Requirement for Chloride Channel Function during the Hepatitis C Virus Life Cycle

Zsofia Igloi, Bjorn-Patrick Mohl, Jonathan D. Lippiat, Mark Harris, Jamel Mankouri

School of Molecular and Cellular Biology and School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom

Hepatocytes express an array of plasma membrane and intracellular ion channels, yet their role during the hepatitis C virus (HCV) life cycle remains largely undefined. Here, we show that HCV increases intracellular hepatic chloride (Cl\(^{-}\)) influx that can be inhibited by selective Cl\(^{-}\) channel blockers. Through pharmacological and small interfering RNA (siRNA)-mediated silencing, we demonstrate that Cl\(^{-}\) channel inhibition is detrimental to HCV replication. This represents the first observation of the involvement of Cl\(^{-}\) channels during the HCV life cycle.

Received 8 October 2014 Accepted 7 January 2015
Accepted manuscript posted online 21 January 2015
Editor: M. S. Diamond
Address correspondence to Jamel Mankouri, J.mankouri@leeds.ac.uk.
*Present address: Bjorn-Patrick Mohl, London School of Hygiene & Tropical Medicine, London, United Kingdom.
Z.I. and B.-P.M. contributed equally to this work.
Copyright © 2015, Igloi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported license. doi:10.1128/JVI.02946-14
At these inhibitory concentrations, NPPB and IAA-94 did not affect cell viability (Fig. 2A, gray bars) or HCV internal ribosome entry site (IRES)-mediated translation (Fig. 2B), suggesting a specific inhibition of virus genome replication. The inhibitory effects of NPPB and IAA-94 were also confirmed in a genotype 1B-derived replicon (SGR-Feo-Con1) (63% / H11006 23% and 56% / H11006 27% inhibition, respectively), suggesting these effects to be conserved across genotypes (Fig. 2E). Surprisingly, DIDS, a well-characterized broadly acting inhibitor of anion exchangers, did not inhibit SGR replication (Fig. 2A to E). We reasoned that HCV genome replication is thus dependent on the function of an NPPB- and IAA-94-sensitive, DIDS-insensitive Cl− channel. Several Cl− channels with this pharmacological profile have been reported (22). Given these effects, we proceeded to examine Cl− homeostasis during the HCV replication cycle. For this, we used the fluorescent indicator N-ethoxycarbonylmethyl-6-methoxyquinolinium bromide (MQAE), a dye quenched by enhanced Cl− influx and the subsequent increase in intracellular chloride concentration [Cl−]i. Figure 2F demonstrates that SGR–Feo–JFH-1-harboring cells display a 42% increase in [Cl−], compared to the level in parental Huh7 cells (gray bars), consistent with an
enhanced basally active Cl⁻ inward conductance. The [Cl⁻]ᵢ increase could be suppressed by prolonged treatment with NPPB (10 μM), IAA-94 (100 μM), and DIDS (100 μM) for 24 h, and luciferase expression was assessed as described for Fig. 1. Results were calculated relative to those for an untreated control. Error bars represent the SEM from three independent experiments. **, significant difference from the value for the untreated control (P < 0.05); NS, no differences at the 0.05 significance level. (B) Huh7 cells were transfected with either the pRZF vector (mock control) or the pRZF vector containing the firefly luciferase gene under the translational control of the HCV or the encephalomyocarditis virus (EMCV) IRES and the promoter-driven Renilla luciferase gene as previously described (10). Four hours posttransfection, cells were treated with DCV, IAA-94, NPPB, and DIDS for 48 h, and luciferase expression was assessed as described for Fig. 1. Error bars represent the SEM from three independent experiments. Values are normalized to the Renilla luciferase values to assess effects on translation. (C) Sample Western blots immunoblotted for NS5A and GAPDH (loading control) are shown. SGR–Feo–JFH-1 cells were treated with DCV, NPPB, IAA-94, or DIDS for 48 h. (D) SGR–Feo–JFH-1 cells were treated with the indicated Cl⁻ blockers; 48 h posttreatment, they were fixed with methanol and permeabilized in 50% methanol-acetone. NS5A was visualized via labeling with sheep anti-NS5A antisera followed by staining with Alexa Fluor 488-conjugated secondary antibodies. Representative confocal images are shown. (E) SGR-Feo-Con1 cells stably expressing a luciferase subgenomic replicon (genotype 1b) were treated with the Cl⁻ channel inhibitors, and luciferase activity was assessed as described for panel A. Error bars represent the SEM of results of stimulations from three independent experiments. **, significant difference from the value for the untreated control (P < 0.05). NS, no differences at the 0.05 significance level. (F) Naive Huh7 and SGR-Neo-JFH-1 cells were seeded into 12-well plates and treated for the indicated times with 10 μM NPPB and then were loaded with 5 mM 6-methoxy-quinolyl acetoethyl ester (MQAE) in Dulbecco modified Eagle medium (DMEM) for 1 h at 37°C. Following incubation, cells were washed three times with DMEM and fluorescent images immediately acquired using the IncuCyte Zoom live-cell imager. Mean fluorescence per cell was calculated from a minimum of three independent experiments performed in triplicate using IncuCyte Zoom live-cell imager software.

