Enhanced Neuronal Damage After Ischemic Insults in Mice Lacking Kir6.2-Containing ATP-Sensitive K⁺ Channels

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Sun, Hong-Shuo, Zhong-Ping Feng, Takashi Miki, Susumu Seino, and Robert J. French. Enhanced neuronal damage after ischemic insults in mice lacking Kir6.2-containing ATP-sensitive K⁺ channels. J Neurophysiol 95: 2590–2601, 2006. First published December 14, 2005; doi:10.1152/jn.00970.2005. Adenosine triphosphate (ATP)–sensitive potassium (KATP) channels, which are weak inwardly rectifying potassium channels, are heterooctamers of pore-forming subunits (Kir6.6) and modulatory sulfonylurea receptors (SURs) (Babenko et al. 1998; Miki et al. 1999; Seino 1999; Shyng and Nichols 1997). KATP channels were first described in cardiac myocytes (Noma 1983) and were later identified and studied in other tissues, including pancreatic β-cells (Ashcroft et al. 1984; Cook and Hales 1984; Rorsman and Trube 1985), skeletal muscle (Light and French 1994; Spruce et al. 1985), smooth muscle (Standen et al. 1989), brain (Ashcroft 1988; Bajgar et al. 2001; Fujimura et al. 1997; Yamamoto et al. 1997), pituitary gland (Bernardi et al. 1988), and sino-atrial node pacemaker cells (Han et al. 1996). In the CNS, Kir6.2-containing KATP channels are found in multiple regions from rat preparations including hippocampus (Pelletier et al. 2000; Zawar et al. 1999; Zhou et al. 2002), cerebellum, and neocortex (Zhou et al. 2002), and from mouse substantia nigra pars reticulata (Yamada et al. 2001) and hypothalamus (Miki et al. 2001). KATP channels are so named because their activity is controlled by intracellular ATP and adenosine diphosphate (ADP) concentrations. An increase in the [ATP] tends to close KATP channels, by an interaction with the Kir6.6 subunits, whereas an increase in [ADP] tends to open the KATP channels, by interactions with the SUR subunits. Under certain conditions, other ligands, such as phosphoinositides, may dramatically affect KATP activity (Hilgemann and Ball 1996). Also, Müller and collaborators (2002) showed anoxic activation in dorsal vagal neurons of juvenile mice to be ATP independent. Opening of KATP channels tends to hyperpolarize the cell, shifting the membrane potential toward the potassium equilibrium potential. Thus KATP channels couple a cell’s metabolic status to its membrane potential, thereby regulating cellular functions, such as insulin secretion from pancreatic β-cells, excitability of neurons and skeletal muscle, and contributing to cytoprotection in cardiac and brain ischemia (Ashcroft et al. 1984; Babenko et al. 1998; Cook et al. 1988; Light et al. 1996, 2001; Misler and Giebisch 1992; Seino and Miki 2003, 2004; Suzuki et al. 1997; Terzic et al. 1995; Zhou et al. 2002). Expression of KATP channels in normally vulnerable cells can confer resistance to metabolic stress (Jovanovic et al. 1998a,b, 1999).

In heart, KATP channels can open under hypoxic or ischemic conditions, leading to a decrease in energy expenditure that is thought to be protective during metabolic stress (Babenko et al. 1998; Carmeliet et al. 1999; Faivre and Findlay 1990). In the CNS, there is evidence that Kir6.2-containing channels in substantia nigra pars reticulata neurons are directly involved in neuroprotection against the effects of hypoxia-induced generalized seizure (Yamada et al. 2001). However, the involvement of Kir6.2 KATP channels in neuroprotection against ischemic insults in other parts of the brain, such as in the hippocampus or neocortex, remains unknown.

Here, we use the Kir6.2 knockout (KO) mice to explore the functional consequences of the presence and activation of...
Kir6.2-containing K<sub>ATP</sub> channels in the CNS. We show the detailed expression pattern of Kir6.2 in mouse hippocampus. Opening of these channels in hippocampal neurons stabilizes the resting potential in the face of anoxic stress and protects neurons in hippocampal slices against the injury. In whole animals, the absence of Kir6.2 was associated with dramatically increased damage after ischemia induced by middle cerebral artery occlusion (MCAO).

**METHODS**

**KO mice**

KO mice (8- to 12-wk-old, male) lacking the Kir6.2 gene (Kir6.2<sup>−/−</sup>) (Miki et al. 1998) were used; KO mice were shipped from Chiba University, and the colony for this study was bred and maintained in the Animal Resource Centre of the Faculty of Medicine, University of Calgary. Knockout of the gene encoding Kir6.2 was verified by PCR genotyping. Basic physiological parameters of knockout mice were not significantly different from those of wild-type (WT) controls. These values included body weight, body temperature, blood glucose, blood gases, pH, blood pressure, heart rate, ECG, respiratory rate, and O<sub>2</sub> consumption. The mice develop normally and are fertile, with no apparent abnormalities in general appearance (Miki et al. 1998; Yamada et al. 2001). The KO and WT mice, as previously described (Li et al. 2000; Miki et al. 1998; Yamada et al. 2001). The brain cytoarchitecture of KO and WT mice appears to be identical (Miki et al. 1998; Yamada et al. 2001). The KO and WT mice, as previously described (Miki et al. 1998), were housed in groups of four mice per cage, with free access to food and water, in a room with an ambient temperature of 20 ± 1°C and a 12:12 h light/dark cycle. All the experimental procedures were approved by the local animal ethics committee of the Animal Resources Centre of the Faculty of Medicine, University of Calgary.

