European Physiology Day
Virtual Meeting
12th October 2021

Functional characterization of Kir2.1 channel in an oxidative stress-related model of aging neuroglia

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OBJECTIVE

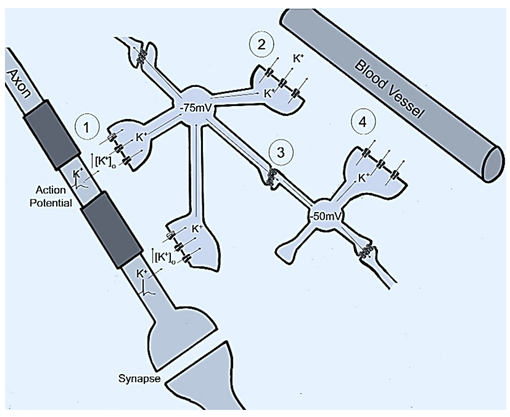

Figure 1. Model of K^+ spatial buffering. Potassium released during action potential propagation is taken up by astrocytes processes at nodes of Ranvier and synapses via strongly rectifying Kir channels, i.e. homomeric Kir2.1 channels, heteromeric Kir4.1/2.1 and Kir4.1/Kir5.1 channels. Potassium is extruded from glia at sites of low K^+ activity via weakly rectifying Kir4.1 homomeric channels. Taken from (3).

Epilepsy is a chronic disease of the brain and its prevalence increases with age. Strikingly, about 50% of all epilepsy cases diagnosed in elderly patients (>65 years) are idiopathic (1). Metabolic changes, including the production of reactive oxygen species, may contribute to epilepsy development (2). The neuronal glia plays a crucial role in epilepsy by controlling neuronal hyperexcitability. One of the key roles of glial cells is the spatial buffering of extracellular K^+ ions that are released by excited neurons and transported through glial inwardly rectifying potassium (Kir) channels from extracellular regions of high K^+ to those of low K^+ to inhibit epileptogenesis (**Figure 1**) (3). However, whether Kir channels can be the target of oxidative stress during aging is not known. Among experimental oxidative stress-related aging models, exposure to D-galactose (D-gal) is considered the most similar to natural aging (4). In the present study, we investigated the effect of D-gal-induced aging on Kir channel function in glioblastoma U87-MG cells.

METHODS

RT-qPCR was utilized to identify the isoform(s) of Kir channels expressed in glioblastoma U87-MG cells. In addition, cell viability and oxidative stress following exposure of cells for 24 hours to 30 or 100 mM D-gal have been assessed by the MTT colorimetric assay and estimation of thiobarbituric acid reactive substances (TBARS) levels –a marker of lipid peroxidation– as well as membrane total sulfhydryl (SH) groups, respectively. The membrane K^+ conductance was measured by the patch-clamp technique in whole-cell configuration. As Kir channels are sensitive to Ba^{2+}, all currents have been subtracted of the Ba^{2+}-insensitive component. Moreover, to confirm the role of Kir2.1, ML-133, a specific inhibitor, was used in patch clamp technique. To obtain equal osmolarity, equimolar amounts of mannitol were used as the control for D-gal. The oxidizing agent TBH70X (1 mM) was used as an alternative to D-gal for inducing oxidative stress.

RESULTS

Screening of all 15 known isoforms of Kir channels by RT-qPCR revealed that the predominant transcript expressed in U87-MG cells corresponds to the Kir2.1 channel (**Figure 2**). Among other Kir channels known to be expressed in neuronal glia, Kir4.1 was 26-fold less expressed and other Kir members, such as Kir5.1, were virtually absent. Additionally, exposure of cells to 10 μM ML-133, a specific inhibitor of Kir2.1, induced a decrease of inwardly rectifying K^+ currents in U-87MG cells (**Figure 3 A**), thus confirming RT-qPCR results. Conversely, application of 10 μM VU013, a specific inhibitor of Kir4.1, failed to inhibit the potassium current in U-87MG cells (**Figure 3 B**).

