Genetic Inactivation of \( \text{Kcnj16} \) Identifies Kir5.1 as an Important Determinant of Neuronal \( \text{PCO}_2/\text{pH} \) Sensitivity*

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The molecular identity of ion channels which confer \( \text{PCO}_2/\text{pH} \) sensitivity in the brain is unclear. Heteromeric Kir4.1/Kir5.1 channels are highly sensitive to inhibition by intracellular pH and are widely expressed in several brainstem nuclei involved in cardiorespiratory control, including the locus coeruleus. This has therefore led to a proposed role for these channels in neuronal \( \text{CO}_2 \) chemosensitivities. To examine this, we generated mutant mice lacking the Kir5.1 (\( \text{Kcnj16} \)) gene. We show that although locus coeruleus neurons from \( \text{Kcnj16}^{+/+} \) mice rapidly respond to cytoplasmic alkalinization and acidification, those from \( \text{Kcnj16}^{-/-} \) mice display a dramatically reduced and delayed response. These results identify Kir5.1 as an important determinant of \( \text{PCO}_2/\text{pH} \) sensitivity in locus coeruleus neurons and suggest that Kir5.1 may be involved in the response to hypercapnic acidosis.

Inwardly rectifying potassium (Kir)\(^{4} \) channels are important for the regulation of the resting membrane potential and the control of cellular electrical activity (1). However, the physiological role of the Kir5.1 channel remains unclear because this subunit does not produce functional K\(^+ \) channels when expressed by itself. Instead, it appears to coassemble selectively with either Kir4.1 or Kir4.2 subunits to form novel heteromeric channels (2–4).

Heteromeric Kir4.1/Kir5.1 channels exhibit several unique biophysical properties compared with homomeric Kir4.1, but most importantly they show a dramatic increase in sensitivity to inhibition by intracellular H\(^+ \) (pHi) while remaining insensitive to extracellular pH (5–9). Although several other Kir channels (Kir1.1, Kir2.3, Kir4.1, and Kir4.2) also exhibit some degree of pH sensitivity, heteromeric Kir4.1/Kir5.1 channels are highly sensitive within the physiological range (Kir4.1/ Kir5.1 pKi\(_d\) = 7.4) (3). These channels therefore provide a link between changes in intracellular pH and control of the resting membrane potential.

Both Kir5.1 and Kir4.1 were originally cloned from the brain and are expressed abundantly in the brainstem, especially in several \( \text{CO}_2 \)-chemosensitive nuclei involved in cardiorespiratory control. In particular, both subunits are coexpressed in locus coeruleus (LC) neurons (10, 11). The LC is a \( \text{CO}_2 \)-chemosensitive region of the pons where more than 80% of neurons respond to hypercapnic acidosis with an increase in firing rate (12–14). This increase in firing rate of LC neurons during hypercapnia is primarily thought to involve changes in intracellular pH, rather than extracellular pH or molecular \( \text{CO}_2 \) (14). Kir4.1/Kir5.1 channels are therefore attractive candidates as potential chemoreceptors in these cells. However, the identity of the channels involved remains unclear as several other types of ion channels have also been implicated (15, 16). One of the problems with dissection of these pathways is the lack of specific blockers for many of the channels involved. Therefore, to gain a greater understanding of the potential contribution of Kir5.1 to these chemosensitive pathways, we created a mutant mouse with a specific deletion of the Kir5.1 (\( \text{Kcnj16} \)) gene. Our results demonstrate that Kir5.1 plays a crucial role in defining the pH sensitivity of LC neurons and may therefore play an important role in their response to hypercapnic acidosis.

**Experimental Procedures**

*Creation of Kir5.1 (\( \text{Kcnj16} \)) Knock-out Mice—Kir5.1 (\( \text{Kcnj16} \)) is encoded by a single exon in the mouse genome. A Kir5.1 DNA probe was used to screen a phage library prepared using genomic DNA from a 129/SvJ mouse. Positive clones containing the Kir5.1 gene were isolated, and a 6.0-kb BamHI fragment encoding the last 59 amino acids of Kir5.1 and associated downstream sequence was cloned into the pBluescript SK\(^+ \) vector. A neomycin resistance gene was then inserted to replace the remainder of the Kir5.1 open reading frame, and a 3.2-kb fragment encoding the sequence upstream of the Kir5.1 exon was then added to create the targeting vector. The linearized vector was then electroporated into ES cells derived from 129/SvJ mice, which were then cultured in the presence of G418. Positive clones were then analyzed by Southern blotting and PCR to identify successfully integrated constructs. Blastocyst-mediated transgenesis was then performed to produce chimeric mice. The generation of chimeric mice using the pK05 vector was performed by Polygene AG (Rümlang, Switzerland). Chimeric mice were then bred with C57BL/6J mice, and a colony carrying the null allele was established by breeding heterozygous mutant mice. The...
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strains were backcrossed with C57BL/6J for more than 10 generations to establish an isogenic strain. An illustration of the gene-targeting strategy is shown in Fig. 1. This strain is now available as a public resource through Mousebook on the Web.

