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CRITICAL ROLE OF CASEIN KINASE 2 IN HEPATITIS C NS5A-MEDIATED INHIBITION OF Kv2.1 K⁺ CHANNEL FUNCTION

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Abstract

Inhibiting injury-induced increases in outward K⁺ currents is sufficient to block cell death in cortical neuronal injury models. It is now known that apoptosis is facilitated in hepatocytes by the same K⁺ channel as in cortical neurons, namely the delayed rectifier K⁺ channel Kv2.1. The hepatitis C virus (HCV) protein NS5A prevents the apoptosis-enabling loss of intracellular potassium by inhibiting Kv2.1 function and thus blocking hepatocyte cell death. Critically, neurons expressing NS5A1b (from HCV genotype 1b), but not NS5A1a, can be protected from lethal injurious stimuli via a block of Kv2.1-mediated potassium currents. Here, we identify a key component unique to NS5A1b, which is necessary for restricting Kv2.1 currents and establishing neuroprotection. By comparing the sequence differences between NS5A1b and 1a we identify putative casein kinase 2 (CK2) phosphorylation regions unique to the 1b genotype. We show that selective inhibition of CK2 in cortical neurons results in loss of NS5A1b's ability to depress outward potassium currents, and, surprisingly, potentiates currents in non-NS5A-expressing cells. As such, our results suggest that NS5A1b-mediated inhibition of Kv2.1 function is critically dependent on its phosphorylation status at genotypic-specific CK2-directed residues. Importantly, inhibiting NS5A viral replicative function with the novel HCV drug Ledipasvir does not impair the ability of this protein to block Kv2.1 function. This suggests that the modulation of NS5A function by CK2 may be a component of HCV unique to the regulation of apoptosis.

1. Introduction

In neuronal pro-apoptotic signaling processes, loss of intracellular potassium is mediated by an increase in voltage-dependent K⁺ currents [1,2,3]. This phenomenon ensures the completion of apoptosis by providing a favorable environment for caspase and nuclease activation and contributing to the apoptotic volume decrease [1,3]. Interfering with the apoptotic K⁺ current surge in vitro can effectively block neuronal cell death following oxidative injury [2,4,5,6]. In mammalian cortical neurons undergoing apoptosis, enhancement in K⁺ current follows the (SNARE)-dependent de novo insertion of Kv2.1 encoded K⁺ channels in the cell membrane [7].

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Apoptosis in liver cells seemingly occurs by the same Kv2.1-mediated pathway as in cortical neurons [8]. To help establish viral persistence in the liver, the HCV protein NS5A prevents loss of intracellular potassium by inhibiting Kv2.1 function and thus blocking apoptosis [8]. Importantly, neurons transfected with a plasmid encoding NS5A genotype 1b, but not genotype 1a, exhibit a decrease in K⁺ current under basal conditions and can be protected from lethal injurious stimuli via a block of Kv2.1-mediated potassium current [9]. NS5A1b co-expression prevents phosphorylation of wild-type Kv2.1 by Src kinase at cytoplasmic residue Y124 [9], which is a necessary step for the membrane insertion of Kv2.1 channels, and the subsequent increase in K⁺ currents [10,11]. NS5A1b also prevents phosphorylation of two other, minor, Src targets at Kv2.1, Y686 and Y810 [9, 12].

Whether the genotype-specific effects of NS5A1b on Kv2.1 channel modulation are due to specific amino acid differences, phosphorylation level, and/or host cell interactions remains to be established. Functions of the viral protein are known to be dependent on specific phosphorylation events [13]. The kinase CK2 has been shown to basally phosphorylate serine and threonine residues on the C-terminus of NS5A genotype 1b [14,15,16]. There is some heterogeneity in the kinase CK2 phosphorylation profiles between genotypes 1a and 1b, with more, and different, sites predicted and different CK2 phosphorylation sites in genotype 1b, particularly within the 70 amino acids that lie most near the C-terminus [14]. Here, we investigate if phosphorylation by CK2 plays a critical role in the effect of NS5A1b on Kv2.1 function. Our results indicate that selective inhibition of endogenous CK2 in cortical neurons results in loss of NS5A1b's ability to depress outward potassium currents. Our results also show that CK2 inhibition leads to increased K⁺ currents in vector-expressing cells. Finally, we test whether the viral replicative functions of NS5A are necessary for inhibition of Kv2.1 channel function with the use of the novel antiviral agent Ledipasvir, a drug whose mechanism of action may influence the phosphorylation status of NS5A [17].

