilera VM, Torres J. MERS coronavirus envelope protein has a single transmembrane domain that forms pentameric ion channels. *Virus Res*. 2015; 201:61–66. [PubMed: 25733052]
- Torres J, Maheswari U, Parthasarathy K, Ng L, Liu DX, Gong X. Conductance and amantadine binding of a pore formed by a lysine-flanked transmembrane domain of SARS coronavirus envelope protein. *Protein Sci*. 2007; 16:2065–2071. [PubMed: 17766393]
- Triantafilou K, Kar S, Vakakis E, Kotecha S, Triantafilou M. Human respiratory syncytial virus viroporin SH: a viral recognition pathway used by the host to signal inflammasome activation. *Thorax*. 2013a; 68:66–75. [PubMed: 23229815]
- Triantafilou K, Kar S, van Kuppeveld FJ, Triantafilou M. Rhinovirus-Induced Calcium Flux Triggers NLRP3 and NLRC5 Activation in Bronchial Cells. *Am J Respir Cell Mol Biol*. 2013b10.1165/rcmb.2013-00320C
- Triantafilou K, Triantafilou M. Ion flux in the lung: virus-induced inflammasome activation. *Trends Microbiol*. 2014; 22:580–588. [PubMed: 24986075]
- Venkatagopalan P, Daskalova SM, Lopez LA, Dolezal KA, Hogue BG. Coronavirus envelope (E) protein remains at the site of assembly. *Virology*. 2015; 478:75–85. [PubMed: 25726972]
- Verdia-Baguena C, Nieto-Torres JL, Alcaraz A, Dediego ML, Enjuanes L, Aguilera VM. Analysis of SARS-CoV E protein ion channel activity by tuning the protein and lipid charge. *Biochim Biophys Acta*. 2013; 1828:2026–2031. [PubMed: 23688394]
- Verdia-Baguena C, Nieto-Torres JL, Alcaraz A, Dediego ML, Torres J, Aguilera VM, Enjuanes L. Coronavirus E protein forms ion channels with functionally and structurally-involved membrane lipids. *Virology*. 2012; 432:485–494. [PubMed: 22832120]
- Wang C, Lamb RA, Pinto LH. Direct measurement of the influenza A virus M2 protein ion channel activity in mammalian cells. *Virology*. 1994; 205:133–140. [PubMed: 7526533]
- Watanabe S, Watanabe T, Kawaoka Y. Influenza A virus lacking M2 protein as a live attenuated vaccine. *J Virol*. 2009; 83:5947–5950. [PubMed: 19321619]

- Whitehead SS, Bukreyev A, Teng MN, Firestone CY, St Claire M, Elkins WR, Collins PL, Murphy BR. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. *J Virol.* 1999; 73:3438–3442. [PubMed: 10074199]
- Wilson L, Gage P, Ewart G. Hexamethylene amiloride blocks E protein ion channels and inhibits coronavirus replication. *Virology.* 2006; 353:294–306. [PubMed: 16815524]
- Wilson L, McKinlay C, Gage P. SARS coronavirus E protein forms cation-selective ion channels. *Virology.* 2004; 330:322–331. [PubMed: 15527857]
- Wong SK, Li W, Moore MJ, Choe H, Farzan M. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J Biol Chem.* 2004; 279:3197–3201. [PubMed: 14670965]
- Wozniak AL, Griffin S, Rowlands D, Harris M, Yi M, Lemon SM, Weinman SA. Intracellular proton conductance of the hepatitis C virus p7 protein and its contribution to infectious virus production. *PLoS pathogens.* 2010; 6:e1001087. [PubMed: 20824094]
- Wu YH, Kuo WC, Wu YJ, Yang KT, Chen ST, Jiang ST, Gordy C, He YW, Lai MZ. Participation of c-FLIP in NLRP3 and AIM2 inflammasome activation. *Cell Death Differ.* 2014; 21:451–461. [PubMed: 24270411]
- Wuytack F, Raeymaekers L, Missiaen L. Molecular physiology of the SERCA and SPCA pumps. *Cell calcium.* 2002; 32:279–305. [PubMed: 12543090]
- Yount B, Roberts RS, Sims AC, Deming D, Frieman MB, Sparks J, Denison MR, Davis N, Baric RS. Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. *J Virol.* 2005; 79:14909–14922. [PubMed: 16282490]
- Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med.* 2012; 367:1814–1820. [PubMed: 23075143]
- Zhou Y, Frey TK, Yang JJ. Viral calciomics: interplays between Ca²⁺ and virus. *Cell calcium.* 2009; 46:1–17. [PubMed: 19535138]

