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Hexamethylene amiloride blocks E protein ion channels and inhibits coronavirus replication

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Abstract

All coronaviruses encode a small hydrophobic envelope (E) protein, which mediates viral assembly and morphogenesis by an unknown mechanism. We have previously shown that the E protein from Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) forms cation-selective ion channels in planar lipid bilayers (Wilson, L., McKinlay, C., Gage, P., Ewart, G., 2004. SARS coronavirus E protein forms cation-selective ion channels. Virology 330(1), 322–331). We now report that three other E proteins also form cation-selective ion channels. These E proteins were from coronaviruses representative of taxonomic groups 1–3: human coronavirus 229E (HCoV-229E), mouse hepatitis virus (MHV), and infectious bronchitis virus (IBV), respectively. It appears, therefore, that coronavirus E proteins in general, belong to the virus ion channels family. Hexamethylene amiloride (HMA) – an inhibitor of the HIV-1 Vpu virus ion channel – inhibited the HCoV-229E and MHV E protein ion channel conductance in bilayers and also inhibited replication of the parent coronaviruses in cultured cells, as determined by plaque assay. Conversely, HMA had no antiviral effect on a recombinant MHV with the entire coding region of E protein deleted (MHVΔE). Taken together, the data provide evidence of a link between inhibition of E protein ion channel activity and the antiviral activity of HMA.
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Keywords: E protein; Coronavirus; Ion channel; Mouse hepatitis virus (MHV); Human coronavirus 229E (HCoV-229E); Infectious bronchitis virus (IBV); Amiloride; Hexamethylene amiloride (HMA); Antiviral compound

Introduction

Coronaviruses (order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus* (Gonzalez et al., 2003)), are enveloped, single-stranded, positive-sense RNA viruses with a genome of about 30 kb. All coronaviruses encode the envelope (E) protein, which is a small hydrophobic membrane protein. While the exact functions and mechanisms of the coronavirus E protein are yet to be fully characterized, the E protein has been shown to be important for coronavirus replication, with roles in viral assembly and morphogenesis (Fischer et al., 1998; Kuo and Masters, 2003; Ortego et al., 2002). In general, while the different coronavirus E proteins share little sequence homology, their basic structures are similar (Shen et al., 2003). All E proteins are small hydrophobic proteins, with a single putative transmembrane α-helix and a hydrophilic C terminal domain (Shen et al., 2003; Siddell, 1995).

Previously, we demonstrated that the E protein from Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) forms cation-selective ion channels in planar lipid bilayers that are about 10-fold more selective for Na^+ than for K^+ ions. The first 40-amino-acid residues of SARS-CoV E protein, encompassing the putative transmembrane (TM) domain, were sufficient for formation of ion channels with similar properties to the full-length peptide (Wilson et al., 2004). This was the first coronavirus E protein shown to belong to the virus ion channel family. Madan et al. (2005) have recently reported that the MHV E protein induces membrane permeability changes in *E.*

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coli and mammalian membranes, indicating that it has viroporin-like activity (Madan et al., 2005). Therefore, it is possible that ion channel formation is a function common to all coronavirus E proteins.

It is not yet known whether the SARS-CoV E protein ion channel activity has an important role in coronavirus replication. However, for the two best characterized virus ion channels – M2 protein from influenza A and Vpu from HIV-1 – ion channel activity has been strongly linked to roles in the virus's life cycles by studies with mutants and channel blocking drugs (Hout et al., 2005a, 2005b; Pinto et al., 1992; Sakaguchi et al., 1996; Schubert et al., 1996). The M2 ion channel activity is inhibited by amantadine and some of its derivatives, and these are currently used in the clinical treatment of influenza A infections (Fleming, 2001; Pinto et al., 1992). We have demonstrated that the Vpu ion channel activity in planar lipid bilayers is blocked by the amiloride derivate HMA, and that HMA also inhibits replication of HIV-1 in cultured human macrophages (Ewart et al., 2002, 2004). For some other viral ion channels, inhibitors have also been discovered (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2005; Premkumar et al., 2004), although evidence of antiviral activity of the compounds has not yet been published. The goals of our research were, therefore, to identify other coronavirus E protein ion channels, find blockers of the channel activity and characterize the effect of channel-blocking compounds on viral replication.

In this paper, we report that E proteins from three coronaviruses – human coronavirus 229E (HCoV-229E, a group 1 coronavirus); mouse hepatitis virus (MHV, group 2), and infectious bronchitis virus (IBV, group 3) – have ion channel activity in planar lipid bilayers. Furthermore, HCoV-229E and MHV E protein ion channel conductance was inhibited by the amiloride derivative HMA, but not by amiloride itself. Channel-blocking activity correlated with inhibition of replication of these viruses, as demonstrated by plaque assays. Conversely, HMA had no antiviral activity against the recombinant MHV with the entire E protein deleted, indicating that E protein is the possible antiviral target.

Results

Peptide characterization

Synthetic peptides corresponding to the E proteins of HCoV-229E, MHV, and IBV, were prepared using solid phase chemistry. The full-length peptides, with predicted molecular weights of 9092.9, 9660.0, and 10475.4 Da, respectively, were excised from SDS-polyacrylamide gels and purified as described in Materials and methods. Western blot analysis of the purified peptides (Fig. 1) showed that all three preparations contained discrete protein bands electrophoresing at approximately the expected size of the full-length products and recognized by the appropriate peptide-specific antibodies. HCoV-229E E protein ran as a single discrete band at about its expected molecular weight, indicating that the sample contained mostly full-length protein and did not contain significantly truncated products. The MHV and IBV E samples both yielded discrete doublet bands on the blots, with in each case the smaller of the two bands running at about the expected molecular weight. It is possible that, due to their highly hydrophobic nature, the proteins may electroporese more slowly than expected, and it may be the larger of the two bands that corresponds to the full-length (or at least very near full-length) protein and the smaller band represents a truncated species. The MHV E protein was detected with an antibody that recognizes an epitope within the first 19 N-terminal amino acids (referred to as, anti-MHV EN). Since, peptide synthesis proceeds from the C-terminus to the N-terminus and because it would require about 6-amino-acid residues to form an epitope to react with the anti-MHV EN antibody, the Western blot indicates the smaller immunoreactive peptide would be truncated at the N-terminus by maximally about 13 amino acids. As predicted from MHV E protein hydropathy plot, the E protein N-terminus consists of about a 14-amino-acid hydrophilic region so even the

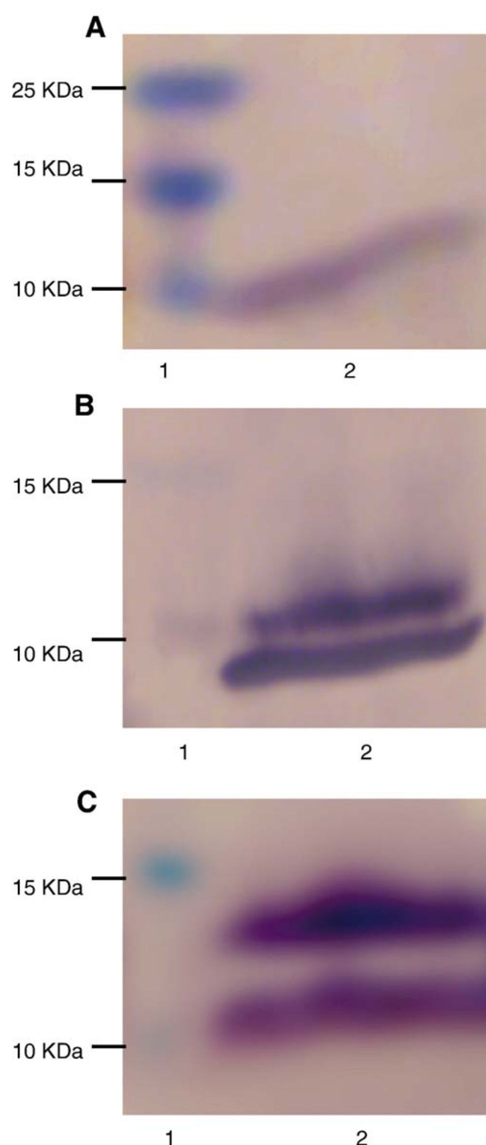

Fig. 1. Western blot analysis of HCoV-229E (group 1), MHV (group 2) and IBV (group 3) synthetic E peptides. Lane 1: molecular weight marker, (A) lane 2: HCoV-229E E peptide, (B) lane 2: MHV E peptide. (C) Lane 2: IBV E peptide.

smaller of the two immunoreactive species would contain an intact TM putative ion channel domain. The IBV E protein also ran as a doublet, and again, it is likely that the larger band represents the full-length product and the smaller band is IBV E protein with several amino acids truncated from the N-terminus. For all three synthetic peptides Western blots confirmed the presence of proteins of at least sufficient length to contain the TM putative ion channel-forming domain.

