Targeted Disruption of Kir2.1 and Kir2.2 Genes Reveals the Essential Role of the Inwardly Rectifying K⁺ Current in K⁺-Mediated Vasodilation

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Abstract—The molecular bases of inwardly rectifying K⁺ (Kir) currents and K⁺-induced dilations were examined in cerebral arteries of mice that lack the Kir2.1 and Kir2.2 genes. The complete absence of the open reading frame in animals homozygous for the targeted allele was confirmed. Kir2.1−/− animals die 8 to 12 hours after birth, apparently due to a complete cleft of the secondary palate. In contrast, Kir2.2−/− animals are viable and fertile. Kir currents were observed in cerebral artery myocytes isolated from control neonatal animals but were absent in myocytes from Kir2.1−/− animals. Voltage-dependent K⁺ currents were similar in cells from neonatal control and Kir2.1−/− animals. An increase in the extracellular K⁺ concentration from 6 to 15 mmol/L caused Ba²⁺-sensitive dilations in pressurized cerebral arteries from control and Kir2.2 mice. In contrast, arteries from Kir2.1−/− animals did not dilate when the extracellular K⁺ concentration was increased to 15 mmol/L. In summary, Kir2.1 gene expression in arterial smooth muscle is required for Kir currents and K⁺-induced dilations in cerebral arteries. (Circ Res. 2000;87:160-166.)

Key Words: arteries □ vasodilation □ potassium channels □ muscle, smooth □ Kir2.1 channel

Local blood flow in the brain is matched to the metabolic needs of nearby neurons. Recent advances in imaging brain activity have taken advantage of this relationship between neuronal activity and local blood supply to map functional organization within the brain.¹ Potential metabolic signals to the cerebral vasculature include carbon dioxide, lactic acid, adenosine, H⁺, and histamine. However, a potent signal that has often been overlooked is an increase in extracellular potassium ions. During periods of cerebral hypoxia, ischemia, or hypoglycemia, extracellular K⁺ concentration ([K⁺]) can rise to >10 mmol/L.² Increases in [K⁺] have also been shown to occur with changes in neuronal¹ and cardiac activity. These modest rises in [K⁺] cause vasodilation of small cerebral and coronary vessels³ and thereby may cause a selective increase in the perfusion of metabolically active tissue.

Although large increases in [K⁺], cause depolarization, the elevation of [K⁺] to 10 to 16 mmol/L hyperpolarizes the membrane potential of arterial smooth muscle.⁴,⁵ This hyperpolarization decreases the open probability of voltage-activated Ca²⁺ channels, which in turn reduces cytosolic Ca²⁺ levels and promotes vasodilation.⁶,⁷ To explain the paradoxical sustained hyperpolarization in response to modest increases in [K⁺], ² hypotheses have been proposed, invoking either the Na⁺,K⁺-ATPase⁸ or Kir channels.⁹,⁶,¹⁰ Modest increases, from 0 mmol/L to <5 mmol/L in [K⁺], cause transient vasodilations of cerebral arteries, which were prevented by inhibitors of the Na⁺,K⁺-ATPase.¹¹ In this case, [K⁺], presumably stimulates the electrogenic Na⁺,K⁺-ATPase, which causes a transient membrane potential hyperpolarization; this decays as Na⁺ is extruded.

The elevation of [K⁺], also activates Kir channels and thereby causes membrane hyperpolarization toward the potassium equilibrium potential (E钾). The membrane potential of myocytes in pressurized arteries is ≈40 mV positive to E钾. Therefore, increased Kir channel activity in 15 mmol/L [K⁺], would cause a sustained hyperpolarization.⁶,⁷ Kir currents have been characterized in intact voltage-clamped cerebral arteriolar⁶ and isolated arterial myocytes.¹¹ Native Kir channels in arterial smooth muscle and cloned Kir2 family members show strong inward rectification, a conductance dependent on [K⁺] and a similar voltage- and time-dependent gating process.⁷,¹¹ Recently, Bradley et al₁² identified transcripts for Kir2.1, but not Kir2.2 or Kir2.3, in isolated smooth myocytes from rat cerebral, coronary, and mesenteric arteries and showed that the cloned Kir2.1 currents most closely resemble those of the native current.

