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Dengue Virus M Protein C-Terminal Peptide (DVM-C) Forms Ion Channels

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Abstract. A chemically synthesized peptide consisting of the C-terminus of the M protein of the Dengue virus type 1 strain Singapore S275/90 (DVM-C) produced ion channel activity in artificial lipid bilayers. The channels had a variable conductance and were more permeable to sodium and potassium ions than to chloride ions and more permeable to chloride ions than to calcium ions. Hexamethylene amiloride (100 μM) and amantadine (10 μM), blocked channels formed by DVM-C. Ion channels may play an important role in the life cycle of many viruses and drugs that block these channels may prove to be useful antiviral agents.

Key words: DVM-C — Virus ion channels — Dengue — Amantadine — HMA

Introduction

The dengue virus belongs to the family of Togoviruses and the subgroup Flaviviridae. The Flaviviruses are a family of at least 66 viruses, 29 of which cause human diseases including dengue, yellow fever, Murray Valley encephalitis and Japanese encephalitis. Dengue occurs mainly in tropical areas of Asia, Oceania, Africa, Australia and the Americas. Some outbreaks of dengue have involved one million or more cases with attack rates of 50–90%. The World Health Organization estimates 50 million cases of dengue infection occur each year, resulting in approximately 24,000 deaths (WHO 1998).

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Although not normally fatal, cases of haemorrhage and death have been described during outbreaks of classic dengue fever in Australia, Greece and Formosa. Dengue fever is an infectious disease carried by mosquitoes and caused by any of four related dengue viruses DEN-1, DEN-2, DEN-3, and DEN-4. A person can be infected by at least two, if not all four types at different times during a life span, but only once by the same type. There is no specific treatment for dengue fever, and most people recover completely within 2 weeks. The majority of deaths that result from dengue infections are due to the development of severe bleeding problems — Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). People who develop DHF have a 5% chance of death but if they go on to develop DSS then the mortality rate can rise as high as 40%.

Flaviviruses are enveloped viruses containing a nucleocapsid that is surrounded by a membrane. The dengue virus contains a single strand of positive sense RNA that is approximately 19,700 nucleotides. Electron micrographs of the virus show a particle that is about 500 Å in diameter containing an electron–dense core, which is surrounded by a lipid bilayer or membrane (Kuhn et al., 2002). There are three structural proteins: C (core), M (membrane) and E (envelope). The E protein is a glycoprotein of about 51–60 kD that exists as a dimer at neutral pH. Ninety such dimers are found in the virus (Kuhn et al., 2002) and the structure of the protein suggests that it is a fusion protein. Kuhn et al. (2002) hypothesize that low pH induces a conformational change in the E protein that initiates fusion.

The M protein or membrane protein is 7–8 kD and consists of 75 amino acids. The M protein is produced by the cleavage of the pre-M glycoprotein during the late phase of viral maturation. The C-terminal end of the protein from amino acids 48 to 70 contains a highly hydrophobic transmembrane region

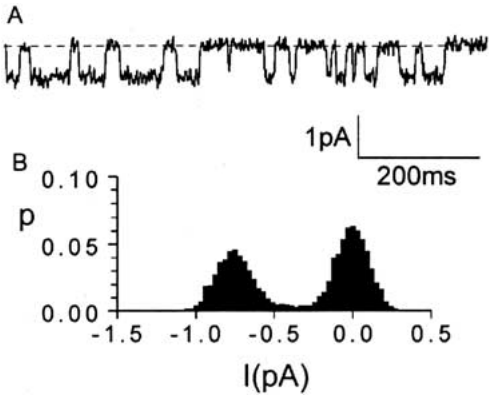

Fig. 1. (*A*) Currents recorded at −100 mV after adding DMV-C to the cis chamber (cis 500 mM KCl and trans 100 mM KCl. The currents open downwards, broken line shows the zero current level. (*B*) All points histogram of current shown in *A*.

that "anchors" the protein in the lipid bilayer. The N-terminal ectodomain of 38 amino acids is on the outside of the membrane (Kuhn et al., 2002). It has been shown that mice immunized with dengue 4 pre-M or M protein were protected from dengue 4 encephalitis challenge (Bray & Lai, 1991). The function of the M protein is still unknown.