**Hippocampal slices and oxygen and glucose deprivation (OGD)**

Adult KO and WT mice (8–12 wk of age; weight 26–35 g) were deeply anesthetized with halothane and decapitated. The brains were rapidly removed, and horizontal slices (250 μm) were cut with a vibratome. Immediately after sectioning, the hippocampal slices were transferred to a holding chamber with artificial cerebrospinal fluid (ACSF) gassed with carbogen (95% O<sub>2</sub>-5% CO<sub>2</sub>) for ≈1 h, at 30 ± 2°C, before OGD or electrophysiological recording (Liu et al. 2003; Wang et al. 2003a,b). The composition of the ACSF was (in mM): 124 NaCl, 3 KCl, 1.25 Na<sub>2</sub>HPO<sub>4</sub>, 2 MgCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, and 10 dextrose (pH 7.4). The ACSF was saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub> at room temperature.

All OGD protocols and electrophysiological recordings (see Electrophysiology below) were carried out at room temperature. OGD for 30 min was used to simulate the experimental ischemia produced in whole animals (Fujimura et al. 1997; Garcia et al. 1999; Yamada et al. 2001; Yamamoto et al. 1997). There were four groups for the OGD experiments used for histochemical analysis. 1) WT control: the slices were exposed to normal conditions with ACSF gassed with carbogen (95% O<sub>2</sub>-5% CO<sub>2</sub>) for 30 min. 2) WT OGD: the slices were incubated in experimental “ischemic” conditions, with ischemia simulated by aspiration of ACSF gassed with carbogen (95% O<sub>2</sub>-5% CO<sub>2</sub>) for 30 min. 3) WT control: the preparation was exposed with carbogen (95% O<sub>2</sub>-5% CO<sub>2</sub>) for 30 min. 4) KO OGD: the preparation was incubated with simulated ischemia as for the WT OGD group. All slices were allowed to recover for 60 min in normal ACSF gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. The injured and surviving neurons were identified by confocal microscopy using double staining [propidium iodide (PI) and neuronal nuclei (NeuN)]. Numbers of injured and surviving neurons under different experimental conditions were counted and compared.

**Focal cerebral ischemia**

The modified monofilament intraluminal MCAO procedure (Clark et al. 1997) was used for the in vivo experiments. Mice were anesthetized with an intraperitoneal (ip) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Body temperature was monitored and controlled at 37 ± 1°C during the procedure. The local cerebral blood flow was also measured using laser Doppler flowmetry (LDF) to confirm adequate decreases in blood flow during the MCAO. In brief, an 8–0 surgical nylon monofilament with rounded tip was gently introduced into the left internal carotid through the external carotid stump, and advanced approximately 10 mm past the carotid bifurcation (Barber et al. 2004). The filament was left in place for 15 or 30 min, and then withdrawn. Reperfusion (24–48 h) was allowed by withdrawal of the filament. Animals recovered from the anesthesia at room temperature. The sham-operated animals for both KO and WT mice were treated similarly except for the actual MCAO. After 24–48 h, the brains of both KO and WT mice were removed and fixed as described above. Neuronal damage was assessed using parallel labeling, in adjacent slices, with Fluoro-Jade (for injured cells) and NeuN (for surviving neurons).

Neurological deficits were evaluated on a standard six-point scale (Clark et al. 1997). Scale of scores: 0—no neurological deficit; 1—retracts left forepaw when lifted by the tail; 2—circles to the left; 3—falls while walking; 4—does not walk spontaneously; 5—dead.

**Histochemistry**

TRIPHENYLTETRAZOLIUM CHLORIDE (TTC) STAINING. In the MCAO experiments, morphometric analysis was carried out using the standard TTC staining technique (Clark et al. 1997; Majdli et al. 2000). The brain was sliced into 1-mm coronal sections with a matrix. The slice was then incubated in 2% TTC in phosphate buffer and stained at 37°C for 30 min. After staining, the slice was stored in 10% phosphate-buffered formalin. Areas not stained red with TTC were considered to be lesions and used to calculate total lesion areas. Video images of individual sections were captured with a digital camera mounted on a microscope, and the area was traced using the National Institutes of Health image system (see Confocal imaging and Cell counting). The infarct volume in cubic millimeters was then calculated by measuring infarct areas on the separate slices, multiplying the areas by slice thickness, and summing for all slices (Majdli et al. 2000). Infarct volumes of both KO and WT were compared to determine any significant difference.

**PROPIDIUM IODIDE (PI) LABELING.** Nonviable cells in hippocampal slices after OGD were labeled with marker of cell death, PI (5 μg/ml) (Bonde et al. 2002; Laake et al. 1999; Macklis and Madison 1990; Müller and Ballanyi 2003; Yin et al. 2002). The numbers of injured cells were determined by using confocal laser microscopy to select the central layer of cells in an acutely stained, unfixed, 30-μm section from the 250-μm slice, thus avoiding surface regions that were damaged by sectioning during slice preparation. Images were viewed with a confocal laser scanning microscope (see also Immunohistochemistry and Confocal imaging).