2. Materials and methods

2.1 Cell culture and transfection

Cortical neuronal cultures were prepared as previously described from embryonic day 17 Sprague-Dawley rats of either sex [18]. At 3–5 weeks in vitro these cultures contain 10–20% neurons [19]. Electrophysiology was performed on 3 to 4-week-old cultures (19–26 days in vitro). Neurons were transfected as previously described using Lipofectamine 2000 (Invitrogen; [6]) according to the manufacturer's instructions with a plasmid encoding NS5A1b-eGFP, or the parent vector eGFP. Cells were used for electrophysiology studies 24 hours after transfection.

2.2 Drug Treatment

5-Oxo-5,6-dihydroindolo-[1,2-a]quinazolin-7-acetic acid (IQA, Casein Kinase II Inhibitor IV, Calbiochem) was used for inhibition of Casein Kinase II. IQA is an indoloquinazoline derivative that selectively suppresses CK2 activity in vitro with an IC₅₀ of 0.39 μM with no effect on 44 other kinases, including CK1 [20]. IQA (10 μM) or, in a separate set of studies, Ledipasvir (3 μM, MedChem Express), was added to growth media 4 hours after

transfection of cortical neuronal cultures. IQA or Ledipasvir were not present in extracellular solution during the electrophysiology studies.

2.3 Electrophysiological measurements

Whole-cell recordings were conducted with 2 M Ω electrodes on transfected neurons positive for eGFP [5]. The extracellular solution contained the following (in mM): 115 NaCl, 2.5 KCl, 2.0 MgCl₂, 1.0 CaCl₂, 10 HEPES, 10 D-glucose; pH 7.2; 0.25 μ M tetrodotoxin. The electrode contained the following (in mM): 100 K-gluconate, 10 KCl, 1 MgCl₂, 1 CaCl₂, 2.2 Mg₂ · ATP, 0.33 GTP, 11 EGTA, 10 HEPES, pH 7.2. Measurements were obtained under voltage clamp with an Axopatch 200B amplifier (Axon Instruments) and pClamp software (Molecular Devices). Partial compensation for series resistance was performed (80%) in all cases; currents were filtered at 2 kHz and digitized at 10 kHz. Delayed-rectifier potassium currents were evoked with a series of 200ms voltage steps from a holding potential of -80 mV to +80 mV, in 10 mV increments. Before depolarization, a single 30ms prepulse to +10mV was used to inactivate A-type K⁺ currents. For analysis and determination of current densities, steady state current amplitudes were measured at +10 mV and 180 ms, then normalized to cell capacitance. Peak conductance (G) was calculated from peak steady-state current amplitudes (I) using the equation $G = I / (V - E_K)$, where E_K is the Nernst K⁺ equilibrium potential. The conductance was plotted against the potential (V) and fitted to a single Boltzmann function $G = G_{\max} / (1 + \exp[-(V - V_{1/2}) / k])$, where $G_{\max}$ is the maximum conductance, $V_{1/2}$ is the potential at which the channel has half-maximal conductance, and k is the parameter that represents the slope of the activation curve.

3. Results

It has been previously shown that cortical neurons expressing HCV NS5A1b exhibit significantly reduced (~60%) delayed rectifier K⁺ current densities under basal conditions, as compared to untransfected or parent vector-expressing cells [9]. Indeed, in this set of experiments, K⁺ current densities were confirmed to be significantly smaller (~50%) in NS5A1b-expressing cells, as compared to parent vector (eGFP) expressing cells under basal conditions (Figure 1a, first vs. second bars). It must be noted that Kv2.1 channel is the primary mediator of delayed rectifier K⁺ currents in the cultured cortical neurons utilized here [6]. This result indicates, as previously described, that NS5A1b inhibits normal Kv2.1 activity, likely by interfering with normal trafficking of channels to the surface of the neurons [9].

