Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride

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Received 13 October 2003; revised 18 November 2003; accepted 19 November 2003

First published online 16 December 2003

Edited by Maurice Montal

Abstract A 63 residue peptide, p7, encoded by hepatitis C virus was synthesised and tested for ion channel activity in lipid bilayer membranes. Ion channels formed by p7 had a variable conductance: some channels had conductances as low as 14 pS. The reversal potential of currents flowing through the channels formed by p7 showed that they were permeable to potassium and sodium ions and less permeable to calcium ions. Addition of Ca^+ to solutions made channels formed by p7 less potassium- or sodium-selective. Hexamethylene amiloride, a drug previously shown to block ion channels formed by Vpu encoded by HIV-1, blocked channels formed by p7. In view of the increasing number of peptides encoded by viruses that have been shown to form ion channels, it is suggested that ion channels may play an important role in the life cycle of many viruses and that drugs that block these channels may prove to be useful antiviral agents.

Key words: Cation-selective ion channels; Hepatitis C virus; p7; Hexamethylene amiloride

1. Introduction

Hepatitis C virus (HCV) is the major cause of non-A and non-B hepatitis. It can eventually cause chronic liver diseases such as cirrhosis, liver failure and liver cancer. Approximately 10 000 to 12 000 deaths occur in the USA per year as a result of HCV infections. The current treatment for HCV is a combination of pegylated α-interferon and a broad spectrum antiviral agent, ribavirin. This treatment, although effective in some cases, can lead to the emergence of resistant viruses [1,2]. There are no effective vaccines against the virus mainly because of the large number of genotypes and variants of the virus.

HCV belongs to the family Flaviviridae and genus Hepacivirus with six major genotypes and approximately 100 subtypes depending on the geographic distribution of the virus. It is a small, enveloped virus containing a positive-sense single-stranded RNA that encodes a polyprotein of 3010–3033 amino acids. The polyprotein is co-translationally or post-translationally cleaved proteolytically into 10 mature proteins. The functions of the various proteins are poorly understood. They include three structural proteins (core, E1 and E2) and six non-structural proteins (NS2–NS5) [3,4]. Located between the structural (E2) and non-structural regions (NS2) of the polyprotein [5,6], and generated by the partial processing of an E2-p7 precursor, is a small membrane protein p7. P7 has been shown to be essential for the production of infectious virus but it does not affect RNA replication [7,8].

P7 is a small hydrophobic protein containing 63 amino acids. The protein is a polytopic membrane protein [9] found in the endoplasmic reticulum and plasmalemma of host cells. It has two hydrophobic segments spanning amino acids 19–32 and 36–58 that are thought to be embedded in the membrane. The N- and C-termini are exposed to the extracellular environment [9]. The C-terminal half of p7 has features typical of a signal peptide while the N-terminal membrane-spanning region looks as though it might form an ion channel. We decided therefore to examine whether the p7 protein of HCV formed an ion channel. While this work was in progress, two other papers have appeared showing that p7 forms an ion channel in lipid bilayers [10,11] but the selectivity of the ion channel was not investigated. It was also found that amantadine [10] and long-alkyl-chain iminosugar derivatives [11] could block the p7 channel. We report here that channels formed by p7 are cation-selective at normal pH but can become less cation-selective when solutions contain calcium ions. In addition, we report that an amiloride derivative, hexamethylene amiloride (HMA), which blocks Vpu ion channels [12], also blocks p7 channels.

