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Supplementary Material

Determination of Pore-Lining Residues in the Hepatitis C Virus p7 Protein

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Supplementary Information for Chew *et al.*

METHODS

Compounds. $MgCl_2$ and $CuCl_2$ were purchased from Sigma-Aldrich (Poole, Dorset, UK). *N*-nonyl deoxynojirimycin (*N*N-DNJ) was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). 1, 2-Dioleoyl-*sn*-glycero-3 phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were from Avanti Polar Lipids (Alabaster, AL, USA). For preparation of stock solutions, $MgCl_2$ or $CuCl_2$ were dissolved in water at concentrations between 5 mM and 40 mM; 10 mM stock solutions of iminosugar derivatives were prepared using methanol: water (1:1, v/v).

Peptide Synthesis. HCV p7 of both genotype (GT) 1a, strain H77, and GT 2a, strain JFH-1, were synthesized using standard Boc methodology (1) and purified via semi-preparative HPLC and their respective masses confirmed by mass spectrometry.

Amino acid sequence of the p7 proteins used in this study:

H77 strain

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         10          20          30          40          50          60
ALENLVILNAASLAGTHGLVSFLVFFCFAWYLKGRWVPGAVYAYLGMWPLLLLLLALPQRAYA
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JFH-1 strain

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         10          20          30          40          50          60
ALEKLVVLHAASAANCHGGLLYFAIFFVAAWHIRGRVVPLTTYCLTGLWPFCLLLMALPRQAYA
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Channel recordings. Artificial bilayer membranes comprising DOPC and POPE were prepared. 5 mg of a 4:1 (w/w) DOPC and POPE mixture were dissolved in 1 ml chloroform, dried under a stream of N_2 gas and re-suspended in *n*-decane at 25 mg/ml. For channel recordings, a Delrin cup chamber system was used with an aperture diameter of 150 μm. Experiments were performed at 24 ± 1 ºC. The chambers separated by the aperture were filled with 1 ml buffer (300 mM KCl, 5 mM K^+-HEPES, pH 7.0). Bilayers were detected using an episodic voltage ramp between ±10mV. A lipid bilayer acts as a thin insulating barrier between the two conducting buffer solutions and as such it is a capacitor. From the measured conductance responses, the bilayer's capacitance can be calculated. A specific capacitance of 1 pF/cm2 in response to applied voltage is indicative of a unilamellar membrane. Formation of the bilayer and induction of channel activity was achieved by one of two methods. For the stirring method, as described for the spontaneous insertion of other small hydrophobic ion channel-forming peptides dissolved in trifluoroethanol (TFE) into lipid bilayers (2-6), aliquots of the peptide containing up to 60 pmol (2 – 5 μl from a stock solution of 80 μg/ml p7 dissolved in TFE) were added to the *cis* chamber while stirring to facilitate collision between peptide and bilayer. For the second method we used an improved pre-treatment protocol for reconstitution of the

peptide, which was adapted from Mehnert *et al.* (7) and includes lipid pre-treatment of the 150 μm aperture prior to contact with the buffer solution. To smooth the aperture's irregularities, a drop of a 5 mg/ml lipid preparation (DOPC: POPE 4:1) was applied and dried under N_2 gas.

Approximately 0.6 μl of the peptide stock solution in TFE (80 μg/ml) was brushed across the aperture together with approximately 0.4 μl of the lipid suspension and incubated at room temperature for 5 min. The buffer level was subsequently raised and lowered until a bilayer was formed. The pre-treatment method resulted in an approximate 10-fold reduction of the amount of peptide required to approximately 6 pmol and the overall quality of channel recordings was improved substantially.

For both methods, p7 was incorporated into the bilayer in the presence of a constant electric field (usually -60 mV). Using the stirring method, ion channel activity of p7 was usually observed 10 - 20 min after addition of peptide (n >40). With the pre-treatment protocol reconstitution and channel activity occurred in most cases within 2 min after negative voltage was applied (n >230). In control experiments where TFE or ethanol alone were added, no ion-channel activity was observed (n = 30, waiting time = 40 min). Channel recordings were performed at constant voltage ranging from -80 mV to +80 mV. When planar lipid bilayers were exposed to p7 (purified from independent batches of synthesis), currents were observed in more than 150 experiments performed on 20 independent experimental days.