Tissue Preparation—This study was carried out by using brainstem tissue dissected from adult Kcnj16+/+ and Kcnj16−/− male mice (P90 ± 10 days). Mice were decapitated after 30 min of deep chloralum (4%, intraperitoneal) anesthesia and the cranium opened to expose the entire brain. The brain was rapidly removed and put into an ice-cold oxygenated solution of 2.5 mM KCl, 26.2 mM NaHCO3, 1 mM Na2HPO4, 2 mM MgSO4, 0.5 mM CaCl2, 11 mM D-glucose, 238 mM sucrose, saturated with 95% O2 and 5% CO2, pH 7.4. Coronal slices (220-μm thickness) were cut from the brainstem (submerged in the same ice-cold solution) using a Vibratome. Slices containing the LC were incubated at room temperature for 30 min in artificial cerebrospinal fluid (aCSF) (125 mM NaCl, 2.5 mM KCl, 26 mM NaHCO3, 1.25 mM Na2HPO4, 1 mM MgCl2, 2.4 mM CaCl2, 11 mM D-glucose, saturated with 95% O2 and 5% CO2, pH ~7.4) and more negative than ~40 mV and the series resistance changed <20% throughout the entire recording period. NH4Cl was dissolved in aCSF to a final concentration of 10 mM, pH ~7.4. Complete exchange of the bath solution occurred in about 1–2 min.

Data Analysis and Statistical Evaluation—The firing rate was obtained by calculating the instantaneous firing frequency (IFF), as 1/spike interval and the time at the end of each interval was used to indicate the time for each IFF. The action potential height was measured from threshold to peak. The spike threshold was defined as the membrane potential at which the first derivative of the membrane potential exceeded 10 V/s. Data were acquired at 10–20 kHz, filtered at 3 kHz, and analyzed with Pulse-fit, Origin7, and Igor. The statistical significance of the differences was calculated by using Student’s t tests and ANOVA, and the difference was considered significant at p < 0.05 (*) and p < 0.01 (**).

RESULTS

Genetic Deletion of Kir5.1 (Kcnj16)—We deleted the Kcnj16 gene in embryonic stem cells by insertion of a neomycin resistance gene which replaced the open reading frame of Kir5.1. Targeted cells were then used to generate chimeric mice and a mutant line containing the null allele in C57BL/6J mice, which were backcrossed for at least 10 generations to
ensure isogenicity. Breeding of heterozygous Kcnj16<sup>+/−</sup> mice generated live pups in a Mendelian ratio. Kcnj16<sup>−/−</sup> mice were also viable, fertile, and exhibited no observable behavioral or physical abnormalities.

**Reduced Response of Locus Coeruleus Neurons to pH in Kirs1−/− Mice—**LC neurons in the pons are confined to an easily identifiable anatomical area at the border of the IVth ventricle. Typical LC neurons are spontaneously active (0.5–5 Hz), display pacemaker-like firing, and possess consistent action potential parameters (17). Thus, the properties of LC neurons recorded in brain slices are remarkably uniform, as is their response to hypercapnic acidosis. Cultured LC neurons also retain their CO<sub>2</sub> chemosensitivity, indicating that external inputs are not essential for this process (18). Thus, LC neurons represent an ideal experimental model system to assess the potential contribution of Kir5.1 to pH sensitivity. Knockout of Kir5.1 means that the remaining Kir.1 subunits can also still form homomeric channels but that this Kir conductance, although effective in controlling the resting membrane potential, will not be pH-sensitive within the physiological range (3).