The E protein from group 1 coronavirus HCoV-229E forms potassium preferring ion channels in planar lipid bilayers

Addition of about 3 μg of purified HCoV-229E E protein to the CIS chamber in the planar lipid bilayer rig resulted in the formation of ion channels in the bilayer. Fig. 2A shows typical current trace in a solution containing a 10-fold gradient of NaCl (500 mM CIS: 50 mM TRANS) at a range of holding potentials. Fig. 2B shows the current–voltage (I–V) plot for this experiment, which reveals a reversal potential of about +26 mV. In nineteen similar experiments where the theoretical equilibrium potential for Na^+ ions was +54 mV, the average measured reversal potential was +22 ± 7 mV (mean ± SEM). This indicates that the channel is weakly selective for Na^+ ions over Cl^- ions. Using ion activities in the Goldman, Hodgkin, and Katz (GHK) equation the P_{Na^+}/P_{Cl^-} ratio was calculated to be 3, indicating that the HCoV-229E E protein ion channel is only 3 times more permeable to Na^+ than Cl^- ions. For these 19 experiments, the average conductance was 19 ± 4 pS, and the maximum conductance measured was 91 pS.

Fig. 2C shows typical current traces for HCoV-229E E protein in asymmetrical KCl solution, in this experiment the K^+ ion current was observed to reverse at +42 mV (Fig. 2D). In thirteen comparable experiments with a theoretical K^+ equilibrium potential of +54 mV, the average measured reversal potential was +38 ± 4 mV. By the GHK equation, HCoV-229E E protein is 12 times more selective for K^+ than for Cl^- ions. For the 13 experiments, the average conductance was 23 ± 5 pS. The ion selectivity for HCoV-229E E protein ion channel is thus, $K^+ > Na^+ > Cl^-$ and the channel is about 4 times more permeable to K^+ than to Na^+ ions (Table 1).

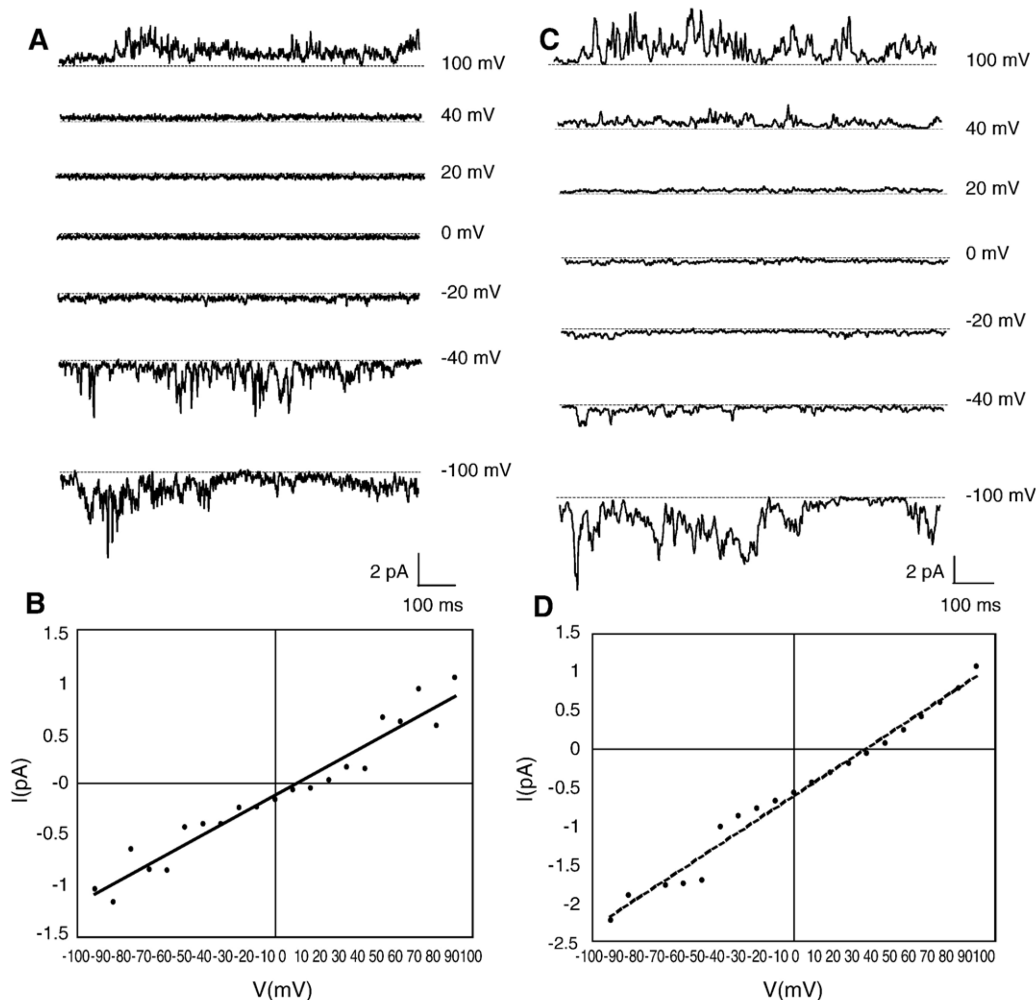

Fig. 2. Group 1 HCoV-229E E protein ion channel activity in planar lipid bilayers. The closed state is shown as a broken line; openings are deviations from the line. The CIS chamber is earthed and the TRANS chamber was held at various potentials between −100 mV and +100 mV, and the data were filtered at 100 Hz. (A) E protein ion channel activity in 10-fold gradient of NaCl (CIS-TRANS). (B) I–V relationship in asymmetrical NaCl solution; average current (I) measured against holding potential (mV). C) E protein ion channel activity in asymmetrical KCl solution. (D) I–V relationship in asymmetrical KCl solution.

Table 1
Relative ion permeabilities of coronavirus E protein ion channels

Source of E protein: parent virus	Taxonomic group	Relative ion permeability		
		$P(Na^+/Cl^-)$	$P(K^+/Cl^-)$	Selectivity series
HCoV-229E	1	3	12	$K^+ > Na^+ > Cl^-$
MHV	2a	138	2	$Na^+ \gg K^+ > Cl^-$
SARS-CoV[a]	2b	90	9	$Na^+ \gg K^+ > Cl^-$
IBV	3	10	3	$Na^+ > K^+ > Cl^-$

[a] See Wilson et al. (2004).

The group 2 coronavirus MHV E protein forms ion channels that are sodium selective

MHV E protein was tested for its ability to form ion channels in planar lipid bilayers, and experiments were done to determine the selectivity of MHV E protein for Na^+ ions over Cl^- ions and for K^+ ions over Cl^-. Fig. 3A shows characteristic current traces for MHV E protein ion channel activity in asymmetrical NaCl solution. In this experiment the ion current reversed at +42 mV (Fig. 3 B). In 14 equivalent experiments, the average reversal potential was +49 ± 1 mV, which is close to the Na^+ equilibrium potential of +54 mV. Thus, the MHV E protein ion channel is highly selective for Na^+ ions and the P_{Na^+}/P_{Cl^-} ratio is 138. For the 14 experiments the conductance varied between 235 and 7 pS, with an average conductance of 61 ± 16 pS.

Fig. 3C illustrates representative current traces of MHV E protein ion channel activity in a 10-fold concentration gradient of KCl. In this experiment, K^+ ion flow was observed to reverse at +25 mV (Fig. 3D), but after eleven similar experiments, the average reversal potential was calculated to be +13 ± 6 mV, indicating that MHV E protein ion channel is only 2 times more selective for K^+ over Cl^- ions. The conductance for the 11 experiments ranged from 60 to 6 pS with an average of 18 ± 5 pS. The ion selectivity order for MHV E protein ion channel was $Na^+ \gg K^+ > Cl^-$, with the channel being about 69 times more permeable to Na^+ than K^+ ions. Thus, the MHV E protein ion channel distinguishes between monovalent cations and is highly selective for Na^+ ions (Table 1).

