

Endothelial K_{ir} channels: Do they act as amplifiers of endothelial cell hyperpolarization?

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Abstract

Objective: We examined whether middle cerebral artery (MCA) endothelial cells express K_{ir} channels, and if so, whether K_{ir} channels act to amplify agonist-stimulated endothelial cell (EC) hyperpolarization. EC hyperpolarization has been shown to directly promote vasodilation. **Methods:** K_{ir} 2.x channel cDNA was evaluated in freshly isolated EC by RT-PCR. K_{ir} channel function was measured in EC by whole cell patch clamp. The role of K_{ir} channels in EC hyperpolarization (voltage-sensitive dye-di-8-ANEPPS) and vasodilation was evaluated in intact pressurized MCA. **Results:** Freshly isolated EC expressed RNA for K_{ir} for K_{ir} 2.1 and 2.2, whereas whole MCA expressed K_{ir} 2.1, 2.2, 2.3, and 2.4. Whole cell patch clamp in EC demonstrated a Ba^{2+} -sensitive inwardly rectifying current. This current exhibited established characteristics of K_{ir} channel currents. Luminal $BaCl_2$ ($75 \mu M$) was used to evaluate the functional role of K_{ir} channels in agonist-mediated ($10 \mu M$ UTP) EC hyperpolarization and vasodilation in pressurized MCA. Hyperpolarization and dilation to luminal UTP were both slightly reduced in the presence of $BaCl_2$. **Conclusion:** We demonstrated that MCA EC express functional K_{ir} channels that are likely of the K_{ir} 2.1 or 2.2 subtype. The K_{ir} channels contribute to UTP-mediated vasodilation and may act through amplification of EC hyperpolarization.

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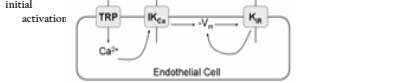
Introduction

Endothelial hyperpolarization promotes vasodilation in a number of small arteries and arterioles. In fact, endothelial hyperpolarization may be the fundamental endothelial event required to produce endothelium-dependent hyperpolarizing factor (EDHF)-mediated dilation.

In rat middle cerebral artery (MCA), EDHF-mediated dilation critically involves the activation of endothelial intermediate conductance K_{Ca} (K_{Ca}) channels and subsequent endothelial cell hyperpolarization. However, we believe that the resulting endothelial hyperpolarization reflects more than the effect of activation of K_{Ca} channels alone. In this study, we examined a potential role of endothelial inward rectifier K^+ (K_{ir}) channels in amplifying K_{Ca} -mediated hyperpolarization (see schematic below).

We propose the following hypotheses:

- 1) MCA endothelial cells possess functional K_{ir} channels.
- 2) Endothelial cell hyperpolarization resulting from the initial activation of K_{Ca} channels amplifies hyperpolarization resulting from the initial activation of K_{ir} channels.



Methods

Experiments were performed with MCA obtained from male Long Evans rats or freshly isolated MCA endothelial cells. Experimental protocols were approved by the Animal Protocol Review committee at Baylor College of Medicine.

MCA Dissection: MCA were harvested, cut into short segments, and digested using a combination of papain and collagenase/dispase (see Marrelli et al. 2009). The artery segments were gently triturated to release smooth muscle and endothelial cells.

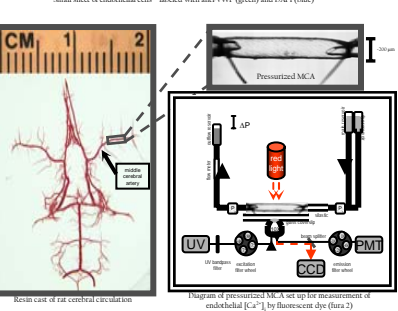
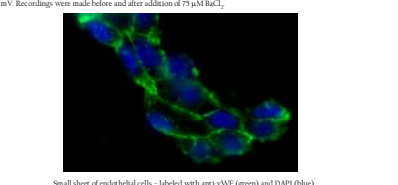
RT-PCR: Endothelial cells (in multi cell planar sheets) were harvested by glass microspipette. Messenger RNA was isolated using the RNeasy Micro kit (Qiagen) followed immediately by first strand cDNA synthesis with the Superscript III reverse transcriptase (Invitrogen). Vector controls and RT (-) controls were run to rule out genomic or solution contamination.

Western Blot: MCA were harvested, frozen, and crushed under liquid N₂ prior to protein extraction. SDS gel lanes were loaded with 5 μ g total protein, size fractionated, and transferred to nitrocellulose membrane. Antibodies were prepared in black solution consisting of 5% NDM and 0.1% Tween. A chemiluminescence system was used to detect immunogenic bands.

