VALIDATION OF CORONAVIRUS E PROTEINS ION CHANNELS AS TARGETS FOR ANTIVIRAL DRUGS

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1. INTRODUCTION

Coronaviruses are divided into three groups, depending on the sequence homology and antigen cross-reactivity. Groups 1 and 2 contain the mammalian coronaviruses, and group 3 consists of the avian coronaviruses. All coronavirus groups encode E protein, a small, 9–12 kDa integral membrane protein. Although, there is little sequence homology between the coronavirus groups, all E proteins share structural homology, they all contain an N-terminus, which consists of a short 7–9 amino acid hydrophilic region, and a 21–29 amino acid hydrophobic transmembrane domain, followed by a hydrophilic C-terminal region. The exact functions and mechanisms of the coronavirus E proteins are yet to be established, although E proteins have been shown to be important for coronavirus replication, mediating viral assembly, and morphogenesis.

Coronavirus E proteins share several characteristics with viral ion channels, which are small hydrophobic virus-encoded proteins. Virus ion channels have a highly hydrophobic domain that forms at least one amphipathic α-helix that oligomerizes to form an ion-conductive pore in membranes. Virus ion channels function to modify the cells permeability to ions and have been shown to mediate viral entry/exit or virus assembly and budding. The first identified viral ion channel, hence the best characterized, is the M2 protein encoded by influenza A. M2 forms proton selective ion channels that mediate viral uncoating and protects acid-sensitive hemagglutinin glycoprotein during transport to the cell surface. Although influenza B does not encode the M2 ion channel, it has been demonstrated to have two ion channel forming proteins, NB and BM2, whose role in viral replication are currently being investigated. Since the identification of the M2 ion channel, several other viruses have been demonstrated to encode viral ion channels. The HIV-1 accessory protein Vpu has also been shown to have ion channel activity, which mediates the release of viral particles from the plasma membrane. Most recently, the 6K proteins from the alphaviruses, Ross River Virus and Barmah Forest Virus, have been shown to form cation-selective ion channels in planar...
lipid bilayers. Additionally, several authors have shown ion channel activity of the hepatitis C virus (HCV) p7 protein.  

The M2 channel is well established as a target for antiviral drug therapy, and blockers of other viral ion channels have been shown to inhibit replication of the parent virus. The M2 ion channel is inhibited by amantadine and some of its derivatives, which are currently used as clinical treatment of influenza A infection. The Vpu ion channel activity in planar lipid bilayers is inhibited by the amiloride derivatives 5-(N,N-hexamethylene)amiloride (HMA) and 5-(N,N-dimethyl)amiloride (DMA), but not by amiloride itself. Furthermore, HMA inhibits Vpu enhancement of Gag-driven virus-like particles (VLP) budding from HeLa cells co-expressing Vpu and Gag and also inhibits HIV-1 replication in cultured primary human macrophages. In addition, the p7 ion channel has been shown to be inhibited by HMA, amantadine, and long-alkyl chain immunosugar derivatives. Thus, a number of precedents have been set for ion channels as targets of potential antiviral compounds.

Due to coronavirus E proteins similarities with viral ion channels and their important role in viral replication, we hypothesized that coronavirus E proteins have ion channel activity, and compounds that block these channels may result in inhibition of viral replication. We report here that representative E proteins from all three coronavirus groups form ion channels. Furthermore, we found that certain amiloride derivatives block E protein ion channel activity and inhibit replication of coronaviruses in cultured cells.

2. MATERIALS AND METHODS

2.1. Peptide Synthesis and Purification

E peptides corresponding to the human coronavirus 229E (HCoV-229E) E protein (MFLKLVDDHALVNVNLLWCVVLIVLLVCITIKLKLCTCMFNCNRTFVYGPK NVVYHIYSYHMIDPFPKRVIDF), GenBank accession number NP_073554, mouse hepatitis virus (MHV)-A59 E protein (MFNLFLTDTVWYVGQIIIFAVCLMVIT IIYVAFLASIKLCLGCGLNTLVSPSRYLYDRSKQLYKYYNEEMLPLLEVDI), GenBank accession number NP_068673, severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) full-length E protein (MYSFVEETGTTLVNSVLLFLAFVF LLVTLAILTARLCAYCCNIVNVSVLYKPTVYVYVSVKNLNSSEGVPDDL), SARS - CoV N-terminal E protein (MYSFVEETGTTLVNSVLLFLAVFVFLLVTLEAIL TALRLC), GenBank accession number NC004718, and infectious bronchitis virus (IBV) Beaudette strain E protein (MTNLNKLNLGNSFLTLAYIFVGFLALYLLGRA LQAFFQQADACCLFWYTWVVPAGKGTAFVYNHTYGGKLNKELETVINEFPK NGWK), GenBank accession number CAC39303, were chemically synthesized and purified, as described previously. The peptides were shown to contain full-length products by a variety of methods including, Western blot analysis with E protein specific antibodies, and mass spectral analysis.  