Highlights

SARS-CoV E protein forms calcium ion channels, a novel highly relevant function

Transport of calcium ions through E protein channel stimulates the inflammasome

Inflammasome derived exacerbated proinflammation causes SARS worsening

E protein ion channel and its driven proinflammation may be targets to treat SARS

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

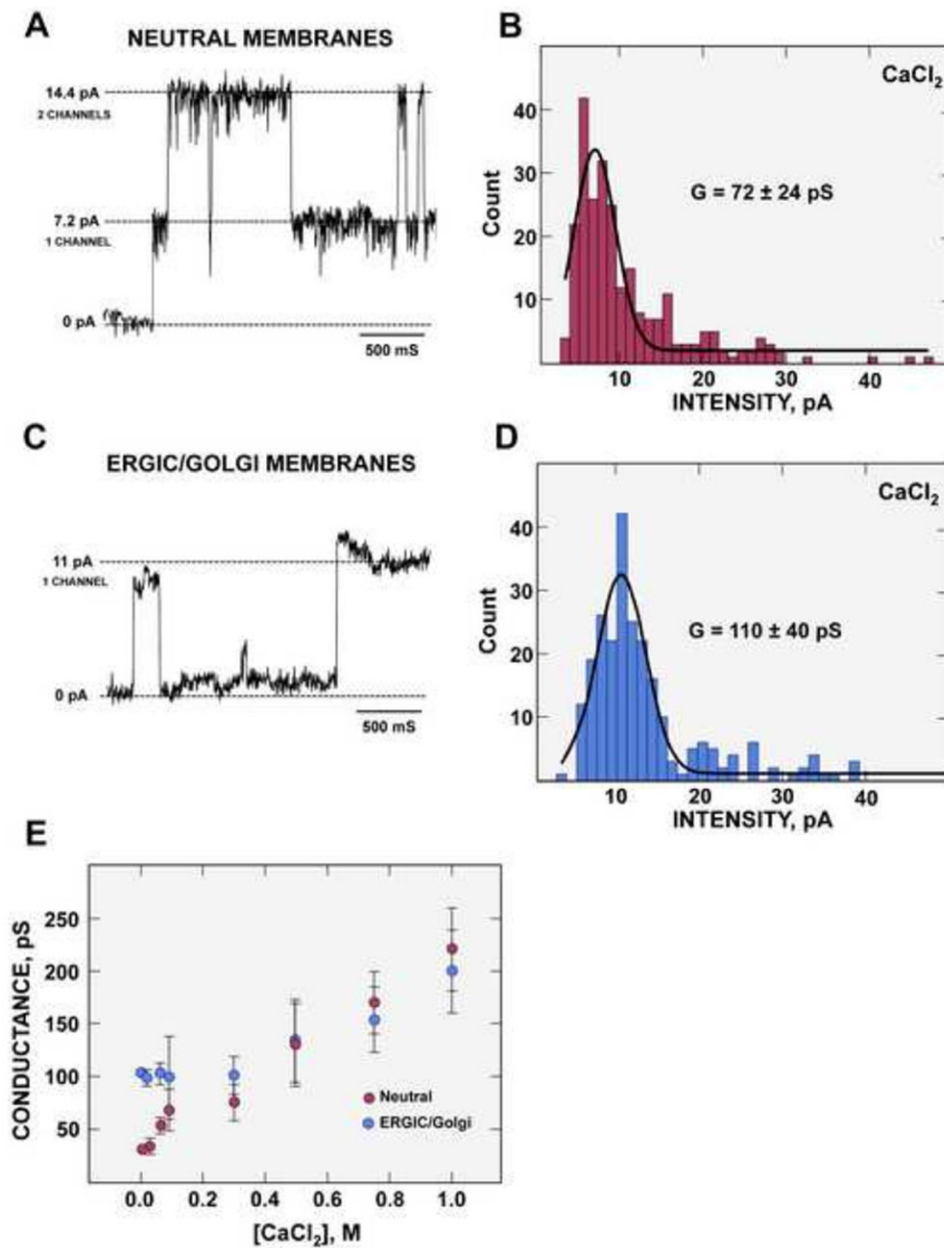


Figure 1. SARS-CoV E protein IC activity in CaCl_2 solutions. (A) Current recordings showing intensity jumps (measured in pA) corresponding to the assembly and disassembly of one or several channels in neutral DPhPC membranes. (B) Histogram representing the intensity of the different current jumps recorded and their respective frequency in neutral membranes. Conductance (G), which is the ratio between current intensity and the applied voltage, is also indicated (measured in pS). Current jumps (C) and their corresponding histogram (D) measured in ERGIC/Golgi membranes. (E) Single-channel conductance variation in solutions containing increasing concentrations of CaCl_2 . Magenta circles represent data from neutral membranes and blue circles values from ERGIC/Golgi membranes. Error bars show standard deviations from three independent experiments.

