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Calcium- and Metabolic State-Dependent Modulation of the Voltage-Dependent Kv2.1 Channel Regulates Neuronal Excitability in Response to Ischemia

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Ischemic stroke is often accompanied by neuronal hyperexcitability (i.e., seizures), which aggravates brain damage. Therefore, suppressing stroke-induced hyperexcitability and associated excitoxicity is a major focus of treatment for ischemic insults. Both ATP-dependent and Ca^{2+}-activated K^+ channels have been implicated in protective mechanisms to suppress ischemia-induced hyperexcitability. Here we provide evidence that the localization and function of Kv2.1, the major somatodendritic delayed rectifier voltage-dependent K^+ channel in central neurons, is regulated by hypoxia/ischemia-induced changes in metabolic state and intracellular Ca^{2+} levels. Hypoxia/ischemia in rat brain induced a dramatic dephosphorylation of Kv2.1 and the translocation of surface Kv2.1 from clusters to a uniform localization. In cultured rat hippocampal neurons, chemical ischemia (CI) elicited a similar dephosphorylation and translocation of Kv2.1. These events were reversible and were mediated by Ca^{2+} release from intracellular stores and calcineurin-mediated Kv2.1 dephosphorylation. CI also induced a hyperpolarizing shift in the voltage-dependent activation of neuronal delayed rectifier currents (I_K), leading to enhanced I_K and suppressed neuronal excitability. The I_K blocker tetraethylammonium reversed the ischemia-induced suppression of excitability and aggravated ischemic neuronal damage. Our results show that Kv2.1 can act as a novel Ca^{2+}- and metabolic state-sensitive K^+ channel and suggest that dynamic modulation of I_K/Kv2.1 in response to hypoxia/ischemia suppresses neuronal excitability and could confer neuroprotection in response to brief ischemic insults.

Key words: **channel; epilepsy; hypoxia; ion channels; hippocampus; neurons; neuroprotection**

Introduction

Hypoxia is the most common cause of neonatal seizures and a risk factor for the later development of epilepsy. In adults, ischemic attack is often accompanied by seizures (Varelas and Mirski, 2001), and the resulting hyperexcitability may aggravate brain damage from ischemic stroke. Indeed, there are many similarities between ischemia and epilepsy regarding brain-damaging mechanisms (Leker and Neufeld, 2003). In each of these pathological conditions, an acute membrane depolarization caused by voltage- and ligand-gated ion channels results in neuronal hyperexcitability, intracellular Ca^{2+} ($[Ca^{2+}]_i$) overload, and neuronal damage, together referred to as excitotoxicity (Lee et al., 1999). On the basis of these similarities, antiepileptic drugs have been tested as neuroprotective agents in animal models of stroke, and some, especially channel blockers, have shown promising effects (Calabresi et al., 2003). Therefore, suppressing neuronal hyperexcitability after ischemic insults might be a fertile therapeutic target for stroke.

Voltage-dependent K^+ (Kv) channels are potent suppressors of neuronal excitability and represent key candidates for physiological and therapeutic modulation of the neuronal hyperexcitability that occurs in hypoxic/ischemic and epileptic patients. Delayed rectifier Kv currents (I_K) are important in regulating somatodendritic excitability in hippocampal and cortical pyramidal neurons (Bekkers, 2000; Du et al., 2000; Korngreen and Sakmann, 2000). In these neurons, the Kv2.1 channel is the major component of somatodendritic I_K (Martina et al., 1998; Murakoshi and Trimmer, 1999; Du et al., 2000; Malin and Nerbonne, 2002; Pal et al., 2003). Interestingly, many studies of somatodendritic I_K, and of Kv2.1, in pyramidal neurons suggest a function in regulating excitability and Ca^{2+} influx during periods of repetitive high-frequency firing (Colbert and Pan, 1999; Bekkers, 2000; Du et al., 2000; Kang et al., 2000; Korngreen and Sakmann, 2000; Misonou et al., 2004) rather than in a more classical role in action potential repolarization. We found previously that increased neuronal activity induced by seizures or glutamate stimulation changes Kv2.1 phosphorylation state and localization and yields increased amplitude of hippocampal neuron I_K/Kv2.1 attributable to hyperpolarizing shifts in voltage-dependent activation (Misonou et al., 2004). Such enhanced activity of Kv2.1 under conditions of hyperexcitability is predicted to provide homeostatic suppression of neuronal activity (Surmeier and Foehring, 2004; Misonou et al., 2005).

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We present here the surprising finding that *in vivo*, brief CO_2 anesthesia results in dephosphorylation of brain Kv2.1 and dispersion of somatodendritic Kv2.1 clusters in neurons throughout the brain. Short periods of hypoxia/ischemia can induce similar modulation of Kv2.1 channels in cultured hippocampal neurons by inducing intracellular Ca^{2+} release, presumably from mitochondria, and calcineurin-mediated Kv2.1 dephosphorylation. The ATP- and Ca^{2+}-mediated modulation of Kv2.1 is associated with increased amplitude of neuronal I_K attributable to a hyperpolarizing shift in the voltage dependence of activation and a hypoxia/ischemia-induced suppression of neuronal excitability. Together with our previous findings of Kv2.1 modulation in seizure animals (Misonou et al., 2004), we suggest that phosphorylation-dependent modulation of the abundant somatodendritic Kv2.1 channel may be a general mechanism to suppress pathological hyperexcitability of central neurons.

Materials and Methods

Preparation of hypoxic/ischemic animals. All animal use procedures were performed in strict accordance with the *Guide for the Care and Use of Laboratory Animals* described by the National Institutes of Health. Rats were subjected to CO_2 inhalation in a closed chamber connected to a CO_2 gas cylinder. Some animals were anesthetized by inhalation of diethyl ether or isoflurane in a closed chamber or by intraperitoneal injection of pentobarbital (50 mg/kg) or a mixture of ketamine and xylazine. Animals were then either decapitated to remove the brains or perfused with 4% paraformaldehyde for immunohistochemistry. To induce complete global ischemia, decapitated heads were incubated for 4 min at room temperature (~25°C). For immunoblotting, a crude whole-brain membrane fraction was prepared from these animal brains, and proteins were analyzed on immunoblotting as described previously (Misonou et al., 2004). For immunohistochemical analyses, sagittal brain sections (each 40 μm thick) were prepared from perfusion-fixed animals (Rhodes et al., 2004). The sections were blocked with 10% goat serum and then stained with a rabbit anti-Kv2.1 antibody (Trimmer, 1991) using Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) as described previously (Rhodes et al., 2004). Fluorescent images were taken with a 24-bit color digital camera installed on a Zeiss (Oberkochen, Germany) Axiovert 200M microscope with a 63×, 1.3 numerical aperture lens and a grating imaging system, Apotome, using Axiovision software.

Materials. Unless stated otherwise, all reagents were obtained from Sigma (St. Louis, MO) or Roche (Indianapolis, IN).

Neuronal culture. Dissociated cultures of embryonic rat hippocampal neurons were prepared as previously described (Misonou et al., 2004).

Biochemical analysis of neuronal proteins. Samples for biochemical analyses were prepared from neuronal cultures as described previously (Misonou et al., 2004). Briefly, neurons were washed twice with HBSS and incubated with drugs as indicated in the figure legends. The cells were then washed twice with ice-cold Locke's solution (in mM: 154 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 5 HEPES, pH 7.4), harvested, and centrifuged at 12,000 × g for 30 min at 4°C. The pellets were extracted by adding sample buffer (2% SDS, 1 mM EDTA, 10% glycerol, 0.001% bromophenol blue, 5% β-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8) and sonicating briefly. Rat brain membranes were prepared and treated with purified phosphatases as described previously (Misonou et al., 2004). Proteins were separated on 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Kv2.1 mouse monoclonal antibody K89/41 (Antonucci et al., 2001) or anti-Kv4.2 mouse monoclonal antibody K57/1 (Rhodes et al., 2004). The blots were incubated with HRP-conjugated secondary antibodies (MP Biomedicals, Irvine, CA) followed by enhanced chemiluminescence reagent (PerkinElmer, Wellesley, MA). Immunoreactive bands were visualized by exposing the blot to x-ray film. Because the Kv2.1 population of relative electrophoretic mobility (M_r) ≈125 kDa represents the major constitutively phosphorylated form of neuronal Kv2.1 (Murakoshi et al., 1997), here we quantified the relative amounts of this species as a measure of Kv2.1 phosphorylation state (Misonou et al., 2004). Immunoreactive bands were quantified after scanning using NIH ImageJ software (http://rsb.info.nih.gov/ij/) with the gel plotting macro. Quantitation was performed on samples from at least three different cultures.