We therefore compared the response of LC neurons to intracellular acidification using both Kcnj16<sup>+/−</sup> and Kcnj16<sup>−/−</sup> adult mice (P90 ± 10 days). The electrical activity of LC neurons were recorded by means of whole-cell patch-clamp recordings in both voltage-clamp and current-clamp modes. The resting membrane potentials of LC neurons recorded from either Kcnj16<sup>+/−</sup> or Kcnj16<sup>−/−</sup> slices oscillated between −49 mV and −58 mV, displayed similar input resistances (366 ± 41 megohms and 402 ± 40 megohms, respectively; n = 9; p > 0.05) as well as basal firing frequencies (3.4 ± 0.5 and 2.9 ± 0.4 Hz, respectively; n = 16; p > 0.05). Intracellular alkalinization and acidification were induced with ammonium chloride (NH<sub>4</sub>Cl, 10 mM). This “NH<sub>4</sub>Cl pre-pulse” technique is a well established method for intracellular acidification that has been shown to decrease the intracellular pH of many neuronal and nonneuronal cell types (19–21). Bath perfusion of aCSF containing NH<sub>4</sub>Cl at pH 7.4 initially causes a transient intracellular alkalinization. However, the subsequent removal of NH<sub>4</sub>Cl causes a rapid intracellular acidification which reverses with time (19, 21).

We recorded the spontaneous firing activity of LC neurons in brain slices before, during, and after the superfusion of aCSF containing 10 mM NH<sub>4</sub>Cl for 3 min. Following NH<sub>4</sub>Cl withdrawal, the firing rate of Kcnj16<sup>+/−</sup> neurons increased remarkably (Fig. 2A). However, in Kcnj16<sup>−/−</sup> mice this increase in firing rate was prominently reduced (Fig. 2B). Fig. 3, A and B, shows the time course of changes in the IFF which occurred upon NH<sub>4</sub>Cl application. Analysis of the firing responses evoked by NH<sub>4</sub>Cl withdrawal and calculated as the difference between the peak and baseline IFF (ΔIFF), demonstrated that LC neurons from Kcnj16<sup>−/−</sup> mice had a 3-fold smaller ΔIFF compared with wild type (WT) (Kcnj16<sup>+/−</sup>; 13.5 ± 3Hz, n = 8; Kcnj16<sup>−/−</sup>; 4.4 ± 1Hz, n = 7; p < 0.01; Fig. 3D).

Interestingly, we also observed that the firing rates transiently decreased ~20% in Kcnj16<sup>+/−</sup> neurons (n = 8) prior to withdrawal of the NH<sub>4</sub>Cl prepulse (Fig. 3, A and C). This effect is similar to the reported effect of NH<sub>4</sub>Cl on LC neurons in rat brain slices due to transient intracellular alkalinization prior to withdrawal of the prepulse (12). However, in Kcnj16<sup>−/−</sup> mice this initial decrease in firing rate was barely detectable (<5%; n = 7; p < 0.01; Fig. 3, B and C).

Furthermore, we also found that the time course of the response to cytoplasmic acidification was delayed in Kcnj16<sup>−/−</sup> mice. The firing frequency of Kcnj16<sup>+/−</sup> neurons reached a maximum value 233 ± 7 s after application of NH<sub>4</sub>Cl (latency of the effect). By contrast, Kcnj16<sup>−/−</sup> neurons reached a peak firing frequency after 269 ± 6 s (p < 0.01; n = 8) (Fig. 3E). The degree of neuronal sensitivity to pH was...
also estimated from the slope of the NH$_4$Cl-induced response (Fig. 3F). This showed that the degree of chemosensitivity measured in $Kcnj16^{(-/-)}$ mice was only 25% of that seen in WT mice.

To confirm that these results were not due to an indirect effect of altering the cytoplasm of LC neurons in the whole-cell recording configuration, we also performed extracellular recordings of LC neuronal activity in response to NH$_4$Cl (Fig. 4). This confirmed that the effect was not dependent upon the recording configuration used.

Reduced Outward and Inward Currents in LC Neurons from Kir5.1-null Mice—We next performed whole-cell voltage-clamp recordings to examine the currents elicited by NH$_4$Cl application. Consistent with previous reports (12) we found that NH$_4$Cl induced an outward current during superfusion and an inward current following NH$_4$Cl withdrawal in WT $Kcnj16^{(+/+)}$ LC neurons when clamped at −60 mV (Fig. 5A). However, in $Kcnj16^{(-/-)}$ neurons these outward currents were barely detectable, and the inward currents were markedly reduced (Fig. 5, C and D). Furthermore, their activation was slower compared with WT $Kcnj16^{(+/+)}$ LC neurons; inward currents in WT mice reached a peak amplitude of 156 ± 32 pA after 236 ± 32 s ($n = 10$), whereas in $Kcnj16^{(-/-)}$ neurons peak currents of 78.6 ± 29 pA were reached after 309 ± 20 s ($n = 6$) (Fig. 5E). Their slope of activation was also reduced (Fig. 5F).