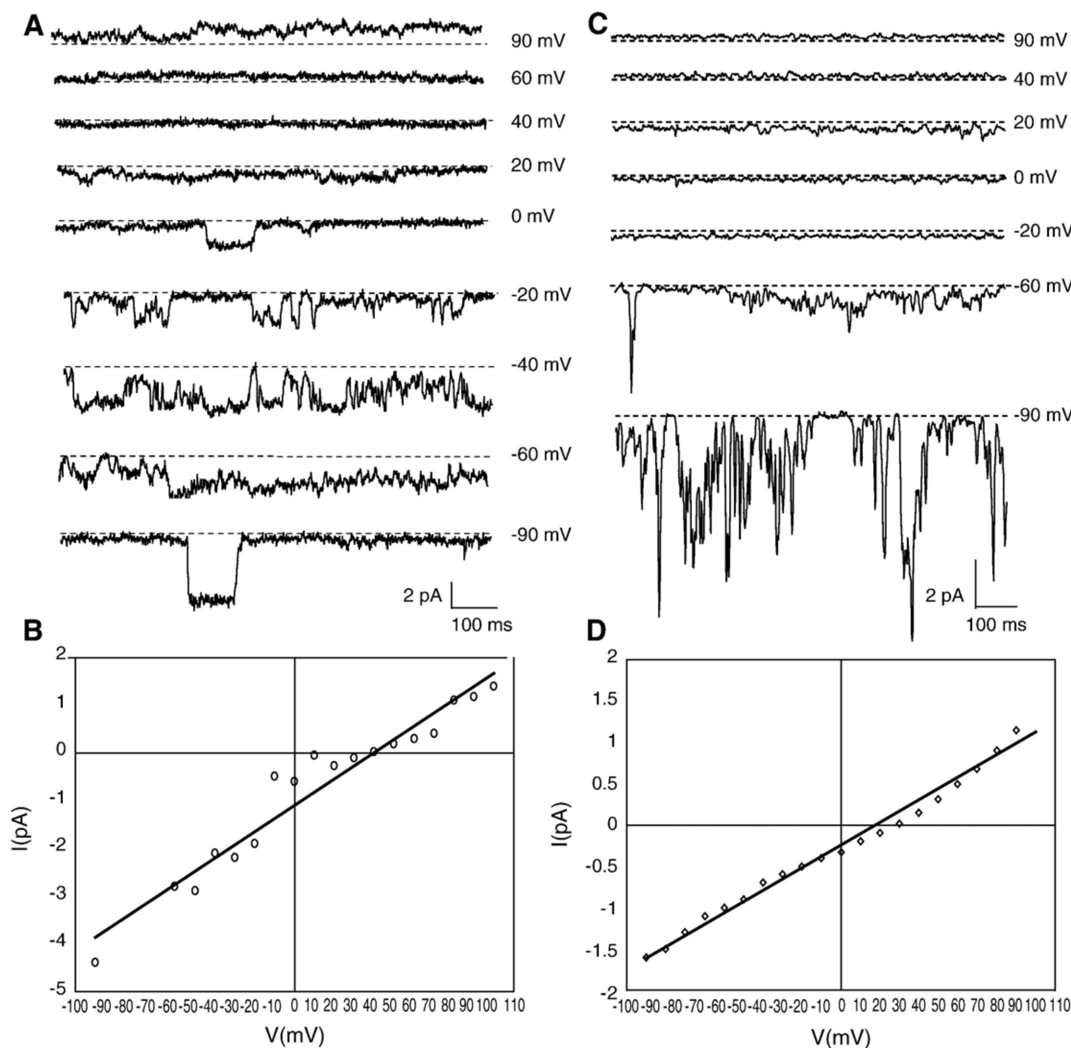

Fig. 3. Group 2 MHV E protein ion channel activity in planar lipid bilayers, as described in the legend to Fig. 2.

The group 3 coronavirus IBV E protein forms ion channels that prefer sodium

IBV E protein also forms ion channels in planar lipid bilayers and experiments conducted in NaCl and KCl solutions demonstrated that IBV E protein channels have a slight preference for Na^+ ions over K^+ ions, similar to E protein channels from the group 2 coronaviruses MHV and SARS-CoV (Wilson et al., 2004). Fig. 4A shows typical current traces of IBV E protein ion channel conductance in asymmetrical NaCl solution. In this experiment, the Na^+ ion flow was observed to reverse at +30 mV (Fig. 4B). In 13 independent experiments, the average reversal potential was +36 ± 5 mV and the IBV E protein P_{Na+}/P_{Cl-} ratio was 10. For the 13 experiments, the conductance was variable, the highest conductance measured was 77 pS, with an average conductance of 24 ± 5 pS. The *I–V* relationship for the IBV E protein in NaCl solution was nonlinear, indicating rectification of the ion channel. The channel conductance was smaller when the TRANS chamber was held at positive potentials relative to the CIS chamber than when the polarity was reversed.

Typical current traces for IBV E protein in asymmetric KCl solution are shown in Fig. 4C; the *I–V* plot indicates that the K^+ ion flow reversed at +28 mV (Fig. 4D). In 20 equivalent experiments, the average reversal potential was +22 ± 3 mV, thus, the IBV E protein was only 3 times more permeable to K^+ than Cl^- ions. The average conductance for the 20 experiments was 22 ± 6 pS. Thus, the IBV E protein ion selectivity series is $Na^+ > K^+ > Cl^-$ and the channel is about 3 times more selective for Na^+ than K^+ ions (Table 1). Rectification of the IBV E protein channel was not seen in KCl solutions.

We currently do not have an explanation as to why the IBV channel shows rectification in Na^+ but not K^+ solutions. Rectification indicates that ions flow more readily through the channel in one direction than the other and the mechanism by which this is achieved in IBV E protein channels is likely to be related to specific properties of the protein quaternary structure forming the gate and selectivity filters of the channel. The in vivo significance of a rectifying channel is that ion conductance will occur preferentially in one direction, i.e., extracellular–intracellular, or vice versa, which could have important implications for the physiological functions of the channel.