To explore the functional role of Kir2.1 and Kir2.2 in the cerebral vasculature, we engineered mice that lack these channels. This strategy permits the study of the effects of the deletion of a single Kir gene on the ionic currents of individual arterial myocytes and the contribution of that gene to K⁺-induced vasodilation. We demonstrate the absence of K⁺ currents in myocytes isolated from cerebral arteries of...
Kir2.1−/− mice but not from control animals. Cerebral arteries from Kir2.1−/− mice fail to dilate in response to elevations of [K+], from 6 to 15 mmol/L. In contrast, control and Kir2.2−/− arteries dilated normally. These results provide compelling evidence for the involvement of the inward rectifier potassium channel, Kir2.1, in potassium-induced dilations of cerebral arteries.

Materials and Methods

Generation of Targeted Mice

Mapped 129S/v genomic plasmids were graciously provided by Drs J.B. Redell and Bruce Tempel (University of Washington). The Kir2.2 gene was isolated from a 129S/v mouse genomic library. The 5′ arm of the Kir2.1 targeting construct was a 5.2-kb SacI fragment of the genomic clone upstream of the Kir2.1 open reading frame. A 3.5-kb PstI fragment downstream of the cDNA coding sequence was used for the 3′ arm (Figure 1A). For Kir2.2, a 2.5-kb NotI-BamHI fragment 900 bases upstream from the Kir2.2 open reading frame formed the 5′ arm, whereas the 3′ arm was a 7.2-kb StuI-SmaI downstream fragment (Figure 1B). In both constructs, the neomycin resistance gene was interposed between the 2 arms and a thymidine kinase cassette was added 3′.

These constructs were electroporated into R1 ES cells (courtesy of Andras Nagy, Mount Sinai Hospital, Toronto, Canada) and selected with 200 μg/mL G418 and 2 μmol/L gancyclovir. Colonies with homologous recombination events were determined with DNA hybridization. DNA from Kir2.1-targeted cells was digested with AflII and probed with a 2.2-kb fragment 3′ to the targeting vector (Figure 1A). DNA from Kir2.2-targeted cells was digested with BamHI and probed with a 0.9-kb fragment 3′ to the Kir2.2 targeting vector (Figure 1B).

Mice that lack Kir2.1 were created through blastocyst injection (University of Cincinnati), and germ line transmission was confirmed. Kir2.2 mice were generated through aggregation with morulae from CD-1 mice.

Physiological Studies

Kir2.1−/− or control littermate mice (<1 day postnatal) were euthanized through exsanguination under deep pentobarbital anesthesia (150 mg/kg i.p.). Basilar, cerebellar, and posterior cerebral arteries were dissected in cold (4°C) oxygenated (95% O2/5% CO2) PSS of 140 mmol/L. Animals were killed by intraperitoneal injection of 150 mg/kg sodium pentobarbital and exsanguination under deep pentobarbital anesthesia. The entire open reading frame that encoded the Kir2.1 gene was removed, which ensured that a truncated version of the protein would not be produced. Twelve of the ~200 neomycin- and gancyclovir-resistant ES cell clones that were isolated and genotyped were heterozygous for the targeted allele. Four independent heterozygous clones were injected to produce chimeric mice. A Southern blot analysis of their offspring confirmed transmission of the targeted allele in the expected 50% ratio. Interbreeding of males and females heterozygous for the Kir2.1-targeted alleles produced the expected mendelian ratios: 40 Kir2.1+/+, 85 Kir2.1+/−, and 43 Kir2.1−/−. Heterozygous mice were bred into the FVB background to establish congenic lines from each of the 4 independent ES cell lines. Subsequent experiments were carried out with 2 of the 4 lines after 5 back crosses to the FVB line.