ATP assay. Neurons were incubated with 5 mM 2-deoxy-D-glucose and 5 mM sodium azide for 5–15 min to induce chemical ischemia (CI), washed twice with ice-cold Locke's solution, and harvested in lysis buffer (1% Triton X-100 and 10 mM Tris-HCl, pH 8.0). As a control, neurons were incubated without the CI reagents, but the reagents were added immediately before the wash step. The suspension was centrifuged at 16,000 × *g* for 10 min at 4°C to remove insoluble materials. The resultant lysates were analyzed for ATP levels by using an ATP assay kit based on a luciferin–luciferase method.

Immunofluorescence staining of neurons. Neurons were incubated with drugs as indicated in the figure legends and then fixed and stained with K89/41 and anti-microtubule-associated protein-2 (MAP-2) mouse monoclonal antibodies and Alexa-conjugated isotype-specific secondary antibodies (Invitrogen) as described previously (Misonou et al., 2004). Fluorescent images were taken with a 24-bit color digital camera installed on a Zeiss Axioskop 2 microscope with a 63×, 1.3 numerical aperture lens, using Axiovision software. Images were transferred to Adobe PhotoShop software (Adobe Systems, San Jose, CA) as JPEG files. Quantitative analyses of Kv2.1 clustering were done using raw images. The percentage of cells exhibiting diffuse Kv2.1 staining was determined by collecting images from 100 cells from each of two independent experiments (Lim et al., 2000).

Ca^{2+} imaging. Neurons were washed twice with HBSS supplemented with 10 mM HEPES, pH 7.4, and then loaded with 5 μM Fluo-4-AM (Invitrogen) for 5 min at room temperature (23–25°C). After washing out excess dye, cells were further incubated for 15 min to allow deesterification of AM ester. Neurons were imaged at 1 Hz using confocal laser scanning microscopy (Zeiss LSM510) with argon laser (488 nm) and a 40×, 1.3 numerical aperture lens. Fluorescence intensity was measured on the NIH ImageJ software (http://rsb.info.nih.gov/ij/). Values were expressed as ratio of fluorescence intensity (F) against the basal intensity (F_0), $\Delta F/F_0$. For the analyses of spontaneous Ca^{2+} transients, frequencies of Ca^{2+} transients with $\Delta F/F_0 > 0.2$ were measured and expressed as percentages of the values in control. Alternatively, total Ca^{2+} loads by Ca^{2+} transients were measured by integrating traces of Ca^{2+} signal for 2 min.

Electrophysiology. I_K current recordings were made with the whole-cell voltage-clamp configuration. Patch pipettes were pulled from borosilicate glass tubing, heat-polished at the tip to give a resistance of 1–2 MΩ when filled with the pipette solution (in mM: 140 KCl, 5 EGTA, 2 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.3). Currents were recorded with a patch-clamp amplifier (EPC-10; HEKA Elektronik, Lambrecht/Pfalz, Germany), sampled at 10 kHz, and filtered at 2 kHz by a digital Bessel filter. All currents were capacitance and series resistance compensated and leak subtracted by standard P/n procedure. External solution contained 0.1 μM tetrodotoxin (TTX), 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.3. CI (5 mM 2-deoxy-D-glucose and 5 mM sodium azide) was applied to neurons for 15 min using a polytetrafluorethylene-glass multiple-barrel perfusion system. Currents were recorded from neurons before and after induction of CI. All recordings were performed at room temperature (23–25°C). The membrane potential was held at −100 mV and depolarized from the holding potential of −100 mV to voltages between −90 and +80 mV in 10 mV increments for 200 ms. Before the start of the test depolarization, a single prepulse to −10 mV was given for 30 ms to inactivate majority of the transient outward K^+ currents. Peak outward sustained K^+ currents (I) were measured 100 ms into the pulse at each depolarizing potential and were converted to conductance (G) using the equation, $G = I/(V - E_k)$. The Nernst K^+ equilibrium potential E_K was calculated as −84 mV. The normalized conductances were plotted against the test potential (V) and fitted to a single Boltzmann equation $G = G_{max}/(1 + \exp[-(V - V_{1/2})/k])$, where G_{max} is the maximum conductance, $V_{1/2}$ is the test potential at which the I_K channels have a half-maximal conductance, and k represents the slope of the activation curve.

Viability assay. Viability of neurons was assessed using the LIVE/DEAD assay kit (Invitrogen). Cells were incubated with CI reagents in

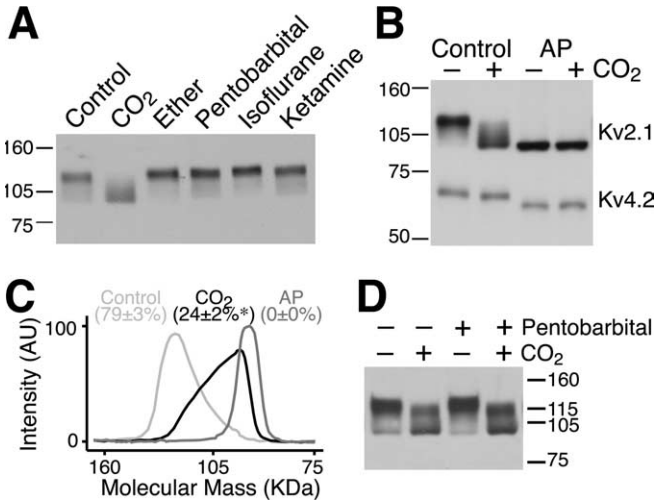

Figure 1. CO_2 inhalation induces dephosphorylation of Kv2.1 *in vivo*. ***A***, Effects of different anesthetics on the phosphorylation state of Kv2.1 *in vivo*. Animals were anesthetized by 2.5 min inhalation of CO_2, diethyl ether (Ether), isoflurane, or by intraperitoneal injection of 50 mg/kg pentobarbital, or 24 mg/kg ketamine and 3.2 mg/kg xylazine (Ketamine) and were then decapitated to prepare brain membrane fractions. Proteins were separated by SDS-PAGE and analyzed for Kv2.1 by immunoblotting. Numbers on the left indicate the mobility of molecular weight standards in kilodaltons. ***B***, Crude membranes were prepared from brains of control and CO_2-treated animals and incubated without (control) or with 0.1 U/ml AP. Proteins were analyzed by immunoblotting for Kv2.1 and Kv4.2. ***C***, Line scan analysis of immunoblots showing dramatic dephosphorylation of Kv2.1 in CO_2-treated animals. Bands of total and phosphorylated (M_r of 25 kDa) Kv2.1 were quantified. Numbers below the labeling indicate the amount of phosphorylated Kv2.1 as a percentage of total Kv2.1 level and are the mean ± SEM from three independent experiments. $^*p < 0.001$ compared with control. AU, Arbitrary units. ***D***, Effects of pentobarbital on CO_2-induced Kv2.1 dephosphorylation. Animals were injected with vehicle or 50 mg/kg sodium pentobarbital and then treated with CO_2 for 2 min. Crude brain membranes were prepared and analyzed by immunoblotting for Kv2.1.

HBSS for 10 min, washed twice with HBSS, and incubated with HBSS in the presence or absence of 5 mM tetraethylammonium (TEA) or 10 nM iberiotoxin for 1 h. Cells were then washed twice with HBSS and brought back to the normal culture condition. Cell viability was assessed after 24 h incubation. Numbers of live cells (calcein-positive cells, in green) and dead cells (ethidium homodimer-1 positive cells, in red) were counted in five areas (~7.5 mm^2 each) imaged with a 2.5× objective in three different cultures, and the viability was expressed as percentage viable cells, relative to control (untreated) cultures. Minor populations of non-neuronal cells in these relatively pure hippocampal cultures were excluded from the count.

Statistical analyses. All data are presented as the mean ± SEM. Normal data were compared by a two-sided *t* test. A *t* value of $p < 0.05$ was considered to be statistically significant.