**FLUORO-JADE STAINING.** In MCAO experiments, the injured neurons and glial cells, were marked with Fluoro-Jade (Colombo and Puissant 2002; Schmued et al. 1997) as described (Wang et al. 2003a). At 24 h after MCAO, animals were deeply anesthetized and transcranially perfused with 0.9% NaCl saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Hippocampal sections (30 μm) were dried overnight for Fluoro-Jade staining (Histo-Chem, Jefferson, AR). The slides were immersed for...
3 min in 100% ethanol, 1 min in 70% ethanol, 1 min in distilled water, and then transferred to a solution containing 0.01% Fluoro-Jade and 0.1% acetic acid (1:10) for 30 min on a shaker. After three 10-min washes, the slides were coverslipped and subsequently imaged.

Immunocytochemistry

PREPARATIONS. Animals were deeply anesthetized by halothane inhalation, and then perfused through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. The brains were quickly removed and placed in the same fixative at 4°C overnight. They were subsequently sectioned into 30-μm-thick slices for immunocytochemical staining. General procedures for immunostaining were as described elsewhere (Wang et al. 2003a).

KIR6.2. The sections (30 μm) were blocked in 3% normal goat serum/0.3% Triton X-100/0.1% BSA in PBS (room temperature for 1 h), followed by incubation with rabbit anti-Kir6.2 antibody, 1:500 (Suzuki et al. 1997), at 4°C overnight. Subsequently, the sections were incubated with the affinity-purified second antibody, goat anti-rabbit Cy3 antibody (1:200; Chemicon, Temecula, CA). Sections were then rinsed, dried, and coverslipped with Dako (Carpinteria, CA) fluorescence mounting medium. Images were viewed with a confocal laser scanning microscope (see Imaging below).

NEUN. To assess the numbers of both surviving and damaged cells in the same hippocampal slices, the top and bottom two to three 30-μm sections from each 250-μm slice were discarded because of possible mechanical induced trauma. The remaining three to five middle sections were kept for examination. For double labeling (after PI staining) the sections (30 μm), from a single hippocampal slice, were further incubated in mouse anti-NeuN (1:100; Chemicon) (Jongen-Relo and Feldon 2002; Mullen et al. 1992; Sharp et al. 2002; Xu et al. 2002; Yang and Donner 2002). These sections were then reacted with conjugate-absorbed goat anti-mouse fluorescein (1:200; Chemicon). Sections were rinsed, dried, and coverslipped with Dako fluorescence mounting medium. Images were viewed with a confocal laser scanning microscope (see Imaging below) to identify surviving neurons, as previously described (Wang et al. 2003a). This strategy of double-staining slices with PI and NeuN was adopted independently in a recent study on ischemic preconditioning in rat hippocampal slices (Hassen et al. 2004).

Confocal imaging

Antibody labeling and double-labeling slides were imaged with a confocal laser scanning microscope (Olympus LSM-GB200) and analyzed with a three-dimensional (3D) constructor (Image-Pro Plus software). In each preparation, the slice was initially scanned to determine a depth halfway between the slice surfaces and the midslice section was used for imaging. The purpose was to eliminate any mechanical damage and consequent distortion of the images. Note that, for the parallel electrical recordings, we used cells at or near the surface of the slice to allow reliable seal formation. In that case, seal formation, attainment whole cell recording, and the initial resting potential, gave objective indications of the condition of the cell. Thus in both histological analysis, and the whole cell recordings, we are confident that the damage observed resulted, largely or completely, from the experimental treatment. We produced 3D digital reconstructions from a series of confocal images taken at 0.5-μm intervals through the region of interest, and optical stacks of 5–10 images were produced for the figures, as described (Wang et al. 2003a; Zhu et al. 2002).

Cell counting

The numbers of injured and surviving cells were measured using the National Institutes of Health image system (Image J) with a computer. Double-stain labeling with PI (or Fluoro-Jade) and NeuN in the hippocampal slice preparation revealed both injured and surviving cells in the same slice of the experiment. The numbers of injured and surviving neurons per slice were counted and compared for different experimental conditions. Cell counts were routinely obtained from a reconstructed volume measuring 400 × 400 × 5 μm (see previous paragraph).

Electrophysiology

Whole cell patch-clamp recording of neurons in hippocampal slices was performed using an IR-DIC Zeiss Axioskop 2 microscope, as described (Liu et al. 2003; Wang et al. 2003a,b). A single slice was transferred to the recording chamber (volume, 1 ml), submerged, and superfused (about 2 ml/min) with ACSF [composition, see Hippocampal slices and oxygen and glucose deprivation (OGD), above] saturated with 95% O2-5% CO2 at room temperature. The internal pipette solution for voltage clamp contained (in mM) 142.5 K-gluconate, 17.5 KCl, 0.2 EGTA, 10 HEPES (pH 7.4; 298 mOsm). Hippocampal CA1 neurons were visualized with differential interference contrast (IR-DIC) optics using infrared lighting and displayed with IR-intensified Nevison camera (Axioskop2FS). Changes in resting membrane potentials of the hippocampal neurons were recorded in current-clamp mode. Raw data were amplified using an Axopatch1-D, sampled at 5 kHz and analyzed with pClamp8 software (Axon Instruments, Foster City, CA).