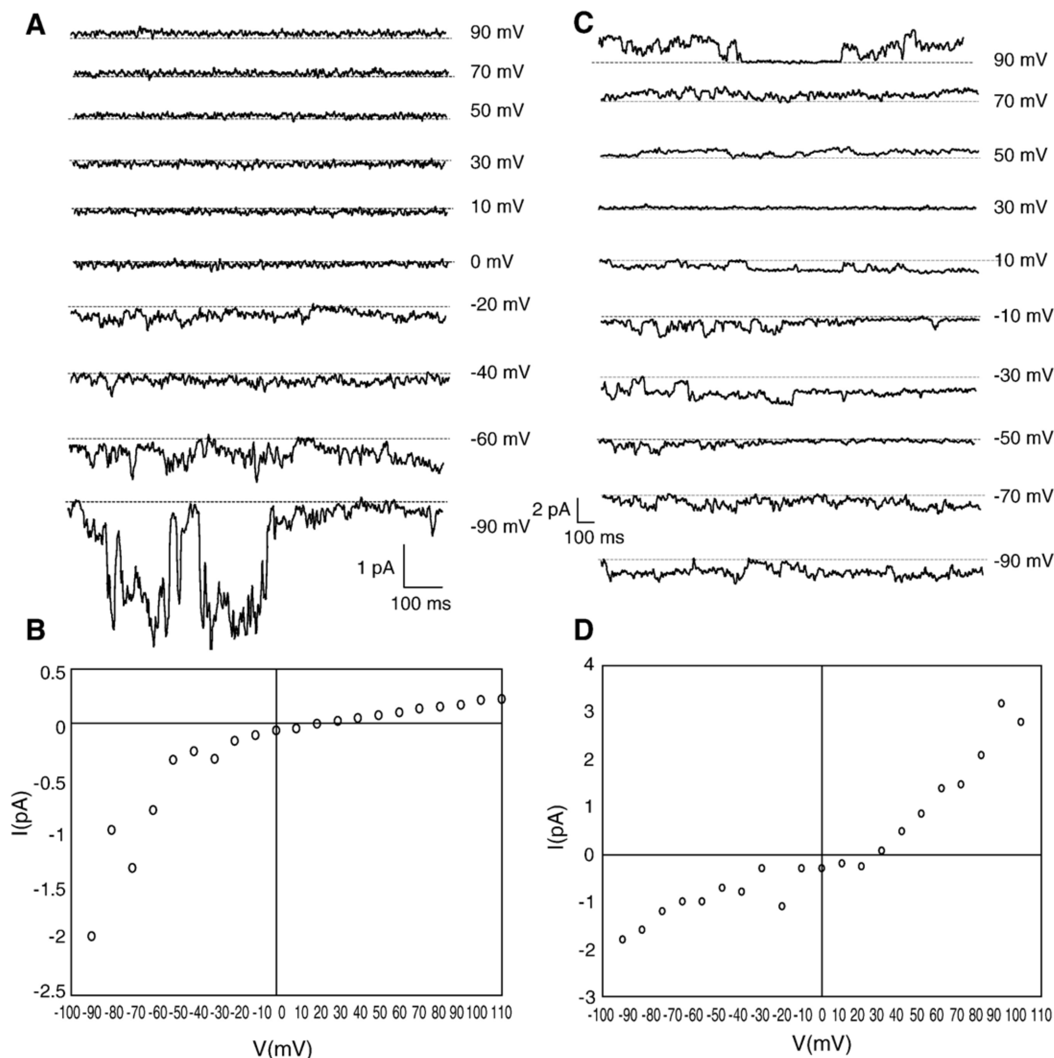

Fig. 4. Group 3 IBV E protein ion channel activity in planar lipid bilayers, as described in the legend to Fig. 2.

Effects of epitope-specific antibodies on E protein ion channel conductance

We have previously shown that ion channel conductance in planar lipid bilayers is dependent on addition of channel-forming peptides and is not due to addition of solvent or buffers alone (Ewart et al., 1996; Premkumar et al., 2004; Wilson et al., 2004). To demonstrate that the ion channel activities we were observing were specifically due to the HCoV-229E or MHV E proteins, rather than a contaminant in samples, purified epitope-specific antibodies recognizing these proteins were utilized. In some previously published cases, such antibodies have been shown to inhibit ion channel activity directly in bilayer experiments. This inhibition depends on binding of the antibody to an exposed epitope and either physical blocking of the ion channel by the antibody or induction of a conformational change in the ion channel structure that inactivates it. However, for many antibodies, simple binding of the antibody may not be sufficient to inactivate the target ion channel. So alternatively, the antibodies can be used to remove the ion channel-forming peptide from solution (Ewart et al., 1996; Melton et al., 2002).

Addition of between 2 and 7 μg of affinity purified antibody, that recognizes the HCoV-229E C-terminal end of the TM domain (anti-HCoV-229E E antibody), to the CIS (n = 8 experiments) or TRANS (n = 12) chambers did not noticeably reduce the HCoV-229E E protein ion channel conductance (data not shown). However, when this antibody was used to remove HCoV-229E E protein from solutions (using a Seize X protein A immunoprecipitation kit) samples of the remaining solution did not form ion channels, even after prolonged periods of stirring for over 15 min (n = 5, results not shown). Depletion of the HCoV-229E E protein from the samples was confirmed by Western blot analysis using the anti-HCoV-229E E polyclonal antibody (data not shown). In contrast, samples containing the purified synthetic HCoV-229E E protein always resulted in ion channel conductance within 15 min of the addition of the peptide to the CIS chamber. Hence, the immunoprecipitation experiments confirmed that the ion channel conductance observed after addition of the purified synthetic HCoV-229E E protein to planar lipid bilayers was dependent on presence of the full-length HCoV-229E E protein.

In the case of the MHV E protein, an antibody directed to the E protein first 19 amino acids of the N-terminus (anti-MHV EN antibody) significantly inhibited ion channel conductance when added to the CIS chamber ($P \ll 0.01$; n = 5, T test to test the difference between the means). These data confirm that the channel-forming species in the samples was the MHV E protein (Fig. 5A1–2). Conversely, addition of the anti-MHV EN antibody to the TRANS chamber did not affect channel conductance (Fig. 5A3–4). This result indicates that the MHV E protein inserts in the bilayer in an orientation-specific manner with the N-terminal domain facing the CIS chamber. Since, the anti-MHV EN antibody significantly inhibited MHV E protein ion channel activity it confirms that the ion channel-forming species contains the MHV E protein N-terminus and is thus likely to be full-length peptide. Another MHV E antibody, the

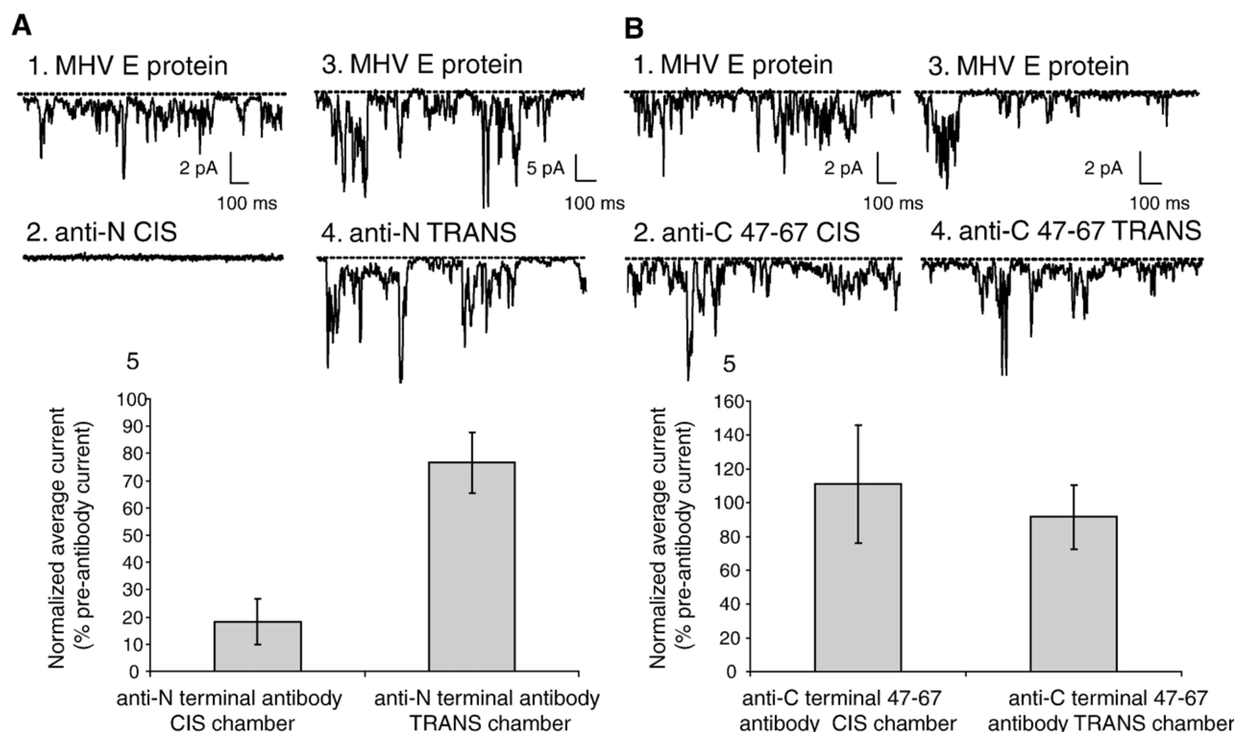

Fig. 5. Antibody treatment of MHV E protein ion channel activity in planar lipid bilayers. (A) Representative current traces at holding potential of −40 mV. (1) MHV E protein ion channel activity before addition of antibody and (2) after addition of anti-MHV EN antibody to the CIS chamber. (3) MHV E protein ion channel activity before addition of antibody and (4) after addition of anti-MHV EN antibody to the TRANS chamber. (5) Normalized average current (percent pre-antibody current) of the MHV E protein ion channel after addition of the anti-MHV EN antibody to the CIS or TRANS chamber. Error bars are SEM. (B) As described in panel A, except anti-MHV C terminal 47–67 antibody was used.

anti-MHV E47–67, which does not bind directly adjacent to the putative TM domain, did not inhibit MHV E protein ion channel conductance when added to the CIS (n = 8) or the TRANS chamber (n = 7) (Fig. 5B1–4). However, similarly to the HCoV-229E E protein, the anti-MHV E47–67 antibody was used to immunoprecipitate the full-length MHV E protein from solution. Western blot analysis of the immunoprecipitate flow-through demonstrated that the MHV E protein had been removed from the solution (data not shown). Addition of the MHV E depleted solution to the CIS chamber did not result in ion channel formation in planar lipid bilayers (n = 5 experiments), even after stirring for up to 1 h and 40 min. These data further demonstrate that the ion channel conductance detected was specifically due to the purified full-length MHV E protein.