Results

CO_2 anesthesia rapidly induces dephosphorylation of rat brain Kv2.1

The delayed rectifier Kv2.1 channel α subunit is a six-transmembrane segment polypeptide with large cytoplasmic N and C termini that contain more than 60 candidate phosphorylation sites. On SDS-polyacrylamide gels, the major forms of Kv2.1 in rat brain and cultured embryonic rat hippocampal neurons exhibit substantially higher M_r (≈125 and ≈115 kDa) than predicted from the deduced Kv2.1 primary sequence (95.3 kDa). *In vitro* alkaline phosphatase (AP) treatment of extracts prepared from either brain or cultured neurons shifts the M_r of Kv2.1 to ≈100 kDa (Misonou et al., 2004) (Fig. 1*B*), suggesting that Kv2.1 is constitutively and highly phosphorylated in both brain and cultured hippocampal neurons.

In response to revised institutional guidelines for animal care, we began to use CO_2 gas to deeply anesthetize animals before they were decapitated. However, we found that Kv2.1 in samples prepared from the brains of CO_2-anesthetized rats exhibited an altered M_r (≈100 kDa) that was similar to that of Kv2.1 in AP-treated samples, and distinct from the mobility of Kv2.1 (≈125 kDa) in previous samples prepared from animals decapitated without CO_2 anesthesia (Fig. 1*A*). When we examined the effects of various forms of anesthesia on the phosphorylation state of Kv2.1 *in vivo*, we found that CO_2 was unique in causing this significant shift in the M_r of Kv2.1 (Fig. 1*A*). The differences in M_r of Kv2.1 in samples from control and CO_2-treated animals were eliminated by AP treatment (Fig. 1*B*,*C*), showing that they were attributable solely to changes in Kv2.1 phosphorylation state. The M_r of two related rat brain Kv channel α subunits, Kv4.2 (Fig. 1*B*) and Kv1.1 (data not shown), which also undergo significant M_r shifts during *in vitro* AP treatment, were not altered by the brief CO_2 anesthesia. We have shown previously that kainate-induced seizures elicit dephosphorylation of Kv2.1 *in vivo* (Misonou et al., 2004). That the CO_2-induced dephosphorylation of Kv2.1 shown here was independent of CO_2-induced seizure activity was shown by the fact that the levels of phosphorylated Kv2.1 were not significantly different ($p = 0.09$; $n = 3$) between rats subjected to CO_2 inhalation in the absence or presence of deep pentobarbital anesthesia (Fig. 1*D*).

CO_2 anesthesia dramatically changes the subcellular localization of neuronal Kv2.1 *in vivo*

Kv2.1 is abundantly expressed in virtually all mammalian brain neurons (Trimmer and Rhodes, 2004) and is present in large somatodendritic clusters (Trimmer, 1991) that disperse in response to kainate-induced seizures (Misonou et al., 2004). To address whether CO_2 anesthesia altered Kv2.1 clustering, pentobarbital-anesthetized rats were subjected to brief (2 min) CO_2 inhalation and then rapidly perfused with paraformaldehyde fixative to minimize postmortem ischemic effects. In brains of control (pentobarbital anesthetized) rats, immunofluorescence staining for Kv2.1 was present as distinct surface clusters on somata and proximal dendrites of neurons in hippocampus and subiculum (Fig. 2), as well as cerebral cortex, thalamus, hypothalamus, and striatum (data not shown). In brains from CO_2-treated animals, these high-density clusters of Kv2.1 were dispersed such that Kv2.1 staining was now uniform on the surface membrane in virtually all neurons in the hippocampal formation (Fig. 2) and in all other brain regions examined (data not shown). In contrast, the localization of other neuronal K^+ channels (Kv4.2, Kv1.4, and BK/Slo) was not significantly altered in the brains of the CO_2-treated animals (data not shown). Optical sectioning (Lanni and Wilson, 2000) of the Kv2.1-stained brain sections revealed that Kv2.1 remained in the neuronal membrane after CO_2 treatment (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material), suggesting that the change in Kv2.1 localization in the brains of CO_2-treated animals was attributable to a lateral dispersion in the plasma membrane. CO_2 anesthesia also led to disruption of the unusually large (2–5 μm diameter) Kv2.1 clusters (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material) associated with muscarinic synapses in motor neurons (Muennich and Fyffe, 2003). These results demonstrate that brief CO_2 inhalation in both conscious and unconscious rats rapidly changes the phosphorylation state and localization of Kv2.1 *in vivo* and, in some cells, their association with specialized synapses. As such, previous results obtained from CO_2-anesthesized animals may have

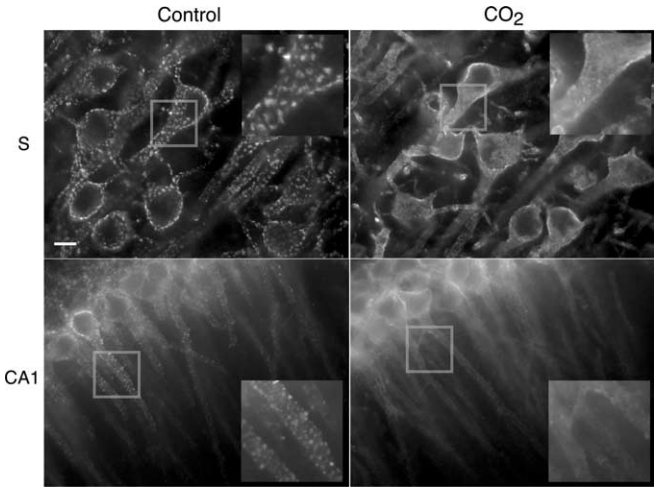

Figure 2. CO_2 dramatically changes localization of Kv2.1 *in vivo*. Rats were intraperitoneally injected with 50 mg/kg pentobarbital and exposed to air (control) or CO_2 for 2 min in a closed chamber. Brain sections from these control and CO_2-treated rats were stained with anti-Kv2.1 antibody. Images were taken by conventional fluorescence microscopy from the subiculum (S) and CA1 region of the hippocampus. Insets are higher-magnification views of Kv2.1 staining corresponding to the boxed areas in the main images. Scale bar, 10 μm.

yielded an underestimation of the full extent of Kv2.1 phosphorylation and clustering in brain neurons. Moreover, these dynamic changes in Kv2.1 localization *in vivo* could impact its role in regulating somatic and dendritic excitability.

Ischemia induces Kv2.1 dephosphorylation in culture and *in vivo*

To begin to investigate the neuronal signaling mechanisms underlying the dramatic effects of CO_2 inhalation *in vivo*, we examined the effects of CO_2 exposure and acidified culture medium, mimicking tissue acidosis, on Kv2.1 in cultured hippocampal neurons. We found that the phosphorylation state of Kv2.1 was not altered during exposure of neurons to pH 6.6 medium or elevated CO_2 for 20 min (Fig. 3*A*) mixed in ambient air to avoid excessive anoxia. However, inhibition of oxidative ATP synthesis, such as occurs in conditions of hypoxia/ischemia, by exposing cultured neurons to CI (5 mM sodium azide and 5 mM 2-deoxy-D-glucose) (Swanson and Benington, 1996) for 10 min induced Kv2.1 dephosphorylation (Fig. 3*B*). The intracellular ATP levels in cultured neurons were decreased to 17.1 ± 2.0% of control levels by 5 min of CI and to 8.8 ± 2.0% by 15 min of CI, consistent with a previous report (Bernstein and Bamburg, 2003). Although blockade of voltage-dependent Na^+ channels with TTX partially but not significantly ($p = 0.08$) inhibited dephosphorylation of Kv2.1 induced by 10 min of CI (Fig. 3*B*), CI for 15 min even in the presence of 1 μM TTX yielded full induction of Kv2.1 dephosphorylation (Fig. 3*C*), suggesting that the CI effects occur essentially independent of membrane depolarization. The CI-induced Kv2.1 dephosphorylation was completely reversible during CI washout (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material). Thus, as shown previously for glutamate-induced Kv2.1 dephosphorylation (Misonou et al., 2004), neurons can reestablish the normal Kv2.1 phosphorylation state after this brief ischemic insult.