2. Materials and methods

2.1. Peptide synthesis

The full-length p7 peptide of HCV H77 1a genotype (ALENLVILNAASLAGTHGLVSFLVFFCFAWYLKGRWVPGAVYAFYGMWPLLLLLLALPQRAYA) was synthesised using the Fmoc method. The synthesis was done on a Symphony Peptide Synthesiser from Protein Technologies Inc. (Tucson, AZ, USA). Rink Resin was used to give C-terminal amides and the coupling was done with HBTU and hydroxybenzotriazole in *N*-methylpyrrolidone. Each of the synthesis cycles used double coupling and a four-fold excess of the amino acids. Temporary α-N Fmoc-protecting groups were removed using 20% piperidine in dimethylformamide. The synthesised p7 was characterised by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (Bruker Daltonics, Billerica, MA, USA) and showed a clear peak at a *m/z* ratio of 7018.4, corresponding to the full-length peptide. There were no other significant peaks in the region above a *m/z* of about 5000. Other peaks were evident in the lower *m/z* range, many of which could be attributed to the matrix substrate used, while others presumably reflect the presence of molecules derived from the peptide synthesis reaction. Given the predominance of the peak corresponding to the full-length p7 peptide, we chose to use the preparation without further purification for examination of ion channel formation.

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0014-5793/03/$30.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01453-4

2.2. Ion channel recording

Lipid bilayer studies were performed as described elsewhere [13,14]. A lipid mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabaster, AL, USA) was prepared on the day of an experiment. The lipid mixture was painted onto an aperture with diameter of 150–200 μm in the wall of a 1 ml delrin cup. The aperture separated two chambers, *cis* and *trans*, both containing salt solutions at different concentrations. The *cis* chamber was connected to ground and the *trans* chamber to the input of an Axopatch 200 amplifier. Normally the *cis* chamber contained 500 mM KCl plus 10 mM HEPES (pH 7.4) and the *trans* 50 mM KCl plus 10 mM HEPES (pH 7.4). All solutions used contained either HEPES or MES to buffer pH. The bilayer formation was monitored electrically by the amplitude of the current pulse generated by a linear voltage ramp (1 V/s). Potentials are expressed *trans* chamber with respect to *cis*. P7 and drugs were added to the *cis* chamber and stirred until channel activity or a change in channels was seen. Currents were filtered at 1 kHz, digitised at 2 kHz and stored on magnetic disk. Reversal potentials were determined either from current–voltage (*I–V*) curves (Fig. 2) or by filtering currents at 200 or 500 Hz and closely scanning current direction with 10–20 mV changes in potential around the potential at which currents reversed direction. If single channel currents were large, variable and not well-defined (as was generally the case), we found this method much more accurate than fitting curves to *I–V* relationships and determining the reversal potential from where the curve crosses the abscissa. When plotting current amplitude against potential (e.g. in Fig. 2) two methods were used. For small, well-defined single channel currents, single channel current amplitude was measured directly (*I* (pA), Fig. 2A). Where currents were large and had several levels, the mean current was measured over a period of 10–30 s (*I′* (pA), Fig. 2B,C). This was obtained by integrating all the current data points and dividing by the number of points.

The p7 peptide was dissolved in 2,2,2-trifluoroethanol (TFE) at 10 mg/ml (1.4 μM). Generally, 10 μl of this was added to the *cis* chamber of the bilayer which was stirred. Channel activity was normally seen within 15–30 min and stirring was then stopped. In 13 control experiments, no channel activity was observed after 10 μl TFE was added to the *cis* chamber for periods of 10–20 min.

2.3. Hexamethylene amiloride

A stock solution of HMA was made by dissolving HMA in dimethyl sulphoxide (DMSO) at a concentration of 500 mM. This solution was diluted to 50 mM HMA using 0.1 M HCl. Normally, 2 μl of the 50 mM HMA was added to the *cis* chamber after channel activity was seen. The *cis* chamber contained 1 ml of solution making the final concentration of HMA 100 μM. The final concentration of DMSO was 0.02% and this concentration does not form channels nor adversely affect bilayer integrity or stability.

3. Results

3.1. P7 forms cation-selective ion channels

Initial experiments were done with 500 mM KCl in the *cis* chamber and 50 mM KCl in the *trans* chamber. When the p7 peptide was added to the *cis* chamber and stirred, channel activity was recorded in 38 of 42 experiments. Generally, large currents with variable amplitude were recorded, as has been reported by others [10,11]. Occasionally, much smaller channel currents were recorded as illustrated in Fig. 1 in which the potential in the *trans* chamber was −80 mV and the currents are downwards. In this experiment, currents reversed at +44.3 mV (Fig. 2A), close to the potassium equilibrium potential in these solutions (+53 mV, using activities in the Nernst equation), indicating that the channels were potassium-selective. An all-points histogram from a longer current record (14.2 s) at this potential in the same experiment is shown in Fig. 1B. The amplitude of the open-channel peak is 1.7 pA. The *I–V* relationship in this experiment is shown in Fig. 2A. The line fitted to the data points crosses the abscissa at +44.3 mV