Unfortunately, we were not able to obtain enough anti-IBV E protein antiserum to conduct ion channel inhibition and immunoprecipitation experiments with the IBV E protein. Nevertheless, the anti-IBV E antibody directed to the C-terminal was used to demonstrate that the synthetic purified IBV E protein contained full-length peptide (Fig. 1).

HMA inhibits coronavirus E protein ion channels

We tested amiloride, plus its derivative, HMA for their ability to inhibit HCoV-229E, MHV, and IBV E protein ion channels in planar lipid bilayers. Once ion channel conductance was detected, 100–200 μM of compound was added to the CIS chamber while stirring to facilitate binding of the compound to the channel. Fig. 6 demonstrates that HMA significantly reduced HCoV-229E E protein ($P \ll 0.01$, n = 5) and MHV E protein ($P \ll 0.01$, n = 5) current across the bilayer, while, HMA had no significant affect on IBV E protein channel conductance (P = 0.14, n = 10). Amiloride itself did not have a significant effect on any of the coronavirus E proteins ion channel activity (data not shown). Furthermore, addition of the compound solvent, 50% DMSO: 50% Methanol, alone did not affect the E protein ion channel conductances (data not shown), indicating that channel inhibition was dependent on the presence of HMA.

HMA inhibits MHV E protein ion channel activity in a dose-dependent manner

To determine if HMA inhibits MHV E protein in a dose-dependent manner, HMA was titrated on MHV E protein channel activity in planar lipid bilayers. Fig. 7 demonstrates that, with increasing concentration of HMA, there is a subsequent decrease in channel activity. The percent inhibition was plotted against the concentration of HMA and from the equation of the line the effective concentration 50 (EC_{50}) of HMA on MHV E protein channel activity in bilayers was calculated to be 10.2 μM. These data further confirm that the inhibition observed with HMA is specifically due to addition of the compounds and not to spontaneous reductions in the channels macroscopic conductance. Occasionally, channel activity in bilayers can go quiet in the absence of inhibitors, this could be due to the lipids encompassing the peptides, reduction in the macroscopic channel activity or open probability. This spontaneous reduction in

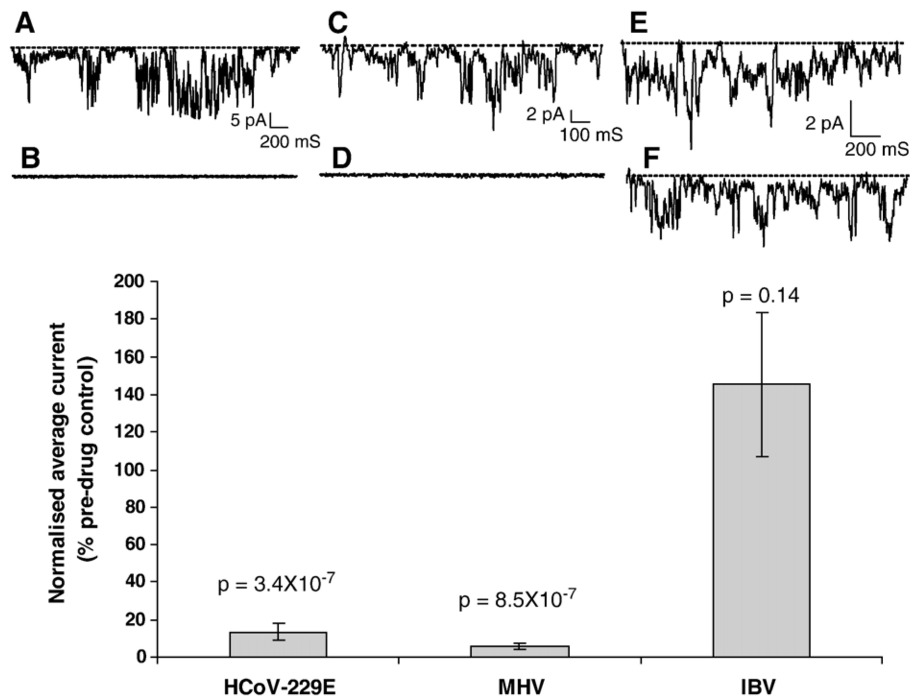

Fig. 6. Coronavirus E protein ion channel activity inhibited by HMA in planar lipid bilayers in asymmetrical NaCl solution. The CIS chamber was earthed, and the TRANS chamber was held at constant voltage. The closed state is shown as a broken line; openings are deviations from the line. (A) HCoV-229E E protein ion channel conductance at −70 mV and (B) after addition of 100 μM of compound to the CIS chamber. (C) MHV E protein ion channel conductance at −40 mV and (D) after addition of 100 μM of compound to the CIS chamber. (E) IBV E protein ion channel conductance at −50 mV and (F) after addition of 100 μM of compound to the CIS chamber. (G) Normalized average current (percent pre-drug control). Error bars are SEM.

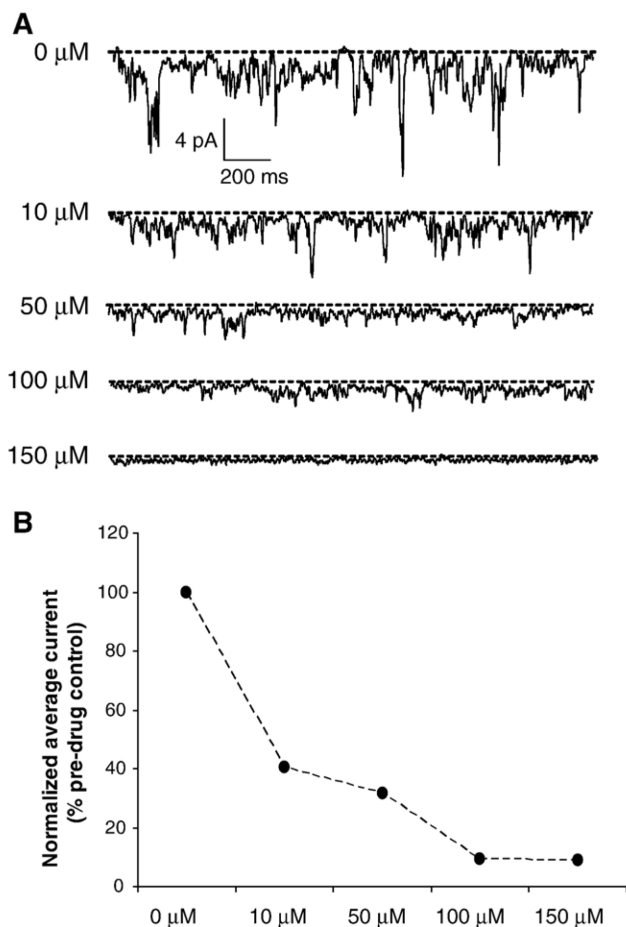

Fig. 7. HMA dose-dependent inhibition of MHV E protein ion channel activity in planar lipid bilayers in asymmetrical NaCl solution. The CIS chamber was earthed and the TRANS chamber was held at constant voltage, −50 mV. The closed state is shown as a broken line; openings are deviations from the line. Scale bar is 4 pA and 200 ms. (A) Addition of increasing concentrations of HMA to the CIS chamber of the bilayer from 10 μM to 150 μM. (B) The measured average currents at the different concentrations of HMA were normalized and data are shown as a percentage of the pre-drug control.

channel activity would not occur in a linear dose-dependent fashion, as observed in Fig. 7A.