The efficacy of CI but not media acidosis nor CO_2 itself on Kv2.1 dephosphorylation in cultured neurons led us to reinvestigate the basis for CO_2-induced Kv2.1 dephosphorylation *in vivo*. Brains from pentobarbital-anesthetized rats were subjected to complete global ischemia for 4 min after decapitation (Namba et al., 2002), which induced Kv2.1 dephosphorylation *in vivo* (Fig. 3*D*). Overdose itself with pentobarbital (150 mg/kg) over a 4 min period did not elicit Kv2.1 dephosphorylation (Fig. 3*D*), indicating that Kv2.1 dephosphorylation is not rapid postmortem changes. Together, these results suggest that the CO_2 effects *in vivo* are primarily attributable to hypoxia but not hypercapnia and that metabolic inhibition by hypoxia/ischemia *in vivo* and by CI *in vitro* induces rapid dephosphorylation of Kv2.1.

Chemical ischemia modulates the phosphorylation state and localization of Kv2.1 through calcineurin activation

We next began to investigate the signaling pathway underlying the CI-induced dephosphorylation of Kv2.1. We found that, unlike the effects of glutamate (Misonou et al., 2004), the CI effects occurred in the absence of extracellular Ca^{2+} (Fig. 4*A*). Antagonists for ionotropic glutamate receptors (GluRs) (AP-5 and CNQX; GluR blockers) that predictably blocked the effects of glutamate (Misonou et al., 2004) did not affect induction by CI (Fig. 4*B*), showing that the CI effects were not mediated by secondary and incidental glutamate release. This conclusion was further supported by the result that blockade of exocytosis by botulinum neurotoxin type B also did not affect CI-induced Kv2.1 dephosphorylation, although the toxin effectively reduced the levels of vesicle-associated membrane protein-2 (data not shown).

The effects of glutamate on Kv2.1 phosphorylation in cultured hippocampal neurons are mediated by calcineurin (Misonou et al., 2004). Interestingly, the CI-induced effects on Kv2.1 dephosphorylation were also significantly ($p = 0.003$) blocked by the calcineurin inhibitors cyclosporin A (Fig. 4*C*) and FK520 (5 μM), a more widely available FK506 analog (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material). These results suggest a CI-induced signaling pathway distinct in its upstream events from that induced by glutamate but similar in its convergence on calcineurin-dependent Kv2.1 dephosphorylation.

Given that CO_2 inhalation *in vivo* and glutamate stimulation *in vitro* (Misonou et al., 2004) lead to both Kv2.1 dephosphorylation and dispersion of Kv2.1 clusters, we next examined the effects of CI on Kv2.1 localization in cultured neurons. Cultured hippocampal pyramidal neurons have distinct Kv2.1 clusters on their somatodendritic membrane (Fig. 4*D*) similar to those seen on pyramidal neurons *in vivo* (Fig. 2). CI caused dispersion of somatodendritic Kv2.1 clusters in cultured neurons (Fig. 4*E*), similar to the effects of glutamate stimulation (Misonou et al., 2004). As such, the majority of neurons treated with CI (74.7 ± 3.6%) showed diffuse somatodendritic Kv2.1 staining compared with a small minority of cells (13.3 ± 1.7%) in control cultures. Note that these cultured neurons have an additional pool of Kv2.1 on the axon initial segment, a culture artifact (Lim et al., 2000) not seen in brain neurons (Fig. 2), and this pool of Kv2.1 was not affected by glutamate or CI treatment. Importantly, the effects of CI on Kv2.1 localization were significantly inhibited by cyclosporin A (31.4 ± 2.2% cells showed a diffuse Kv2.1 pattern; $p < 0.001$ compared with CI-treated cells in the absence of cyclosporin A) (Fig. 4*F*), supporting a role for calcineurin in the CI-induced effects on Kv2.1 phosphorylation and localization.

Chemical ischemia increases $[Ca^{2+}]_i$ by inducing Ca^{2+} release from intracellular stores

Given the lack of a requirement for extracellular Ca^{2+} in the CI-induced effects on Kv2.1 and that increased $[Ca^{2+}]_i$ is neces-

sary and sufficient to induce the calcineurin-dependent dephosphorylation of Kv2.1 during glutamate stimulation (Misonou et al., 2004), we next investigated whether the CI effects were mediated by Ca^{2+} release from intracellular stores. Ca^{2+} imaging of cultured hippocampal neurons was performed using Fluo-4 and confocal laser scanning microscopy, focusing on changes in Fluo-4 fluorescence intensity on the soma and proximal dendrites in which Kv2.1 is localized. Hippocampal neurons exhibited spontaneous $[Ca^{2+}]_i$ transients whose frequency and amplitude were suppressed by 1 μM tetrodotoxin (data not shown). CI was then induced, leading to a gradual increase in Fluo-4 signal intensity over 5 min (Fig. 5*A*,*B*), as reported previously (Chow and Haddad, 1998). As shown in Figure 5*B*, CI induced a large and sustained increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. This CI-induced increase in $[Ca^{2+}]_i$ exhibited slower kinetics than that induced by glutamate stimulation in the presence of extracellular Ca^{2+}, as reported for cultured cortical neurons (Chow and Haddad, 1998). However, the CI-induced increases in $[Ca^{2+}]_i$ reached levels comparable with those seen during glutamate stimulation within 5 min of CI induction (Fig. 5*B*).

To pharmacologically investigate the source of intracellular Ca^{2+} release, we examined the effects of thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+}-ATPase caffeine to induce Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which induces Ca^{2+} release from mitochondria by eliminating the H^+ gradient (Yang et al., 2003). Neither thapsigargin nor caffeine induced significant Kv2.1 dephosphorylation (data not shown). However, treatment with FCCP alone resulted in an increase in $[Ca^{2+}]_i$ similar to that obtained with CI (Fig. 5*C*), as well as dephosphorylation of Kv2.1 (supplemental Fig. 2*C*, available at www.jneurosci.org as supplemental material) in the absence of extracellular Ca^{2+}. Importantly, FCCP treatment after induction of CI yielded no additional increases in $[Ca^{2+}]_i$ (Fig. 5*C*), suggesting that the CI- and FCCP-induced Ca^{2+} release was from the same intracellular pool (i.e., mitochondria). Together, these data suggest that CI induces Ca^{2+} release from intracellular stores, presumably from mitochondria, and that the resultant increase in $[Ca^{2+}]_i$ is sufficient to induce calcineurin-dependent dephosphorylation of Kv2.1.

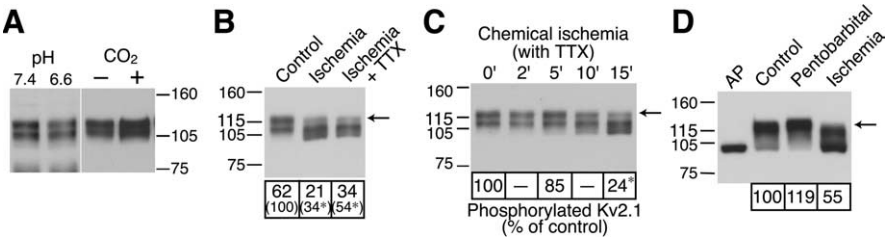

Figure 3. Ischemia induces dephosphorylation of Kv2.1 *in vitro* and *in vivo*. ***A***, Cultured hippocampal neurons were incubated in buffer at pH 7.4 or 6.6 or treated in a chamber filled with CO_2 gas at 37°C for 20 min. Proteins were solubilized in SDS sample buffer, fractionated on SDS-PAGE, and analyzed for Kv2.1 by immunoblotting. ***B***, Neurons were incubated with CI reagents (5 mM 2-deoxy-D-glucose and 5 mM sodium azide; Ischemia) in the presence or absence of 1 μM TTX for 10 min. Bands of total and phosphorylated (M_r of 125 kDa, indicated by an arrow) Kv2.1 were quantified. Values in boxes indicate the amount of phosphorylated Kv2.1 (M_r of 125 kDa) as a percentage of total Kv2.1 and are the mean values from five independent experiments (control, 62.0 ± 4.7%; ischemia, 21.0 ± 4.5%; ischemia plus TTX, 33.5 ± 0.5%). Values in parentheses denote the level of phosphorylated Kv2.1 shown as a percentage of the control level. Statistical significance between the control and experimental values are shown by asterisks ($p < 0.01$, $n = 5$). ***C***, Neurons were incubated with CI reagents (Chemical ischemia) for 2, 5, 10, or 15 min in the presence of 1 μM tetrodotoxin. The levels of phosphorylated Kv2.1 were as follows: control, 100.0 ± 7.6%; 15 min of CI, 23.5 ± 3.1%. *$p < 0.01$ ($n = 3$). ***D***, Animals were decapitated (Control) or injected with 150 mg/kg pentobarbital. To induce complete global ischemia, decapitated heads were incubated for 4 min at room temperature (Ischemia). Crude brain membranes were prepared from these animals, and proteins were analyzed by immunoblotting for Kv2.1. A fraction of crude membranes was incubated with 0.1 U/ml AP. The levels of phosphorylated Kv2.1 were as follows: control, 100.0 ± 3.3%; global ischemia, 55.4 ± 0.7%. Numbers to left or right denote mobility of molecular weight standards in kilodaltons.