The complete absence of the Kir2.1 open reading frame in animals homozygous for the targeted allele was confirmed with Southern blot analysis (Figure 1C). Soon after birth, the Kir2.1−/− pups exhibit a gradual swelling of their stomach and small bowel with air. In addition, most of the Kir2.1−/− pups become cyanotic and gasp for breath. All Kir2.1−/− animals die within 12 hours after birth. The only apparent gross abnormality of the Kir2.1−/− animals is a complete cleft of the secondary palate (Figure 2). This defect is 100% penetrant in the homozygous pups regardless of strain background and is never observed in their heterozygous littermates. The cleft prevents the knockout pups from nursing and probably leads to dehydration. In addition, the cleft likely allows the aspiration of oral secretions and thereby causes the visible respiratory problems. The cleft is wide, and the bones of the nasal cavity are visible. Although there is some narrowing of the maxilla in the Kir2.1−/− pups, there are no defects in any of the other bones and cartilage derived from the first pharyngeal arch. In addition, derivatives of more posterior pharyngeal arches, including the hyoid bone and the thyroid and cricoid cartilage, are of normal size and morphology. Thus, the craniofacial defect is limited to palatogenesis.

Kir2.1 and Kir2.2 share a very high level of sequence homology, and their expression patterns overlap in both skeletal and cardiac myocytes. To guard against the possibility that our analysis of the Kir2.1 knockout would be complicated by a compensatory upregulation of Kir2.2, we also generated a Kir2.2 knockout. As with Kir2.1, the entire open reading frame of Kir2.2 was removed (Figure 1B). Eight of ~150 neomycin- and gancyclovir-resistant ES cell clones were heterozygous for the targeted allele. Two of these heterozygous clones were used to generate chimeric mice. Interbreeding of males and females heterozygous for the Kir2.2-targeted alleles produced the expected mendelian ratios: 34 Kir2.2+/+, 70 Kir2.2+/−, and 30 Kir2.2−/−. Two FVB congenic lines, representing both of the independent ES cell clones, were established, and subsequent experiments were carried out with these lines after 5 back crosses to the FVB line.
DNA analysis confirmed the absence of the Kir2.2 open reading frame in animals homozygous for the targeted allele (Figure 1C). In contrast to Kir2.1\(^{2/2}\) mice, Kir2.2\(^{2/2}\) mice appear normal as adults. A histological analysis of their hearts and brains revealed no abnormalities.

Inward Rectifier Currents Are Absent in Cerebral Artery Myocytes Isolated From Kir2.1\(^{2/2}\) Mice

To explore the role of Kir2.1 channels, K\(^{+}\) currents were measured in isolated myocytes from cerebral arteries of control (wild type and heterozygous littermates) and Kir2.1\(^{2/2}\) mice. The control and Kir2.1\(^{2/2}\) myocytes were similar in size, based on cell capacitance (control 5.5 ± 0.3 pF, n = 25; Kir2.1\(^{2/2}\) 5.7 ± 0.6 pF, n = 23) but smaller than myocytes isolated from similar arteries of adult mice (12.1 ± 0.8 pF, n = 5).

Ba\(^{2+}\)-sensitive inward K\(^{+}\) currents in 140 mmol/L [K\(^{+}\)]\(_{o}\) were evoked with voltage ramps from -2100 to +40 mV at 0.3 mV/ms (Figure 3A). Myocytes from control arteries exhibited significant inward currents negative to the potassium equilibrium potential (\(\approx 0\) mV). Current density (\(-8.7±2.8\) pA/pF at \(-100\) mV, n = 9) was similar to previous measurements made with adult rat cerebral myocytes (\(\approx 8\) pA/pF)\(^{11}\). In contrast, inward currents were not detected in Kir2.1\(^{2/2}\) myocytes (\(-0.4±0.2\) pA/pF at \(-100\) mV, n = 9, significantly different from control, \(P<0.01\)) (Figures 3A and 3B).

To determine whether other K\(^{+}\) currents were affected by ablation of the Kir2.1 gene, voltage-dependent K\(^{+}\) currents were examined. Currents in 6 mmol/L [K\(^{+}\)]\(_{o}\) were elicited by a series of 10-mV depolarizing steps (\(-60\) to +50 mV) from a holding potential of \(-70\) mV (Figures 4A and 4B). Steady-state currents were measured at the end of the 1.5-second voltage step and were plotted, normalized to cell capacitance, as a function of the depolarizing voltage (Figure 4C). In marked contrast to the absence of Kir currents, there were no significant differences in the outward current ampli-
Figure 3. Kir channel currents are absent in myocytes isolated from cerebral arteries of neonatal Kir2.1−/− mice. A, Current-voltage relationship of Kir measured as a Ba2+/K+ sensitive current in cerebral artery myocytes from representative control and Kir2.1−/− animals. Currents were obtained during voltage ramps from −100 to +40 mV at 0.3 mV/ms in symmetrical 140 mmol/L K+ in the presence and absence of 100 μmol/L Ba2+. B, Summary of 100 μmol/L Ba2+/K+ sensitive current at −100 mV obtained from control (n=9 cells) and Kir2.1−/− (n=9) myocytes (P<0.01).