HMA, but not amiloride, inhibits MHV replication in cultured cells

To determine if amiloride and HMA inhibit MHV replication in cultured cells, they were tested for their ability to reduce the number of MHV plaques in L929 cells without plaque overlay. MHV infection in L929 cells without plaque overlay, results in the newly emerged virus diffusing from the original infected cell and starting a new plaque. Therefore, in the presence of antiviral compound, there is a reduction in plaque number. L929 cells were infected with MHV at a multiplicity of infection (MOI) of 0.01 and treated with various concentrations of HMA or amiloride (see Materials and methods). The number of plaques per well was counted, and the percent reduction in plaque number was calculated from the no compound control. The percent reduction was plotted against the compound concentration, and the EC_{50} was calculated from the equation of the line. The EC_{50} of HMA on MHV replication was calculated to be 3.91 μM (Table 2), in reasonable agreement with the EC_{50} measured in bilayer experiments. Amiloride did not inhibit MHV replication in cultured cells (Table 2), which correlates with the inability of amiloride to inhibit ion channel activity in bilayers.

HMA inhibits MHV but not MHVΔE replication

To investigate the plaque phenotype of MHV in the presence of HMA, L2 cells with a plaque overlay was used. The plaque overlay on the MHV infected L2 cells slows diffusion of the newly emerged virus, which then infect adjacent cells, resulting in larger plaques than in the L929 cells. HMA was tested on MHV infection in L2 cells at 20 μM, which is significantly above the EC_{50} measured in L929 cells (Table 2), but removed from toxicity (as determined by MTT cytotoxicity assay). To determine if the MHV E protein was the antiviral target, HMA was also tested for its ability to inhibit the recombinant MHV with the entire E protein deleted (MHVΔE). MHVΔE replicates to low titer and has a small plaque phenotype in L2 cells (Kuo and Masters, 2003). Therefore, it is expected if compounds were targeting the MHV E protein, replication of MHVΔE would not be affected. Furthermore, if HMA was targeting the MHV E protein the plaque phenotype of MHV wild-type in the presence of HMA would be comparable to the MHVΔE small plaque phenotype.

Fig. 8 shows that the MHV wild-type virus in mouse L2 cells has a large plaque phenotype of about 3–4 mm in diameter in the absence of antiviral compound (Fig. 8A1). In the presence of 20 μM HMA the plaque size is reduced to about 1 mm (Fig. 8A2), about the same size as the MHVΔE plaques (Fig. 8B1). Conversely, 20 μM of amiloride did not reduce MHV plaque size (Fig. 8A3). Moreover, none of the amiloride derivatives tested notably inhibited MHVΔE plaque formation (Fig. 8B1–3), indicating that MHV E protein is the probable antiviral target.

HMA inhibits HCoV229E replication in cultured cells

HMA, which blocked HCoV-229E E protein ion channel conductance in planar lipid bilayers, also inhibited replication of HCoV-229E in cultured cells (Fig. 8C2). The EC_{50} of HMA on HCoV-229E was calculated to be 1.34 μM (Table 2). Additionally, amiloride, which did not block HCoV-229E E protein ion channel current amplitude, had no antiviral activity on HCoV-229E replication in cultured cells (Fig. 8C3).

Table 2
EC_{50} of HMA on MHV wild-type and HCoV-229E replication in cultured L929 cells

Coronavirus	HMA EC_{50}	Amiloride EC_{50}
MHV wild type	3.91 μM	NA
HCoV-229E	1.34 μM	NA

NA = not active against viral replication.

Discussion

Previously, we reported that the SARS-CoV E protein is a member of the viral ion channel family (Wilson et al., 2004), and in this paper, we extend the list of known coronavirus ion channels by three. Our data demonstrate that the E proteins from HCoV-229E, MHV, and IBV – representative of coronavirus taxonomic groups 1, 2, and 3, respectively – form cation-selective ion channels in planar lipid bilayers. In the cases of HCoV-229E and MHV, epitope-specific antibodies were used to confirm that the channel-forming species in test samples was indeed the purified synthetic E protein. The anti-HCoV-229E E antibody, which binds on the C-terminal side of the transmembrane helix, did not inhibit channel activity in bilayers. However, when this antibody was used to immunoprecipitate the E protein, the residual supernatant did not form ion channels. The anti-MHV EN antibody inhibits the MHV E ion channel activity when added to one chamber (but not the other) of the bilayer rig, indicating that the N-terminus of the peptide is exposed on the CIS side of the bilayer. Further, this shows that the peptides are aligned in a parallel, rather than antiparallel, conformation in the presumed homo-oligomer that forms the ion channel.

Herein, we also show that HMA inhibits the ion channels formed by HCoV-229E and MHV E proteins (see Fig. 6). HMA, an amiloride analogue, also blocks other viral ion channels including; Vpu from HIV-1 (Ewart et al., 1996); p7 from hepatitis C virus (HCV) (Premkumar et al., 2004) and M protein from Dengue (Premkumar et al., 2005). Such broad-spectrum inhibition by HMA suggests that the drug-binding sites of these channels from diverse viruses share structural similarities. Another example of one compound inhibiting different ion channels is amantadine, which blocks both influenza A M2 (Pinto et al., 1992; Sugrue and Hay, 1991), HCV p7 (Griffin et al., 2003) and Dengue M protein channels (Premkumar et al., 2005). HMA does not block the IBV E protein ion channel suggestive of a more divergent structure of this group 3 coronavirus E protein. In this regard, models of the membrane topology of the IBV and MHV E proteins are quite different (Corse and Machamer, 2000; Maeda et al., 2001). The rectified *I–V* plot observed in NaCl solutions (Fig. 4B) is also indicative of unique properties of the IBV E protein channels that warrant further investigation.

For some viral ion channels, like influenza A M2 and HIV-1 Vpu, ion channel activity is known to be important for virus replication (reviewed in Fischer and Sansom, 2002; Gonzalez and Carrasco, 2003). Amantadine's clinical use against influenza A infections is the clearest evidence of this and the mechanism of action of the M2 channel in virus replication has been well characterized. We have shown that HMA – a blocker of the Vpu ion channel – inhibits replication of HIV-1 in cultured human primary macrophages (Ewart et al., 2004), but in this case, the underlying mechanistic links remain to be uncovered. In this manuscript, we report that in addition to blocking E

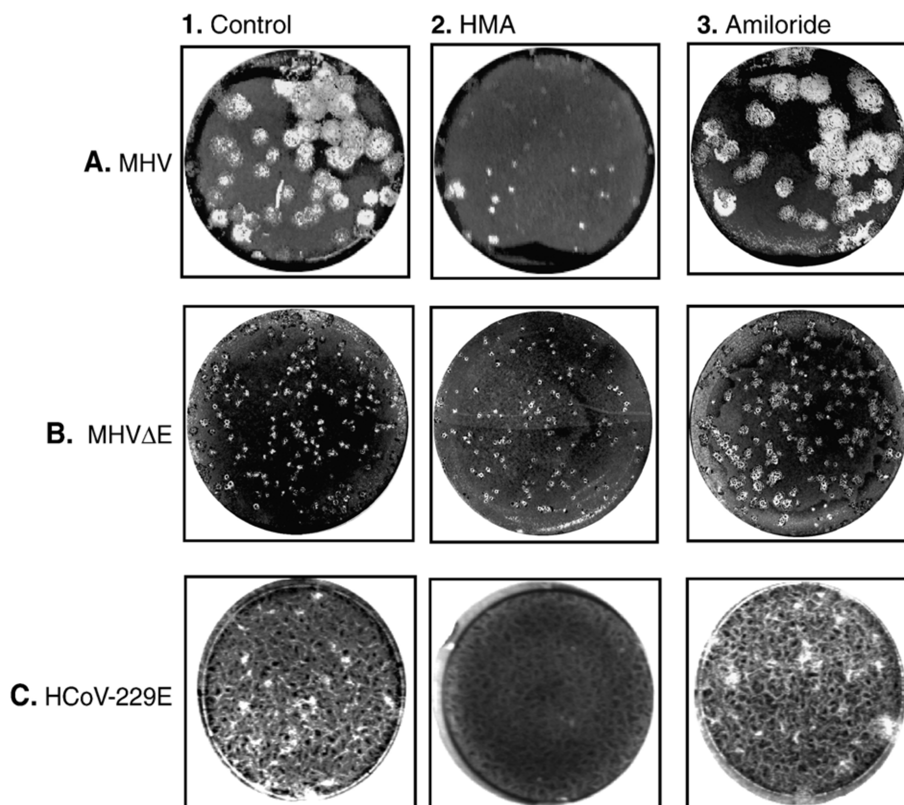

Fig. 8. Plaque assay of amiloride and HMA inhibition of HCoV-229E, MHV, and MHVΔE replication in cultured cells. (A) MHV wild-type in mouse L2 cells. (1) No compound control and with (2) 20 μM HMA or (3) 20 μM amiloride. (B) MHVΔE recombinant virus in mouse L2 cells. (1) No compound control and with (2) 20 μM HMA or (3) 20 μM amiloride. (C) HCoV229E in human MRC-5 cells. (1) No compound control and with (2) 20 μM HMA or (3) 20 μM amiloride.

protein ion channels, HMA also inhibits replication of HCoV-229E and MHV. Conversely, HMA at 20 μM does not inhibit the recombinant virus, MHVΔE, with the entire E protein deleted (see Figs. 6–8). Taken together, the data imply that the antiviral target of HMA is the E protein, and further, that the E protein ion channel activity is important for coronavirus replication. Other groups have previously provided evidence for the importance of E protein for replication of MHV and transmissible gastroenteritis coronavirus (TGEV), a group 1 coronavirus (Kuo and Masters, 2003; Ortego et al., 2002). Deletion of the open reading frame encoding the E protein from the genome of MHV results in a strongly attenuated virus with a small plaque phenotype (Kuo and Masters, 2003). This indicates that, while the E protein has important roles in the MHV life cycle, it is not essential for in vitro replication of MHV. In contrast, the E gene is thought to be essential for replication of TGEV (Ortego et al., 2002).