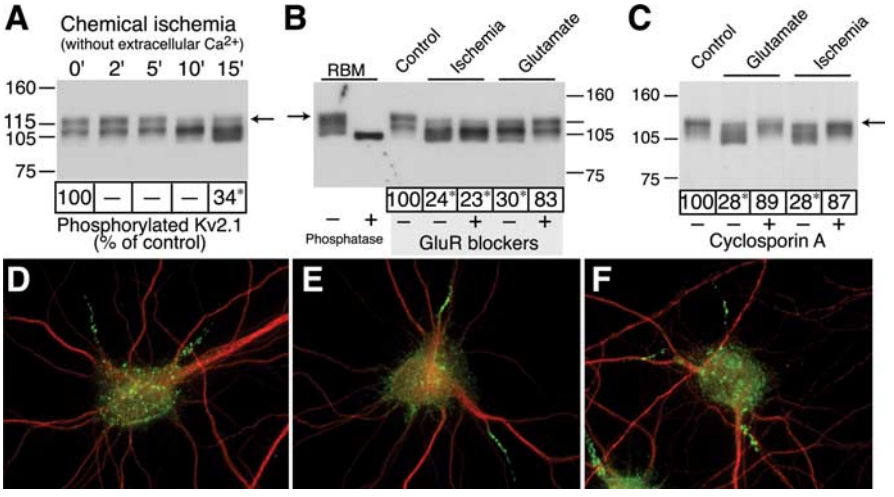

Figure 4. Chemical ischemia changes Kv2.1 phosphorylation state and localization by driving Ca^{2+} from the intracellular stores and activating calcineurin. ***A***, Neurons were incubated with CI reagents for 2, 5, 10, and 15 min in the absence of extracellular Ca^{2+}. Values in boxes indicate the level of phosphorylated Kv2.1 (marked by an arrow) shown as a percentage of the control level (control, 100 ± 6.4%; 15 min of CI, 34.1 ± 7.2%) ($n = 3$). ***B***, Neurons were incubated with 20 μM glutamate or CI reagents (Ischemia) for 15 min, in the presence or absence of 100 μM AP-5 and 10 μM CNQX (GluR blockers) in the presence of 1 μM tetrodotoxin. The levels of phosphorylated Kv2.1 were as follows: control, 100 ± 6.8%; ischemia, 24.2 ± 2.2%; ischemia plus GluR blockers, 22.7 ± 2.4%; glutamate, 30.4 ± 4.6%; glutamate plus GluR blockers, 83.4 ± 1.7% ($n = 3$). RBM, Rat brain membrane fraction. ***C***, Neurons were incubated with 20 μM glutamate or CI reagents for 15 min, in the presence or absence of 20 μM cyclosporin A in the presence of 1 μM tetrodotoxin. The levels of phosphorylated Kv2.1 were as follows: control, 100 ± 6.8%; glutamate, 28.4 ± 2.8%; glutamate plus cyclosporin A, 89.1 ± 5.5%; ischemia, 28.2 ± 5.4%; ischemia plus cyclosporin A, 86.5 ± 9.7% ($n = 3$). Proteins were solubilized in SDS sample buffer, fractionated on SDS-PAGE, and analyzed for Kv2.1 by immunoblotting. Statistical significance between control and experimental values (***A***, ***B***) or between values in the presence and absence of cyclosporin A (***C***) are shown by asterisks ($p < 0.01$). Numbers to left or right denote mobility of molecular weight standards in kilodaltons. ***D–F***, Neurons were incubated without (***D***) or with (***E***) CI reagents, or CI reagents and 20 μM cyclosporin A (***F***) for 15 min. Cells were fixed with 4% paraformaldehyde and stained with anti-Kv2.1 antibody (green) and anti-MAP-2 antibody (red).

Chemical ischemia alters the voltage-dependent activation of neuronal I_K and neuronal activity

Whole-cell patch-clamp recording from cultured hippocampal neurons revealed that CI led to large hyperpolarizing shifts (control, $V_{1/2}$ of +16.6 ± 0.4 mV; CI, $V_{1/2}$ of −4.3 ± 0.4 mV; $p <$ 0.001 vs control; $n = 5$) in the voltage dependence of activation of

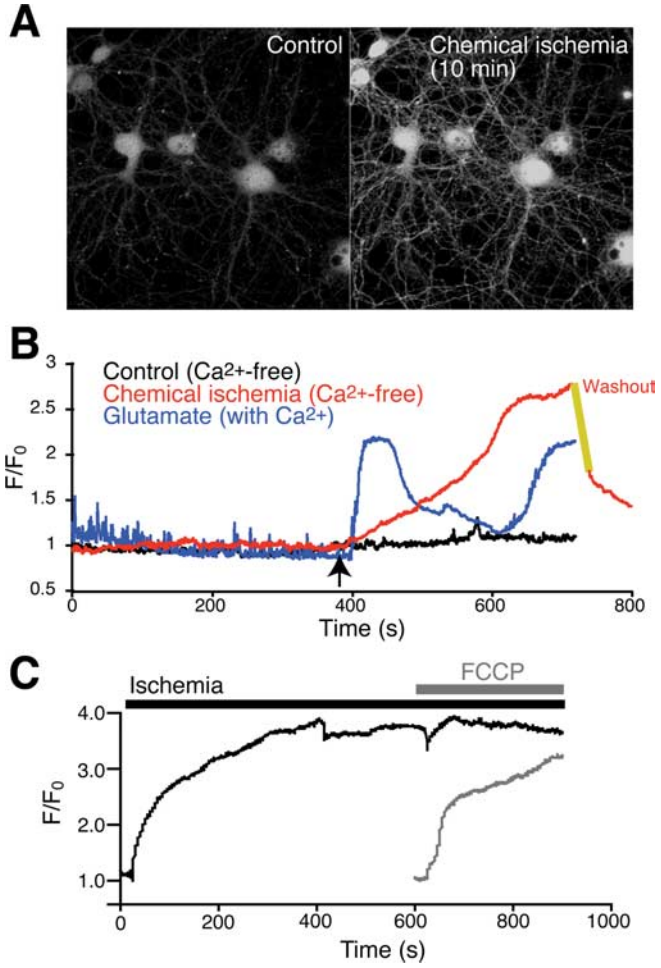

Figure 5. Chemical ischemia induces Ca^{2+} release from intracellular stores. ***A***, Neurons were loaded with 5 μM Fluo-4 and incubated with CI reagents (5 mM 2-deoxy-D-glucose and 5 mM sodium azide) for 10 min in the presence of 1 μM tetrodotoxin. ***B***, CI-induced increase of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. CI reagents or glutamate (10 μM) were added at the point indicated by an arrow. Neurons were incubated with glutamate in the presence of extracellular Ca^{2+}. Thick yellow line represents the absence of recording during the washout period. ***C***, Role of mitochondria as a source of released Ca^{2+}. Neurons were incubated without (gray trace) or with (black trace) CI reagents for 10 min and then incubated with 1 μM FCCP for 5 min. Imaging rate, 1 Hz.