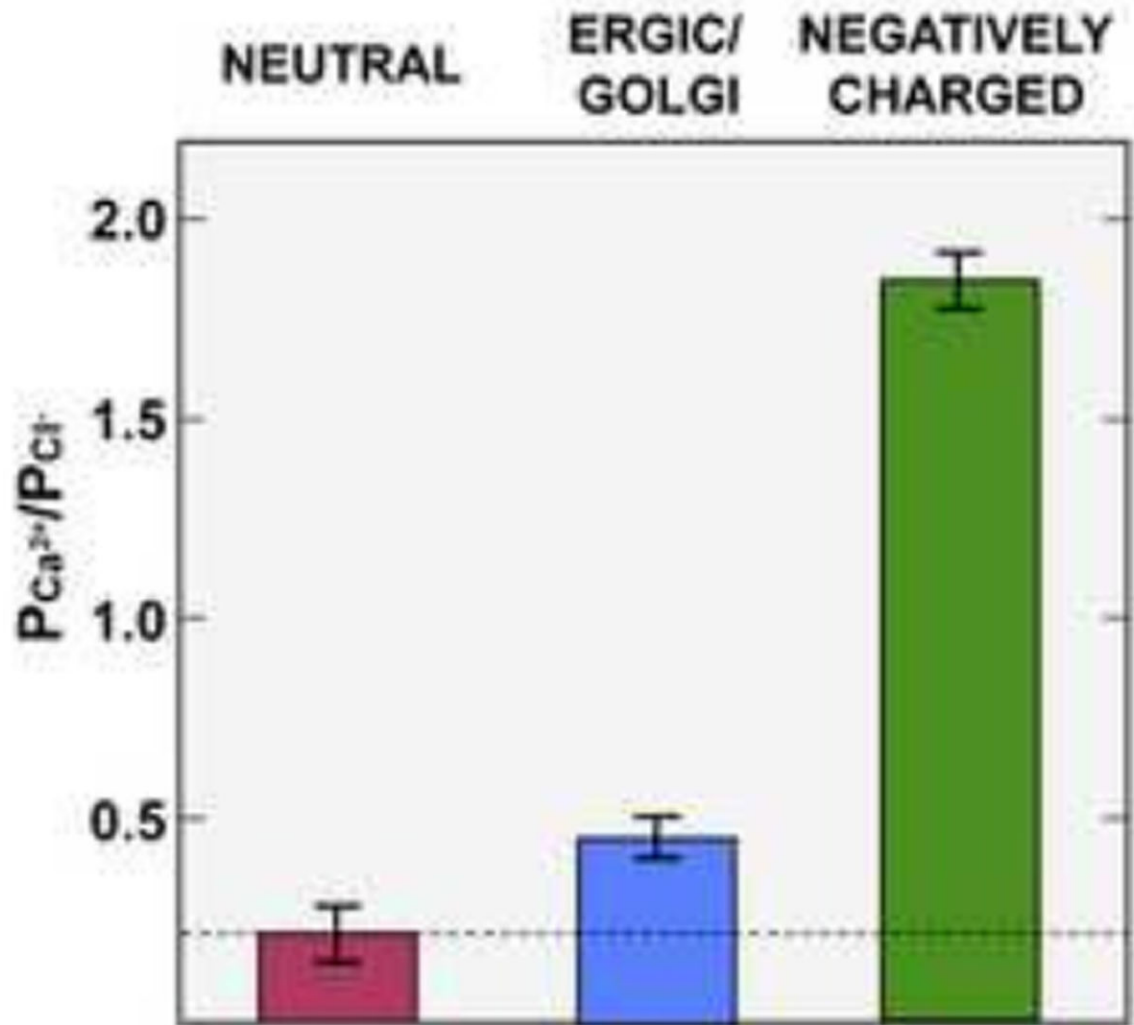


Figure 2. Ca^{2+} selectivity of SARS-CoV E protein channel. Permeability ratios $P_{Ca^{2+}}/P_{Cl^{-}}$ in neutral DPhPC (magenta column), ERGIC/Golgi (blue column) or negatively-charged DPhPS membranes (green column). Dotted line represents the permeability ratio value for a hypothetical neutral pore. Values above the line represent cation selectivity, and those below correspond to anion selectivity. Error bars show standard deviations.

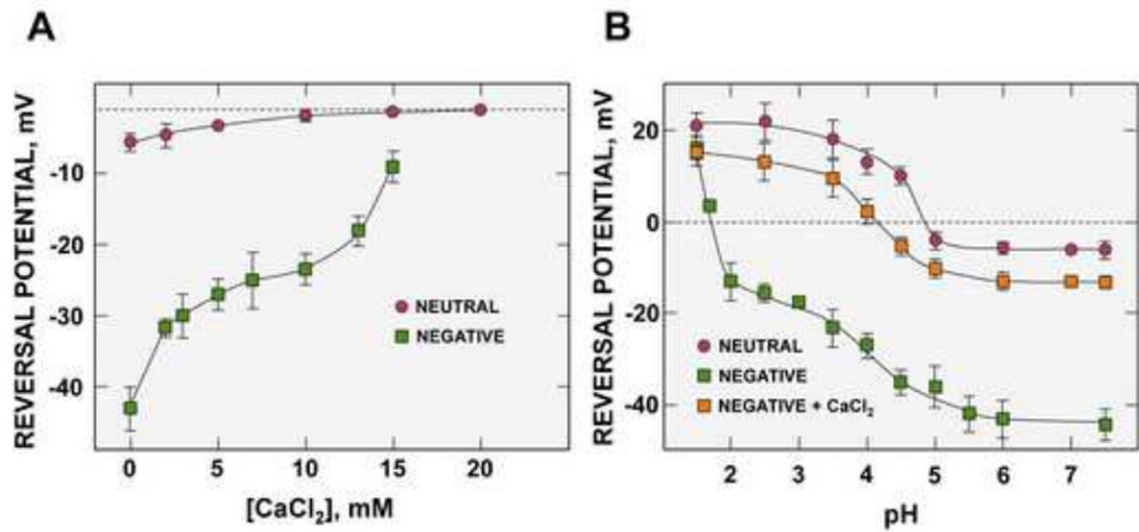


Figure 3.

Modulation of SARS-CoV E protein channel selectivity by Ca²⁺. (A) Reversal potential (E_{rev}) was measured in asymmetric (500 mM | 50 mM) KCl solutions upon addition of millimolar CaCl₂ concentrations. Two series of experiments were performed: in neutral DPhPC (magenta circles) and negatively charged DPhPS membranes (green squares). Dotted line shows E_{rev} value corresponding to a non-selective ion channel. (B) Effect of pH on E protein E_{rev} measured under the same KCl conditions above mentioned in neutral (magenta circles) and negatively charged membranes in the absence (green squares) or the presence of 15 mM CaCl₂ (orange squares). Dotted line shows the E_{rev} value corresponding to a non-selective ion channel. Error bars represent standard deviations from three independent experiments.