2.2. Ion Channel Recording

The HCoV-229E, MHV, SARS-CoV, and IBV purified E proteins were resuspended to 1 mg/ml in 2,2,2-trifluoroethanol (TFE) and their ability to form ion channels was
tested on a Warner bilayer rig (Warner instruments, Inc. Hamden, CT), as described previously.13,14

2.3. Amiloride Derivatives Ion Channel Inhibition

A 50 mM stock solution of compounds was prepared in 50% DMSO, 50% methanol, which was further diluted for use in ion channel inhibition studies and in the antiviral assays.

To determine if the amiloride derivatives blocked SARS-CoV E protein ion channel conductance in planar lipid bilayers, after ion channel current amplitude was detected, 100–200 µM of compound was added to the CIS chamber while stirring. The current across the bilayer was recorded prior to addition of SARS-CoV E protein, after detection of ion channel conductance, and after addition of the compound. T-test (Microsoft Excel) was used to test the difference between the normalized mean currents before and after addition of the compound.

2.4. Testing Amiloride Derivative Antiviral Activity

The virus plaque phenotype in the presence or absence of antiviral compound was studied in L2 cells (ATCC). The L2 cells were plated in 6-well plates and grown to confluence, then infected with a MOI 0.01 of MHV-A59 (ATCC) or MOI 0.1 of MHVΔE or MHVSARS E (kind gift from Paul Masters, Wadsworth Center, Albany, NY) for 1 hour. The virus was removed and replaced with 1% seaplaque overlay in MEM supplemented with 10% FCS and 20 µM or 0 µM of antiviral compound in 50% DMSO 50% methanol. After 48 hours incubation at 37°C/5% CO2, the cells were stained with 0.1% crystal violet in 20% methanol.

To determine the Selectivity Index (SI) of the antiviral compounds the effective concentration 50 (EC50) was calculated by plaque assay on L929 cells (ATCC) and the toxicity concentration 50 (TC50) was calculated by MTT cytoxicity assay. The SI was calculated by dividing the TC50 by the EC50 (SI = TC50/EC50).

3. RESULTS AND DISCUSSION

3.1. Ion Channel Recordings

HCoV-229E, MHV, IBV, SARS-CoV -full-length and -N-terminal E proteins were tested for their ability to form ion channels in planar lipid bilayers. Experiments were done to determine E proteins ion selectivity for Na⁺ over Cl⁻ and K⁺ over Cl⁻ ions. Figure 1 shows typical ion channel conductance of representatives from coronavirus E proteins from group 1, 2, or 3, demonstrating that E protein ion channel activity is a general property of all coronavirus groups. Interestingly, the E proteins from the different coronavirus group had divergent ion channel selectivity. The group 1 coronavirus HCoV-229E E protein is K⁺ selective, whereas the group 2 MHV and SARS-CoV E proteins, as well as the group 3 coronaviruses, IBV E protein were more selective for Na⁺ ions. The different E protein ion channel selectivity may reflect subtly divergent roles of the E protein groups in the coronavirus life cycle, although this remains to be established. In support of this idea is
that the group 1 \( \text{K}^+ \) selective E proteins are essential for viral replication,\(^\text{15}\) whereas group 2 (\( \text{Na}^+ \) selective) E proteins are important, but not essential for coronavirus replication.\(^\text{16}\) Further data to support this theory was presented at this conference (see Refs. 17 in this proceedings book), Masters et al., 2006 demonstrated that the \( \text{Na}^+ \) selective E proteins from group 2, bovine coronavirus and SARS-CoV, as well as the group 3 IBV could substitute for the \( \text{Na}^+ \) selective MHV E protein and enhance replication of recombinant MHV virus. On the contrary, the group 1 transmissible gastroenteritis virus E protein, which our data suggest may be \( \text{K}^+ \) selective, could not substitute for the MHV E protein in recombinant viruses.\(^\text{17,18}\) Because, the group 1 and 2 coronavirus E proteins share more sequence homology than the group 2 and 3 E proteins,\(^\text{18,19}\) the ability of the different E protein groups to substitute for the MHV E protein could be more dependent on their ion channel selectivity than the sequence homology.