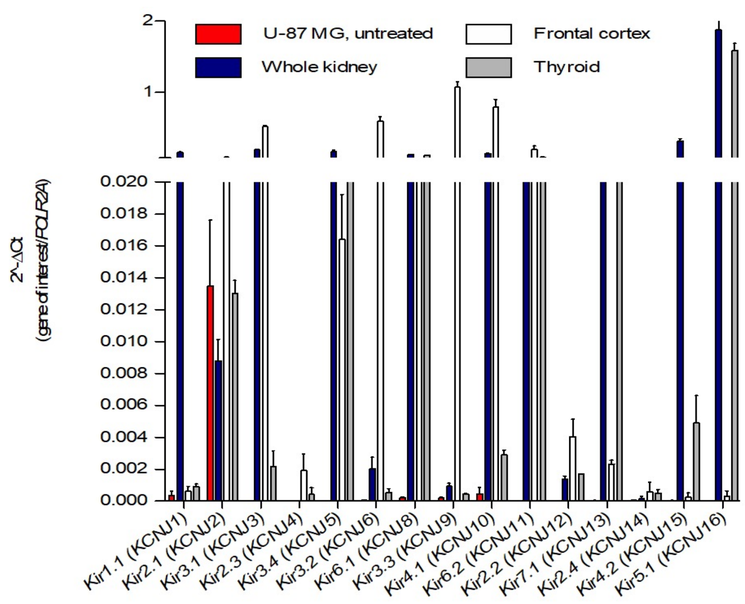

Figure 2. Kir transcript expression in U87-MG cells. One ug total RNA from three independent subcultures was reverse transcribed and probed for expression of the transcripts of all 15 known Kir isoforms by qPCR on the RotorGene (QIAGENE) platform. Human whole kidney, frontal cortex and thyroid served as positive controls.

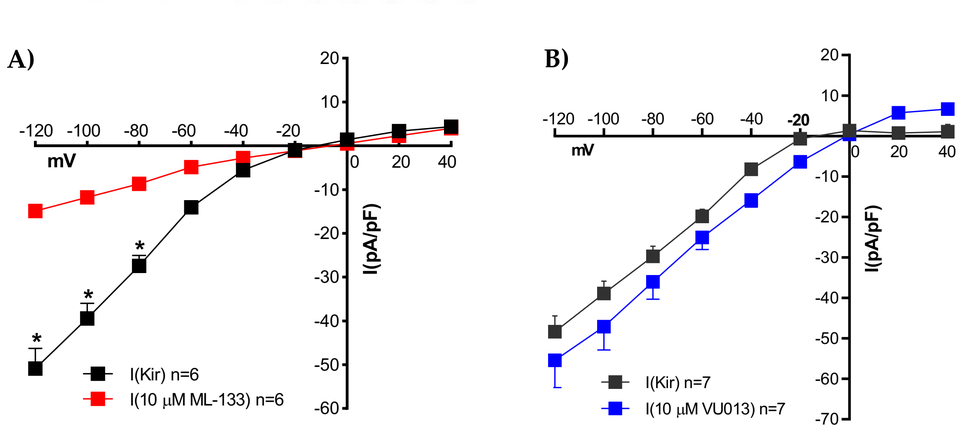

Figure 3. ML-133 reduced, while VU013 did not affect the Ba^{2+}-sensitive K^+ conductance in U87-MG cells. The whole cell inwardly rectifying Ba^{2+}-sensitive K^+ current was studied in U87-MG cells endogenously expressing Kir2.1. The voltage protocol consisted of voltage steps from -120 to 40 mV in 20 mV increments from a holding potential of -60 mV. The duration of the voltage steps was 400 ms. **A**) Exposure to ML-133 inhibited the K^+ current, while **B**) VU013 was ineffective. *p<0.05, unpaired Student's t-test. (n) refers to the number of cells.