Membrane potential changes of single neurons from each experimental group were recorded at room temperature during exposure to different experimental conditions. There were three sets of experiments, in which separate slices were exposed to control (normoxic) conditions, or to a 5- or 30-min period of OGD, as described in Hippocampal slices and oxygen and glucose deprivation (OGD). The experimental groups were: 1) WT; 2) WT slices in the presence of the KATP channel blocker, tolbutamide (0.5 mM; Sigma, St. Louis, MO); and 3) KO. After the period of OGD, the preparations were switched back to normal ACSF gassed with carbogen. The time courses of the resting membrane potential were recorded throughout.

Statistics

We present data as means ± SE. Group data were compared using ANOVA and multiple-comparison tests (SigmaStat3.0 of SPSS, Chicago, IL). For all tests, the difference was considered as statistically significant if P < 0.05.

RESULTS

We first studied the expression pattern of the Kir6.2 KATP channels in the CNS. We then examined the importance of KATP channels in protection from the damaging effects of ischemia in studies of WT and KATP-deficient KO mice subjected to oxygen and glucose deprivation in vitro, and experimental focal ischemia in vivo. The protective mechanism by which KATP channels act was studied electrophysiologically in hippocampal slices from the same groups of mice.

Localization of Kir6.2 channels in hippocampus by staining with anti-Kir6.2 antibody

Using immunocytochemical staining, we studied Kir6.2 expression patterns in the hippocampal sections from WT mice, consistent with the previous finding in a rat preparation (Zhou et al. 2002). Confocal imaging demonstrated that the fluorescence-labeled Kir6.2 protein was expressed throughout the hippocampus of adult WT mice (Fig. 1). A variety of cell types
were Kir6.2 positive, including pyramidal neurons in the CA1 and CA3 regions, granule cells in the dentate gyrus, and interneurons in the hilus. In contrast to the WT mice, Kir6.2 proteins were not detected in the hippocampus of the KO mice. This is the first detailed description of the distribution of these plasmalemmal K<sub>ATP</sub> channels in mouse hippocampus. Wide-spread expression of Kir6.2 in the cortex of WT, but not KO animals, is also confirmed in the online Fig. 1 (see Supplementary Material).<sup>1</sup>

**Neuronal injury in hippocampal slices from the KO mice after OGD**

To examine the cytological consequences of oxygen and glucose deprivation in the hippocampal sections, we used PI to mark the injured neurons and NeuN to label the surviving neurons. The fluorescent labeling was visualized using confocal imaging microscopy and the incidence of the injured and surviving neurons in the WT and KO preparations was compared. For these studies, hippocampal slices were exposed for 30 min to OGD and allowed to recover for 60 min in normal ACSF buffer gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. As shown in Fig. 2, the double PI and NeuN staining demonstrated that, without OGD insult, the majority of neurons in both the WT and KO mice were labeled by NeuN (green), indicating few injured cells. After OGD, only a few neurons were damaged in hippocampal slices from the WT mice, as shown by the large number of cells that were NeuN positive (Fig. 2). In contrast, substantial neuronal injury was evident in all regions of hippocampus from the KO mice, as shown by the fact that most neurons were PI positive (red), and few neurons survived after exposure to OGD (Fig. 2). The populations of the damaged and

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<sup>1</sup> The Supplementary Material for this article (a figure and two tables) is available online at http://jn.physiology.org/cgi/content/full/00970.2005/DC1.
surviving neurons from both WT and KO groups are compared in Fig. 3. The number of PI positive cells was significantly higher \((P < 0.05)\) in the KO preparations after OGD exposure than in either the KO control or the WT control or WT OGD groups (Fig. 3). In addition, the number of NeuN positive cells was significantly lower \((P < 0.05)\) in the KO OGD group than in the others (Fig. 3). The counts of injured and surviving neurons in different regions of hippocampus under each experimental condition are tabulated in the on-line Supplementary, Tables 1 and 2. Consistent with these observations, severe injury occurred in hippocampal slices from WT mice that were incubated with K\(_{\text{ATP}}\) channel blocker tolbutamide \((196,230 \pm 20,346 \text{ cells/mm}^3, n = 11)\), were stained with PI. Overall, the results indicate that Kir6.2, an integral subunit of plasma membrane K\(_{\text{ATP}}\) channels, is essential to provide neuronal protection during hypoxic “ischemia” in vitro.