Very little is known about the life cycle of the virus in the human host. Replication of the dengue virus seems to follow a path similar to that of the influenza viruses. Like the alphaviruses and influenza viruses, the virus enters cells in an endocytotic vesicle. The pH in the endocytic vesicles becomes lower, causing a conformational change in the E protein from a dimer to a trimer, which results in the fusion of the host cell membrane and the viral envelope followed by release of the viral nucleocapsid into the host cytoplasm and then translation of the genome. The M2 protein in the influenza A virus functions as a proton channel that may be responsible for uncoating of the viral genetic material at low pH (Sugrue et al., 1990; Sugrue & Hay, 1991). The M protein of the dengue virus may have a similar function.

In this paper we show that a synthesized peptide of the C-terminus of M protein of the Dengue virus type 1 strain Singapore S275/90 (Fu et al., 1992) forms ion channels in artificial lipid bilayers. The channels were blocked by the amiloride derivative 5-(N,N-hexamethylene) amiloride (HMA, 100 μM). HMA has previously been shown to block ion channels formed by Vpu of HIV and to prevent budding of virus-like particles from HeLa cells expressing HIV-1 Gag and Vpu proteins (Ewart et al., 2002). Amantadine (10 μM), a drug that has been shown to block channels formed by M2 protein of influenza A viruses and channels formed by HCV protein p7, also blocked DVM-C ion channels.

Materials and Methods

Peptide Synthesis

A peptide corresponding to the C-terminal 40 amino acids of the M protein of the Dengue virus type 1 strain Singapore S275/90 (Fu et al., 1992) (ALRHPGFTVIALFLAHAIGTSITQKGIIFILL MLVTPSMA) was synthesized using the Fmoc method. The synthesis was done on a Symphony Peptide Synthesizer (Protein Technologies, Tucson, Arizona) used to give C-terminal amides. The coupling was done with HBTU and hydroxybenzotriazole in N-methylpyrrolidone. Each of the synthesis cycles used double coupling and a 4-fold excess of the amino acids. Temporary α-N Fmoc-protecting groups were removed using 20% piperidine in DMF.

Recording Currents

Lipid bilayer studies were performed as described elsewhere (Miller, 1986; Sunstrom et al., 1996). A lipid mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidyl-serine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabaster, Alabama) was used. The lipid mixture was painted onto an aperture 150–200 μM in diameter in the wall of a 1 ml delrin cup. The aperture separated two chambers, cis and trans, both containing salt solutions at different concentrations. All solutions contained 10 mM HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) to maintain the pH of solutions at 7.4. The cis chamber was connected to ground and the trans chamber to the input of an Axopatch 200 amplifier. The potentials were corrected, where applicable, for liquid junction potentials using JPCalc. Normally the cis chamber contained 500 mM NaCl and the trans 50 mM NaCl with pH set at 7.4 with HEPES.

The bilayer formation was monitored electrically by the amplitude of the current pulse generated by a current ramp: as the bilayer thins, capacitance increases. The bilayers were formed in the presence of the asymmetric solutions as quickly as possible. After adding peptide, the solutions in both chambers were stirred continuously until channel activity was seen. The stirring would counteract any possible leakage of solutions during formation of the bilayer. The potentials were measured in the trans chamber with respect to the cis. The protein was added to the cis chamber and stirred until channel activity was seen. The currents were filtered at 1000 Hz, digitized at 5000 Hz and stored on magnetic disk. We measured either the amplitude of individual channel openings (*see* Figs. 1 and 2), or the mean current during a long period of at least 30 s (Figs. 3 and 4). It was necessary to measure mean current when there were no discrete channel openings (the usual situation). The mean current is the average of all data points when the base line is set at 0 pA. All experiments were carried out at room temperature at about 20°C.