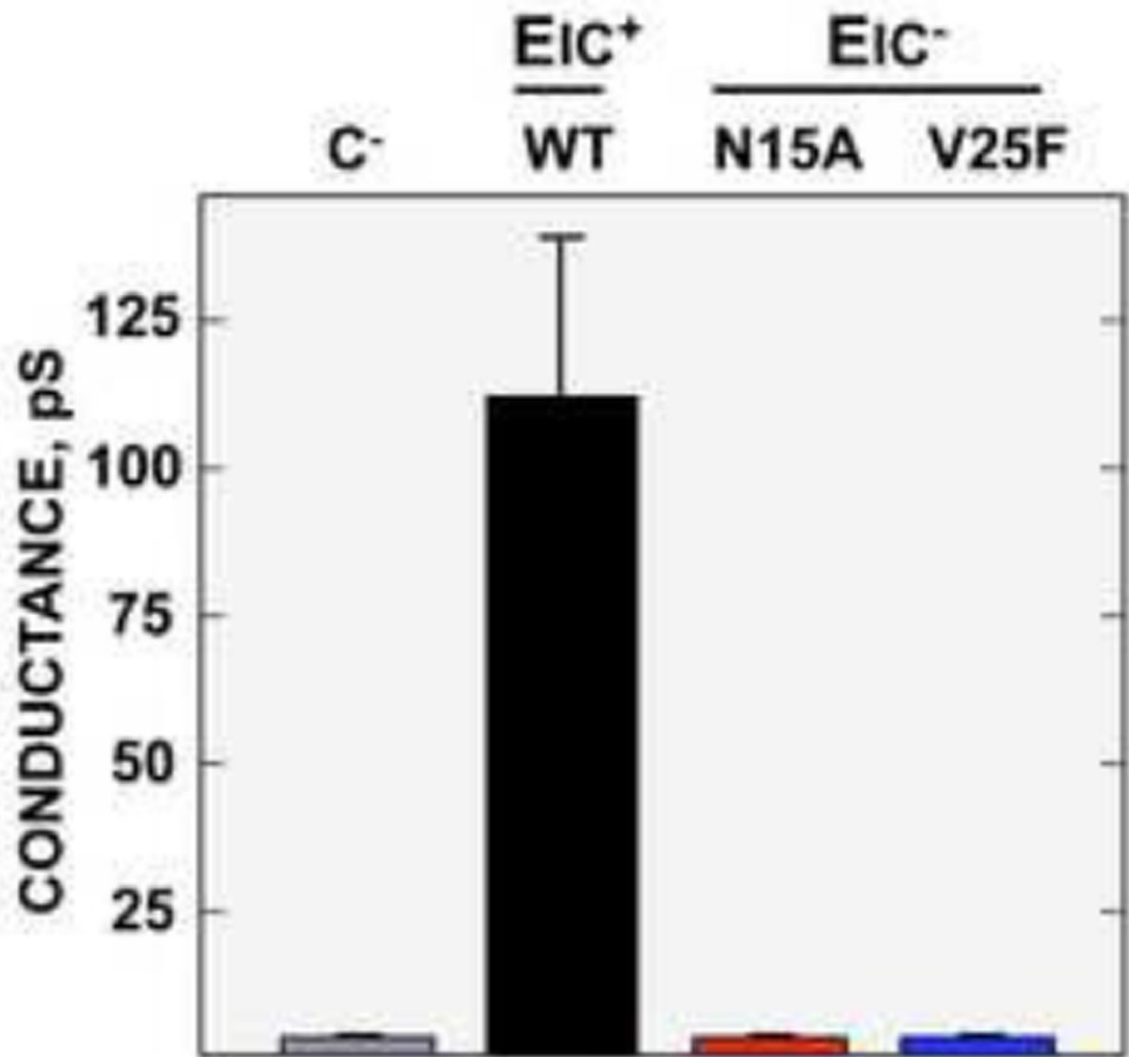


Figure 4. Mutations inhibiting SARS-CoV E protein conductance in CaCl₂. Single channel conductance was measured in 100 mM CaCl₂, in the absence of any peptide (C-, grey column), or in the presence of wildtype SARS-CoV E protein transmembrane domain peptide (WT, black column), or the mutant peptides N15A (red column) and V25F (blue column). Error bars represent standard deviations.