D-gal (30 and 100 mM) or TBH70X had no obvious cytotoxicity, but 100 mM D-gal or TBH70X activated oxidative stress pathways, namely significantly enhanced TBARS levels and reduced the abundance of membrane SH groups (**Figure 4)**

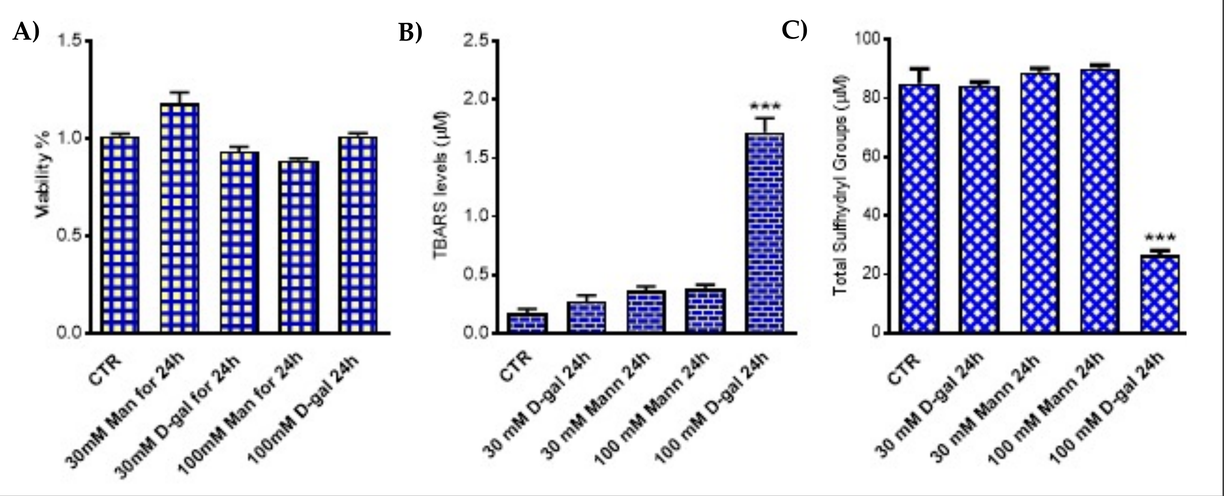

Figure 4. *In vitro* effects of increasing concentrations of D-gal (30 and 100 mM) for 24 hours, and 1 mM TBH70X for 2 hours on **A**) cell viability (MTT assay), **B**) thiobarbituric acid reactive substance (TBARS) and **C**) total sulfhydryl groups. ***p<0.001 versus control, 30mM D-gal, 30-100 mM Mannitol, and 1 mM TBH70X as determined by one way ANOVA followed by Bonferroni's post hoc test (n=3).

Interestingly, 100 mM D-Gal exposure was associated with a pronounced decrease of Ba^{2+}-sensitive inwardly rectifying K^+ currents, most likely mediated by Kir2.1 (**Figure 5 B**) ($p<0.05$, n=5). Importantly, 30 mM D-gal and 30-100 mM mannitol failed to elicit oxidative stress and, accordingly, had no significant effect on the Ba^{2+}-sensitive K^+ current (n=5) (**Figure 5 A**) .