The DVM C-terminal peptide (DVM-C) was dissolved in 2,2,2-trifluorethanol (TFE) at 0.05 mg/ml to 1 mg/ml. 10 μl of this was added to the cis chamber of the bilayer, which was stirred. The transmembrane potential was held at 0 mV and channel activity was normally seen within 15–30 min. Solutions containing the same concentration of TFE did not induce channel activity (Premkumar et al., 2004).

Channel activity appeared after the addition of the peptide to the cis chamber of the bilayer setup. The contents of both the chambers were stirred until channel activity was seen within 15–30 minutes of adding the peptide. In control experiments in which no peptide was added and the chambers were stirred, no activity was seen for periods over 30 min. Only when peptide was added to the cis chamber was channel activity seen. The reversal potential of

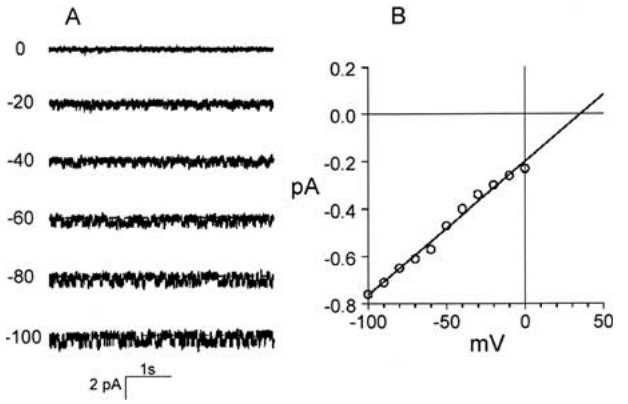

Fig. 2. (*A*) Currents recorded over a range of potentials in a bilayer after the addition of DMV-C to the cis chamber. The cis chamber contained 500 mM KCl and the trans chamber contained 100 mM KCl. The potential during each trace is shown on the left. (*B*) Plot of current (ordinate) amplitude generated by DVM-C against potential (trans with respect to cis, abscissa) across the bilayer. The cis chamber contained 500 mM KCl and the trans chamber contained 100 mM KCl. The line is the best fit of a straight line to the data points. The slope is 5.6 pS and current is zero at +35.6 mV.

currents seen with DVM-C ion channels was determined by changing the potential across the bilayer. The most accurate way we found to do this was to gradually turn the holding potential potentiometer of the Axopatch clockwise and anticlockwise through a range of potentials to determine the potential at which currents reversed. Potential was changed at a rate less than 100 mV per 30 s to avoid significant capacitive current. In some experiments the reversal potential was obtained by changing the holding potential in 10 mV steps for at least 30 s at each potential and plotting a mean current-voltage graph. The reversal potential was then calculated from the graph. Ion equilibrium potentials were calculated from the Nernst equation using ion activities. Relative ion permeabilities were calculated from the Goldman-Hodgkin-Katz equation also using ion activities. Because the mean current recorded was a function of both channel-open probability and single-channel current, the method was not as accurate as the first method. The calculations for the reversal potentials of the channels were corrected for junction potential.

HMA Block: Solutions of 50 mM HMA were prepared by first making a 500 mM solution in DMSO. This solution was further diluted to 50 mM HMA using 0.1 M HCl. 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 same concentration of DMSO/HCl in solutions not containing peptide did not cause channel activity (Premkumar et al., 2004). The solutions in both the cis and trans chambers were buffered with HEPES. The addition of HMA would not greatly alter the pH of the cis chamber.

Amantadine Block: Solutions of 1 mM amantadine were made in distilled water. 10 μl of this solution was added to the cis chamber containing 1 ml solution after channel activity was seen, making the final concentration of amantadine 10 μM.