Immunofluorescence: The MCA were harvested and placed in an isolated recirculation bath. The artery was then cannulated with two glass microspipettes through which the artery was pressurized (95 mm Hg) and luminally perfused (100 μ l/min). The tissue bath was placed on the stage of an inverted microscope and the artery imaged on a video screen. All vessel experiments were performed in the presence of 1 μ M NMDA and indomethacin to inhibit NMDA and cyclooxygenase, respectively.

Endothelial Membrane Potential Measurement: The endothelium was selectively loaded in pressurized MCA with a voltage sensitive fluorescent dye, di-8-ANEPPS (see Marrelli et al. 2003). The dye was excited at 475 nm and detected at 560 and 620 nm wavelengths. The resulting F_{560}/F_{620} ratio reflects changes in endothelial membrane potential, where a decrease corresponds to hyperpolarization.

Whole Cell Patch Clamp of Endothelial Cells: Whole cell voltage-clamp measurements were performed with freshly isolated MCA endothelial cells in the ruptured configuration. The bath solution contained 30 mM K⁺ and the pipette solution contained 145 mM K⁺, resulting in a predicted E_K of 30 mV. The voltage was ramped from -100 to +40 mV from a holding potential of -50 mV. Recordings were made before and after addition of 75 μ M $BaCl_2$.



Results

Which K_{ir} channel isoforms are expressed in rat MCA?

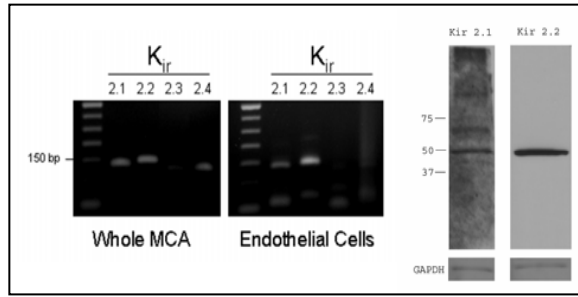
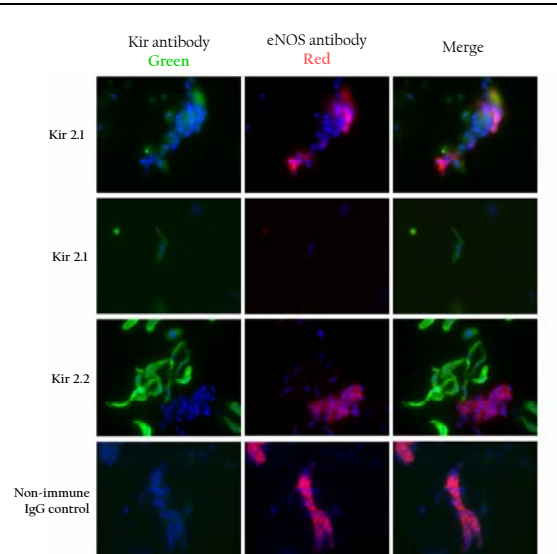


Figure 1: (Left) RT-PCR demonstrating message for K_{ir} 2.x channel isoforms in whole middle cerebral artery (MCA) and in freshly isolated MCA endothelial cells. The predicted product sizes are as follows: K_{ir} 2.1 (136 bp), 2.2 (145 bp), 2.3 (120 bp), and 2.4 (127 bp). (Right) Western blot analysis of K_{ir} 2.1 and 2.2 channels in whole MCA. Lanes were loaded with 5 μ g total protein. The calculated mass of K_{ir} 2.1 and 2.2 is 48.2 and 48.4 kDa, respectively. K_{ir} channel antibodies were purchased from Sigma.

Which cell types in the MCA express K_{ir} 2.1 or K_{ir} 2.2 channel protein?



Inhibition of endothelial K_{ir} channels results in attenuated endothelial cell hyperpolarization in pressurized MCA

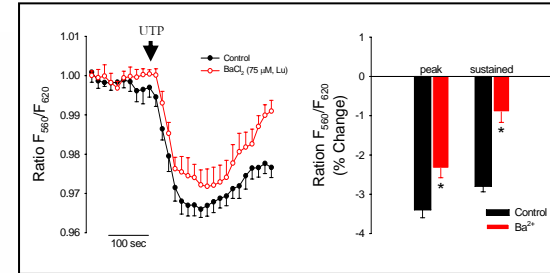


Figure 4 (top): Measurement of endothelial cell hyperpolarization in response to luminal UTP (10^{-5} M) in pressurized MCA. In one group, $BaCl_2$ ($75 \mu M$) was included in the luminal perfusate to inhibit endothelial K_{ir} channels (red open circles). Hyperpolarization is reflected by a decrease in the F_{560}/F_{620} ratio. (Left) Summary of individual responses to luminal UTP ($n=7$ and 5 for control and $BaCl_2$, respectively). (Right) Summary of peak and sustained hyperpolarization to UTP in the absence or presence of luminal $BaCl_2$. Peak and sustained hyperpolarizations are significantly reduced by the presence of luminal $BaCl_2$ ($P<0.05$, t-test).