Data analysis. Currents were recorded using a MultiClamp 700A system from Axon Instruments (Union City, US) and the data were filtered with a Bessel-8-pole low-pass-filter at 100 Hz. Shut events shorter than 15 ms were considered part of a burst and excluded from single ion channel analysis. Data were analyzed using Clampfit and a user-written Excel algorithm. Currents were measured to an error of ±0.5 pA. Thus at a voltage of +100 mV, this would equate to an error of 5 pS. However, after a Gaussian filter is used, this error was reduced to ± 0.1 pA. This proved sufficiently sensitive not only to detect the lowest reported conductance states, but also to find new "sub-conductance" states.

For the calculation of the current-voltage curves of the different p7 conductance states, current histograms comprising between 3000 and 7600 single channel opening events were generated for each of the traces. The histograms showed three predominant conductance states the values of which were used for analysis in I/V plots.

To give a detailed distribution of the conductance states in form of conductance histograms, we represented each of the N_{ev} events of a recorded trace by a dwell-time weighted Gaussian distribution function, so that the conductance-histogram function $f(\kappa)$ is:

$$f(\kappa) = \frac{A}{\sqrt{2\pi\sigma}} \sum_{i=1}^{N_{ev}} t_{dw,i} \exp\left[-\left(\frac{\kappa - \kappa}{\sigma} \right)^2 \right].$$

A is a normalization constant set so that $\int_0^\infty f(\kappa)d\kappa = 1$. For the *i*th event the maximum of a single Gaussian function is equal to its mean conductivity $\kappa_i = I_i/V$ (measured current value divided by the applied voltage). The width σ is set to a small value, uniform for all events. The frequency of channel openings was normalized (total area under the curve: 50).

RESULTS

An outline of the experiments conducted is presented in Table 1.

Table 1- Summary of electrophysiology experiments performed in planar lipid bilayers of lipid composition DOPC and POPE (4:1). The electrolyte solution in both the *cis* and *trans* chamber is 300 mM KCL, 5 mM K^+=HEPES, pH 7.0 at room temperature. The whole series of experiments was done using both H77 and JFH-1 strains of HCV p7. Bilayers were stable in all cases and 10 traces were recorded for each condition.

Experiment conditions	Comments
p7 only	
p7 with 1mM of Mg^{2+}	
p7 with 500 µM of Cu^{2+}	70% inhibition
p7 with 50 µM of Cu^{2+}	90% inhibition
p7 with 500 µM of Cu^{2+} followed by change in pH to 6.2	90% inhibition followed by recovery of 6%.
p7 with 500 µM of Cu^{2+} followed by change in pH to 5.7	90% inhibition followed by recovery of 9 %
p7 with 500 µM of Cu^{2+} followed by change in pH to 5.2	90% inhibition followed by recovery of 26 %
H17A p7 mutant with 500 µM of Cu^{2+}	

Our group has previously incorporated p7 into a lipid bilayer composed of DOPC/POPE (4:1). This resulted in ion channels which give rise to burst like firing patterns, with opening states typically lasting several hundred milliseconds. The characteristics of the p7 ion channel activity were found to be very similar to those described before (Whitfield *et al*., submitted). Typical recordings show two main opening states and one sub-conductance state (see main text Figure 1B for the H77 strain and Figure SI-2 for the JFH-1 strain). The mean conductance values were 25.5 ± 1.8 pS and 21.5 ±1.7 pS for the two main states, respectively, and 11.5 ± 1.9 pS for the sub-conductance state. Mean currents were calculated over a 400 s period once channel activity had been established. Relative mean currents were calculated for 100 s intervals with respect to that initial 400 s period. Relative mean current versus time data for this strain is shown in Figure SI-3 and is very similar to that observed for the H77 strain. The current-voltage relationship of p7 conductance states observed exhibited ideal ohmic behaviour as shown in Figure SI-4 (for JFH-1) throughout the voltage range tested -80 mV to +80mV, confirming previous work that the ion channel and related states are not voltage gated.