In order to assess whether basal phosphorylation by CK2 is required for NS5A1b's modulatory effect on Kv2.1 activity, we used IQA (Casein Kinase II Inhibitor IV) to reduce CK2 activity in cortical neurons expressing NS5A1b. We used the specific, ATP-competitive inhibitor IQA to limit constitutive activation of CK2 in neurons[20,21]. K⁺ currents in NS5A1b-expressing cells treated with IQA were significantly larger than the currents in untreated NS5A1b-expressing cells (Figure 1A, second vs. fourth bar). Interestingly, IQA-treated cells not expressing NS5A1b showed a significant increase in K⁺ current density as compared to non-treated, vector-expressing cells (Figure 1A, third vs. first bar; Figure 1C). The percent increase in current between untreated and IQA-treated NS5A1b-expressing cells (~50%) was significantly larger than the percent increase between

untreated and IQA-treated control, vector-expressing cells (~20%) (Figure 1B). The difference in percent increase suggests that while CK2 inhibition by itself has some effect on Kv2.1 activity, CK2 inhibition also has an additional, separate, significant effect on Kv2.1 activity in NS5A1b expressing cells. These results suggest that phosphorylation of NS5A1b by CK2 is necessary for Kv2.1-modulatory properties of NS5A1b. Additionally, these results suggest that constitutively active CK2 by itself may downregulate Kv2.1 activity under basal conditions, or that the CK2 inhibitor may have induce a shift in the voltage-gated activation of Kv2.1.

To determine whether K^+ channel activation in cortical neurons is altered in the presence of the CK2 inhibitor IQA, the conductance-voltage relationship of peak potassium current recorded from vector transfected neurons in the two treatment groups (DMSO or 10 μ M IQA) was plotted and fit to a Boltzmann distribution (Figure 1D). We found that treatment with IQA did not lead to a significant shift in the voltage-dependency of K^+ channel activation ($V_{1/2}$, vehicle, 23.9 ± 1.4 mV; IQA, 21.4 ± 1.2 mV; $n = 12$ and 13 cells, respectively). As such, CK2 constitutively inhibits Kv2.1 channel activity in neurons, albeit by a yet undefined process.

Finally, we evaluated whether treatment with the anti-HCV agent, Ledipasvir, would interfere with NS5A1b's ability to modulate Kv2.1-mediated K^+ current under basal conditions. K^+ currents in NS5A1b-expressing cells treated with Ledipasvir were not significantly different from currents in non-treated NS5A1b-expressing cells (Figure 2). K^+ currents in the Ledipasvir treated and non-treated groups of NS5A1b-expressing cells were significantly different from currents in control, vector-expressing treated and non-treated cells (Figure 2). These results suggest that the viral replicative properties of NS5A are not necessary for inhibition of Kv2.1 K^+ currents.

4. Discussion

We report here that CK2 inhibition in neurons interferes with the NS5A1b mediated modulation of delayed-rectifier, Kv2.1-mediated basal K^+ currents. Interestingly, we also found that suppression of CK2 in cortical neurons significantly increases K^+ currents in comparison to vehicle-treated cells. Further analysis showed that there is no shift in the voltage-dependency of Kv2.1 channel activation in neurons with reduced CK2 activity. Finally, we establish that treatment with Ledipasvir does not modify NS5A1b's inhibitory effect on basal K^+ currents.

The Kv2.1-mediated K^+ current surge is a required step for apoptosis in cortical neurons, which can be blocked by NS5A1b expression [9]. Our results indicate that host cell modification, namely phosphorylation by CK2, of NS5A1b plays a critical role in enabling the viral protein's Kv2.1-modulatory properties. These results point to the potential importance of the sequence of the last 70 amino acids in domain 3 of the protein, where a difference in CK2 phosphorylation profiles and amino acid sequences between 1a and 1b genotypes has been suggested [14,22,23]. CK2 is present in the brain at levels higher than any other organ, and is localized specifically to neurons within the CNS [16,21]. CK2 is a pleiotropic protein with over 300 known substrates, and is constitutively active within the