Figure 2 (left): Immunofluorescence analysis of K_{ir} 2.1 and K_{ir} 2.2 channel expression in freshly dispersed cells of rat MCA. Endothelial cells were identified based on immunoreactivity to eNOS antibody (red). Smooth muscle cells were identified based on the elongated spindle-shaped nucleus (blue). Faint immunoreactivity to K_{ir} 2.1 (green) was detected in endothelial cells and smooth muscle cells (rows 1 and 2). Strong immunoreactivity to K_{ir} 2.2 (green) was detected in smooth muscle cells, but not in endothelial cells (row 3). Control experiments were performed in which non-immune IgG was substituted for the primary antibody (green, row 4).

Endothelial cells possess Ba^{2+} -sensitive inwardly rectifying K^+ channels

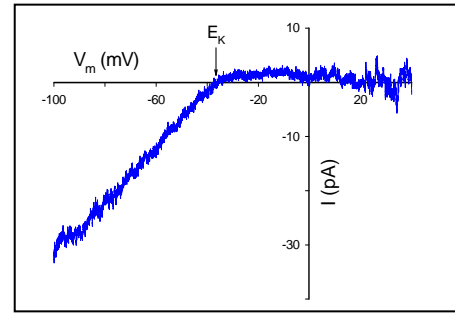


Figure 3: Whole cell patch clamp of an MCA endothelial cell demonstrating a Ba^{2+} -sensitive inwardly rectifying K^+ current. The current trace above was obtained by subtracting the trace with Ba^{2+} from the pre- Ba^{2+} control trace. The bath solution contained 30 mM K⁺ in order to elicit more prominent inward currents. The predicted E_K in these conditions is -38 mV (arrow). Note the outward 'hump' between -40 mV and 0 mV that is characteristic of K_{ir} channels.

Inhibition of endothelial K_{ir} channels makes EDHF-dependent dilations transient in pressurized MCA

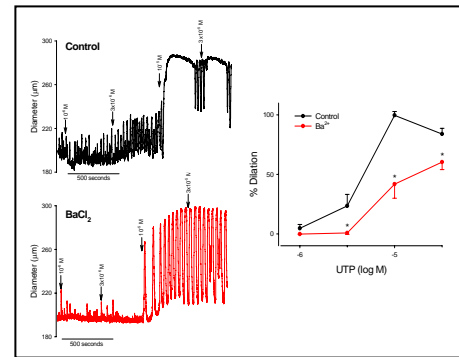


Figure 5: (Left) Representative diameter traces of pressurized MCA in response to luminal UTP in the absence (black) or presence of luminal $BaCl_2$ ($75 \mu M$, red). The concentration and point of delivery of UTP is indicated by the arrows. Note that the presence of $BaCl_2$ converted the sustained dilation to UTP into an oscillatory response. (Right) Summary of UTP-mediated dilations in the absence or presence of $BaCl_2$. The diameters were obtained by performing a time average over 300 seconds. Luminal $BaCl_2$ results in significantly reduced EDHF-mediated dilations in pressurized MCA (2 way RM-ANOVA).

Summary & Conclusions

These data demonstrate that:

- MCA endothelial cells express message for Kir 2.1 and Kir 2.2, however, protein was detected only for Kir 2.1.
- MCA smooth muscle cells express Kir 2.1 and Kir 2.2 protein.
- MCA endothelial cells possess Ba^{2+} -sensitive inwardly rectifying K^+ currents characteristic of K_{ir} channels.
- Inhibition of endothelial K_{ir} channels results in reduced endothelial cell hyperpolarization to UTP in pressurized MCA.
- Inhibition of endothelial K_{ir} channels results in attenuated EDHF-mediated dilations in pressurized MCA.

From these data, we conclude that 1) endothelial cells possess functional K_{ir} channels and 2) that these K_{ir} channels contribute to endothelial cell hyperpolarization and EDHF-mediated dilation. Our data suggests that MCA endothelial cells express K_{ir} 2.1 channels, however, we cannot exclude the possibility of less abundant expression of K_{ir} 2.2 channels as well.

Acknowledgements

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