FIGURES

Figure SI-1. Sequence alignment for 26 representative strains after Steinman *et al.* (8)

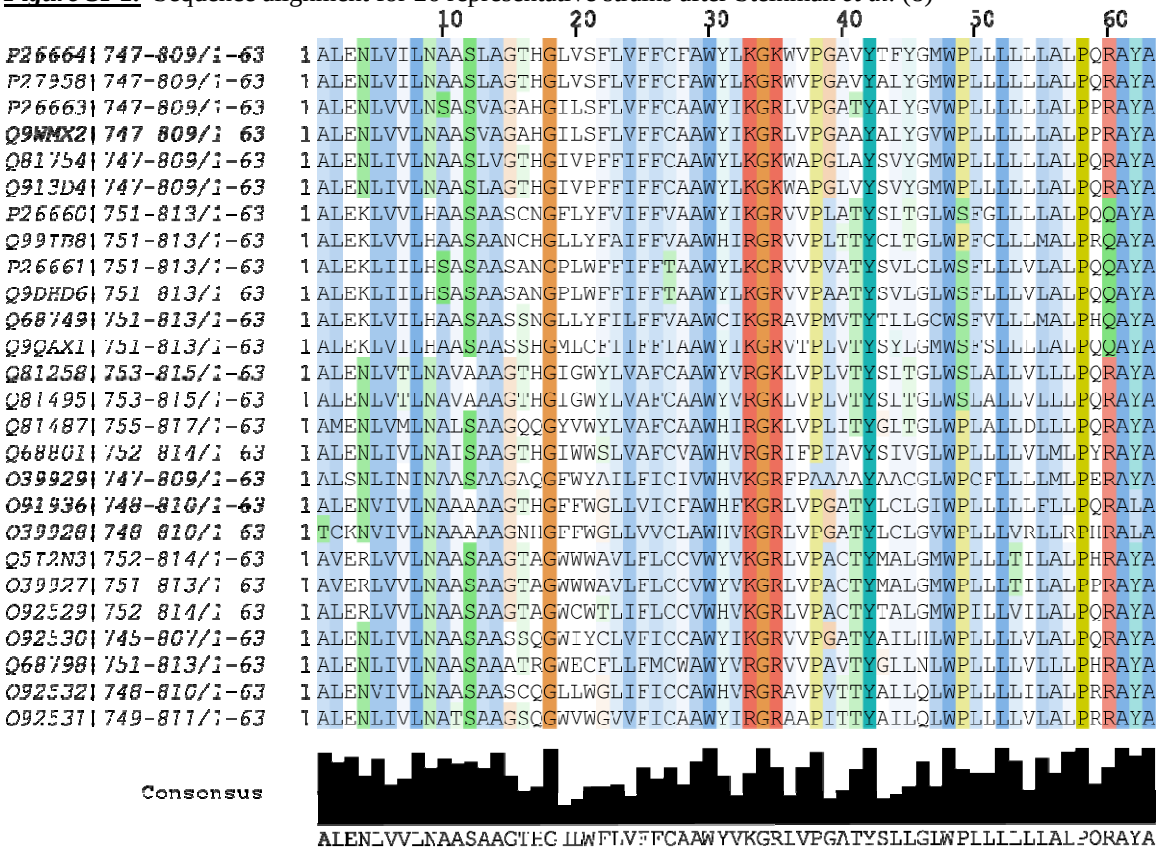


Figure SI-2. Typical current trace recordings for the JFH-1 p7 ion channels obtained in 4:1 (w/w) DOPC/POPE mixture at -60 mV. Channel opening events are observed as upward deflections.