I_K, without any significant change in the slope of the activation curve (Fig. 6*A*,*B*). These CI-induced changes are comparable with those induced by glutamate stimulation (Misonou et al., 2004). As a result of the CI-induced change in voltage-dependent activation, increased I_K amplitude was observed at each test potential (Fig. 6*A*,*B*). These CI-induced shifts in voltage-dependent activation of I_K were blocked by pretreatment with FK520 ($V_{1/2}$ of $+13.1 \pm 0.5$ mV) (Fig. 6*B*), suggesting that the calcineurin-dependent dephosphorylation of Kv2.1 was responsible for the altered biophysical properties of I_K. Previous studies have shown that the bulk (≥80%) of I_K in hippocampal and cortical pyramidal neurons is contributed by Kv2.1 (Murakoshi and Trimmer, 1999; Du et al., 2000; Pal et al., 2003). We found that the broad-spectrum K^+ channel blocker TEA when used at 5 mM [the K_i for TEA block of Kv2.1 is 1–5 mM based on studies of recombinant rat brain Kv2.1 in heterologous cells (Shi et al., 1994; Immke et al., 1999)] effectively blocked (90.0 ± 0.2% inhibition) I_K in control neurons. These data suggest that TEA effectively blocks the major Kv2.1 component of I_K. Interestingly, a similar level of TEA inhibition was obtained for the increased I_K in neurons after induction of CI (92.0 ± 1.4% inhibition), suggesting that the bulk of the enhanced I_K in ischemic neurons can be attributed to Kv2.1. A mixture of iberiotoxin (IbTx, 10 nM), a specific blocker of large-conductance Ca^{2+}-activated BK K^+ channels, and apamin (500 nM), a specific blocker of small-conductance Ca^{2+}-activated SK channels, yielded minimal block (12.0 ± 13.3% inhibition) of the total outward K^+ current in these neurons.

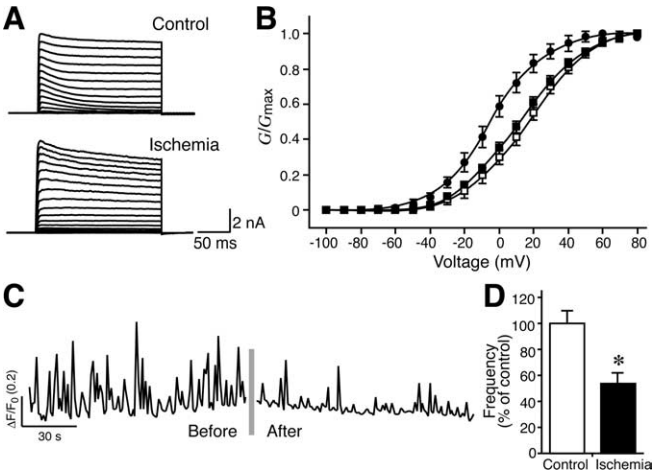

Figure 6. Chemical ischemia alters the properties of I_K current and suppresses neuronal excitability. ***A***, Representative I_K currents in a cultured hippocampal neuron recorded under whole-cell voltage clamp. The membrane potential was held at −100 mV and depolarized from the holding potential of −100 mV to voltages between −90 and +80 mV in 10 mV increments for 200 ms. A 30 ms prepulse to −10 mV was given before each test depolarization to eliminate transient K^+ current. ***B***, The plot shows the conductance–voltage (*G–V*) relationship of peak I_K currents recorded from neurons before (open square), after (filled circle) CI, and after CI in the presence of 5 μM FK520 (filled square). ***C***, Suppression of spontaneous Ca^{2+} transients after CI. Neurons were loaded with 5 μM Fluo-4. Representative spontaneous Ca^{2+} bursts in the soma before and after CI. Imaging rate, 1 Hz. ***D***, Spontaneous Ca^{2+} bursts with $\Delta F/F_0 > 0.2$ in the soma for 2 min were analyzed in cells before (Control) and after (Ischemia) the treatment with CI reagents. *$p < 0.01$ ($n = 8$).

Because such large changes in the activation properties of neuronal I_K would be predicted to dramatically alter neuronal excitability (Surmeier and Foehring, 2004), we next examined whether CI changes the electrical activity of neurons, using spontaneous $[Ca^{2+}]_i$ transients as an indirect readout of activity. As shown in Figure 6*C*, control hippocampal neurons exhibited regular spontaneous $[Ca^{2+}]_i$ transients. Neurons subjected to CI (10 min) and washout (10 min, which restores resting $[Ca^{2+}]_i$) (Fig. 5*B*) showed a significantly (≈50%; $p = 0.005$ compared with control) depressed frequency of spontaneous $[Ca^{2+}]_i$ transients of $\Delta F/F_0 > 0.2$ (Fig. 6*C*,*D*). The overall Ca^{2+} load (obtained by integrating changes in spontaneous $[Ca^{2+}]_i$ transients over a 2 min period) was also significantly reduced (supplemental Fig. 3*A*, available at www.jneurosci.org as supplemental material). Furthermore, FK520 pretreatment reversed this suppression ($p = 0.04$) (supplemental Fig. 3*A*, available at www.jneurosci.org as supplemental material), indicating that the postischemic suppression of neuronal excitability results from calcineurin-dependent events.

A neuroprotective mechanism conferred by TEA-sensitive and iberiotoxin-insensitive K^+ channels

To further understand the role of I_K/Kv2.1 modulation in the suppression of neuronal activity, we examined whether TEA could reverse the suppression of neuronal excitability induced by CI. As shown in Figure 7, neurons incubated with 5 mM TEA (which blocks >90% of I_K in these cultured hippocampal neu-

rons; see above) showed initial burst activity followed by recovery of relatively normal spontaneous Ca^{2+} transients. However, TEA treatment of neurons that had been subjected to CI for 10 min elicited Ca^{2+} overload (Fig. 7) (supplemental Fig. 3*B*,*C*, available at www.jneurosci.org as supplemental material). In contrast to the dramatic effects of TEA, 10 nM IbTx did not have any noticeable effect on CI-induced $[Ca^{2+}]_i$, at least within the 4 min window of imaging (data not shown). These results show that, after brief ischemic insults, neurons exhibit hyperexcitability that can be suppressed by TEA-sensitive IbTx-insensitive I_K currents.

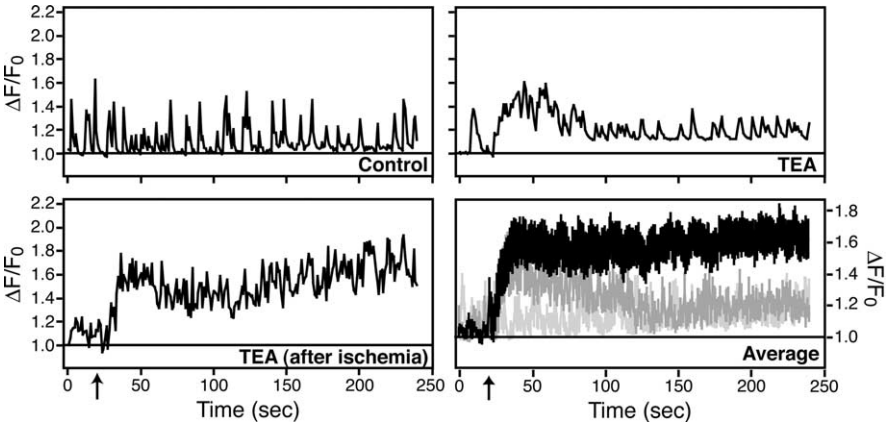

Figure 7. Tetraethylammonium induces Ca^{2+} overload in cultured neurons after ischemic insults. Neurons were loaded with 5 μM Fluo-4, incubated without or with CI reagents (5 mM 2-deoxy-D-glucose and 5 mM sodium azide) for 10 min, washed, and then incubated without or with 5 mM TEA. Representative traces of Ca^{2+} signals in cells treated with vehicle (Control), TEA-treated cells (TEA), and cells treated with TEA after ischemic insults (TEA after ischemia) are shown. The traces in the right bottom panel shows the means ± SEM of each data point in control (light gray), TEA (gray), and TEA after ischemia (black) from five independent cultures. The arrow indicates the time of TEA addition.

To examine whether I_K-mediated suppression of excitability would affect neuronal viability after brief ischemic insults, we assessed the neuronal damage induced by CI in the presence of TEA. Neurons were subjected to CI for 10 min, washed, and then incubated with or without TEA for 1 h. Cell viability was quantified after 24 h incubation under normal culture conditions. As shown in Figure 8, brief CI in itself did not affect neuronal viability, suggesting that neurons have protective mechanisms against this extent of ischemic insult. However, brief exposure of cells to 5 mM TEA just after CI resulted in significant neuronal damage, whereas TEA treatment in itself had little effect on cell viability (Fig. 8*B*). IbTx (10 nM) did not affect neuronal viability in the presence (Fig. 8*B*) or absence (data not shown) of ischemic insult. These results suggest that TEA-sensitive and IbTx-insensitive I_K channels, of which a large component is Kv2.1, confer protection against ischemic neuronal damage by suppressing neuronal hyperexcitability.