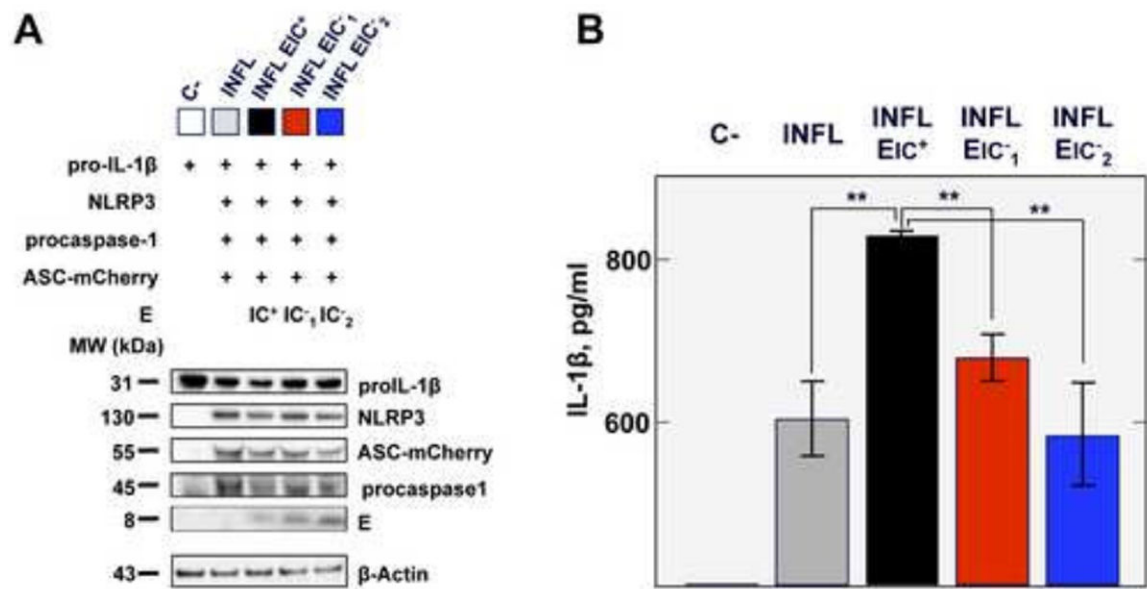


Figure 5.

Inflammasome activation through SARS-CoV E protein ion channel activity. The components of the NLRP3 inflammasome (INFL), NLRP3 (NLRP3-HA), ASC (ASC-mCherry), procaspase-1 (procaspase-1-Myc) and pro-IL-1 β were transfected in Vero E6 cells, in the absence or presence of SARS-CoV E protein with (IC⁺) or without (IC⁻) ion channel activity. EIC₁⁻ represents the N15A mutant and EIC₂⁻ indicates the V25F mutant. As a negative control, cells were transfected solely with pro-IL-1 β (C-). (A) Western blot showing the presence of the inflammasome complex components and E protein within Vero E6 cells lysates. (β -Actin was also detected as a loading control. (B) Levels of active IL-1 β present in the cell supernatant. Error bars represent standard deviations from three independent experiments. Statistically significant data are indicated with two asterisks (Student's t-test p value <0.01).