Figure 4. Voltage-dependent K+ channel currents are similar in control and Kir2.1−/− myocytes. A and B, Outward membrane currents elicited by a series of 10-mV depolarizing steps (−60 to +50 mV) from a holding potential of −70 mV in control (A) and Kir 2.1−/− (B) cerebral artery myocytes. Intracellular K+ was 140 mmol/L, and extracellular K+ was 6 mmol/L. C, Summary of the current-voltage relationship of voltage-dependent K+ channel currents in myocytes obtained from control (n=9) and Kir 2.1−/− (n=10) cerebral arteries.

Figure 5. U46619-constricted arteries from Kir2.1−/− mice fail to dilate in response to elevation of [K+]o. Neonatal control (A) and Kir2.1−/− (B) cerebral artery segments constrict in response to the thromboxane A2 mimic U46619. Constriction was reversed by the application of 15 mmol/L K+ in control (A) but not Kir2.1−/− (B) arteries. C, Summary of the percent K+-induced dilation in U46619-constricted cerebral arteries from control and Kir 2.1−/− mice (n=4 each). U46619 concentration was 0.1 to 0.3 μmol/L, *P<0.05.

tude between control and Kir2.1−/− myocytes (current density at +50 mV: 27.9±7.8 pA/pF, control, n=9; 34.0±11.0 pA/pF Kir2.1−/−, n=10).

Elevations in [K+]o Dilated Control but Not Kir2.1−/− Arteries

The neonatal lethality of the Kir2.1−/− mice required that techniques for the analysis of vascular reactivity be adapted to newborn mouse pups. Neonatal arteries were delicate, and sometimes both Kir2.1−/− and control animals exhibited spontaneous fluctuations in diameter. Neonatal control arteries constricted to the thromboxane A2 mimic U46619 (0.1 to 0.3 μmol/L): arterial diameter decreased from 115.3±11.3 to 65.3±5.3 μm (n=3). This constriction provided a background on which the effects of vasodilatory agents could be examined. Forskolin (1 μmol/L), an activator of adenylate cyclase, dilated cerebral arteries from neonatal controls, reversing by 43.7±15.0% (n=3) the constrictions observed in the presence of U46619.

U46619 also constricted cerebral arteries isolated from Kir2.1−/− neonatal mice from 105.0±12.4 to 70.7±9.7 μm (n=4). Forskolin at 1 μmol/L also dilated Kir2.1−/− arteries, reversing 47.0±21.8% (n=3) of the constriction to U46619. Elevation of [K+]o, from 6 to 15 mmol/L dilated U46619-constricted arteries from control mice by 71.5±3.1% (n=3) (Figures 5A and 5C). In marked contrast, elevations of [K+]o did not alter the diameter of arteries from Kir2.1−/− mice (Figures 5B and 5C), indicating a role of Kir2.1 channels in K+-induced dilatations.