Previously, it has been demonstrated that the transmembrane (TM) domain of several ion channels, including M2 and Vpu, form channels with similar properties as the full-length proteins.\(^\text{18,19}\) Therefore, we tested the ability of the SARS-CoV E protein N-terminal first 40 amino acids, which encompass the putative TM domain for its ability to form ion channels in planar lipid bilayers. Indeed, the SARS-CoV N-terminal peptide formed ion channels that had similar properties to the full-length E protein. Thus, the hydrophilic C-terminal domain is dispensable for ion channel activity in planar lipid bilayers.\(^\text{13}\) Several studies are currently being conducted to determine if E proteins TM domain ion channel activity is important for coronavirus replication (see Refs. 20 and 21 in this proceedings book). Intriguingly, these studies have shown that substitution or mutation of the E protein TM domain is detrimental for viral replication, suggesting that E protein ion channel activity could be important for coronavirus replication.

### 3.2. Amiloride Derivatives Inhibit SARS-CoV E Protein Ion Channel Conductance

Because it is possible that E protein ion channel activity is important for coronavirus replication and a precedent has been set that ion channels are suitable targets of antiviral therapy, we tested amiloride, plus, its derivatives HMA, and 5-(\( \text{N}-\text{Methyl-N-}...
isobutyl)amiloride (MIA) for their ability to inhibit SARS-CoV E protein ion channel conductance. Table 1 demonstrates that HMA reduced the conductance across the bilayer by about 80%, MIA by about 70%, while amiloride itself did not significantly reduce the current across the bilayer (Table 1).

3.3. Amiloride Derivatives Inhibit Replication of MHV and MHVSARS E, but Not MHVΔE

We found that HMA and MIA inhibited the SARS-CoV E protein ion channel conductance in planar lipid bilayers; therefore, we wanted to test their ability to inhibit coronavirus replication. Due to the safety issues and difficulties of working with the SARS-CoV, we decided to use the recombinant MHV virus that expresses the SARS-CoV E protein in place of the MHV E protein (MHVSARS E). The MHVSARS E virus replicates efficiently in cultured mouse cells, but has a slightly smaller plaque phenotype than the wild-type MHV.17 As a control we also used the MHV recombinant virus with the entire E protein deleted (MHVΔE), which replicates to low titre in mouse cells and has a small plaque phenotype.22 MHV wild-type virus has a plaque phenotype of about 3–4 mm in the absence of antiviral compound, but in the presence of 20 µM HMA or MIA the plaque size is reduced to about 1 mm. Similarly, 20 µM of HMA or MIA reduced the MHVSARS E plaque size from about 2–3 mm to 1 mm. In contrast, neither HMA nor MIA significantly affected MHVΔE plaque phenotype. Comparable with the ion channel conductance study, amiloride did not have any significant effect on MHV, MHVSARS E or MHVΔE replication (Table 1). The selectivity index (SI) of HMA and MIA on MHV and MHVSARS E replication were greater than 10, indicating that the antiviral activity of the compounds are notably removed from toxicity (Table 1). Further, the antiviral activity of these compounds against MHV expressing the homo- or heterologous E proteins, but not against the ΔE construct, together with direct observations of SARS-CoV E channel blockage, is supportive of our hypothesis that the mechanism of action of the compounds is via inhibition of E protein ion channel activity.

Table 1. Summary of amiloride derivatives inhibition of, SARS-CoV E protein in planar lipid bilayers and MHV, MHVSARS E, and MHVΔE replication in cultured cells.

| Compound | Structure | Normalized % of SARS CoV E current (± S.E.M) | MHV replication | MHVSARS E replication | MHVΔE replication |
|----------|-----------|-----------------------------------------------|-----------------|------------------------|-------------------|
| Amiloride | ![Amiloride Structure](image) | 147 ± 42 (n=6, p=0.16) | No significant inhibition | No significant inhibition | No significant inhibition |
| HMA      | ![HMA Structure](image) | 21 ± 9 (n=5 p=0.0003) Blocks | Inhibits SI=37 | Inhibits SI=31 | No significant inhibition |
| MIA      | ![MIA Structure](image) | 33 ± 15 (n=5 p=0.006) Blocks | Inhibits SI=77 | Inhibits SI=33 | No significant inhibition |
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