Consistent with the idea that this initial alkalinization-induced outward current might be caused by activation of a potassium channel in WT mice, we found that it reversed at the potassium equilibrium potential ($-106 ± 1.9$ mV; $n = 6$). However, the subsequent inward currents seen upon NH$_4$Cl withdrawal did not show a reversal potential (Fig. 6).

Decreased Response of LC Neurons to CO$_2$ in Kir5.1-null Mice—We next examined whether the reported response of LC neurons to hypercapnic acidosis (11–16) was similar to the effects we observed with NH$_4$Cl. We therefore tested the effects of aCSF bubbled with either 5% CO$_2$ (control) or 15% CO$_2$ (hypercapnia) and analyzed the effect on the spontaneous discharge rate of these neurons. All WT $Kcnj16^{(+/+)}$ neurons tested responded to hypercapnia with a significant increase in their spontaneous firing rate. This effect reversed quickly after returning to 5% CO$_2$ (Fig. 7A, upper). The ΔIFF response of $Kcnj16^{(+/+)}$ neurons to 15% CO$_2$ was 1.48 ± 0.2 Hz ($n = 5$) (Fig. 7D). By contrast, $Kcnj16^{(-/-)}$ neurons responded to 15% CO$_2$ with a reduced ΔIFF of 0.81 ± 0.1 Hz ($p < 0.05$) (Fig. 7A, lower, and D). The rate of this response was also flattened compared with WT neurons (Fig. 7, B and C), and Fig. 7E shows that the slope of this response was ~50% of that seen in $Kcnj16^{(+/+)}$ neurons.

**DISCUSSION**

We have generated a Kir5.1 knock-out strain of mice and used these mice to investigate the role of Kir5.1 in the cellular pathways that underlie the chemosensitivity of locus coeruleus neurons. Our results clearly demonstrate that Kir5.1 plays a key role in determining the PCO$_2$/pH sensitivity of these neurons.

**Generation of Kir5.1-null Mice**—In addition to being expressed in the brain, Kir5.1 is found in a wide variety of peripheral and epithelial tissues (1, 5, 22). However, $Kcnj16^{(-/-)}$ mice exhibited no obvious physical or behavioral deficits. Furthermore, despite expression of Kir5.1 in both ovaries and...
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spermatozoa (22, 23), male and female Kcnj16\textsuperscript{\textminus/\textminus} mice were fertile. The seemingly normal development of Kcnj16\textsuperscript{\textminus/\textminus} mice is in marked contrast to Kir4.1 knock-out mice, which die within 10–14 days due to abnormal cerebellar development (24). The isogenic strain of Kir5.1\textsuperscript{\textminus/\textminus} mice we have created therefore now provides an excellent resource for future studies to address the potential role of Kir5.1 in other tissues where it is also expressed.

Kir5.1 as a Potential CO\textsubscript{2} Chemoreceptor in Locus Coerulescent Neurons and the Role of pH—Previous studies have shown that intracellular acidification, induced by CO\textsubscript{2}, markedly increases the firing rate of LC neurons (12, 14–16). We were also able to observe this effect in LC neurons from our Kcnj16\textsuperscript{\textminus/\textminus} mice. However, we found that this response was dramatically reduced and much slower in Kcnj16\textsuperscript{\textminus/+} neurons. Furthermore, we also observed a reduction in H\textsuperscript{+}-induced inward currents (Fig. 5) in Kcnj16\textsuperscript{\textminus/+} neurons and therefore propose that intracellular acidification, generated by either CO\textsubscript{2} or NH\textsubscript{4}Cl prepulse, increases the firing rate of LC neurons by inhibition of Kir4.1/Kir5.1 channels and subsequent depolarization of the cell membrane (see Fig. 8). Although the NH\textsubscript{4}Cl-evoked inward current does not reverse at E\textsubscript{K}, a concurrent decrease in potassium conductance and increase in other cation conductances would result in an inward current, as has been described for muscarinic- and substance P-evoked inward currents in LC neurons (25, 26) and proposed as a possible mechanism of action for NH\textsubscript{4}Cl (12). Indeed, a number of studies suggest that the firing rate response of LC neurons to acidic stimuli is complex and may involve multiple different ion channels including L-type Ca\textsuperscript{2+} channels activation (27). NH\textsubscript{4}Cl acidification may also act predominantly at an electronically distant part of the cell, and such remote regions may not become sufficiently polarized to reverse the current flow. On the other hand, our conclusion is strongly supported by the fact that NH\textsubscript{4}Cl-induced outward current is due to an increase in K\textsuperscript{+} conductance that is almost absent in Kcnj16\textsuperscript{\textminus/+} neurons. These findings indicate that the NH\textsubscript{4}Cl-induced outward current in LC neurons is mediated predominantly by the activation of Kir4.1/Kir5.1 channels.