Together, these results suggest that hypoxia/ischemia *in vivo* and *in vitro* induces calcineurin-mediated Kv2.1 dephosphorylation that leads to dramatic changes in its localization and function. Such modulation of Kv2.1 represents a novel protective mechanism activated in response to brief ischemia insults.

Discussion

The major findings of this study are that brief ischemic insults *in vivo* and *in vitro* induce dephosphorylation of Kv2.1, loss of Kv2.1 clustering, and a large hyperpolarizing shift in the voltage-dependent activation of Kv2.1/I_K channels. The ischemia-induced modulation of Kv2.1 is distinct from that induced in central neurons by seizures *in vivo* and by glutamate stimulation *in vitro* (Misonou et al., 2004) in that it occurs in the absence of extracellular Ca^{2+} and is not dependent on ischemia-induced seizures *in vivo* or glutamate release *in vitro* (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The effects of ischemia *in vivo* are also more widespread and are observed throughout the brain, whereas the effects induced by kainate-induced seizures are restricted to the limbic system (Misonou et al., 2004). Despite these distinctions, increased $[Ca^{2+}]_i$ and calcineurin remain central to both responses. Moreover, both the glutamate- and ischemia-induced effects on Kv2.1 are reversible, in that removal of either stimulus leads to recovery of phosphorylated Kv2.1 and Kv2.1 surface clusters and restoration of the relatively depolarized voltage-dependent activation typical of control neurons. Although release of Ca^{2+} from intracellular stores, specifically mitochondria as opposed to glutamate-stimulated Ca^{2+} influx via ionotropic glutamate receptors, seems to be critical to the ischemia-induced modulation of Kv2.1, sustained increases in $[Ca^{2+}]_i$ leading to activation of calcineurin remain key. As predicted for the effects of glutamate stimulation (Surmeier and Foehring, 2004; Misonou et al., 2005), brief ischemic insult and enhanced activation of Kv2.1 leads to a suppression of neuronal activity (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The K^+ channel blocker TEA eliminates CI-induced suppression of neuronal excitability, and TEA treatment after brief and otherwise nonlethal exposure to CI leads to cell death. These findings show that neurons can use different upstream signaling pathways to achieve reversible calcineurin-dependent regulation of Kv2.1 localization and function in response to both hyperexcitability and ischemia. These studies also provide evidence that the activity of Kv2.1, although classically defined as a strictly voltage-dependent delayed rectifier K^+ channel, is also dependent on metabolic state and $[Ca^{2+}]_i$.

Potential roles for Kv2.1 modulation in brain ischemia

Neurons need protection against excessive hyperactivity, which could lead to pathological excitotoxic conditions. It is widely accepted that neurons have developed a diverse array of immediate and long-term homeostatic protective mechanisms that respond to abnormal hyperactivity. Hypoxia and ischemia cause membrane depolarization, and the generalized seizures that can be evoked by such metabolic stress can aggravate brain damage (Calabresi et al., 2003). However, different neurons have distinct immediate and long-term protective mechanisms "on demand" to suppress the hyperexcitability induced by brief hypoxia/ischemia. One immediate mechanism is the depression of neuronal excitability during hypoxia/ischemia-induced activation of neuronal K_{ATP} channels (Ballanyi, 2004), especially in neurons in anoxia-tolerant brain regions such as substantia nigra pars reticulata (Yamada et al., 2001), dorsal vagal neurons, and cerebellar Purkinje cells (Ballanyi, 2004). However, many brain neurons (e.g., hippocampal pyramidal neurons) express lower levels of functional K_{ATP} channels (Zawar and Neumcke, 2000; Griesemer et al., 2002), such that brief hypoxia and ischemia in hippocampal slices induces immediate seizure-like hyperactivity followed by a delayed and prolonged suppression of neuronal activity (Kawasaki et al., 1990; Yamamoto et al., 1997). In cultured

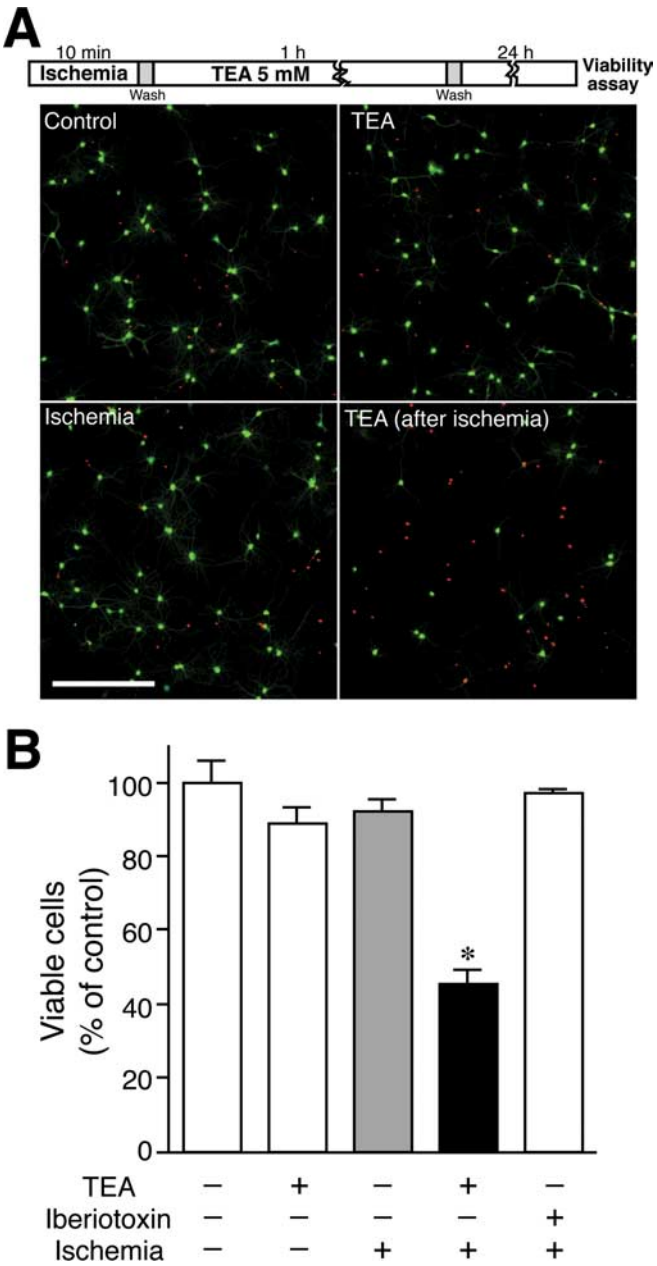

Figure 8. Tetraethylammonium aggravates neuronal damage after ischemic insults. ***A***, Neurons were incubated with CI reagents (5 mM 2-deoxy-D-glucose and 5 mM sodium azide) for 10 min, washed, and then incubated with 5 mM TEA for 1 h. After the incubation, cells were kept in the normal culture condition for 24 h and subjected to the cell viability assay as illustrated in the top schematic. All treatments were done in the absence of tetrodotoxin. Representative images taken with a 2.5× objective were shown in the bottom panels. Scale bar, 500 μm. ***B***, The number of total and viable cells were counted (total of >200 cells were counted in each sample) and shown as percentages of viable cells in control. Iberiotoxin (10 nM) was added as described for TEA. Data are the means ± SEM ($n = 5$). *$p < 0.01$.