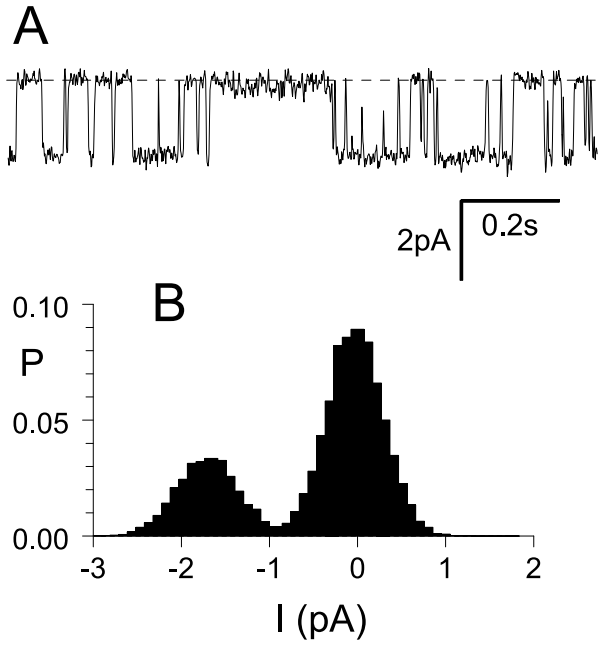

Fig. 1. A: Currents recorded after addition of p7 to the *cis* chamber (KCl, 500 mM *cis*, 50 mM *trans*). Downward currents shown were recorded at a potential of −80 mV. The broken line shows the zero (closed channel) current level. B: All-points histogram of the current shown in A recorded for 14.2 s.

and its slope is 14 pS indicating a channel conductance of 14 pS.

In most experiments, currents seen after addition of p7 to the *cis* chamber were much larger. Records of large currents recorded over a range of potentials in another experiment are shown in Fig. 3A. The currents were upwards at +20 mV and downwards at +60 mV, reversing close to +40 mV. An *I–V* curve of mean currents recorded with 10 mV steps in potential in another similar experiment is shown in Fig. 2B. The current reversed at +35.5 mV and the slope of the fitted line was 11.2 pS. In 10 similar experiments, the average reversal potential was +34.0 ± 2.2 mV (mean ± 1 S.E.M.) indicating that the channels were about seven times more permeable to K^+ than to Cl^-.

Similar results were obtained when both chambers contained NaCl. After addition of p7 to the *cis* chamber and stirring, ion channel activity was seen in 12 of 16 bilayers. Currents recorded in an experiment when the *cis* chamber contained 500 mM NaCl and the *trans* chamber 50 mM NaCl are shown in Fig. 3B. In this experiment, currents reversed between +30 and +50 mV, closer to the Na^+ equilibrium potential (+53 mV) than the Cl^- equilibrium potential (−53 mV), indicating that channels were much more permeable to Na^+ than to Cl^-. The mean reversal potential measured in five experiments was +38.0 ± 2.0 mV indicating that the channels were about 10 times more permeable to Na^+ than to Cl^-.

Currents were generated by p7 in seven of eight bilayers when the *cis* chamber contained 150 mM $CaCl_2$ and the *trans* chamber contained 50 mM $CaCl_2$. Mean current is shown plotted against potential in one of these experiments (Fig. 2C). The reversal potential was −13.4 mV and the slope of the fitted line was 10.5 pS. In two other similar experiments in which potential was changed over a wide range and the re-