Together, these data confirm that SGR–Feo–JFH-1 cells possess enhanced transepithelial Cl⁻ transport through an NPPB-sensitive Cl⁻ channel.

Since SGR–Feo–JFH-1-harboring cells express only the HCV
nonstructural proteins NS3 to NS5B, it was important to determine whether Cl⁻ channel inhibition suppressed HCV replication in the context of virus-infected cells (23). We initially used a monocistronic full-length HCV chimeric genotype 2a virus, J6/JFH1, which is fully infectious in cell culture and expresses Renilla luciferase, herein termed J6/JFH-1 RLuc (24). Assays were performed by virus infection (multiplicity of infection [MOI] of 0.5) in the presence of each channel modulator, and luciferase expression was analyzed 48 h postinfection (p.i.). Figure 3A shows that NPPB and IAA-94 treatment significantly decreased J6/JFH-1 RLuc activity (67% ± 20% and 63% ± 5% inhibition, respectively) confirming a dependence on Cl⁻ influx during the virus life cycle. When these assays were performed in the presence of DIDS (100 μM), J6/JFH-1 RLuc activity also decreased by 77% ± 4% at concentrations that did not affect SGR–Feo–JFH-1 replication (Fig. 3B). To verify these data, we directly infected Huh7 cells with full-length JFH-1 virus (25) in the presence of each Cl⁻ inhibitor and measured the production of infectious virions by focus-forming assay. As shown in Fig. 3B, virus yields were significantly lower in IAA-94-, NPPB-, and DIDS-treated cells (87% ± 14%, 81% ±
This was paralleled by a decrease of both NS5A and core protein expression in virus lysates as assessed by Western blot analysis (Fig. 3C). No effects on JFH-1 virus production were observed when TEA or KCl was assessed in these assays (Fig. 3D). We subsequently performed time-of-addition focus reduction assays using JFH-1 virus inoculum to assess the effects of DIDS over the time course of HCV infection. Cells were treated with each inhibitor 24 h p.i., and virus production was assessed 72 h p.i. Figure 3E shows that DCV, NPPB, and IAA-94 reduced JFH-1 virus production when added postinfection (92% ± 9%, 81% ± 23%, and 72% ± 22% inhibition, respectively), consistent with a block of HCV replication. DIDS however, failed to reduce virus production relative to that in the untreated wells, consistent with a lack of inhibition of HCV replication. To further determine which steps of the HCV life cycle are impaired by DIDS, we examined the effects of each Cl⁻ channel inhibitor on virus entry by adding them to JFH-1 inoculum during the initial 3 h of virus infection (26). The HCV-neutralizing mouse monoclonal E2 antibody AP33, a characterized inhibitor of HCV entry, was included in these assays for verification (27). Figure 3F shows that, while AP33 (50 μg/ml) inhibited HCV entry by 72% ± 11%, IAA-94, NPPB, and DIDS did not impede...
viral entry. These observations suggest that a DIDS-sensitive Cl⁻ channel can inhibit early postentry virion trafficking and/or early replication events but does not inhibit virus entry or replication following the establishment of infection.