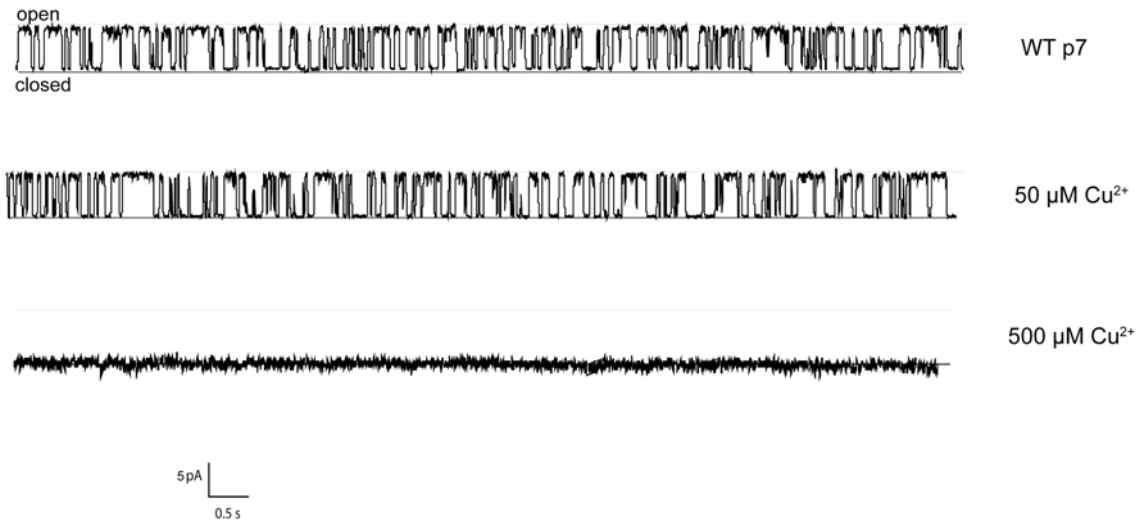
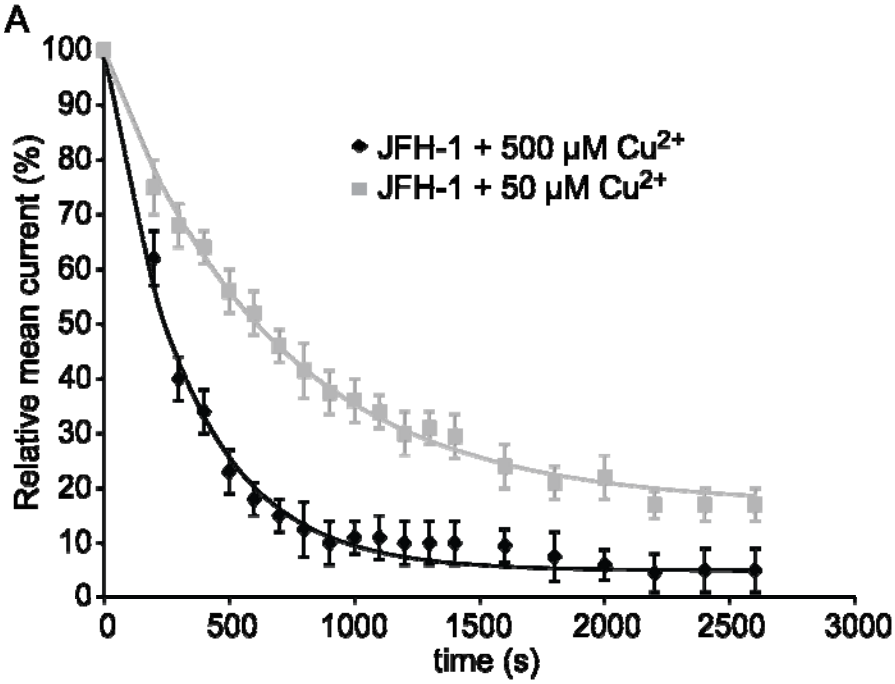


Figure SI-3. (A) Time course of inhibition for the JFH-1 strain p7 ion channel protein by 50 μM (grey squares) and 500 μM (black diamonds) Cu^{2+}. (B) Response of the channels to change in pH to 6.2 (black diamonds), 5.7 (grey squares), and 5.2 (hollow squares).