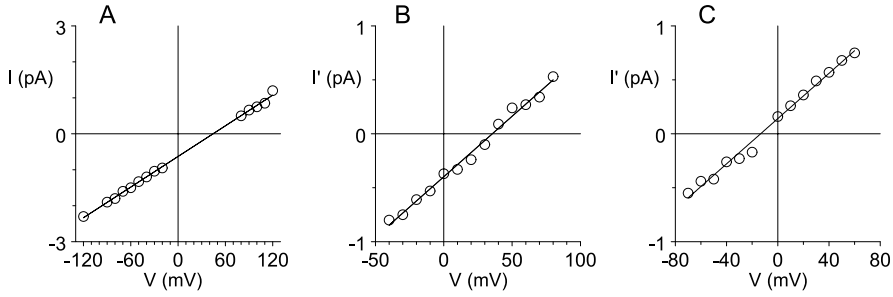

Fig. 2. Ion selectivity of channels formed by p7. A: Plot of the amplitude of currents recorded in the experiment illustrated in Fig. 1 (ordinate) against potential (*trans* with respect to *cis*, abscissa) across the bilayer. The line is the best fit (least squares) of a straight line to the data points. The slope is 14 pS and current was zero at +44.3 mV. B: Plot of mean current generated by p7 against potential (*cis* 500 mM KCl, *trans* 50 mM KCl) in an experiment in which currents were larger than in Fig. 1. The line is the best fit (least squares) of a straight line to the data points. The slope is 11.2 pS and current is zero at +35.5 mV. C: Plot of mean current generated by p7 against potential (*cis* 150 mM $CaCl_2$, *trans* 50 mM $CaCl_2$). The line is the best fit (least squares) of a straight line to the data points. Current reversed at −13.4 mV and the slope of the line is 10.5 pS.