The collection of features listed below distinguishes our hippocampal slice experiments from previous studies, such as those by the following authors: Fujimura et al. (1997); Garcia et al. (1999); Yamada et al. (2001); and Yamamoto et al. (1997). These significant details include the use of 1) adult mice; 2) thinner hippocampal slices \((250 \mu\text{m})\) to help optimize oxygenation; 3) cell death assessment by confocal imaging, of the middle layer of the slice rather than surface layers, thus avoiding cells mechanically damaged during slice preparation (see NeuN in Immunocytochemistry); and 4) double labeling to identify separately the surviving neurons (stained with NeuN) and damaged cells, presumably including glial cells, stained with PI in the same hippocampal slice, thus improving the accuracy of cell death and survival assessment.
Altered membrane potentials after ischemic insults in KO mice

To determine whether expression of Kir6.2 channels affects the membrane potentials of neurons undergoing ischemic insult, we exposed the hippocampal slices from KO and WT mice to OGD. The membrane potentials in the hippocampal CA1 pyramidal neurons were examined using whole cell patch-clamp recordings. Under normal conditions, the resting membrane potentials of hippocampal CA1 pyramidal neurons in KO mice did not differ from those in the WT mice (−67.4 ± 0.58 mV in the KO mice, n = 18; and −67.8 ± 1.10 mV in the WT mice, respectively, n = 25/group, P > 0.1) (Fig. 4). In response to a brief, transient episode of OGD (5 min), the membrane potentials in the WT mice first transiently hyperpolarized (from −68.1 ± 1.08 to −76.9 ± 1.64 mV, a change of −8.8 ± 1.12 mV; n = 17). In contrast, neurons in KO mice were reversibly depolarized (from −67.3 ± 0.58 to −58.9 ± 1.07 mV, a change of 8.4 ± 0.89 mV; n = 18) (Fig. 4). Application of tolbutamide, an SUR1-specific K<sub>ATP</sub> channel blocker (Gribble et al. 1998), eliminated the hyperpolarization in WT mice, leaving only a depolarizing response (from −67.1 ± 2.68 to −59.3 ± 2.34 mV, a change of 7.9 ± 1.20 mV; n = 8).

When the hippocampal slices were exposed to OGD for a longer time (30 min), CA1 neurons of the WT mice showed a biphasic response; a slight depolarization followed the initial hyperpolarization and, after returning to control conditions, the membrane potential recovered partially to a value near its basal level (−60.4 ± 1.99 mV, n = 17). In contrast, neurons from KO mice exposed to the prolonged OGD showed strong depolarization without initial hyperpolarization, and the membrane did not repolarize after oxygen and glucose were restored (−14.1 ± 1.18 mV, n = 18) (Fig. 5). Tolbutamide changed the membrane potential responses of the WT neurons from slight depolarization to a large, irreversible depolarization (−19.9 ± 3.28 mV, n = 8) in prolonged OGD (Fig. 5).

Consistent with the nearly reversible changes in resting potential after a brief, 5-min OGD (Fig. 4), no detectable tissue damage was detected with PI/NeuN double staining for either WT or KO slices after a 5-min OGD. For WT mice, this is as expected from WT data in Figs. 2 and 3, which show negligible damage after the more severe, 30-min OGD exposure. For KO

**FIG. 4.** Transient membrane potential changes in CA1 neurons resulting from a brief (5-min) OGD. A: individual membrane potential traces showing responses to a brief episode of OGD. KO neuron depolarized during OGD, whereas the WT neuron hyperpolarized. Response of the WT neuron in the presence of K<sub>ATP</sub> channel blocker tolbutamide (WT + KCB) resembled the response of the KO neuron. Horizontal bars represent 5 min of OGD. B: mean values of the resting potential before OGD, and of maximum or minimum of the potential transient after a brief episode of OGD, for 3 different groups. Mean values of the membrane potential before OGD were significantly different from the maxima/minima at the end of OGD, and the changes for the KO and WT + KCB groups were significantly different from those for the WT. *P < 0.05; n = 18 (KO); n = 17 (WT); n = 8 (WT + KCB). Labeling: KO, knockout; WT, wild-type; KCB, K<sub>ATP</sub> blocker (tolbutamide).
mice, no detectable changes were observed in KO slices subjected to a 5-min OGD (on-line data supplement, Fig. 2). In vivo studies: neuronal injury after focal ischemia in Kir6.2 null (KO) mice

All KO mice died within 24 h of a 30-min episode of MCAO (n = 10). In contrast, all WT mice survived the same treatment without neurological or morphological damage (n = 10). When the MCAO time was reduced to 15 min, there was 50% mortality for the KO mice at 24 h after the MCAO (n = 20); 10 mice died (score 5, according to the neurological deficit scale defined in METHODS, Focal cerebral ischemia) and none of the 10 surviving mice walked spontaneously (score 4). All WT mice remained alive for 24 h after the 15-min MCAO. WT MCAO mice were killed for analysis at 24 h so that they could be rigorously compared with the KO MCAO group; the WT MCAO group showed no neurological deficits (score 0), nor any morphological changes in the brain. No visible bleeding occurred in the brain or around the operated area in any group. Thus the poor survival of the KO group after OGD cannot be attributed to surgical damage.

At a gross anatomical level, triphenyltetrazolium chloride (TTC) stain revealed damaged areas in the neocortical region of brains of the KO mice that had been exposed to 15-min MCAO (Fig. 6). The damaged area of the KO mouse brain was not stained with TTC and remained a white color, whereas the normal, unaffected area of the KO mouse brain stained red with TTC. In contrast, the WT mouse brains from the 15-min MCAO experiment showed no visible damage in any area of the brain; all areas were evenly stained with red TTC (Fig. 6). Mean infarct volume for the KO mouse brains was 11.67 ± 1.28 mm³ (n = 11) compared with 0 mm³ (n = 10) in the WT mouse brains (P < 0.01).