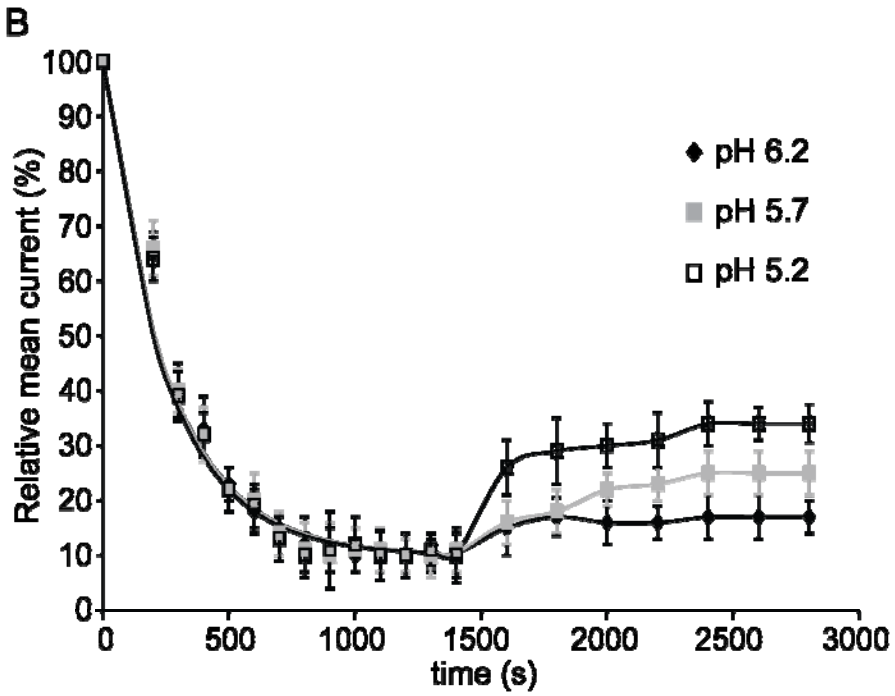
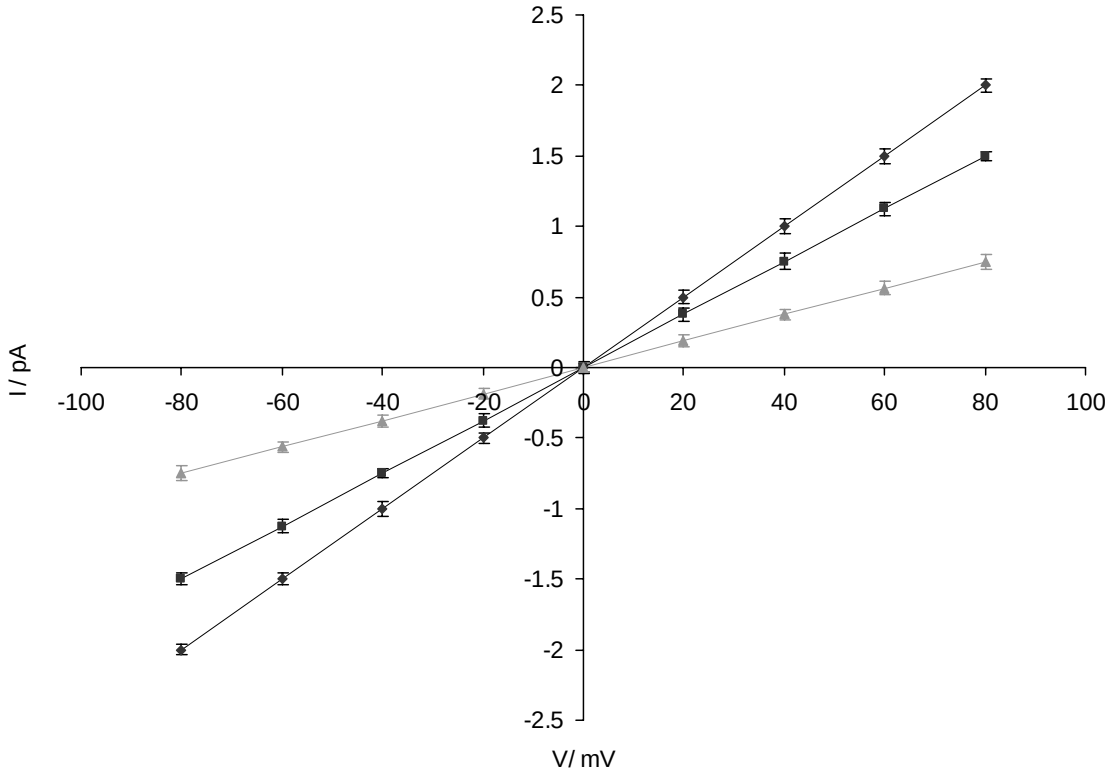


Figure SI-4. Graph of current versus voltage for the wild type (WT) JFH-1 p7 ion channel in a lipid bilayer of DOPC and POPE (4:1) where typical recordings show two main states (back diamonds and black squares) and one sub conductance state (grey triangles). Symbols in the graph depict the recorded currents (mean ± standard error for 10 trials).



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