Results

Experiments were done with 500 mM NaCl or KCl in the cis chamber and 50 mM NaCl or KCl in the trans chamber and the pH was buffered at 7.4 with Na or K HEPES. When the DVM-C peptide was added to the cis chamber and stirred, channel activity was recorded in 157 of 212 experiments. Generally, large currents with variable amplitude were recorded, presumably because of aggregation of the DVM-C peptide leading to larger ion channels or the synchronous opening of many ion channels formed by DVM-C. However, occasionally low-conductance channels were seen, as illustrated in Fig. 1 in an experiment in which the cis chamber contained 500 mM KCl and the trans chamber contained 100 mM KCl. It can be seen that there were discrete, low-amplitude currents with an amplitude of about 8 pA with a potential across the bilayer of −100 mV. Current amplitude varied with bilayer potential (Fig. 2*A*) but became too small between 0 and +70 mV for their amplitude to be measured with any confidence. Extrapolation of the linear fit to the current amplitudes gave a reversal potential between +30 and +40 mV (Fig. 2*B*), which is close to the K^+ equilibrium potential (+38 mV), indicating that channels were more permeable to K^+ than to Cl^-. The channels in this experiment had a conductance of about 6 pS.

Normally currents were much larger, as illustrated in Fig. 3. In experiments, where the chambers contained 500 mM (cis) and 50 mM (trans) NaCl, the currents reversed between +19 and +39 mV, which is close to the Na^+ equilibrium potential (+53 mV), indicating that channels were more permeable to Na^+ than to Cl^-. The mean reversal potential measured in 10 experiments was +27.8 ± 1.4 mV (after correcting for liquid junction potentials), indicating that the channels were about 4 times more permeable to Na^+ than to Cl^- (Goldman Hodgkin Katz equation). Channel activity was also recorded in 43 of 63 bilayers when the cis chamber contained 500 mM NaCl and the trans chamber 100 mM NaCl. The currents reversed between +12 and + 23 mV (Na^+ equilibrium potential +38 mV). Currents recorded in a typical experiment over a range of potentials in an experiment in which the cis chamber contained 500 mM NaCl and the trans chamber 100 mM NaCl are shown in Fig. 3*A*. In 12 experiments the mean reversal potential was 16.9 ± 0.9 mV (after correcting for liquid junction potentials), indicating the channels were about 3.3 times more permeable to Na^+ than to Cl^-. A plot of the mean current recorded with 10 mV step potentials in a similar experiment is shown in Fig. 4*A*. The current reversed at +19.1 mV (after correcting for the liquid junction potential) with the slope of the fitted line being 9.8 pS.

In experiments in which there was 500 mM KCl in the cis chamber and 50 mM KCl in the trans chamber, ion channel activity was seen in 53 of 63 bilayers. The currents reversed between +20 and +40 mV. In 14 such experiments, the average reversal potential was