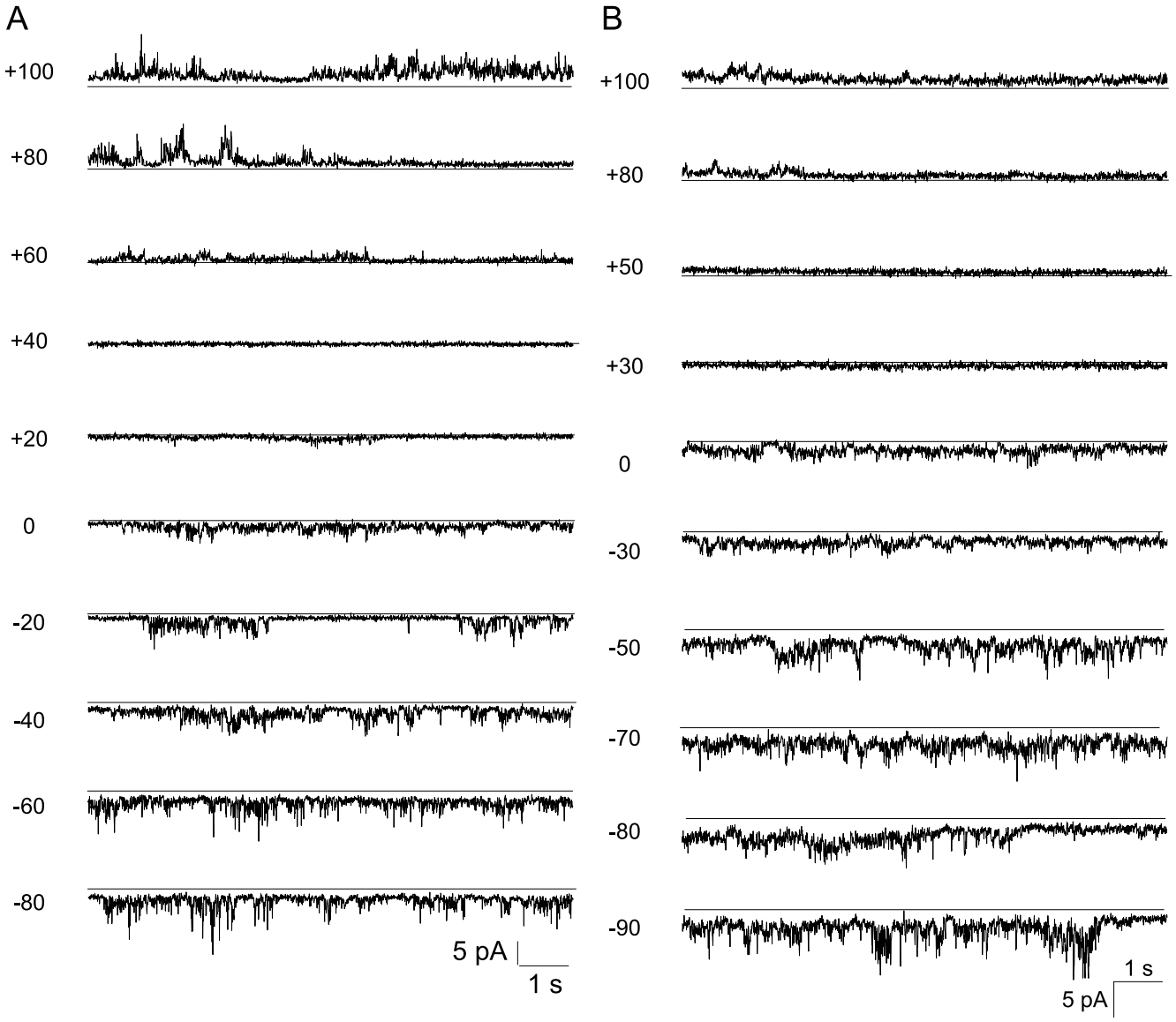

Fig. 3. Currents recorded over a range of potentials in two bilayers after addition of p7. A: The *cis* chamber contained 500 mM KCl and the *trans* chamber contained 50 mM KCl. B: The *cis* chamber contained 500 mM NaCl and the *trans* chamber contained 50 mM NaCl. The potential during each trace is shown at the left of the trace.

versal potential could be clearly defined, reversal potentials were −15.2 and −7.1 mV. These negative reversal potentials indicate that the ratio of cation to anion permeability is less with Ca^{2+} than with K^+ or Na^+. In other experiments, we used a set of solutions in which Ca^{2+} concentration was kept low while Cl^- concentration was relatively high. The *cis* chamber contained 30 mM choline chloride and 10 mM $CaCl_2$ while the *trans* chamber contained 50 mM $CaCl_2$. After adding p7 to the *cis* chamber and stirring, channel activity was seen in 10 of 12 bilayers. In eight of these experiments, currents reversed between 0 and +5 mV, between the Cl^- equilibrium potential (+17.4 mV) and the Ca^{2+} equilibrium potential (−20.3 mV). Finally, in six experiments in which the *cis* chamber contained 500 mM KCl and the *trans* chamber contained 50 mM KCl, the reversal potential of currents generated by p7 was shifted to less positive potentials in each case when 10 mM $CaCl_2$ was added to the *cis* chamber. The average reversal potential in the six bilayers shifted from +32 ± 4.0 mV to +15 ± 5.6 mV indicating that the ratio of potassium to chloride permeability had decreased from 6.0 to 2.2. This could only be caused by a decrease in the ratio of potassium to chloride permeability. We concluded that in solutions containing Ca^{2+}, channels were much less cation-selective and that significant current could be carried by chloride ions.

3.2. The p7 channel is blocked by HMA

We have previously reported that channels formed by the HIV-1 protein, Vpu, can be blocked by HMA [12]. We found that the channels formed by the p7 peptide were also blocked by HMA, as illustrated in Fig. 4. The traces in Fig. 4A were obtained at 0 mV: the *cis* chamber contained 500 mM KCl and the *trans* 50 mM KCl. Addition of the p7 peptide produced the channel activity shown in Fig. 4A. Addition of 2 μl of 50 mM HMA to the *cis* chamber followed by stirring resulted in suppression of the channel activity as illustrated in the traces in Fig. 4B. The HMA did not completely block the channel activity and this was more often observed with larger currents. Inhibition of channel activity by 100 μM HMA produced by the p7 peptide was recorded in all nine out of nine experiments. HMA (500 μM) also blocked channels conducting sodium ions (500 mM *cis*/50 mM *trans*) in two out of two experiments.