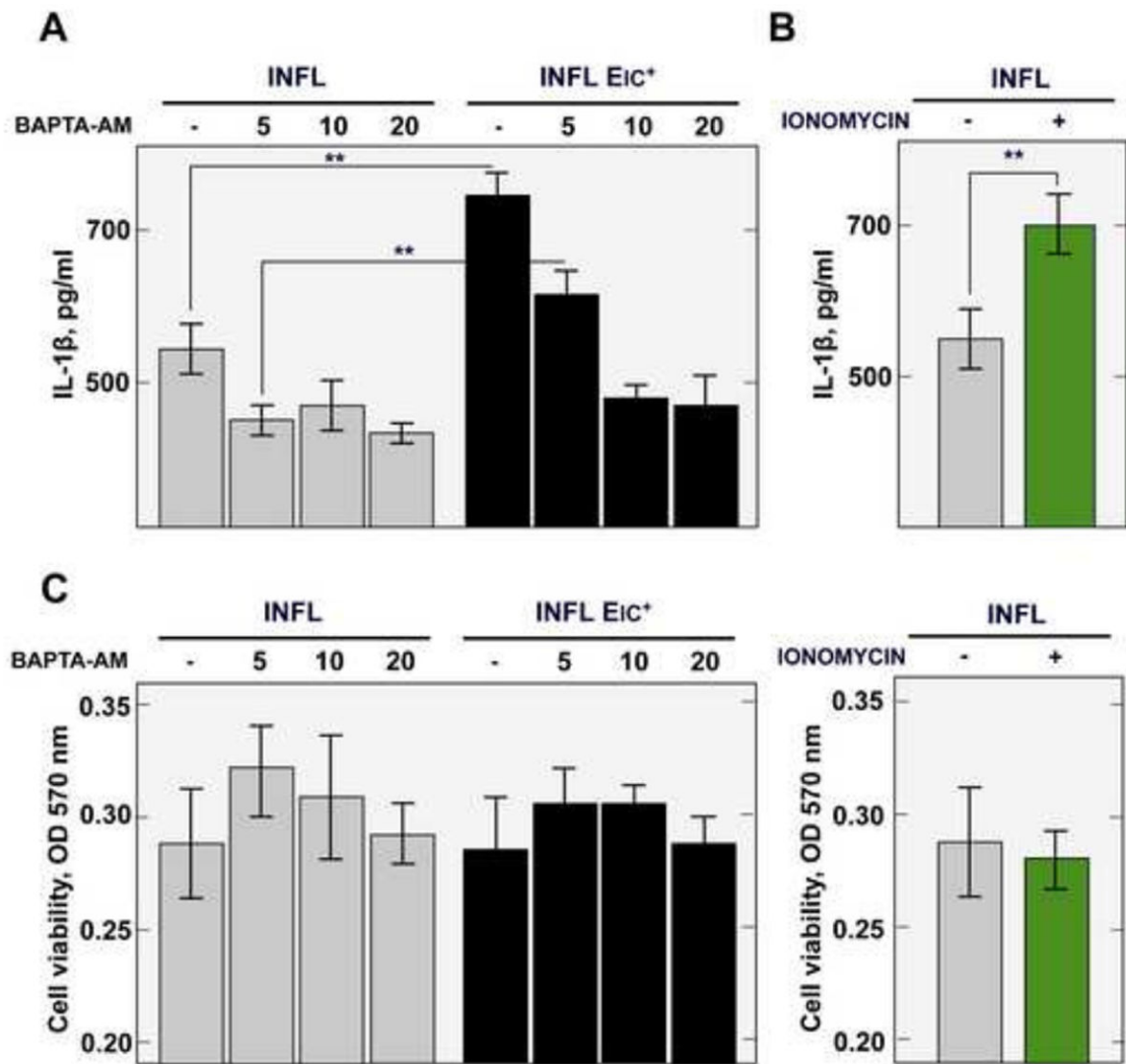


Figure 6.

Inflammasome activation through SARS-CoV E protein Ca^{2+} channel activity. (A) The inflammasome complex (INFL) was reconstituted in cells without or with wildtype E protein (Eic⁺) in the presence of increasing concentrations (μM) of the cell permeable Ca^{2+} chelator BAPTA-AM. DMSO was added as negative control (-). The levels of active IL-1 β present in the cell media were measured by ELISA. (B) Activation of the inflammasome by the Ca^{2+} ionophore ionomycin. Error bars represent standard deviations from three independent experiments; statistically significant data are indicated with two asterisks (Student's t-test p value <0.01). (C) Cell viability MTT assay. Optical densities (OD) representing cellular metabolic activity under the different experimental conditions were measured at 570 nm.