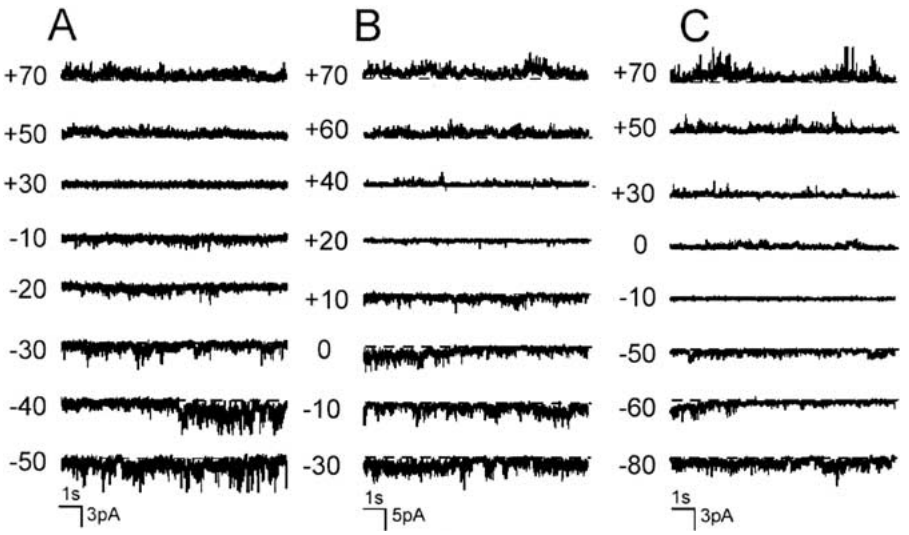

Fig. 3. Currents recorded over a range of potentials in three bilayers after the addition of DMV-C to the cis chamber. (*A*) The cis chamber contained 500 mM NaCl and the trans chamber contained 100 mM NaCl. (*B*) The cis chamber contained 500 mM KCl and the trans chamber contained 50 mM KCl. (*C*) The cis chamber contained 150 mM $CaCl_2$ and the trans chamber contained 50 mM $CaCl_2$. The potential during each trace is shown at the left of the trace.

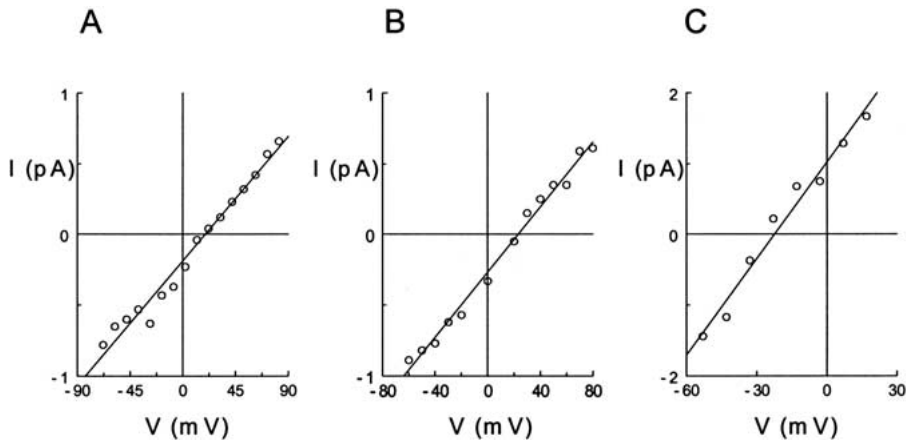

Fig. 4. Ion selectivity of channels formed by DVM-C peptide. Plots of mean currents (ordinate) generated by DVM-C against potential (trans with respect to cis, abscissa) across the bilayer. (*A*) The cis chamber contained 500 mM NaCl and the trans chamber contained 100 mM NaCl. The line is the best fit (least squares) of a straight line to the data points. The slope is 9.8 pS and current is zero at +19.1 mV (*B*) The cis chamber contained 500 mM KCl and the trans chamber contained 50 mM KCl. The line is the best fit of a straight line to the data points. The slope is 11.5 pS and current is zero at +23.3 mV. (*C*) The cis chamber contained 150 mM $CaCl_2$ and the trans chamber contained 50 mM $CaCl_2$. The line is the best fit of a straight line to the data points. The slope is 4.5 pS and current is zero at −22.4 mV.

+25.9 ± 1.6 mV, indicating that the channels were about 4 times more permeable to K^+ than to Cl^{-1}.

The channels do not seem to be permeable to calcium ions. When the cis chamber contained 150 mM $CaCl_2$ and the trans chamber 50 mM $CaCl_2$, channel activity was seen in 38 of 68 bilayers. In three experiments in which the reversal potential was defined with a high degree of accuracy, the currents reversed at −22.1 ± 3.1 mV (after correcting for the liquid junction potential) (Figs. 3*C* and 4*C*), closer to the chloride equilibrium potential (−26 mV) than the calcium equilibrium potential (+13 mV), indicating that the channels were more permeable to chloride than to calcium ions.