In some experiments, staining with Fluoro-Jade or NeuN was used to reveal more details regarding the damaged versus normal brain tissues from KO mice, or from WT mice, after a 15-min episode of MCAO. There were detectably damaged areas of the brains (indicated by Fluoro-Jade staining) after 15 min of MCAO in the KO mice. However, there was no detectable damage in the brains from WT mice that were exposed to the same treatment. We assessed the numbers of damaged and surviving cells in adjacent sections of the same regions of the brain for both experimental groups (KO and WT MCAO), using the side contralateral to the MCAO as a control. Sections from a KO mouse are shown in Fig. 7. Data from both WT and KO groups are summarized in Table 1. Substantial numbers of neurons degenerated and few neurons survived in the MCAO-affected areas of brains of the KO MCAO group. The numbers of damaged or surviving cells of the MCAO-affected areas in the brains were significantly different (P < 0.05) from those of the contralateral middle cerebral artery perfused areas in the KO mice. No damage was detected in the relevant middle cerebral artery (MCA) perfusion area in the contralateral side of the brains of these KO mice. Furthermore, there were many surviving cells and no visibly injured cells, revealed by Fluoro-Jade staining, in the MCAO field of the brains from the WT mice.

DISCUSSION

We used Kir6.2-deficient KO mice to study the neuroprotective role of plasma membrane KATP channels, examining the

**FIG. 6.** Triphenyltetrazolium chloride (TTC) staining slices of brains from KO and WT mice after 15 min of middle cerebral artery occlusion (MCAO). KO mouse brains showed extreme damage (unstained area) in neocortex region, whereas there was no detectable damage in the WT mouse brains. Damaged area is not stained with TTC and is grayish-white in color, whereas the normal unaffected area is stained red. Thickness of the brain slice was 1 mm. Scale bars: 2 mm.
response to OGD in vitro in hippocampal slice, and to experimental ischemia (MCAO) in vivo. We show for the first time that 1) Kir6.2 protein is expressed in neurons of all regions in mouse hippocampus, 2) OGD induces extreme neuronal injury in hippocampus of KO mice, 3) opening of Kir6.2-containing plasma membrane KATP channels is responsible for the hyperpolarization observed after hypoxia, and 4) Kir6.2 is essential for neuroprotection after in vivo focal ischemia.

**Kir6.2 expression of the hippocampus of the mouse brain**

In the rat brain, Kir6.2 KATP channels have been detected in hippocampal neurons using electrophysiological approaches (Pelletier et al. 2000; Zawar et al. 1999) or in mRNA levels

**TABLE 1. Numbers of damaged cells (Fluoro-Jade stained) and surviving (NeuN stained) neurons of cortex in the 15-min MCAO side and contralateral normal side, from KO and WT mice**

<table>
<thead>
<tr>
<th></th>
<th>Fluoro-Jade Stain (Damaged Cells)</th>
<th>NeuN Stain (Surviving Cells)</th>
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<tr>
<td></td>
<td>MCAO</td>
<td>Contralateral</td>
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<td></td>
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<td>MCAO</td>
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<tr>
<td>WT</td>
<td>0 ± 0</td>
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</tr>
<tr>
<td>KO</td>
<td>191,750 ± 15,870*</td>
<td>0 ± 0</td>
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Values are means ± SE, n = 5 animals in each of the four groups. Numbers of cells/mm³ (counts rounded to the nearest 10). *Statistically significant differences from control, P ≤ 0.05.
using single-cell RT-PCR (Zawar et al. 1999). Recently, the channel protein was detected in rats using the anti-Kir6.2 antibody (Zhou et al. 2002). However, only gross anatomical distribution of the channel protein was provided in that study. Complementing those findings, we showed, using immunohistochemistry combined with confocal imaging, that Kir6.2 protein was expressed in neurons of all the hippocampal regions in the adult WT mouse brain. The specific cell types that expressed Kir6.2 included pyramidal neurons in the CA1 and CA3 regions, granule cells in the dentate gyrus, and interneurons in the hilus area. This is the first demonstration of robust expression of plasmalemmal Kir6.2 K\textsubscript{ATP} channel in hippocampus of the mouse brain. A parallel analysis with the same antibody clearly showed K\textsubscript{ATP} channel protein expression in the neocortex of adult WT mice (on-line data supplement Fig. 1). Thus the Kir6.2 protein is widely distributed in central neurons of the hippocampus and cortex in the adult mouse. In contrast, the KO mice did not express Kir6.2, confirming the KO animal model for the following studies on neuroprotection.

**Effect of simulated “ischemia” (OGD) on hippocampal slices**

In vitro, 30-min oxygen and glucose deprivation (OGD) resulted in extensive cell damage in all regions of hippocampal slices from KO mice. The damaged areas included the CA1, CA3, dentate gyrus, and hilus regions of the hippocampus. On the other hand, the WT OGD group, and the control groups of both KO and WT, showed minimal levels of damage, with nearly 100% survival in all cell types and regions of the hippocampus. Thus in this in vitro model, neurons of KO mice were much more susceptible to metabolic stress than were those of WT mice.