cell [14]. Thus, differences in CK2 phosphorylation profiles between the HCV genotypes likely stem from the number and location of specific phosphorylation sites, rather than activity of the kinase itself. NS5A contains three domains that are separated by two sequences consisting of repetitive amino acids [24]. Position 1 in the amino acid sequence of NS5A1b corresponds to position 1973 in the HCV 1b genome sequence [24]. Domain III of NS5A1b is comprised of residues 356–447 [13]. Residues 228–278 and 378–447 are required for basal phosphorylation [25]. Analysis of NS5A 1a and 1b C-terminus sequences [14] indicates that Ser386, Thr 406, and Ser383 residues are predicted CK2 phosphorylation sites unique to NS5A genotype 1b. It may be these CK2 directed sites that enable NS5A1b to proceed in its inhibition of Kv2.1 activity. It is possible that a conformational change occurs with NS5A phosphorylation, which may enable the viral protein's further interaction with host cell proteins [24]. In future studies, experiments should test whether NS5A1b domain 3 is sufficient to reduce Kv2.1 function during apoptosis.

Of note, the functional significance of NS5A phosphorylation by CK2 in HCV infection has remained unresolved [24]. Mutations affecting basal phosphorylation and deletions of all of the CK2 phosphorylation sites in NS5A did not have a significant impact on viral RNA replication in a replicon system [25]. Although these residues may not be required for viral replication, our results suggest that, in our system, phosphorylation by host-cell CK2, possibly in the aa 383–406 region [14], enables NS5A1b to interfere with cellular apoptosis [9]. Interestingly, our results also confirm that the amino acid sequence that is required for NS5A1b's ability to modulate Kv2.1 activity is distinct from the residues bound and inhibited by Ledipasvir. Although Ledipasvir's exact mechanism of antiviral action is still unclear, one avenue by which NS5A inhibitors may work is prevention of hyperphosphorylation [17]. It is thought that a tightly regulated control of NS5A phosphorylation vs. hyperphosphorylation is required for efficient viral function and replication [17]. Also it is now known that Ledipasvir inhibits NS5A through direct binding and that a drug associated resistance mutation (Y93H) in NS5A1b-domain I reduces the binding affinity, and presumably the inhibitory effect, of Ledipasvir[26]. Thus, the work presented here will aid in future studies aimed at determining the precise sequence required for the anti-apoptotic effect of NS5A1b.

Surprisingly, we found that treatment with the CK2 inhibitor, IQA, inherently increases Kv2.1 mediated K^+ currents in vector-expressing cells. One explanation for this phenomenon would be a hyperpolarizing shift in the voltage-dependency of Kv2.1 channel activation after treatment with IQA, whether from the compound itself, or from blockage of an indirect effect of CK2 on Kv2.1 channel activation. However, we found Kv2.1 channel voltage-gated activation to be the same in vehicle-treated and IQA-treated neurons. Another potential explanation for our result is an indirect effect of CK2 on Kv2.1 activity via a signaling pathway. CK2 is a widely expressed, conserved serine-threonine kinase, the signaling function of which is extensive. The majority of the kinase's substrates are implicated in signal transduction, gene expression, and transcription-related functions [20]. Holoenzymic CK2 is a tetramer of catalytic (CK2 α) and regulatory (CK2 β) subunits. It is constitutively active either with or without its non-catalytic β subunits [20]. Several studies have shown that the α subunit of CK2 associates with protein phosphatase 2A (PP2A), and in some systems enhances the activity of PP2A via phosphorylation. [27,28,29]. PP2A is

regulated by posttranslational modifications to the C subunit such as phosphorylation [27,30,31]. In turn, Yokoyama & Miller [27] reported that Src kinase function is inhibited by PP2A; PP2A directly binds to regulatory and catalytic domains of Src. This may be a potential pathway involving CK2 which indirectly downregulates Src phosphorylation of Kv2.1 via PP2A activation, and thus diminishes Kv2.1 activity under normal, steady-state conditions. Therefore, as CK2 has widespread function in different signaling cascades, it is possible that suppression of CK2 activity in our system indirectly led to increased Kv2.1 activity via this pathway.