We have previously found that channels formed by the HIV-1 protein, Vpu, and by HCV protein p7 can be blocked by hexamethylene amiloride (HMA) (Ewart et al., 2002; Premkumar et al., 2004). We found that the channels formed by the DVM-C peptide were also blocked by HMA, as illustrated in Fig. 5. The traces in Fig. 5*A* were obtained at 0 mV, the cis chamber contained 500 mM KCl and the trans 50 mM KCl. Addition of the DVM-C peptide produced the channel activity shown in Fig. 5*A*. Addition of 2 μl of 50 mM HMA to the cis chamber followed by stirring resulted in disappearance of the channel activity as illustrated by the traces in Fig. 5*B*. Block of channel activity produced by the DVM-C

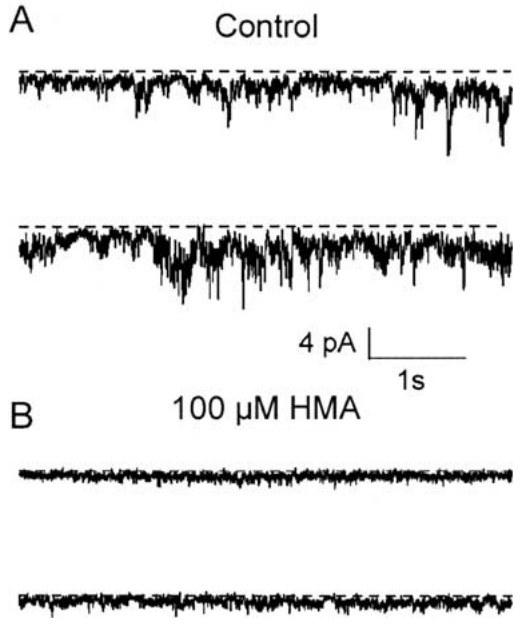

Fig. 5. Currents blocked by 100 μM HMA. (*A*) Currents recorded at 0 mV after addition of DVM-C to the cis chamber. The cis chamber contained 500 mM KCl and the trans chamber contained 50 mM KCl. (*B*) Current traces after HMA had been added to the cis chamber.

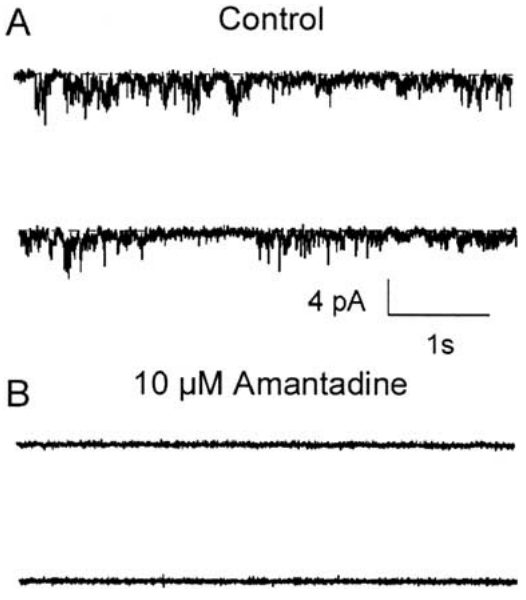

Fig. 6. Currents blocked by 10 μM Amantadine. (*A*) Currents recorded at 0 mV after addition of DVM-C to the cis chamber. The cis chamber contained 500 mM KCl and the trans chamber contained 50 mM KCl. (*B*) Current traces after Amantadine had been added to the cis chamber.

peptide with 100 μM HMA was recorded in 8 out of 11 experiments. The channels were not blocked by the diluent DMSO/HCl for HMA. In 5 experiments where the DMSO/HCl at concentrations normally present in the HMA solution was added to channels formed by DVMC peptide, no block in the channel activity was seen.