In LC neurons it has been proposed that it is intracellular acidification that mainly underlies the increase in the firing rate (14, 27). Interestingly, Kir4.1/Kir5.1 channels only respond to changes in intracellular pH and are insensitive to changes in extracellular pH (6). This is in marked contrast to the effect of hypercapnic acidosis on many K\textsubscript{2}P channels which have also been identified as chemoreceptors in other brainstem areas and which respond to changes in extracellular pH (28). In LC neurons which express both TASK-1 and Kir4.1/Kir5.1 channels (29, 30) mechanisms will therefore exist to sense changes in both extracellular and intracellular pH, and such redundancy may be functionally advantageous. The presence of a residual response in LC neurons lacking Kir5.1 is also perhaps not surprising, especially given the presence of other chemosensitive channels (15). However, our results demonstrate that despite this redundancy Kir5.1 clearly plays a major role in defining the response of these neurons to CO\textsubscript{2}.

Potential Role of Kir5.1 Subunits in the Ventilatory Responses to Hypercapnia—The activity of LC neurons is thought to play a critical role in attention, learning and memory, stress-induced responses (e.g. “fight or flight” response), anxiety, and certain pain sensations (31, 32). The LC also excerts an excitatory influence on the CO\textsubscript{2} stimulation of breathing (15). In healthy individuals, the primary function of pH-sensitive neurons in the LC may be to produce an aversive or anxiety response to elevated CO\textsubscript{2} levels. Indeed, LC neurons receive and send inputs to the medullary respiratory network and, although controversial, contribute to the respiratory responses to hypercapnia. A number of studies have proposed that besides the LC, several other chemosensitive areas of the brainstem (including the nucleus of the solitary tract; the retrolateral medulla, and the pre-Bötzinger complex) (15, 33, 34) may also need to be stimu-
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FIGURE 7. LC neurons from Kcnj16+/−/− mice show abnormal response to hypercapnic acidosis. A, representative current clamp recordings showing the response of WT (upper) and Kcnj16+/−/− (lower) neurons to 15% CO2 and after returning to control solution (5% CO2). B and C, time course of IFF (Hz) change upon 5-min application of 15% CO2 for Kcnj16+/+/+ (B) and Kcnj16+−/−/− (C) neurons. D, ΔIFF (Hz) values calculated as the difference between the peak (15% CO2) and the base-line (5% CO2) spontaneous firing frequency for Kcnj16+/+/+ and Kcnj16+−/−/− neurons. E, regression line slope (%) of the effect induced by 15% CO2 superfusion and calculated as detailed in Fig. 3, for Kcnj16+/+/+ and Kcnj16+−/−/− neurons. Data are means ± S.E. (error bars) of 5–8 independent experiments for each group. Statistical significance was calculated by using unpaired Student’s t test and ANOVA.

FIGURE 8. Model for the role of Kir5.1 in LC neuronal excitability and modulation by NH4+/ and CO2. NH4+ ions enter the neuron by means of transporters and permeable ion channels. NH4+/ (pKa 9.2 at pH 7.4) is the lipophilic and which diffuses freely across cell membrane. NH4+ then accumulates in the neurons, becomes protonated, and transiently alkalinizes the cytoplasm (NH4+ + H2O ⇌ NH3 + OH−). During the washout of NH4Cl from the recording chamber, NH3 diffuses out of the neuron, and the NH3, which was either generated inside or entered the neuron, rapidly acidifies the cytoplasm (NH3 + H2O ⇌ NH4+ + OH−). This increased [H+] inhibits Kir4.1/Kir5.1 activity, depolarizing the cell and increasing the neuronal firing rate. By contrast, CO2 diffuses freely across cell membrane during hypercapnia and, by means of carbonic anhydrases (CA), is rapidly converted to bicarbonate and H+ which inhibits Kir4.1/Kir5.1 channels. In Kir5.1 knock-out animals, this chemosensitive signaling mechanism is absent resulting in a reduced response to both NH4Cl and hypercapnic acidosis.

defining the chemosensitive response of distinct cells of the central nervous system. We conclude that the physiological pH sensitivity of heteromeric Kir4.0/Kir5.1 channels allows cells expressing these channels to link changes in CO2 levels to changes in neuronal activity and therefore act as highly sensitive PCO2/H+ chemoreceptors.

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