Accumulated evidence from a number of studies, including those cited in the following sections, suggests that most mature brain structures are vulnerable to hypoxic–ischemic damage and that K\textsubscript{ATP} channels contribute to neuronal protection. The Kir6.2-dependent neuroprotective effect, which we observed during hypoxia in the hippocampus, is similar to that reported in a study of substantia nigra pars reticulata (SNr) neurons, in which the same Kir6.2 KO mice were used to test the involvement of Kir6.2-containing channels in protecting SNr neurons against the effects of hypoxia-induced generalized seizure (Yamada et al. 2001). Our present study provides additional evidence that neuronal Kir6.2 K\textsubscript{ATP} channels in the brain are important in protecting the cells in experimental ischemia. The combination of the electrophysiological data with the cytological analysis suggests that protection is mediated, in whole or in part, at the level of the surface membrane.

**In vivo studies on focal cerebral ischemia (MCAO)**

Mice lacking Kir6.2 were much more vulnerable to MCAO than were the WT mice. KO mice surviving at 24 h after a 15-min MCAO showed severe neurological deficits on a six-point standard scale (Clark et al. 1997). We observed no anatomical damage (see next paragraph), nor any neurological deficits, in WT mice subjected to 15 min of MCAO.

Gross morphological studies, using TTC staining to assess the damaged area from the neocortex of the MCAO mice, identified large infarct volumes in the KO mice within 24 h after a 15-min episode of MCAO. In contrast, the TTC staining revealed no detectable damage in the WT mouse brain following the same procedure. Staining with Fluoro-Jade and NeuN was used to identify damaged and surviving neurons, respectively. These more quantitative, histochemical methods verified that there were substantial numbers of degenerating neurons and few surviving neurons in brains of the KO, 15-min MCAO group in this whole animal model. Many surviving cells, but no injured cells, were apparent in the brains from the WT animals (see Table 1). These combined anatomical and cytochemical analyses do not exclude the possibility that more subtle signs of damage might be detected in controls (WT animals or KO contralateral tissue) using other methods such as electron microscopy. Also, longer-term studies may reveal further damage arising from immune responses, even extending to the contralateral side (Clarkson et al. 2005). However, the data in Figs. 6 and 7 clearly show that MCAO-induced damage is minimal, if not negligible, in the contralateral, perfused tissue of the KO animals. Similarly, there was no observable damage on either side of the WT MCAO brains (on-line data supplement Fig. 3), on the timescale of our experiments. This contrasts dramatically with the damage in the KO mice on the side subjected to MCAO. Thus K\textsubscript{ATP} channels containing Kir6.2 are necessary for neuroprotection during in vivo experimental ischemia, as well as for resistance to in vitro metabolic stress.

**Other tissues and the cyto-location of K\textsubscript{ATP} channel-mediated protection**

In heart, plasma membrane K\textsubscript{ATP} channels have the subunits of SUR2A/Kir6.2 (Babenko et al. 1998; Miki et al. 1999; Seino 1999). However, the K\textsubscript{ATP} channels of neuronal plasmalemma share the subunit composition of those in pancreatic beta cells, SUR1/Kir6.2. There is no specific and selective opener targeted only to the predominant neuronal plasma membrane Kir6.2 K\textsubscript{ATP} channels. Thus the use of Kir6.2 KO mice for this study provided the most specific and discriminating strategy to explore the role of these channels. Activation of ATP-sensitive potassium channels in the heart is associated with ischemic preconditioning (IPC) and with acute cardioprotection. It has been suggested that specific activation of mitochondrial K\textsubscript{ATP} channel initiates IPC and prevents mitochondrial dysfunction associated with Ca\textsuperscript{2+} overload during reperfusion. An alternative, or complementary, hypothesis is that activation of plasmalemmal K\textsubscript{ATP} channels protects by stabilizing the resting membrane potential during ischemic stress (Gross and Fryer 1999), although this hypothesis, alone, appears not to account for all aspects of cardioprotection (Nichols and Lederer 1991). Pharmacological criteria have been used to infer that plasmalemmal and mitochondrial channels may have separate, specific roles in different aspects and phases of the response to acute metabolic stress (Dzeja et al. 2001; Guminia et al. 2003; Light et al. 2001; Ozcan et al. 2002). Busija and collaborators have used the MCAO model in rats to test the neuroprotective effect of diazoxide, applied as a mitochondrial K\textsubscript{ATP} channel opener, during transient focal cerebral ischemia (Domoki et al. 1999; Rajapakse et al. 2002; Shimizu et al. 2002). Diazoxide reduced the infarct volume by 40% (based on TTC staining) and 5-hydroxydecanol (5-HD),
used as a selective mitochondrial K\textsubscript{ATP} inhibitor, completely blocked diazoxide's effect. A study of rat brain, reported that mitochondria contain six- to sevenfold more K\textsubscript{ATP} channels per milligram of mitochondrial protein than those of liver or heart (Bajgar et al. 2001). In brain, however, diazoxide would activate SUR1-containing channels in the plasmalemma. Thus conclusions regarding the functional roles of mitochondrial K\textsubscript{ATP} channels depend heavily on the selectivity of a single inhibitory agent, 5-HD. This issue will not be fully resolved until there is a confirmed subunit composition for mitochondrial K\textsubscript{ATP} channels. At present, their molecular identity—and even their very existence—remains controversial (Ardehali and O'Rourke 2005; Das et al. 2003; Garlid 2000; Zhou et al. 1999, 2005).