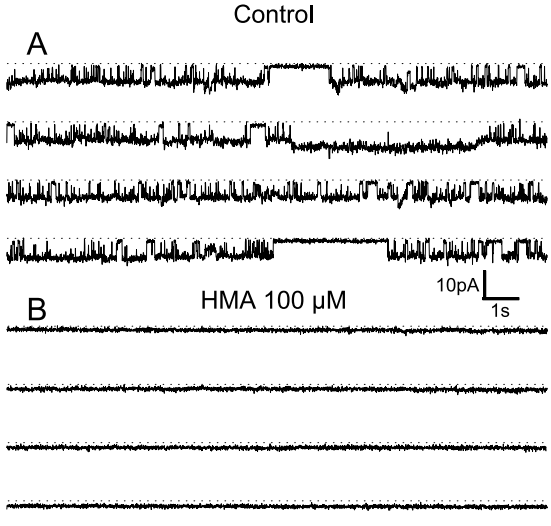

Fig. 4. Block of currents by HMA ($V = 0$ mV). A: Current traces recorded after addition of p7 to the *cis* chamber containing 500 mM KCl. The *trans* chamber contained 50 mM KCl. B: Current traces recorded after HMA had been added to the *cis* chamber and stirred.

4. Discussion

We have confirmed that HCV p7 forms ion channels in planar lipid bilayer membranes [10,11]. As found previously, the channels had a very variable conductance and this is generally found when channel-forming virus proteins are inserted into planar bilayer membranes [13,15–19]. We assume that the larger channels were caused by aggregates of p7, either because aggregation of the p7 peptide leads to a larger ion channel or the aggregated ion channels can open synchronously (for a review, see [20]). It is sometimes possible, by adding very small amounts of protein and stopping stirring when channels are first seen, to obtain much smaller single channel currents [16] (Fig. 1) that we assume to be the unitary channels.

We have extended the recent studies of p7 ion channel activity [10,11] to show that the p7 channels are selective for monovalent cations (Na^+ and K^+) over monovalent anions (Cl^-). In the previous work, *cis* and *trans* chambers contained the same solutions so that it was not possible to determine ion selectivity of the channels. Our results suggest that calcium reduces the cation selectivity of channels. When the Ca^{2+} concentrations in the *cis* and *trans* chambers were 150 mM and 50 mM, respectively, currents reversed between −7 and −15 mV, closer to the Cl^- equilibrium potential of −28 mV than the calcium equilibrium potential of +14 mV: the channels appear to be more permeable to Cl^- than to Ca^{2+} but they must still be conducting Ca^{2+}. In other experiments, when 10 mM $CaCl_2$ was added to a *cis* chamber containing 500 mM KCl, the reversal potential shifted towards the Cl^- equilibrium potential indicating that channels had become either more permeable to Cl^- or less permeable to K^+. We assume that the calcium ions interact with p7 and modify the structure of the channels, affecting the selectivity filter. A similar conversion of cation-selective channels formed by the influenza B virus protein to anion-selective channels at low pH has been reported previously [13].

HMA, a drug that blocks HIV-1 Vpu ion channels and depresses budding of virus-like particles in cells expressing HIV-1 Gag and Vpu [12], also blocks the ion channels formed by p7. Therefore, it is possible that there is some similarity between the binding sites for HMA in Vpu and p7, though there is little sequence identity between the two peptides.

There is now an increasing list of virus proteins that have been shown to form ion channels in lipid bilayers or cells, including some influenza virus proteins, M2 [15,21] and NB [22], HIV-1 proteins Vpr [17] and Vpu [16,18] and the 6K protein of alphaviruses [19] and now HCV p7. It has been suggested that these virus ion channels be called 'viroporins' [23] but the term suggests large conductance, non-selective channels like porins in bacterial membranes. The virus ion channels are quite different. They can select between cations, between anions and cations, and can have a very low conductance as shown in Fig. 1 and Fig. 2A.

It seems likely that formation of ion channels may be an important role in the life cycle of viruses that encode them and that drugs that block these ion channels may affect virus replication.

Acknowledgements: We are grateful for support from the Australian Research Council.

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