HMA inhibited MHV replication in cultured cells with an EC_{50} of 3.94 μM and the EC_{50} of HMA on MHV E protein ion channel activity in planar lipid bilayers was calculated to be 10.2 μM. The two EC_{50}s are in good agreement, particularly given the vast differences in the physico-chemical environments the channels are exposed to in the bilayer compared to infected cells and the fact that membrane insertion and channel assembly are not regulated by the same natural processes in the artificial bilayer.

The E protein channels described here have different ion selectivities (see Table 1), but all E proteins tested hitherto prefer monovalent cations over chloride ions. The channel formed by the HCoV-229E E protein (group 1 coronavirus) is about four times more selective for K^+ than Na^+, while the channels from MHV (group 2) and IBV (group 3) prefer Na^+ ions. The two E proteins from the group 2 coronaviruses tested thus far, MHV and SARS-CoV (subgroups 2a and 2b, respectively) are the most similar and have the highest selectivity for sodium. Others have previously noted a relatively high degree of amino acid sequence similarity between MHV and SARS-CoV E proteins, especially in the TM domain (Shen et al., 2003). As more E protein channels are characterized, it will be interesting to see how the channel properties vary with sequence divergence and whether ion selectivity properties co-segregate with taxonomic groupings.

Complementation studies suggest that the E proteins from coronavirus groups 2 and 3 may be functionally more similar than for the group 1 E proteins. In a recombinant MHV (group 2) construct, the IBV E protein (group 3) – as well as the other group 2 E proteins from bovine coronavirus and SARS-CoV – could substitute for the MHV E protein and enhance replication of the recombinant viruses. On the other hand, the TGEV (group 1) E protein could not functionally replace the MHV E protein (Lili Kuo and Paul Masters, personal communication). In similar experiments, group 1 and group 2 E proteins were not able to substitute for each other in the formation of heterologous virus-like particles (VLPs) (Baudoux et al., 1998). It is possible that the functional similarities and differences may be related to the ion channel selectivities of the E protein channels, which we found to be more similar between groups 2 and 3 E channels (prefer Na^+ ions) than group 1 (prefers K^+ ions). Of course, more ion channel selectivity and complementation studies are required to confirm these early observations.

In summary, the data presented here demonstrate that coronavirus E proteins belong to the growing family of virus ion channels. HMA blocks the HCoV-229E and MHV E protein channels, which correlated with its ability to inhibit coronavirus replication in cultured cells. Conversely, HMA did not affect MHVΔE recombinant virus replication in cultured cells. Taken together, the data suggest that E protein ion channel activity is important for coronavirus replication and E protein blockers could have potential anticoronaviral therapeutic use.

Materials and methods

Peptide synthesis and purification

Peptides were synthesized corresponding to the E protein sequences from three coronaviruses in the GenBank database (Table 3). The peptides were synthesized at the Biomolecular Research Facility, John Curtin School of Medical Research, on a SYMPHONY/MULTIPLEX (Protein Technologies inc. Woburn, MS) multiple peptide synthesizer using FMOC chemistry and solid phase peptide synthesis.

The full-length peptides were purified from any truncated products using ProteoPLUS kit (Qbiogene, Inc., CA), following manufactures instructions. Briefly, E peptides were dissolved in loading buffer (60 mM Tris–HCl pH 8.3, 6 M urea, 5% SDS, 10% glycerol, 0.2% Bromophenol blue, and 100 mM β-mercaptoethanol) and approximately 3 μg of the E peptides were run with molecular weight markers (MBI Fermentas, Hanover, MD) on a 4–20% gradient polyacrylamide gels (Gradipore, NSW, Australia). The band corresponding to the full-length peptides, of about 9.1, 9.7, or 10.5 kDa for HCoV-229E, MHV, or IBV E proteins, respectively, were excised from the gel, placed in a ProteoPLUS tube, and subjected to electrophoresis. The purified peptides were dried in a Speedy-Vac and resuspended in 2,2,2-trifluoroethanol (TFE). The peptide concentration was determined by Bradford assay (Pierce, Rockford, IL), following manufacturer's instructions. Attempts to characterize the purified E peptides by Mass spectrometry were unsuccessful, possibly due to the hydrophobic nature of the peptides.

Raising and purifying antibodies against HCoV-229E and MHV E protein

Three synthetic peptides were also made for production of antibodies recognizing the HCoV-229E and MHV E proteins. The sequences of these shorter peptides are indicated as underlined bold-face segments of the full-length sequences in Table 3. The antigenic peptides were chemically synthesized and coupled to a poly-lysine core via their terminal cysteine to prepare multiple antigenic peptides (MAP) (Lu et al., 1991). Individual, New Zealand White rabbits were immunized with the MAP conjugated peptides with initial immunization of 200 μg peptide in Freund's complete adjuvant (Imject®, Pierce, Rockford, IL). Boosters of 200 μg of peptide were given every

Table 3
Synthetic E protein peptides and antigenic peptides

Source virus	Peptide sequence	GenBank accession
HCoV-229E	MFLKLVDDHALVVNVLLWCVVLIVILL**VCITIIKLIKLCFTCHMFC**NRTVYGPIK NVYHIYQSYMHIDPFPKRVIDF	NP_073554
MHV-A59	**MFNLFLTDTVWYVGQIIFIFAVC**LMVTIIVVAFLASIKLCIQLCGL**CNTLVLSPS IYLYDRSKQLY**KYYNEEMRLPLLEVDDI	NP_068673
		AY278741
IBV-Beaudette strain[a]	MTNLLNKSLDENGSFLTALYIFVGFLALYLLGRALQAFVQAADACCLFWYTW VVVPGAKGTAFVYNHTYGKKLNKPEL**ETVINEFPKNGWKQ**	CAC39303

[a] IBV E peptide for raising C-terminal antibody described in Corse and Machamer (2000), J.Virol.

2 weeks in Freund's incomplete adjuvant (Imject®, Pierce, Rockford, IL), until sufficient antibody titers were attained. About 15 ml of blood was collected from the ear vein 2, 4, 6, 8, and 10 weeks post-immunization and antisera prepared. Antiserum was assayed for antibody production by Western blotting with the appropriate full-length E peptide. Epitope-specific antibodies were purified from antisera by SulfoLink™ columns coupled to the equivalent peptide (Pierce, Rockford, IL), following manufactures instructions.

Western blots

For Western blot analysis, samples were prepared in loading buffer (60 mM Tris–HCl pH 8.3, 6 M urea, 5% SDS, 10% glycerol, 0.2% Bromophenol blue, and 100 mM β-mercaptoethanol) and run with molecular weight markers (MBI Fermentas, Hanover, MD) on 4–20% gradient polyacrylamide gels (Gradipore, NSW, Australia). Peptides were transferred to polyvinylidene difluoride membranes (Invitrogen, Vic, Australia), using a semi-dry transfer apparatus (Amersham Biosciences, Vic, Australia). Nonspecific sites were blocked with skim milk proteins in Tris-buffered saline containing 1% Tween-20. Peptides were detected with purified primary antibody to HCoV-229E E protein TM domain C-terminal (anti-HCoV-229E E); MHV E protein N-terminal antibody (anti-MHV EN); and IBV E protein anti-C-terminal antibody (anti-IBV EC) (Corse and Machamer, 2000) (kind gift from Carolyn Machamer, John Hopkins University, Baltimore). The primary antibodies were detected with goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (Dako, NSW). Color development was visualized with Western blue-stabilized substrate for alkaline phosphatase (Promega, NSW, Australia).