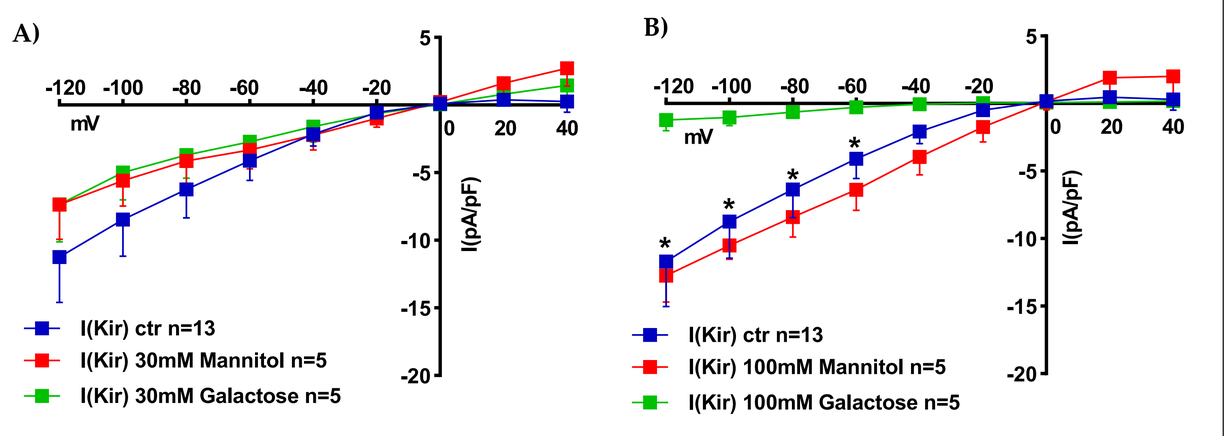

Figure 5. D-gal inhibits Kir2.1 channel function. The voltage protocol consisted of voltage steps from -120 to 40 mV in 20 mV increments from a holding potential of -60 mV. The duration of the voltage steps was 400 ms. **A**) Treatment with 30 mM D-gal for 24 hours did not suppress the Kir2.1current **B**) Treatment with 100 mM D-gal for 24 hours suppressed the Kir2.1current. Kir2.1 currents were isolated from the total currents by subtracting currents measured in the same cell after application of 1mM $BaCl_2$, an efficient blocker of Kir2.1. *p<0.05, unpaired Student's t-test. (n) refers to the number of cells.

Exposure of cells for 5 minutes or pre-incubation for 2 hours with 1 mM TBH70X significantly reduced the Ba^{2+}-sensitive K^+ currents compared to control, thus supporting the concept that the activity of Kir2.1 is highly sensitive to oxidative stress (**Figure 5 A-B**).

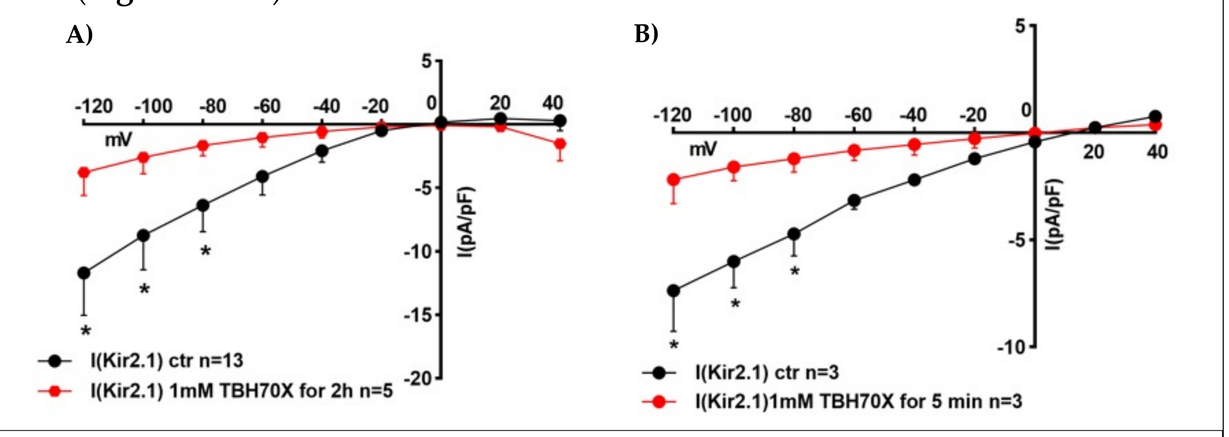

Figure 6. Oxidation due to TBH70X application inhibits Kir2.1 channels. Whole cell current was studied in U87-MG cells endogenously expressing Kir2.1. The voltage protocol consisted of voltage steps from -120 to 40 mV in 2 0mV increments from a holding potential of -60 mV. The duration of the voltage steps was 400 ms. Exposure to 1mM TBH70X for 5 minutes or 2 hours inhibited the Kir2.1 current (**A-B**). *p<0.05, unpaired Student's t-test. (n) refers to the number of cells.

CONCLUSIONS

-These findings reveal a novel Kir2.1 channel modulation that is likely to occur in oxidative stress.

-We suggest that inhibition of Kir2.1 in neuronal glia may alter the extracellular K^+ buffering and contribute to oxidative stress-related neuronal hyperexcitability and epileptogenesis during aging.

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