To further investigate the role of Kir 2.1 channels, the effects of [K+]o, on pressure-induced constrictions were examined. Pressure-induced constriction (“myogenic tone”) is a major contributor to vascular resistance and the regulation of blood flow in vivo.8 Cerebral arteries from neonatal control and Kir2.1−/− mice also exhibit pressure-induced constrictions. The elevation of intravascular pressure to 40 mm Hg constricted cerebral arteries by 24.8±3.7% (n=5) in control mice and 26.8±3.8% (n=8) in Kir2.1−/− mice. The passive diameters, obtained in the presence of Ca2+-free PSS and 1 μmol/L nisoldipine, of control (95.8±12.5 μm) and Kir2.1−/− (88.6±9.1 μm) cerebral arteries were not significantly different at an intravascular pressure of 40 mm Hg.
The responses of pressurized cerebral arteries from control and Kir2.1\(^{-/-}\) mice to elevated [K\(^+\)]\(_o\) differed significantly from each another, which is consistent with observations in U46619-constricted arteries. Control pressurized arteries dilated to an increase in [K\(^+\)]\(_o\) from 6 to 15 mmol/L (Figures 6A and 6E), reversing 52.8±7.8\% (n=5) of the pressure-induced constriction. The addition of a blocker of Kir channels, BaCl\(_2\) (50 \mu mol/L), had no significant effect on diameter in 6 mmol/L K\(^+\) but prevented K\(^+\)-induced dilations (Figure 6E). In contrast, pressurized Kir2.1\(^{-/-}\) arteries did not dilate to 15 mmol/L [K\(^+\)]\(_o\) (Figures 6B and 6E). The failure of pressurized Kir2.1\(^{-/-}\) arteries to dilate in response to [K\(^+\)]\(_o\) did not correspond to a general loss of reactivity. Forskolin (1 \mu mol/L) reversed pressure-induced constrictions in both control and Kir2.1\(^{-/-}\) cerebral arteries (percent dilations: control 96.3±1.9\%, n=3; control: Kir2.1\(^{-/-}\) 77.3±6.9\%, n=6) (Figures 6C through 6E).

**Elevations in [K\(^+\)]\(_o\), Dilated Pressurized Cerebral Arteries From Control and Kir 2.2\(^{-/-}\) Mice**

To assess a possible role of Kir2.2 channels in the mouse vasculature, cerebral artery diameter was examined in Kir2.2\(^{-/-}\) adult mice and their wild-type littermates or age-matched FVB mice. Cerebral arteries from control and Kir2.2\(^{-/-}\) mice constricted to the same degree to intravascular pressure. At 80 mm Hg, control arteries constricted from 146.6±8.9 to 117.0±6.2 \mu m (n=7), and Kir2.2\(^{-/-}\) arteries constricted from 143.2±13.1 to 117.9±11.1 \mu m (n=7). In contrast to the results with Kir2.1\(^{-/-}\) arteries, increased [K\(^+\)]\(_o\) from 6 to 15 mmol/L caused similar dilations of cerebral arteries from control (46.3±5.4\%, n=7) and Kir 2.2\(^{-/-}\) mice (42.5±4.9\%, n=7) (Figure 7).

**Discussion**

We explored the role of Kir2.1 in giving rise to Kir currents and in mediating K\(^+\)-induced dilations of cerebral arteries by studying lines of mice in which the genes that encode Kir2.1 or Kir2.2 have been removed. In neonatal cerebral arteries, the Kir2.1 gene proved essential for the inward rectifier current and for vasodilation in response to elevated [K\(^+\)]\(_o\). These findings are consistent with a causal relationship between Kir and K\(^+\)-induced vasodilation. The Kir2.1 and Kir2.2 knockout mice have also provided an opportunity to study the roles that Kir2.1 and Kir2.2 play in the heart (J.J. Zaritsky and T.L. Schwarz, unpublished data, 1999). In the future, these mice should enable an examination of Kir2.1 and Kir2.2 in other tissues in which either channel is found, such as skeletal muscle, smooth muscle of the gastrointestinal tract, macrophages, and the central nervous system.

**Cleft Plate Phenotype of Kir2.1\(^{-/-}\) Mice**

The perinatal lethality of the Kir2.1 knockouts could derive from a defect in any of the organ systems that express this channel. However, because these animals do not evince arrhythmias or skeletal paralysis and because the appearance of the animals indicates dehydration and respiratory problems, the lethality is most likely the result of the cleft palate. The presence of a cleft palate in 100% of the Kir2.1\(^{-/-}\) animals suggests intriguing possible roles for the Kir2.1 protein. Although cleft palates can be associated with other
Role of Kir2.1 Channels in Cerebral Artery Smooth Muscle