Ion channel recording

The purified HCoV-229E, MHV, and IBV E proteins were resuspended to 1 mg/ml in TFE and their ability to form ion channels was tested on a Warner bilayer rig (Warner instruments, Inc. 1125 Dixwell Avenue, Hamden, CT 06514), as described previously (Wilson et al., 2004). Briefly, a lipid mix of 3:1:1 1-palmitoyl-2-oleolyl phosphatidyl ethanolamine:1-palmitoyl-2-oleolyl phosphatidyl serine:1-palmitoyl-2-oleolyl phosphatidyl choline in chloroform were dried under N_2 gas and resuspended in n-decane. Bilayers were painted across a circular hole of approximately 100 μm diameter in a Delrin cup separating aqueous solutions in the CIS and TRANS chambers. For testing E protein selectivity for Na^+ and Cl^- ions, the solution in the CIS chamber consisted of 500 mM NaCl, and 5 mM HEPES (pH 7.2), while the solution in the TRANS chamber contained 50 mM NaCl and 5 mM HEPES (pH 7.2). For testing E protein selectivity for K^+ and Cl^- ions, the solution in the CIS chamber consisted of 500 mM KCl and 5 mM HEPES (pH 7.2), while the solution in the TRANS chamber contained 50 mM KCl and 5 mM HEPES (pH 7.2). Currents were amplified using a Warner model BD-525D amplifier with sampling rate of 5 kHz and filtered at 1 kHz before being digitally recorded directly using

the Data Collect software developed by Mr. Bernie Keys (BioResearch Electronics, Canberra, Australia).

Small aliquots (about 3 μg) of the E peptides dissolved in TFE were added to the CIS chamber, while stirring to facilitate spontaneous insertion of the hydrophobic peptides into the lipid bilayer. Typically, when present, ion channel activity was detected after about 5–15 min of stirring. The CIS chamber was earthed and the TRANS chamber was held at a range of potentials between +100 mV to −100 mV. The voltage (V_m) vs. the current (I) were plotted (I–V plot) to determine the reversal potential (V_r), which indicates the ion selectivity of the channel. The theoretical V_r for a Na^+ or K^+ selective channel in our experimental set up was +54 mV, whereas the theoretical V_r for a Cl^- channel was −54 mV (using activities in the Nernst equation). The V_r was used in the Goldman–Hodgkin–Katz (GHK) equation to determine the relative permeability ratios (P_{Na^+}/P_{Cl^-} or P_{K^+}/P_{Cl^-}) of the ion channel.

Epitope-specific antibody inhibition of E protein ion channel activity

To determine antibody blocking, after ion channel activity was detected, affinity-purified polyclonal antibody was added to the CIS or TRANS chamber to a final concentration of about 50–100 μg/ml, while stirring for 30 s. Bilayer currents were recorded before ion channel formation, after ion channel formation, and after the addition of the purified antibody. A *T* test (Microsoft Excel) was used to determine if there was significant difference between the mean current recorded before and after addition of the epitope-specific antibody.

Affinity purified anti-HCoV-229E E and anti-MHV E47–67 antibodies were used to immunoprecipitate the HCoV-229E and MHV E proteins, respectively, using the Seize X protein A immunoprecipitation kit (Pierce, Rockford, IL), following manufactures instructions. Briefly, the antibodies were bound and cross-linked to protein A column and 1 mg/ml of corresponding E protein was immunoprecipitated, from the solution. The flow-through, which should not contain any E protein, and eluate, which contains the immunoprecipitated E protein were analyzed by Western blot. The HCoV-229E and MHV E protein flow-through were tested for their ability to form ion channels in planar lipid bilayers as described above.

Amiloride derivative inhibition of E protein ion channels

Stock solutions of amiloride and HMA (Sigma, Australia) at 50 mM were prepared in 50% DMSO:50% methanol. To determine if the amiloride derivatives blocked the E proteins ion channel conductance in planar lipid bilayers, after ion channel currents were detected, 100–200 μM of compound was added to the CIS chamber while stirring to facilitate binding of the compound to the channel. The current across the bilayer was recorded prior to addition of the E protein, after detection of ion channel conductance, and after addition of the compound. A *T* test (Microsoft Excel) was used to test the difference between the normalized mean currents before and after addition of the compounds.

Viruses and cells

Mouse L2, L929, and 17Cl1 cells lines (ATCC) were grown in DMEM (Invitrogen, Vic, Australia) supplemented with 10% FCS (Invitrogen, Vic, Australia). MHV-A59 (ATCC) and MHV with the entire E protein deleted (referred to as MHVΔE) (kind gift from Paul Masters, Wadsworth centre, Albany), were amplified in 17Cl1 cells. The MHV wild-type and MHVΔE recombinant virus were plaque assayed in L2 cells with plaque overlay for observation of plaque phenotype or assayed in L929 cells without overlay for the easy counting of plaque numbers.

Human MRC-5 cell lines (ATCC) were grown in DMEM (Invitrogen, Vic, Australia) supplemented with 10% FCS (Invitrogen, Vic, Australia). The HCoV-229E (ATCC) was two times plaque purified in MRC-5 cells and plaque assayed in MRC-5 cells with plaque overlay.

Antiviral plaque assay

For determining if HMA or amiloride had antiviral activity on MHV and for calculating the compounds EC_{50} the plaque assay was done in L929 cells, without plaque overlay. MHV infection in L929 cells without plaque overlay results in the newly emerged plaques disseminating from the original infected cell and starting a new plaque. Therefore, in the presence of antiviral compound, there is a reduction in plaque number, which can easily be counted. The L929 cells were infected with MHV wild-type at a multiplicity of infection (MOI) 0.01 for 1 h at 37 °C in 5% CO_2, then 10 μM, 7.5 μM. 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.3125 μM, or 0 μM of amiloride or HMA were added. After 24-h incubation at 37 °C in 5% CO_2, the culture media were removed, and the cells were stained with 0.1% crystal violet in 20% methanol. The plaque number per well was counted, and the percent reduction was calculated from the no compound control. The compound concentration was plotted against the percent reduction in plaques, and the EC_{50} was calculated from the equation of the line.

The plaque phenotype of MHV wild-type or MHVΔE in the presence or absence of antiviral compound was studied in mouse L2 cells with plaque overlay (1% Seaplaque in MEM, 10% FCS). The L2 cells were plated in 6-well plates and grown to confluence, then infected with MHV wild-type at a MOI of 0.01 or MHVΔE (MOI 0.1) for 1 h. Higher MOI of MHVΔE was used so that the number of plaques per well were comparable between the MHV wild-type and MHVΔE, which has a lower titration. After 1-h incubation, the virus was removed and replaced with 1% seaplaque overlay in MEM supplemented with 10% FCS and 20 μM or 0 μM (no compound control) of test-antiviral compound dissolved in 50% DMSO: 50% methanol. After 48-h incubation at 37 °C in 5% CO_2, the cells were stained with 0.1% crystal violet in 20% methanol and the average plaque size determined.

HCoV-229E was plaque assayed in human MRC-5 cells. The MRC-5 cells were grown to confluence in 6-well plates and

then infected with HCoV-229E at a MOI of 0.01 for 1 h at 35 °C in 5% CO_2. After 1-h incubation 5 μM, 2.5 μM, 1 μM or 0 μM (no compound control) of test-antiviral compound dissolved in 50% DMSO: 50% methanol was added to each well. The assay was incubated for 6 days at 35 °C in 5% CO_2, until plaques were visible and then stained with 0.1% crystal violet in 20% methanol.

The concentrations of the compounds used in the antiviral assays were not significantly toxic as determined by the MTT cytotoxicity assay (Sigma, Australia), following manufactures instructions. Note, that for the MRC-5 cells the compounds were found to be considerable more toxic, which is most likely due to the elongated nature of the MRC-5, resulting in greater absorbance of the compounds. Therefore, the amiloride derivatives in the HCoV-229E plaque assay were used at a lower concentration, which was not toxic for the MRC-5 cells.

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