A mechanism of neuroprotection in CA1 neurons: hypoxic hyperpolarization

Resting membrane potentials of CA1 cells recorded under control conditions from KO and WT hippocampal slice preparations were not significantly different. This parallels observations on SNr neurons in KO and WT mice (Yamada et al. 2001). CA1 hippocampal pyramidal neurons in slices from KO mice were irreversibly depolarized after prolonged OGD. This emphasizes that hippocampal neurons from the KO mice were more sensitive to ischemic injury than those of WT mice and suggests that Kir6.2 expression provides neuronal resistance to ischemic insult by preventing excessive depolarization.

Previous studies have demonstrated the phenomenon of hypoxic hyperpolarization of the neuronal cell membrane during transient experimental ischemia in different neurons including hippocampal CA1 cells, striatal neurons, and SNr neurons (Fujimura et al. 1997; Garcia et al. 1999; Yamada et al. 2001; Yamamoto et al. 1997). The hyperpolarization was assumed to counteract the general depolarization induced by hypoxia. Pharmacological agents such as tolbutamide, a K\textsubscript{ATP} channel blocker acting by SUR1 sulfonylurea receptors (Gribble et al. 1998), prevented the hypoxia hyperpolarization and led to depolarization during hypoxia. A study using the K\textsubscript{ATP} channel opener diazoxide demonstrated a reduction in the infarct volume after transient focal cerebral ischemia in rats (Shimizu et al. 2002). Although these studies are important and suggestive, it is unlikely that, in brain, diazoxide acts exclusively on a single K\textsubscript{ATP} channel type. Interpretation of pharmacological data is further complicated by the possibility of plasticity in the response, depending on conditions (D'Hahan et al. 1999). Thus, further studies will be needed to clearly define the particular roles of different subtypes of the K\textsubscript{ATP} channel in ischemic tolerance, such as testing of different mouse strains, which lack other tissue-specific isoforms, or different subunits of the K\textsubscript{ATP} channels (Ashcroft et al. 1984; Babenko et al. 1998; Cook et al. 1988; Light et al. 1996, 2001; Misler and Giebisch 1992; Seino and Miki 2003, 2004; Suzuki et al. 1997; Terzic et al. 1995; Zhou et al. 2002).

Our use of the KO mice lacking Kir6.2 clearly demonstrates the neuroprotective role of the Kir6.2 K\textsubscript{ATP} subunit in experimental ischemia in vivo, and OGD in vitro. Self-consistent data from membrane potential recordings and analyses of cellular damage suggest that the protective, hyperpolarizing response and the resistance to damaging effects of OGD shown by the WT neurons are both associated with the expression of Kir6.2. Because our markers of damaged cells, PI and Fluoro Jade, stain glial cells as well as neurons (see Methods), we cannot preclude the possibility that K\textsubscript{ATP} channels of the glial cells contribute to the overall protective response to anoxia/ischemia in CNS tissue. Indeed, functional and immunohistochemical studies have indicated the presence of K\textsubscript{ATP} channels, including those containing Kir6.2, in glia (Ballanyi et al. 1996; Zawar et al. 1999; Zhou et al. 2002). Nevertheless, our electrophysiological recordings from CA1 neurons revealed that K\textsubscript{ATP} channels in the neurons directly regulate the cellular excitability against the effects of OGD, suggesting the involvement of K\textsubscript{ATP} channels of the neurons per se in neuroprotection against anoxia/ischemia.

In conclusion, compared with heart, there is a relatively short history of studies in brain on the cytoprotective role of K\textsubscript{ATP} channels during ischemia/hypoxia (Bernardi et al. 1988; Blondeau et al. 2000; Ferrer et al. 1995; Fujimura et al. 1997; Garcia et al. 1999; Macklis and Madison 1990; Majid et al. 2000; Yamamoto et al. 1997). Even so, as early as 1982 Hansen and collaborators reported that the anoxia-induced hyperpolarization resulted from an increase in potassium conductance, based on its block by aminopyridines and TEA (Hansen et al. 1982). A study of hippocampal CA1 neurons in rat showed that either glibenclamide or tolbutamide reduced the amplitude of the hypoxic hyperpolarization (Fujimura et al. 1997). Subsequently, the pace of investigations in brain has quickened (Ballanyi 2004; Minami et al. 2004). Most of these studies have been confined to rat brain slice preparations.

Our present study offers new comprehensive insight into the neuroprotective function of the membrane K\textsubscript{ATP} channels. A strength of our study is that it provides data bearing on the role of Kir6.2-containing K\textsubscript{ATP} channels, collected in parallel using the same KO and WT mice, at the whole animal, tissue, and cellular levels. We present clear and consistent evidence that Kir6.2-containing, plasmalemmal K\textsubscript{ATP} channels mediate a key neuroprotective response to acute ischemia and OGD. Their opening hyperpolarizes hippocampal neurons and enhances neuronal survival in the face of ischemic or anoxic attack. In the future, K\textsubscript{ATP} channel modulators may prove to be clinically useful as neuroprotective agents, as part of a combination therapy for stroke management.

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