hippocampal neurons, the suppressed neuronal activity in response to hypoxia/ischemia is attributable to an increase in overall K^+ current. Previous studies have attributed this increase to large-conductance Ca^{2+}-activated BK channels, based on the fact that the increases in K^+ current are dependent on increased $[Ca^{2+}]_i$ (Yamamoto et al., 1997; Erdemli et al., 1998; Tanabe et al., 1999), although more recent studies used more definitive pharmacological blockade (Runden-Pran et al., 2002). That ischemia also modulates Kv2.1 in a Ca^{2+}-dependent manner, through the activation of the protein phosphatase calcineurin, suggests that Kv2.1/I_K may also contribute to ischemia-induced increases in neuronal K^+ current. Moreover, the bulk of whole-cell I_K in hippocampal neurons under both control and CI conditions, as well as the CI-induced suppression of neuronal excitability, are blocked by TEA doses that yield effective blockade of Kv2.1 and BK channels but not by the specific BK channel blocker IbTx. Previous studies showed that knockdown of Kv2.1/I_K in cultured hippocampal slices by antisense oligonucleotides leads to neuronal hyperexcitability, but only under periods of high-frequency stimulation (Du et al., 2000). Conversely, when Kv2.1/I_K is potentiated by arachidonic acid, neurons are protected from 4-aminopyridine-induced hyperexcitability (Colbert and Pan, 1999). These findings suggest that Kv2.1 can indeed act as a potent suppressor of neuronal hyperexcitability. Together with the results presented here, we suggest that, like K_{ATP} and BK channels, Kv2.1 can also couple metabolic state and $[Ca^{2+}]_i$ to membrane excitability under periods of hypoxia/ischemia. However, for Kv2.1, such coupling occurs via a more indirect and slower mechanism than for the intrinsically ATP- and Ca^{2+}-sensitive K^+ channels, acting through dynamic and reversible calcineurin-dependent changes in phosphorylation state of the channel protein (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Given the extremely high level of Kv2.1 expression in the somata and proximal dendrites of hippocampal (and virtually all other central) neurons, this Kv channel may represent a key but previously unrecognized contributor to the metabolic state- and Ca^{2+}-dependent regulation of neuronal excitability.

Short- versus long-term effects of Kv2.1 modulation

Given the dynamic regulation of Kv2.1 function by reversible changes in phosphorylation state, it is not surprising that the role of Kv2.1/I_K in neuroprotection is complex. Increases in delayed rectifier K^+ channel activity in response to severe hypoxia and ischemia have been observed previously in CA1 hippocampal neurons (Chi and Xu, 2000, 2001; Zou et al., 2005), in large aspiny neostriatal neurons (Deng et al., 2005), in substantia nigra neurons (Jiang and Haddad, 1993), and in dorsal vagal motor neurons (Cowan and Martin, 1992). In cultured cortical neurons, increased TEA-sensitive I_K in response to proapoptotic treatments (long-term serum deprivation or staurosporine treatment) is critical to the induction of apoptosis, such that TEA is antiapoptotic (Yu et al., 1997, 1999b; Wang et al., 2000; Wei et al., 2003). Models linking I_K-mediated K^+ efflux to altered $[K^+]_i$ homeostasis, cell shrinkage, and proapoptotic cytochrome *c* release from mitochondria, as well as to direct K^+ regulation of caspase, have been proposed (Yu et al., 1999b; Wei et al., 2003; Hribar et al., 2004). Knockdown experiments in cultured cortical neurons revealed that Kv2.1 is the specific TEA-sensitive Kv channel underlying the proapoptotic I_K activity (Pal et al., 2003). However, we and others (Katsuki et al., 2005) found that TEA treatment exacerbated, not attenuated, neuronal injury induced by brief ischemia. Differences in the severity of insults and the modes of cell death involved, as well as the specific experimental system under study, may impact the effects of I_K/Kv2.1 modulation on neuronal survival. The bulk of the previous studies (Yu et al., 1997, 1999a,b; Pal et al., 2003; Wei et al., 2003) showing proapoptotic roles for I_K/Kv2.1 exposed neurons to relatively long (several hours to days) insults to cause extensive and sustained K^+ efflux, cell shrinkage, and neuronal apoptosis. In our *in vitro* ischemia model, acute and brief CI (~15 min) in itself was not sufficient to kill neurons but together with TEA treatment in-

duced hyperexcitability and cell death, presumably by an excitotoxic non-apoptotic pathway. Because brain ischemia can kill neurons via both apoptosis and necrosis (Zhang et al., 2004), the complex neuroprotective and/or proapoptotic roles proposed for I_K/Kv2.1 may also reflect Kv2.1 modulation in these different contexts. Our results are also consistent with a previous study that TEA does not protect against necrotic neuronal death induced by brief ischemic insult but provides neuroprotection against apoptotic cell death induced by longer, more intense insults (Wei et al., 2003). In addition, the timing of TEA addition relative to that of the ischemic insult can dramatically impact the neuroprotective effects of TEA *in vivo* (Huang et al., 2001).

We suggest that Kv2.1 might have a dual role in response to neuronal insults: an immediate protective role against acute hyperexcitability induced by seizures (Misonou et al., 2004) and brief (~2–15 min) hypoxia attributable to calcineurin-dependent dephosphorylation, and altered localization and function, leading to homeostatic suppression of neuronal hyperexcitability. Removal of the ischemic (or excitotoxic) insult leads to a dynamic reversal of the calcineurin-dependent dephosphorylation and a recovery of the normal Kv2.1 phosphorylation state, clustering, and voltage-dependent activation. However, sustaining Kv2.1 in the dephosphorylated state under conditions of prolonged insult may lead to excessive K^+ efflux, cell shrinkage, and apoptotic cell death. Thus, the role of Kv2.1 in neuronal survival in response to ischemic and other insults may be biphasic (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Brief and reversible activation of Kv2.1 by dephosphorylation may be advantageous attributable to neuroprotective suppression of neuronal excitability, but sustained activation may eventually lead to deleterious effects via lowered $[K^+]_i$.

Possible mechanisms of ischemia-induced modulation of Kv2.1

Electron microscopic analysis has shown that plasma membrane Kv2.1 clusters on the somata and proximal dendrites of pyramidal neurons lie over subsurface cisternae (Du et al., 1998), intracellular ER-derived membranes rich in inositol triphosphate receptors and ryanodine receptors, and associated with high concentrations of mitochondria (Berridge, 1998; Paspalas and Goldman-Rakic, 2004). We found that, in cultured hippocampal neurons plasma membrane, Kv2.1 clusters overlap clusters of ryanodine receptors and the luminal Ca^{2+}-binding protein calsequestrin (Antonucci et al., 2001; Misonou et al., 2005), suggesting a specialized neuronal Ca^{2+} signaling domain that may also contain a high density of calcineurin (Cameron et al., 1995). That FCCP, but not thapsigargin and caffeine, mimicked the effects of CI on modulation of Kv2.1, and that FCCP and CI treatments together yielded no additional increase in $[Ca^{2+}]_i$ than achieved with CI alone suggest that mitochondria, especially ER-associated mitochondria (Berridge, 1998; Paspalas and Goldman-Rakic, 2004), are good candidates for the source of rapid intracellular Ca^{2+} release induced by ischemia. A number of recent studies have revealed the critical role for neuronal mitochondria in regulating Ca^{2+}-dependent signaling events (Yang et al., 2003; Li et al., 2004). Moreover, mitochondrial function can be disturbed under such pathological conditions, for example, Ca^{2+} is released from mitochondria through the $2Na^+/Ca^{2+}$ exchanger in hippocampal neurons during oxygen–glucose deprivation (Zhang and Lipton, 1999). Ca^{2+} stores in the cistern itself are an additional source of CI-induced Ca^{2+} release, as occurs in ischemic spinal cord neurons (Ouardouz et al., 2003). The localization of Kv2.1 at sites close to subsurface cisterns and associated mitochondria may allow for more efficient functional coupling of Ca^{2+} released from these intracellular stores to modulation of plasma membrane Kv2.1 channels. Moreover, such assemblies may allow for local "cluster-by-cluster" or quantal regulation of discrete pools of Kv2.1 in a manner distinct from the more graded response expected for a more uniformly distributed channel population. Dispersion of these Kv2.1 clusters during calcineurin-mediated dephosphorylation could provide a mechanism for more efficient reversibility and recovery, by removing Kv2.1 from this region of local Ca^{2+} and calcineurin signaling. The coupling of neuronal metabolism and $[Ca^{2+}]_i$ to dynamic changes in Kv2.1 localization and function via regulation of the Kv2.1 phosphorylation state provides an effective and reversible mechanism for homeostatic regulation of central neuron excitability under conditions of ischemic and excitotoxic stress.

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