The data presented here will help to identify the components of the NS5A1b protein that are absolutely necessary for restricting the role of the Kv2.1 channel and thus for establishing neuroprotection. NS5A is a multifunctional protein, originally made in host cells to promote viral replication [8,23], thus some components of NS5A1b in domains 1 and 2 are likely unnecessary for neuroprotection and could even present potential complications such as activation of detrimental cellular processes. Experimental verification of NS5A1b derived sequences will provide the foundation for design of novel neuroprotective therapeutic probes in future studies.

5. Conclusions

Our results indicate that NS5A1b-mediated inhibition of Kv2.1 function is critically dependent on its phosphorylation status at genotype-specific CK2-directed residues and that Ledipasvir does not interfere with NS5A1b's modulation of Kv2.1-mediated K⁺ currents. NS5A1b, although it has evolved in a virus to aid in producing chronic infections, could provide the basis for development of a novel protective therapy for injuries that lead to an apoptotic K⁺ surge in injured neurons.

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Highlights

- Neurons expressing NS5A1b have decreased Kv2.1-mediated K⁺ currents
- Casein kinase 2 inhibition blocks K⁺ current modulation by NS5A1b
- Casein kinase 2 inhibition potentiates K⁺ currents
- Ledipasvir treatment does not block NS5A1b's modulation of Kv2.1 function

Figure 1.

NS5A1b modulates outward K^+ currents; CK2 inhibition by IQA blocks current modulation by NS5A1b and potentiates currents in vector-expressing cells. **(A)**Top: Whole-cell K^+ currents in rat cortical neurons in culture, evoked with a series of 10 mV 200-ms depolarizing steps (-80 to $+80$ mV). Scales are 7 nA and 25 ms. Neurons were transfected with empty vector eGFP or NS5A1b. The two rightmost traces represent neurons treated with 10 μ M IQA, CK2 inhibitor. Bottom: Bars represent mean ($\pm$ s.e.m.; $n=12-19$ cells per group) current densities at $+10$ mV. $*p<0.05$, ANOVA/Bonferroni: vector vs. vector/IQA (first vs. third bar); $*p<0.05$, ANOVA/Bonferroni: vector vs. NS5A1b (first vs. second bar); $***p<0.001$, ANOVA/Bonferroni: NS5A1b vs. NS5A1b/IQA (second vs. fourth bar). **(B)** Average K^+ current increase by treatment with IQA (CK2 inhibitor), expressed as a percentage increase ($\pm$ s.e.m.) of the average currents present in untreated neurons of each group; $n=12-19$ cells per group (60 cells total); $**p<0.01$, unpaired t test, increase of current in NS5A1b expressing neurons treated with IQA is significantly different from increase seen in vector expressing group. **(C)**Treatment with CK2 inhibitor (10 μ M IQA) induces a voltage-gated K^+ current surge in cortical neurons. Shown is the steady state current-voltage relationship from representative traces. **(D)** Main plot shows the conductance-voltage (G-V) relationship of peak potassium current recorded from vector-

transfected neurons in the vehicle and IQA treatment groups. Data points represent the mean $(G/G_{\max}) \pm \text{s.e.m.}$ from 12–13 neurons.

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Figure 2.

Ledipasvir treatment does not affect NS5A1b's inhibition of outward K^+ currents. Top: Whole-cell K^+ currents in rat cortical neurons in culture were evoked with a series of 10 mV 200-ms depolarizing steps (-80 to $+80$ mV). Traces are representative whole cell recordings in each treatment group, indicated in the bottom panel. Scale bars are 4nA and 25ms. Neurons were transfected with the empty vector eGFP or NS5A1b. The rightmost group represents neurons also incubated with 3 μ M Ledipasvir, a known inhibitor of NS5A's viral replication function. Bottom: Bars indicate mean ($\pm$ s.e.m.; $n=14-20$ cells per group) current densities (pA/pF) at $+10$ mV. ** $p<0.01$, ANOVA/Bonferroni: vector vs. vector/NS5A1b (first vs. second bar); ** $p<0.01$, ANOVA/Bonferroni: vector vs. NS5A1b/Ledipasvir (first vs. third bar).