Given these data, we investigated the molecular identity of the Cl⁻ channel(s) required during the HCV life cycle. To date, nearly 40 different genes that, when expressed, increase Cl⁻ conductance have been cloned. These include the Cl⁻ intracellular-channel (CLIC) proteins cyclic AMP (cAMP) (CFTR)-, calcium (CaCC)-, voltage-activated Cl⁻ channels and Cl⁻ /H⁺ exchangers (CLCs) as well as ligand-gated Cl⁻ channels (GABAA, GABA(B), and glycine). In hepatocytes, CLIC-1, CLIC-2, CLIC-3, CLIC-5, and CLIC-7 are expressed (9). We confirmed this by reverse transcription-PCR (RT-PCR) analysis (primer sequences are available upon request) and silenced this expression through small interfering RNA (siRNA) transfection (Fig. 4A). Figure 4B shows that CLIC-2, CLIC-3, CLIC-5, and CLIC-7 silencing significantly suppressed SGR–Feo–JFH-1 replication (52% ± 6%, 31% ± 16%, 48% ± 2%, and 50% ± 10% inhibition of luciferase activity, respectively). CLIC-1 knockdown displayed no discernible effects. Since some of these CLC channels and transporters are sensitive to NPPB and IAA-94; this confirmed the importance of Cl⁻ influx during HCV replication.

We finally investigated the effect of Cl⁻ channel silencing on J6/JFH-1 RLuc virus infection. Consistently with what occurred with SGR–Feo–JFH-1, silencing of CIC-2 and CIC-3 inhibited luciferase expression by 50% ± 21% and 52% ± 17%, respectively, confirming their requirement for virus replication. However, when CIC-5 and CIC-7 were silenced, no effects on J6/JFH-1 RLuc luciferase activity were observed despite their inhibitory effects on SGR–Feo–JFH-1 replication. Conversely, silencing of CLIC-1 decreased J6/JFH-1 RLuc luciferase activity by 46% ± 11% (Fig. 4C) despite a lack of effect on the replication of SGR–Feo–JFH-1 (Fig. 4F).

It is interesting to address what might be the molecular mechanisms underpinning the differential effects of CLIC-1, CIC-5, and CIC-7 silencing. While data on the biological role of CIC-1 is limited, its activity has been shown to be required for the regulation of endosomal/lysosomal pH (9, 28). This may explain our observations, since the acidic late endosome/lysosome pH is crucial for induction of HCV glycoprotein (E1/E2) membrane fusion during early HCV postentry events to allow HCV genome release (29). The fact that CIC-5 and -7 are dispensable for J6/JFH-1 RLuc infectivity suggests that their function may be compensated for by HCV core/NS2 expression. CIC-5 is a known 2Cl⁻/H⁺ exchanger rather than a Cl⁻ channel, the function of which is to control endosomal acidification (30, 31). The HCV viroporin p7 is thought to form cationic intracellular channels that promote a global loss of organelle acidity (32, 33). p7 activity may thus prevent the buildup of an excess positive charge in specific organelles, a principle typically achieved by the import of Cl⁻ via anion transporters, including CIC-5.

Considering our findings together, we have confirmed the role of several Cl⁻ channel proteins during the HCV life cycle. Of note, we have identified CIC-2 and CIC-3, whose activities are required during HCV replication. Endosomal acidification and [Cl⁻]i accumulation are significantly impaired in hepatocytes from CIC-2/3 knockout mice (20), and fractionation studies have suggested that these channels reside in early/late endosomes (34). The organization, composition, and functions of membrane structures induced by positive-strand RNA viruses remain largely ill defined but are generally accepted to require endosome integrity to recruit endosomal host cell factors and concentrate virus proteins to produce viral factories. Here, for the first time, we implicate host cell Cl⁻ influx through CLC channels/transporters during this process. The challenge will now be to define the specific virus-host interactions that require Cl⁻ channel functionality.

ACKNOWLEDGMENTS

We thank John McAlvann (Centre for Virus Research, University of Glasgow) for the SGR-JFH-1-luc construct and Takaji Wakiha (National Institute for Infectious Diseases, Tokyo, Japan) for pJFH-1. This work was funded by a Royal Society University Research Fellowship to I.M. (UF100419) and a Wellcome Trust Senior Investigator Award to M.H. (096670/MA).

REFERENCES


13. Wyles DL, Kaithara KA, Korbba BE, Schooley RT, Beadle JR, Hostetler KY. 2009. The octadecylxoyethyl ester of (S)-9-[3-hydroxy-2-