The anti-influenza-A drug amantadine, that has been shown to block M2 ion channels (Duff & Ashley, 1992; Pinto, Holsinger & Lamb, 1992; Tosteson et al., 1994), 28-amino-acid NB peptide that forms channels (Fischer, Pitkeathly & Sansom, 2001) and HCV p7 ion channels (Griffin et al., 2003), also blocked the DVM-C channels. Recently amantadine at a concentration of 1 μM has been shown to block the HCV protein p7 ion channels (Griffin et al., 2003). The DVM-C channels were blocked by 10 μM amantadine in 6 experiments in which the cis chamber contained 500 mM KCl and the trans chamber 50 mM KCl (Fig. 6). Fig. 6*A* shows traces of the channel at 0 mV before the addition of amantadine and Fig. 6*B* shows the traces after addition of 10 μM amantadine and stirring. We were unable to show reversal of the blocks by amantadine and HMA, as we usually lost the bilayer before this could be done.

Discussion

We have found that the DVM-C peptide from dengue virus forms ion channels in lipid bilayer membranes. The channels are selective for monovalent cations over monovalent anions and are relatively impermeable to calcium ions. Hexamethylene amiloride (HMA), a drug that blocks HIV-1 Vpu ion channels and depresses budding of virus-like particles in cells expressing Gag and Vpu (Ewart et al., 2002), also blocks the ion channels formed by DVM-C. It is possible that there is some similarity between the binding sites for HMA in Vpu and DVM-C, though there is little sequence identity between the two peptides.

The dengue virus M protein can be added to an increasing list of virus proteins that have been shown to form ion channels in lipid bilayers or cells. These virus ion channels have been called "viroporins" (Carrasco, 1995) 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.

References

Bray, M., Lai, C.J. 1991. Dengue virus premembrane and membrane proteins elicit a protective immune response. *Virology* **185:**505–508

Carrasco, L. 1995. Modification of membrane permeability by animal viruses. *Adv. Virus. Res.* **45:**61–112

Duff, K.C., Ashley, R.H. 1992. The transmembrane domain of influenza A M2 protein forms amantadine-sensitive proton channels in planar lipid bilayers. *Virology* **190:**485–489

Ewart, G.D., Mills, K., Cox, G.B., Gage, P.W. 2002. Amiloride derivatives block ion channel activity and enhancement of virus-like particle budding caused by HIV-1 protein Vpu. *Eur. Biophys. J.* **31:**26–35

Fischer, W.B., Pitkeathly, M., Sansom, M.S. 2001. Amantadine blocks channel activity of the transmembrane segment of the NB protein from influenza B. *Eur. Biophys. J.* **30:**416–420

Fu, J., Tan, B.H., Yap, E.H., Chan, Y.C., Tan, Y.H. 1992. Full-length cDNA sequence of dengue type 1 virus (Singapore strain S275/90). *Virology* **188:**953–958

Griffin, S.D., Beales, L.P., Clarke, D.S., Worsfold, O., Evans, S.D., Jaeger, J., Harris, M.P., Rowlands, D.J. 2003. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett.* **535:**34–38

Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., Baker, T.S., Strauss, J.H. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108:**717–725

Miller, C. 1986. Ion Channel Reconstitution. Plenum Press, New York

Pinto, L.H., Holsinger, L.J., Lamb, R.A. 1992. Influenza virus M2 protein has ion channel activity. *Cell* **69:**517–528

Premkumar, A., Wilson, L., Ewart, G.D., Gage, P.W. 2004. Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. *FEBS Lett.* **557:**99–103

Sugrue, R.J., Bahadur, G., Zambon, M.C., Hall-Smith, M., Douglas, A.R., Hay, A.J. 1990. Specific structural alteration of the influenza haemagglutinin by amantadine. *Embo J.* **9:**3469–3476

Sugrue, R.J., Hay, A.J. 1991. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology* **180:**617–624

Sunstrom, N.A., Premkumar, L.S., Premkumar, A., Ewart, G., Cox, G.B., Gage, P.W. 1996. Ion channels formed by NB, an influenza B virus protein. *J. Membr. Biol.* **150:**121–132

Tosteson, M.T., Pinto, L.H., Holsinger, L.J., Lamb, R.A. 1994. Reconstitution of the influenza virus M2 ion channel in lipid bilayers. *J. Membr. Biol.* **142:**117–126