Despite the early death of Kir2.1−/− pups, we were able to examine K+ currents from isolated arterial myocytes. Although Kir currents from control myocytes were similar to those observed in previous studies of adult myocytes, they were notably absent in myocytes from Kir2.1−/− animals. Voltage-dependent K+ currents in Kir2.1−/− myocytes appeared unaffected, indicating that the ablation of the Kir2.1 gene had no secondary effect on voltage-dependent K+ channels. Thus, the Kir2.1 gene is necessary for the Kir currents in cerebral arteries. This result is consistent with earlier reports that the native Kir current in arterial myocytes had biophysical and pharmacological properties similar to the cloned Kir2.1 channel.5,7,11,12 We cannot exclude the possibility that another subunit coassembles with Kir2.1 to form these channels, but because the loss of the current is complete in the Kir2.1−/− animals, it is not necessary to invoke any additional channel genes. The normal reactivity of arteries from Kir2.2−/− mice is also consistent with the hypothesis that Kir2.1 is the predominant Kir channel in cerebral artery myocytes.

The Kir2.1−/− mice thus provide a model in which the physiological significance of Kir currents can be examined. Previous work has suggested that Kir might contribute to sustained vasodilation to elevations of K+.5,6,10 Although elevations in [K+]o usually depolarize cells and therefore would be expected to cause the constriction of a blood vessel, modest increases in [K+]o (6 to 15 mmol/L) cause cerebral and coronary arteries to dilate.5,7,10 The conductance of Kir channels increases with increasing [K+]o, probably due to a decrease in polyamine block of the channel pore.14 The elevation of [K+]o would increase the small outward current through these channels, and the membrane potential would be driven toward EK.7 Thus, an elevation in [K+]o, to 15 mmol/L shifts EK from −80 to −60 mV and simultaneously hyperpolarizes the membrane potential of the myocytes from −45 mV to −60 mV.5 This hyperpolarization decreases the open probability of voltage-activated Ca2+ channels and thereby reduces cytosolic Ca2+ levels and vascular tone.8

Despite the short lifespan of the Kir2.1−/− mice, we were able to use cerebral arteries from these animals to probe the role of Kir. Consistent with a causal relationship between Kir and K+-induced dilations, elevations in [K+]o, did not dilate cerebral arteries from Kir2.1−/− animals, although neonatal arteries from control mice did dilate. The arteries in the mutants remained responsive to forskolin and to changes in Ca2+ influx. Thus, although other vasodilatory mechanisms remained intact, the removal of the Kir2.1 gene and inwardly rectifying currents resulted in the selective absence of K+-induced vasodilation.

In contrast, experiments that compared arteries from Kir2.2 knockout with control arteries failed to identify any differences. Both sets of adult vessels exhibited similar pressure-induced constrictions and both dilated when exposed to an external solution that contained 15 mmol/L K+ or to 0 mmol/L Ca2+. Thus, it is unlikely that the Kir2.2 gene plays a role similar to that of Kir2.1 in the regulation of vascular tone.

The alternative mechanism that had been proposed to account for K+-induced vasodilation suggested that modest increases in [K+]o, would increase the electrogenic Na+,K+-ATPase activity, causing hyperpolarization.9,10 However, previous studies had shown that the inhibition of the Na+,K+-ATPase with ouabain or dihydro-ouabain did not prevent dilations in response to increased [K+]o, >5 mmol/L.5,10 The present study strengthens the argument that the Na+,K+-ATPase does not play a major role in the vasodilation caused by increasing [K+]o, from 6 to 15 mmol/L, because no residual dilatory mechanism was detected once the inward rectifier current had been removed. Thus, the Na+,K+-ATPase alone was not sufficient for K+-induced dilations in these conditions. However, we cannot rule out the possibility that transient changes in Na+,K+-ATPase activity contribute to the regulation of cerebral artery diameter in response to changes in [K+]o, <5 mmol/L.

The availability of a knockout mouse that lacks K+-dependent vasodilation will permit the examination of the role of K+ as a messenger for the homeostatic regulation of blood flow in active tissue. Elevations in [K+]o, in the range used in the present study have been observed in the brain and myocardium.2,4,15 These observations, combined with the ability of K+ to dilate blood vessels, make K+ an attractive candidate messenger to communicate the local state of activity to blood vessels.

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